EVELINE CATERINE SANDRI

EFEITO DE UM AGONISTA DOS RECEPTORES ATIVADOS POR PROLIFERADORES DE PEROXISSOMO GAMA (PPARγ) SOBRE OS EFEITOS ANTI-LIPOGÊNICOS DO ÁCIDO LINOLEICO CONJUGADO (CLA) *TRANS*-10, *CIS*-12 NA GLÂNDULA MAMÁRIA DE OVELHAS LACTANTES

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

Orientador : Prof. Dr. Dimas Estrasulas de Oliveira

Co-orientador: Dr. Kevin J. Harvatine

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Aos meus pais, Lauri e Justina,
e ao Rafael.

Dedico

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À minha família, pelo amor incondicional, especialmente meus pais Lauri e Justina, que nunca mediram esforços para que eu pudesse alcançar meus objetivos e aos meus irmãos Eduardo e Estefânia, pelo companheirismo e apoio.

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“Se você quer ser bem sucedido, precisa ter dedicação total, buscar seu último limite e dar o melhor de si”

(Ayrton Senna)

RESUMO

O ácido linoleico conjugado *trans*-10, *cis*-12 é conhecido por inibir a síntese de gordura na glândula mamária de diversas espécies animais. O objetivo deste estudo foi analisar o efeito do PPARγ sobre a lipogênese mamária e expressão gênica, através de um agonista químico específico e sua resposta ao CLA *trans*-10, *cis*-12. Vinte e quatro ovelhas em lactação, com 70 ± 3 dias em lactação (DEL) e peso corporal (PC) de 60 ± 0,45 kg, foram distribuídas aleatoriamente em um dos quatro tratamentos, por 7 dias: 1) Controle (100 mL/dia de solução salina estéril, intravenosa); 2) Tiazolidinediona (TZD) (4mg/kg de PC/dia em 100 mL de solução salina estéril, intravenosa); 3) CLA (27g/dia de CLA desprotegido da bio-hidrogenação ruminal, com 29,9% de *trans*-10, *cis*-12, dosado oralmente); 4) TZD+CLA. Comparado ao Controle, a gordura do leite foi 22,3% menor no tratamento CLA (P=0,05), tendeu a ser 20,7% menor no tratamento TZD+CLA (P=0,06) e o TZD não afetou o teor de gordura (P=0,39). O teor de lactose e as produções de leite e dos componentes não foram afetados pelos tratamentos. O teor de proteína foi menor no CLA comparado ao TZD (P=0,01) e tendeu a ser maior com o TZD comparado ao Controle (P=0,08). Na glândula mamária, o CLA reduziu a expressão do PPARγ, SREBP1 e SCD1, porém o TZD não estimulou a expressão destes. No tecido adiposo, a expressão do PPARγ não foi afetada pelos tratamentos, enquanto que o SREBP1 teve maior expressão nos tratamentos TZD, CLA E TZD+CLA e a SCD1 teve maior expressão com TZD+CLA, comparada aos demais tratamentos. Concluindo, o CLA afetou negativamente a expressão dos genes envolvidos na síntese de lipídeos e o TZD não estimulou a expressão gênica e lipogênese na glândula mamária.

Palavras-chave: Expressão gênica. Gordura do leite. Lipogênese. Tiazolidinediona.

ABSTRACT

The *trans*-10, *cis*-12 conjugated linoleic acid is known to inhibit fat synthesis in the mammary gland of many animal species. The objective of this study was to analyze the effect of PPARγ on mammary lipogenesis and gene expression, through a specific chemical agonist and its response to *trans*-10, *cis*-12 CLA. Twenty four 70 ± 3 days in milk (DIM) and body weight (BW) 60 ± 0.45 kg lactating ewes were randomly assigned to one of the four treatments for 7 days: 1) Control (100 mL/day of sterile saline solution, intravenous); 2) Thiazolidinedione (TZD) (4mg/kg of BW/day in 100 mL of sterile saline solution, intravenously); 3) CLA (27g/d orally-dosed rumen-unprotected 29.9% *trans*-10, *cis*-12 CLA); 4) TZD+CLA. Compared to Control, milk fat was 22.3% lower in CLA (P=0.05), tended to be 20.7% lower in TZD+CLA (P=0.06) and did not change in the TZD treatment (P=0.39). The lactose content and milk yield and production of components were not affected by treatments. The protein content was lower in the CLA compared to TZD (P=0.01) and tended to be higher with the TZD compared to Control (P=0.08). In the mammary gland, CLA reduced expression of PPARγ, SREBP1 and SCD1, but TZD did not stimulate the expression of these genes. In adipose tissue, PPARγ expression was not affected by treatments, whereas the SREBP1 had more expression in TZD treatment, CLA and TZD + CLA and the SCD1 had more expression with TZD+CLA, compared to the other treatments. In conclusion, the CLA negatively affected the expression of genes involved in lipid synthesis and the TZD was unable to stimulate gene expression and lipogenesis in mammary gland.

Keywords: Gene expression. Milk fat. Lipogenesis. Thiazolidinedione

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LISTA DE ABREVIATURAS E SIGLAS

ACC	Acetil-CoA-carboxilase
ACS	Acil-CoA-sintase
AGPAT	Acil glicerol-3-fosfato aciltransferase
aP2	Proteína adipócita 2
CLA	Ácido linoleico conjugado
CD36	Grupo de diferenciação 36
DGAT1	Diacilglicerol aciltransferase 1
DGL	Depressão da gordura do leite
DMBA	Dimetilbenz(a)antraceno
ERK1/2	Quinase reguladora do sinal extracelular1/2
FABP	Proteína de ligação ao ácido graxo
FAS	Ácido graxo sintase
FATP1	Proteína transportadora de ácido graxo
GLUT4	Transportador de glicose estimulado pela insulina
GPAT	Glicerol 3-fosfato aciltransferase
INSIG	Proteína indutora de insulina
LPL	Lipoproteína lipase
MFD	Milk fat depression
mTOR	Mammalian target of rapamycin
NRC	Nutrient requeriments council
PEPCK	Fosfoenolpiruvato carboxiquinase
PPAR	Receptores ativados por proliferadores de peroxissomo
PPER	Elementos de resposta ao proliferador de peroxissomo
RXR	Receptor retinoide X
SCAP	Proteína ativadora de clivagem do SREBP
SCD	Estearoil-CoA-dessaturase
SPOT14	Hormônio responsivo a tireoide
SREBP	Proteína de ligação ao elemento regulatório esterol
TNFα	Fator de necrose tumoral alpha

TZD	Tiazolidinediona
UCP	Proteína desacopladora mitocondrial

SUMÁRIO

1 INTRODUÇÃO

Atualmente, os estudos com animais lactantes têm focado no desenvolvimento de ações que visam aumentar a eficiência produtiva e a qualidade dos produtos lácteos. Como o potencial genético dos ruminantes, principalmente vacas leiteiras, continua a melhorar, avanços em estratégias de alimentação mais eficientes são de extrema importância (BIONAZ, 2014). Para isso, tem-se observado grandes progressos na biologia da lactação, a fim de explicar melhor os processos envolvidos no metabolismo dos principais componentes do leite.

A síntese de gordura no leite recebeu particular interesse por ser o constituinte mais afetado pela dieta (BIONAZ e LOOR, 2011) e devido a sua influência nas propriedades de fabricação e qualidade organoléptica do leite e seus derivados (BIONAZ e LOOR, 2008). Mesmo conhecendo-se sobre a bioquímica da síntese de lipídeos no leite, os sistemas regulatórios e de sinalização celular na glândula mamária não são totalmente claros.

Descrita há muito tempo, a depressão da gordura do leite (DGL) caracteriza-se pelo decréscimo no teor e produção de gordura do leite observado tipicamente em ruminantes alimentados com dietas altamente fermentáveis e/ou que contenham altas concentrações de ácidos graxos poliinsaturados. Estudos mais recentes demonstraram que o CLA *trans*-10, *cis*-12, intermediário da bio-hidrogenação ruminal do ácido linoleico, é um potente inibidor da síntese de gordura e por isso tem sido extensivamente estudado (BAUMAN et al., 2008).

Os mecanismos pelo qual o CLA *trans*-10, *cis*-12 causa redução na síntese de lipídeos do leite envolve, pelo menos em parte, a redução da expressão de genes e o coordenado decréscimo na atividade das enzimas envolvidas nos processos

de síntese e o recrutamento de fatores de transcrição lipogênicos (BAUMAN et al., 2006; KADEGOWDA et al., 2009).

Além dos fatores que causam DGL por meio da alteração da atividade gênica, deve-se considerar a possibilidade de se aumentar o teor e a produção de gordura no leite utilizando-se determinadas substâncias conhecidas pela possibilidade de estimular positivamente a ação dos genes e fatores de transcrição lipogênicos. Para isso, há alguns agonistas disponíveis e estudos sugerem que o uso destes, inclusive em animais *in vivo*, pode aumentar a expressão gênica e ser um importante regulador da síntese de gordura do leite.

Pesquisas com esses agonistas em animais em lactação, aliadas a técnicas de análise molecular envolvendo a expressão gênica, podem promover um conhecimento maior em torno dos mecanismos relacionados à regulação nutricional da lipogênese mamária em ruminantes.

O presente trabalho visa auxiliar na compreensão dos mecanismos que regulam a lipogênese em ruminantes, através da análise da expressão de fatores de transcrição e genes envolvidos na síntese de gordura na glândula mamária de ovelhas lactantes suplementadas com CLA e um agonista químico específico dos receptores ativados por proliferadores de peroxissomo gama (PPARγ).

2 REVISÃO BIBLIOGRÁFICA

2.1 O ÁCIDO LINOLEICO CONJUGADO

2.1.1 Identificação do CLA

O termo ácido linoleico conjugado (CLA) refere-se a uma classe de isômeros posicionais e geométricos do ácido linoleico, com duplas ligações conjugadas, ou seja, separadas apenas por uma ligação simples carbono-carbono e que podem apresentar configurações *cis* ou *trans* (PARIZA et al., 2000; HAYASHI, 2003).

O CLA encontrado no leite e gordura da carne de ruminantes provém de duas principais fontes (GRIINARI e BAUMAN, 1999). A primeira é originária do processo de bio-hidrogenação ruminal parcial do ácido linoleico (C18:2) à ácido esteárico (C18:0). Ela inicia com a isomerização da dupla ligação *cis*-12 a *trans*-11 para formar o isômero *cis*-9, *trans*-11; em seguida, há a redução da ligação *cis*-9 para formar o ácido vacênico (C18:1 *trans*-11) e a etapa final é a hidrogenação da ligação *trans*-11, convertendo o ácido vacênico em ácido esteárico (BAUMAN et al., 2003). Em condições de decréscimo no pH ruminal e consequente mudança no padrão de fermentação, há a formação do isômero *trans*-10, *cis*-12, que é originado por um processo similar, mas envolvendo enzimas e bactérias diferentes. A enzima *cis*-9, *trans*-10 isomerase forma *trans*-10, *cis*-12 na primeira reação e a *cis*-12, *trans*-11 isomerase forma o *trans*-10 C18:1 na reação seguinte e a reação final é a redução da ligação *trans*-10 para formar o ácido esteárico (KHANAL e DHIMAN, 2004). A segunda forma de biossíntese do isômero *cis*-9, *trans*-11 consiste na conversão do ácido vacênico a CLA por meio da enzima delta-9-dessaturase ou estearoil-CoA-dessaturase 1 (SCD1), encontrada no tecido adiposo e glândula mamária de animais em lactação. Ela introduz uma dupla ligação *cis*-9 no ácido

vacênico formando o isômero *cis*-9, *trans*-11 (KHANAL e DHIMAN, 2004; BAUMAN et al., 1999).

