RAFAELLA HORSTMANN

MILHO GELATINIZADO E SINALIZAÇÃO VIA RECEPTOR ATIVADO POR PROLIFERADORES DE PEROXISSOMO GAMA (PPARγ) NA SÍNTESE DE PROTEÍNAS DO LEITE DE OVELHAS.

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade do Estado de Santa Catarina – UDESC, como requisito parcial para obtenção do título de Doutor em Ciência Animal, área de concentração em Produção Animal.

Orientador: Dimas Estrasulas de Oliveira

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RESUMO

HORSTMANN, Rafaella. **Milho gelatinizado e sinalização via receptor ativado por proliferadores de peroxissomo gama (PPARγ) na síntese de proteínas do leite de ovelhas.** 2024. 57. Tese (Doutorado em Ciência Animal – Área: Produção Animal) – Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência Animal, Lages, 2024.

A investigação dos mecanismos ligados a produção de sólidos no leite tem considerável relevância científica e econômica. Os nutrientes e/ou possíveis aditivos presentes na dieta podem modular positivamente a síntese de sólidos no leite de ruminantes. O objetivo deste trabalho foi avaliar potenciais moduladores *in vivo* e *in vitro* da síntese de proteína no leite, a fim de obter informações sobre a regulação nutricional e molecular na glândula mamária de ovelhas. Nesse sentido, um primeiro estudo avaliou a utilização de milho gelatinizado na dieta de ovelhas leiteiras sobre a abundância de RNAm dos genes que codificam as principais proteínas do leite. Um segundo estudo testou o uso de um agonista sintético (tiazolidinediona – TZD) de receptores ativados por proliferadores de peroxissomo gama (PPARγ) no cultivo *in vitro* de explantes de tecido mamário de ovelhas, sobre a abundância de RNAm de genes ligados à síntese de proteína no leite. Os dados foram analisados utilizando o procedimento MIXED do pacote estatístico SAS University Edition. A significância foi declarada com $P < 0,05$ e tendência $P < 0,10$. Para o primeiro experimento, foram utilizadas doze ovelhas Lacaune multíparas, com 140 ± 3 dias em lactação, com período de 5 d de adaptação e 10 d de coleta de dados. Os tratamentos foram: (1) Controle, dieta basal com milho grão ou (2) SFC, dieta basal com milho gelatinizado. As dietas basais foram compostas de silagem de milho, milho moído e farelo de soja. A dieta SFC aumentou significativamente o CMS, a produção de leite e lactose em 17.3% ($P = 0.01$), 17.2% ($P = 0.03$) e 23.8% ($P = 0.05$), respectivamente, comparado ao Controle. A produção de proteína do leite foi aumentada em 14,6% ($P = 0,004$), e a produção de caseína aumentou 23,9% ($P = 0,04$) com a dieta SFC comparada ao Controle. Apesar dos efeitos positivos sobre a produção de leite promovidos pela dieta SFC, a abundância de RNAm dos genes ligados a síntese de proteína no leite não foi modificada pelo tratamento. Para o segundo estudo, foram obtidos por biópsia explantes de glândula mamária de 2 ovelhas primíparas Lacaune x Texel (DEL 54 ± 1,5; PV 69,7 ± 0,25 kg; ECC 2,5 ± 0,25). Os explantes de tecido mamário foram incubados em placas de 6 poços, em estufa a 37 ºC e atmosfera modificada com 5% de CO_2 por 24 horas, usando seis replicatas por tratamento. Os tratamentos aplicados aos tecidos foram: CON (Controle, 6 ml de meio basal + 9 µL de DMSO) e TZD (2,4-Tiazolidinediona, 6 ml de meio basal + 9 µL de 75 µM TZD). O tratamento com TZD regulou positivamente a expressão da proteína do soro α-lactoalbumina (LALBA) em 37,5% ($P = 0,02$) e apresentou tendência de aumento na expressão de α-s1-caseína ($P = 0,07$). Além disso, não houve efeitos de tratamento na abundância relativa de RNAm do sinalizador mTOR, assim como dos genes que codificam as caseínas e a β-lactoglobulina. O cultivo com TZD regulou positivamente a expressão de LALBA na glândula mamária ovina, sem afetar seu receptor PPARγ ou outros genes-alvo da síntese de proteína na glândula mamária.

Palavras-chave: nutrigenômica; produção de leite; teor de energia; expressão gênica.

ABSTRACT

HORSTMANN, Rafaella. **Steam-flaked corn and peroxisome proliferator-activated receptor gamma (PPARγ) signaling in the milk protein synthesis of dairy ewes**. 2024. 57. Thesis (Doctorate in Animal Science - Area: Animal Production) - Santa Catarina State University. Post Graduate Program in Animal Science, Lages, 2024.

Research on the mechanisms related to milk solids yield has considerable scientific and economic value. The nutrients and possible additives added to diets can positively modulate the synthesis of solids in ruminant milk. The objective of this study is to evaluate potential *in vivo* and *in vitro* modulators of protein synthesis, to obtain information about nutritional and molecular regulation in the ewes' mammary gland. In this regard, a first paper evaluated the use of steam flaked corn (SFC) in the diet of dairy ewes on the mRNA abundance of milk protein genes. The second paper tested a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, in the *in vitro* culture of dairy ewes' mammary tissue explants, on the mRNA abundance of milk protein target genes. Data were analyzed using the MIXED procedure in SAS University Edition. Significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$. For the first experiment, twelve multiparous Lacaune ewes, 140 ± 3 days in milk (DIM), 63 ± 0,5 kg of body weight (BW) and milk yield (MY) of 0,96 ± 0,2 kg/d, were assigned to the following treatments: (1) Control, basal diet with corn grain or (2) SFC, basal diet with steam-flaked corn. The experimental period was 15d, 5d of diet and management adaptation and 10d of data collection. The SFC diet significantly increased DMI, milk and lactose yields by 17.3% ($P = 0.01$), 17.2% ($P = 0.03$) and 23.8% ($P = 0.05$), respectively, compared to Control. Milk protein yield also increased by 14.6% ($P = 0.004$), and casein yield increased by 23.9% ($P = 0.04$) with the SFC diet compared to the Control. Despite improved milk performance, the SFC diet did not affect the mRNA abundance of milk protein genes. For the second experiment, mammary explants of two primiparous Lacaune x Texel ewes rearing their single lambs at days in milk (DIM) 54 ± 1.5, with body weight (BW) 69.7 ± 0.25 kg and body condition score (BCS) 2.5 ± 0.25 were obtained by biopsy. Mammary explants were incubated in 6-well plates in air at 37 °C and humidified atmosphere with 5% CO_2 for 24 hours, using six replicates per treatment. The treatments applied were CON (Control, 6 ml of basal medium + 9 µL of DMSO) and TZD (2,4-Thiazolidinedione, 6 ml of basal medium + 9 µL of 75 µM TZD). The TZD treatment upregulated the expression of whey protein α-lactalbumin (LALBA) by 37.5% ($P = 0.02$) and showed a tendency towards increased expression of α-s1-casein ($P = 0.07$). Also, there was no treatment effect on the mammary mRNA relative abundance of mTOR, as well as on the genes coding for caseins and β-lactoglobulin. The TZD culture increasead the mRNA abundance of LALBA, without affecting its receptor PPARγ or other target milk protein genes in ewes' mammary explants.

Keywords: nutrigenomics; milk yield; energy content; gene expression.

LISTA DE ABREVIATURAS

AA	Aminoácidos
AAE	Aminoácidos essenciais
AGNE	Ácidos graxos não esterificados
BACT	Actin beta
BCS	Body condition score
BHBA	Beta-hidroxibutirato
BLG	β-lactoglobulina/β-lactoglobulin
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
BW	Body weight
cDNA	Complementary DNA
CEM	Células epiteliais mamárias
CMS	Consumo de matéria seca
CP	Crude protein
CSN1S1	αS1-caseína/Casein-α S1
CSN1S2	αS2-caseína
CSN2	β-caseína/Casein-β
CSN3	k-caseína/Casein-κ
DM	Dry matter
DMI	Dry matter intake
DIM	Days in milk
DMSO	Dimethyl sulfoxide
DNA	Ácido desoxirribonucleico
DNAse	Deoxyribonuclease
ECC	Escore de condição corporal
ECM	Energia corrigida para a produção de leite a 3,5%
ELL	Energia líquida de lactação
FA	Fatty acid
FAME	Fatty acid metil esters
FASN	Ácido graxo sintase/ Fatty acid synthase
FCM	Gordura corrigida para a produção de leite a 3,5%

FDN	Fibra em detergente neutro
GA	Gentamicin-amphotericin
GSK3	Vias de glicogênio sintase quinase-3
hEGF	Human epidermal growth factor
LALBA	α-Lactoalbumina/α-lactalbumin
ME	Metabolizable energy
MEBM	Mammary epithelial cell basal medium
mRNA	Ácido ribonucleico mensageiro/Messenger ribonucleic acid
mTOR	Proteína-alvo da rapamicina em mamíferos
mTORC1	Complexo 1 do alvo da rapamicina em mamíferos/Mammalian target of rapamycin complex 1
MUFA	Monounsaturated fatty acids
MY	Milk yield
NDF	Neutral detergente fiber
NFC	Non-fiber carbohydrate
PB	Proteína bruta
PBS	Phosphate-buffered saline
PM	Proteína microbiana
PNDR	Proteína não degradada no rúmen
PPARγ	Receptores ativados por proliferadores de peroxissomo gama/Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
RER	Retículo endoplasmático rugoso
RNA	Ácido ribonucleico/Ribonucleic acid
RPS18	Ribossomal protein S18
RT-qPCR	Quantitative real-time polymerase chain reaction
SEM	Standard error of the mean
SFA	Saturated fatty acid
SFC	Steam-flaked corn
SREBF1	Sterol regulatory element-binding transcription factor 1
TMR	Total mixed ration
TZD	2,4-Thiazolidinediona/2,4-Thiazolidinedione
UFA	Unsatured fatty acid

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SUMÁRIO

1 INTRODUÇÃO

Efeitos relacionados a nutrição, genética e ao ambiente quanto a produção de sólidos no leite são discutidos e revisados por muitos estudos, destacando sua importância para a indústria leiteira (Piccioli-Cappelli et al., 2022), assim como a importância social, econômica e nutricional do leite para a saúde humana (Hue-Beuavais et al., 2021). Muitos fatores intrínsecos e extrínsecos regulam a quantidade e a qualidade dos sólidos no leite, como a gordura e a proteína (Piccioli-Cappelli et al., 2022).

