UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

FACULDADE DE CIÊNCIAS AGRÁRIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

EXTRATO AQUOSO DE *Moringa oleifera* COMO ADITIVO PARA OVELHAS DA RAÇA PANTANEIRA EM LACTAÇÃO

RENATA ALVES DA CHAGAS

Tese apresentada ao Programa de Pós-graduação em Zootecnia – Área de Concentração: Produção Animal, como parte das exigências para obtenção do título de Doutor (a) em Zootecnia.

Dourados – MS

Fevereiro de 2023

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Orientador: Prof. Dr. Fernando Miranda de Vargas Junior

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RENATA ALVES DA CHAGAS

Tese apresentada como parte dos requisitos exigidos para obtenção do título de DOUTORA EM ZOOTECNIA

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Aos meus pais, pelo incentivo e apoio de sempre.

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Travessia

"Há um tempo em que é preciso
Abandonar as roupas usadas
Que já têm a forma do nosso corpo...
E esquecer os nossos caminhos
Que nos levam
Sempre aos mesmos lugares

É o tempo da travessia
E se não ousarmos fazê-la
Teremos ficado para sempre
À margem de nós mesmos."

Fernando Pessoa

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LISTA DE ABREVIATURAS

ABTS - 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic)

ADF – Acid detergente fibre

AEMO – Aqueous extract of *Moringa oleifera*

AGCC – Ácidos graxos de cadeia curta

AGV – Ácidos graxos voláteis

BCFA – Branched chain fatty acids

BW – Body weight

CLA – Conjugated linoleic acid

CP – Crude protein

DIM – Days in milk

DM – Dry matter

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EE – Ether extract

FAME – Fat acid methyl ester

FRAP - ferric reducing antioxidant power

HPLC – High-performance liquid chromatography

iNDF – Indigestible neutral detergnet fibre

LCE – Leite corrigido energeticamente

LCFA - Long chain fatty acids

LCG – Leite corrigido para gordura

MUFA - Monounsaturated fatty acids

MUN – Milk urea nitrogen

NDF – Neutral detergente fibre

OBCFA - odd-chain fatty acids

OM – Organic matter

PUFA – Polyunsaturated fatty acids

SCFA - Short chain fatty acids

SEM – Standard error mean

SFA - Saturated fatty acids

TBARS - thiobarbituric acid-reactive substances

VFA – Volatile fatty acids

RESUMO

CHAGAS, R.A. Extrato aquoso de *Moringa oleifera* como aditivo para ovelhas da raça Pantaneira em lactação. 2022. p.91. Tese – Doutorado em Zootecnia – Faculdade de Ciências Agrárias – Universidade Federal da Grande Dourados, Dourados, MS, 2022.

Os objetivos dessa tese foram I) abordar sobre a utilização de aditivos na nutrição de ruminantes e o uso de aditivos naturais na fermentação ruminal, metabolismo de ruminantes e ação no leite (Capítulo I); II) estudar o efeito do extrato aquoso de *Moringa oleifera* (AEMO) como aditivo para ovelhas em lactação e qual a sua influência na fermentação ruminal, parâmetros metabólicos e desempenho de ovelhas em lactação (Capítulo II); III) avaliar o efeito da suplementação de AEMO para ovelhas em lactação sobre a produção e composição do leite, perfil de ácidos graxos e antioxidantes do leite (Capítulo III). Capítulo I: existe um potencial já comprovado na literatura que os aditivos naturais em substituição a aditivos comumente utilizados que podem substituir estes sem acumular resíduos no leite ou na carne. Os aditivos naturais possuem compostos com capacidade antioxidante e atividade antimicrobiana que pode beneficiar a saúde animal e dos humanos. Capítulo II: Foram utilizadas doze ovelhas distribuídas em quatro quadrados latinos 3x3 replicados, com períodos de 14 dias (coleta nos últimos cinco dias de cada período). Os tratamentos fornecidos diariamente foram: 20 ml de água como controle, 20 ml de AEMO (20-AEMO) e 40 ml de AEMO (40-AEMO). Foram realizadas duas ordenhas diárias. O concentrado foi fornecido na base de 3% do peso corpóreo e feno ad libitum. O consumo, digestibilidade e pH ruminal não foram afetados pelo AEMO, mas houve uma tendência de redução do acetato, aumento do propionato e consequentemente redução da produção de metano em 40-AEMO. A produção de leite foi menor para 20-AEMO, assim como: gordura, proteína e lactose. A produção de proteína foi menor para 40-AEMO. Os parâmetros sanguíneos e urinários não foram afetados pelo uso do AEMO. Recomenda-se estudos com diferentes concentrações de extrato para elucidar os efeitos na fermentação ruminal e na síntese de compostos no leite. Capítulo III: Utilizou-se o mesmo protocolo experimental do capítulo II. Realizou-se a caracterização do extrato aquoso de *Moringa oleifera*, determinou-se a produção e composição do leite, perfil de ácidos graxos de cadeia curta e longa, e antioxidantes presents no leite. A produção de leite foi influenciada pelo extrato AEMO, onde o 20-AEMO foi inferior ao Controle, porém com maior teor de sólidos totais. Para os teores

de proteína e caseína, observou-se redução nos animais suplementados com 40-AEMO. Os ácidos graxos de cadeia curta detectados no leite das ovelhas, não diferiram entre os tratamentos. O ácido graxo c10-15:1 aumentou com o 40-AEMO. Os antioxidantes e a oxidação avaliados no leite das ovelhas, não foram influenciados pela adição de AEMO. O extrato aquoso de *Moringa oleifera* é rico em teores antioxidantes, porém na dose utilizada demonstrou ser pouco expressivo quanto ao efeito no perfil lipídico do leite em relação a ácidos graxos importantes para saúde humana. O teor antioxidante do extrato não foi suficiente para transferir quantidades expressivas de antioxidantes para o leite.

Palavras-chave: modulador ruminal; fermentação; desempenho; ácidos graxos; produção de leite.

ABSTRACT

CHAGAS, R. A. Aqueous extract of Moringa oleifera as an additive for lactating Pantaneira ewes.

The objectives of this thesis were I) to address the use of additives in ruminant nutrition and the use of natural additives in ruminal fermentation, ruminant metabolism and action in milk (Chapter I); II) to study the effect of aqueous extract of Moringa oleifera (AEMO) as an additive for lactating ewes and what is its influence on ruminal fermentation, metabolic parameters and performance of lactating ewes (Chapter II); III) to evaluate the effect of AEMO supplementation for lactating ewes on milk production and composition, fatty acid profile and milk antioxidants (Chapter III). Chapter I: There is already proven potential in the literature for natural additives to replace commonly used additives that can replace these without accumulating residues in milk or meat. Natural additives have compounds with antioxidant capacity and antimicrobial activity that can benefit animal and human health. Chapter II: Twelve ewes distributed in four replicated 3x3 Latin squares were used, with periods of 14 days (collection in the last five days of each period). The treatments given daily were: 20 ml of water as a control, 20 ml of AEMO (20-AEMO) and 40 ml of AEMO (40-AEMO). Two daily milkings were performed. The concentrate was provided on the basis of 3% of body weight and hay ad libitum. Intake, digestibility and ruminal pH were not affected by AEMO, but there was a tendency to reduce acetate, increase propionate and consequently reduce methane production in 40-AEMO. Milk production was lower for 20-AEMO, as well as: fat, protein and lactose. Protein production was lower for 40-AEMO. Blood and urinary parameters were not affected by the use of AEMO. Studies with different extract concentrations are recommended to elucidate the effects on ruminal fermentation and on the synthesis of compounds in milk. Chapter III: The same experimental protocol as in Chapter II was used. The characterization of the aqueous extract of Moringa oleifera was carried out, the production and composition of the milk, the profile of short and long chain fatty acids, and antioxidants present in the milk were determined. Milk production was influenced by the AEMO extract, where the 20-AEMO was lower than the Control, but with a higher total solids content. For protein and casein contents, there was a reduction in animals supplemented with 40-AEMO. Short-chain fatty acids detected in ewes' milk did not differ between treatments. The c10-15:1 fatty acid increased with 40-AEMO.

Antioxidants and oxidation evaluated in sheep's milk were not influenced by the addition of AEMO. The aqueous extract of Moringa oleifera is rich in antioxidant contents, but at the dose used it proved to be little expressive in terms of the effect on the lipid profile of milk in relation to fatty acids that are important for human health. The antioxidant content of the extract was not sufficient to transfer expressive amounts of antioxidants to the milk.

Keywords: ruminal modulator; fermentation; performance; fatty acids; milk production.

CONSIDERAÇÕES INICIAIS

O uso de aditivos é evidenciado devido a melhora no desempenho e produção de animais ruminantes, trazendo benefícios relacionados a modificação da fermentação ruminal, desta forma, são também chamados de moduladores ruminais. O processo de modulação do rúmen, engloba a ação de maior síntese microbiana e alteração na proporção dos ácidos graxos de cadeia curta, desencadeando redução na formação de metano; na menor degradação de proteína verdadeira; e na biohidrogenação de ácidos graxos insaturados. Dentre os aditivos, os ionóforos são os mais utilizados na nutrição de ruminantes.

De maneira geral, os aditivos possuem benefícios para ruminantes, porém, há relatos que aditivos químicos podem deixar vestígios na carne e leite (JOUANY E MORGAVI, 2007) e com isto, é possível que ocasionem em danos à saúde de consumidores. Além do acúmulo de resíduos nos produtos, há estudos relatando que os aditivos acarretam resistência pelos microrganismos do rúmen, o que também gera preocupação, posto que não há conhecimento dos genes ligados a esse mecanismo em bactérias resistentes a antibióticos (ALMEIDA et al., 2021). A preocupação dos técnicos em relação a estes aditivos deve-se a toxicidade, efeitos carcinogênicos, tumores gênicos ou à genotoxicidade que estes podem ocasionar (MEENAKSHI et al., 2009). Como alternativa para estes aditivos, é levantada a hipótese do uso de plantas em função da composição de compostos bioativos que possuem benefícios comprovados em estudos (TUMER et al., 2015), bem como, de sua ação como potenciais modulares ruminais (LIU et al., 2015; VASTA et al., 2019).

Biocompostos, refere-se a uma gama de produtos potenciais capazes de atuar na fermentação ruminal, e assim melhorar o desempenho animal (CALSAMIGLIA et al., 2007). Dentre os compostos encontrados em plantas, a moringa se destaca, pela sua potencialifdade em poder atuar como um modulador ruminal, por apresentar polifenóis, taninos, compostos fenólicos, saponinas e outros (MUKUNZI et al., 2011). O tanino melhora a síntese microbiana e atua na diminuição de H_2, reduzindo produção de metano (BROUCEK, 2018). As saponinas atuam como inibidoras do crescimento de protozoários ruminais, através da ação detergente que estas possuem, causando a solubilização dos lipídeos que envolvem as células, e por consequência, a morte destas (MAKKAR et al., 1998). Os flavonóides atuam como os taninos e saponinas, havendo relatos da ação de

flavonóides na redução do pH ruminal, na alteração da proporção de propionato e na degradação de proteínas (BROUDISCOU E LASSALAS, 2000; YAGHOUBI et al., 2007; BALCELLS et al., 2012; SERADJ et al., 2014). Os terpenoides possuem atividade antibacteriana no organismo (GUIMARÃES et al., 2019). Os alcaloides modificam a população microbiana do rúmen, e possuem efeito farmacológico atuando como antimicrobiano e anticancerígeno (SOLTAN et al., 2018).

Há estudos utilizando a moringa de diversas formas, apresentando resultados positivos com ruminantes em lactação. Cohen-Zinder et al. (2015) utilizaram a moringa de forma ensilada e observaram maior rendimento de leite, LCG (leite corrigido para gordura) e LCE (leite corrigido energeticamente); sendo o leite caracterizado com 20% mais de atividade antioxidante. Kholif et al. (2017) utilizaram extrato aquoso de moringa para cabras, e observaram um maior consumo de ração e melhor digestibilidade dos nutrientes bem como, um aumento da fermentação ruminal. Kholif et al. (2016) ao utilizarem folhagem de moringa em substituição a trevo Berseem em cabras, observaram melhora no consumo de ração, maior digestibilidade, melhora na fermentação e na produção, composição e perfil de ácidos graxos do leite. Sánchez et al. (2006) forneceram folhagens frescas de moringa junto ao feno de *B. Brizantha* em vacas, e observaram melhora no consumo, aumento da digestibilidade e aumento na produção de leite.

Ainda que sejam encontrados trabalhos utilizando a moringa, e um trabalho utilizando extrato aquoso em cabras leiteiras, dentro das pesquisas realizadas, não há trabalhos publicados com a utilização de extrato aquoso de moringa em ovelhas em lactação.

Inicia-se esse trabalho com uma revisão bibliográfica (Capítulo I) para evidenciar os possíveis benefícios da utilização da *Moringa oleifera* como aditivo para ovelhas em lactação. Já o capítulo II, teve por objetivo estudar o efeito do extrato aquoso de *Moringa oleifera* como aditivo e qual a sua influência na fermentação ruminal, nos parâmetros metabólicos e no desempenho de ovelhas em lactação. Tendo como hipótese, que os compostos bioativos do extrato aquoso de *Moringa oleifera* atuarão sobre os microrganismos ruminais, alterando as vias de fermentação e consequentemente melhorando o desempenho de ovelhas em lactação. No Capítulo III, teve-se como objetivo avaliar o efeito da suplementação de extrato aquoso de *Moringa oleifera* na

produção, composição, perfil de ácidos graxos no leite de ovelhas, bem como a presença de antioxidantes do leite e queijo. Neste caso, a hipótese é que o extrato atuará sobre as vias de biohidrogenação no rúmen e consequentemente no perfil lipídico do leite e queijo, bem como, na possível transferência dos antioxidantes para o leite e queijo.

CAPÍTULO I

USO DE ADITIVOS NA NUTRIÇÃO DE RUMINANTES E POTENCIAL DA *Moringa oleifera* COMO MODULADOR RUMINAL NO DESEMPENHO E METABOLISMO DE RUMINANTES

1. REVISÃO DE LITERATURA

1.1. Uso de aditivos na produção de ruminantes

A utilização de aditivos na produção de ruminantes é preconizada, devido a benefícios nutricionais relacionados a modulação ruminal. Os aditivos são fornecidos com o intuito de obter maior síntese microbiana, e alteração na formação de ácidos graxos de cadeia curta (AGCC) através da digestão da fibra. Bem como, redução na formação de metano, na degradação da proteína verdadeira do alimento, na biohidrogenação de ácidos graxos insaturados e na degradação do amido no rúmen (ZEOULA et al., 2008).