Embora vários isômeros do CLA sejam formados durante os processos mencionados, o CLA *trans*-10, *cis*-12 e o CLA *cis*-9, *trans*-11 têm recebido maior atenção devido suas ações metabólicas. Numerosas propriedades têm sido atribuídas ao CLA, incluindo ação como agente anticarcinogênico, antiaterosclerótico, antiadipogênico, antidiabetogênico e modulador da resposta imune (BELURY, 2002; LEE et al.,1994; COOK et al., 1993; PARIZA, 1979; HOUSEKNECHT et al., 1998). O CLA *cis*-9, *trans*-11, o mais abundante em alimentos derivados de ruminantes, é responsável por inibir a ação tumorigênica do dimetilbenzeno(a)antraceno (DMBA) em câncer de pele, estômago e mama (HA et al., 1987; HA et al., 1990; IP et al., 1991). Por sua vez, o CLA *trans*-10, *cis*-12 foi identificado como um efetivo agente inibidor da síntese de gordura na glândula mamária e tecido adiposo em várias espécies (BAUMGARD et al., 2000; OSTROWSKA et al., 2003).

2.1.2 Efeitos fisiológicos do isômero *trans*-10, *cis*-12

A DGL naturalmente ocorre quando vacas são alimentadas com dietas altamente fermentáveis e/ou suplementadas com óleos vegetais e/ou de peixe (BAUMAN e GRIINARI, 2003). Griinari et al. (1998) demonstraram que um ambiente ruminal alterado, induzido por uma alimentação de alto concentrado ou dietas com baixa fibra, está associado com uma mudança no perfil de ácido *trans*-octadienóico da gordura do leite.

Várias teorias têm sido propostas para explicar a DGL, mas muitas delas têm se mostrado inadequadas, principalmente aquelas que se baseiam em uma limitação no fornecimento de precursores lipogênicos (BAUMAN e GRIINARI, 2003; GRIINARI e BAUMAN, 2006). A mais aceita, a teoria da bio-

hidrogenação, propõe que a DGL induzida pela dieta refere-se à inibição da síntese lipídica por ácidos graxos específicos que são intermediários da bio-hidrogenação de ácidos graxos poliinsaturados presentes na dieta e que são produzidos somente sob certas condições de fermentação ruminal (BAUMAN e GRIINARI, 2001). O primeiro desses intermediários a ser identificado como um potente inibidor da síntese foi o CLA *trans*-10, *cis*-12 (BAUMAN et al., 2008).

Baumgard et al. (2000) constataram com a infusão abomasal dos dois principais isômeros em vacas em lactação, que o CLA *trans*-10, *cis*-12 reduziu 42 e 44% o teor e a produção de gordura no leite, respectivamente, e que o CLA *cis*-9, *trans*-11 não teve efeito sobre a gordura do leite, demonstrando claramente que o CLA *trans*-10, *cis*-12 é o responsável pela DGL. A habilidade do CLA *trans*-10, *cis*-12 em regular a síntese de gordura no leite também tem sido observada em outros mamíferos, tais como ratos (LOOR et al., 2003), suínos (BOMTEMPO et al., 2004; POULOS et al., 2004), ovelhas (OLIVEIRA et al., 2012; BALDIN et al., 2013), cabras (FERNANDES et al., 2014) e humanos (MASTERS et al., 2002).

Além da ação na glândula mamária, outros trabalhos têm mostrado que o isômero *trans*-10, *cis*-12 tem efeito também na composição corporal de diversos modelos animais. Em suínos, a inclusão de doses crescentes de CLA na dieta dos animais demonstrou um aumento na deposição de tecido magro e redução na deposição de gordura no tecido adiposo (OSTROWSKA et al., 1999; OSTROWSKA et al., 2003), e o CLA também aumentou os níveis de ácidos graxos saturados e reduziu os monoinsaturados, o que sugere seu envolvimento nos processos de síntese e dessaturação de ácidos graxos no tecido adiposo (BEE, 2000).

Em humanos, o tratamento de pré-adipócitos isolados do tecido adiposo com o CLA *trans*-10, *cis*-12 preveniu o acúmulo de triglicerídeos, enquanto que o CLA *cis*-9, *trans*-11

aumentou consistentemente o acúmulo de gordura (BROWN et al., 2001). Ainda, Gaullier et al. (2004) avaliaram durante um ano um grupo de pessoas recebendo uma suplementação de CLA (mistura dos isômeros *cis*-9, *trans*-11 e *trans*-10, *cis*-12) e após 6 meses já observaram redução na gordura corporal e aumento na massa corporal magra.

Dois principais mecanismos têm sido propostos para explicar os efeitos do CLA nas mudanças da composição corporal. Primeiro, pela redução na captação de gordura e aumento na liberação de gordura nos adipócitos e segundo, pelo aumento na β-oxidação dos ácidos graxos no tecido muscular.

O CLA consistentemente altera a composição dos ácidos graxos da membrana, por alterar os níveis de ácidos graxos monoinsaturados, através da redução do índice de dessaturação, que indica um decréscimo na atividade da enzima SCD1, alvo do CLA. Uma proporção de ácidos graxos saturados e monoinsaturados é importante na manutenção da fluidez da membrana e qualquer alteração nessas taxas pode intervir em uma variedade de respostas fisiológicas, como taxa metabólica, sensibilidade a insulina e obesidade, todos influenciados pelo CLA (NTAMBI et al., 2000).

2.1.3 Regulação da expressão dos genes lipogênicos

Se o CLA altera o metabolismo lipídico de um modo geral, isso se deve ao efeito direto ou não na regulação gênica, seja no mRNA ou na atividade de enzimas e fatores de transcrição, seja na modificação do metabolismo como um todo (JOSÉ, 2005).

A síntese de gordura no leite requer a atividade coordenada de enzimas envolvidas na captação de metabólitos, lipogênese *de novo*, transporte, dessaturação e esterificação de ácidos graxos. Baumgard et al. (2002) mediram a expressão gênica da acetil-CoA-carboxilase alfa (ACCα), ácido graxo

sintase (FAS), SCD1, lipoproteína lipase (LPL), proteína de ligação à ácido graxo (FABP), glicerol 3-fosfato aciltransferase (GPAT) e acilglicerol-3-fosfato aciltransferase (AGPAT) em vacas recebendo CLA *trans*-10, *cis*-12 e este reduziu a expressão do mRNA de todas as enzimas avaliadas. Esses dados comprovaram que, pelo menos em parte, o mecanismo pelo qual o CLA *trans*-10, *cis*-12 inibe a síntese de gordura inclui o decréscimo na expressão de genes que codificam enzimas envolvidas na captação e transporte de ácidos graxos circulantes, síntese *de novo*, dessaturação e síntese de triglicerídeos.

Mach et al. (2013) utilizaram dados da expressão gênica e perfil de ácidos graxos de vacas suplementadas com uma fonte de ácidos graxos insaturados de um estudo anterior para identificar a associação entre a expressão de genes relacionados ao metabolismo lipídico e as concentrações de ácidos graxos no leite. Um grupo de 51 genes teve correlação negativa com o CLA *trans*-10, *cis*-12, *cis*-11, *trans*-9 e outros ácidos graxos *trans* e foram positivamente associados com altas concentrações de ácidos graxos sintetizados *de novo*, como palmitato e ácidos graxos de cadeia curta. Os principais genes identificados nesse grupo foram ACCα, FAS, diacilglicerol aciltransferase 1 (DGAT1) e os fatores de transcrição receptores ativados por proliferadores de peroxissomo gama **(**PPARγ) e proteína de ligação ao elemento regulatório do esterol 1 (SREBP1).

Outros trabalhos realizados com vacas ou ovelhas suplementadas com o CLA *trans*-10, *cis*-12 ou usando o cultivo de células mamárias epiteliais bovinas também mostraram que o isômero reduziu a expressão dos principais genes envolvidos no metabolismo lipídico em todos os casos (PETERSON et al., 2003; PETERSON et al., 2004; KADEGOWDA et al., 2010; HUSSEIN et al., 2013; HARVATINE et al., 2006). Além disso, esses mesmos autores verificaram inibição na expressão do fator de transcrição

SREBP1, importante regulador da expressão de genes envolvidos na síntese lipídica. O SREBP1 é sintetizado no retículo endoplasmático, onde fica ancorado pela proteína indutora de insulina (INSIG). Para efetuar a transcrição, a proteína ativadora de clivagem do SREBP1 (SCAP) transloca o SREBP1 até o complexo de Golgi, onde se torna ativa pela clivagem da porção N-terminal. Uma vez ativa, se desloca até o núcleo e liga-se a sequência de DNA da região promotora do gene alvo (HUSSEIN et al., 2013).

Recentemente, tem-se mostrado que a ativação de outro fator de transcrição, o PPARγ, pode regular positivamente os genes lipogênicos em células mamárias (POSTIC et al., 2007). Baseado nisso, os efeitos dos ácidos graxos *trans* podem ser controlados através de reguladores transcricionais na glândula mamária, semelhante ao que ocorre em outros tecidos lipogênicos (KADEGOWDA et al., 2010).

2.2 PPARγ

2.2.1 Identificação

Os receptores nucleares controlam o metabolismo afetando a expressão do mRNA de genes alvos, incluindo enzimas metabólicas (DESVERGNE et al., 2006). Eles representam um importante sistema regulatório nas células, tecidos e órgãos, tendo papel central na coordenação metabólica de todo o organismo.

O PPAR compreende um grupo de receptores nucleares com três isoformas, codificadas por diferentes genes: PPARα, PPARβ e PPARγ. Os PPARs são fatores de transcrição dependentes de ligantes, que regulam a expressão dos genes alvos através da ligação aos elementos de resposta do proliferador de peroxissomo (PPERs) dos genes regulados. O receptor liga-se ao PPRE como um heterodímero formado junto com o receptor retinóide X (RXR). Com a ligação de um

agonista, a conformação do PPAR é alterada e estabilizada, permitindo a ligação com o gene alvo e promovendo a transcrição do mesmo (BERGER e MOLLER, 2002).

Duas isoformas do PPARγ são expressas em nível de proteína e diferenciam-se somente pelo número de aminoácidos. O PPARγ 1 é a forma predominante em humanos e é expresso no tecido adiposo e em outros tecidos nos quais tem função importante, particularmente no intestino e células imunes (ROGUE et al., 2010). O PPARγ 2 está expresso em altos níveis no tecido adiposo (MICHALIK et al., 2006).

2.2.2 Efeitos biológicos do PPARγ

O PPARγ tem sido identificado em humanos e ratos como regulador direto da proliferação, maturação e diferenciação das células adiposas (LEHRKE e LAZAR, 2005; TONTONOZ e SPIEGELMAN, 2008).

