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OVINOCULTURA DE LEITE EM CONDIÇÕES DE DESAFIO: IMPACTOS DA NUTRIÇÃO SOBRE DESEMPENHO ZOOTÉCNICO E SAÚDE ANIMAL

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OVINOCULTURA DE LEITE EM CONDIÇÕES DE DESAFIO: IMPACTOS DA NUTRIÇÃO SOBRE DESEMPENHO ZOOTÉCNICO E SAÚDE ANIMAL

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Zootecnia, Área de Concentração Ciência e Produção Animal, da Universidade do Estado de Santa Catarina (UDESC), como requisito parcial para obtenção de grau de **Mestre em Zootecnia**
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OVINOCULTURA DE LEITE EM CONDIÇÕES DE DESAFIO: IMPACTOS DA NUTRIÇÃO SOBRE DESEMPENHO ZOOTÉCNICO E SAÚDE ANIMAL

Elaborada por

Davi Fernando Alba

como requisito parcial para obtenção do grau de

Mestre em Zootecnia

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Zootecnia
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OVINOCULTURA DE LEITE EM CONDIÇÕES DE DESAFIO: IMPACTOS DA NUTRIÇÃO SOBRE DESEMPENHO ZOOTÉCNICO E SAÚDE ANIMAL

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Chapecó, 22 de novembro de 2019

A exploração de ovinos para a produção de leite e para fabricação de derivados, como o queijo, sorvetes e iogurtes é uma realidade crescente no Brasil. No entanto, diversos fatores podem estar presentes nos sistemas de produção afetando a saúde dos animais e consequentemente a produção de leite. Dentre os principais problemas enfrentados pelas ovelhas destaca-se o estresse térmico, o balanço energético negativo no período de transição e a mastite. Visando reduzir os diferentes fatores estressantes para ovelhas em lactação, a presente dissertação teve como objetivo avaliar variáveis relacionadas à saúde animal, desempenho produtivo e qualidade de leite de ovelhas Lacaune expostas a condições desafiadoras, isto é, animais em estresse térmico, com mastite ou em período de transição, assim como verificar se a adição de aditivos na dieta poderia minimizar os efeitos negativos causados pelos diferentes desafios. Para isso, quatro experimentos distintos foram realizados. No experimento I, usamos 30 ovelhas (20 com mastite subclínica e 10 usadas como controle) em duas etapas do experimento, sendo que em um primeiro momento identificamos os agentes etiológicos envolvidos, a produção e qualidade do leite, assim como realizado testes de sensibilidade a antimicrobianos; já no segundo momento avaliamos a eficácia de ceftiofur no tratamento de mastite por duas vias de administração (intramuscular e intramamaria), além de verificado a presença da droga no leite pós-tratamento. Na etapa I desse estudo destacamos que ovelhas com mastite subclínica apresentavam uma resposta leucocitária elevada, maior peroxidação lipídica no soro e no leite, menor produção e qualidade de leite, assim como verificamos que o gênero *Staphylococcus spp.* foi o mais comum dentre os microorganismos envolvido nos casos de mastite. Na segunda etapa detacamos que as duas vias de tratamento tiveram baixa eficácia na cura da mastite, assim como ambas as vias deixam resíduos no leite por maior período que o descrito na bula dos produtos. No experimento II, 30 ovelhas da raça Lacaune no pico de produção foram usadas no estudo durante o verão, quando os animais sofrem com estresse térmico por calor. Animais foram divididos em três grupos (controle; suplementadas com 1% de farinha de resíduo de uva (FRU) e suplementadas com 2% de FRU) com 10 ovelhas cada. Entre os resultados destacamos que a adição de FRU na dieta das ovelhas teve efeitos benéficos à saúde animal, isto é, houve redução dos níveis de oxidantes e aumento do sistema antioxidante no soro e no leite, além de apresentar uma resposta antinflamatória nos animais que receberam 2% FRU. Esses resultados tiveram papel importante para o aumento na produção de leite e redução na contagem de células somáticas (CCS) no leite. No experimento III, houve a adição de biocolina vegetal (BV) na dieta de ovelhas no período de transição, sendo usado 24 ovelhas em período pré-parto (aproximadamente 20 dias), divididas em dois grupos (um sem BV e outro recebendo 5g de BV/animal/dia). No período conhecido como de transição (20 dias pré e pós-parto) não verifiquei diferença

entre grupos nos parâmetros resposta avaliados, isto é, a BV não teve os efeitos hepatoprotetores e antioxidantes esperados; mas após o pico de produção, a adição de BV na dieta das ovelhas estimulou aumento de globulinas, reduziu a atividade de enzimas hepáticas, reduziu reações oxidativas no soro em consequência da resposta antioxidante. Também acredito que esses benefícios da BV na dieta estão relacionados a maior percistencia de lactação das ovelhas, assim como maiores níveis de glutationas (GST e GPx) no leite. O experimento IV usou 30 ovelhas em pico de produção e em condições de estresse por calor, divididas em três grupos homogêneos (controle, 5g BV/animal/dia e 10g BV/animal/dia). O estudo teve duração de 20 dias, onde destaco como principais resultados a maior eficiência produtiva dos animais, devido a um aumento na produção de leite, assim como melhorá da qualidade do leite, isto é, houve redução da CCS e oxidantes. Analisando os parâmetros resposta referente a saúde animal, novamante destaco o aumento de globulinas e variáveis antioxidantes séricas, efeitos positivos ao animal em situação de estresse. De modo geral, verifiquei que as condições de estresse avaliadas aqui, como mastite e estresse térmico afetam negativamente a saúde animal, a produtividade e qualidade do leite, assim verificamos que a FRU e BV são aditivos em potencial para minimizar esses efeitos negativos. A BV na dose usada no periodo de transição não mostrou-se eficiente, porém nos periodo de lactação foi capaz de manter a persistência da lactação.

Palavras-chave: Mastite subclínica, estresse térmico, aditivos, bem-estar animal.

ABSTRACT
Master's Dissertation
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

MILK FARMING IN CHALLENG CONDITIONS: IMPACTS OF NUTRITION ON ZOOTHENIC PERFORMANCE AND ANIMAL HEALTH

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The exploiration of sheep farming in the production of milk for the production of dairy products such as cheese, ice cream and yogurt is a growing reality in Brazil. However, several factors may be present in production systems affecting animal health and consequently milk production. Among the main problems faced by sheep we highlight the thermal stress, the negative energy balance in the transition period and mastitis. Aiming to reduce the different stressors for lactating sheep, this dissertation aimed to evaluate variables related to animal health, productive performance and milk quality of Lacune ewes exposed to stress conditions, that is, animals in thermal stress, with mastitis or In the transition period, as well as checking if adding additives to the diet could minimize the negative effects caused by different stresses. For this, four distinct experiments were performed. In experiment I, we used 30 sheep (20 with subclinical mastitis and 10 used as controls) in two stages of the experiment. First, we identified the etiological agents involved, milk yield and quality, as well as antimicrobial susceptibility tests. In the second stage we evaluated the efficacy of ceftifur in the treatment of mastive by two routes of administration (intramuscular and intramammary), and verified the presence of the drug in milk after treatment. In step I of this study we highlight that sheep with subclinical mastitis had a high leukocyte response, higher serum and milk lipid peroxidation, lower milk yield and quality, as well as the genus Staphylococcus spp. It was the most common among the microorganisms involved in cases of mastitis. In the second stage we found that both treatment routes had low efficacy in curing mastitis, as both routes leave residues in the milk for a longer period than described in the package insert. In experiment II, 30 lacaune ewes at peak production were used in the study during the Brazilian summer, when the animals suffer from heat stress. Animals divided into three groups (control; supplemented with 1% grape residue flour (FRU); and supplemented with 2% FRU) with 10 sheep each. Among the results we highlight that the addition of FRU in the sheep diet had beneficial effects on animal health, that is, the reduction of oxidant levels and increase of the antioxidant system in whey and milk, besides presenting an anti-inflammatory response in the animals that received 2 % FRU. These results probably played an important role in increasing milk production and reducing somatic cell count (SCC) in milk. In experiment III, there was the addition of plant biocolin (BV) in the sheep diet during the transition period, and 24 ewes were used in the pre-partum period (approximately 20 days), divided into two groups (one without BV and another receiving 5g of BV / animal / day). During the transitional period (20 days before and after delivery) we did not find any difference between groups in the evaluated response parameters, ie, BV did not have the expected hepatoprotetotes and antioxidant effects; but after peak production, the addition of BV to sheep diets stimulated

increased globulins, reduced liver enzyme activity, reduced serum oxidative reactions as a result of the antioxidant response. We also believe that these dietary benefits of BV are related to higher lactation rates of sheep, as well as higher levels of glutathione (GST and GPx) in milk. Experiment IV used 30 sheep at peak production and under heat stress conditions, divided into three homogeneous groups (control, 5g BV / animal / day and 10g BV / animal / day). The study lasted 20 days, where we highlight as main results the higher productive efficiency of the animals, due to an increase in milk production, as well as improved milk quality, ie, reduced CCS and oxidants. Analyzing the response parameters related to animal health, novamante highlight the increase of globulins and serum antioxidant variables, positive effects to the animal under stress. Overall, we found that stress conditions evaluated here as mastitis and heat stress negatively affect animal health, milk yield and quality, thus found that FRU and BV are potential additives to minimize these negative effects. BV at the dose used in the transition period was not efficient, but in lactation periods it was able to maintain lactation persistence.

Keywords: Subclinical mastitis, heat stress, additives, animal welfare.

SUMÁRIO

1. CAPÍTULO I

REVISÃO DE LITERATURA

1.1. PANOMARA DA OVINOCULTURA DE LEITE NO BRASIL

O setor de ovinocultura está em constante crescimento, pois segundo dados coletados em 2017, o rebanho ovino em nível mundial era composto por 1.202.430.935 animais, sendo a China o maior produtor, seguida pela Austrália e Índia; já o Brasil ocupava o 19º lugar com 17.976.367 animais (FAO, 2017). Quando olhamos o cenário nacional, notamos que o rebanho ovino possui destaque nas regiões Nordeste e Sul do Brasil, ao passo que as regiões Centro-Oeste e Sudeste não possuem um setor desenvolvido nesse agronegócio. O censo do Instituto Brasileiro de Geografia e Estatística (IBGE) realizado em 2017 revelou um rebanho ovino de 13.770.344 animais no país, número menor do que o contabilizado pela Organização das Nações Unidas (FAO). Destes, a Região Nordeste possui 9.032.191 animais, seguida pela Região Sul com 3.304.397 (Embrapa, 2018a).