Considerando esses aspectos, estudos prévios descreveram a relação positiva entre a disponibilidade de carboidratos fermentáveis no rúmen, produção de proteína microbiana (Oba e Allen, 2003) e a secreção de proteína verdadeira no leite (Broderick, 2003). Aparentemente, a disponibilidade de aminoácidos e a energia da dieta afetam a síntese de proteína no leite (Osorio et al., 2016). Num estudo conduzido por Daniel et al. (2016), altas concentrações de aminoácidos essenciais (AAE) na circulação sanguínea ocasionadas por aumentos no suprimento de proteína microbiana (PM) estimularam a síntese de proteína no leite de vacas.

Aumentos na fração degradável de carboidratos servem como substratos para a produção de proteína microbiana, melhorando a síntese de proteína pela glândula mamária (Stelwagen, 2022b). Os grãos de cereais utilizados na alimentação de ruminantes podem ser processados termicamente para melhorar sua utilização pelos animais. O *steam-flaked* é uma dessas técnicas, que otimizam a utilização de amido (Ahmadi et al., 2020) pela aplicação de calor e umidade aos grãos, desnaturando a matriz proteica no entorno dos grânulos e gelatinizando o amido (Oba e Kammes-Main, 2023).

Além disso, Osorio et al. (2016) reportaram que o efeito da energia sobre a síntese de proteína no leite pode também ser impulsionada por aumentos nos níveis de glicose e insulina. Em nível molecular, há uma clara relação entre a disponibilidade AAE e a sinalização de insulina na síntese de proteína do leite na glândula mamária, através de mudanças na expressão gênica (Bionaz e Loor, 2011; Loor et al., 2013), atividade da proteína-alvo da rapamicina em mamíferos (mTOR) e proteínas relacionadas (Cant et al., 2018). Um composto amplamente usado como antidiabético por melhorar a sensibilidade à insulina é a 2,4-Thiazolidinediona,

reconhecida por ser agonista do fator de transcrição PPARγ em ruminantes (Hosseini et al., 2017).

O PPARγ, desempenha um importante papel como regulador da síntese lipídica em ruminantes (Bionaz et al., 2013) e possui papel ligado a sensibilidade à insulina (Olefsky e Saltiel, 2000). No entanto, sua função na síntese de proteína no leite de ruminantes ainda não é estabelecida, o que nos motiva a buscar por mais informações a respeito do seu modo de ação no metabolismo mamário. Em um experimento in vivo, Sandri et al. (2018) reportaram aumentos no teor de proteína no leite no tratamento com TZD e sugeriram que isso possa ter ocorrido através de uma regulação positiva de PPARy, contribuindo para a síntese de proteína via mTOR. Neste sentido, nosso intuito foi avaliar potenciais moduladores *in vivo* e *in vitro* da síntese de proteína no leite, a fim de obter informações sobre a regulação nutricional e molecular na glândula mamária de ovelhas.

CAPÍTULO I

2 REVISÃO DE LITERATURA

2.1 Síntese de proteína no leite

A proteína é um dos compostos de maior importância no leite de ovelhas (Molik et al., 2012), que ainda contempla lipídeos, lactose, vitaminas, minerais, ácidos nucleicos e células em sua composição (Hue-Beuavais et al., 2021). As proteínas do leite têm majoritariamente origem na glândula mamária, sendo sintetizadas no epitélio mamário e secretadas no lúmen alveolar. As proteínas do leite de origem mamária podem ser classificadas como caseínas e do soro (Stelwagen, 2022b), compostos que diferem em composição, estrutura e propriedades (Molik et al., 2012). A família das caseínas inclui αS1-caseína (CSN1S1), αS2-caseína (CSN1S2), β-caseína (CSN2) e k-caseína (CSN3), e as proteínas do soro são compostas por α-lactoalbumina (LALBA) e β-lactoglobulina (BLG) (Gomez-Cortes e Amigo, 2022).

A biossíntese de proteína do leite envolve etapas de absorção de AA, transcrição e tradução gênica (Stelwagen, 2022b), modificações pós-tradução e por fim, secreção das proteínas sintetizadas no lúmen alveolar (Monks e Mather, 2022). Esse processo complexo ocorre através das células epiteliais mamárias envolvendo diferentes vias de secreção, sofrendo influência de fatores nutricionais, hormonais, gênicos e inerentes aos animais de forma individual, como o estágio de lactação (Wang et al., 2017), mudando seu teor (Stelwagen, 2022b). Em ovelhas, o teor de proteína do colostro pode chegar a 19,4% (Kessler et al., 2019) enquanto durante a lactação esse teor tende a estar entre 4,7 e 7,2%, apresentando valores superiores ao final da lactação.

Para a síntese de proteína no leite, são exigidos os AAE absorvidos em proporções variáveis, dependendo dos níveis de energia e proteína microbiana da dieta e proteína não degradada no rúmen (PNDR), assim como o consumo e status de saúde dos animais (Cant et al., 2018). No início da lactação, a glândula mamária tem prioridade sobre outros órgãos quanto a absorção de AA (Galindo et al., 2015) e vacas leiteiras de alta produção chegam a mobilizar cerca de 1 kg de proteína tecidual para suprir a demanda de AA pela glândula mamária (Bell et al., 2000).

Alguns fatores que afetam a síntese de proteína no leite estão relacionados a disponibilidade de aminoácidos e energia da dieta (Osorio et al., 2016). Mais

especificamente, o suprimento de proteína metabolizável, a produção ruminal de proteína microbiana e a relação entre proteína bruta (PB) e energia líquida de lactação (ELL), são geralmente responsáveis pela síntese de proteína verdadeira no leite (Nudda et al., 2020). Nesse processo, a captação e absorção de AA no tecido mamário correspondem a aproximadamente 90% da síntese de proteína no leite (Cant et al., 1993). Esses substratos se movimentam pela membrana basolateral das células intermediados por vários sistemas de transporte de AA específicos, dependentes e independentes de sódio (Stelwagen, 2022b).

Assim, fica clara a influência de substratos energéticos, hormônios e AA específicos sobre o fluxo sanguíneo, a absorção mamária de nutrientes e, consequentemente, a síntese de proteína no leite (Arriola Apelo et al., 2014). O processo de síntese de proteína no leite a partir de aminoácidos na glândula mamária não difere daquele que ocorre em outros tecidos no organismo (Stelwagen, 2022a). No entanto, os mecanismos intracelulares pelos quais hormônios e metabólitos estimulam a síntese de sólidos, como a proteína no leite, ainda é pouco compreendida (Nichols et al., 2019).

2.1.1 Caseínas

As proteínas de maior proporção no leite são as caseínas (76-83% do total) e sendo consideradas fosfoproteínas sintetizadas pelas células epiteliais mamárias sob influência multi-hormonal (Martin et al., 2022). Sua síntese ocorre no retículo endoplasmático rugoso (RER), sendo transportada ao complexo de Golgi, onde é concentrada e empacotada por vesículas secretórias para ser exportada ao lúmen alveolar (Nickerson e Akers, 2022). O tipo e a concentração de caseínas variam entre as espécies, no leite seu estado nativo ocorre na forma de micelas (Rocha-Mendoza; Jiménez-Flores, 2022). São descritas cinco variantes de αs1-caseína (A-E) no leite ovino, responsáveis pela variação quantitativa do teor de caseína, as variantes diferem quanto ao grau de fosforilação pela substituição de alguns aminoácidos, sendo a αs2-caseína a fração de maior heterogeneidade (Martin et al., 2022).

2.1.1 Proteínas do soro

As proteínas do soro do leite podem ser classificadas como α-lactoalbumina (LALBA), β-lactoglobulina (BLG), imunoglobulinas, albumina sérica bovina (BSA) e

outras proteínas menores (Guha et al., 2021). No leite ovino, elas somam de 17-24% do total de proteínas (El-Salam e El-Shibiny, 2013). Há alguns anos, as proteínas do soro, possuíam valor de mercado limitado e eram descartadas como resíduos ou utilizadas na alimentação animal. Atualmente, as proteínas do soro do leite estão sendo reconhecidas pelo seu valor biológico, devido a propriedades bioativas e nutricionais (Stelwagen, 2022b).

A α-Lactoalbumina é o componente mais comum da proteína do soro, presente no leite de todos os mamíferos e contribuindo de forma significativa para a síntese de lactose (Guha et al., 2021). A LALBA ovina é considerada homóloga à LALBA bovina (Gomez-Cortes e Amigo, 2022). Trata-se de uma metaloproteína que contém um átomo de cálcio por molécula, e uma importante fonte de peptídeos bioativos com funções antibactericidas, prebióticas e imunoestimuladoras (Ho e Bansal, 2022).