Como principal regulador do metabolismo lipídico, uma função importante do PPARγ é permitir a liberação dos ácidos graxos das proteínas transportadoras e promover sua captação celular. Além da captação, o PPARγ promove a armazenagem lipídica no tecido adiposo, onde regula a diferenciação dos adipócitos e síntese de ácidos graxos através do controle da expressão de enzimas lipôgenicas tais como SCD1 (WAY et al., 2001; RISERUS et al., 2005), a esterificação de ácidos graxos nos triglicerídeos, pela regulação direta da glicerol quinase, e controla a expressão das proteínas da família de pirilipinas envolvidas na organização estrutural das gotículas lipídicas (GUAN et al., 2002; DALEN et al., 2004).

A ação do PPARγ se dá também sobre a expressão da proteína adipócita 2 (aP2) (TONTONOZ et al., 1994), fosfoenolpiruvato carboxiquinase (PEPCK) (TONTONOZ et al., 1995), acil-CoA-sintase (ACS) (SCHOONJANS et al., 1995), proteína transportadora de ácido graxo 1 (FATP1) (MARTIN et al., 1997) e grupo de diferenciação 36 (CD36)

(SFEIR et al., 1997), e de genes que controlam a homeostase energética celular, aumentado a expressão das proteínas desacopladoras mitocondriais 1, 2 e 3 (UCP-1, UCP-2, e UCP-3, respectivamente) (KELLY et al., 1998) e reduzindo a leptina, proteína que inibe a alimentação e aumenta o metabolismo catabólico dos lipídeos (KALLEN e LAZAR, 1996; DE VOS et al., 1996).

O PPARγ tem papel importante como regulador da sensibilidade a insulina, porém os mecanismos envolvidos nesse processo ainda não são totalmente elucidados (DESVERGNE et al., 2004; EVANS et al., 2004). Possivelmente, a sensibilidade a insulina é adquirida pela ativação do PPARγ no tecido adiposo, o qual impede o redirecionamento dos lipídeos para o músculo e fígado, onde o acúmulo de gordura causa efeitos prejudiciais (FEIGE et al., 2006).

Estudos sobre a expressão do PPARγ têm demonstrado que quando há uma severa resistência a insulina no tecido muscular, isto pode ser resultado da ausência anormal do fator de transcrição (HEVENER et al., 2003). Já mutações que evitam a fosforilação e conseqüente inativação do PPARγ aumentam sua atividade e previnem a ocorrência de obesidade ocasionada pela resistência a insulina nos tecidos (RANGWALA et al., 2003).

Uma das formas na qual o PPARγ aumenta a sensibilidade a insulina é pela transativação do transportador de glicose estimulado pela insulina (GLUT4), que promove o fluxo intracelular da glicose (BROWN e MCINTOSH, 2003). Além disso, no tecido adiposo de roedores observou-se que agonistas do PPAR inibem a expressão do fator de necrose tumoral alfa (TNFα), uma citocina pró-inflamatória que é associada à resistência a insulina (HOTAMISLIGIL et al., 1993) e que diminui a transdução do seu sinal neste tecido (HOTAMISLIGIL et al., 1994). Além das suas propriedades metabólicas, o PPARγ tem ação anti-inflamatória,

antiaterosclerótica e pode ser supressor de tumores (LEHRKE e LAZAR, 2005).

Apesar de possuir maior expressão no tecido adiposo de ruminantes, o metabolismo lipídico na glândula mamária desses animais parece ser controlado, pelo menos em parte, pelo PPARγ, uma vez que se observou aumento na sua expressão na glândula mamária de vacas, entre a prenhez e lactação (BIONAZ et al., 2013; BIONAZ e LOOR, 2008).

Essa idéia foi suportada por Kadegowda et al. (2009), os quais verificaram que a ativação do PPARγ em células mamárias bovinas com o uso de agonistas sintéticos aumentou a expressão de genes envolvidos na síntese de triglicerídeos, síntese de ácidos graxos, captação e transporte de ácidos graxos, tais como ACCα, FAS, AGPAT, DGAT1, SREBP1 E INSIG1. Da mesma forma, em células mamárias de cabras também tratadas com o agonista, observou-se ação parecida, com aumento na expressão dos genes LPL, FAS, ACCα, FABP, SREBP1 e SCD1 e nas células em que a expressão do PPARγ foi bloqueada, a atividade gênica foi reduzida em até 67% (SHI et al., 2013).

Os resultados mencionados sugerem que esses genes são alvos do PPARγ nas células mamárias de ruminantes e, dessa forma, pode representar um importante ponto de controle da síntese de gordura no leite desses animais.

2.2.3 Resposta do PPARγ a agonistas naturais e sintéticos

A análise estrutural do PPARγ mostrou que os ligantes, ao unirem-se ao receptor, modificam sua conformação e o tornam ativo (XU et al., 1999). Essa mudança de conformação remove o complexo co-repressor do heterodímero PPAR/RXR e atrai o complexo co-ativador, essencial para a interação com o processo transcricional (PÉGORIER et al., 2004).

A diversidade de funções nas quais o PPARγ está envolvido é refletida pela diversidade de ligantes que podem

ligar-se a ele. Os PPARs são ativados por uma grande quantidade de lipídeos derivados da dieta ou provenientes dos processos de sinalização intracelular, o que inclui ácidos graxos saturados e insaturados e derivados como prostaglandinas e leucotrienos (KREY et al., 1997; BERGER E MOLLER, 2002).

Ligantes naturais do PPARγ, tais como ácidos graxos poliinsaturados *cis* ou prostaglandinas, têm, relativamente, uma menor afinidade de ligação comparada aos ligantes sintéticos (KENNEDY et al., 2008). Em contraste, ácidos graxos saturados e certos ácidos graxos *trans,* como o CLA, comprometem a sensibilidade a insulina, possivelmente por reduzir a expressão do PPARγ e vários de seus genes alvos (BROWN et al., 2003; BROWN et al., 2004; KANG et al., 2003; GRANLUND et al., 2003).

Em não-ruminantes, os principais ligantes endógenos são o ácido linoleico, ácido linolênico, ácido araquidônico e seus derivados (FORMAN et al., 1996). Embora em determinados estudos *in vitro* os ácidos graxos insaturados tenham mostrado maior efeito em relação aos saturados, ambos aumentam a transativação do PPARγ (ESCHER e WAHLI, 2000; DESVERGNE e WAHLI, 1999).

Experimentos com cultivos de células epiteliais mamárias e renais de bovinos (MAC-T e MDBK, respectivamente) demonstraram que em ruminantes os ácidos graxos de cadeia longa induziram a expressão de genes comprovadamente alvos do PPARγ e os ácidos graxos saturados tiveram maior ação que os insaturados (BIONAZ et al., 2013). Isto sugere uma adaptação evolucionária do PPARγ nos ruminantes em resposta aos ácidos graxos saturados, os quais são mais abundantes na circulação destes animais, comparados aos não-ruminantes, devido a extensa bio-hidrogenação ruminal dos ácidos graxos insaturados (ZACHUT et al., 2010; OR-RASHID et al., 2009; PELTIER et al., 2008; MA et al., 1995).

Vários agonistas sintéticos são disponíveis hoje e para o PPARγ o mais comumente usado é o tiazolidinediona (TZD) (BIONAZ et al., 2013). Os TZDs foram desenvolvidos inicialmente para melhorar as ações antidiabéticas dos agentes hipolipidêmicos e incluem o troglitazone, rosiglitazone e pioglitazone, que possuem atividade antidiabética e promovem sensibilidade a insulina em humanos com diabetes tipo 2 ou com deficiência na tolerância a glicose (MOLLER e GREENE, 2001; WILLSON et al., 2000). Em animais, um dos primeiros estudos desenvolvidos com o uso do agonista TZD demonstrou que a injeção *in vivo* do agonista reverteu parcialmente a resistência a insulina induzida pelo TNFα em novilhas (KUSHIBIKI et al., 2001). Outros estudos também verificaram que o tratamento com o agonista rosiglitazone aumentou a expressão da LPL no tecido adiposo e de genes conhecidos pelo envolvimento na síntese de gordura no leite em células MAC-T (MUHLHAUSLER et al., 2009; KADEGOWDA et al., 2009).

Na ausência do TZD ou outro potencial ligante sintético, o PPARγ recruta co-repressores para seus genes alvos (LEHRKE e LAZAR, 2005). Deste modo, camundongos tiveram os genes alvos no tecido adiposo deprimidos pela redução no conteúdo do PPARγ (KUBOTA et al., 1999; MILES et al., 2000).

2.3 REGULAÇÃO DO PPARγ PELO CLA

Pelas evidências de que o PPARγ atua na expressão de genes envolvidos na síntese lipídica de diversos tecidos, entre eles glândula mamária, e que tem a capacidade de ligar-se e tornar-se ativo por ácidos graxos, incluindo o CLA, a administração deste poderia mudar a expressão do receptor na glândula mamária. Contudo, ao contrário do que se tem observado em não-ruminantes, em que o CLA é um ativador do PPARγ, em ruminantes este parece não ser ativado pelo CLA,

especialmente nas células epiteliais mamárias (KADEGOWDA et al. 2009).

Existem algumas divergências em relação ao papel do PPARγ na regulação da síntese de gordura no leite e sua associação aos mecanismos do CLA sobre a depressão da gordura do leite (BAUMAN et al., 2008). No entanto, em tecidos extramamários onde a família dos fatores de transcrição do PPAR são altamente expressos e são reguladores chave da diferenciação de tecidos específicos, eles podem ser importantes nas respostas funcionais provocadas pelo CLA *trans*-10, *cis*-12.

Como mencionado anteriormente, o CLA *trans*-10, *cis*-12 pode ter efeito direto sobre a expressão do PPARγ e seus genes alvos. Pelas evidências de que CLA *trans*-10, *cis*-12 previne o acúmulo de gordura em pré-adipócitos humanos e que induz a resistência a insulina (BROWN et al., 2001; BROWN et al., 2003), tem-se sugerido que esses efeitos são exercidos pela depressão na expressão ou atividade do PPARγ. Para testar esta hipótese, Brown e McIntosh (2003) avaliaram os efeitos dos dois principais isômeros sobre o PPARγ de adipócitos humanos e verificaram que o CLA *trans*-10, *cis*-12 foi responsável pela redução na expressão do PPARγ 1 e 2 e dos genes alvos (aP2, LPL, GLUT4), enquanto que o CLA *cis*-9, *trans*-11 aumentou a expressão do fator de transcrição e dos genes alvos.

Estudos desenvolvidos por Liu et al. (2007) e Purushotham et al. (2007) mostraram que o agonista rosiglitazone atenuou a resistência a insulina em camundongos alimentados com uma mistura de isômeros do CLA (*cis*-9, *trans*-11 e *trans*-10, *cis*-12), porém, posteriormente Kennedy et al. (2008) verificaram que a suplementação conjunta do rosiglitazone e CLA *trans*-10, *cis*-12 em cultivos de células adipócitas não preveniu a supressão do PPARγ pelo CLA e o agonista não foi capaz de superar sua ação anti-adipogênica,

evidenciando o antagonismo entre o isômero *trans*-10, *cis*-12 e o PPARγ.