A criação de ovinos é norteada pela finalidade dos animais, direcionados para produção de carne, leite, lã ou pele. Dessa forma, cada finalidade possui diferentes exigências a serem atendidas pelo produtor. Para a determinação do sistema de produção deve-se considerar a raça a ser utilizada de acordo com o clima, levando em conta a interação genótipo/ambiente, mão de obra requerida no manejo a ser empregado, bem como cuidados com úbere e ordenha dos animais (Selaive e Osório, 2014).

Uma característica da criação de ovinos é a precocidade em comparação aos bovinos. Mais animais nascem em um ano, já que o tempo de gestação de uma ovelha é de 152 dias (5 meses), enquanto que o de uma vaca é de 280 a 290 dias (9 meses). Isto facilita a troca de animas na ocorrência de doenças, também possibilita uma seleção maior das matrizes utilizadas e consequente melhoria do desempenho, além de antecipar o retorno do investimento na criação (Morris e Kenyon, 2014). A atividade pode ser utilizada em propriedades com mão de obra familiar, visando incrementar a renda das famílias (Selaive e Osório, 2014; Epagri, 2019).

A comercialização de ovinos gerou ao Brasil uma movimentação de R$641.015,00 em 2017, com 3.372.707 animais. Com isso, houve um aumento econômico da atividade de 229,25% em relação ao censo anterior (2006). A produção de lã no Brasil chegou a 7.134.000 Kg, com um total de 2.232.606 animais tosquiados, sendo que apenas o Sul do

país foi o responsável por quase toda essa produção, isto é, 7.059.000 kg (Embrapa, 2018b).

A produção de leite de ovelha é uma atividade desenvolvida em pequena escala no Brasil. O início da produção leiteira no país se deu pela raça Lacaune no sul do país (Guimarães e Souza, 2014). Em estudo elaborado por Bianchi (2018) com dados de 15 propriedades no Brasil, observou-se que a produção comercial de leite de ovelha tem se desenvolvido a partir da década de noventa, o que demonstra que é uma atividade agropecuária com poucos anos de produção. Em 2011, a atividade computava uma produção de aproximadamente 509.000 litros (Rohenkohl et al., 2011). Segundo a Associação Brasileira de Criadores de Ovinos Leiteiros (ABCOL), em 2015 o rebanho de ovinos leiteiros no país era de pouco mais de 6800 matrizes, com produção de 700 mil litros de leite por ano (Bianchi, 2017). Nos anos de 2015 a 2017 das principais unidades significativamente produtoras de leite de ovelha no Brasil, quatro estavam na localizadas no estado do Rio Grande do Sul, três unidades em Santa Catarina, uma no Paraná, duas em São Paulo, uma no Rio de Janeiro, três em Minas Gerais eem Brasília (Bianchi, 2018).

Uma das raças mais exploradas para a atividade leiteira é a Lacaune. As fêmeas da raça podem atingir produção de até 4,5 litros de leite por dia no pico de produção (Brito et al., 2006), mas isso depende da idade da ovelha e das condições alimentares e ambientais oferecidas ao animal. Estudos realizados com ovelhas Lacaune no Oeste de Santa Catarina verificaram produção em torno de 2 litros de leite/animal/dia no pico de produção (Bianchi et al., 2018; Santos et al., 2019).

Mesmo a produção por animal ser em menor quantidade, o leite ovino tem elevado teor de sólidos (Sakul e Boylan, 1992; Katsiari et al., 2002; Pavic et al., 2002), o que favorece a produção de derivados, principalmente o queijo. Além disso, a produção de queijos permite agregação de valor ao produto final, o que pode aumentar a renda dos agricultores.

Um dos momentos mais críticos para as ovelhas leiteiras é o final da gestação, parto e início da lactação, também conhecido como período de transição, onde os animais podem desenvolver doenças metabólicas. Uma das mais importantes é a toxemia da prenhez (Marteniuk e Herdt, 1988; Santos et al., 2011; Schlumbohm e Harmeyer, 2008), que estão relacionadas a maiores demandas de glicose sérica no final da gestação (principalmente quando a gestação é de mais de um cordeiro), mas que não consegue ser suprida apenas pela alimentação, o que estimula a lipólise sobrecarregando o fígado das ovelhas, gerando transtornos metabólicos.

Além disso, estudos têm apontado que a toxemia da prenhez, além de alterações metabólicas, pode diminuir a imunidade dos animais (Lacetera et al., 2001; Hefnawy et al., 2010), o que também pode predispor a outras doenças infecciosas, como a mastite. Já o tratamento dos casos de mastite ovina, muitas vezes é realizado com medicamentos para uso em bovinos, o que pode deixar resíduos de antimicrobianos no leite devido a períodos de retenção diferentes entre as espécies (Pengov e Kirbis, 2009).

Somado a isso, animais mantidos em ambientes com altas temperaturas demandam de maior gasto de energia para dissipação de calor devido ao estresse térmico, o que também afeta a produção dos animais (Morrison, 1983; Marai et al., 2007), assim como o consumo de alimentos (Costa et al., 1992). Para minimizar os efeitos do balanço energético no final da gestação, são necessários cuidados com a dieta dos animais, disponibilizando alimentos concentrados que consigam fornecer maiores quantidade de glicose (Van Saum, 2000; Brozos et al., 2011). Salienta-se que a dieta deve ser formulada de acordo com idade dos animais, de modo a atender as exigências nutricionais, principalmente em ovelhas no pico de lactação, para obter o máximo desempenho produtivo.

1.1.1. IMPACTOS DA MASTITE NA OVINOCULTURA

Um dos principais problemas que comprometem a ovinocultura é a mastite. Doença que compromete a glândula mamária das ovelhas diminuindo a produção (Santos et al., 2007). A mastite é o resultado da resposta imunológica a algum tipo de agressão sofrida pelo tecido mamário, afetando o bem-estar dos animais, diminuindo a eficiência produtiva e aumentando o custo de produção (Nogueira et al., 2013), além de aumentar custos com tratamentos e reposição de matrizes descartadas devido a doença.

De acordo com Mutinati et al. (2013), a gestação e a produção de leite exigem bastante da ovelha, que acaba perdendo peso, reduzindo resposta imunológica e ocasionando o que se chama de estresse oxidativo. A diminuição da capacidade imunológica frente aos agentes patogênicos, somada aos danos celulares provocados pelo estresse oxidativo podem favorecer o desenvolvimento da mastite.

As cabras e ovelhas sadias apresentam níveis basais de CCS muito mais elevado do que os verificados em vacas sadias (~300.000 cel./ml para cabras, ~200.000 cel./ml para ovelhas e ~70.000 cel./ml para vacas), desse modo o nível de células somáticas em leite proveniente de úberes com infecção é, geralmente, muito mais elevado em caprinos

e ovinos do que em vacas (Junior et al., 2015). Brito et al. (2006) afirmam que a detecção da mastite através da contagem de células somáticas e o "California Mastitis Test" (CMT) é uma importante ferramenta para o diagnóstico precoce de mastite, resultando em menores perdas econômicas.

Nas mastites clínicas, em ovelhas leiteiras e nas ovelhas destinadas à produção de carne os principais microrganismos isolados em casos isolados ou em surtos são *Staphylococcus aureus, Staphylococcus* coagulase negativos, *Mannheimia haemolytica* (Bergonier e Berthelot, 2003). Analisando 124 amostras de leite provenientes de 62 ovelhas, 26,6% mostraram-se microbiologicamente positivas, onde *Staphylococcus* coagulase negativo foi o principal microrganismo isolado, detectado em 19 glândulas mamárias (57,6%), *Staphylococcus aureus, Micrococcus* sp., *Streptococcus α hemolítico* e *Streptococcus agalactiae* foram isolados, respectivamente, em cinco (15,2%), cinco (15,2%), três (9%) e uma (3%) glândulas mamárias (Coutinho et al., 2006).

Silva e Silva (2010) examinaram 352 glândulas mamárias, das quais 5,97% apresentaram mastite clínica (MC) e por meio do California Mastitis Test, 7,39% das metades mamárias apresentaram mastite subclínica (MSC). Na MC as bactérias isoladas foram *Staphylococcus* spp. coagulase negativo (42,9%); *Staphylococcus aureus* (9,52%); *Streptococcus* spp. (4,76%) e *Escherichia coli* (4,76%). Na MSC as bactérias isoladas foram *Staphylococcus* spp. coagulase negativo (26,9%); *Staphylococcus aureus* (15,4%); *Streptococcus* spp. (7,69%); *Escherichia coli* (7,69%) e *Citrobacter freundii* (11,5%). Em um compilado de dados de pesquisas, os principais microrganismos isolados de casos de mastite em pequenos ruminantes no Brasil foram *Staphylococcus* spp., *Staphylococcus* coagulase negativa., *S. warneri, S. caprae, S. sciuri, S. epidermidis, S. aureus, Streptococcus* spp., *S. agalactiae, Micrococcus* spp., *Corynebacterium* spp., *Corynebacterium bovis, Bacillus* spp., *Pasteurella multocida, Pseudomonas* spp, *Escherichia coli, Klebsiella* spp., *Serratia* spp (Peixoto, 2010). Esses dados evidenciam que os principais microrganismos envolvidos na mastite em ovinos pertencem ao gênero *Staphylococcus* spp.

A sensibilidade a antimicrobianos é muito variável e depende de diversos fatores. Dentre eles estão à cepa bacteriana, dose correta dos produtos, uso ou aplicações de acordo com o recomendado na bula do medicamento e testes de sensibilidade antes da aplicação. Estudos verificaram que a sensibilidade a antimicrobianos de microrganismos isolados de amostras de leite de ovelhas com mastite oscila entre 20 a 100% (Coutinho et al., 2006) ou 50 a 100% (Silva e Silva, 2010).

Além de reduzir a produção, a mastite pode comprometer a qualidade dos produtos derivados do leite. Rovai et al. (2015) utilizaram leite de ovelhas sadias e com mastite para avaliar a produção, valores de CCS, propriedades de coagulação, o rendimento da produção de queijo, os constituintes do queijo (gordura, proteína) observando que houve diferença significativa entre a produção entre uma glândula saudável e uma com mastite, e entre os valores de CCS no mesmo animal; além da diminuição da gordura do queijo do leite de animais com mastite; no entanto, quando misturados o leite de animais sadios e doentes, não ocorreu diferença significativa. Esses dados evidenciam a importância do diagnóstico precoce dos animais com mastite, visto que se a proporção de animais com a doença no rebanho for elevada, podem ocorrer perdas significativas na produção (litros de leite/animal/dia) e dos derivados do leite (quilos de queijo).