Durante a fermentação das hexoses, que são os compostos responsáveis pela liberação de energia para o animal, ocorre a liberação de hidrogênio no rúmen. Esse hidrogênio, pode ser utilizado durante a produção de ácidos graxos de cadeia curta e produção de matéria orgânica microbiana. No entanto, o excesso de produção de hidrogênio, é utilizado pelas bactérias metanogênicas e eliminado na forma de metano (BAKER, 1999).

A intensidade da liberação do metano depende de alguns fatores, como: o consumo de alimento, o tipo de animal e a digestibilidade do que é consumido. Desta forma, a quantidade de carboidrato ingerido, bem como o tipo deste, a manipulação da microbiota do rúmen, ingestão de alimento, processamento de volumoso e adição de lipídeos, são fatores que influenciam na quantidade de metano produzido no rúmen (JOHNSON e JOHNSON, 1995). Ainda pode-se incluir o uso do hidrogênio pelas bactérias no processo de biohidrogeinização dos ácidos graxos insaturados (SÁ et al., 2014).

A forma mais fácil de manipular o rúmen, é por meio de aditivos fornecidos na ração ou naturalmente presente em alimentos; sendo os estudos com aditivos focados em aumentar a eficiência da conversão dos nutrientes contidos nos alimentos em produtos como carne e leite, e reduzir o impacto que o metano causa no meio ambiente (RIBEIRO JUNIOR et al., 2011). Os aditivos são comumente conhecidos como melhoradores ruminais ou melhoradores de desempenho, e há uma gama de produtos que podem ser utilizados em ruminantes, como os ionóforos (lasalocida, narasina, monensina etc.), tampões, antibióticos não ionóforos, enzimas fibrolíticas, leveduras, lipídeos, própolis, entre outros (OLIVEIRA et al., 2005). Os ionóforos são utilizados como moduladores

ruminais há cerca de 40 anos, dentre eles a monensina é o mais utilizado (ALMEIDA et al., 2021).

A fermentação ruminal é alterada com a ação dos ionóforos, através da seleção de bactérias Gram-negativas, alterando a proporção de ácidos graxos de cadeia curta (AGCC) e quantidade de nitrogênio amoniacal no rúmen. Estes processos afetam o metabolismo de proteína e energia pelo animal (MARINO E MEDEIROS, 2015). O método de ação dos inóforos deve-se a diferença na membrana celular entre bactérias Gram-negativas e Gram-positivas. As bactérias Gram-negativas são menos susceptíveis a ação dos ionóforos, pois possuem uma dupla camada lipídica (Figura 1) composta por porina, que são canais de proteína, possuindo um tamanho de 600 Da (OLIVEIRA et al., 2005), e a maioria dos ionóforos são maiores que as porinas, o que evita a passagem dos mesmos (NAGARAJA, 1997).

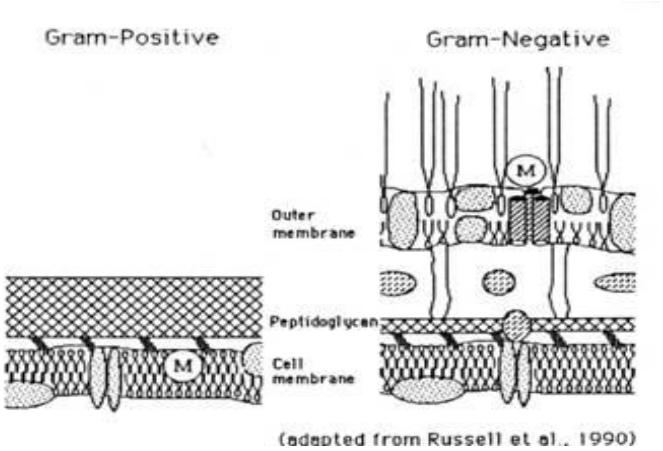

Figura 1. Diferença entre a estrutura da camada lipídica de Gram-positivas e Gram-negativas (Adaptado de Russell et al., 1990)

Ainda assim, há susceptibilidade de algumas bactérias Gram-negativas em situações de alta concentração de ionóforos (NAGARAJA, 1997). Já as bactérias Gram-positivas, não possuem essa dupla camada lipídica, tornando mais propícia a entrada da monensina (OLIVEIRA et al., 2005). A monensina possui um grupo carboxílico exposto, que geralmente é próton negativo, o que favorece a entrada da monensina nas bactérias Gram-positivas (PRESSMAN, 1976). A molécula de monensina, é basicamente um poliéster carboxílico. Essa molécula associa-se a íons metálicos que facilita a entrada através da membrana das células de bactérias Gram-positivas (PRESSMAN, 1976). A ligação dos íons metálicos e prótons, permite que a monensina atue como metal/próton antiporte (LANA E RUSSELL, 2001), ou seja, aumenta o transporte de íons de potássio

para fora da célula, causando um desbalanço de íons intracelular. O grupo carboxílico da monensina, possui uma constante dissociação (pKa) levemente alcalino, e as bactérias Gram-positivas possuem um pH mais acidificado, o que facilita a ação de inibição da monensina (CHOW E RUSSELL, 1990).

Os ionóforos causam a diminuição da degradação de proteína no rúmen, acarretando a passagem de uma proporção maior de proteína verdadeira (LANA et al., 2000). Isso é possível devido à queda da atuação do grupo de bactérias hiper-produtoras de amônia, que atuam na degradação de aminoácidos e peptídeos no rúmen (MARINO E MEDEIROS, 2015).

O aumento da amônia ruminal é consequência da degradação ruminal de peptídeos e da desaminação de aminoácidos, devido ao processo de hidrólise que acontece no rúmen (CALDAS NETO et al., 2008). As bactérias *Clostridium sticklandii*, *Peptostreptococcus anaerobicus* e *Clostridium aminophilum* estão presentes no rúmen e utilizam somente aminoácidos como fonte energética, com consequente aumento da produção de amônia, porém essas bactérias são sensíveis aos ionóforos (RUSSEL, 1987). Este autor identificou que houve uma redução de 50% na produção de amônia ruminal, concomitante ao fornecimento de monensina, devido a diminuição de 10 vezes das bactérias fermentadoras de aminoácidos, com um aumento da proteína bacteriana. No entanto, há relatos de que bactérias proteolíticas podem desenvolver resistência aos ionóforos, essa hipótese é condizente com resultados de estudos "*in vitro*" e "*in vivo*", que evidenciaram minimização da redução da amônia ao longo do tempo (VAN NEVEL E DEMEYER, 1977; WALLACE et al., 1981; WHETSTONE et al., 1981; NEWBOLD et al., 1990).

Os ionóforos atuam na retenção de energia fermentada no rúmen, causando a maior proporção de propionato em relação ao acetato, causando diminuição de perda energética através do metano (SALMAN et al., 2006). Os ionóforos não atuam diretamente contra as bactérias metanogênicas (archaea), mas atuam na diminuição de H_2 e formato, devido ao aumento de propionato, sendo realizado o redirecionamento do hidrogênio que seria utilizado na produção de metano (OLIVEIRA et al., 2005).

Outro benefício da utilização dos ionóforos é a diminuição de distúrbios metabólicos, como o timpanismo e acidose, pois este aditivo atua diminuindo a concentração de ácido lático e na menor produção de mucopolissacarídeos que estabilizam a espuma, sendo as bactérias produtoras de metano, as maiores responsáveis

pela produção destas substâncias (ZANINE et al., 2006; MARINO E MEDEIROS, 2015). Bactérias produtoras de ácido lático (*Streptococcus bovis* e *Lactobacillus spp*.) são Gram-positivas, logo, susceptíveis a ação de ionóforos. Desta forma a monensina é utilizada na redução de incidência de acidose lática (NAGARAJA et al., 1997).

Há uma adaptação gradativa dos microrganismos do rúmen a alguns ionóforos (ALMEIDA et al., 2021). Essa adaptação faz com que a resposta ao uso da monensina reduza, diminuindo os efeitos sobre a produção de metano (CALLAWAY et al., 2003). E essa resistência aos aditivos, causa preocupação à saúde pública, uma vez que não há identificação dos genes ligados a esse mecanismo em bactérias resistentes a antibióticos (ALMEIDA et al, 2021). Assim, os consumidores podem ser expostos a bactérias resistentes por meio do consumo de produtos de origem animal. Havendo evidências que a carne e leite, contém quantidades altas de bactérias e seus genes de resistência (MARSHALL E LEVY, 2011). Há alguns estudos que evidenciam o aumento de bactérias resistentes a antibióticos entre animais de produção (CABELLO, 2006; LEE, 2003; PERRETEN, 2005; WITTE, 2000).

De acordo com Yokoyama et al. (1985), os ionóforors além de afetar a fermentação ruminal, podem ter efeito sobre a microbiota intestinal e o metabolismo do animal, pois boa parte da monensina suplementada pode ser absorvida no intestino. A monensina que escapa do rúmen, é transportada para o intestino, onde parte pode ser absorvida e metabolizada pelo animal ou ser excretada nas fezes (DONOHO, 1984). O que é absorvido, pode ser empregado no produto final, como carne e leite, o que é um problema, visto por consumidores (RIBEIRO, 2014). Todo o produto que é passível de deixar resíduo no produto final pode causar problemas de toxicidade a saúde humana, sendo estabelecido um limite de máximo de resíduo (LMR) pela Comissão do Codex Alimentarius (órgão da OMS) (SALMAN et al., 2006). De acordo com Palermo Neto (1998), o LMR de monensina é de no máximo 15 µg/kg. Ainda que haja muitos benefícios de se utilizar aditivos na nutrição de ruminantes, há estes relatos, que o mesmo deixa vestígios no produto final, o que gera preocupação nos consumidores, que estão cada vez mais exigentes quanto a qualidade dos produtos que consomem (JOUANY E MORGAVI, 2007).

1.2. Aditivos naturais na produção de ruminantes

Há um interesse dos consumidores de se obter alimentos cada vez mais saudáveis e que não causem riscos à saúde. Devido aos aditivos, comumente utilizados na nutrição de ruminantes, serem passiveis de deixar resíduos no produto final a utilização de extratos vegetais na alimentação animal vem ganhando destaque. Em função disto, existem diversos estudos avaliando aditivos naturais em substituição aos ionóforos, permitindo maior qualidade e segurança alimentar para o produto final (AGUIAR, 2009).

Os aditivos, além de atuarem no processo fermentativo do rúmen, também possuem como função a manutenção da saúde do intestino, com atuação na absorção de nutrientes ingeridos, e/ou que são produzidos no rúmen (OLIVEIRA et al., 2019). E devido as recorrentes preocupações da população no mundo todo em relação as superbactérias resistentes a determinados medicamentos e com a necessidade relevante de alternativas para os aditivos comumente utilizados, na pecuária como um todo, que podem gerar resíduos no produto final, surgiram os aditivos naturais como potenciais substitutos (FLEES et al., 2021).

Antibióticos vêm exercendo sua função no desempenho de animais de produção, de forma crucial, desde 1920 (CASTANON, 2007). Entretanto, devido a resistência antimicrobiana que surgiu como um alerta à saúde humana, aumentando a conscientização pública (MARSHALL E LEVY, 2011; TANG et al., 2017), fato que levou a União Europeia e os Estados Unidos a proibirem o uso de antibióticos na produção de animais de produção, em 2006 e 2017, respectivamente (CASTANON, 2007; TANG et al., 2017). No Brasil, há restrições na utilização de antibióticos, mas os promotores de crescimento ainda podem ser utilizados para melhorar o desempenho de animais de produção. Porém, como há restrição em alguns países, há a necessidade de adequação dessas substâncias que possam substituir estes antibióticos (FERREIRA E ASTOLFI-FERREIRA, 2006).

Alguns biocompostos presentes nas plantas, tem comprovado sua eficiência como moduladores da fermentação ruminal (LIU et al., 2015; VASTA et al., 2019). Estes compostos possuem vários princípios ativos e modos de ação antimicrobiana, oferecendo baixo risco e resistência bacteriana, apresentando vantagens em relação a aditivos químicos (ACAMOVIC e BROOKER, 2005), atuando como potenciais substitutos (FLEES et al., 2021). Além disso, os aditivos naturais possuem muitos compostos com

capacidade antioxidante e alta atividade antimicrobiana que podem beneficiar a saúde animal e humana (NIKMARAM et al., 2018).

Os biocompostos naturais de plantas, são metabólitos vegetais, não nutritivos que exercem funções importantes nas plantas, como a proteção contra organismos herbívoros, microrganismos, insetos e patógenos (BODAS et al., 2012). A extração destes biocompostos e utilização como aditivos naturais na alimentação animal é uma ótima opção, visto que não acumulam resíduos químicos no produto final (carne ou leite), e que não representam risco a saúde dos consumidores (FLEES et al., 2021). Há uma gama de plantas, que podem ser fornecidas ou utilizadas para produzir extratos que atuam como aditivos naturais para os ruminantes.

A premissa de se utilizar extratos naturais de plantas, é a mesma que utilizar aditivos químicos na produção animal, para modificar a fermentação ruminal e melhorar o aproveitamento do alimento, aumentando a eficiência de produção. Exemplos de compostos que podem atuar na modificação ruminal, são os taninos e as saponinas (RIBEIRO JUNIOR et al., 2011). Esses compostos, assim como outros aditivos, devem ser utilizados em doses adequadas, uma vez que o excesso destes, podem prejudicar a saúde animal e causar efeitos adversos na população microbiana ruminal (RIBEIRO JUNIOR et al., 2011).

A utilização de extratos fitoquímicos como aditivos possui benefícios satisfatórios, no entanto, deve-se ter cuidado com a dosagem utilizada, e, com a pureza do extrato, pois os extratos podem apresentar teores antinutricionais, e deve-se ter um equilíbrio entre a redução do metano e a utilização dos nutrientes pelo animal (ALMEIDA et al., 2021). O equilíbrio para esse processo acontecer de forma saudável, é complexo, uma vez que as plantas possuem concentrações de compostos diferentes, e entre a mesma planta, pode ocorrer diferenças, dependendo da época de colheita, local etc. E a presença de fitoterápicos nos extratos naturais, é dependente da forma de extração, como a temperatura, umidade do ar e tempo, bem como, pelo solvente utilizado na extração. Há mais de 200.000 compostos secundários nas plantas (HARTMANN, 2007), o que explica a complexidade de se ter um equilíbrio, no fornecimento de extratos para animais.