Uma possível forma pelo qual o CLA *trans*-10, *cis*-12 afeta diretamente o PPARγ seria pela competição com ligantes endógenos ou diminuição da síntese destes ligantes (BROWN e MCINTOSH, 2003). Kennedy et al. (2008) propuseram ainda que o CLA *trans*-10, *cis*-12 pode suprimir a atividade do PPARγ pela sua fosforilação via quinase reguladora do sinal extracelular 1/2 (ERK1/2), a qual reduz a afinidade aos ligantes e/ou recrutamento de cofatores, inibição da heterodimerização com o RXR e alteração na ligação do PPRE aos genes alvos, porém, a ação do CLA como ligante do PPARγ ainda não é totalmente esclarecida (HERRMANN et al., 2009).

3 ARTIGO

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) AGONIST THIAZOLIDINEDIONE (TZD) DOES NOT STIMULATE LIPOGENESIS AND LIPOGENIC GENE EXPRESSION AND FAIL TO OVERCOME *TRANS*-10, *CIS*-12 CONJUGATED LINOLEIC ACID (CLA) INHIBITION IN LACTATING EWES

ABSTRACT

The *trans*-10, *cis*-12 CLA is known to promote depression in milk fat and its mechanism of action is by regulating the expression of genes and transcription factors involved in lipid synthesis. The PPARγ is one of the transcription factors responsible for the processes of adipogenesis and lipogenesis and is activated by specific natural or synthetic ligands such as TZD. In this study, we evaluated the effect of PPARγ in lipid synthesis in lactating ewes through a specific chemical agonist and its response to supplementation of *trans*-10, *cis*-12 CLA. Twenty four lactating ewes with 70 ± 3 DIM and BW 60 ± 0.45 kg were randomly assigned one of the four treatments for 7 days: 1) Control - 100 mL/day of sterile saline solution, intravenous; 2) TZD (4mg/kg of BW/day in 100 mL of sterile saline solution, intravenous); 3) CLA (27g/d orally-dosed of rumen-unprotected with 29.9% of *trans*-10, *cis*-12 CLA); 4) TZD+CLA. Milk fat content was 22.3% lower in CLA (P=0.05), tended to be 20.7% lower in TZD+CLA (P=0.06) and the TZD did not affect the fat content (P=0.39). The lactose content, milk yield and production of components were not affected by treatments. The protein content was lower in the CLA compared to TZD (P=0.01) and tended to be higher with the TZD compared to control (P=0.08). In the mammary gland, CLA decreased expression of PPARγ, SREBP1 and

SCD1, and the TZD did not stimulated the expression of these genes. In adipose tissue, the expression of PPARγ were not affected, whereas SREBP1 had more expression in TZD, CLA and TZD+CLA treatments and SCD1 had higher expression in TZD+CLA, compared to the other treatments. In conclusion, CLA negatively affected the expression of genes involved in lipid synthesis and the TZD was unable to increase gene expression and lipogenesis in mammary gland.

Keywords: Gene expression. Milk fat depression. Peroxisome proliferator-activated receptor gamma.

3.1 INTRODUCTION

The conjugated linoleic acid (CLA) comprises a mixture of octadecadienoic acid isomers, found in meat, milk and dairy products from ruminants being *cis*-9, *trans*-11 and *trans*-10, *cis*-12 the most studied isomers. The CLA acts on several biological processes and the *trans*-10, *cis*-12 isomer particularly, is able to inhibit milk fat synthesis. Feeding CLA supplements has been shown to reduce milk fat synthesis in lactating cows (BAUMGARD et al., 2002), mice (LOOR et al., 2003), pigs (BONTEMPO et al., 2004; POULOS et al., 2004), ewes (OLIVEIRA et al., 2012), goats (BALDIN et al., 2013; FERNANDES et al., 2014) and humans (MASTERS et al., 2002).

Baumgard et al. (2000) first showed that *trans*-10, *cis*-12 CLA is the isomer responsible for inhibits milk fat synthesis in dairy cows. Later, Baumgard et al. (2002) described that the mechanism involves, at least in part, a down-regulation of gene expression codifying enzymes involved in the milk fat synthesis.

The PPARγ is activated by natural (e.g. fatty acids and eicosanoids) or synthetic ligands (e.g. TZD) that initiate heterodimerization with retinoid X receptor (RXR) followed by

their binding to response element in the target genes (KENNEDY et al., 2008). Specific *trans* polyunsaturated fatty acids such as *trans*-10, *cis*-12 CLA appear to reduce PPARγ expression in ruminants (KADEGOWDA et al., 2009). In the other way, the TZD activates the PPARγ and promotes upregulation of lipogenic genes.

There are several studies using specific agonists in ruminants, most of them performed with cattle and fewer studies with ewes and goats (BIONAZ et al., 2013). These studies also suggest that PPARγ expression can be manipulated by the use of these synthetic agonists both *in vivo* and *in vitro* research.

Our central hypothesis is that there may be a change in the expression of PPARγ in the mammary gland of lactating ewes during the administration of agonist and it can increases milk fat synthesis and inhibit the anti-lipogenic effects of *trans*-10, *cis*-12 CLA, through specific chemical agonist TZD and its response to *trans*-10, *cis*-12 CLA.

3.2 MATERIAL AND METHODS

3.2.1 Animals, design and treatments

All procedures were approved by the Santa Catarina State University Ethical Committee, protocol nº 01.38.14 and performed at Pinheiro Seco farm, Bom Retiro, SC (27º47'57.11"S and 49º29'14.65"W). Twenty-four crossbred Lacaune/East Friesan lactating ewes with 70 ± 3 days in milk (DIM) and body weight (BW) of 60 ± 0.45 kg were randomly assigned to one of the following treatments: 1) Control (100mL/day of sterile saline solution, intravenously); 2) TZD (4mg/kg of BW/day in 100 mL of sterile saline solution, intravenously); 3) CLA (27g/d rumen-unprotected with 29.9% of *trans*-10, *cis*-12 CLA and 29.8% of *cis*-9, *trans*-11 CLA, orally-dosed); 4) TZD+CLA. The amounts of TZD and CLA

were based in the papers of Smith et al. (2007) and Oliveira et al. (2012), respectively. In treatment 4, the infusion of TZD started one day before CLA dosing in an attempt to allow TZD to stimulate PPARγ gene expression before the effects of CLA starts.

3.2.2 Management and feeding

All animals grazed paddocks of festuca (*Festuca arundinacea* Schreb.) and white clover (*Trifolium repens* L.) with free access to water during the day and were housed at night in collective pens where they received, in a dry matter basis, 1 kg/d of corn silage plus 0.9 kg/d of a concentrate mixture containing soybean meal (39%), ground corn (56%) and a commercial vitamin/mineral mix (5%). Also, they had free access to water and a mineral salt. The corn silage and concentrate were expected to complement to meet or exceed the needed nutrients excepting those provided by pasture according the Nutrient Requirements Council (NRC, 2007). Ewes were milked twice a day at 06:00h and 14:30h and all treatments were provided before the afternoon milking.

3.2.3 Experimental period, sampling and analyses

The experimental period lasted 7 days and on the last day, individual milk samples from the a.m. and p.m. milkings were proportionally collected and stored at 4°C with a preservative (bromopol tablet; D & F Control Systems Inc., San Ramon, CA, USA). Milk fat, protein, lactose, and total solids were determined by infrared analysis (AOAC, 2000; method 972.160) and somatic cell count by flow cytometry. Milk yield was measured on d 0 and 7 of experimental period.

3.2.4 Mammary and adipose tissue biopsies

Mammary biopsies were taken between 1 to 4 h after the a.m. milking on d 7 of experimental period. Lidocaine hydrochloride subdermal block (2 mL/ewe) was administered above the incision site. A 0.5 cm incision was made in the skin at the midpoint of the rear quarter where a coaxial needle with a trocar was introduced. 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 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 1mL of Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories, Grand Island, NY, USA) and stored in liquid nitrogen until RNA extraction. Immediately after removal of the biopsy needle, a purse string suture was placed around the incision with number 1 Nylon. Animals were observed for two days post-biopsy and milked by hand to remove blood cloths. The biopsy procedure resulted in minimal bleeding and milk appeared normal in 2 to 4 milkings following the biopsy. 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 block was administered in a circular pattern surrounding the incision site (2 mL/ewe). Once the block was effective, an 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 irrigated and closed with number 1 Nylon using a blanket

stitch. After biopsies of adipose and mammary tissues, flunixin meglumine (1.1 mg/kg of BW) was administered.

3.2.5 RNA extraction, synthesis of complementary DNA (cDNA) and quantitative real time PCR (qRT-PCR)

RNA extraction, synthesis of complementary DNA (cDNA) and quantitative real time PCR (qRT-PCR) were done at Santa Catarina State University biochemistry laboratory.

Total mRNA was extracted from both mammary and adipose tissues samples using the RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, Germantown, MD, USA) with on-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). Agarose electrophoresis was used to determine RNA integrity. Total RNA was transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with random primers.

PCR amplification was performed in triplicates in a 48 wells reaction plate (MicroAmp™, Applied Biosystems, Waltham, MA, USA) with 15µL volume reaction, 30 ng of cDNA and SYBR Green Select Master Mix (Applied BioSystems, Foster City, CA, USA) in a StepOne Real-Time machine (Applied BioSystems, Foster City, CA, USA). The level of expression of ribosomal protein S18 (RPS18) gene was used to normalize the amount of message in all samples. 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. Message level of the sample was determined, in relation to a dilution curve of pooled cDNA from mammary or adipose tissue.

3.2.6 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 and transcription factors was measured: PPARγ, SREBP1 and SCD1. The primer sequences of measured genes are listed in Table 1.

Table 1 - Ovine primers used in real-time PCR analysis

Gene	Forward primer[1]	Reverse primer
SREBP1	CCAGCTGACAGCTCCATTGA	TGCGCGCCACAAGGA
PPARγ	CCAAGAATATCCCCGGCTTT	AGGCCAGCATCGTGTAAATGA
SCD1	CCGCCCTGAAATGAGAGATG	CATGAGGATGATGTTTCTCCAAAC
S18	GCCTTTGCCATCACTGCAAT	TGAGCTCTCCTGCCCTCTTG

Source: author production
[1] Primers are reported as 5' to 3' sequence.

3.2.7 Statistical analysis

The experimental design was completely randomized. Gene expression data were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA, version 9.2, 2009) and the means compared by LSMEANS at 5% significance level. The "housekeeping" gene 18S (18S ribosomal subunit) was used as a covariate in the model. Data outside the range of -2.0 to +2.0 of the Studentized Residual were considered "outliers" and excluded from the analysis.

Milk yield and concentration and yield of milk components were analyzed by the MIXED procedure, using the animal as a random effect and the production of the day "zero" as a covariate. Means were compared using the LSMEANS procedure at 5% significance level. A trend was considered when $0.05 < P < 0.10$.

3.3 RESULTS

3.3.1 Milk composition

Milk production and milk components are presented in Table 2. There was no effect of treatment on milk yield and the yield of the components and lactose content. Milk protein content was 17.5% lower in CLA compared to TZD (P=0.01) and tended to be 11.8% higher in TZD (P=0.08) compared to Control.

Total solids content was 10.3% (P=0.04) and 15% (P=0.004) lower in CLA compared to Control and TZD, respectively. Compared to Control, milk fat concentration decreased 22.3% in the CLA treatment and tended to be 20.5% lower in the TZD+CLA treatment (Table 2).