As características sensoriais do queijo podem ser afetadas pelo armazenamento do leite com mastite quando este não é processamento logo após a ordenha, onde o queijo produzido com esse leite apresentou pior aceitação por ter sabor menos palatável (ser mais ácido, amargo) e ainda ter a textura menos resistente (Rovai et al., 2015). Como na maioria das fazendas produtoras de leite, o processamento não ocorre logo após a ordenha, a presença de leite com mastite e microrganismos pode comprometer a produção dos derivados, e consequentemente diminuir a aceitação pelo consumidor.

Uma questão que dificulta o tratamento de mastite em ovinos é a falta de formulações com antimicrobianos direcionadas a espécie. Isso se torna ainda mais difícil quando se busca tratamentos por via intramamária, devido a escassez de bisnagas com tamanho adequado para a aplicação do produto pelo esfíncter do teto das ovelhas. Isso restringe os produtores a usar medicamentos destinados ao tratamento de mastite em bovinos, o que pode causar outros problemas, como resíduos de antibiótico no leite, apontado por Pengov e Kirbis (2009), que também verificaram que o período de retirada do leite (descarte) é maior para ovelhas do que para vacas, pois o período de retenção dos produtos foram maiores para ovelhas.

Em rebanhos com casos frequentes de mastite, ressalta-se a necessidade fazer o isolamento bacteriano a fim de identificar quais os microrganismos presentes na fazenda produtora, bem como, testar a sensibilidade a diferentes classes de antimicrobianos antes de realizar a aplicação nos animais. Esse procedimento reduz os custos com tratamentos, visto que o produtor utiliza apenas produtos previamente selecionados, aumentando as

chances de eficácia e diminui a possibilidade de usar drogas para as quais já existe resistência bacteriana.

1.1.2. AMBIENTE E ESTRESSE TÉRMICO

A região Sul do Brasil é caracterizada por estações do ano definidas, sendo que o inverno é frio e úmido e o verão quente e úmido. Essa variação climática exige dos animais de produção uma adaptação constante às variações de umidade e temperatura. O conjunto de mecanismos utilizados pelos animais para manter a temperatura corporal é denominado termorregulação, sendo fundamental para a adaptação dos animais aos diferentes ambientes (Souza e Batista, 2012). Os autores apontam que quando as temperaturas são muito severas, os animais tem dificuldade de dissipar calor, podendo desenvolver uma quadro de estresse térmico. Ames (2014) cita que as necessidades de direcionamento de energia para ganhos ou perdas de calor podem comprometer a produção animal.

Um termo utilizado sobre a temperatura ideal para os animais de produção foi apresentado por Mount (1974), a termoneutralidade. De acordo com o autor, seria a faixa de temperatura ambiental onde o animal necessita gastar a menor quantidade de energia para regulação da temperatura, onde o mesmo esteja em uma zona de conforto ideal, em plena saúde e potencial produtivo. A faixa correspondente a termoneutralidade pode ser observada na Figura 1.

Figura 1. Zona de termoneutralidade. Adaptado de Neto Carvalho, Revista leite Integral, 2012.

Estudos com ovinos e caprinos no Brasil verificaram que além do ambiente, fatores como a cor da pele e o tamanho dos pelos podem afetar a temperatura corporal dos animais (Acharya et al., 1995; Leitão et al., 2013). A zona de conforto térmico para fêmeas da raça Santa Inês foi em torno de 25°C em ambiente com umidade relativa de 65% (Eustáquio Filho, et al., 2011). No entanto, em ambientes abertos (campos e pastagens) não é possível controlar umidade e temperatura, o que pode expor os animais a situações de estresse. Marai et al. (2007) apontam que ovinos expostos a altas temperaturas tem diminuição na taxa de ingestão de alimentos, comprometendo sua produção e reprodução, visto que a temperatura afeta negativamente as funções biológicas. O estresse térmico também foi verificado em ovinos transportados em caminhões, principalmente quando os mesmos ficavam estacionados, devido ao menor fluxo de ar (Fisher et al., 2005).

Buscando a melhoria dos sistemas de produção, Wojtas et al. (2014) observaram parâmetros fisiológicos de ovinos em diferentes temperaturas e umidades, bem como

efeitos do aumento da ventilação no ambiente, e verificaram que o aumento da velocidade do ar melhorou a condição dos animais mesmo em temperaturas mais elevadas. A partir desses dados, verificou-se que a construção de locais para produção de ovinos devem ser planejados de modo a aproveitar as correntes de ar. Chauhan et al. (2014) observaram que o estresse térmico prejudica as respostas do sistema antioxidante das ovelhas, no entanto, este efeitos negativos são amenizado quando adicionados alimentos com propriedade antioxidante na dieta. O mesmo foi observado por Santos et al. (2019), isto é, com a adição de óleo de açaí na dieta de ovelhas em lactação em estresse térmico os autores verificaram que os animais melhoraram a produção de leite e tiveram ativação do sistema antioxidante, mesmo em condições adversas. A adição de farinha de resíduos de uva na dieta de galinhas de postura em estresse térmico melhorou a eficiência produtiva e a qualidade dos ovos (Reis et al., 2019). Esses dados mostram que a adequação das instalações, ou mesmo, a adição de produtos (via alimentar) que minimizem os impactos do estresse térmico podem ser utilizados para melhorar a saúde e o desempenho dos animais de produção.

Diante do exposto verifica-se a necessidade de ajuste dos ambientes para o melhor desenvolvimento da ovinocultura. Além disso, fatores relacionados às raças utilizadas também podem comprometer o sistema produtivo, visto que as necessidades e capacidade de adaptação ambiental podem ser diferentes. A disponibilização de água e alimentos de qualidade, junto a um ambiente adequado (temperatura, umidade e velocidade do ar) é essencial para a exploração máxima da atividade.

1.2. NUTRIÇÃO DE OVELHAS LEITEIRAS

A eficiência produtiva de ovelhas está relacionada a diversos fatores, dentre eles, a genética, a idade, o desenvolvimento corporal e principalmente aos alimentos disponíveis durante a lactação. O manejo das fêmeas destinadas a produção de leite deve iniciar logo ao nascimento, estendendo-se até as mesmas chegarem a idade reprodutiva, onde cada cabanha deve estabelecer calendário de vacinação contra doenças, aplicação de vermífugos, de acordo com as necessidades locais e do sistema de produção utilizado. Por se tratarem de animais destinados a produção de leite para consumo humano, os cordeiros são separados das mães precocemente, sendo alimentados parcial ou totalmente de forma artificial (Monteiro et al., 2014), isto é, com leite de ovelha ou sucedâneos. Bôas

et al. (2003) apontam que os cordeiros têm um crescimento expressivo nas primeiras semanas de vida. No entanto a separação dos cordeiros da mãe exige mais atenção aos animais, visto que se deve fornecer alimentação suficiente e de boa digestibilidade, para garantir esse crescimento. Uma forma de manter o desenvolvimento dos cordeiros é a suplementação com concentrado (Bôas et al., 2003), que ajuda a fornecer energia e proteína aos animais. O desenvolvimento dos animais até a idade reprodutiva depende de vários fatores, dentre eles a genética, o ambiente e o sistema de produção onde estão inseridos (Simplício e Maia, 2014).

Em relação as matrizes, Kahn et al. (1999) apontam que final da gestação e durante a lactação, há um aumento das necessidades de energia metabolizável, que pode se intensificar conforme o aumento da produção de leite. Os autores citam também que a suplementação nutricional das ovelhas pode melhorar a resistência a parasitoses e melhorar a produtividade. Uma característica comum em ovinos é o desenvolvimento de gestações gemelares, com dois ou mais fetos em ovelhas multíparas. Estudo elaborado por Macedo Junior et al. (2011), apontaram que ovelhas com gestações gemelares com 2 fetos apresentaram maior produção de leite, sendo que este leite possui maior valor energético. Consequentemente, como a produção de leite foi maior, os autores citam que a necessidade energética dessas ovelhas é superior quando comparado as com gestações simples. Esse é um fator que deve ser considerado em sistemas de produção, onde ovelhas, de mesma idade, raça e peso, podem ter demandas nutricionais distintas, mas que devem ser consideradas para manter a produção e saúde do rebanho. Principalmente, por que animais com maior produtividade podem consumir mais reservas corporais (gordura e musculatura), o que pode comprometer gestações posteriores.

Para melhorar a saúde e produtividade de ovelhas em lactação, diversos estudos têm sido realizados utilizando aditivos e suplementos. Dentre os estudos recentes com ovinos, podem ser citados Santos et al. (2019) que adicionaram óleo de açaí na dieta de ovelhas Lacaune em lactação, o qual melhorou o status antioxidante e aumentou a produção de leite; Jaguezeski et al. (2019) que adicionaram nanocapsulas de curcumina na dieta de ovelhas, o que aumentou os níveis de antioxidantes e reduziu a peroxidação lipídica no leite, bem como reduziu os leucócitos totais; Biazus et al. (2018) que suplementaram ovelhas com difenil disseleneto e verificaram aumento da atividade antioxidante e de gordura no leite, bem como ação antinflamatória nos animais. Custódio et al. (2017) adicionaram um produto homeopático na dieta de ovelhas prenhez durante o período de transição, que reduziu os níveis de corpos cetônicos e enzimas hepáticas,

melhorando a saúde das ovelhas. Os dados da literatura apontam melhoras na saúde animal, bem como, do leite de ovelhas, o que ressalta a importância do desenvolvimento e utilização de produtos naturais visando melhorias na saúde e produção animal.

1.2.1. FONTES DE COLINA NATURAL COMO SUPLEMENTO NA NUTRIÇÃO ANIMAL

Para que ovinos possam ter um bom desenvolvimento e posteriormente uma boa vida produtiva, é necessário formular uma dieta completa, de onde o organismo possa extrair os macro e micronutrientes desde o nascimento. A colina é uma nutriente importante para as funções celulares, seja em sua forma íntegra, ou em seus metabolitos, e está diretamente relacionada a mecanismos de sinalização transmembrana, neurotransmissão e transporte de lipídios (Zeisel e Blusztajn, 1994). Tem grande importância para a função hepática, sendo essencial para síntese de lipoproteínas e fosfolipídios, atuando também no transporte e quebra de gorduras, prevenindo o desenvolvimento do fígado gorduroso (Saeed et al., 2017).

Em aves e suínos (denominados não ruminantes), já existem valores de exigência para a suplementação de colina na dieta (Rostagno et a., 2005), comumente utilizada na forma de cloreto de colina (Pompeu et al., 2011; Farina, 2014; Trindade Neto et al., 2009; Robles-Huaynate et al., 2014). Em ruminantes, como os ovinos, a suplementação de colina na forma de cloreto de colina na dieta é ineficaz, pois está é degradada no rúmen e convertida em metano (Neill et al. 1978; Neill et al., 1979).