1.3. Efeitos de aditivos naturais no rúmen

O tanino, é um composto biológico geralmente encontrado nas cascas, sementes, flores e frutas (PING et al., 2012; BRAGA et al., 2018; SARTORI et al., 2018; TENG et al., 2019); sendo encontrado em elevadas proporções nos constituintes das plantas (SOUZA et al., 2019). São compostos fenólicos solúveis em água e precipitadores de proteínas (SILVA e SILVA, 1999; LIMA et al., 2010). Estes compostos, além de precipitadores, também possuem capacidade estabilizadora, sendo divididos em taninos condensados e taninos hidrolisáveis (SILVA et al., 2020). Os taninos hidrolisáveis são estruturados por glicoses centrais ou polióis ligados a uma ou mais frações gálicas ou elágicas (SHIMOZU et al., 2017), sendo facilmente hidrolisadas com ácidos, bases ou enzimas (GRASEL et al., 2016b). Os taninos condensados normalmente são ligados covalentemente à catequina e epicatequina (CHAI et al., 2018), sendo compostos classificados quimicamente como polímeros de flavonóides (ASHOK e UPADHYAYA, 2012). A solubilidade dos taninos condensados é devido a forma oligomérica, que é solúvel em água, porém, quando estão na estrutura de flavonóides são insolúveis em água (MARTINS et al., 2020). Ambas as formas possuem grande capacidade de complexação, o que os caracteriza como potenciais inibidores de enzimas (SILVA e SILVA, 1999), e portanto, como aditivos na nutrição de ruminantes (GRAINGER et al., 2009; GRIFFITHS et al., 2013; Brito et al., 2013).

Os taninos atuam na diminuição de produção de H_2, e assim, reduz produção de metano (BROUCEK, 2018). Os taninos são compostos por polifenóis, com diversos pesos moleculares e complexidade, sendo substâncias que devem ser utilizadas moderadamente, para ter efeito benéfico. O uso irracional pode resultar em efeitos adversos, dependendo da espécie e estado fisiológico do animal, ou dieta fornecida (KUMAR E SINGH, 1984). Porém, os efeitos negativos são resultantes da ingestão de alta quantidade de taninos pelos animais, se o consumo for moderado há impactos positivos (AGUERRE et al., 2016).

As saponinas são outro grupo de compostos encontrados em plantas, que agem como modulador ruminal, e atuam como inibidor do crescimento de protozoários ruminais. Este composto possui ação detergente, e pode ser responsável pela morte dos protozoários no rúmen (MAKKAR et al., 1998). A saponina solubiliza os lipídeos que envolvem os protozoários, causando alteração na sua permeabilidade e consequente morte da célula (KLITA et al., 1996; WALLACE et al., 1981). Os protozoários são

responsáveis por boa parte da liberação de nitrogênio no rúmen, o que acarreta o aumento da amônia ruminal e diminui o fluxo de nitrogênio microbiano para o duodeno (RIBEIRO JUNIOR et al., 2011). Os protozoários beneficiam a metanogênese, uma vez que produzem hidrogênio quando ocorre a fermentação de carboidratos, servindo de cápsula para cerca de 30% das *Archaeas* produtoras de metano (JOUANY, 1996).

Devido a ação detergente das saponinas, essa leva, a redução da amônia, aumento da utilização do nitrogênio da dieta, mudança no perfil de ácidos graxos de cadeia curta, aumento da síntese microbiana e com todas essas características, consequentemente, a redução de formação de metano (RIBEIRO JUNIOR et al., 2011). A alteração do perfil dos ácidos graxos de cadeia curta, é umas das principais ações da saponina, devido ao aumento da proporção de propionato e diminuição na proporção acetato:propionato (RIBEIRO JUNIOR et al., 2011). A diminuição do acetato e butirato, e o aumento do propionato, se dá pela diminuição da atividade fermentativa dos protozoários (JOUANY, 1994).

Hess et al. (2003), observaram redução de 54% na contagem de protozoários e redução de 20% na produção de metano, num experimento *in vitro* utilizando a fruta tropical *Sapindus saponária* na proporção de 100 mg/g, rica em saponina. Wang et al. (1998), também realizando um experimento *in vitro*, observaram uma redução de 15% na produção de metano com a utilização de extrato de *Yucca schidigera*, na proporção de 4,4% de saponina com a inclusão de 0,5 mg ml^{-1} na solução tampão, em relação ao grupo controle. De acordo com Ribeiro Junior et al. (2011) há dificuldade em se obter resultados concretos quando utiliza-se saponina como modulador da fermentação ruminal, uma vez que há uma diversidade alta da estrutura destes compostos, o que depende da espécie vegetal utilizada, podendo se encontrar uma gama de diferentes saponinas em uma mesma planta. Desta forma há a necessidade de mais estudos para avaliar a real eficácia na fermentação ruminal com a utilização de saponinas.

Os flavonóides, assim como os taninos e as saponinas, são compostos que podem atuar na modulação ruminal, e assim como as saponinas, podem ser encontradas muitas variações de flavonóides, em uma mesma planta, o que dificulta a determinação de resultados mais precisos, quanto ao efeito dos flavonóides no organismo (LIN et al., 2018). Alguns estudos, avaliados *in vitro* e *in vivo*, verificaram que extratos vegetais contendo flavonóides, atuaram sobre a redução do pH ruminal, na proporção de

propionato e degradação de proteínas (BROUDISCOU E LASSALAS, 2000; YAGHOUBI et al., 2007; BALCELLS et al., 2012; SERADJ et al., 2014). Os flavonóides podem modificar o produto da fermentação, bem como, a concentração e a composição dos microrganismos que consomem ácido lático, como a bactéria *Megasphaera elsdenii*, e as *Archaeas* (SERADJ et al., 2014), o que pode resultar na diminuição da formação de gases, como o metano. Patra e Saxena (2010) observaram ações diretas dos flavonóides, sobre metanogênicas, e protozoários relacionados a produção de metano no rúmen. Kim et al. (2015) constataram que os flavonóides não interferem no crescimento microbiano, mas reduzem a produção de metano, podendo ser um potencial regulador da fermentação ruminal em animais de produção.

Os terpenoides, também conhecidos como isoprenoides, possuem atividade antibacteriana, possuindo interação com a membrana celular, essa interação causa modificações na estrutura da membrana das bactérias, ocasionando em fluidificação e expansão (GUIMARÃES et al., 2019). Essa modificação torna a membrana instável, resultando no vazamento de íons pela membrana celular, causando diminuição no gradiente iônico transmembranar (GRIFFIN et al., 1999; CALSAMIGLIA et al., 2007). As bactérias podem combater esses efeitos que os terpenoides causam na membrana, no entanto, há um gasto alto de energia para essa atividade, e assim, ocorre um crescimento bacteriano lento. Essa taxa de crescimento das bactérias, tem por consequência a menor taxa da população de determinados microrganismos em ruminantes, alterando o poder fermentativo no rúmen, e com possível consequência na melhora do desempenho animal (CALSAMIGLIA et al., 2007).

Os alcaloides podem modificar a população microbiana ruminal e por consequência, melhorar o desempenho animal (SOLTAN et al., 2018), devido a composição de nitrogênio que estes compostos possuem, e pelo seu efeito farmacológico que atua como antimicrobiano e anticancerígeno (THAMBIDURAI et al., 2017). Os alcaloides piperidínicos atuam com alta citotoxicidade nas bactérias Gram-positivas, bloqueando os canais de cálcio na membrana da célula (SANTOS et al., 2013). Estes alcaloides também possuem propriedades anfotéricas, que permitem uma melhor interação com a membrana celular e também inibindo seus canais (SANTOS et al., 2013). Devido a essas atividades os alcaloides podem atuar na modulação da fermentação ruminal (OLIVEIRA, 2020). É possível que os aditivos alternativos não abordem todos

os patógenos bacterianos contra os quais os aditivos comumente utilizados englobam. Ainda que seja uma limitação, podem acarretar menores efeitos colaterais (GEBREYES et al., 2017).

Os compostos fenólicos de modo geral, atuam de diferentes formas no organismo do animal, onde, compostos de baixo peso molecular, são mais ativos na população microbiana do rúmen, diferindo nos mecanismos que envolvem a atividade antimicrobiana (BODAS et al., 2012). Os mesmos autores citam que os efeitos de fitoterápicos nos microrganismos do rúmen modificam a produção de amônia e AGCC.

A busca por melhor sustentabilidade na produção de ruminantes inclui a eficiência alimentar como pilar, bem como, a redução da emissão de metano (BEAUCHEMIN et al., 2020), objetivando a melhora da alimentação e manejo animal (BAYAT et al., 2021). Estudos demonstram que extratos vegetais melhoram o desempenho animal, com principal ação na redução de emissões de metano (YANG et al., 2007; KOLLING et al., 2018; OLIJHOEK et al., 2019). Beauchemin et al. (2020) citam que diversas estratégias alimentares podem influenciar na redução de metano, variando de 0 a 80%, no entanto, são estratégias onerosas, com disponibilidade limitada em alguns casos e com efeitos muito variáveis. Deste modo, os extratos vegetais podem atuar de forma satisfatória e combater estas limitações.

Há muitas evidências que os extratos naturais podem atuar como moduladores ruminais, diminuindo a produção de metano ou alterando a degradação de compostos nitrogenados. No entanto, o efeito de extratos de plantas é depende de diversos fatores, como a época de colheita da planta, a espécie, a forma de extração, pois isso irá influenciar na concentração de compostos no extrato. E por fim, outro fator que influencia a ação dos compostos no rúmen, é a dosagem fornecida aos animais (OLIVEIRA, 2020).

1.4. Potencial da moringa como aditivo para ruminantes

A *Moringa oleifera* é muito utilizada como fonte de alimento, em regiões tropicais e subtropicais, devido suas propriedades e benefícios nutricionais, antioxidantes e fitoquímicos inerentes, também por sua resistência de sobreviver em condições climáticas diversas (FALOWO et al., 2018). Todas as partes da planta da moringa, são comestíveis e contêm compostos que são importantes para o bem-estar de humanos e são funcionais

para animais de produção (KADHIM e AL-SHAMMAA, 2014), havendo possibilidade de utilizar a planta de diversas formas com possíveis resultados positivos.

A moringa é uma árvore tradicional que tem mostrado potencial em complementar e alterar medicamentos de uma forma natural (TILOKE et al., 2013). Tradicionalmente, a *Moringa oleifera* é utilizada para o tratamento de hiperglicemia, inflamação, infecções bacterianas/virais e câncer, devido ao teor de antioxidantes e compostos bioativos que possuem eficácia na medicina (TUMER et al., 2015).

A *Moringa oleifera* é muito utilizada na alimentação animal, no mundo todo, pois possui produção elevada de biomassa, alto valor nutricional, benefícios fitoquímicos e antioxidantes (FALOWO et al., 2018; DONG et al., 2019). Os benefícios fitoquímicos, que são basicamente, a composição de compostos com funções benéficas no organismo do ser humano ou do animal, podem ter ação antioxidante, alti-helmíntica, anticoccidiana e ação antibacteriana (SALEM et al., 2014; PEDRAZA-HERNÁNDEZ et al., 2019). Os microrganismos ruminais podem utilizar os compostos advindos da moringa e utilizar como fonte de energia, possuindo o benefício de não acumular resíduos no produto final (SALEM et al., 2014, PEDRAZA-HERNÁNDEZ et al., 2019).

A *Moringa oleifera* possui grande potencial para ser utilizada como modulador ruminal em ruminantes, pois possui uma gama de compostos metabólicos, que utilizados de forma controlada, podem impactar na fermentação ruminal (SALEM et al., 2014; PEDRAZA-HERNÁNDEZ et al., 2019). Há estimativa da moringa possuir mais de 200 compostos nas suas diferentes partes, como a folha, caule, raiz e sementes. Estes compostos são divididos em hidrocarbonetos, cetonas, ácidos graxos, álcoois, aldeídos, terpenos (FALOWO et al., 2018), ácido ascórbico, flavonóides, fenólicos e carotenóides (ANWAR et al., 2005; MAKKAR E BECKER, 1996). Mukunzi et al., (2011), ressalta o alto teor de polifenóis que a moringa possui.

Al-Juhaimi et al. (2019) ao utilizarem feno de alfafa, e folhas de moringa (*Moringa peregrina Forssk* e *Moringa oleifera Lam.*) secas, moídas e transformadas em pellets para cabras, e observaram maior teor de gordura do leite com a dieta de *Moringa oleifera Lam*. Os teores de sólidos totais e energia do leite foram menores com a dieta de *Moringa peregrina Forssk.* Quanto ao teor oxidante do leite, os mesmos autores observaram que o alto teor de fenólicos totais da moringa melhorou a saúde das cabras, preveniu a oxidação e assim, aumentou o tempo de vida útil de produtos feitos com o

leite. Segundo Mbikay (2012) fala que as folhas da moringa podem ser utilizadas para eliminar os radicais livres devido a sua alta atividade antioxidante.

1.5. Efeito da moringa no metabolismo de ruminantes

Parte dos aditivos químicos fornecidos aos animais pode passar de forma intacta pelo rúmen a atuar também no intestino e no metabolismo pós-absortivo (YOKOYAMA et al., 1985). Acredita-se que o mesmo ocorra com os aditivos naturais, pois há relatos de biocompostos nos produtos finais, porém não há relatos dos efeitos pós-ruminal para aditivos naturais. Desta forma, os aditivos que escapam do processo de fermentação ruminal terão ação a nível intestinal ou do metabolismo animal.

Compostos fitogênicos possuem propriedades antimicrobianas e anti-helmínticas, que podem melhorar o aproveitamento do alimento, havendo influência no produto final (VALDES et al., 2015).

Babiker et al. (2017) estudaram a moringa fornecida de forma peletizada em substituição parcial ao feno de alfafa e observaram que a moringa possui alto teor de compostos fenólicos e antioxidantes, e isso pode combater a oxidação de certos nutrientes no organismo do animal, melhorando o desempenho e saúde animal. Segundo Sreelatha e Padma (2009), a moringa possui um grande potencial de atividade antioxidante contra radicais livres, prevenindo a oxidação de biomoléculas.

Babiker et al. (2017) observaram redução no teor de colesterol e glicose nas ovelhas e cabras alimentadas com moringa, podendo ser atribuído ao efeito funcional dos compostos fenólicos e a atividade antioxidante da moringa. Segundo Saxena et al. (2013) a síntese e absorção do colesterol podem diminuir quando há presença de fitoquímicos es antioxidantes. Já em relação a glicose, Iqbal et al. (2012), explicam que é um dos teores precursores para a síntese de colesterol no intestino delgado ou fígado.

Babiker et al. (2017) observaram aumento no malonaldeído no sangue e no leite de ovelhas, podendo este fato ser devido à alta atividade catalase do soro que previne a degradação de hidroperóxidos de malonaldeído. De acordo com Nascimento et al. (2013) o malonaldeído, entre outros aldeídos, são os produtos finais da peroxidação lipídica, sendo o malonaldeído reconhecido como um biomarcador geral altamente danoso para o organismo.