Table 2 - Treatment effects on milk yield and composition of lactating ewes

	Treatments[1]					
Variable	**Control**	**TZD**	**CLA**	**TZD+CLA**	**SEM[2]**	**P-Value[3]**
Milk yield (kg)	0.63	0.49	0.57	0.44	0.069	0.21
Fat (%)	6.14^{ab}	6.70^{a}	4.77^{c}	4.88^{bc}	0.45	0.02
Fat (kg)	0.038	0.034	0.035	0.033	0.005	0.93
Protein (%)	5.09^{ab}	5.70^{a}	4.70^{b}	5.35^{ab}	0.23	0.05
Protein (kg)	0.032	0.031	0.033	0.035	0.005	0.95
Lactose (%)	4.71	4.48	4.73	4.61	0.12	0.53
Lactose (kg)	0.033	0.025	0.034	0.031	0.007	0.81
Total solids (%)	16.96^{ab}	17.9^{a}	15.21^{c}	15.99^{bc}	0.56	0.02
Total solids (kg)	0.11	0.10	0.11	0.11	0.017	0.93

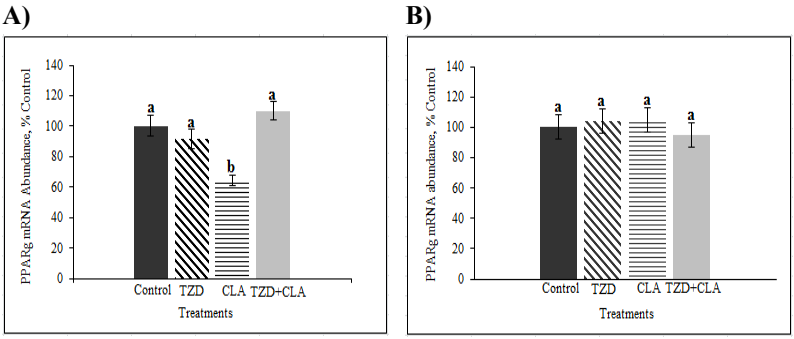
Source: author production.
[1] Control - 100mL/day of sterile saline solution; TZD - 4mg/kg of BW/day in 100 mL of sterile saline solution; CLA - 27g/d rumen-unprotected (29.9% of *trans*-10, *cis*-12 and 29.8% of *cis*-*9*, *trans*-11);
[2] Standard Error Mean.
[3] $P<0.05$.

3.3.2 Expression of lipogenic enzymes in mammary gland and adipose tissue

Only one data from each treatment were excluded from statistical analysis as outliers. In the mammary gland, CLA decreased the PPARγ gene expression by 35.6% (P=0.02) when compared to Control, by 41.4% (P=0.004) when compared to TZD+CLA and tended to be 29.5% (P=0.06) lower when compared to TZD. In contrast, compared to Control, TZD did not stimulate PPARγ gene expression

(P=0.59, Figure 1A). In adipose tissue, the treatments did not affect the expression of PPARγ (P=0.85, Figure 1B).

Figure 1 - PPARγ gene expression in the mammary gland (A) and adipose tissue (B) of ewes supplemented with TZD[1], CLA[2] and TZD+CLA, compared to the Control[3]



Source: author production

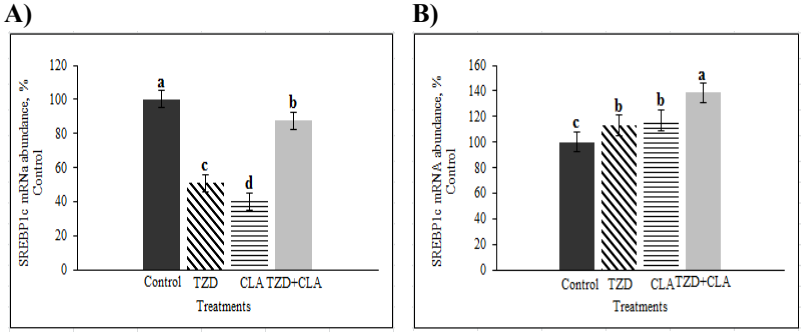
[1] TZD - 4mg/kg of BW/day in 100 mL of sterile saline solution.

[2] CLA - 27g/d rumen-unprotected (29.9% of *trans*-10, *cis*-12 and 29.8% of *cis*-9, *trans*-11);

[3] Control - 100mL/day of sterile saline solution.

In the mammary gland, CLA reduced SREBP1 gene expression by 60%, 21.2% and 54.3% compared to Control (P=0.0001), TZD (P=0.01) and TZD+CLA (P=0.0001), respectively. Similarly, TZD decreased by 49.2% SREBP1 gene expression compared to Control (P=0.0001, Figure 2A). In adipose tissue, CLA increased the expression of SREBP1 by 17.1% compared to Control (P=0.007), it was not different when compared to TZD (P=0.47, Figure 2B) and TZD+CLA increased the expression by 38.5%.

Figure 2 - SREBP1 gene expression in the mammary gland (A) and adipose tissue (B) of ewes supplemented with TZD[1], CLA[2] and TZD+CLA, compared to the Control[3]



Source: author production

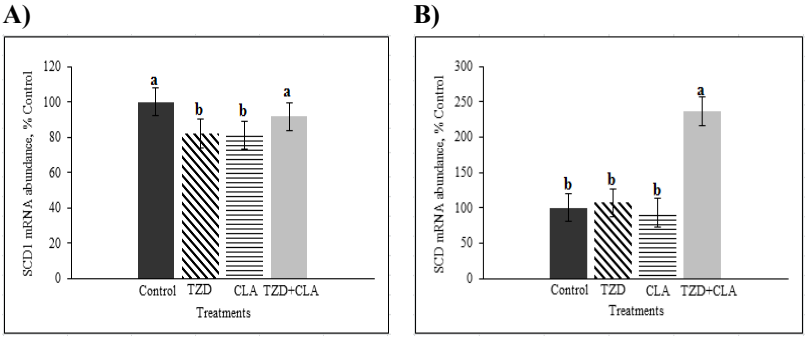
[1] TZD - 4mg/kg of BW/day in 100 mL of sterile saline solution.

[2] CLA - 27g/d rumen-unprotected (29.9% of *trans*-10, *cis*-12 and 29.8% of *cis*-9, *trans*-11);

[3] Control - 100mL/day of sterile saline solution.

The SCD1 expression in the mammary gland, compared to Control, was lower in animals supplemented with CLA and TZD (P=0.0005 and P=0.001, respectively), whereas the expression with TZD+CLA tended to be lower (P=0.06, Figure 3A). There was no difference between CLA and TZD treatments (P=0.82). In adipose tissue (Figure 3B), SCD1 had higher expression in TZD+CLA when compared to the other treatments (increase of 136.3% in relation to Control).

Figure 3 – SCD1 gene expression in the mammary gland (A) and adipose tissue (B) of ewes supplemented with TZD[1], CLA[2] and TZD+CLA, compared to the Control[3]



Source: author production

[1] TZD - 4mg/kg of BW/day in 100 mL of sterile saline solution.

[2] CLA - 27g/d rumen-unprotected (29.9% of *trans*-10, *cis*-12 and 29.8% of *cis*-9, *trans*-11);

[3] Control - 100mL/day of sterile saline solution.

3.4 DISCUSSION

As demonstrated by previous studies, the effects of CLA on milk fat synthesis were also observed in this study. Others research groups have shown that in lactating cows, the abomasal infusion of a mixture of CLA isomers or purified *trans*-10, *cis*-12 CLA, consistently reduces the concentration and yield of milk fat (CHOUINARD et al., 1999; LOOR and HERBEIN, 1998). In our study ewes showed less pronounced milk fat depression (MFD) when compared to CLA studies in dairy cows. In part, this can be explained by the fact that different from the cow, MFD does not commonly occur in goats and ewes (SHINGFIELD et al., 2010). Comparison of the changes in milk fat concentration and secretion with similar diets and/or supplementation with *trans*-10, *cis*-12 CLA, suggests that in small ruminants the ruminal biohydrogenation pathways are more stable to diet-induced changes. These

differences may relate to feeding behavior, ruminating, buffering, kinetics of digestion and passage rate (CHILLIARD et al., 2003; PULINA et al., 2006; BERNARD et al., 2009; FERNANDES et al., 2014), which ultimately results in less exposure of the mammary gland to *trans* fatty acids that inhibit milk fat synthesis.

This study found no change in milk production or synthesis of other milk components, which is a common phenotype during CLA-induced MFD. However, reduction of milk fat by *trans*-10, *cis*-12 CLA may increase milk production and/or milk protein during early lactation as reported by Medeiros et al. (2010) in grazing dairy cows or in underfeeding situations (LOCK et al., 2006).

In contrast, the treatment with TZD tended to increase the protein content. 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. A role for insulin in milk protein synthesis was suggested to be through the control of gene expression of milk proteins and regulation of translation via the mammalian target of rapamycin (mTOR) pathway (BIONAZ et al., 2012). Given the importance of insulin on milk protein synthesis, the TZD, which increases insulin sensitivity by binding to PPARγ, may stimulate insulin activity in mammary cells and consequently increase protein synthesis via mTOR. Future studies may be conducted in order to further elucidate these regulatory mechanisms of synthesis and allow possible interventions to increase the milk protein content.

Milk fat synthesis involves several biochemical pathways that include fatty acid uptake and transport, *de novo* fatty acid synthesis, desaturation and esterification. Noteworthy, *trans*-10, *cis*-12 CLA is capable of causing changes in the expression of genes encoding enzymes involved in most of the pathways listed above (SHINGFIELD et al., 2010). On the other hand, the expression of lipogenic enzymes

is stimulated by a class of transcription factors that are the primary regulators of lipid synthesis. One of those is the PPARγ, investigated in this study.

PPARγ expression is normally high in adipose tissue and low in the mammary gland (BIONAZ et al., 2013). This possibly explains why the agonist TZD did not stimulate the activity of PPARγ in adipose tissue in this study. As a transcription factor-dependent ligand, many polyunsaturated fatty acids (e.g. CLA) are natural PPARγ ligands that induce changes in gene expression and lipogenic rates in non-ruminants (BENSINGER and TONTONOZ, 2008; BERGER and MOLLER, 2002). In ruminants, however, PPARγ seems to respond differently to CLA isomers, especially in mammary epithelial cells.

Kadegowda et al. (2009) used a MAC-T cell line expressing low PPARγ (BIONAZ et al., 2013) and observed an activation of PPARγ by agonist rosiglitazone with parallel increase in the expression of ACCα, FAS, AGPAT, DGAT1, INSIG1 and SREBP1. This suggested that those genes may be a PPARγ target in bovine mammary cells. Contrarily, treatment with *trans*-10, *cis*-12 CLA inhibited activation of PPARγ target genes (KADEGOWDA et al., 2009). Overall, we observed agreeing results in this study and additionally, demonstrated *in vivo* that the agonist TZD was unable to stimulate lipogenesis and increasing gene expression in the mammary gland (i.e. occurrence of MFD in the TZD+CLA treatment and reduction in the expression of PPARγ, SREBP1 and SCD1 in the TZD treatment).