Buscando melhorias de produção em ruminantes e, sabendo que a colina era degradada no rúmen, foi necessária a busca por outras maneiras de suplementação do nutriente. Neste sentido, diversos estudos foram realizados utilizando fontes de colina protegidas da degradação ruminal (RPC), para avaliar se a suplementação do nutriente traria efeitos benéficos para bovinos (Cooke et al., 2007; Bryant et al., 1999; Bindel et al., 2000; Guretzky et al., 2006) e ovinos (Tsiplakou et al., 2016; Michailoff et al., 2017).

A maioria dos estudos com a RPC focaram em aumento da produção (ganho de peso) e avaliação da função hepática. A colina protegida melhorou o ganho de peso de novilhas em terminação (Bindel et al., 2000), bovinos em terminação alimentados com quatro níveis de colina protegida (0; 0,25; 0,5 e 1,0 % do concentrado) que tiveram maior ganho de peso na dose de 0,25%; enquanto que cordeiros a suplementação com as mesmas doses do produto não tiveram efeito sobre o ganho de peso (Bryant et al., 1999),

todavia a suplementação de colina protegida melhorou o ganho de peso de bovinos no início da terminação (Pinotti et al., 2009).

Em vacas em lactação, a suplementação de RPC tem sido estudada buscando aumento da produção de leite, bem como aumento dos sólidos (proteína, lactose e gordura). A adição RPC em diferentes doses aumentou a produção de leite por animal/dia de acordo com o aumento dos níveis de suplementação, parecendo ser um limitador de produção (Erdman e Sharma, 1991), pode prevenir ou atenuar os casos de lipidose hepática em vacas com restrição alimentar (Cooke et al., 2007). Estudo com suplementação de RPC em vacas holandesas sugere que a colina pode aumentar a taxa de síntese de lipoproteínas de densidade muito baixa e a secreção de lipídios esterificados do fígado, sendo que o desempenho das vacas é responsivo ao aumento da oferta de colina durante o período pré e pós-parto (Piepenbrink e Overton, 2003). A adição de RPC aumentou a produção de leite e a concentração de colina no leite (Piotti et al., 2003), dado que reforça que a produção de leite pode estar relacionada a biodisponibilidade de colina.

A discussão sobre a importância de aditivos na dieta animal, bem como, as novas perspectivas mundiais voltadas para o uso de fontes renováveis de alimentos, estimularam estudos com extratos de plantas com ação no metabolismo animal, melhorando a saúde e desempenho (Rochfort et al., 2008; Vasta e Luciano, 2011; Geraci et al., 2012; Hassan et al., 2013; Valero et al., 2014). Sabendo disso, um dos produtos que foram desenvolvidos é a biocolina, uma fonte vegetal de colina (BV), composta por extratos vegetais a base de *Trachyspermum amni, Citrullus colocynthis, Achyranthus aspera* e *Azadirachta,* e que possui baixa capacidade higroscópica (Rohr, 2018). De acordo com a literatura, a BV contém a colina em forma de conjugados de colina, principalmente na forma de fosfatidilcolina, uma molécula que apresenta resistência natural contra a degradação ruminal (Godínez-Cruz et al., 2015). Estudos com a BV foram realizados em frangos de corte (Calderano et al., 2015) que verificaram que a BV pode substituir o cloreto de colina na dieta. Mena-Bustamante (2018) adicionou a BV na dieta de galinhas de postura, onde a mesma teve bons resultados em relação a taxa de postura e qualidade dos ovos. A adição de BV na dieta de ovelhas em período pré-parto melhorou o peso das ovelhas, bem como aumentou a produção de leite após o parto (Crosby et al., 2017). Já em bovinos de corte, a BV na dieta aumentou o ganho de peso dos animais (Fernandes et al., 2008). As rotas de síntese de colina e fosfatidilcolina podem ser observadas na Fígura 2, onde hipotetizamos o local onde a colina vegetal agiria.

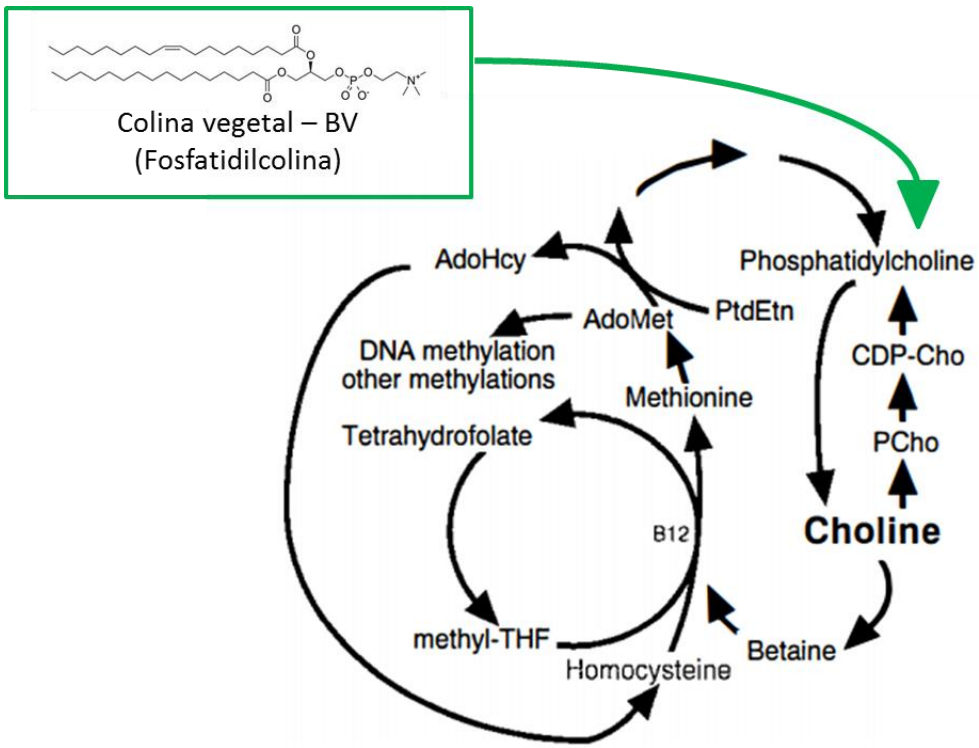

Figure 2. O metabolismo da colina, folato e metionina estão intimamente relacionados (setas em preto). A hipótese é que a colina vegetal (em verde) seria absorvida pelo intestino animal já na forma de fosfadilcolina, poupando a transformação de colina em fosfatidilcolina. Adaptado de Zeisel & Blusztajn (1994).

Os estudos com essa nova fonte de colina ainda são recentes, no entanto, têm apresentados bons resultados. A principal dificuldade de utilizar a colina na dieta de ruminantes é o fato desta ser degradada no rúmen; se a BV não for degradada pelos microrganismos ruminais, será uma fonte viável de suplementação, mas para isso são necessários mais estudos a fim de verificar se a mesma é absorvida pelos animais e qual a dose recomendada para cada espécie.

1.2.2. USO DOS RESÍDUOS AGROINDUSTRIAIS DA UVA NA SUPLEMENTAÇÃO ANIMAL

Uma das atividades agrícolas desenvolvida em regiões do Brasil e a viticultura, com a maior produção destinada ao processamento da uva para produção de vinho (vinicultura). Atualmente, o Brasil ocupa o 17º lugar na produção de vinho no mundo (Embrapa, 2018). Em 2015 a produção brasileira mostrou crescimento comparado aos

anos anteriores, onde foram produzidas mais de 1.499.353 toneladas de uvas, das quais 1.025,475 produzidas na região Sul (Mello, 2015).

As uvas podem ser consumidas in natura, ou serem processadas para fabricação de geleias, sucos e vinho. De toda uva produzida em 2015, mais de 52% foi destinada a produção de produtos derivados (Mello, 2015). Alguns produtos podem utilizar a totalidade da fruta, como as geleias, no entanto, bebidas como o suco e o vinho, utilizam apenas a parte líquida da fruta, sendo produzidas quantidades significativas de resíduos.

A produção de vinho pode ser obtida através da moagem dos cachos de uva íntegros, ou apenas dos grãos, sendo que isso vai depender das tecnologias empregadas na produção. De modo geral, cerca de 3 a 7% do peso do cacho é composta pelos ramos que dão sustentação aos grãos; já após a moagem dos grãos, o bagaço composto pelas sementes e casca da uva equivalem a 15% do peso dos grãos (Cataluña, 1984), dessa forma a cada quilograma de uva processada, são produzidos em torno de 200 gramas de resíduos. A produção de vinhos produz grande quantia de material orgânico que são despejados no meio ambiente, sendo o bagaço o principal tipo de resíduo (Embrapa, 2018c). Os autores citam ainda que podem ser produzidos óleos utilizados em cosméticos, extratos ricos em compostos fenólicos ou fibras alimentares que trazem benefícios para a saúde.

A extração de fibras com potencial antioxidante do bagaço de uva é possível, sendo que esta pode ser utilizada como alimento, sendo uma forma que minimiza os impactos da produção desse resíduo ao meio ambiente (Costa et al., 2019). Estudos comparando farinha de uva e seu extrato líquido mostrou que a farinha pode ser indicada para aumentar teores de fibra na dieta, bem como, o extrato líquido servir como fonte de antioxidantes, visto que o mesmo foi adicionado a um produto que teve boa aceitação (Beres et al., 2019). Alimentos funcionais com propriedades antioxidantes como a uva e o vinho podem trazer benefícios a saúde humana, minimizando efeitos metabólicos e dos radicais livres, sendo que já existem estudos avaliando esses efeitos no sistema cardiovascular (Siochetta, 2018). Como os compostos bioativos estão presentes nos resíduos, a adição desses na dieta de animais de produção também podem trazer melhorias na saúde e consequentemente na produção.

Eleonora et al. (2014) adicionou diferentes doses de bagaço de uva na dieta de cordeiros e verificaram que níveis mais altos de inclusão do resíduo na dieta diminuiu o ganho de peso dos animais devido a diminuição da digestibilidade, uma consequência da grande quantidade de fibras, taninos e lignina. Estudos com adição de silagem de bagaço

de uva em diferentes níveis de inclusão na dieta de vacas observaram que a capacidade antioxidante do leite pode ser melhorada através do resíduo na dieta (Santos et al., 2014). Esses estudos apontam que o resíduo pode ser utilizado na alimentação animal, no entanto a quantidade de inclusão na dieta total deve ser melhor estudada. Os efeitos esperados pela utilização de resíduos da uva estão relacionados aos compostos bioativos, porem ainda existem poucos dados concretos; sabe-se que os polífenois auxiliam na função antioxidante, pois sua adição nas dietas melhora estabilidade oxidativa da carne dos animais, mas é difícil determinar níveis ideais de inclusão, devido a grande variação dos resíduos, além disso deve-se verificar a aceitação dos produtos com esses compostos; pois de acordo com a literatura seu uso pode afetar as características sensoriais dos produtos (Brenes et al., 2016).