A moringa possui alto teor de terpenóides, antraquinonas e glicosídeos (GOPALAKRISHNAN et al., 2016). Os terpenóides possuem ação antimicrobiana, sendo um potente composto a favor do controle de populações microbianas para indústria farmacêutica, e também possuem ação antioxidante, que combatem os radicais livres e inibem a peroxidação lipídica, inibem a modificação de proteínas e os danos ao DNA (SAITO et al., 2004; TOSCAN, 2010). A antraquinona em pequenas quantidades atua como laxante e em grandes quantidades atua como purgativo (LEÃO, 2015). Esse composto é empregado terapeuticamente como catártico, por irritar o intestino grosso, que aumenta a mobilidade intestinal, e diminui a absorção de água (IZZO et al., 1999). A antraquinona também age como antioxidante e captadora de radicais livres, podendo apresentar atividade antiprotozoária (LEÃO, 2015). Os glicosídeos, por exemplo, são compostos antimicrobianos naturais, ao qual, seu efeito biológico depende da concentração e tipos estruturais, podendo ser tóxicos, antinutricionais ou benéficos para saúde dependendo da concentração ingerida (FAHEY et al., 2001; GUIL-GUERRERO et al., 2016).

Desta forma, aditivos naturais possuem muitos benefícios aos animais de produção, e concomitantemente, aos consumidores de produtos derivados de animais. Assim, há possibilidade de que, aditivos naturais fornecidos a animais ruminantes, e que não sofram influência da fermentação ruminal, tragam benefícios a nível intestinal e assim, participem do metabolismo pós fermentativo destes animais.

1.6. Efeito da moringa na produção de leite

Os alimentos saudáveis e produtos naturais são foco de interesse devido a melhora do bem-estar estar de uma forma geral que estes fornecem, tal como na prevenção de doenças e na adição de substâncias promotoras da saúde em dietas com inclusão de aditivos naturais (ALENISAN et al., 2017).

Um dos benefícios da utilização de aditivos naturais é devido o potencial antioxidante que este pode incorporar ao leite (MARLES E FARNSWORTH, 1995; PRASAD et al., 2012). Há uma grande preocupação dos consumidores por alimentos em que foram adicionados antioxidantes sintéticos. Desta forma, há recomendações para utilização de antioxidantes de fontes naturais, ao invés de antioxidantes sintéticos que

possuem restrições quanto aos efeitos tóxicos e cancerígenos (ZAMBONIN et al., 2012; ABDEL-HAMEED et al., 2014). Marles e Farnsworth (1995), realizaram uma revisão com diversos estudos que avaliaram o consumo frequente de antioxidantes de fontes naturais em humanos, e a influência que isso causou, como a diminuição da incidência de determinados tipos de câncer, hipertensão, diabetes e doenças cardiovasculares, principalmente em países em desenvolvimento, onde boa parte da população possui recursos limitados e acesso restrito a tratamentos modernos. Produtos derivados do leite, bem como, o leite em si, é um dos alimentos mais completos que existem, possuindo grande potencial de atividade antioxidante, devido a presença de moléculas com esta ação, como caseína e proteínas do soro do leite (PIHLANTO, 2006; SUETSUNA et al., 2000).

Como o leite possui atividades com potencial para agir no combate da oxidação aos radicais livres, e uma forma de potencializar essa ação no organismo dos animais, é o fornecimento de plantas ricas em antioxidantes naturais e compostos fenólicos que são progressivamente aplicados na fabricação de alimentos lácteos, como forma de melhorar as características nutricionais e terapêuticas (SHORI E BABA, 2013; SHORI E BABA, 2014; KARAASLAN et al., 2011; MARTINS et al., 2014; BERTOLINO et al., 2015). Os antioxidantes naturais, derivados de plantas, podem controlar a formação desencadeada de radicais livres, com consequência no aumento da capacidade antioxidante, podendo também, substituir antioxidantes sintéticos com efeitos colaterais, como impactos no fígado e ação carcinogênica (MEENAKSHI et al., 2009).

Outro potencial da utilização de aditivos naturais em animais de produção é a modificação no perfil lipídico ou características químicas do leite. A utilização de extrato aquoso de *Moringa oleifera* em cabras leiteiras, melhorou a concentração de ácidos graxos de cadeia curta, com possível influência do aumento da digestibilidade, recorrente da utilização do extrato aquoso (KHOLIF et al., 2017). Sendo o ácido propiônico produzido em maior proporção no rúmen, o que é considerado benéfico para produção de leite (KHOLIF, et al., 2016). Devido o ácido propiônico ser o principal ácido graxo de cadeia curta gliconeogênico, fundamental para a biossíntese da lactose (LINN, 1988).

Kholif et al. (2017) obtiveram uma maior concentração de albumina sérica com a inclusão de extrato aquoso de moringa, podendo indicar a melhora do estado nutricional e fisiológico das cabras.

Este fator pode ter sido influenciado pelo aumento de ingestão de proteína bruta e digestibilidade da matéria orgânica (KHOLIF et al., 2015). Desta forma, é possível verificar que a utilização de aditivos naturais traz respostas no metabolismo e influencia a qualidade do produto final.

A parcial substituição do feno de alfafa pela folha de moringa atrasou a diminuição da lactação em ovelhas e cabras, sendo possível que a alta degradação proteica das folhas de moringa, tenham influenciado esse resultado, já que a degradação da folha de moringa é comparável ao farelo de soja, que levam a um melhor aproveitamento do alimento pelos animais (BABIKERA et al., 2017). Zarkadas et al. (1995), explica que esse prolongamento da fase de lactação é devido a alta concentração de aminoácidos que a moringa possui.

Babikera et al. (2017) obtiveram aumento no valor malonaldeído no leite de ovelhas e cabras alimentadas com folha de moringa, podendo ser explicado pelo alto teor de fenólicos da moringa, o que impede a oxidação lipídica do leite, limitando a formação desse composto. Outro fator positivo da folha de moringa, foi o aumento da vitamina C no leite de ovelhas e cabras, pois o leite destas duas espécies é pobre nessa vitamina.

Este fator pode ser pela presença de macro e microminerais adequados, e também por vitaminas contidas nas folhas de moringa, que aumentam a o crescimento e atividade microbiana do rúmen, melhorando assim, a qualidade do leite (GEBREGIORGIS et al., 2012).

1.7 Influência da *Moringa oleífera* na produção de queijos

A valorização da produção de leite ovino está geralmente relacionada com a produção de queijos, e isto se dá por dois aspectos, primeiro pelo rendimento queijeiro do leite de ovelha ser elevado comparativamente com o leite de outras espécies e em segundo pela excelente aceitação do consumidor. A produção leiteira é influenciada em quantidade e qualidade de constituintes e rendimentos queijeiros conforme o grupo racial utilizado e o desenvolvimento e aplicação de técnicas que aperfeiçoem o manejo e alimentação. Em relação a alimentação, as folhas da moringa apresentam grande potencial como forrageira, pois são ricas em proteína e aminoácidos solúveis (BAKKE et

al., 2010), podendo melhorar a síntese microbiana no rúmen, auxiliando no aumento de proteína do leite, e consequentemente do queijo.

Gerônimo e Gomes (2015), avaliaram a inclusão de extrato aquoso de *Moringa oleifera* diretamente na massa de queijos tipo Minas Frescal, e concluíram que a moringa melhora a característica de umidade desse tipo de queijo, conferindo maior frescor, o que melhora a qualidade do queijo tipo Minas Frescal, inclusive perante a legislação.

Além da composição química das folhas da moringa serem de boa qualidade, também possuem alto teor de antioxidante (YANG, 2006; SAINI et al., 2014), sendo o teor de fenólicos mais substancial, dentre os antioxidantes encontrados na planta (YANG, 2006). A moringa também é rica em composição de taninos (PINA et al., 2018), composto antioxidante que possui diversos benefícios para saúde, quando em quantidades ideias. Santillo et al. (2022) utilizando tanino na alimentação de vacas leiteiras, observaram um aumento da atividade antioxidante nos queijos produzidos com o leite das mesmas, concluindo que as moléculas antioxidantes da dieta podem modificar as do leite e consequentemente, dos queijos, conferindo valor nutricional agregado aos produtos lácteos. O efeito antioxidante atribuído a moringa, por seus diversos constituintes, pode ser vantajoso no aumento do tempo de prateleira de queijos de menor teor de cura ou com maior concentração de ácidos graxos trazendo estabilidade para estes quanto a uma possível rancificação. As folhas da moringa foram estudadas quanto a qualidade de estabilidade oxidativa comparada com antioxidante sintético comumente utilizado, e se mostrou semelhante com esse produto (REDDY et al., 2005).

A moringa possui benefícios na qualidade de queijos, e tanto pode ser utilizada diretamente na massa dos queijos (GERÔNIMO E GOMES, 2015), quanto via alimentação ou suplementação para ovelhas produtoras de leite (MENCI et al., 2021a), com o objetivo de melhorar o teor nutricional e a atividade antioxidante. Sendo necessário mais estudos com a avaliação da moringa, nessas duas formas de administração, para efetiva conclusão do benefício da moringa na produção de queijos produzidos com leite ovino.

Para produção de queijos é necessário a qualidade em passos básicos e importantes que são realizados, como a qualidade da coagulação do leite. Esse processo se dá pelo rompimento das micelas de caseína, que representam cerca de 80% das proteínas contidas no leite. As caseínas-κ são partidas pelo coagulante utilizado na produção, e liberam as

caseínas α e β, incluindo também, as partículas de para-κ-caseinato, que são precipitadas, e formam o famoso coágulo. O coagulante utilizado nesse processo, é crucial, pois este pode ser responsável pelo alto nível de degradação proteica, o que pode conferir menor tempo de prateleira, sabor amargo e menor rendimento do queijo (FOX e LAW, 1991; FOX, 1997). A quimosina é o principal coagulante utilizado na manufatura de queijos, no entanto, devido a alta produção, este produto tem se tornado escasso e oneroso (SOLORZA-FERIA et al., 2011). Com isso, há pesquisas (UCHIKOBA e KANEDA, 1996; ASAKURA et al., 1997; LO PIERO et al., 2002) que avaliaram a utilização de compostos de plantas naturais como potenciais coagulantes que podem servir como alternativa à quimosina. Desta forma, os compostos da moringa, além de possuírem potenciais antioxidantes que podem melhorar a vida útil e qualidade de queijos, podem auxiliar no processo de concentração e estabilidade da caseína, para um processo adequado de coagulação.

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CAPÍTULO II

EXTRATO DE *Moringa oleifera* COMO MODULADOR NA FERMENTAÇÃO RUMINAL E O DESEMPENHO DE OVELHAS EM LACTAÇÃO

Capítulo submetido em Journal of Dairy Science

INTERPRETIVE SUMMARY: *Moringa* extract to modulate rumen fermentation and lactation performance of ewes

By Chagas et al. Aqueous extract of *Moringa oleifera* can work as a natural rumen modulator, replacing chemical additives commonly used in ruminant production. Natural additives are beneficial for not accumulating residues in the final product that would cause harm to human health. Moringa compounds act directly on rumen fermentation, altering short chain fatty acids production, decreasing methane production, and promoting microbial protein synthesis, which can change milk composition.

Running head: Moringa extracts on ewe performance

Moringa extract to modulate rumen fermentation and lactation performance of ewes

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ABSTRACT

This study aimed to evaluate the supplementation of aqueous extract of *Moringa oleifera* (**AEMO**) as a natural ruminal modulator to improve lactation performance of ewes. Twelve ewes were used in replicated 3 × 3 Latin square, with periods of 14 days (assessments on the last five days of each period). Treatments were: 20 ml of water as Control, 20 ml of aqueous extract (**20-AEMO**), and 40 ml of aqueous extract (**40-AEMO**). Ewes were milked twice a day (7:30 am and 2:30 pm). Diet corresponds to concentrate (at 3% of BW) and hay *ad libitum*. Was determined the intake, fermentative parameters, digestibility, metabolic parameters, and milk production and composition. Intake and digestibility were not affected by AEMO. Milk production and the components fat, protein and lactose were lower when ewes were supplemented with 20-AEMO; protein production was lower when the highest extract level (40-AEMO) was used. Ruminal pH did not differ among treatments, but there was a tendency to reduce acetate, increase propionate, hence reducing methane production in 40-AEMO. Blood and urinary parameters were not affected by AEMO. Inclusion of moringa extracts as an additive in lactating ewes diet does not affect intake and nutrients digestibility, but affect ruminal fermentation and microbial synthesis, altering methane emission, metabolizable protein, and consequently milk production. Therefore, it's recommended studies with different extract concentrations to investigate possible effects on rumen fermentation and synthesis of milk compounds.

Key words: digestibility, fermentation, methane, natural additives

1. INTRODUCTION

Ionophores act on the rumen microorganism population favoring its proper functioning (Alves et al., 2019). This chemical additive is commonly used in animal production and affects Gram-positive bacteria, allowing most of the substrate to be used by Gram-negative bacteria (Marino and Medeiros, 2015), which results in higher propionic acid production, increasing the energy supply for the animal (Asmare, 2014). Consequently, the ionophore action considerably decreases methane and carbon dioxide emissions (Almeida et al., 2021), reducing the environmental impact caused by these gases. In addition, ionophores decrease acidosis, as the rumen pH remains more stable due to reduced hydrogen release (Callaway et al., 2003). However, the continuous use of ionophore in the animal diet can cause long-term problems such as: microorganism resistance in the animal (Almeida et al., 2021); residues in meat and milk that can bring microorganism resistance to the human being (Jouany and Morgavi, 2007); and mainly the possible accumulated residues eliminated via excreta in the environment (Blackwell et al., 2007).

In view to increase sustainably in the ruminant production, natural additives are an excellent alternative to replace antibiotics and ionophores. Plants rich in fatty acids (Aly et al., 2016), vitamins (Glover-Amengor et al., 2017), and bioactive compounds that have antimicrobial action (Valdes et al., 2015) have the potential to be used for natural additive production. *Moringa oleifera* and its extracts have these characteristics, thus it has been studied as an option to improve animal performance and efficient use of dietary nutrients (Cohen-Zinder et al., 2016; Kholif et al., 2018a). All parts of Moringa are edible and can be used in several ways (Giuberti et al., 2021).

Studies report the benefits of using different parts of Moringa on ruminant performance (Sultana et al., 2015; Kholif et al., 2018a; Parra-Garcia et al., 2019). Extracts from Moringa leaves were evaluated through in vitro assays and characterized as promising strategies to increase quality of ruminant products and health-related characteristics (Giuberti et al., 2021). Aqueous extract of *Moringa oleifera* (**AEMO**) (125 g of DM/L) improved animal performance of lactating goats receiving 10, 20, or 30 ml of AEMO, in synergy with the increased digestibility (Kholif et al., 2018a). Oral administration of AEMO in sheep

had beneficial effects against *Fasciola gigantica* and *Clostridium novyi*, common parasite and bacteria found in the gastrointestinal tract that causes infection (Shanawany et al., 2019).