Na glândula mamária, LALBA forma um complexo com a enzima galactosiltransferase, atuando como unidade regulatória (Ho e Bansal, 2022), com papel fundamental na síntese de lactose no leite (Stelwagen, 2022b). A ligação de glicose e galactose é o passo final da síntese de lactose na glândula mamária, e a glicose é seu único precursor (Stelwagen, 2022a). Ela forma, em conjunto com a β-1,4-galactosiltransferase, o complexo lactose sintase, responsável pela catalisação da formação de lactose a partir de glicose e UDP-galactose, ocorrendo no complexo de Golgi das células epiteliais mamárias (Martin et al., 2022).

A β-lactoglobulina (BLG) é uma proteína globular que pertence à família das lipocalinas, pequenas proteínas com capacidade de ligação a pequenas moléculas hidrofóbicas (Selvaggi et al., 2014). Ela representa cerca de metade das proteínas do soro em ruminantes e está presente no leite da maioria dos mamíferos (O'Mahony e Fox, 2013). Diferente de LALBA, que possui função enzimática na síntese de lactose, BLG aparentemente possui funções ligadas ao transporte de retinol e ácidos graxos (Selvaggi et al., 2014). No entanto em ovelhas, os genes que codificam α-LA (LALBA) e BLG, têm sido identificados em regiões do cromossomo associadas a características ligadas a performance de produção e componentes do leite (Padilla et al., 2018), indicando que possam ainda haver funções não identificadas dessas proteínas.

2.2 Regulação gênica da síntese de proteína no leite

A síntese de proteína na glândula mamária é iniciada por indutores da expressão gênica e se traduz em um processo que envolve a interação entre fatores de transcrição induzidos pela ação de hormônios e outros compostos, e regiões promotoras ou intensificadoras do DNA (Stelwagen, 2011). Nesse processo, as células epiteliais mamárias (CEM) são quase que exclusivamente responsáveis pela expressão gênica (Bionaz et al., 2012) e a sua diferenciação leva à síntese e secreção de componentes específicos no leite, como as proteínas (Hue-Beuavais et al., 2021).

O “start” da expressão de um gene que codifica a formação de uma nova proteína é iniciado pela enzima RNA polimerase no núcleo da célula, formando um molde de RNA (RNAm) complementar ao DNA, o chamado processo de transcrição (Stelwagen, 2011; Stelwagen, 2022b). Assim que a transcrição ocorre, os RNAm são exportados do núcleo, formando complexos com os ribossomos, que levam ao processo de tradução, onde os polipeptídios sintetizados formam as proteínas (Seamans e Cashman, 2022).

As proteínas, resultado da tradução de moléculas de RNA, desempenham diversas funções biológicas nas células (Loor et al., 2022). No entanto, a taxa mamária de tradução total desses RNAm pode ser afetada por mudanças na expressão de genes ligados a síntese proteica no leite, por alterações na tradução de RNAm pela fosforilação de fatores de tradução, por modificações na abundância de componentes tradutores nas células secretoras e/ou por alterações no número de células secretoras (Cant et al., 2018). Entretanto, o processo de tradução e formação da proteína do leite não necessariamente significa que ela seja uma proteína funcional, para que isso aconteça são necessárias algumas modificações pós-traducionais que garantem que suas estruturas tridimensionais sejam dobradas adequadamente (Stelwagen, 2022b). Ainda, em alguns casos, para se tornarem biologicamente ativas, as proteínas podem ser ligadas a outros grupamentos funcionais como os carboidratos, lipídeos, fosfatos e acetatos (Seamans e Cashman, 2022). Em ruminantes, a síntese de proteína no leite é um processo de tradução de RNAm dos genes CSN1S1, CSN1S2, CSN2, CSN3, LALBA e BLG (Cant et al., 2018).

2.2.1 A interação insulina-mTOR

Estímulos externos às células como os nutrientes, hormônios e fatores de crescimento, assim como, mecanismos de controle internos regulam a expressão gênica na síntese de proteína do leite (Loor et al., 2022; Wang et al., 2017). A regulação da síntese de proteína no leite ocorre via pós-transcricional por uma via de sinalização de insulina-mTOR (Osorio et al., 2016). Brevemente, mTOR é uma proteína quinase que controla o metabolismo celular (Panwar et al., 2023), podendo ser ativada por fatores de crescimento, aminoácidos e pela carga energética intracelular (Cant et al., 2018). E é por meio dessa via, que uma célula pode decidir com base no seu status de AA e energia, sua resposta aos estímulos do organismo, aumentando sua síntese de proteína global, crescimento e progressão à mitose (Cant et al., 2018). A cascata de sinalização mTOR consiste em dois complexos denominados mTORC1 e mTORC2, formados por múltiplas subunidades e com diferentes funções (Panwar et al., 2023). O complexo mTORC1 controla a taxa de tradução global de RNAm em muitos tipos de células, incluindo as do epitélio mamário (Burgos et al., 2010, 2012; Appuhamy et al., 2011).

A eficiência de tradução é regulada por várias cascatas de sinalização que influenciam a atividade de mTORC1, ISR (resposta integrada ao estresse) e GSK3 (vias de glicogênio sintase quinase-3; Cant et al., 2018). Tanto mTORC1 quanto ISR foram descritas como reguladores da síntese de proteína mamária *in vitro* (Burgos et al., 2010, 2012; Appuhamy et al., 2011) e *in vivo* (Rius et al., 2010; Toerien et al., 2010). No entanto, estudos realizados no início da lactação indicam que a síntese proteica no leite pode não ser regulada exclusivamente por mTOR (Rius et al., 2010; Toerien et al., 2010; Ma et al., 2019).

Ao que parece, a disponibilidade de energia contribui significativamente para a síntese de proteína no leite bovino, já a disponibilidade de AA parece não ter o mesmo nível de contribuição (Burgos et al., 2010). No estudo conduzido por Nichols et al. (2017) aumentos no suprimento de AAE para a glândula mamária não causaram efeitos sobre a expressão de CSN2 e LALBA, no entanto, os autores observaram um aumento de 256 g/d na produção de proteína do leite, decorrente de um aumento de 0,40 pontos percentuais no teor de proteína e um aumento de 14% na produção de leite. De maneira oposta, um estudo com ratos em restrição alimentar ou jejum, demonstrou que o RNAm de genes que codificam proteínas no leite tem a síntese recuperada pela realimentação, o que indica que pelo menos em

parte, a síntese de proteína no leite é regulada por meio de adaptações transcriptômicas, e não somente pela disponibilidade de energia (Burgos et al., 2010).

Ainda, Osorio et al. (2016), destacaram que o efeito agonista da energia sobre a síntese de proteína no leite parece ser impulsionado também por aumentos na disponibilidade de insulina e glicose, e não somente pela maior disponibilidade de energia para o mecanismo de tradução. A insulina atua como reguladora da síntese pelo controle da expressão gênica de proteínas no leite e pela regulação da tradução via mTOR (Bionaz et al., 2012; Osorio et al., 2016). Já foi demonstrado, por exemplo, que a associação de prolactina, insulina e glicocorticóides aumentam a síntese *de novo* e a secreção de α-lactalbumina e caseínas, em cultivos de explantes (Akers; Capuco, 2022).

2.3 O papel regulador de PPARγ e o uso de agonistas sintéticos

A nutrição impacta a síntese de proteína do leite via regulação da tradução de RNAm (Pszczolkowski e Arriola Apelo, 2020) e essas mudanças ocasionadas por nutrientes dietéticos são mediadas por fatores de transcrição (FTs) nas células (Osorio et al., 2016). Os FTs são proteínas que controlam a transcrição da informação genética, elas se ligam a sequências específicas do DNA para a formação do RNA (Loor et al., 2022). A função dos fatores de transcrição é desempenhada de forma isolada ou em conjunto com outras proteínas, atuando como ativadores ou repressores da RNA polimerase de determinados genes (Loor et al., 2022).

Um importante fator de transcrição da espécie ovina é a família de receptores ativados por proliferadores de peroxissomo (PPAR, que inclui as isoformas α, β e γ), que atua como mediador das mudanças transcricionais adaptativas que ocorrem em função da lactação na glândula mamária ovina (Osorio et al., 2016). Especificamente PPARγ, é descrito como um importante regulador da síntese lipídica em ruminantes (Bionaz et al., 2013) e possui papel ligado a sensibilidade à insulina (Olefsky e Saltiel, 2000).

O papel regulador de PPARγ na síntese de proteína no leite em ruminantes ainda não é estabelecido. No entanto, sua expressão pode ser manipulada *in vivo* e *in vitro* com o uso de agonistas sintéticos, como por exemplo, a Tiazolidinediona (TZD; Bionaz et al., 2013). A 2,4-Thiazolidinediona é o composto molecular básico

da síntese de todas as moléculas de TZD conhecidas por serem agonistas de PPARγ em ruminantes (Sandri et al., 2018; Hosseini et al., 2017; Schoenberg et al., 2011) e amplamente utilizado como antidiabético por promover aumentos na sensibilidade à insulina. Além disso, os TZDs podem causar efeitos secundários em outros tipos de tecidos que não exclusivamente adiposos, como a glândula mamária. Sandri et al. (2018) num experimento *in vivo* com o usou o TZD na tentativa de inibir efeitos antilipogênicos de *trans*-10, *cis*-12 CLA em ovelhas leiteiras e reportaram aumentos no teor de proteína do leite com o tratamento, que pode ter ocorrido através de uma regulação positiva de PPARγ, contribuindo positivamente para a síntese de proteína via mTOR.

Dado a função reguladora da insulina na síntese de proteína no leite, os resultados reportados nos impulsionam a buscar mais informações a respeito dos mecanismos que permeiam esses processos. Ainda que possua efeito sobre a sensibilidade à insulina, no trabalho conduzido por Rosa et al. (2017) com injeções de TZD em cabras leiteiras foram observados apenas efeitos modestos na expressão de genes alvo de PPARy em células epiteliais mamárias (CEM), sem efeitos para o tecido adiposo, o que pode ter sido reflexo da condição nutricional ou inflamatória dos animais, já que o experimento avaliou animais com mastite subclínica.