In addition to PPARγ, SREBP1 is another family of transcription factors regulating lipogenic enzymes involved in milk fat synthesis in the mammary gland. More specifically, SREBP1c regulates enzymes involved in fat synthesis and it is the predominant transcript expressed in the mammary tissue, especially in early lactation. However, because of limited amount of available sequence, qRT-PCR does not distinguish

the isoforms 1a and 1c and thus, results usually refer to them collectively as SREBP1 (HARVATINE and BAUMAN, 2006; RUDOLPH et al., 2007). Supporting the results of this study, others have observed a reduction in the expression of SREBP1 in bovine mammary epithelial cells treated with *trans*-10, *cis*-12 CLA (KADEGOWDA et al., 2013; KADEGOWDA et al., 2009; PETERSON et al., 2004), or in cows under diet-induced MFD and/or supplemented with *trans*-10, *cis*-12 CLA (HARVATINE and BAUMAN, 2006). Furthermore, Hussein et al. (2013) showed a 30% reduction in the SREBP1 expression in ewes treated with CLA. The effect of the *trans*-10, *cis*-12 CLA on SREBP1 is believed to be indirect, and unlike other transcription factors such as PPARγ, fatty acids and cholesterol do not bind to SREBP1, but instead induce changes on the expression of this transcription factor (PÉGORIER et al., 2004). A possible mechanism in which *trans*-10, *cis*-12 CLA reduces the transcription of SREBP1 is through competitive binding with other transcription factors that positively regulate SREBP1.

Kadegowda et al. (2009) observed that SREBP1 increased expression when MAC-T cells were treated with the agonist rosiglitazone. Our results however, do not support upregulation of SREBP1 *in vivo* by the agonist TZD, suggesting that it may be targeted PPARγ, once the expression of PPARγ also was not stimulated by the agonist.

One of the effects of CLA that has been consistently observed is its ability to alter the fatty acid composition of tissues by reducing the levels of monounsaturated fatty acids, which are synthesized by the enzyme SCD1 (LEE et al., 1995). Bionaz and Loor (2008) evaluated mRNA expression of genes associated with lipid synthesis in the mammary tissue during lactation cycle and SCD1 mRNA abundance was the highest among all genes measured. Kinsella (1972) suggested that in growing ruminants SCD1 is more expressed in adipose tissue, whereas during lactation SCD1 is highly expressed in the

mammary gland, where it plays a crucial role in the provision of monounsaturated for triglycerides synthesis.

Relative to SCD1 gene expression, treatment with *trans*-10, *cis*-12 CLA reduced SCD1 expression in MAC-T cells. Contrarily, the PPARγ agonist increased SCD1 expression, confirming that SCD1 is a PPARγ target gene (KADEGOWDA et al., 2009). Despite the demonstrated relationship between PPARγ and SCD1, in our study the agonist repressed expression of SCD1 in the mammary gland, which is in accordance with what was showed before by Kurebayashi et al. (1997) and Kim et al. (2000) in adipose cells, whereas in adipose tissue the TZD+CLA treatment caused a overexpression of it.

The class of TZDs comprises three major forms - troglitazone, rosiglitazone, and pioglitazone - and all can activate PPARγ. However, unlike rosiglitazone and pioglitazone, troglitazone represses both SCD1 gene and protein expression. It is still unclear how the three TZDs are able to exert different effects on SCD1 expression, but it may be due to differences in conformation of PPARγ isoforms, potency of the ligand and differences in conformational arrangement of the different ligands (PATON and NTAMBI, 2009). These contradictory findings may be also due to differences in the application of TZD, cell types, tissues and animal models (KAHN et al., 2000; LI and LAZAR, 2002).

Moreover, many of the differences in our results compared to other studies might be due to changes in the forms of action of the agonist TZD and CLA on cell culture under controlled conditions and cells *in vivo*, like the conditions used in this study. In addition to the *in vivo* effects, it is noteworthy that there are no studies with agonists in ewes and due to the physiologic aspects of these animals, the lipogenic mechanisms and response to TZD may be different.

Another feature to consider is the time for metabolism and clearance of TZD. Arévalo-Turrubiarte et al. (2012)

measured the concentration of the agonist in blood, liver and muscle of cattle and found no evidence for the presence of TZD and its metabolites in blood and muscle. Furthermore, TZD has 3-4 hours elimination period (HAUSMAN et al., 2009), which explains its absence in blood samples collected later than 3-4h after administration. In our study, this could partially explain the absent effects of TZD on the evaluated genes as we collected biopsy samples in a period greater than 12 hours after the last administration of TZD. Thus, further studies are needed to better characterize the metabolism and effects of TZD agonist.

Opposite to the mammary gland, CLA had no effect on PPARγ expression in adipose tissue. However, we observed greater expression of SREBP1 and SDC1 in adipose tissue in TZD, CLA and TZD+CLA treatments. Brown et al. (2003) evaluated effects of the main CLA isomers on human adipocytes and found that *trans*-10, *cis*-12 CLA reduced insulin-stimulated glucose uptake and the PPARγ expression. On the contrary, *cis*-9, *trans*-11 CLA stimulated the expression of PPARγ and several other target genes, suggesting that the *trans*-10, *cis*-12 CLA is indeed anti-adipogenic and *cis*-9, *trans*-11 CLA promotes adipogenesis. Choi et al. (2000) examined the effects of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA on gene expression and fat composition of mouse preadipocytes (3T3-L1). The results showed that only treatment with *trans*-10, *cis*-12 CLA reduced the expression of the SCD1, and other genes, such as SCD2, FAS and PPARγ, were not significantly affected. In some conditions, CLA may mimic the effects of TZD via activation of PPARγ (PARK et al., 1999), particularly when a mixture of isomers is used due to the fact that *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA act differently on lipid metabolism.

Also with respect to adipose tissue, the increased lipid synthesis in adipose tissue during MFD may be an indirect response due to the reduction in energy otherwise used for milk

fat synthesis. Harvatine et al. (2009) confirmed this when observed that in cows abomasally infused *trans*-10, *cis*-12 CLA, the expression of enzymes involved in lipid synthesis (FAS, SCD1 and FABP1) and regulatory elements (SREBP1, SPOT14 and PPARγ) increased in this tissue whereas fat synthesis decreased in the mammary gland.

3.5 CONCLUSION

The TZD agonist effects have not been previously reported *in vivo* with lactating ewes and our study showed that it did not stimulate milk fat synthesis and was incapable to overcome the anti-lipogenic effects of CLA in lactating dairy ewes. The PPARγ, SREBP1 and SCD1 expression in mammary gland was reduced by CLA, confirming its negative effects on the expression of lipogenic genes, and TZD did not stimulate the expression of these genes. No change in the expression of PPARγ was observed in the adipose tissue, whereas SREBP1 and SCD1 were increased in this tissue with TZD, CLA and TZD+CLA treatments.

3.6 REFERENCES

AOAC. Official Methods of Analysis, Association of Official Analytical Chemists, Arlington, VA, USA. 2000.

ARÉVALO-TURRUBIARTE, M. et al. Effect of 2,4-Thiazolidinedione on Limousin cattle growth and on muscle and adipose tissue metabolism, **PPAR Research,** 2012.

BALDIN, M. et al. A rumen unprotected conjugated linoleic acid (CLA) supplement inhibits milk fat synthesis and improves energy balance in lactating goats, **Journal of Animal Science**, v. 91, p. 3305-3314, 2013.

BAUMGARD, L. H. et al. Identification of the conjugated linoleic acid isomer that inhibits fat synthesis, **American Journal Physiology Regulatory Integrative Comparative Physiology**, v. 278, p. 179-184, 2000.

BAUMGARD, L. H. et al. *Trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows, **Journal of Dairy Science**, v. 85, p. 2155-2163, 2002.

BENSINGER, S. J.; TONTONOZ, P. Integration of metabolism and inflammation by lipid-activated nuclear receptors, **Nature,** v. 454, p. 470–477, 2008.

BERGER, J.; MOLLER, D. E. The mechanisms of action of PPARs, **Annual Review of Medicine**, v. 53, p. 409–435, 2002.

BERNARD, L. et al. Effect of plant oils in the diet on performance and milk fatty acid composition in goats fed diets based on grass hay or maize silage, **The British Journal of Nutrition**, v. 101, p. 213–224, 2009.

BIONAZ, M.; LOOR, J. J. Gene networks driving bovine milk fat synthesis during the lactation cycle, **BMC Genomics,** v. 9, p. 366, 2008.

BIONAZ, M. et al. Milk protein synthesis in the lactating mammary gland: insights from transcriptomics analyses, **Intech Open Science,** 2012.

BIONAZ, M. et al. Functional role of PPARs in ruminants: potential targets for fine-tuning metabolism during growth and lactation, **PPAR Research,** 2013.

BONTEMPO, V. et al. Dietary conjugated linoleic acid positively affects immunologic variables in lactating sows and piglets, **The Journal of Nutrition**, v. 134, p. 817-824, 2004.

BROWN, J. M. et al. Isomer-specific regulation of metabolism and PPARγ signaling by CLA in human preadipocytes, **Journal of Lipid Research,** v. 44, p. 1287-1300, 2003.

CHILLIARD, Y. et al. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis, **Journal of Dairy Science**, v. 86, p. 1751–1770, 2003.

CHOI, Y. et al. The *trans*-10, *cis*-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes, **The Journal of Nutrition,** v. 130, p. 1920-1924, 2000.

CHOUINARD, P. Y. et al. Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows, **The Journal of Nutrition**, v. 129, p.1579–1584, 1999.

FERNANDES, D. et al. Milk fat depression and energy balance in stall-fed dairy goats supplemented with increasing doses of conjugated linoleic acid methyl esters, **Animal**, v. 8, p. 587-595, 2014.

HARVATINE, K. J.; BAUMAN, D. E. SREBP1 and Thyroid Hormone Responsive Spot14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA, **The Journal of Nutrition,** v. 136, p. 2468-2474, 2006.

HARVATINE, K. J. et al. Expression of enzymes and key regulators of lipid synthesis is upregulated in adipose tissue

during CLA-induced milk fat depression in dairy cows, **The Journal of Nutrition,** v. 139, p. 849-854, 2009.

HAUSMAN, G. J. et al. The biology and regulation of preadipocytes and adipocytes in meat animals, **Journal of Animal Science**, v. 87, p. 1218–1246, 2009.

HUSSEIN, M. et al. Conjugated linoleic acid-induced milk fat depression in lactating ewes is accompanied by reduced expression of mammary genes involved in lipid synthesis, **Journal of Dairy Science**, v. 96, p. 3825–3834, 2013.

KADEGOWDA, A. K. G. et al. Peroxisome proliferator-activated receptor-γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents, **Journal of Dairy Science**, v. 92, n. 9, p. 4276–4289, 2009.

KADEGOWDA, A. K. G. et al. *Trans*-10, *cis*-12 Conjugated linoleic acid-induced milk fat depression is associated with ihibition of PPARγ signaling and inflammation in murine mammary tissue, **Journal of Lipids**, 2013.

KAHN, C. R. et al. Unraveling the mechanism of action of thiazolidinediones, **The Journal of Clinical Investigation**,v. 106, p. 1305-1307, 2000.

KENNEDY, A. et al. *Trans*-10, *cis*-12 Conjugated linoleic acid antagonizes ligand-dependent PPARγ activity in primary cultures of human adipocytes, **The Journal of Nutrition**, v. 138, p. 455-461, 2008.