Dentre os componentes com ação funcional da uva e derivados estão os compostos fenólicos, um deles o resveratrol (Sautter et al., 2005; Abe et al., 2007), produzido naturalmente pelas plantas como mecanismo de proteção a infecções fúngicas ou danos por radiação ultravioleta (Langcake e Pryce, 1976). Pesquisadores apontam que o resveratrol previne alterações vasculores e o envelhecimento (Delmas et al., 2005), em ratos com trombose induzida a adição de resveratrol na dieta reduziu em 30% o ateroma (Fukao et al., 2004); já Floreani et al. (2003) verificaram que o resveratrol na dieta de porquinhos da Índia promoveu ação cardiopretatora do coração, melhorando atividade da enzima catalase e redução a produção de radicais livres. Em macrófagos de ratos, o resveratrol minimizou a resposta a estímulos pró-inflamatórios, com redução da produção de óxido nítrico e prostaglandina E2 (Leiro et al., 2004). Em ratos com tumor hepático, o resveratrol teve efeitos antioxidantes, hipolipidêmico e tendencioso efeito anti-tumoral (Miura et al., 2003). Estudos recentes têm apontado efeitos benéficos do resveratrol em tratamento de doenças que afetam o sistema nervoso, como a infecção por *Toxoplasma gondii* (Bottari et al., 2018) e por *Trypanosoma cruzi* (Fracasso et al., 2019), visto que o mesmo melhorou a viabilidade das células nervosas, tendo um efeito neuroprotetor. Esses estudos colaboram com a ideia de que o resveratrol melhora a saúde, tendo efeitos anti-inflamatórios e imunomoduladores, efeitos desejáveis para potencializar a produção animal.

Mattos et al. (2016) em revisão sobre potencial antimicrobiano e antioxidante de compostos fenólicos da vinificação, encontraram diversos estudos utilizando extratos oriundos da uva, como aditivos naturais na indústria de alimentos, a fim de evitar o crescimento microbiano e a oxidação lipídica. Compostos extraídos dos resíduos de uva

podem ter ação benéfica à saúde, além de serem utilizados como aditivos; para isso novos métodos para o beneficiamento desses produtos estão sendo estudados, de modo a aproveitar os compostos, diminuindo problemas ambientais e pensando em questões econômicas com a sua utilização (Beres et al., 2017). Os resíduos do processamento da uva e do vinho podem ser utilizados em diferentes finalidades, visto que possuem grande potencial antioxidante relacionado a propriedades benéficas a saúde, um potencial como volumoso pelo teor de fibras na alimentação de ruminantes, ou ainda servir como adubo orgânico.

1.3. PARÂMETROS RESPOSTA DA SAÚDE ANIMAL

Para monitorar a saúde animal, ocorrência de doenças ou avaliar efeitos de produtos ou manejos empregados, podem ser utilizadas diferentes técnicas ou análises laboratoriais, como hemograma, dosagem de enzimas e hormônios. Essas análises são comumente utilizadas para avaliar a saúde dos animais (Campigoto et al., 2019; Sousa et al., 2019; Da Rosa et al., 2019). Em relação a variáveis mensuradas para verificar efeitos produtivos em ovinos, são comumente utilizados o ganho de peso diário, produção de leite, relação entre quantidade de alimentos consumido e ganho de peso ou produção de leite (Manso et a., 1998; Vignola et al., 2009; Santos et al., 2019), porém é importante avaliar parâmetros resposta para compreender os mecanismos envolvidos nas alterações verificadas.

O hemograma é utilizado para avaliar os principais componentes do sangue, principalmente leucócitos, eritrócitos e plaquetas (Failace e Fernandes, 2015). É através desse exame que se pode identificar o desenvolvimento de processos inflamatórios/infecciosos, anemia ou problemas imune dos animais. O hemograma é um exame comumente usado na rotina hospitalar humana e na clínica de pesquenos animais; porém em animais de produção esse exame é raramente usado no campo, apesar dos importantes dados fornecidos.

A função hepática e renal, assim como o metabolismo (lipídico, proteico e de carboidratos) pode ser avaliada facilmente no soro de animais, investigando variáveis respostas, como o colesterol, triglicerídeos, proteína total, albumina, ureia, glicose, entre outros. Isso é possível, pois existem parâmetros de referência conhecidos sobre essas variáveis nas diferentes espécies (Boyd, 1984). Em casos de onde há suspeita de doenças hepáticas, pode-se mensurar enzimas relacionadas à função e saúde desse órgão como a

alanina aminotransferase, aspartato aminotransferase, gama glutamil transferase referente ao fígado e substâncias como a creatinina e ureia relacionadas à função dos rins, por exemplo. Em estudos com aditivos, suplementos e produtos com ação farmacológica, essas variáveis são fundamentais para verificar os efeitos dos produtos sobre a função de órgãos alvo ou onde estes serão metabolizados, pois alguns níveis de inclusão ou doses utilizadas, podem ser tóxicas, ou causar danos aos órgãos, mesmo que não afete o comportamento e ingestão de alimentos nos animais a curto prazo, como o observado por Melchert et a. (2009) na utilização de Levamisol em gatos, onde os mesmos apresentaram sinais de intoxicação, já Baldissera et al. (2016) avaliaram os efeitos de um medicamento antiparasitário em ratos e verificaram que o produto afetava negativamente o sistema antioxidante dos animais.

Outras variáveis que podem ser mensuradas para avaliar a saúde dos animais são os níveis de antioxidantes e oxidantes, assim como danos provocados pelos radicais livres em células ou tecidos (Barbosa et al., 2010). Os antioxidantes são moléculas com potencial de doação de elétrons, essenciais para o funcionamento dos sistemas, visto que atuam combatendo as substâncias com ação oxidante no organismo, mantendo assim, a homeostase celular (Halliwell, 1990; Sies, 1997). Quando há um desequílibrio entre os níveis de antioxidantes, com aumento dos oxidantes, pode-se desenvolver estresse oxidativo (Rahal et al., 2014), o que pode comprometer a estrutura de membranas celulares, função de órgãos e tecidos, afetando negativamente a saúde e produção animal.

O sistema antioxidante é formado por duas vias; em uma delas, estão as enzimas presentes no organismo com ação antioxidante (superóxido dismutase, catalase, glutationa transferase, glutationa peroxidase, glutationa redutase) e na outra estão substâncias com ação antioxidantes que podem ser obtidas da alimentação, como tocoferóis, carotenoides (Sies, 1993; Sies, 1997). Diversos estudos sugerem que a adição de substâncias com características antioxidantes na alimentação humana trazem benefícios a saúde (Vannucchi et al., 1988; Moraes e Cola, 2006; Pereira e Cardoso, 2012) principalmente em longo prazo, visto que os oxidantes podem causar a morte celular e acelerar o envelhecimento (Hirata et al., 2004; Gava e Zanoni, 2005).

Atualmente estudos mostram que os antioxidantes melhoram o funcionamento celular e consequentemente melhoram a produção animal, isso porque reduzem reações oxidativas e radicais livres no organismo animal (Molosse et al., 2019; Reis et al., 2019; Cazarotto et al., 2019). Além disso, a adição de antioxidantes na dieta de animais aumentou os níveis de antioxidantes nos produtos, o que pode aumentar o tempo de

prateleira (Jaguezeski et a., 2018; Gali et. al., 2018; Migliorini et al., 2019; Fortuoso et. al., 2019).

1.4. OBJETIVO

1.4.1. OBJETIVO GERAL

A presente dissertação teve como objetivo avaliar variaveis relacionadas a saúde animal, desempenho produtivo e qualidade de leite de ovelhas Lacaune expostas a condições de estresse, isto é, animais em estresse térmico, com mastite ou em período de transição; assim como verificar se adicionando aditivos na dieta poderíamos minimizar os efeitos negativos causados pelos diferentes tipos de estresse.

1.4.2. OBJETIVOS ESPECÍFICOS

Quatro objetivos específicos foram planejados:

1. Avaliar os efeitos da mastite subclínica sobre o hemograma, bioquímica sérica, estatus antioxidante e oxidante, produção e composição do leite, além de identificar os agentes causadores da infecção e sua sensibilidade a antimicrobianos.

2. Avaliar se a suplementação de ovelhas leiteiras em estresse térmico com farinha de casca e semente de uva altera positivamente os parâmetros de produção e qualidade do leite, resposta imune e perfil oxidativo.

3. Avaliar os efeitos da inclusão de uma fonte de colina vegetal na dieta de ovelhas no período de transição sobre a produção, função hepática, qualidade do leite e saúde dos animais.

4. Verificar os efeitos da adição de uma fonte de colina vegetal na dieta de ovelhas no pico de lactação sobre a produção e qualidade do leite, função hepática, estatus antioxidante e saúde do animal.

2 - CAPÍTULO II

ARTIGOS e/ou MANUSCRITO

Os resultados desta dissertação são apresentados na forma de 2 artigos e 2 manuscritos, com sua formatação de acordo com as orientações das revistas aos quais foram submetidos:

2.1 – ARTIGO I

Subclinical mastitis in Lacaune sheep: causative agents, impacts on production, quality milk, oxidative profiles and treatment efficacy of ceftiofur

Davi Fernando Alba [1], Gilneia da Rosa[1], Dalana Hanauer[2], Thalia Festa Saldanha[3], Carine F. Souza[4], Matheus D. Baldissera[4], Daiane Silva dos Santos[5], Ana Paula Piovezan[6], Lilian Kolling Girardini[6], Aleksandro Schafer da Silva[5*]

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Subclinical mastitis in Lacaune sheep: causative agents, impacts on production, quality milk, oxidative profiles and treatment efficacy of ceftiofur