Apesar dos avanços recentes nos estudos sobre a regulação da síntese de proteína no leite, o entendimento sobre os mecanismos intracelulares e suas interações ainda necessita de maiores esclarecimentos (Cant et al., 2018). Compreender os mecanismos intracelulares que influenciam a expressão de genes que codificam proteínas na síntese de proteína no leite de ruminantes pode resultar em ferramentas que nos permitam futuramente manipular os teores de sólidos no leite.

2.4 Outros efeitos associados ao TZD e a influência na lactação

O início da lactação representa um período de desafio fisiológico aos animais. Nessa fase, observa-se uma resistência à insulina, que desencadeia a mobilização das reservas corporais com o intuito de aumentar o suprimento energético destinado à glândula mamária, não estando a energia dos componentes da dieta diretamente

associada a produção de leite (Allen e Piantoni, 2014). O que não ocorre na segunda metade do período lactacional, onde o direcionamento de energia é alterado, a concentração de insulina aumenta e a produção de leite é associada ao consumo de energia (Allen e Piantoni, 2014).

Outros efeitos associados ao tratamento com TZD incluem alterações na dinâmica dos marcadores sanguíneos, como adiponectina, insulina, glicose, ácidos graxos não esterificados (AGNE) e beta-hidroxibutirato (BHBA; Hosseini et al., 2017). O TZD demonstrou melhora na sensibilidade à insulina em tecidos periféricos como o tecido adiposo, assim como ajudou na manutenção do ECC no pós-parto de vacas leiteiras (Schoenberg et al., 2011; Smith et al., 2009). No tecido adiposo subcutâneo de vacas secas, a injeção intravenosa de 4 mg TZD/kg regulou positivamente a expressão de PPARy e seus alvos FASN e SREBF1, que atuam como os principais reguladores da adipogênese e lipogênese (Hosseini et al., 2015).

2.5 Regulação nutricional e a produção de proteína no leite

A nutrição desempenha papel crucial sobre alterações em qualidade e quantidade da produção de sólidos no leite (Palmquist e Jenkins, 2017). Como os substratos para a síntese de proteína no leite são oriundos da corrente sanguínea, a dieta determina em grande parte a quantidade e o tipo de aminoácidos disponíveis para a absorção mamária (Stelwagen, 2022b). Diferentes fontes de energia afetam a produção e composição do leite em função dos efeitos relacionados a síntese de proteína microbiana, produção de ácidos graxos voláteis e ao metabolismo pós-absortivo (Morris et al., 2020).

Na formulação de dietas de animais lactantes, o amido e a gordura são utilizadas como fonte de energia (Morris et al., 2020). O amido é o carboidrato de maior importância digerido no intestino delgado, já que representa a única fonte exógena de glicose para os ruminantes (Sutton; Reynolds, 2022). Falando especificamente da energia derivada do amido, animais de alta produção geralmente recebem dietas ricas em grãos e tem o milho como principal ingrediente. No entanto, ao contrário dos suplementos lipídicos que podem ser transferidos diretamente para a síntese de gordura no leite (Rico et al., 2014; Nichols et al., 2019), a energia do amido contido no milho que passa pelo rúmen sem ser digerida e que pode ser diretamente aproveitada pelo organismo, é em torno de 35 – 45% (Morris et al., 2020).

Uma forma de impactar positivamente a síntese de proteína no leite, é prover um aumento da fração de carboidratos degradáveis na dieta, seja pelo provimento de mais energia aos microrganismos ruminais, melhorando a produção de proteína microbiana como substrato para a glândula mamária ou aumento o suprimento de substratos energéticos para a glândula mamária, permitindo maior absorção de aminoácidos (Stelwagen, 2022b). Isso porque, aumentos nos níveis de amido da dieta incrementam o suprimento energético, dando suporte ao metabolismo e ao fluxo de proteína microbiana ruminal (Roman-Garcia et al., 2016). Diferente das vacas, as ovelhas são menos sensíveis a condições de baixo pH no rúmen, podendo ser bem adaptadas a dietas ricas em amido (Molle; Landau, 2017).

Para ilustrar como a composição da dieta influencia a produção de proteína no leite, a meta-análise de Angelez-Hernandes et al. (2020) investigou a relação entre forragem e concentrado na composição do leite ovino, e os autores observaram que o teor de proteína do leite foi maior com menor inclusão de forragem na dieta. De acordo com Jenkins e McGuire (2006), isso pode ser atribuído a uma maior síntese de propionato e proteína microbiana, devido ao maior consumo de carboidratos de rápida fermentação, resultando em aumentos na concentração de insulina e, consequentemente, em maiores produções de leite e proteína. Em outro estudo realizado com ovelhas em meio da lactação (89 dias pós-parto), foi observado que ao aumentar a porcentagem de amido na dieta de 23 para 36% não resultou em aumento na produção de leite, mas aumentou o teor de proteína e reduziu o teor de ureia no leite (Cannas et al., 2013).

2.5.1 Uso de milho termicamente tratado como modulador da síntese de proteína no leite

A forma química e física do amido na dieta, bem como, a quantidade e o nível de consumo total da dieta, influenciam a quantidade de amido a ser digerida no intestino delgado (Sutton; Reynolds, 2022). Em função disso, diversas técnicas de processamento mecânicas ou térmicas têm sido desenvolvidas para aumentar a digestibilidade do amido de grãos de cereais, melhorando a capacidade microbiana e enzimática de hidrólise dos grânulos de amido (Malekkhahi et al., 2022).

Uma dessas técnicas de processamento térmico utilizada para otimizar a utilização do amido pelos ruminantes é conhecida como *steam-flaking* (laminação a vapor, tradução literal; Ahmadi et al., 2020). A técnica promove aumentos na

digestibilidade do milho, submetendo os grãos ao calor e a umidade, desnaturando a matriz proteica que protege os grânulos e gelatinizando o amido (Oba e Kammes-Main, 2023).

Além disso, o *steam-flaking* pode melhorar a produção de ácido propiônico e de PM, o que consequentemente, pode favorecer a disponibilidade de energia e de AA para a síntese de leite (Oba e Allen, 2003; Carmo et al., 2014, dando mais suporte da lactação em vacas leiteiras em comparação a dietas compostas por milho grão (Cooke et al., 2008). Piantoni e VandeHaar (2023) destacaram recentemente em sua revisão que aumentos na oferta de substratos para a síntese de PM e AA podem aumentar a produção de leite e/ou de proteína do leite.

Trabalhos prévios destacaram que dietas ricas em amido aumentaram a produção e o teor de proteína no leite, em comparação a dietas ricas em FDN (Piccioli-Cappelli et al., 2014; Cantalapiedra-Hijar et al., 2015; Doorenbos et al., 2017). E ainda, que a síntese de proteína no leite pode ser resultado de aumentos na concentração de insulina promovida por dietas ricas em amido, o que pode ter regulado de forma positiva a atividade mTORC1 (Arriola Apelo et al., 2014). Razzaghi et al. (2021) destacaram que dietas com milho *steam-flaked* apresentaram tendência (P = 0.07) em aumentar a produção de proteína do leite em vacas, dos 5 aos 64 dias em lactação. No entanto, resultados diferentes foram reportados por Ahmadi et al. (2020), que não reportaram diferenças no CMS, produção de leite com 3,5% de gordura e energia corrigida para a produção de leite a 3,5% (FCM e ECM), gordura ou teor de proteína no leite em dietas contendo milho grão e milho *steam-flaked* na dieta de vacas leiteiras. Nesse sentido, o processamento do milho com o objetivo de favorecer o teor de amido digestível na dieta é de grande interesse, embora seus efeitos sobre a produção de leite e sólidos não sejam bem estabelecidos na literatura (RafieeYarandi et al., 2019).

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3 HIPÓTESES

I. A utilização de milho gelatinizado na dieta de ovelhas leiteiras aumenta a síntese de proteína no leite e a abundância de mRNA de genes ligados à síntese de proteína no leite na glândula mamária de ovelhas.

II. A sinalização via PPARγ promove aumento na abundância de mRNA de genes que codificam proteínas do leite.

4 OBJETIVO GERAL

Avaliar potenciais moduladores *in vivo* e *in vitro* da síntese de proteína no leite, a fim de obter informação sobre a regulação nutricional e molecular na glândula mamária de ovelhas.

4.1 OBJETIVOS ESPECÍFICOS

I. Avaliar se a utilização de milho gelatinizado na dieta de ovelhas modula síntese de proteínas e expressão de genes que codificam as proteínas do leite.

II. Avaliar se um agonista sintético de PPARγ estimula a expressão de genes que codificam proteínas do leite no tecido mamário de ovelhas.