KIM, Y. et al. Differential regulation of the stearoyl-CoA desaturase genes by thiazolidinediones in 3T3-L1 adipocytes, **Journal of Lipid Research,** v. 41, p. 1310-1316, 2000.

KINSELLA, J. E. Stearyl CoA as a precursor of oleic acid and glycerolipids in mammary microsomes from lactating bovine: possible regulatory step in milk triglyceride synthesis, **Lipids,** v. 7, p. 349-355, 1972.

KUREBAYASHI, S. et al. Thiazolidinediones downregulate stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes, **Diabetes,** v. 46, p. 2115-2118, 1997.

LEE, K. N. et al. Dietary conjugated linoleic acid changes fatty acid composition in different tissues by decreasing monounsaturated fatty acids, **IFT Annual Meeting**: Book of Abstracts, p. 183, 1995.

LI, Y.; LAZAR, M. A. Differential gene regulation by PPARgamma agonist and constitutively active PPARgamma2, **Molecular Endocrinology,** v. 16, p. 1040-1048, 2002.

LOCK, A. L. et al. A conjugated linoleic acid supplement containings *trans*-10, *cis*-12 reduces milk fat synthesis in lactating sheep, **Journal of Dairy Science,** v. 89, p. 1525-1532, 2006.

LOOR, J. J. et al. Effects of dietary *cis*-9, *trans*-11–18:2, *trans*-10, *cis*-12–18:2, or vaccenic acid (*trans*-11–18:1) during lactation on body composition, tissue fatty acid profiles, and litter growth in mice, **British Journal of Nutrition**, v. 90, p. 1039-1048, 2003.

LOOR, J. J.; HERBEIN, J. H. Exogenous conjugated linoleic acid isomers reduce bovine milk fat concentration and yield by inhibiting de novo fatty acid synthesis, **The Journal of Nutrition**, v. 128, p. 2411–2419,1998.

MASTERS, N. et al. Maternal supplementation with CLA decreases milk fat in humans, **Lipids**, v. 37, p. 133-138, 2002.

MEDEIROS, S. R. et al. Effects of dietary supplementation of rúmen-protected conjugated linoleic acid to grazing cows in early lactation, **Journal of Dairy Science**, v. 93, p. 1126-1137, 2010.

NRC. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids, Washington, DC, 2007.

OLIVEIRA, D. E. et al. An unprotected conjugated linoleic acid supplement decreases milk production and secretion of milk components in grazing dairy ewes, **Journal of Dairy Science**, v. 95, p. 1437-1446, 2012.

PARK, Y. et al. Changes in body composition in mice during feeding and withdrawal of dietary conjugated linoleic acid, **Lipids,** v. 34, p. 243–248, 1999.

PATON, C. M.; NTAMBI, J. M. Biochemical and physiological function of stearoyl-CoA desaturase, **American Journal Physiology Endocrinology Metabolism,** v. 297, p. 28-37, 2009.

PÉGORIER, J. P. et al. Control of gene expression by fatty acids, **The Journal of Nutrition**, v. 134, p. 2444–2449, 2004.

PETERSON, D. G. et al. The inhibitory effect of *trans*-10, *cis*-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1, **The Journal of Nutrition**, v. 134, p. 2523–2527, 2004.

POULOS, S. P. et al. Conjugated linoleic acid during gestation and lactation does not alter sow performance or body weight gain and adiposity in progeny, **Animal Research**, v. 53, p. 275-288, 2004.

PULINA, G. et al. Effects of nutrition on the contents of fat, protein, somatic cells, aromatic compounds, and undesirable substances in sheep milk, **Animal Feed Science and Technology**, v. 131, p. 255–291, 2006.

RUDOLPH, M. C. et al. Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine, **Physiological Genomics**, v. 28, p. 323–336, 2007.

SAS Institute Inc. SAS/STAT: User's guide. Version 9.2.ed. Cary, NC, 2009. 240p.

SHINGFIELD, K. J. et al. Role of *trans* fatty acids in the nutritional regulation of mammary lipogenesis in ruminants, **Animal,** v. 4, p. 1140-1166, 2010.

SMITH, K. L. et al. Prepartum 2,4-thiazolidinedione alters metabolic dynamics and dry matter intake of dairy cows, **Journal of Dairy Science**, v. 90, p. 3660–3670, 2007.

4 REFERÊNCIAS DISSERTAÇÃO

BALDIN, M. et al. A rumen unprotected conjugated linoleic acid (CLA) supplement inhibits milk fat synthesis and improves energy balance in lactating goats, **Journal of Animal Science**, v. 91, p. 3305-3314, 2013.

BAUMAN, D. E. et al. Biosynthesis of conjugated linoleic acid in ruminants, **Proceedings of the American Society of Animal Science**, 1999.

BAUMAN, D. E. et al. New perspectives on lipid digestion and metabolism in ruminants, **Proceedings Cornell Nutrition Conference**, p. 175-189, 2003.

BAUMAN, D. E. et al. Major advances associated with the biosynthesis of milk, **Journal of Dairy Science,** v. 89, p. 1235-1243, 2006.

BAUMAN, D. E. et al. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model, **The Journal of Nutrition**, v. 138, p. 403-409, 2008.

BAUMAN, D. E.; GRIINARI, J. M. Regulation and nutritional manipulation of milk fat: low-fat milk syndrome, **Livestock Production Science**, v. 70, p. 15–29, 2001.

BAUMAN, D. E.; GRIINARI, J. M. Nutritional regulation of milk fat synthesis, **Annual Review of Nutrition**, v. 23, p. 203–27, 2003.

BAUMGARD, L. H. et al. Identification of the conjugated linoleic acid isomer that inhibits fat synthesis. **American Journal Physiology Regulatory Integrative Comparative Physiology**, v. 278, p. 179-184, 2000.

BAUMGARD, L. H. et al. *Trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows, **Journal of Dairy Science**, v. 85, p. 2155-2163, 2002.

BEE, G. et al. Dietary conjugated linoleic acids alter adipose tissue and milk lipids of pregnant and lactating sows, **The Journal of Nutrition**, v. 130, p. 2292–2298, 2000.

BELURY, M. A. Inhibition of carcinogenesis by conjugated linoleic acid: potential mechanisms of action, **The Journal of Nutrition**, v. 132, p. 2995–2998, 2002.

BERGER, J.; MOLLER, D. E. The mechanisms of action of PPARs, **Annual Review of Medicine**, v. 53, p. 409–435, 2002.

BIONAZ, M. Nutrigenomics Approaches to Fine-Tune Metabolism and Milk Production: Is This the Future of Ruminant Nutrition?, **Advances in Dairy Research,** v. 2, 2014.

BIONAZ, M. et al. Functional role of PPARs in ruminants: potential targets for fine-tuning metabolism during growth and lactation, **PPAR Research,** 2013.

BIONAZ, M.; LOOR, J. J. Gene networks driving bovine milk fat synthesis during the lactation cycle, **BMC Genomics,** v. 9, p. 366, 2008.

BIONAZ, M.; LOOR, J. J. Gene networks driving bovine mammary protein synthesis during the lactation cycle, **Bioinformatics and Biology Insights,** v. 5, p. 83-98, 2011.

BONTEMPO, V. et al. Dietary conjugated linoleic acid positively affects immunologic variables in lactating sows and piglets, **The Journal of Nutrition**, v. 134, p. 817-824, 2004.

BROWN, J. M. et al. Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines, **The Journal of Biological Chemistry**, v. 279, p. 26735–26747, 2004.

BROWN, J. M. et al. Isomer-specific regulation of metabolism and PPARγ signaling by CLA in human preadipocytes, **Journal of Lipid Research,** v. 44, p. 1287-1300, 2003.

BROWN, J. M. et al. *Trans*-10, *cis*-12, but not *cis*-9, *trans*-11 conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells isolated from human adipose tissue, **The Journal of Nutrition**, v. 131, p. 2316–2321, 2001.

BROWN, J. M.; MCINTOSH, M. K. Conjugated linoleic acid in humans: regulation of adiposity and insulin sensitivity, **The Journal of Nutrition,** v. 133, p. 3041-3046, 2003.

COOK, M. E. et al. Immune modulation by altered nutrient metabolism: Nutritional control of immune-induced growth depression, **Poultry Science**, v. 72, p. 1301-1305, 1993.

DALEN, K. T. et al. Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma, **Diabetes,** v, 53, p. 1243–1252, 2004.

DESVERGNE, B.; WAHLI, W. Peroxisome proliferator-activated receptors: nuclear control of metabolism, **Endocrine Reviews**, v. 20, p. 649–688, 1999.

DESVERGNE, B. et al. Be fit or be sick: peroxisome proliferator-activated receptors are down the road, **Molecular Endocrinology**, v. 18, p. 1321–1332, 2004.

DESVERGNE, B. et al. Transcriptional regulation of metabolism, **Physiological Reviews**, v. 86, p. 465–514, 2006.

DE VOS, P. et al. Thiazolidinediones repress gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma, **The Journal of Clinical Investigation**, v. 98, p. 1004–1009, 1996.

ESCHER, P.; WAHLI, W. Peroxisome proliferator-activated receptors: insight into multiple cellular functions, **Mutation Research**, v. 448, p. 121–138, 2000.

EVANS, R. M. et al. PPARs and the complex journey to obesity, **Nature Medicine**, v. 10, p. 355–361, 2004.

FEIGE, J. N. et al. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions, **Progress in Lipid Research,** v. 45, p. 120-159, 2006.

FERNANDES, D. et al. Milk fat depression and energy balance in stall-fed dairy goats supplemented with increasing doses of conjugated linoleic acid methyl esters, **Animal**, v. 8, p. 587-595, 2014.

FORMAN, B. M. et al. The peroxisome proliferator-activated receptors: ligands and activators, **Annals of the New York Academy of Sciences**, v. 804, p. 266–275, 1996.

GAULLIER, J. M. et al. Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy

overweight humans, **American Journal Clinical Nutrition**, v. 79, p. 1118–1125, 2004.

GRANLUND, L. et al. *Trans*-10, *cis*-12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARg modulator, **Journal of Lipid Research**, v. 44, p. 1441–1452, 2003.

GRIINARI, J. M. et al. *Trans*-octadecenoic acids and milk fat depression in lactating dairy cows, **Journal of Dairy Science**, v. 81, p. 1251–1261, 1998.

GRIINARI, J. M.; BAUMAN, D. E. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants, **Advances in Conjugated Linoleic Acid Research**, v. 1, p. 180–200, 1999.

GRIINARI, J. M.; BAUMAN, D. E. Milk fat depression: concepts, mechanisms and management applications, **Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress**, p. 389–417, 2006.

GUAN, H. P. et al. A futile metabolic cycle activated in adipocytes by antidiabetic agents, **Nature Medicine**, v. 8, p. 1122–1128, 2002.

HA, Y. L. et al. Anticarcinogens from fried ground beef heat-altered derivatives of linoleic acid, **Carcinogenesis**, v. 8, p. 1881–1887, 1987.

HA, Y. L. et al. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid, **Cancer Research**, v. 50, p. 1097–1101, 1990.

HARRELL, R. J. et al. Effects of conjugated linoleic acid on milk composition and baby pig growth in lactating sows, **Journal of Animal Science**, v. 78, p. 137–138, 2000.