However, based on available knowledge, few studies are reporting the effect of AEMO on lactating ewe performance. Thus, sought to evaluate the effect of AEMO as an additive on rumen fermentation, metabolic parameters, and performance of lactating ewes. The hypothesis was that AEMO compounds will act on ruminal microorganisms, altering the fermentation pathways, hence improving the lactating ewe performance.

2. MATERIALS AND METHODS

The experiment was carried out at the Federal University of Grande Dourados, located in Dourados-MS, Brazil (22°11'55" S, 54°56'7" W, and 452 m altitude), with a tropical climate (wet summers and dry winters). During the trial, the average temperature was 26.4ºC, with maximum of 30.3ºC and minimum of 22.7ºC, according to the Embrapa Agropecuaria Oeste meteorological station. All procedures were approved and supervised by the Animal Use Ethical Committee of the Federal University of Grande Dourados (Protocol 4.536.527).

2.1 Aqueous extracts of *Moringa oleifera*

Fresh *Moringa oleifera* leaves were harvested and stored at -20 ºC until extract preparation. The harvest was carried out from young and mature trees, in autumn, with sunny weather conditions, in the morning (7 am to 12 pm). Leaves were chopped (approximately 1 cm) and diluted in distilled water based on the DM ratio (163.3 g of DM/L). The extract was heated at 30 °C for 24 h, followed by solid particle removal. The aqueous extract was frozen in daily portions and thawed at 4 °C overnight before use.

The extracts were submitted to phytochemical prospecting (Matos, 2009) to confirm the secondary metabolite classes (Wagner e Bladt, 2009). Was confirmed triterpenes and steroids presence by hydrolysis of dry methanolic extract. This procedure was done with potassium hydroxide (0.5 mol/L) and submitted to reflux for 1 hour. Then, the compounds were extracted with ethyl ether and submitted to the Liebermann-Burchard reaction. To determine the presence of secondary metabolite classes, we assess the intensity reactions (Fontoura et al., 2015), classified as: negative reaction (- = 0 %), low intensity (+ = 10 %), medium intensity (++ = 50 %), and high intensity (+++ = 100 %). The extracts were solubilized at 1 mg/ml in methanol to analyze phenolic compounds, flavonoids and tannins, and all analyses were performed in triplicates.

The content of phenolic compounds was determined based on the Folin-Cionalteau colorimetric method (Djeridane et al., 2006). For this, thestored solution of each sample (100 µL) was mixed with the other reagents and incubated in a dark environment at 23±2ºC for 2 hours. Their absorbances were recorded at a wavelength of 760 nm. The results were expressed as mg of gallic acid per L of extract (mg GAE L^{-1}). The flavonoid content was determined according to the methodology described by Djeridane et al. (2006). For this, the stored solution of each sample (1000 µL) was mixed with the other reagents and incubated in a dark environment at 23±2ºC for15 min. Their absorbances were recorded at a wavelength of 430 nm. The results were expressed as mg of *flavonoids* rutin per L of extract (mg RUE L^{-1}).

The tannins content was determined using the Folin-Denis spectrophotometric method (Pansera et al., 2003). The sample (2 ml) was mixed with 2 ml of Folin-Denis reagent and 2 ml of 8% sodium carbonate. After 2 hours of reaction, the mixture was read in a spectrophotometer at a wavelength of 725 nm, using water as blank sample. Results were expressed as mg of tannic acid equivalent per L of extract (mg TAE L^{-1}).

2.2 Milk antioxidant activity

Milk antioxidant activity was analyzed by measuring the radical scavenging activities of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), flavonoid and polyphenol contents, and lipid oxidation (thiobarbituric acid-reactive substances, TBARS).

The ABTS radical scavenging activity (%) is a measure of the milk ability to scavenge the 2,2-azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid radical, determined by colorimetry according to Re et al. (1999). DPPH radical scavenging activity (%) was quantified by colorimetry based on antioxidant-mediated electron transfer, which reduces DPPH (purple color) to diphenyl-picrylhydrazine (yellow color) according to Bondet et al. (1997). FRAP was determined according to Zhu et al. (2002).

Polyphenols were determined with Folin-Ciocalteu reagent and gallic acid as a reference standard, according to Singleton and Rossi (1965). Milk lipid oxidation was based on TBARS, determined according to Souza et al. (2011) methodology.

2.3 Animals and experimental design

Twelve Pantaneira ewes with 87 ± 4.33 days in milk (DIM) and 46.7 ± 4.19 kg BW were used. The ewes were placed in individual pens (2 m^2) with rice husk bedding. The ewes were weighed and had the body condition evaluated according Diffay et al (2004) every 14 days. The ewes were milked at 07:30 am and 14:30 pm. Oat hay (*Avena sativa*) and water were provided *ad libitum* (considering 10% of orts). The concentrate was supplied at the rate of 3% of BW. Formulated with soybean meal, ground whole corn, wheat meal, urea, limestone, manganese sulfate, zinc sulfate, iron sulfate, potassium iodate, sodium selenite, vitamin A, Vitamin D3, Vitamin E, Cobalt Sulfate and Q.S.P to meet ewe requirements. The chemical composition of diet is shown in Table 1. The diet was provided in two portions, 60% after morning milking and 40% after afternoon milking. The animals received the same diet during the entire experimental period, varying only the concentration of supplemented extract.

The experiment was conducted using a replicated 3 × 3 Latin square design with three treatments, three 14-d periods (9 d for adaptation and 5 d for data collection), and 12 ewes. Ewes were blocked into four groups of three based on milk production and weight assigned to a square and then randomly assigned to treatment sequence within each square. Daily treatments used were placebo with 20 ml of water as Control, 20 ml of aqueous moringa extract (20-AEMO), and 40 ml of aqueous *Moringa* extract (40-AEMO). Treatments were daily and orally administered after morning milking with a dosing gun to ensure they received the entire portion.

2.4 Evaluation of intake and digestibility

Feed intake was obtained daily by the difference between offer and leftover. Leftover were weighed before the morning meal. Diet ingredients and leftover were sampled on the 11^{th}, 12^{th}, and 13^{th} d of each period. Was performed fecal spot sampling at 6h and 12 h of d-11, at 8 and 14 h of d-12, and at 10 and 16 h of d-13, and composited by animal and period. The samples were stored (-18ºC) until analysis.

All samples were pre-dried at 65ºC in a forced-ventilation oven for 48 hours and then weighed to obtain the pre-dry weight. The samples were ground using a Wiley mill with a 1-mm screen. Then, were analyzed for DM (ID 934.01), ash (ID 930.05), CP (ID 981.10), and ether extract (EE, ID 920.39) contents according to AOAC (1990). NDF and ADF contents were performed according to Van Soest et al. (1991).

For digestibility assessment, we used indigestible NDF (iNDF) from fecal, leftovers and feed samples as an internal marker, and it was assessed as described by Huhtanen et al. (1994). To determine iNDF, we weighed 25 mg/cm^2 of feed, orts, and feces in filter bags measuring 5 x 5 cm. We incubated the samples in duplicate for 288 h in cattle with rumen cannulas. Then, the bags were removed and washed in running water until they were completely clean and submitted to fiber analysis in neutral detergent (Van Soest et al., 1991).

2.5 Rumen

Ruminal fluid was collected on the 14th day of each experimental period using an esophageal tube 3 hours after feeding. Immediately after each collection, the ruminal fluid pH was determined with a digital potentiometer (Mpa-210, MS Tecnopon instrumentação, Brazil) calibrated with pH 7.0 and 4.0 buffers (AOAC, 1992). A subsample was stored at -80 ºC for further short chain fatty acids (SCFA) analysis.

For SCFA analysis, samples were centrifuged at 7000 rpm for 5 minutes and filtered through a nylon filter (0.22 µm). The analysis was performed with high-performance liquid chromatography (HPLC, Shimadzu, Prominence model). The HPLC was equipped with a model SPD-20 ultraviolet detector programmed to operate at a wavelength of 210 nm. In addition, the system was equipped with an Aminex HPX-87H column with dimensions of 300 x 7.8 nm and a particle diameter of 9 µm at 30°C. The injection volume was 20 µL. The mobile phase consisted of 5 mM H_2SO_4 in the isocratic mode for 37 minutes. Methane was calculated using the equation of Moss et al. (2000).

2.6 Milk production

Milk was weighed in the last five days of each period. On the 10th, 11th, and 12th day of each experimental period, a composite sample (50 ml) from the two milking of each day was performed. Milk contents of fat, protein, casein and ureic nitrogen were determined according to Silva et al. (1997), lactose was determined according to Teles et al. (1978), and total solids according to ISO 6731 (2010). Defatted solids were determined by the difference between total solids and fat content.

2.7 Blood

Blood was sampled on the 13th day of each experimental period, 4 hours after feeding. Vacutainer tubes with potassium fluoride were used for urea nitrogen analysis and heparin for glucose analysis. The tubes were centrifuged at 4000 rpm for 15 minutes, and then the plasma was stored at -18ºC until analysis. Glucose and urea were quantified by the colorimetric method (Gold Analyze Test, reaction time: 10 minutes at 37ºC).

2.8 Urine

Spot urine sampling was performed at the same time as feces sampling. It was diluted (1:5) in sulfuric acid solution (50%) and stored (-18ºC) for determination of allantoin, creatinine, uric acid, and xanthine + hypoxanthine. A separate sample without acidification was stored (-18ºC) for urea determination.

Creatinine was quantified using the colorimetric method (Gold Analyze Test, reaction time: 90 seconds at 37ºC, standard: 1 x 5 ml). Creatinine data were used to estimate urine excretion (Kozloski et al., 2005). Urea was quantified using the commercial kit (Gold Analyze Test, reaction time: 10 minutes at 37ºC). Allantoin was quantified according to the method described by Young and Conway (1942), cited by Chen and Gomes (1992). Xanthine + hypoxanthine was determined by the method described by Chen and Gomes (1992), with minor changes, which consists of evaluating the uric acid by calorimetry kit (Teste Vida Biotecnologia).

2.9 Statistical Analysis

Means were calculated for all measurement of each variable during the sampling period for each ewe. The resulting mean data were analyzed using the Mixed procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC) according to a replicated 3 × 3 Latin square design. The model includes square, period, and treatment as fixed effects and ewe within the square as random effect.

$$Y_{ijkl} = \mu + S_i + P_j + T_k + E_{l(i)} + E_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of i^{th} square, P_j = fixed effect of j^{th} period, T_k = fixed effect of k^{th} treatment, $E_{l(i)}$ = random effect of l^{th} ewe within i^{th} square, and E_{ijkl} = is the residual error term. The least-square means were tested using the Tukey, and significance was declared at $P \leq 0.05$.

3. RESULTS

The phenolic compounds, flavonoids, and tannins had medium intensity (Table 1). Coumarins, triterpenes, steroids, cyanogenic heterosides, cardioactive heterosides, reducing sugars, and saponins had low intensity. On the other hand, the alkaloids had a negative reaction to the characterization intensity. The content of phenolic compounds, flavonoids, and tannins in AEMO were 137.48 mg/g, 87.71 mg/g, and 11.10 mg/g, respectively. The AEMO lipid oxidation content (TBARS) was 17.24 mmol/kg of fat. The antioxidants evaluated from the aqueous extract were ABTS, DPPH, polyphenols, and FRAP, with 230.26 ET µM, 39.30 %, 73.23 mg EAG/L, and 109.35 mg EAG/L, respectively.

3.1 Intake and digestibility

The AEMO supplementation for lactating ewes, regardless of dose (20-AEMO or 40-AEMO), did not influence DM and OM intake (Table 3), maintaining an average of 1530 g/d and 1440 g/d, respectively. The same was observed for CP, EE, NDF, and total carbohydrate intake, which were not affected by AEMO supplementation (Table 3), with averages of 260 g/d, 47 g/d, 301 g/d, and 1130 g/d, respectively.

The digestibility of DM, OM, CP, EE, NDF, total carbohydrate, and total digestible nutrients were not influenced by AEMO supplementation for lactating ewes (Table 3), maintaining averages of 57.17; 61.79; 54.69; 82.85; 24.60; 62.49; and 62.86%, respectively. Was observed a weak tendency ($P = 0.13$) for increased NDF digestibility when 20-AEMO was supplemented.

3.2 Rumen

The AEMO supplementation did not influence ruminal pH, maintaining an average of 5.69. Similarly, there was no effect of AEMO on total SCFA production (Table 4), with a mean of 108 mmol/L. The molar proportion of acetic, propionic, butyric, and valeric acids did not change with the inclusion of AEMO, maintaining the averages of 50.77, 37.83, 7.81, and 1.02%, respectively. Consequently, the acetate:propionate ratio was not influenced by any AEMO level (Table 3), with an average of 1.42. Iso-valeric acid increased ($P = 0.03$) by 1.08 percentage units with 20-AEMO

supplementation for lactating ewes. No differences were observed among treatments for methane emission (Table 4), maintaining the average emission of 17.24 mmol/L, and ratio of 15.99 between methane and short-chain fatty acids.

3.3 Milk production

Milk production in g/day reduced (9.47 %, $P < 0.01$) with 20-AEMO supplementation, remaining similar between 40-AEMO and control (Table 5). 20-AEMO supplementation also caused a decrease ($P<0.01$) in the production of fat (1.7%), protein (10.3%), and lactose (11.3%) compared to the control. Supplementation of 40-AEMO decreased ($P<0.01$) milk protein production (Table 5). Other milk parameters such as casein, total solids, defatted solids, and milk urea nitrogen (MUN), were not influenced ($P > 0.05$) by AEMO supplementation for lactating ewes, maintaining averages of 14 g/d, 102 g/d, 76 g/d and 17 g/d, respectively (Table 5).

3.4 Blood and urine

Blood glucose and urea levels were not influenced ($P>0.05$) by AEMO supplementation for lactating ewes. Even so, numerically, 40-AEMO presented the lowest urea concentration (-7.2 mg/dL, Table 6). Furthermore, the urinary parameters evaluated urea, allantoin, uric acid, and xanthine+hypoxanthine were not influenced by the addition of AEMO (Table 6), maintaining the averages of 11 g/d, 10.90 mmol/d, 2.35 mmol/d, and 0.18 mmol/d, respectively. However, allantoin concentration decreased ($P=0.41$) by 50% when the lactating ewes were supplemented with AEMO.

4. DISCUSSION

The DM and nutrient intake observed in this study are evidence that the compounds of *Moringa oleifera* did not influence or affect these variable. However, Kholif et al. (2018a) using AEMO with a lower concentration (125g of DM/L), observed an increase in DM intake with the increase in AEMO supplied. Therefore, according to Leone et al. (2015), the possible effects of AEMO on intake may have

different intensities depending on the variations in its phytogenic compound concentrations depending on different leaf maturity at harvest or the crop location (Leone et al., 2015).