CAPÍTULO II

STEAM-FLAKED CORN IMPROVES MILK AND PROTEIN YIELD WITHOUT MODULATE THE mRNA ABUNDANCE OF MILK PROTEIN GENES IN DAIRY EWES

STEAM-FLAKED CORN IMPROVES MILK AND PROTEIN YIELD WITHOUT MODULATE THE mRNA ABUNDANCE OF MILK PROTEIN GENES IN DAIRY EWES

ABSTRACT

Increasing the digestibility of carbohydrates in the diet can enhance microbial protein yield and the substrates supply, facilitating the absorption of amino acids, improving the milk protein synthesis. We hypothesize that the use of steam-flaked corn in the diet of lactating ewes increases the mRNA abundance of milk protein genes and, in turn, improves milk protein synthesis. Twelve multiparous Lacaune ewes, at 140 ± 3 days in milk (DIM), with a body weight (BW) of 63 ± 0.5 kg and a milk yield (MY) of 0.96 ± 0.2 kg/d, were randomly assigned to the following treatments: (1) Control, basal diet with corn grain or (2) SFC, basal diet with steam-flaked corn. The experimental period was 15d, consisting of 5d for diet and management adaptation and 10d of data collection. Data were analyzed using the MIXED procedure in SAS University Edition. The model included treatment as a fixed effect and animals as a random effect. Milk yield, composition, and feed intake were analyzed using repeated measures. Significance was declared at $P < 0.05$, and tendencies at $P < 0.10$. The SFC diet significantly increased dry matter intake (DMI), milk and lactose yields increased by 17.3% ($P = 0.01$), 17.2% ($P = 0.03$) and 23.8% ($P = 0.05$), respectively. Milk protein yield increased by 14.6% ($P = 0.004$), and casein yield increased by 23.9% ($P = 0.04$) with the SFC diet compared to the Control. Despite improved milk performance, the SFC diet did not affect the abundance of mRNA encoding milk protein genes. The SFC diet leads to significant improvements in milk yield and components. However, these effects do not appear associated to milk protein gene expression in dairy ewes' mammary gland at the end of lactation cycle.

Keywords: nutritional modulation; gene expression; milk protein synthesis.

INTRODUCTION

Milk protein biosynthesis is a complex process that involves steps of amino acid absorption, gene transcription and translation (Stelwagen, 2022b), post-translational modifications, and finally, secretion of proteins synthesized in the alveolar lumen (Monks and Mather, 2022). This process occurs in mammary epithelial cells and involves different secretion pathways, influenced by nutritional, hormonal, genetic, and individual animal factors, such as the stage of lactation (Wang et al., 2017). Previous studies have shown that nutrition plays a crucial role in regulating gene expression in ruminants. Recognizing how nutrients can interact with and influence the molecular mechanisms underlying physiological processes has caused a paradigm shift in nutritional science, leading to the emergence of nutrigenomics (Seamans and Cashman, 2022).

The use of steam-flaked corn in the nutrition of dairy ruminants has increased in the past years. This method enhances the digestibility of corn in the rumen by denaturing the protein matrix of starch granules, increasing the corn starch gelatinization (Oba and Kammes-Main, 2023). The denaturation of this matrix allows for greater microbial attachment and improved starch digestion, thereby increasing the energy available for microbial protein synthesis (Theurer et al., 1999). Consequently, the enhanced energy and microbial protein availability lead to higher

concentrations of milk protein, as well as increased milk and protein yields in lactating dairy cows (Theurer et al., 1999; Oba and Allen, 2003; Carmo et al., 2014).

Dairy cows and ewes exhibit analogous physiological and metabolic traits, mainly related to the main pathways of amino acid synthesis, lipid metabolism, cell proliferation and others, described by transcriptome analysis (Loor et al., 2022), in addition to cellular mechanisms for milk secretion (Monks and Matter, 2022). Regarding dietary considerations, ewes may be more adapted than cows to low pH in rumen provided by high-starch diets (Molle and Landau, 2017), although this is not completely established (Harmon and Swanson, 2020).

The response of dairy ewes to energy supplementation is influenced by multiple factors, including nutritional quality, ruminal fermentation, and physiological status (Miccoli et al., 2022). Most studies that evaluated the use of SFC in the diet have focused on dairy cows, without assessing gene expression in response to supplementation (Malekkhahi et al., 2021; Ahmadi et al., 2020; Martins et al., 2019; Rafiee-Yarandi et al., 2019; Razzaghi et al., 2021). This highlights the lack of information regarding dairy ewes and the molecular mechanisms at the level of gene expression involved in the response to the energy content of diets, especially in relation to the impact on milk protein synthesis.

This is the first attempt to evaluate the effect of steam-flaked corn on the diet of dairy ewes and its impact on the modulation of mRNA abundance of genes related to milk protein synthesis, milk yield, and milk content. We hypothesize that the use of SFC in the diet of lactating dairy ewes will positively modulate the mRNA abundance of milk protein genes, thereby increasing milk protein content.

MATERIAL AND METHODS

Animals, experimental design and treatments

All procedures were approved by the Santa Catarina State University Ethical Animal Committee, protocol no. 3162250216. The experiment was performed in Bom Retiro, SC (27º47'50" S and 49º29'21" W) on a commercial dairy sheep farm. Twelve multiparous Lacaune ewes, DIM 140 ± 3, BW 63 ± 0.5 kg, MY 0.96 ± 0.2 kg/d were randomly assigned to either (1) Control, basal diet with corn grain, or (2) SFC, basal diet with steam-flaked corn. The basal diet was composed of corn silage and soybean meal (Table 1). Diets were formulated and adjusted weekly based on refusals, to meet the nutritional requirements according to the SRNS (Tedeschi et al., 2010). Ewes were housed in collective pens per treatment to receive the experimental diets in TMR, with *ad libitum* access to freshwater. The animals were fed twice a day and received an average of 5.8 kg (as fed) on the Control diet and 6.4 kg (as fed) on the SFC diet. The experimental period lasted 15d, 5d of diet and management adaptation and 10d of data collection.

Table 1. Experimental diets ingredients and chemical composition.

	Treatments[1]	
Item	CON	SFC
Ingredient (%)		
Corn silage	68.8	70.7

Soybean meal	18.3	15.6
Corn grain	12.9	
Steam-flaked corn		13.7
Chemical composition, % of DM		
DM	41.0	40.3
CP	13.4	13.4
NDF	48.4	51.8
NFC[2]	31.8	32.0
Ether extract	3.1	3.1
Ash	4.4	4.4
ME, Mcal/kg of DM	2.503	2.512

[1] CON: basal diet; SFC: basal diet with steam-flaked corn.
[2] NFC = 100 – (NDF + CP + ether extract + ash).

Feeding management, milk and blood sampling and analysis

The TMR was offered after milking, and refusals were weighed before feeding. Ewes were milked twice daily at 0600 and 1330 h. Milk yield was recorded, and milk samples were collected individually at both milkings on days 0, 3, 4, 6, and 7 of data collection period. One milk sample was stored at 4 °C with preservative (Bronopol; D & F Control Systems Inc., USA) for fat, protein, lactose, casein, and total solids analysis by infrared spectroscopy (DairySpec FT; Bentley Instruments Inc., Chaska, MN, USA). On the last day of treatment (10 d), a milk sample without preservative was stored at -20°C for FA profile. Milk fat cake was obtained by centrifuging refrigerated milk at 3000 rpm for 15 min at 4°C. Approximately 50 mg was then methylated following the methodology of Kramer et al. (1997) and quantified by gas chromatography according to the methodology of Kramer et al. (2002). Fatty acid methyl esters were identified by comparison with three references (Supelco FAME Blend #C4-C24, CLA *trans*-9, *cis*-11 #16413, and CLA *trans*-10, *cis*-12 #04397; Sigma Aldrich), and were identified according to their reported elution order under the same chromatographic conditions. Peripheral capillary blood glucose was measured in the vessels using a portable device, and the reading was taken on day 10 prior to the biopsy procedure.

Mammary tissue biopsies

Mammary biopsies were performed on day 10 after morning milking. Before the procedure, 4 mL of lidocaine hydrochloride was administered above the incision site. A coaxial needle (Hospifer, Porto Alegre, RS, Brazil), with a trocar was introduced into the middle point of udder halve. The biopsy was performed using a EstaCore disposable cannula biopsy (Geotek Medikal - EstaCore disposable cannula biopsy/GEC 1412, Yenimahalle-Ankara, Turkey). A biopsy needle was inserted through the coaxial cannula and approximately 35 mg of mammary tissue sample was collected. Samples were rinsed with saline solution, placed in cryotubes containing 1 ml of Dulbecco's PBS (Gibco Laboratories) and immediately stored in liquid nitrogen until RNA extraction. Immediately after the biopsy, the incision was sutured with number 1 Nylon, and

flunixin meglumine (1.1 mg/kg of BW) was administered. Ewes were observed and manually milked to remove post-procedure blood clots.

RNA isolation, complementary DNA synthesis and quantitative RT-qPCR

Total RNA extraction of mammary tissue samples was carried out using the RNeasy Lipid Tissue Mini kit (Qiagen Sciences, Germantown, MD, USA) with DNAse treatment (RNase-free DNase set, Qiagen Sciences, Germantown, MD, USA) "on column." A spectrophotometer (NanoDrop ND2000; NanoDrop Technologies, USA) was used to measure the RNA concentration and isolation quality ($A_{260/280}$ ratio = 2.09 ± 0.05). Total RNA was transcribed to complementary DNA using the kit GoScript™ Reverse Transcription Mix - Random Primers - A2801 (Promega Corporation, Madison, WI, USA). The RT-qPCR was performed in triplicates in a 48-well reaction plates (MicroAmp™, Applied Biosystems, USA) with 15 µl volume reaction with 30 ng of cDNA and 7.5 µl GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) in a StepOne Real-Time machine (Applied Biosystems), using primers for the genes of interest. Data were analyzed using StepOne 2.1 software (Applied BioSystems, Waltham, MA, USA), generating dissociation curves to determine the presence of a single product at the end of the reaction. Regression equations were generated with the RT-qPCR cycle threshold values, to determine the reaction efficiency. Primers sequences were obtained from published studies and are presented in Table 2. The mRNA abundance of mammalian target of rapamycin (mTOR), αS1-casein (CSN1S1), αS2-casein (CSN1S2), β-casein (CSN2), κ-casein (CSN3), α-lactoalbumin (LALBA) and β-lactoalbumin (BLG) was measured. Gene transcript abundance was calculated using a 7-point calibration curve generated from serial dilution of a cDNA pool, expressed as mRNA copy number relative to the geometric mean of housekeeping genes (β-actin and RPS18).