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Abstract

Mastitis is a major disease affecting dairy sheep. It is caused by microorganisms that generate inflammation of the mammary gland in response to tissue invasion. This syndrome affects the welfare of ewes, as well as the production and quality of the milk, thereby reducing its productive efficiency. Because mastitis causes inflammation process, it also increases the production of free radicals that cause lesions via lipoperoxidation, causing damage to proteins, cells and tissues. One way to minimize the impact of the disease is antimicrobial treatment. Nevertheless, the continuous use of antimicrobials contributes to microbial resistance, in addition to producing residues in the milk and derivatives if not given during the grace period. Therefore, the objective of this study was to evaluate the consequences of subclinical mastitis on ewe health, milk production, milk composition and quality. We also evaluated the susceptibility of the bacteria *in vitro* using disk diffusion antibiograms. Finally, we performed two-way testing of efficacy of treatment in Lacaune ewes using the same agents. In the first stage of the study, 30 lactating ewes (± 90 days) were used, 10 of which were negative on the CMT (California Mastitis Test) used as control group (CG) and 20 sheep with subclinical mastitis diagnosed by CMT (MG). Samples were collected and several analyses were performed on the milk and blood. We found that ewes in the MG had higher lipid peroxidation in serum and milk, as well as lower production, with reduction of the total dry extract in milk. There were 15 isolates of *Staphylococcus hyicus*, four isolates of each *S. epidermidis* and *S. intermedius*, and two isolates of *Corynebacterium* spp. The primary hematological result was leukocytosis in ewes with mastitis. Based on the antibiogram, we chose ceftiofur for *in vivo* tests. In this stage, we divided the sheep with subclinical mastitis into two subgroups of 10 ewes each, to receive drug by two routes: intramuscular (IM) and intramammary (IMM). In the IMM group, of the 10 CMT-positive ewes at the beginning of the experiment, seven were already negative by the racket test 120 hours after the last application (70% efficacy). In the IM group, of the 10 positive ewes, only four were negative after 120 hours of the final application, a low efficacy treatment (40%). We evaluated antimicrobial residues in the milk of treated animals. We found this material within 5 days after treatment in the two forms used; despite the fact that the product's stated withholding period is 3 days. We conclude that ewes with mastitis produce less milk of lower quality. We also conclude that, although ceftiofur is 100% effective *in vitro*, when used in ewes with mastitis, the efficacy did not exceed 70%, and was more efficient when administered *via* the intramammary route.

Keywords: oxidative stress, subclinical mastitis, milk sheep, antimicrobial resistance, *Staphylococcus* spp.

1. Introduction

According to the 2017 agricultural census, sheep flocks in Brazil included approximately 13,770,344 head, of which more than 20% were found in the southern region [1]. The total includes sheep destined for production of meat, wool and milk. In 2009, the United Nations Food and Agriculture Organization (FAO) observed that sheep's milk was the fourth most-produced type worldwide, accounting for 1.3% of total production [2]. Since then, sheep milk production has expanded, especially in Brazil [3]. Even so, the production and industrial processing of sheep milk remains small-scale in Brazil. Data collected directly from companies and websites suggest national output of 509,000 liters per year [4]. Milk yield is high, which is to say that milk solids levels are high, and Lacaune sheep produce more than 15% solids [5]; the same was observed by Blagitz et al. [6] in Santa Inês sheep; Selaive and Osório [7] point out that sheep milk can reach up to 18.5% solids. This property is important in cheese production. Nevertheless, the solids content of the milk may be affected by the feed supplied to the animals and udder health status.

One of the main problems encountered in sheep farming is mastitis, usually caused by microbiological agents that generate severe local inflammation, compromising the mammary gland and significantly reducing milk production [8]. The strong immune response in the affected ewes affects their welfare and increases the cost of production on account of the need for treatment and disposition [9], as well as mortality in more severe cases serious. Ewes with mastitis may have altered milk composition in terms of fat, protein, and somatic cell counts. This is detrimental to industrial processing, altering coagulation characteristics and cheese production [10], as well as sensory characteristics of the product.

Clinical mastitis is easily identified by changes in milk visible in the dark-bottomed mug test. Subclinical mastitis is characterized by decreased production at the level of ownership and productive yield [11]. An easy way to identify subclinical mastitis is measurement of somatic cell counts (SCC), as well as the California Mastitis Test (CMT) [5].

Mastitis has great economic importance in dairy flocks [12 - 15]. These studies identified several etiologic agents in milk samples, and found that many of the bacteria

were resistant to drugs used on farms. Cortinhas et al. [16] used ceftiofur hydrochloride for intramammary treatment of clinical mastitis in cows and cured more than 70%. Similarly, Oliver et al. [17] used intramammary infusions to treat subclinical mastitis in cows, curing 65.8% with 8 days of treatment. Neto et al. [18] used ceftiofur hydrochloride to treat dry cows 30 days before delivery; nevertheless, antimicrobial residues were detected in 10% of the milk of animals during the first postpartum days. Cristina et al. [19] used ceftiofur intramuscularly in cows to evaluate the residue in milk, and found that it remained for at least 12 hours after application. Based on these data, we aimed to treat subclinical mastitis in sheep using ceftiofur intramuscularly and intramammary to evaluate the efficacy of the product used by two routes, as well as to measure its excretion in milk.

Although mastitis is a localized pathology, in clinical cases in ewes, it can have up to 40% mortality when left untreated, especially for mastitis caused by *Staphylococcus aureus* [8]. The same author found that inoculation of the pathogens in the mammary gland caused lost production and one ewe died due to the severity of the infection. In addition, the infectious process can produce metabolites from oxidative reactions and inflammation such as free radicals that circulate throughout the ewe's body, as well as depositing into cells and/or organs, thereby causing injury. In these infectious/inflammatory processes, there is a need for greater energy production, because phagocytosis, regulation of cell growth, intercellular signaling and synthesis of biological substances are important for containing the infection [20]. Excess free radicals cause cell damage by lipid peroxidation (LPO), resulting in oxidation of cell membranes, damaging proteins and DNA, and causing changes in cellular and tissue function, ultimately compromising animal health [21].

We designed the present study because of the importance of mastitis for the production of sheep milk, the lack of information regarding this disease in sheep, and because of the need for more effective treatment alternatives. Therefore, the objective of this study was to evaluate the consequences of subclinical mastitis on ewe health, milk production, milk composition and quality. We also evaluated, in vitro, the susceptibility of isolated pathogens using disc diffusion antibiograms, and later *in vivo* tests, to test the two-way efficacy of treatment for ewes using the same agent.

2. Materials and Methods

The project was approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Santa Catarina, protocol number 8278020918. The experiment was carried out in a dairy sheep farm located in the municipality of Chapecó-SC. The study was divided into two stages, as described below.

2.1. STAGE I

2.1.1. Animals and installation

The farm where the experiment was carried out generates sheep milk for the production of derivatives, mainly cheeses, in addition to marketing animals for breeding and meat production. On this farm, antimicrobial therapy has not been used in dairy sheep to treat mastitis for at least five years; this is a requirement of the company that processes and industrializes milk. However, the percentage of ewes with mastitis in the flock was high, affecting approximately 20%, leading to a considerable volume of milk being discarded, thereby reducing the productive efficiency of the farm and resulting in premature ewe culling. In the experimental period, the shed contained approximately 120 Lacaune lactating ewes (approximate weight 70 kg), all in a confined system, of which 30 were selected during the lactation period of approximately 90 days. The ewes were divided into two groups: 20 mastitic ewes (MG), diagnosed using the California Mastitis Test (CMT) and 10 CMT mastitis-negative ewes, used as control group (CG). MG ewes had a mastitis history of more than 30 days and were already in a separate bay from the other productive ewes; however, they did not present with signs such as fever, loss of appetite, apathy, dyspnea or difficulty in locomotion. The ewes were housed in a covered shed, separated by group in two 24 m^2 bays, in wood-shaving covered floors and access to water *ad libitum*. The ewes received the same feed, divided twice a day (7:00 a.m. and 5:00 p.m.), concentrate (17% crude protein), corn silage, and hay.

2.1.2. Milk measurement

On day 0 of the experiment, the milking of the animals was mechanized and performed twice (06:00 and 17:00 hours). Individual milked volume was measured using a "Milk Meter" (True Test®, Auckland, New Zeland).

2.1.3. Sample collection

For culturing and antimicrobial susceptibility testing, on day 0, a milk sample of each ewe was collected in a sterile bottle, after cleaning the teat (papilla), disinfection with 70% alcohol and discarding the first three strips. Another 40 mL from each ewe were collected using WB HI/Pullout Tru-Test © equipment, which allowed collection of a milk sample from the complete milking of each ewe. Of the 40 mL, 2 mL were stored in microtubes for evaluation of antioxidant and antioxidant status biomarkers.

After morning milking, while the ewes were fasting, we manually collected blood samples from jugular vein using Vacutainer tubes. Approximately 4 mL of blood were placed in tubes containing EDTA (ethylenediamine tetra acetic acid) for erythrogram and leukogram; another 4 mL were placed in tubes without anticoagulant to obtain serum for biochemical analyzes and levels of oxidants and antioxidants.

2.1.4. Milk analysis

2.1.4.1. Isolation and identification of microorganisms and antimicrobial susceptibility testing

Samples were cultured in blood agar supplemented with 5% defibrinated sheep blood, MacConkey Agar and Sabouraud Agar. The plates were incubated at 37 ° C for 24 to 72 h and the microorganisms were identified according to morphological characteristics as described by the National Mastitis Council [22] and Markey et al. [23].

Sensitivity profiles of the microorganisms were determined using the disc diffusion method in Müller Hinton Agar, as described by Clinical and Laboratory Standards Institute [24]. We tested disks (LABORCLIN®) impregnated with the following antimicrobials: amoxicillin + clavulanic acid (10 μg), ceftiofur (30 μg), cefalexin (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), streptomycin (10 μg), gentamicin (10 μg), marbofloxacin (5 μg), neomycin (30 μg), oxacillin (1 μg), penicillin (10 IU), tetracycline (30 μg) and trimethoprim + sulfamethoxazole (25 μg). Plates were incubated in a bacteriological oven for 18–24 h at 37 °C. Subsequently, the halos were read and the sensitivity profile of the isolates was determined.

2.1.4.2. Centesimal composition and somatic cell count (CCS) in milk

The centesimal composition of fat, protein, lactose and total dry extract was determined using an infrared analyzer (LactoStar Funke Gerber®) and SCC using a

digital counter (Ekomilk Scan Somatic Cell Analyzer®), field equipment used for SCC counting in cow's milk.

2.1.4.3. Oxidant/antioxidant status analysis

Glutathione peroxidase (GPx) activity was measured using tert-butyl hydroperoxide as the substrate [25]. Enzyme activity was determined by monitoring the disappearance of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in a medium containing potassium phosphate buffer (100 mM) + EDTA (1 mM), pH 7.7. Results were expressed as U GPx/mg protein. Superoxide dismutase activity (SOD) was determined according to the principle of auto-oxidation of pyrogallol, which is inhibited in the presence of SOD. The variation of optical density was determined kinetically for two minutes at 420 nm, at ten-second intervals, according to the methodology described by Beutler [26]. Activity was expressed as U SOD/mg protein.