In this work, the hypothesis was raised that ewes would improve ruminal fermentation efficiency and thus would better use the feed provided. Therefore, an increase in digestibility was expected, since moringa bioactive compounds increase digestibility due to improved ruminal fermentation (Soltan et al., 2018). An explanation of no effects on DM digestibility in this trial could be result of an overdose of AEMO concentration (163.3 g of DM/L). Since Kholif et al. (2018a) observed an increase in DM digestibility when AEMO at a lower concentration (125 g of DM/L) was administered to lactating goats.

The numerical differences observed in the SCFA proportions indicate a change in rumen fermentation. Moreover, was verified a numerical reduction of acetic acid (P = 0.15) for treatments with AEMO and a numerical increase of propionic acid (P = 0.20) in 40-AEMO treatment. This balance between the proportion of propionic acid and acetic acid in the total SCFA is consistent with the numerical methane reduction (P = 0.30) that was observed in this study. According to Russell and Houlihan (2003), ionophores act on Gram-positive bacteria, increasing the proportion of Gram-negative bacteria, which are more resistant to these substances, causing an increase in propionic acid and a decrease in methane emissions. In this essay, when ewes were supplemented with 40-AEMO, was observed this propensity that AEMO must mitigate methane emissions.

Another factor contributing to rumen fermentation alteration theory is the decreased allantoin excretion in the urine. Allantoin is directly linked to microbial protein production, where the greater the microbial synthesis, the greater the allantoin output in the urine (Giesecke et al., 1994). This is evidence that moringa compounds influences rumen microorganisms and microbial synthesis. Some studies (Cardoso-Gutierrez et al., 2021; Naikoo et al., 2021) report that certain plant compounds have an antimicrobial effect. Moringa has some of these compounds, such as polyphenols and tannins (Özcan, 2018). The antimicrobial effect of these compounds can lead to rupturing the bacterial cell membrane, disintegrating it, and causing ions leakage (Bodas et al., 2012). The amount of 40 ml of AEMO may

have affected rumen bacteria due to the antimicrobial effect, impairing ruminal degradation and microbial protein synthesis.

The use of 20 ml of extract per day (20-AEMO) decreased the milk production and constituents (fat, protein and lactose), indicating a change in nutrient availability for milk synthesis. Nutrient availability restriction may occur due to a decrease in rumen degradation and consequent reduction in the availability of propionate, acetate, and amino acids, which are the main precursors for milk lactose, fat, and protein synthesis (Osorio et al., 2016). However, when 40 ml of extract (40-AEMO) was used, milk production was not affected, although it has reduced milk protein. Limitations on protein production may be due to the effect of AEMO on rumen degradation and consequently on microbial synthesis, which justifies the lower milk protein production. The amino acid composition of the microbial protein is similar to the amino acid requirement for milk production (Tedeschi et al., 2015), which indicates that ewes receiveing AEMO had a deficiency in amino acid balance. When the animal absorbs a greater quantity of amino acids, it will consequently increase the use these molecules by splanchnic tissues (Omphalius et al., 2019), increasing its catabolism. These events will reduce the efficiency of protein synthesis in milk (Kim and Lee, 2021), and increase urinary N excretion. Loss can also occur if there is an imbalance in rumen fermentation which leads to an increase in ammonia, which when absorbed is directed to the liver and excreted in urea form (Cruz et al., 2020). The 40-AEMO probably harmed ruminal fermentation and protein efficiency post-absorption since there was a lower milk protein production for treatment 40-AEMO and greater urea urinary excretion.

5. CONCLUSIONS

The inclusion of aqueous extract of *Moringa oleifera* as an additive in lactating ewes does not affect the intake and digestibility of nutrients. Furthermore, it can affect ruminal fermentation and microbial synthesis, altering methane emission and consequently milk production. Therefore, it's recommended more studies with different extract concentrations to investigate possible relationships between rumen fermentation and the synthesis of compounds in milk.

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Table 1. Bioactive compounds and antioxidants from aqueous extracts of *Moringa oleifera* provided as a natural additive for lactating ewes

Bioactive compounds	Aqueous extracts of *Moringa oleifera*
Phenolic compounds	++
Flavonoids	++
Tannins	++
Coumarins	+
Triterpenes and steroids	+
Cyanogenic heterosides	+
Cardioactive heterosides	+
Reducing sugars	+
Saponins	+
Alkaloids	-
Phenolic compound content (mg/L)	2416.7
Flavonoids (mg/L)	1547.2
Tannins (mg/L)	195.8
Antioxidants	
ABTS (ET µM)	230.26
DPPH (%)	39.30
Polyphenols (mg EAG/L)	73.23
FRAP (mg EAG/L)	109.35
Oxidation	
TBARS (mmol/kg of fat)	17.24

The presence of secondary metabolic compounds was classified as: negative reaction (- = 0 %), low intensity (+ = 10 %), medium intensity (++ = 50 %), and high intensity (+++ = 100 %).

Table 2. Chemical composition of concentrate and oat hay provided according to the 3% BW of lactating ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO)

Nutrients	Concentrate	Oat hay
DM, %	85.76	84.57
OM, %	95.49	93.17
CP, %	15.62	8.67
Ether Extract, %	5.51	0.60
NDF, %	14.77	56.85
ADF, %	3.75	29.43
Total Carbohydrate[2], %	74.35	83.43

Table 3. Dry matter and nutrient intake and digestibility of lactating ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO)

	Treatments[1]			SEM	*P*-value
	Control	20-AEMO	40-AEMO		
Con : For Ratio, kg[3]	80:20	81:19	82:18	67.35	0.47
Intake					
DM, g/d	1554.2	1515.1	1522.0	62.46	0.80
OM, g/d	1468.3	1417.2	1437.7	55.78	0.65
CP, g/d	264.1	256.1	262.7	9.30	0.66
Ether Extract, g/d	48.5	47.5	49.0	1.42	0.57
NDF, g/d	318.7	297.2	289.8	29.30	0.57
Total Carbohydrate[2], g/d	1154.8	1112.1	1124.7	46.04	0.64
Digestibility					
DM, %	55.84	55.98	59.70	5.673	0.63
OM, %	60.63	60.43	64.33	5.160	0.58
CP, %	52.49	52.65	58.93	6.901	0.43
Ether Extract, %	81.49	83.48	83.59	4.961	0.88
NDF, %	33.37	37.82	37.14	6.415	0.88
Total Carbohydrate[3], %	61.55	61.23	64.71	5.031	0.64
Total Digestible Nutrients, %	61.74	61.66	65.19	4.954	0.60

[1]Treatments: 20-AEMO: 20 ml of aqueous extract of *Moringa oleifera*; 40-AEMO: 40 ml of aqueous extract of *Moringa oleifera*.

[2]Con : For Ratio: Concentrate : Forage Ratio.

[3]Total Carbohydrate (100-(ash + EE + CP)), (Sniffen et al., 1992).

Table 4. Ruminal parameters of lactating ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments[1]			SEM	*P*-value
	Control	20-AEMO	40-AEMO		
Ruminal parameters					
pH	5.69	5.74	5.64	0.163	0.82
Total SCFA, mmol/L	111.1	105.9	108.6	10.85	0.87
Acetic acid, %	54.34	50.23	47.74	3.423	0.15
Propionic acid, %	36.26	35.36	41.89	3.742	0.20
Butyric acid, %	7.79	8.44	7.20	2.455	0.88
Iso-valeric acid, %	1.21[b]	2.29[a]	0.91[b]	0.510	0.03
Valeric acid, %	0.60	1.68	0.78	0.694	0.24
Acetate:Propionate ratio	1.70	1.46	1.10	0.342	0.22
Methane, mmol/L	19.13	18.12	14.47	3.087	0.30
Methane / VFA ratio	17.50	17.05	13.42	2.591	0.25

[a,b,c]Within a row, means without a common superscript are different based on the treatment effect ($P<0.05$).

[1]Treatments: 20-AEMO: 20 ml of aqueous extract of *Moringa oleifera*; 40-AEMO: 40 ml of aqueous extract of *Moringa oleifera*.

Table 5. Milk composition of ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments[1]			SEM	*P*-value
	Control	20-AEMO	40-AEMO		
Milk production, g/d	625.1[a]	565.9[b]	617.0[a]	17.75	<0.01
Fat, g/d	26.35[b]	25.90[b]	31.04[a]	1.754	<0.01
Protein, g/d	40.40[a]	36.23[b]	35.99[b]	1.492	<0.01
Casein, g/d	15.48	14.76	14.73	0.607	0.37
Lactose, g/d	23.93[a]	21.22[b]	23.77[a]	0.841	<0.01
Total solids, g/d	104.63	99.08	102.34	4.086	0.39
Defatted solids, g/d	80.05	74.29	74.60	4.107	0.29
Milk ureic nitrogen, mg/d	18.49	16.60	17.98	1.115	0.22

[a,b,c]Within a row, means without a common superscript are different based on the treatment effect ($P<0.05$).

[1]Treatments: 20-AEMO: 20 ml of aqueous extract of *Moringa oleifera*; 40-AEMO: 40 ml of aqueous extract of *Moringa oleifera*.

Table 6. Glucose and urea concentration in blood, and urinary parameters of lactating ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments[1]			SEM	*P*-value
	Control	20-AEMO	40-AEMO		
Blood					
Glucose, mg/dL	67.3	65.5	66.0	2.17	0.68
Ureic Nitrogen, mg/dL	66.5	64.0	59.3	4.50	0.29
Urinary parameters					
Urea, g/d	11.92	11.90	13.58	1.230	0.31
Allantoin, mmol/d	16.45	7.49	8.78	7.115	0.41
Ureic acid, mmol/d	2.46	2.20	2.40	0.439	0.82
X + H[2], mmol/d	0.20	0.23	0.12	0.139	0.73

[1] Treatments: 20-AEMO: 20 ml of aqueous extract of *Moringa oleifera*; 40-AEMO: 40 ml of aqueous extract of *Moringa oleifera*.

[2]X + H: Xanthine + Hypoxanthine.

CAPÍTULO III

EFEITO DA SUPLEMENTAÇÃO DE EXTRATO AQUOSO DE *Moringa oleifera* NA COMPOSIÇÃO, ÁCIDOS GRAXOS E ATIVIDADE ANTIOXIDANTE DO LEITE DE OVELHAS

Capítulo redigido conforme as normas da Revista Journal of Dairy Science

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INTERPRETIVE SUMMARY: Effect of aqueous *Moringa oleifera* extract as an additive to ewes on milk and cheese composition, fatty acids, and antioxidant activity

By Chagas et al. The aqueous extract of *Moringa oleifera* has an interesting antioxidant profile to be used as a rumen modulator in lactating ewes. Natural additives have the benefit of not accumulating in the final product, reducing the risks to human health. Studies with doses of aqueous extracts of Moringa oleifera can influence the lipid profile of milk and cheese in relation to fatty acids and antioxidants beneficial to human health.

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Effect of aqueous M*oringa oleifera* extract as an additive to ewes on milk and cheese composition, fatty acids, and antioxidant activity

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ABSTRACT

This study aims to evaluate the aqueous extract of *Moringa oleifera* (**AEMO**) as an additive for lactating ewes on milk and cheese production, composition, fatty acids profile, and antioxidants. Twelve ewes were used in replicated 3x3 Latin square, with periods of 14 days (collection on the last five days of each period). Treatments used were: 20 ml of water as Control, 20 ml of aqueous extract (**20-AEMO**), and 40 ml of aqueous extract (**40-AEMO**). Ewes were milked twice a day (7:30 am and 2:30 pm). The concentrate was supplied on a 3% live weight basis and hay ad libitum. We characterized the AEMO biocompounds and determined milk and cheese production and composition, fatty acid profile, and antioxidants of ewes. Milk production was influenced by the AEMO, where the 40-AEMO was equal to the Control, while the 20-AEMO was inferior to both. The 20-AEMO showed higher total solids content. Was observed a reduction in protein and casein contents when 40-AEMO was used. AEMO as an additive for lactating ewes did not influence fat, lactose, defatted solids and milk ureic nitrogen, as well as no chemical parameter of the cheese was changed with the use of AEMO. The short-chain fatty acids detected in the milk and cheese of ewes did not differ between treatments. The c10-15:1 fatty acid increased with AEMO supplementation, with 40-AEMO having the highest mean, differing from the Control. The antioxidants evaluated in sheep's milk and lipid oxidation were not influenced by AEMO supplementation for lactating ewes. The DPPH content was changed in the cheese when the ewe's received AEMO. The aqueous extract of *Moringa oleifera* is rich in antioxidants, but at the doses used, it did not prove to be an advantageous additive for lactating ewes. Furthermore, the extract was not very expressive regarding the effect on the lipid profile of milk and cheese concerning fatty acids important for human health and antioxidants.

Key words: bioactive composite, free radicals, lipid profile, microbial protein, rumen modulator

1. INTRODUCTION

Sheep milk stands out worldwide due to its different physicochemical characteristics compared to other milk-producing species (Corrêa et al., 2014). Sheep milk is rich in vitamins, polyphenols, enzymes, proteins, fats, and minerals, making milk with greater nutritional value than other species (Cabiddu et al., 2019; Scano et al., 2019). Since sheep's milk is rich in solids and mainly rich in lipids, it is essential to emphasize the quality of the fat that comprises this milk. Ewes milk is rich in unsaturated chain fatty acids, which benefits the reduction of bad cholesterol (Høstmark and Haug, 2013). In addition, it has a significant content of conjugated linoleic acid (CLA), a fatty acid known to have a cancer-fighting effect (Prandini et al., 2007; Cieslak et al., 2010). However, Eifert et al. (2006) determined that the correlation between dietary fatty acids and milk is low. This fact is probably due to the biohydrogenation that takes place in the rumen.

Ruminal fermentation can be altered with the use of additives in the diet, which act in different ways on microorganisms in the rumen, protecting and stabilizing the environment, so that microorganisms continue to operate symbiotically (Guan et al., 2006). The improvement of ruminal efficiency with the use of additives is important, but at the same time these can leave residues in the milk, and mainly in the environment. This has been a cause for concern and for the search for alternatives or minimization of possible impacts. Thus, natural additives are an option, being strategic and promising to increase the quality and characteristics of final ruminant products (Giuberti et al., 2021).

Moringa oleifera has several biocompounds, such as secondary metabolites, phenolic acids, tannins, flavonoids, and saponins, among others (Anwar et al., 2007; Brunelli et al., 2010). These compounds can act directly on the population of microorganisms in the rumen, altering fermentation and modifying the formation of end products, such as short chain fatty acids (SCFA) (Bodas et al., 2012). With this change in the production of SCFAs, it is possible that milk production, or milk solids, will change depending on the diet of the animals. In

addition, *Moringa oleifera* has several benefits, including a considerable load of antioxidants (Falowo et al., 2018; Dong et al., 2019), which can pass into milk and make this product, as well as cheese, food with higher biological value. The aqueous extraction of Moringa leaf can reduce the anti-nutritional content of non-beneficial compounds (Xu et al., 2019) and concentrate compounds such as saponins and tannins that can act as rumen additives (Sultana et al., 2015) for sheep in lactation.