Table 2. Ovine primers used in the quantitative real-time RT PCR (RT-qPCR) analysis.

Gene	Primers*	Access
CSN1S1	F: CCTAATCCCATTGGCTCTGA R: TGACTCTTCACCACAGTGGC	Tsiplakou et al. (2015)
CSN1S2	F:CAGGTTAAGAGAAATGCTGGCC R:TGGTGGAGAGCTGCTCTCTGTT	Tsiplakou et al. (2015)
CSN2	F: CCTCTTACTCAAACCCCTGTGG R:GGACTCCCATTATTTCAGGCTG	Tsiplakou et al. (2015)
CSN3	F: CCTGCCATCAATACCATTGCT R:TGCTTCGGTGGTAGGTGTACTG	Tsiplakou et al. (2015)
mTOR	F: GCCCCCGATCGTGAAGTTA R: TGAAATCCAGAGACTCCGT	Sandri et al. (2018)
LALBA	F: CACCTGCTGTCTTTGCTGCTT R: AGCAAAACGAGGTAGCCAAGG	Tsiplakou et al. (2015)
BLG	F: GATCCCTGCGGTGTTCAAGAT R: CAGCACTGTTTTCCATGCAGAA	Sandri et al. (2018)
BACT	F: GCCTTTGCCATCACTGCAAT	Ticiani et al. (2016)

RPS18	R: TGAGCTCTCCTGCCCTCTTG F: GCCCTGAGGCTCTCTTCCA R: CGGATGTCGACGTCACACTT	Ticiani et al. (2016)

*Primers are listed as 5' to 3' sequence. CSN1S1: αS1-casein; CSN1S2: αS2-casein; CSN2: β-casein; CSN3: κ-casein; mTOR: mammalian target of rapamycin; LALBA: α-lactoalbumin; BLG: β-lactoalbumin; BACT: β-actin; RPS18: ribosomal protein S18.

Statistical analysis

Data were analyzed using the MIXED procedure in SAS University Edition. The model included treatment as a fixed effect and animals as a random effect. Milk yield, composition, and feed intake were analyzed as repeated measures over time. Milk yield and composition on day 0 and the geometric mean of housekeeping genes (ribosomal protein S18 and β-actin) were used as covariates in their respective analyses. Studentized residuals outside ± 2.0 were considered outliers and excluded from the analysis. Significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$.

Results

Dry matter intake (DMI), milk yield, and milk composition are presented in Table 3. The DMI, milk and lactose yields increased by 17.3% (P = 0.01), 17.2% (P = 0.03) and 23.8% (P = 0.05), respectively, with the SFC compared to the Control. No differences were observed in peripheral blood glucose levels between treatments (50.5 vs. 51.4, Control and TZD, respectively; P = 0.8). Milk protein yield increased by 14.6% (P = 0.004), and casein yield increased by 23.9% (P = 0.04) with the SFC diet compared to the Control. There was a tendency for increased fat (P = 0.10) and total solids (P = 0.07) yields with the SFC diet. There was no effect of treatment on milk composition traits, but there was a tendency for reduced lactose content (P = 0.09).

Table 3. Effect of steam-flaked corn diet (SFC) on feed intake, milk yield and composition of lactating ewes.

	Treatments			
Variable	CON	SFC	SEM	P-value
Dry matter intake, kg/d	1.912	2.236	0.009	0.012
Yield, kg/d				
Milk	0.889	1.042	0.008	0.028
Lactose	0.039	0.048	0.00008	0.051
Protein	0.049	0.056	0.00004	0.004
Casein	0.046	0.057	0.0001	0.037
Fat	0.061	0.068	0.0001	0.096
Total Solids	0.161	0.178	0.0002	0.069
Milk composition, %				
Lactose	4.744	4.514	0.002	0.095
Protein	5.770	5.774	0.005	0.986

Casein	5.160	5.470	0.006	0.315
Fat	6.874	6.965	0.007	0.793
Total Solids	18.458	18.584	0.009	0.777

CON: control diet (basal); SFC: steam-flaked corn diet; SEM: Standard error of the mean; P-value: level of significance (P < 0.05).

The milk fatty acid profile is shown in Table 4. The SFC diet fairly impacted on milk fatty acids, increasing C18:1 *trans*-11 (P = 0.03) in 70.2% and decreasing C14:0 in 44.4%. Furthermore, the SFC treatment exhibited a tendency for increase the C18:2 n-6 (P = 0.10) in 36.6% and the sum of PUFA (P = 0.10) in 42.3%. There was also a tendency for a decrease in the sum of others FA with the SFC treatment compared to Control.

Table 4. Effect of steam-flaked corn diet (SFC) on milk fatty acid.

	Treatments			
Variable	CON	SFC	SEM	P-value
FA (g/100 g)				
C6	1.238	0.176	0.070	0.175
C8	1.384	0.337	0.075	0.209
C10	5.761	3.120	0.274	0.374
C12	4.748	4.687	0.066	0.940
C14	10.422	5.826	0.165	0.017
C14:1	1.200	1.215	0.012	0.922
C16	29.524	30.160	0.106	0.586
C16:1	1.658	1.585	0.004	0.138
C18	10.315	14.187	0.265	0.189
C18:1 *trans*-9	0.503	0.741	0.016	0.182
C18:1 *trans*-11	1.362	2.323	0.037	0.033
C18:1 *cis*-9	18.828	18.207	0.113	0.658
C18:1 *cis*-11	0.627	0.699	0.009	0.445
C18:2 n-6	2.574	3.480	0.049	0.100
C18:3 n-3	0.946	1.385	0.047	0.383
cis-9, *trans*-11-CLA	0.400	0.564	0.012	0.186
C20:4 n-6	0.185	0.256	0.005	0.158
Others FA	7.250	6.054	0.063	0.098
∑ SFA	64.152	62.287	0.329	0.596
∑ MUFA	24.423	25.569	0.268	0.688
∑ PUFA	4.122	5.866	0.091	0.097

CON: control diet (basal); SFC: steam-flaked corn diet. Others FA: sum of FA not identified in the chromatography analysis; ∑ SFA, ∑ MUFA, ∑ PUFA: sum of SFA, MUFA and PUFA, respectively; SEM: Standard error of the mean; Level of significance (P < 0.05).

The results for mRNA relative abundance of genes involved in milk protein synthesis are shown in Table 5. Despite the positive effects on milk performance induced by the SFC diet, there were no treatment effects on mRNA relative abundance for the tested milk protein genes (Table 5).

Table 5. Effect of steam-flaked corn diet (SFC) on mRNA relative abundance related to genes encoding casein (CSN1S1, CSN1S2, CSN2, and CSN3), genes encoding the serum proteins α-lactalbumin (LALBA) and β -lactoglobulin (BLG), and mTOR.

	Treatments			
Gene	CON	SFC	SEM	P-value
mRNA relative abundance, %				
LALBA	100	59.988	4.430	0.303
BLG	100	99.192	1.234	0.956
CSN1S1	100	100.952	1.669	0.956
CSN1S2	100	83.908	1.764	0.407
CSN2	100	96.624	1.320	0.821
CSN3	100	98.022	2.443	0.922
mTOR	100	95.351	0.963	0.664

CON: control diet (basal); SFC: steam-flaked corn diet; SEM: Standard error of the mean; Level of significance ($P < 0.05$).

Discussion

Contrary to our initial hypothesis, the SFC treatment did not modulate the mRNA abundance of mammary milk protein genes. However, despite the lack of effects on gene expression, it was possible to improve the performance in the final of lactation cycle. In the current study, the SFC treatment increased DMI, milk, lactose, and protein yield (Table 3). Similarly, Razzaghi et al. (2021) also reported increases in milk yield with SFC diet. Other studies reported no corn flaking effect on DMI (Razzaghi et al., 2021; Ahmadi et al., 2020) and milk production (Mathew et al., 2011; Rafiee-Yarandi et al., 2019).

The increase in milk yield is expected by the enhancement in the energy availability by SFC, providing a better starch digestion and increased glucose supply (Razzaghi et al., 2021) which is a primary precursor for lactose synthesis (Stelwagen, 2022a). Furthermore, the increase in DMI may also have contributed to the increase in milk yield with the SFC diet. In contrast, Rafiee-Yarandi et al. (2019) reported greater production efficiency with the use of SFC in the diet of dairy cows. The authors observed that even with lower DMI, SFC increases milk yield compared to finely ground corn. Although it is expected an increase in lactose content by the increase in the availability of glucose from SFC, we observed only an increase in lactose yield, which is associated to the increase in milk yield. Razzaghi et al. (2021) also did not found an increase in milk protein content but reported an increase in milk protein yield with SFC diets on early lactation dairy cows. The authors attributed this result to the higher supply of gluconeogenic precursors and greater microbial protein synthesis resulting from expanded starch digestibility. Like the lactose yield, the protein yield can be also associated to the higher milk yield.

These results demonstrate, at least in transcription level, that the mTOR signaling pathway, and the milk protein genes (CSN1S1, CSN1S2, CSN2, CSN3, LALBA, and BLG) are not responsive to nutritional manipulation through the inclusion of SFC in the diet. Similarly to our results, besides an enhancement of milk protein and casein yield, Nichols et al. (2017) reported that the CSN2 and LALBA expression of protein genes in mammary tissue of cows did not respond to the abomasal

infusion of essential amino acids. In this scenario, the authors suggest an increase in protein and casein yield may be due to an increase in amino acid substrates available to the mammary gland for milk protein synthesis. It is important to highlight that in this study, the inclusion of SFC in the diet was 13.7% DM, which is different from that reported by Ahmadi et al. (2020), where the inclusion of SFC in the diet was 37.4%, this emphasizes the variability in composition between diets and may impact the results observed.