Levels of lipid peroxidation (LPO) were measured using the methodology proposed by Monserrat et al. [27]. Results were expressed μmol CHP/mL milk. Nitric oxide levels were measured indirectly as nitrite/nitrate (NOx) levels according to the technique described by Tatsch et al. [28], where 50 μL of sample were pipetted into a reaction cuvette with 50 μL VCl_3. Subsequently, 50 μL of Griess reagent was added and incubated at 37°C. The readout was performed in 96-well microplates using a SpectraMax I3 (Molecular Devices) plate reader, wavelength 550 nm. The results were expressed as μmol/L. The levels of reactive oxygen species (ROS) in plasma were analyzed by the method described by Ali et al. [29]. The volume of 10 μL of serum were incubated with 12 μL of dichlorofluorescein per 1 mm at 37°C for 1 h in the dark. Fluorescence was determined using 488 nm for excitation and 520 nm for emission and the results are expressed as U DCF / mL.

Non-protein thiol (NPSH) levels were measured using the DTNB (5, 5′-dithiobis (2-nitrobenzoic acid); Sigma) method as described by Sedlak and Lindsay [30]. NPSH content in the samples was measured after deproteinization with trichloroacetic acid (TCA 50%). Absorbance readings (405 nm) were performed using a spectrofluorimeter (Biotek, Synergy HT).

2.1.5. Blood analyses

2.1.5.1. Hemogram

Total erythrocyte and leukocyte counts as well as hemoglobin concentration was performed using a semi-automated cell counter (CELM model CC530). The differential leukocyte count was performed using blood smears stained by the method of Romanowsky [31] and visualized using light microscopy. The hematocrit was obtained using microhematocrit capillary tubes by centrifuging at 11,000 g for 5 minutes.

2.1.5.2. Serum biochemistry

Tubes without anticoagulant were centrifuged (5100 *g* for 10 minutes) for serum separation. The supernatants were transferred to Eppendorf tubes stored at –20 °C until analysis. Levels of total proteins (TP), albumin, triglycerides, cholesterol and urea were measured using a semi-automatic analyzer (Bio-2000 BioPlus®) and commercial kits (Analisa®). Globulin levels were calculated as the difference between total protein and albumin.

2.1.5.3. Oxidant and antioxidant status

The method for measuring serum LPO [27], ROS [29], NOx [28] and NPSH [30] levels, as well as the GPx [25] and SOD [26] activities were the same as for milk described in section 2.1.4.3.

2.1.6 Udder conformation

Udders were evaluated according to the recommendations of Feitosa [32], where external inspection was performed. Udders were classified as normal when the two mammary glands were similar and were arranged in the anatomical position expected for the ovine species, and were considered abnormal when there was discrepancy between the glands, lesions or increased volume such that it would hinder milk production and milking. Udders were also evaluated for the presence (positive) or absence (negative) of masses/nodules by palpation after milking.

2.2. STAGE II

2.2.1. Experimental design

After microbial isolation and antimicrobial susceptibility testing in the first stage of this study, the second stage of the project was started. MG ewes were divided into two

subgroups of 10 ewes, in order to form two homogenous groups, blocked based on the isolated pathogens. One subgroup (IM) received intramuscular treatment and the other subgroup (IMM) received intramammary treatment. Only one antimicrobial drug available in commercial formulation was chosen for both routes of application. Based on the susceptibility test of step 1 of this experiment, we chose ceftiofur, a 3rd generation cephalosporin, active against gram-positive and gram-negative bacteria, including those producing beta-lactamases, as follows: 5 grams of ceftiofur hydrochloride in 100 mL of vehicle) for intramuscular application; and Spectramast®LC (125 mg of ceftiofur hydrochloride in 10 mL of vehicle) for intramammary application.

In intramuscularly treated ewes, each ewe received 1.5 mL of ceftiofur once daily for three consecutive days (24-hour interval). The dose was calculated according to the manufacturer's recommendation for the product, which contains 1 mL for each 50 kg of body weight. In ewes with intramammary treatment, the commercially-available product for the treatment of mastitis in dairy cattle was used. Considering the difference between the size of the mammary gland and the milk production, the product was fractionated in four doses of 2.5 mL. The product (2.5 mL) was injected into the interior of each mammary gland through the teat canal, once daily after morning milking for three consecutive days (24-hour intervals).

2.2.2. Sample collection

On day 4 (24 hours after the last application) and on day 9 (5 days after the last application) individual milk samples were collected from each ewe (including 5 mL off the top of the harvested milk, representing the fat portion, totaling 10 mL), stored in sterile vials and sent for analysis of antimicrobial residues. On day 9, milk and blood samples from the ewes were also collected for analysis of milk composition and hemogram/biochemical variables, respectively.

2.2.3. Treatment efficiency

Efficacy of the treatments was measured according the results of CMT and SCC on day 4 (24 hours after the last application) and on day 9 (120 hours after the last application) compared to day 0 (before antimicrobial application).

2.2.4. Antimicrobial residue in milk

To verify the presence or absence of antimicrobial residues, we used the commercial kit Eclipse 50 – Cap-Lab®. The test measures inhibition of microbial growth, using a microtiter plate whose wells contain specific culture medium with *Geobacillus stearothermophilus* spores and an acid-base indicator. After the application of 50 µL of milk, the plates were incubated at 65 ºC and the spores germinate and multiply by acidifying the medium and resulting in the indicator changing from blue to yellow-green. If the milk sample contains an antimicrobial concentration higher than the detection limit of the test, the growth of the microorganism is inhibited such that there will be no acid production, nor consequent modification of the color of the medium. The threshold for detection of ceftiofur residues in parts per billion (PPB) was 100 µg/mL.

2.2.5. Milk composition and SCC

The percentage of fat, lactose, protein and total solids, as well as SCC was evaluated according to methodology previously described in stage I.

2.2.6. Hemogram and biochemical analysis

The hemogram and serum biochemistry levels were also evaluated according to methodology previously described in step I.

2.3. Statistical analysis

The data were subjected to normality testing (Shapiro-Wilk). Data that did not present normal distribution (LPO and SCC in milk; total leukocytes, lymphocytes, neutrophil, monocyte and eosinophil in blood; and LPO and GPx in serum) were transformed to logarithms to normalize them. Data were subsequently subjected to comparison of means using two-way ANOVA for comparisons between groups and analysis over time. Significance was considered when $P < 0.05$. The statistical analyses were performed using R-language, v.3.1 (R Development Core Team 2012).

3. Results

3.1. Stage I

3.1.1. Isolates and antimicrobials

Of the 30 samples collected (20 ewes from MG and 10 from CG), there was no growth and microbial isolation in five. In the remaining 25 milk samples, there was

growth of *Staphylococcus hyicus* (n = 15, corresponding to 60% of the isolated agents), *S. epidermidis* (n = 4, corresponding to 16% of the isolated agents), *S. intermedius* (n = 4, corresponding to 16% of the isolated agents) and *Corynebacterium* spp. (n = 2, corresponding to 8% of the isolated agents) (Table 1).

Most of the isolated microorganisms were sensitive to the 13 antimicrobials tested, as detailed in Table 1. Only three isolates of *S. hyicus* were resistant to oxacillin, one isolate of *S. intermedius* was resistant to streptomycin, marbofloxacin and oxacillin, and an *S. intermedius* isolate was resistant to tetracycline, just as a *S. hyicus* isolate was resistant to enrofloxacin and tetracycline.

3.1.2. Production, composition and quality of milk

Production (liters/ewe/day), protein content, fat and total solids were lower in the MG than in CG, except for lactose levels that were greater in the MG (Table 2). With respect to SCC, levels were significantly greater in the MG group (Table 2).

Reactive oxygen species (ROS) and LPO levels, as well as GPx and SOD activities were also greater in the milk of the MG group. The NOx levels were lower in the MG group. Non-protein thiol (NPSH) levels were not significantly different between groups (Table 2).

3.1.3. Hemogram

Numbers of erythrocytes, hematocrits, and hemoglobin levels did not significantly differ between groups ($P > 0.05$), whereas total leukocyte values were significantly higher in the MG group as a consequence of a significant increase in neutrophils and lymphocytes ($P < 0.05$). The numbers of monocytes and eosinophils were similar in both groups ($P > 0.05$) (Table 3).

3.1.4. Serum biochemistries and oxidative profile

Urea levels were greater in the MG group than in the CG group ($P < 0.05$). The other biochemical variables (glucose, cholesterol, triglycerides, total protein and albumin) did not significantly differ between groups. Levels of LPO, NOx, and NPSH as well as SOD activity were greater in the MG than in the CG. Levels of ROS and GPx activity were not significantly different between groups ($P >0.05$).

3.1.5 Udder conformation

Among the 20 ewes in the MG group, 10 (50%) had some alteration of the udder conformation (Figure 1). Of the 10 ewes in the CG group, only one (10%) had conformational alterations. With respect do masses/nodules, of the 20 ewes in the MG group, 7 (35%) had alterations. Of the 10 ewes in the CG group, one (10%) had nodules.

3.2. Stage II

3.2.1. Efficacy of treatment and clinical evolution

Treatment efficacy is displayed in Table 4. At the beginning of the experiment, 10 ewes in the IM group were positive by the CMT. On day 4 (24 h after the final application) three ewes were had negative CMTs. On day 9, (5 days after the final application), four ewes were negative, with a low efficacy (40%). In the IMM group, of the 10 ewes that were positive on CMT on day 1, on day 4 (24 h after the final application), five ewes were negative on CMT. On day 9 after application, seven ewes were negative, giving an efficacy of 70% for the treatment.

3.2.2. Residual antimicrobial in milk

The results of residual antimicrobial (ceftiofur) in milk are displayed in Table 4. In the IM group, residues were detected in two samples 24 hours after the last application and in two samples 120 hours after last application. For the IMM treatment, 24 hours after the last application residue was detected in all samples, 120 hours after the last application, only one sample had residue.

3.2.3. Milk quality and composition

Milk composition did not significantly differ in terms of percentage of protein and fat between the IM and IMM and CG groups. Lactose was higher in the treated groups (IM and IMM) than in the control group. SCCs were similar in the treated groups (IM and IMM), but were significantly higher than those of the CG group.

3.2.4. Hemogram and serum biochemistries

Blood count variables did not significantly differ between the treated groups (IM and IMM) and GC, except for the number of neutrophils, which was higher in both treated groups. There were no significant differences in terms of biochemical variables (glucose, cholesterol, triglycerides, total protein and albumin) between groups.

4. Discussion

The most prevalent species of microorganism in this study were *Staphylococcus* spp., particularly *S. intermedius*, *S. hyicus* and *S. epidermidis*, corresponding to 92% of the bacterial growth. These species have been reported in the literature as the most prevalent and of greatest importance in the etiology of subclinical mastitis in ruminants ([33], [34], [35]). Drescher et al. [36] also observed that *Staphylococcus* spp. were commonly involved in cases of subclinical mastitis in sheep in the west of Santa Catarina, as observed in the present study.