Therefore, to our knowledge, there are no literature reports on AEMO effects on milk and cheese fatty acids and antioxidants of ewe's. And this led us to evaluate AEMO effects as an additive for lactating ewes on milk and cheese production and composition, fatty acids profile, and antioxidants. In this work, the hypothesis was that AEMO will act on rumen microorganisms, altering the biohydrogenation pathways and, consequently, the milk and cheese lipid profile and transfer antioxidants to milk and cheese.

2. MATERIALS AND METHODS

The experiment was carried out at the Federal University of Grande Dourados, located in Dourados-MS, Brazil. All procedures were approved and supervised by the Animal Use Ethical Committee of the Federal University of Grande Dourados (Protocol 4.536.527). Chagas et al. (unplished data) describe in more detail local climate conditions, ewes' management, food supply and composition.

2.1 Aqueous extracts of Moringa oleifera

Fresh *Moringa oleifera* leaves were harvested and stored at -20 ºC until extract preparation. The harvest was carried out from young and mature trees, in autumn, with sunny weather conditions, in the morning (7 am to 12 pm). Leaves were chopped and diluted in distilled water based on the MS ratio (163.3 g of DM/L). The extract was heated at 30 °C for 24 h, followed by solid particle removal. The aqueous extract was frozen in daily portions and thawed at 4 °C overnight before use.

The extracts were submitted to phytochemical prospecting (Matos, 2009), to confirm the secondary metabolite classes (Wagner e Bladt, 2009). Was confirmed the presence of triterpenes and steroids by hydrolysis of the dry methanolic extract. This procedure was done with potassium hydroxide (0.5 mol/L) and submitted to reflux for 1 hour. Then, the compounds were extracted with ethyl ether and then submitted to the Liebermann-Burchard reaction. To determine the presence of secondary metabolite classes, the reactions of characterization intensities (Fontoura et al., 2015) were classified as: negative reaction (- = 0 %), low intensity (+ = 10 %), medium intensity (++ = 50 %), and high intensity (+++ = 100 %). The extracts were solubilized at the concentration of 1 mg/mL in methanol to analyze phenolic compounds and flavonoids (Djeridane et al., 2006). Was determined the content of phenolic compounds based on the Folin-Ciocalteau colorimetric method (Djeridane et al., 2006). The tannin content was determined using the Folin-Denis spectrophotometric method, with tannic acid as a reference (Ibe et al., 2013; Pansera et al., 2003).

2.2 Experimental design and animal diets

Twelve Pantaneira ewes with 87 ± 4.33 days in milk (DIM), and 46.7 ± 4.19 kg of BW were used. The experiment was conducted using a replicated 3x3 Latin square design with three treatments, three 14-day periods (9 days for adaptation and 5 days for data collection) and 12 sheep. Ewes were blocked into four groups of three based on milk production and BW, assigned to a square, and then randomly assigned treatment sequence within each square. Treatments correspond to dally oral supplementation, Control: 20 ml of water as placebo; 20-AEMO: 20 ml of aqueous extract of *Moringa oleifera*; and 40-AEMO: 40 ml of aqueous extract of *Moringa oleifera*.

2.3 Collection of milk

Milk was weighed in the last five days of each period and converted to mL basis (1.03 g/mL). On the 10th, 11th, and 12th day of each experimental period, a composing sample (50ml) of two milkings of each day was performed. During the last five days of each period, the milk was stored separately by treatment for cheese production, on the days of collection for chemical composition, the remainder was stored.

2.4 Cheese manufacture and yield

The milk was sieved, pasteurized (72°C for 20 seconds), taken to an ice bath, NaCl (25 g/L) added and cooled (38°C). Yeast (0.02 g/L) and rennet (1 ml/L) were added. The mass was cut after 50 min of coagulation; after 10 min the mass was disintegrated and allowed to stand for 10 min. For the draining of the whey, the mass was sieved, weighed in total, to determine the yield, and later weighed in portions of 350 g, packed in specific forms. The cheeses were stored in a maturation chamber at a temperature of 15 ± 0.33 °C and a humidity of 69 ± 8.79% for 14 days.

Cheese yield was based on the total solids content of the cheese (difference between the total liters and the moisture content of the cheeses), in relation to each milk used, determined by the calculation by Saboya et al. (1998) and Furtado (2005a).

2.5 Composition of milk and cheese

Milk contents of fat, protein, casein and ureic nitrogen were determined according to Silva et al. (1997), lactose was determined according to Teles et al. (1978), and total solids according to International standard (2010). Defatted solids were determined by the difference between total solids and fat content.

The moisture, protein, and ash analyses were performed according to the AOAC (1990). For fat determination a Van Gulik butymeter suitable for cheese with 40% graduation was used, according to the methodology of Silva et al. (1997), using the equation of Santos et al. (2011). The water activity (WA) was determined at 25 ± 0.1 °C using a water analyser (AquaLab, Washington). To determine the pH of the cheeses, three assessments were performed at different points in the cheese, shortly after the maturation room was removed, using a digital potentiometer (TESTO-205).

2.6 Milk and cheese fatty acids

Fat acid methyl ester (FAME) from milk and cheese fat samples was prepared by direct transesterification using potassium hydroxide in methanol (2 M; Rego et al., 2009), with the addition of methyl nonadecanoate (1 mL of a 1 mg/mL solution) as internal standard (Alves et al., 2017). FAME was analyzed by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC

2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m x 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA) capillary column. The chromatographic conditions were as follows: injector and detector temperatures were set as 220 and 250°C, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 50°C was held for 1 min, increased at 50°C/min to 175°C and held for 35 min, increased at 2°C/min to 220°C and held for 30 min. Identification of FAME was achieved by comparison of the FAME retention times with those of standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA) and by mass spectrometry using a GC-MS Shimadzu 2010-Plus (Shimadzu, Kyoto, Japan). Integration of peaks was performed using GCsolution Version 2.41.00 (Shimadzu, 2000-2011). Theoretical relative FID response correction factors for FAME were used to correct peak areas, according to Ackman (2002).

2.7 Milk and cheese antioxidant activity

Milk and cheese were analyzed for antioxidant activity by measuring the radical scavenging activities of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the content of polyphenols. In milk, was evaluated ferric reducing antioxidant activity (FRAP) and flavonoid content. In both samples, was determined lipid oxidation (thiobarbituric acid-reactive substances, TBARS).

The ABTS radical scavenging activity (%) is a measure of the ability of milk to scavenge the 2,2-azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid radical, determined by colorimetry according to Re et al. (1999). DPPH radical scavenging activity (%) was quantified by colorimetry based on antioxidant-mediated electron transfer, which reduces DPPH (purple color) to diphenyl-picrylhydrazine (yellow color) according to Bondet et al. (1997). FRAP was determined according to Zhu et al. (2002).

The total flavonoid content was expressed as g% of quercetin determined according to Buriol et al. (2009). Polyphenols were determined with Folin-Ciocalteu reagent and gallic acid as a reference standard, according to Singleton and Rossi (1965). Lipid oxidation of milk was based on TBARS, determined according to the methodology of Souza et al. (2011).

2.8 Statistical Analysis

Means were calculated for all measurement of each variable during the sampling period for each ewe. Production data were analyzed using the Mixed procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC) according to a replicated 3 × 3 Latin square design. The model includes square, period, and treatment as a fixed effect and ewe within the square as a random effect.

$$Y_{ijkl} = \mu + S_i + P_j + T_k + C_{l(i)} + E_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of i^{th} square, P_j = fixed effect of j^{th} period, T_k = fixed effect of k^{th} treatment, $C_{l(i)}$ = random effect of l^{th} ewe within i^{th} square, and E_{ijkl} = is the residual error term.

Cheese data were analyzed using the SAS Mixed procedure (Version 9.2, SAS Institute Inc., Cary, NC) according to a completely randomized design. The model contain fixed effects of treatments (Control, 20-AEMO, and 40-AEMO) and random effect of block.

$$Y_{ij} = \mu + B_i + T_j + E_{ij}$$

The least square means were tested using the Tukey, significance was declared at $P \leq 0.05$.

3. RESULTS

The classification of the presence of phenolic components (Tabela 1), flavonoids, and tannins showed medium intensity. Coumarins, triterpenes, steroids, cyanogenic heterosides, cardioactive heterosides, reducing sugars, and saponins showed low intensity. The alkaloids, on the other hand, showed a negative reaction to the intensity of characterization. The content of phenolic compounds, flavonoids, and tannins in AEMO was 137.48 mg/g, 87.71 mg/g, and 11.10 mg/g, respectively. The AEMO lipid oxidation content (TBARS) was 17.24 mmol/kg of fat. The antioxidants evaluated from the aqueous extract were ABTS, DPPH, polyphenols, and FRAP, showing 230.26 ET μM, 39.30 %, 73.23 mg EAG/L, and 109.35 mg EAG/L, respectively.

3.1 Milk and cheese composition

Milk production was influenced (P<0.01) by AEMO dosage, where 40-AEMO was similar to Control, while 20-AEMO treatment was inferior to both (Table 3). Despite lower milk production, the 20-AEMO treatment showed higher (P=0.04) total solids content. Was observed a reduction (P<0.01) for protein and casein contents when 40-AEMO was supplemented. AEMO supplementation for lactating ewes did not influence fat and lactose, maintaining levels of 4.25 and 3.76%, respectively (Table 3).

The daily AEMO supply did not influence the parameters of fat, protein, ash, moisture, water activity and cheese yield, maintaining levels of 16.19, 3.2, 12.79, 39.70, 0.91 and 63.95%, respectively (Table 3).

3.2 Milk and cheese fatty acids

The short chain fatty acids detected in the milk of the ewes, which were: 4:0, 6:0, 8:0, 9:0, 10:0, 11:0, and 12:0, did not differ between treatments, showing averages of 1.49, 1.67, 1.91, 0.27, 8.94 and 0.81 % of total fatty acids (Table 4). Was observed an effect (P=0.04) for *a*13:0 and a trend (P=0.12) for the myristic (14:0), where the 20-AEMO treatment presented an increase for these fatty acids, and the Control showed the lowest averages.

Was observed a tendency (P=0.12) for the sum of even fatty acids to be superior on 20-AEMO than Control. The *c*10-15:1 fatty acid increased (P=0.05) with AEMO supplementation, with 40-AEMO having the highest mean, differing from the Control (Table 4). The extracts 20-AEMO and 40-AEMO did not influence to the point of completely modifying the lipid profile of the milk, with the other fatty acids being detected with no difference between the treatments and the Control.

The sum of fatty acids of cheeses produced with ewe's milk receiving doses of 20 and 40 ml of AEMO had no influence (P>0.05) on its composition, equating with the lipid profile of the Control treatment.

3.3 Antioxidant activity in milk and cheese

Antioxidants evaluated in sheep's milk were not influenced (P>0.05) by the addition of AEMO as an additive for lactating sheep. ABTS, DPPH, polyphenols, FRAP and flavonoids concentrations were evaluated, obtaining means of 154.64, 8.94, 22.66, 3.05 and 1.54, respectively. As with antioxidants, the addition of AEMO did not influence (P>0.05) the lipid oxidation of milk (Table 5).

The ABTS antioxidants and polyphenols evaluated in cheese produced with ewe's milk receiving 20 and 40 ml of AEMO, showed no difference compared to the control treatment. Means for ABTS and polyphenols of 152.57 and 39.75, respectively, were observed. Was observed influence (P<0.05) of the AEMO for the antioxidant DPPH, being both treatments with the extract, superior to the Control treatment. As with the lipid oxidation of milk, the addition of AEMO did not influence (P>0.05) the lipid oxidation of cheeses (Table 5).

4. DISCUSSION

The aqueous extract of the moringa leaf in this study is rich in phenolic compounds and flavonoids, compared to the content found by Özcan (2018) in moringa seeds. Corroborating with the studies report, various parts of the plant can be rich in bioactive compounds (Kadhim & Al-Shammaa, 2014) desirable in natural additives. High levels of phenolic compounds in the aqueous extract of Moringa oleifera indicate a high power of antioxidant activity and can be used as a supplement to prevent diseases related to oxidative stress in animals (Xu et al., 2019; Nobósse et al., 2018), and may make milk and cheese a products rich in bioactive compounds. In addition, phenolic compounds can react with free radicals, forming stable radicals (Soares et al., 2008). Thus, the higher the content of phenolic compounds, the greater the antioxidant activity of the extract.

The aqueous extract of *Moringa oleifera* had an insignificant amount of fatty acids, which possibly allowed the low influence on ewe's milk and cheeses. Kholif et al. (2018a) noticed a greater amount of hexadecenoic acid (C16:0) in the aqueous extract evaluated in their study, which corroborates the result of the present work, however, this same authors observed a greater amount of (α-) linolenic acid (C18:3)

and linoleic acid (C18:2), in the present study, stearic (C18:0) and oleic (*c*9-18:1) acids were more expressive than those found by Kholif et al. (2018a).

Although 20-AEMO treatment had the lowest quantitative milk production, was found protein and casein content similar to the control treatment. This result indicates that the 20-AEMO milk was more concentrated due to the lower proportion of water concerning the other treatments. The amount of water is generally regulated by the lactose content (Peruzzi et al., 2016). As the lactose content was constant between treatments, some other factors, such as minerals, could possibly influence it. 20-AEMO promoted the highest total solids content in milk, which did not happen in the study by Babiker et al. (2017), using pelleted moringa in lactating goats, or by Sánchez et al. (2006), using fresh moringa in lactating cows. No studies are using AEMO for sheep as a basis for comparison of results, and only one study in goats evaluated metabolic performance (Kholif et al., 2018a) and the chemical and fatty acid composition of milk (Kholif et al., 2018b). However, Cohen-Zinder et al. (2015), assessing moringa silage in dairy cows, did not observe an increase in milk protein with the use of moringa.

20-AEMO and the control maintained the same protein content. However, 40-AEMO caused a reduction in this parameter, indicating a change in the availability of nutrients for milk synthesis. The restriction in the availability of nutrients may have occurred due to the decrease in rumen protein degradation and consequently in microbial synthesis, which results in low amino acid absorption and justifies the lower production of protein in milk (Chagas et al., unpublished data). Tedeschi et al. (2015) determined that the amino acids that make up the microbial protein are similar to the amino acid requirement for milk production, which may indicate that the ewes that received the extract had an imbalance of amino acids. When there is a greater absorption of amino acids by the animal, it can lead to an increase in the use of amino acids by splanchnic tissues (Omphalius et al., 2019), increasing amino acid catabolism and reducing the efficiency of protein synthesis in milk (Kim and Lee, 2021)—indicating that 40-AEMO impaired rumen fermentation and post-absorption protein efficiency, due to the reduction of milk protein in this treatment.