In the current study, the SFC had significant effect on milk FA profile. The SFC diet promote an enhancement in milk FA profile of vaccenic acid (C18:1 *trans*-11) and a reduction on myristic acid (C14:0) profile. These changes indicate that SFC can modulate the FA milk profile, possibly through alterations in ruminal fermentation and lipid metabolism. However, the effects with the use of SFC on milk fatty acid profile are controversial. Mathew et al. (2011) reported no effects of SFC on the milk fatty acid profile in dairy cows. Malekkihahi et al. (2021), observed no treatment effects on milk fatty profiles of *de novo* and preformed FA. The inconsistency in results with SFC diet may be attributed to variations in starch intake, the lactation cycle, milk production level, and dietary composition (Rafiee-Yarandi et al., 2019).

In contrast with other studies, where the increase in milk protein content induced by the SFC diet reduced milk fat content in cows (Rafie-Yarandi et al., 2019; Razzaghi et al., 2021), we did not observe a milk fat content reduction. Petit et al. (2005) attributed the decrease in milk fat content to reductions in short- and medium-chain FA and increases in UFA in the milk fat profile. Although in our study the SFC did not cause a reduction in milk fat content, we observe a C14:0 decrease in the milk fatty acid profile. The C14:0 is quantitatively one of the five most important fatty acids in ewes' milk fatty, together with C16:0, C18:1 cis-9, C10:0, and C18:0, account for >65% of total FAs in sheep's milk (Gomez-Cortes and Amigo, 2022). The observed increase in the C18:1 *trans*-11 in the milk fatty acid profile, an intermediate of linoleic (C18:2 n-6) and linolenic (C18:3 n-3) in ruminal metabolism (Bauman and Griinari, 2003) may be related to greater digestibility of starch provided by SFC. Our findings indicate that SFC can be an effective dietary strategy to enhance milk yield without adversely affecting milk composition. However, the lack of changes in gene expression highlights the complexity of milk synthesis regulation and the need for further research to fully understand these mechanisms.

Conclusion

The SFC leads to an improvement in milk yield and components in late lactation ewes. However, this enhancement does not appear associated to milk protein gene expression in the mammary gland, but rather to an increase in milk protein substrate supply. Our hypothesis that the use of SFC in the diet of ewes would increase mRNA abundance of milk protein genes, thereby enhancing protein synthesis, was rejected.

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CAPÍTULO III

2,4-THIAZOLIDINEDIONE UPREGULATED THE mRNA ABUNDANCE OF α-LACTALBUMIN IN MAMMARY TISSUE OF LACTATING DAIRY EWES

2,4-THIAZOLIDINEDIONE UPREGULATED THE mRNA ABUNDANCE OF α-LACTALBUMIN IN MAMMARY TISSUE OF LACTATING DAIRY EWES

ABSTRACT

The regulatory role of PPARγ in milk protein synthesis in ruminants is not yet established. Despite this, its ability to increase insulin sensitivity may be beneficial for enhancing milk protein synthesis. In this study, we investigated the effects of the PPARγ agonist TZD on milk protein gene expression in the mammary gland, hypothesizing that TZD would upregulate mRNA levels of these genes in the mammary gland of lactating dairy ewes. Mammary explants of two primiparous Lacaune x Texel ewes rearing their single lambs at days in milk (DIM) 54 ± 1.5, with body weight (BW) 69.7 ± 0.25 kg and body condition score (BCS) 2.5 ± 0.25 were obtained by biopsy. Mammary explants were incubated in 6-well plates in air at 37°C and humidified atmosphere with 5% CO_2 for 24 hours, using six replicates per treatment. The treatments applied were CON (Control, 6 ml of basal medium + 9 µL of DMSO) and TZD (2,4-Thiazolidinedione, 6 ml of basal medium + 9 µL of 75 µM TZD). Data were analyzed using the MIXED procedure in SAS University Edition. The model included the mean, fixed treatment effects, random explant effects within treatment, and residual error. The geometric mean of housekeeping genes (RPS18 and BACT) was used as covariates in their respective analyses. Studentized residuals outside ± 2.0 were considered outliers and excluded from the analysis. Significance was declared at $P < 0.05$, and tendencies at $P < 0.10$. The TZD treatment upregulated the expression of whey protein α-lactalbumin (LALBA) by 37.5% ($P = 0.02$) and showed a trend towards increased expression of α-s1-casein ($P = 0.07$). Also, there was no treatment effect on mammary mRNA relative abundance of PPARy, mTOR, CSN1S1, CSN1S2, CSN2, CSN3, LALBA and BLG. The TZD culture increased the mRNA abundance of LALBA, without affecting its receptor PPARγ or other target milk protein genes in ewes' mammary explants.

Keywords: peroxisome proliferator-activated receptor gamma agonist; milk protein genes; whey protein.

INTRODUCTION

The peroxissome proliferator-activated receptor gamma (PPARγ) is an important transcription factor in sheep, acting as a regulator of lipid synthesis in ruminants (Bionaz et al., 2013). Its activation typically promotes positive effects on adipocyte functionality, including significant increases in insulin sensitivity and lipoprotein metabolism (Wang et al., 2018). Insulin acts as a milk protein synthesis modulator by regulating protein expression and the translation mechanism via mTOR (Bionaz et al., 2012), its availability supports milk protein synthesis (Osorio et al., 2016). In a review, Pszczolkowski and Arriola Apelo (2020) highlighted insulin not only as a pivotal signal of systemic energy status but also as a key regulator of amino acid metabolism, controlling the supply, uptake, and utilization by the mammary gland for protein synthesis. In mammary explants, the combination of insulin, prolactin, and glucocorticoids can enhance the *de novo* synthesis of fatty acids and the secretion of α-lactalbumin and caseins (Akers and Capuco, 2022).

The thiazolidinedione (TZD) is commonly recognized as PPARγ agonists, used as antidiabetic agents and a potent mediator of increases in insulin sensitivity (Wang et al., 2018; Hosseini et al., 2017). In a study using a PPARγ agonist, Sandri et al. (2018) reported increases in milk protein content of ewes treated with TZD associated with an upregulation of milk protein genes in mammary gland. According to the authors, this may have occurred through a positive regulation of PPARγ, contributing to the milk protein synthesis via mTOR. However, the regulatory role of PPARγ in milk protein synthesis in ruminants is not yet established. This emphasizes the need to further explore its mechanism of action in mammary metabolism.

There is potential to evaluate TZD as a PPARγ agonist in different species and animal tissues. Studies testing TZD include neutrophil cells in dairy cows (Revelo and Waldron, 2010) and adipocytes in rats (Wang et al., 2018), dairy goats (Rosa et al., 2017), dairy ewes (Sandri et al., 2018), and dairy cows (Hosseini et al., 2017), *in vitro* and *in vivo*, respectively. Studies in models that reproduce mammary function similarly at *in vivo* can facilitate the understanding of milk synthesis molecular mechanisms (Wheeler et al., 1995). For dairy ewes, mammary explants have been presented as a useful model in cultures of up to 24 h in molecular studies to evaluate mRNA abundance (Urio et al., 2018). However, this is the first time that the effects of the PPARγ agonist TZD on milk protein gene expression in the mammary gland were investigated. We hypothesized that the culture of TZD, through its modulatory effects on cell signaling pathways, would upregulate the mRNA abundance of milk protein genes in mammary gland explants from dairy ewes.

MATERIAL AND METHODS

Animals

Santa Catarina State University Ethical Animal Committee approved all animal management and care procedures by protocol number 832827082. Two primiparous Lacaune x Texel ewes rearing their single lambs were harvested at days in milk (DIM) 54 ± 1.5, with body weight (BW) 69.7 ± 0.25 kg and body condition score (BSC) 2.5 ± 0.25, to obtain mammary explants. The ewes received a diet formulated to meet their nutritional requirements according to SRNS (Tedeschi et al., 2010), consisting of corn silage, soybean meal, ground corn, and mineral salt.

Mammary tissue sampling

For the mammary biopsy procedure, ewes were separated from the flock in the morning, ewes were manually milked, and biopsies were performed between 1 and 4 hours after milking. Briefly, prior to the procedure, a local subdermal anesthetic block was performed by administering 8 ml of lidocaine hydrochloride above the incision site. A coaxial needle (Hospifer, Porto Alegre, RS, Brazil), with a trocar was introduced into the middle point of udder halve. The biopsy was performed using a semi-automatic Biopsy Cannula (Geotek Medikal – GSN1410, Yenimahalle-Ankara, Turkey). A biopsy needle was inserted through the coaxial cannula and approximately 40 mg of mammary tissue sample were harvested. Samples were rinsed with a saline solution, placed in tubes containing culture medium, and stored on ice for approximately 2 hours until *in vitro* culture. Immediately after removal of the biopsy instrument, the incision was closed using

number 1 Nylon. The biopsy procedure resulted in minimal bleeding, and ewes were kept separated from lambs, observed, and manually milked to remove post-procedure clots for 5 days. After biopsy procedures, meloxicam (0.025 ml/kg of BW), enrofloxacin (3 ml/kg of BW), and dipyrone monohydrate (0.15 ml/kg of BW) were administered.