HARVATINE, K. J.; BAUMAN, D. E. SREBP1 and Thyroid Hormone Responsive Spot14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA, **The Journal of Nutrition**, v. 136, p. 2468-2474, 2006.

HAYASHI, A. A. **Efeito da suplementação com ácido linoléico conjugado (CLA) na composição do leite, do perfil de ácidos graxos e na atividade de enzimas lipogênicas em ratas lactantes.** 2003. 83 f. Dissertação (Mestre em Agronomia) – Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, São Paulo, 2003.

HERRMANN, J. et al. Isomer-specific effects of CLA on gene expression in human adipose tissue depending on PPARγ2 P12A polymorphism: a double blind, randomized, controlled cross-over study, **Lipid in Health and Disease,** v. 8, p. 1-12, 2009.

HEVENER, A. L. et al. Muscle-specific PPARg deletion causes insulin resistance, **Nature Medicine**, v. 9, p. 1491–1497, 2003.

HOTAMISLIGIL, G. S. et al. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance, **Science**, v. 259, p. 87–91, 1993.

HOTAMISLIGIL, G. S. et al. Tumor necrosis factor alpha inhibits signaling from the insulin receptor, **Proceedings of the National Academy of Sciences of USA**, v. 91, p. 4854–4858, 1994.

HOUSEKNECHT, K. L. et al. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat, **Biochemical and Biophysical Research Communications**, v. 244, p. 678-682, 1998.

HUSSEIN, M. et al. Conjugated linoleic acid-induced milk fat depression in lactating ewes is accompanied by reduced expression of mammary genes involved in lipid synthesis, **Journal of Dairy Science**, v. 96, p. 3825–3834, 2013.

IP, C. et al. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid, **Cancer Research**, v. 51, p. 6118–6124, 1991.

JOSÉ, A. A. F. B. V. **Efeito do ácido linoleico conjugado *trans*-10, *cis*-12 na regulação da lipogênese e expressão gênica em culturas de tecido adiposo de suínos em crescimento.** 2005. 75 f. Tese (Doutor em Agronomia) - Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, São Paulo, 2005.

KADEGOWDA, A. K. G. et al. Dietary *trans* fatty acid isomers differ in their effects on mammary lipid metabolism as well as lipogenic gene expression in lactating mice, **The Journal of Nutrition**, v. 140, p. 919–924, 2010.

KADEGOWDA, A. K. G. et al. Peroxisome proliferator-activated receptor-γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents, **Journal of Dairy Science**, v. 92, n. 9, p. 4276–4289, 2009.

KALLEN, C. B.; LAZAR, M. A. Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-

L1 adipocytes, **Proceedings of the National Academy of Sciences of USA**, v. 93, p. 5793–5796, 1996.

KANG, K. et al. *Trans*-10,*cis*-12 CLA inhibits differentiation of 3T3–L1 adipocytes and decreases PPAR gamma expression, **Biochemical and Biophysical Research Communications**, v. 303, p. 795–799, 2003.

KELLY, L. J. et al. Peroxisome proliferator-activated receptors γ and α mediate *in vivo* regulation of uncoupling protein (UCP1, UCP2, UCP3) gene expression, **Endocrinology**, v. 139, p. 4920–4927, 1998.

KENNEDY, A. et al. *Trans*-10, *cis*-12 Conjugated linoleic acid antagonizes ligand-dependent PPARγ activity in primary cultures of human adipocytes, **The Journal of Nutrition**, v. 138, p. 455-461, 2008.

KHANAL, R. C.; DHIMAN, T. R. Biosynthesis of conjugated linoleic acid (CLA): a review, **Pakistan Journal of Nutrition**, v. 3, p. 72-81, 2004.

KREY, G. et al. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay, **Molecular Endocrinology**, v. 11, p. 779–791, 1997.

KUBOTA, N. et al. PPARgamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance, **Molecular Cell**, v. 4, 597–609, 1999.

KUSHIBIKI, S. et al. Insulin resistance induced in dairy steers by tumor necrosis factor alpha is partially reversed by 2,4-thiazolidinedione, **Domestic Animal Endocrinology**, v. 21, p. 25–37, 2001.

LEE, K. N. et al. Conjugated linoleic acid and atherosclerosis in rabbits, **Atherosclerosis**, v. 108, p. 19-25, 1994.
LEHRKE, M.; LAZAR, M. A. The many faces of PPAR γ, **Cell**, v. 123, n. 6, p. 993–999, 2005.

LIU, L-F. et al. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and heptatic steatosis in high fat-fed mice, **American Journal of Physiology – Gastrointestinal and Liver Physiology**, v. 292, p. 1671–1682, 2007.

LOOR, J. J. et al. Effects of dietary *cis*-9, *trans*-11–18:2, *trans*-10, *cis*-12–18:2, or vaccenic acid (*trans*-11–18:1) during lactation on body composition, tissue fatty acid profiles, and litter growth in mice, **British Journal of Nutrition**, v. 90, p. 1039-1048, 2003.

MA, J. et al. Short-and long-term repeatability of fatty acid composition of human plasma phospholipids and cholesterol esters, **The American Journal of Clinical Nutrition**, v. 62, p. 572–578, 1995.

MACH, N. et al. Relationship between milk fatty acid composition and the expression of lipogenic genes in the mammary gland of dairy cows, **Livestock Science**, v. 151, p. 92-26, 2013.

MARTIN, G. et al. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARα and PPARγ activators, **The Journal of Biological Chemistry**, v. 272, p. 28210–28217, 1997.

MASTERS, N. et al. Maternal supplementation with CLA decreases milk fat in humans, **Lipids**, v. 37, p. 133-138, 2002.

MICHALIK, L. et al. International union of pharmacology. LXI. Peroxisome proliferator-activated receptors, **Pharmacological Reviews**, v. 58, p. 726–741, 2006.

MILES, P. D. et al. Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency, **The Journal of Clinical Investigation,** v. 105, p. 287–292, 2000.

MOLLER, D. E.; GREENE, D. A. Peroxisome proliferator-activated receptor (PPAR) γ agonists for diabetes, **Drug Discovery—Advances in Protein Chemistry**, p. 181–212, 2001.

MUHLHAUSLER, B. S. et al. Rosiglitazone increases the expression of peroxisome proliferator activated receptor-γ target genes in adipose tissue, liver, and skeletal muscle in the sheep fetus in late gestation, **Endocrinology**, v. 150, p. 4287–4294, 2009.

NTAMBI, J. M. et al. Effects of conjugated linoleic acid (CLA) on imunne responses, body composition and stearoyl-CoA desaturase, **Symposium Dietary Supplements for Health, Body Composition and Performance**, 2000.

OLIVEIRA, D. E. et al. An unprotected conjugated linoleic acid supplement decreases Milk production and secretion of Milk components in grazing dairy ewes, **Journal of Dairy Science**, v. 95, p. 1437-1446, 2012.

OR-RASHID, M. M. et al. Plasma fatty acid proile of gestating ewes supplemented with docosahexaenoic acid, **Canadian Journal of Animal Science**, v. 89, p. 138–138, 2009.

OSTROWSKA, E. et al. Dietary conjugated linoleic acids increase lean tissue and decrease fat deposition in growing pigs, **The Journal of Nutrition**, v. 129, p. 2037–2042, 1999.

OSTROWSKA, E. et al. Dietary conjugated linoleic acid differentially alters fatty acid composition and increases conjugated linoleic acid content in porcine adipose tissue, **British Journal of Nutrition**, v. 90, p. 915–928, 2003.

PARIZA, P. W. et al. Effects of temperature and time on mutagen formation in panfried hamburger, **Cancer Letters**, v. 7, p. 63-69, 1979.

PARIZA, M. W. et al. Mechanisms of action of conjugated linoleic acid: evidence and speculation, **Proceedings of the Society for Experimental Biology and Medicine**, v. 223, p. 1-13, 2000.

PÉGORIER, J. P. et al. Control of gene expression by fatty acids, **The Journal of Nutrition**, v. 134, p. 2444S–2449S, 2004.

PELTIER, S. et al. Fatty acid proile of plasma and liver lipds in mice depleted in long-chain polyunsaturated (n-3) fatty acids, **International Journal of Molecular Medicine**, v. 22, p. 559–563, 2008.

PETERSON, D. G. et al. Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis, **The Journal of Nutrition**, v. 133, p. 3098–3102, 2003.

PETERSON, D. G. et al. The inhibitory effect of *trans*-10, *cis*-12 CLA on lipid synthesis in bovine mammary epithelial cells

involves reduced proteolytic activation of the transcription factor SREBP-1, **The Journal of Nutrition**, v. 134, p. 2523–2527, 2004.

POSTIC, C. et al. ChREBP, A transcriptional regulator of glucose and lipid metabolism, **Annual Review Nutrition**, v. 27, p. 179-192, 2007.

POULOS, S. P. et al. Conjugated linoleic acid during gestation and lactation does not alter sow performance or body weight gain and adiposity in progeny, **Animal Research**, v. 53, p. 275-288, 2004.

PURUSHOTHAM, A. et al. Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid, **Journal Lipid Research**, v. 48, p. 444–452, 2007.

RANGWALA, S. M. et al. Genetic modulation of PPARgamma phosphorylation regulates insulin sensitivity, **Developmental Cell,** v. 5, p. 657–663, 2003.

RISERUS, U. et al. Rosiglitazone increases indexes of stearoyl-CoA desaturase activity in humans: link to insulin sensitization and the role of dominant-negative mutation in peroxisome proliferator-activated receptor-gamma, **Diabetes**, v. 54, p. 1379–1384, 2005.

ROGUE, A. et al. Gene expression changes induced by ppar gamma agonists in animal and human liver, **PPAR Research**, 2010.

SCHOONJANS, K. et al. Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter,

The Journal of Biological Chemistry, v. 270, p. 19269–19276, 1995.

SFEIR, Z. et al. Regulation of FAT/CD36 gene ex-pression: further evidence in support of a role of the protein in fatty acid binding/transport, **Prostaglandins and Leukotrienes Essential Fatty Acids**, v. 57, p. 17–21, 1997.

SHI, H. et al. PPAR γ regulates genes involved in triacyglycerol synthesis and secretion in mammary gland epithelial cells of dairy goats, **PPAR Research,** 2013.

TONTONOZ, P. et al. mPPARgamma 2: tissue-specific regulator of an adipocyte enhancer, **Genes & Development**, v. 8, p. 1224–1234, 1994.

TONTONOZ, P. et al. PPARγ 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene, **Molecular and Cellular Biology**, v. 15, p. 351–357, 1995.

TONTONOZ, P.; SPIEGELMAN, B. M. Fat and beyond: the diverse biology of PPARγ, **Annual Review of Biochemistry**, v. 77, p. 289–312, 2008.

WAY, J. M. et al. Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues, **Endocrinology**, v. 142, p. 1269–1277, 2001.

WILLSON, T. M. et al. The PPARs: from orphan receptors to drug discovery, **Journal of Medicinal Chemistry**, v. 43, p. 527–550, 2000.

XU, H. E., et al. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors, **Molecular Cell**, v. 3, p. 397–403, 1999.

ZACHUT, M. et al. Efects of increased supplementation of n-3 fatty acids to transition dairy cows on performance and fatty acid proile in plasma, adipose tissue, and milk fat, **Journal of Dairy Science**, v. 93, p. 5877–5889, 2010.