The chemical composition of cheese produced with ewe's milk depends on several factors, such as: stage of lactation, breed and animal feed (Nespolo et al., 2009). Other factors that can influence the proximate composition of cheeses, mainly the moisture content, would be the period of maturation and pressing of the same (Scott et al., 2002), being the maturation period responsible for adding flavor, color and texture to the cheeses (Sales, 2015). The maturation time of the cheeses was 14 days, the same period for cheeses from the Araxá region (Sales, 2015), a period recognized by Ordinance nº1736 of 27/06/2017 (IMA, 2013). The feeding of the ewe's remained the same for all treatments, in order to detect the difference in the different dosages of AEMO, as well as the maturation period was stable for all treatments, however, the dosages were not sufficient to modify the proximate composition of cheeses.

The pH observed in the cheeses was high (6.44 on average) in relation to what would be ideal in artisanal cheeses (between 5.2 and 5.8 according to Freitas Filho et al., 2009), to avoid the proliferation of contaminating bacteria. Since the pH is dependent on the dairy culture used in the preparation of cheeses (Walstra and Jenness, 1984), and in this study, dairy culture was not used, to determine the action of AEMO in the process of maturation and preservation of cheeses, making it possible to determine that AEMO did not preserve the quality of the product.

According to the mechanism by which the *de novo* synthesis occurs, was noticed a low contribution of fat from rumen microorganisms, as BCFA values are very low concerning SCFA in all treatments. Branched-chain fatty acids are mainly saturated fatty acids (SFAs), having one or more methyl branches in the carbon chain (Ran-Ressler et al., 2014). Therefore, they can be called branched and odd-chain fatty acids (OBCFA), with ruminant milk rich in OBCFA (Gómez-Cortés and Fuente, 2020), making the cheese also rich in OBCFA. Vlaeminck et al. (2006) carried out a meta-analytic study on the primary fatty acids that make up the OBCFAs in milk, comprising: tetradecanoic (iso – 14:0), pentadecanoic (15:0, iso – 15:0, and anteiso) isomers. – 15:0), hexadecanoic (iso – 16:0) and heptadecanoic (17:0, iso – 17:0 and anteiso – 17:0). OBCFA are normally synthesized in the process of microbial fermentation in the rumen (Gómez-Cortés and Fuente, 2020). The main precursors of microbial synthesis of branched-chain fatty acids (BCFA) are leucine, isoleucine, and valine, branched-chain amino acids from the diet (Gómez-Cortés and Fuente, 2020).

Most of the even-numbered SFAs in milk fat are carried out by *de novo* synthesis in the epithelial cells of the mammary gland (Palmquist, 2006). Its synthesis occurs from circulating molecules of acetate and β-hydroxybutyrate, which are generated in the rumen in the fermentation process of dietary carbohydrates (Gómez-Cortés and Fuente, 2020). The enzymes responsible for *de novo* synthesis in the mammary gland are Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The first step of the synthesis comprises the activation of acetate to acetyl-CoA, followed by the condensation of two molecules of acetyl-CoA to form malonyl-CoA, this step being catalyzed by ACC. In sequence, FAS regulates chain elongation of the newly synthesized fatty acid, then the products of the *de novo* synthesis would be even-chain fatty acids. However, in the rumen environment, if the initial substrate is propionate, methylmalonate, or volatile branched-chain fatty acid (isovaleric, isobutyric, and 2-methylbutyric), then the products of the *de novo* synthesis would be odd-chain fatty acids (Gómez-Cortés and Fuente, 2020). Garnsworthy et al. (2006) determined that fatty acids synthesized *de novo* by the mammary gland comprise fatty acids with a chain length of 4 to 14 carbons. There was a change in the production of 14:0 for ewes that received AEMO, with 20-AEMO being more expressive. This may indicate that AEMO in low concentrations can stimulate *de novo* synthesis in the sheep's mammary gland.

In monounsaturated fatty acids (MUFA), shift trans-10 displacement occurred in all ewes (Table 4). The AEMO did not help reverse the possible situation of acidosis, causing it to provide a greater production of *t*10-18:1. All ewes altered the biohydrogenation pathways due to the high concentrate diet, presenting acidosis due to the drop in ruminal pH (Chagas et al., unpublished data). In diets with a higher amount of fiber permeate the main biohydrogenation intermediate is *t*11-18:1, but when a diet rich in starch is provided, the main biohydrogenation intermediate becomes *t*10-18:1 (Bessa et al., 2005). The flow of *t*11-18:1 is desirable, as it acts as a substrate for D9-desaturase, resulting in the formation of conjugated linoleic acid (CLA) *c*9,*t*11-18:2, when there is a greater accumulation of *t*10-18 :1, it is associated that it is the main cause of milk fat depression syndrome (Griinari et al., 1998) and has adverse effects on human health (Aldai et al., 2013). In this way, was noticed that the AEMO did not benefit the t11-18:1 flow, making the lipid profile of milk, and consequently, of cheeses, indifferent to the control.

In this experiment, the supplementation levels of phenolic compounds were 0.448 g/d for 20-AEMO and 0.896 g/d for 40-AEMO. For tannins, 0.036 g/d for 20-AEMO and 0.072 g/d for 40-AEMO. Milk production was similar to the control treatment. Still, the antioxidant content of the milk was stable between treatments, demonstrating that the extract provided was insufficient to pass these levels to the milk. Kholif et al. (2016) provided fresh, hay, and ensiled moringa to lactating goats, and characterized the treatments as the composition of phenolic contents (48 g/kg, 44 g/kg, and 40 g/kg, respectively) and tannins (22 g/kg, 20 g/kg, and 17 g/kg, respectively), with this improvement in nutrient digestibility and increased in quantitative milk production were obtained, with the animals that received the treatments with fresh and ensiled moringa being the most productive.

Scherer and Godoy (2009) emphasize that there is no universal explanation to determine the assessment of the capacity of DPPH, being strongly pointed out as an index of antioxidant activity of the evaluated sample, since the antioxidant content is given by a set of antioxidant analyzes carried out in compared to the oxidative process of a given food (Cieśla et al., 2012; Moon and Shibamoto, 2009), with antioxidant activity responsible for fighting free radicals, which provide faster lipid oxidation (Arnao, 2000). Thus, we realized that the AEMO, in different dosages, allowed the cheeses, a greater stability to free radicals, in relation to the Control.

5. CONCLUSIONS

The aqueous extract of *Moringa oleifera* is rich in antioxidant levels, which can improve the shelf life of milk and dairy products. The 20 ml dosage reduced milk production but increased the solids content compared to the control diet. The 40 ml dose maintained the production but reduced the protein content. The extract is not very expressive regarding the effect on the lipid profile of milk concerning important fatty acids for human health. The extract's antioxidant content was insufficient to transfer significant amounts of antioxidants to the milk, however, it influenced the higher antioxidant intensity of DPPH in the cheeses.

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Table 1. Bioactive compounds and antioxidants from aqueous extracts of *Moringa oleifera* provided as a natural additive for ewes lactating

Bioactive compounds	Aqueous extracts of *Moringa oleifera*
Phenolic compounds	++
Flavonoids	++
Tannins	++
Coumarins	+
Triterpenes and steroids	+
Cyanogenic heterosides	+
Cardioactive heterosides	+
Sugars reductions	+
Saponins	+
Alkaloids	-
Phenolic compound content (mg/g)	137.48
Flavonoids content (mg/g)	87.71
Tannins content (mg/g)	11.10
Antioxidants	
ABTS (ET µM)	230.26
DPPH (%)	39.30
Polifenols (mg EAG/L)	73.23
FRAP (mg EAG/L)	109.35
Oxidation	
TBARS (mmol/kg of fat)	17.24

The presence of secondary metabolic compounds was classified as: negative reaction (- = 0 %), low intensity (+ = 10 %), medium intensity (++ = 50 %), and high intensity (+++ = 100 %).

Table 2. Fatty acids of the aqueous extract of *Moringa oleifera*, oxidative content and antioxidant content of the aqueous extract, hay and concentrate

	Aqueous extract of *Moringa oleifera*	Oat hay	Concentrate
Fat acids			
Total (mg/g of DM)	1.76	4.82	29.64
16:0 (% of FA)	41.02	33.90	16.40
18:0 (% of FA)	15.59	6.86	3.07
*c*9-18:1 (% of FA)	18.75	15.76	30.15
*c*11-18:1 (% of FA)	4.15	1.13	0.89
18:2 n-6 (% of FA)	8.05	22.74	46.18
18:3 n-3 (% of FA)	11.38	11.73	2.12
20:0 (% of FA)	0	3.16	0.53
22:0 (% of FA)	0.21	3.02	0.31
24:0 (% of FA)	0.84	1.69	0.31

Table 3. Milk yield, cheese yield and composition of the milk and cheese of ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments			SEM[1]	*P*-value
	Control	20-AEMO	40-AEMO		
Milk					
Milk production (mL)	609.5[a]	551.7[b]	605.1[a]	17.440	<0.01
Fat (%)	3.97	4.34	4.45	0.416	0.47
Protein (%)	6.58[a]	6.42[a]	5.99[b]	0.124	<0.01
Casein (%)	2.56[a]	2.66[a]	2.34[b]	0.099	<0.01
Lactose (%)	3.76	3.72	3.80	0.087	0.60
Total solids (%)	16.79[b]	17.56[a]	16.71[b]	0.368	0.04
Defatted Solids (%)	12.82	13.21	12.25	0.483	0.14
Milk ureic nitrogen (mg/dL)	2.85	3.15	2.82	0.193	0.18
Cheese					
Fat (%)	15.75	16.75	16.08	3.474	0.95
Protein (%)	47.02	44.43	47.15	1.248	0.15
Ash (%)	13.03	12.47	12.87	0.707	0.73
Moisture (%)	40.59	39.51	39.01	2.267	0.78
Aw (%)	0.92	0.92	0.91	0.010	0.74
Cheese yield (g TS/L)	65.19	65.36	61.32	9.158	0.88
pH	6.46	6.42	6.45	0.018	0.18

Table 4. Sum of short, breached and long chain fatty acids (% of FA) of milk and cheese from ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments			SEM[1]	*P*-value
	Control	20-AEMO	40-AEMO		
Milk					
SCFA (%)	21.57	22.78	22.87	0.932	0.31
iso-BCFA (%)	0.81	0.84	0.83	0.057	0.86
anteiso-BCFA (%)	1.95	1.97	1.92	0.104	0.89
Odd-Chain SFA (%)	4.39	3.81	4.15	0.348	0.27
Even-Chain SFA (%)	69.11^{b}	70.86^{a}	70.05^{ab}	0.807	0.12
MUFA *cis* (%)	17.84	16.83	17.14	0.811	0.45
MUFA *trans* (%)	2.45	2.24	2.63	0.345	0.54
18:2 n-6 (%)	2.85	2.80	2.64	0.149	0.37
18:3 n-6 (%)	0.10	0.11	0.11	0.007	0.49
CLA *c*9,*t*11 (%)	0.16	0.16	0.15	0.037	0.96
PUFA (%)	3.41	3.40	3.24	0.149	0.46
Cheese					
SCFA (%)	21.76	23.18	22.96	2.169	0.79
iso-BCFA (%)	0.46	0.47	0.51	0.081	0.85
anteiso-BCFA (%)	0.79	0.79	0.73	0.062	0.58
Odd-Chain SFA (%)	4.46	4.01	4.28	0.274	0.34
Even-Chain SFA (%)	68.36	70.44	70.00	2.853	0.75
MUFA *cis* (%)	19.27	18.19	18.02	2.059	0.81
MUFA *trans* (%)	2.62	2.19	2.88	0.816	0.71
18:2 n-6 (%)	2.91	2.91	2.54	0.295	0.42
18:3 n-3 (%)	0.16	0.17	0.15	0.021	0.75
18:3 n-6 (%)	0.13	0.11	0.11	0.009	0.12
CLA *c*9,*t*11 (%)	0.20	0.17	0.20	0.022	0.36
PUFA (%)	4.00	3.88	3.56	0.285	0.37

SCFA: Short chain fatty acids; BCFA: Branched chain fatty acids; PUFA: Polyunsaturated fatty acids; LCFA: Long chain fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids

Table 5. Antioxidant activity of milk from ewes receiving 10 ml/d of water (Control), 20 ml/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 ml/d of aqueous extract of *Moringa oleifera* (40-AEMO) as natural additives

	Treatments			SEM[1]	*P*-value
	Control	20-AEMO	40-AEMO		
Milk antioxidants					
ABTS (ET µM)	158.03	157.40	148.51	11.378	0.65
DPPH (%)	9.36	9.02	8.45	1.170	0.74
Polyphenols (mg EAG/L)	24.25	21.83	21.92	2.084	0.43
FRAP (mg EAG/L)	3.01	3.92	2.23	2.178	0.74
Flavonoids (mg/L)	1.77	1.16	1.68	1.011	0.81
Cheese antioxidants					
ABTS (ET µM)	146.68	149.30	161.72	24.193	0.81
DPPH (%)	4.59[b]	5.49[a]	5.18[a]	0.190	0.02
Polyphenols (mg EAG/L)	41.21	39.63	38.41	4.207	0.81
Milk oxidation					
TBARS (mmol/kg of fat)	126.59	104.58	118.12	17.887	0.47
Cheese oxidation					
TBARS (mmol/kg of fat)	19.11	15.71	16.90	6.402	0.86

Considerações finais

Há carência na realização de estudos que abordem a utilização do extrato aquoso de *Moringa oleifera* como aditivo para ovelhas na fase de lactação, visto que os compostos da moringa podem atuar como potentes aditivos naturais.

O estudo teve como principal objetivo, demonstrar o potencial uso do extrato aquoso de moringa como aditivo para ovelhas em fase de lactação e verificar seus possíveis efeitos no desempenho, produção e composição de leite e queijo dessas ovelhas.

O extrato aquoso de *Moringa oleifera*, como aditivo em ovelhas em lactação, não mostrou efeito no consumo e na digestibilidade dos nutrientes. No entanto, afetou a fermentação ruminal e a síntese microbiana, alterando a emissão de metano e consequentemente a produção de leite. A menor dosagem de extrato diminuiu a produção de leite, mas aumentou o teor de sólidos em comparação com a dieta controle. A maior dosagem, manteve a produção, mas reduziu o teor de proteína. O extrato se mostrou pouco expressivo quanto ao efeito no perfil lipídico e antioxidantes do leite. Desta forma, é recomendado que outros estudos, com diferentes concentrações de extrato, sejam realizados, para investigar possíveis relações entre a fermentação ruminal e a síntese de compostos no leite.