Tissue culture and treatments

Mammary explants were incubated in 6-well plates (Nunclon, Nunc, Roskilde, Denmark) in air at 37 °C and humidified atmosphere with 5% CO_2 for 24 hours, using six replicates per treatment. The culture medium contained mammary epithelial cell basal medium (99.2%, MEBM, Lonza Walkersville, MD, USA), and supplements and growth factors required for the culture (MEGM SingleQuots Kit, Lonza Walkersville, MD, USA) with bovine pituitary extract (BPE, 0.4%), human epidermal growth factor (hEGF, 0.1%) insulin (0.1%), hydrocortisone (0.1%), and gentamicin-amphotericin (GA-1000, 0.1%). 2,4-Thiazolidinedione (TZD, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in free DNAse/RNAse dimethyl sulfoxide (DMSO, HiMedia Laboratories Private Limited, Mumbai, MH, India) to reach a stock solution concentration of 50 mM. The treatments applied were CON (control, 6 ml of basal medium + 9 µL of DMSO) and TZD (2,4-Thiazolidinedione, 6 ml of basal medium + 9 µL of TZD stock solution to reach a final concentration of 75 µM TZD/well). After 24 h of culture, explants were stored in cryotubes and kept in liquid nitrogen until RNA isolation.

RNA isolation, complementary DNA synthesis and quantitative RT-qPCR

Total RNA was prepared with the homogenization of approximately 40 mg of mammary tissue. Isolation was carried out 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). A spectrophotometer (NanoDrop ND2000; NanoDrop Technologies, USA) was used to measure the RNA concentration and isolation quality ($A_{260/280}$ ratio = 2.1 ± 0.02). Total RNA was transcribed to complementary DNA using the kit GoScript™ Reverse Transcription Mix - Random Primers - A2801 (Promega Corporation, Madison, WI, USA). The RT-qPCR was performed in triplicates in a 48-well reaction plates (MicroAmp™, Applied Biosystems, USA) with 15 µl of volume reaction with 30 ng of cDNA and 7.5 µl GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) in a StepOne Real-Time (Applied Biosystems, Waltham, MA, USA), using primers for target genes. Data were analyzed using StepOne 2.1 software (Applied BioSystems, Waltham, MA, USA), generating dissociation curves to determine the presence of a single product at the end of the reaction. Regression equations were generated with the RT-qPCR cycle threshold values, to determine the reaction efficiency. Primers sequences were obtained from published studies and are presented in Table 6. The mRNA abundance of following genes was measured: peroxisome proliferator-activated receptor gamma (PPARγ), mammalian target of rapamycin (mTOR), αS1-casein (CSN1S1), αS2-casein (CSN1S2), β-casein (CSN2), κ-casein (CSN3), α-lactoalbumin (LALBA) and β-lactoalbumin (BLG). Gene transcript abundance was

calculated using a 7-point calibration curve generated from serial dilution of a cDNA pool, expressed as mRNA copy number relative to the geometric mean of housekeeping genes (β-actin and RPS18).

Table 6. Ovine primers used in the quantitative real-time RT PCR (RT-qPCR) analysis.

Gene	Primers	Access
CSN1S1	F: CCTAATCCCATTGGCTCTGA R: TGACTCTTCACCACAGTGGC	Tsiplakou et al. (2015)
CSN1S2	F: CAGGTTAAGAGAAATGCTGGCC R: TGGTGGAGAGCTGCTCTCTGTT	Tsiplakou et al. (2015)
CSN2	F: CCTCTTACTCAAACCCCTGTGG R: GGACTCCCATTATTTCAGGCTG	Tsiplakou et al. (2015)
CSN3	F: CCTGCCATCAATACCATTGCT R: TGCTTCGGTGGTAGGTGTACTG	Tsiplakou et al. (2015)
PPAR-γ	F: CCAAGAATATCCCCGGCT R: AGGCCAGCATCGTGTAAA	Hussein et al. (2013)
mTOR	F: GCCCCCGATCGTGAAGTTA R: TGAAATCCAGAGACTCCGT	Sandri et al. (2018)
LALBA	F: CACCTGCTGTCTTTGCTGCTT R: AGCAAAACGAGGTAGCCAAGG	Tsiplakou et al. (2015)
BLG	F: GATCCCTGCGGTGTTCAAGAT R: CAGCACTGTTTTCCATGCAGAA	Sandri et al. (2018)
BACT	F: GCCTTTGCCATCACTGCAAT R: TGAGCTCTCCTGCCCTCTTG	Ticiani et al. (2016)
RPS18	F: GCCCTGAGGCTCTCTTCCA R: CGGATGTCGACGTCACACTT	Ticiani et al. (2016)

*Primers are listed as 5' to 3' sequence. CSN1S1: αS1-casein; CSN1S2: αS2-casein; CSN2: β-casein; CSN3: κ-casein; PPAR-γ: peroxisome proliferator-activated receptor gamma; mTOR: mammalian target of rapamycin; LALBA: α-lactoalbumin; BLG: β-lactoalbumin; BACT: β-actin; RPS18: ribosomal protein S18.

Statistical analysis

Data were analyzed using the MIXED procedure in SAS University Edition (SAS, 2017). The model included the mean, fixed treatment effects, random explant effects within treatment, and residual error. The geometric mean of housekeeping genes (ribosomal protein S18 and β-actin) was used as covariates in their respective analyses. Studentized residuals outside ± 2.0 were considered outliers and excluded from the analysis. Significance was declared at $P < 0.05$, and tendencies at $P < 0.10$.

Results and discussion

Effects of TZD treatment for relative mRNA abundance of genes coding caseins, milk serum proteins, transcription factor, and signaling milk protein in mammary tissue are shown in Table 7. Compared to the Control, TZD treatment upregulated the expression of whey protein α-lactalbumin (LALBA) by 37.5% (P = 0.02) and showed a tendency towards increased expression of α-s1-casein (P = 0.07). There was no treatment effect on mammary mRNA relative abundance of PPARy, mTOR, CSN1S1, CSN1S2, CSN2, CSN3 and BLG.

Table 7. Relative mRNA abundance of genes related to milk protein synthesis in the mammary tissue explants of dairy ewes treated in vitro with TZD.

	Treatments			
Gene	CON	TZD	SEM	P-value
mRNA relative abundance, %				
LALBA	100	137.516	0.836	0.016
BLG	100	96.092	0.685	0.654
CSN1S1	100	122.928	0.683	0.072
CSN1S2	100	116.282	1.976	0.436
CSN2	100	73.825	1.504	0.202
CSN3	100	113.402	1.354	0.436
mTOR	100	86.421	0.216	0.216
PPARγ	100	88.619	2.857	0.723

CON: control, 6 ml of basal medium + 9 µL of DMSO; TZD: 2,4-Thiazolidinedione, 6 ml of basal medium + 9 µL of 75 µM TZD; SEM: Standard error of the mean; Level of significance (P < 0.05).

The TZD culture increased the mRNA abundance of LALBA, without affecting its receptor PPARγ or other target milk protein genes in mammary explants. Our results suggest that TZD plays a modulatory role in LALBA expression. The α-lactalbumin has been associated with enhancements in milk yield due to its regulatory role in milk lactose synthesis (Stelwagen, 2022b). In the mammary gland, α-LA forms a complex with the enzyme galactosyltransferase (Ho & Bansal, 2022) and is considered an important genetic marker for milk production traits in cattle (Martin et al., 2022).

The study of Sandri et al. (2018) reported that the TZD intravenous infusion of 4 mg/kg of BW/day in lactating ewes induced an increase of 18.8% in milk protein content, compared to Control. Furthermore, in contrast to our results, the expression of milk protein genes CSN1S1, CSN1S2, CSN2, CNS3 and BLG were increased by TZD, compared to Control. The authors also reported no treatment effect of TZD on LALBA expression. The lack of effects on the mammary mRNA relative abundance of PPARγ, mTOR, CSN1S1, CSN1S2, CSN2, CSN3, and BLG suggests that the regulation of these genes may be more complex or TZD dose dependent. Currently, there is no consensus on the appropriate dose of TZD to be administered. In general, doses used in cell studies range from 1 µM to 4 µM (Wang et al., 2018; Anderson et al., 2014), while in vivo studies have used intravenous injection doses ranging from 4 to 8 mg of TZD/kg BW (Hosseini et al., 2015; Rosa et al., 2017). Although not significant, the tendency for increased expression of CSN1S1 suggests that there may be a modulatory effect of TZD

under different experimental conditions. This opens new perspectives for future investigations into the regulation of gene expression during lactation.

In addition to its known role in improving milk production, exploring increases in LALBA levels could promote enhancements in the nutraceutical properties of milk. In humans and cattle, partially unfolded LALBA has been associated with tumoricidal functions when bound to oleic acid, a complex known as “HAMLET/BAMLET” (human/bovine α-lactalbumin made lethal to tumor cells) (Ho and Bansal, 2022). Although an increase in the number of gene transcripts does not necessarily imply their translation into a functional protein, and we did not evaluate expression at the protein level or tested the tumoricidal functions, new research focused into role of LALBA as a nutraceutical component could enhanced ewes’ dairy products.

It is important to acknowledge limitations regarding the number of samples and dose of TZD used in this study, reinforcing the need for further investigations testing different levels of TZD and its influence on target milk protein genes in different phases of the lactation cycle. As well as investigating specific mechanisms through which TZD can modulate the expression of LALBA and other milk proteins.

Conclusion

The TZD culture enhanced the mRNA abundance of LALBA, without affecting its receptor PPARγ or other target milk protein genes in mammary explants. The results suggest that TZD treatment may influence the transcripts abundance of specific genes during lactation.

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7 CONSIDERAÇÕES FINAIS

Nossos resultados sugerem que a utilização de milho gelatinizado promove aumentos na produção de leite e na produção de proteína no leite ao final da lactação. No entanto, esse aumento não parece estar relacionado a aumentos na abundância de RNAm de genes ligados à síntese de proteína no leite de ovelhas leiteiras ao final do ciclo de lactação. Além disso, o cultivo *in vitro* de tecido mamário com TZD aumenta a abundância de RNAm de LALBA, sem afetar seu receptor PPARγ ou outros genes ligados a síntese de proteína.