Among the limitations of the study is that there is a paucity of products that treat mastitis in sheep, especially for intramammary application; instead, we needed to adapt treatments used in cattle that may have different levels of efficacy when the dose is extrapolated one species to another. The time of application of the antimicrobial was determined according to the recommendations for cattle, where the product (IMM) should be applied every 24 hours and can be used in treatments of 2 to 8 days, while the injectable product (IM) is recommended for use every 24 hours for three days. To standardize the application during the study, we used both products for three days. Another limitation was the quantification of SCC with field equipment made to measure cells in cow's milk; This is because equipment for sheep milk analysis is one or the other; furthermore, reference values have not yet been officially established in Brazil. One other important additional limitation is the lack of established antimicrobial resistance breakpoints for ewe mastitis pathogens.

Among the milk samples used for microbial isolation, bacterial growth was observed in six samples from the control group (60%), and the isolated microorganisms were the same ones that were present in milk samples from ewes with subclinical mastitis measured by the CMT test. According to Kulkarni and Kaliwal [37], these agents are part of the normal microbiota of the skin and udder. Several species of *Staphylococcus* are commonly found in the tear ducts and on the skin of domestic ruminants, whereupon they are introduced to the mammary gland via the act of suction performed by calves or lambs, without infection of the mammary parenchyma. It is important to emphasize that *Staphylococcus* spp. has opportunistic behavior in mammary gland infections, since mastitis caused by commensal organisms occurs when the immunity of the host is compromised or when hygienic sanitary conditions are not favorable.

It is also worth mentioning the isolation of *Corynebacterium* spp. in two samples. This microorganism is found in soil and water, and it acts as a secondary pathogen in

subclinical mastitis, suggesting transmission at the time of milking, both from the hands of the milker, or from equipment and utensils that have not been not properly disinfected [38]. Mastitis related to this bacterial genus has also been attributed to excessive sucking by the lamb, or by the contact of the skin of the teat with contaminated pastures and beds post-milking [39].

Subclinical mastitis caused by *Corynebacterium* spp. occur rarely and are less worrying because they generally cause a considerable increase in SCCs, facilitating early diagnosis, as does infection by *Staphylococcus* spp. that causes significant losses in milk production by permanent destruction of cells of the glandular epithelium. Consequently, the injured tissue is replaced by fibrous tissue that acts as a form of protection against the invading organism, causing reduction in phagocytosis by neutrophils and the spread of infection via the bloodstream [40].

Corroborating the results of the present study, Bolsanello et al. [39], in a study on the etiology of mastitis in Bergamacia sheep, reported that 61.1% of the isolates were *Staphylococcus* spp. and only 11.2% were *Corynebacterium bovis*. Zafalon et al. [41] isolate *Corynebacterium spp*. in only 0.2%. Nevertheless, the literature considers this agent to be the one with the highest prevalence in the southeast and center-west regions, according to a national data survey performed by Acosta et al. [42]. Unlike *Staphylococcus aureus* infections, other staphylococcal infections, such as those found in this study, are more easily treated and eliminated. Nevertheless, knowledge of the antimicrobial susceptibility profile of the causative agents of this infection is important for treatment success [42].

In this study, the sensitivity profile of the isolated microorganisms did not show great variation. Most of the isolated agents were sensitive to all antimicrobials, a fact explained by the non-use of antibiotics on the farm where the study was carried out for at least five years. Nevertheless, some isolates were resistant to oxacillin, streptomycin, marbofloxacin, tetracycline and enrofloxacin. According to the literature, oxacillin-resistant microorganisms are more resistant to other antimicrobials, especially penicillin, cefepime and gentamicin, compared to other oxacillin-sensitive microorganisms [38]. It is important to perform antibiograms prior to the treatment of animals to increase efficacy and to prevent the spread of bacterial resistance; nevertheless, *in vitro* results are not always reproduced *in vivo*, as occurred in the second stage of this study. Acosta et al. [42] found that the main antimicrobial drugs with resistance problems were penicillin (80%), ampicillin (67%), amoxicillin (67.4%) and neomycin (80%) when tested against

microorganisms that cause mastitis in ruminants. In a study carried out in the west of Santa Catarina by Drescher et al. [36] there was a great variation in susceptibility of microorganisms isolated from sheep milk with subclinical mastitis, where novobiocin had the lowest percentage of sensitivity (10.46%), followed by erythromycin (16.29%), lincomycin (17.43%) and amoxicillin (19.77%). However, there is a great diversity of agents involved in cases of subclinical mastitis in sheep, as well as different levels of resistance between regions or even properties, as can be observed by correlating the results of the study with those already mentioned in the literature.

In Brazil today, commercial treatment of mastitis in sheep is usually the systemic route, because it is rare for companies to produce tubes specific for sheep on account of low demand. Santana et al. [43] noted that various antimicrobials have been recommended for intramammary mastitis therapy in sheep; however, these recommendations were based on cattle. The great diversity of organisms responsible for subclinical mastitis and the increasing resistance of these isolates to conventional antimicrobials highlight the need to organize therapeutic protocols with support in microbial sensitivity tests, because only some drugs are licensed and are available for use in small ruminants. Thus, sheep farmers use anti-mastitic agents and other drugs for other species, creating a high risk to the safety of these ewes, as well as to their effectiveness, because little is known about the great majority of the drugs in dairy sheep.

Ceftiofur was 100% effective in *in vitro* tests and, because it is found on the local market in two different routes of administration, it was chosen for this study. The efficacies of IM and IMM treatment were different, being greater for IMM (70%). This efficacy is nevertheless considered low; therefore, further studies should be developed to define the best dose as well as to produce commercially suitable delivery devices for ovine species. We recorded low efficacy for the IM route, one of the main routes used to control mastitis in sheep, probably to producers having difficulty finding proper tubes for sheep in the Brazilian market. The use of ceftiofur hydrochloride for the treatment of clinical mastitis in cows had an efficacy of more than 70% [16]. In a study with cows with subclinical mastitis, 65.8% were cured with intramammary application over 8 continuous days [17]; these efficacy values were similar to those in the present study, with intramammary ceftiofur over 3 consecutive days.

According to the manufacturer, the commercial product based on ceftiofur when applied by IMM has a withholding period of 72 hours for cows; that is, the minimum period that the milk of the animal should not be used. This same product was used for

sheep and found that even after 120 hours of the last application, one animal (10%) tested positive for antibiotic residue in milk. This highlights the fact that commercial products produced for cattle and other species have different withholding periods. In the technical recommendations of the commercial product based on ceftiofur used in the IM application, there is no mention of withholding period for the use of cow's milk for human consumption. Nevertheless, in sheep's milk, ceftiofur residues were detected in 3 milk samples 120 hours after application.

The presence of antimicrobial residues in the milk represents a risk to the consumer, and is a serious problem for economic and public health [44]. The economic consequences relate to undesirable effects in the manufacture of dairy products [45]. Antimicrobial residues can influence the quality of the derivatives by inhibiting the fermentation of lactic acid bacteria in the production of yogurt, cheese and butter, causing serious damage to the dairy industry. Public health problems have been pointed out since the 1950s [46], when antimicrobial residues such as penicillin in milk sensitized non-allergic individuals, and caused allergic reactions in previously sensitized patients. In the 1990s, Costa [47] and Albuquerque et al. [48] reported the selection of resistant bacterial strains in the environment caused by the exaggerated use of antimicrobials in animals and in the human gastrointestinal tract after ingestion of antimicrobial residues present in food; therefore, countries have created policies to reduce food contamination by antimicrobials, including banning their use in animal feed. Brazil published decree 171 in December 2018 [49].

During the invasion of the mammary gland by the microorganisms, the number of the defense cells, primarily neutrophils, is increased in order to combat the infectious process, resulting in reduced production and changes in milk composition, concomitant with increased somatic cell counts [50]. This process was observed in the MG, where the ratio of SCC and neutrophils was higher than that of the CG, suggesting that infection with the isolated agents caused severe damage to the glandular tissue, even in subclinical infection. Coelho et al. [51] demonstrated that milk with high somatic cell counts results in alterations of the derivatives, where the cheese presented lower protein content, higher humidity and lower industrial yield, reducing the shelf life of the product. Rovai et al. [10] found that milk from sheep with mastitis also have higher SCC, lower coagulation capacity, and higher serum and protein losses during the coagulation process, where the final product (cheese) was softer and more elastic, probably due to the higher water content. In order to avoid further deterioration of the milk, Rovai et al. [10] recommended

that milk from mastitis be processed faster, because the degradation of this milk is greater than the milk of healthy ewes.

Serum NOx levels were higher in sheep with mastitis, whereas in milk, these levels were lower. Although these ewes had chronic mastitis, NOx levels remained high in the blood, probably as a vigilant defense mechanism, keeping bacterial growth controlled, even though they caused only subclinical mastitis. The increase in nitric oxide is normal in acute conditions because of its role in the inflammatory processes, and its metabolism increases considerably in the context of greater flow of epithelial cells and macrophages in processes such as those. Jungi [52] points out that nitric oxide has antimicrobial action, since activated macrophages synthesize NO. We do not have an explanation for the observed difference in NOx levels in whey and milk; however, they may be due to low levels of nitrite/nitrate excretion in milk.

As previously mentioned, the number of neutrophils was higher in ewes with mastitis, a characteristic of the bacterial agents involved in the infection. Pinheiro-Junior et al. [53] in cases of acute mastitis caused by *Corynebacterium pseudotuberculosis*, reported a large release of common adrenocortical substances at the beginning of the infection, reflecting an increase of total leukocytes in infected animals; however, with the chronicity of the disease, the values tend to remain within physiological ranges for the cows [54], possibly explaining why difference was observed between the other variables in the current study.

Increased lipid peroxidation in serum and milk of GM ewes may reflect the inflammatory process caused by mastitis. Ebrahimi et al. [50], reported an increase of neutrophils in order to combat the infectious process. This increase in circulating leukocytes results in higher production of oxidants such as ROS, which also act to combat invasive microorganisms [55]. However, the exacerbated increase in the production of reactive oxygen species can cause lipoperoxidation of cell membranes, consequently causing cellular and tissue damage. It is important to note that high concentrations of ROS in many cells induce the expression of genes whose products exhibit antioxidant activity [56]. In healthy animals, there is constant redox signaling between ROS production and the elimination capacity of ROS. If the initial ROS increase is relatively small, the antioxidant response may be sufficient to compensate for the increase of ROS and to redefine the original long-term equilibrium. These mechanisms tend to maintain a stable state called redox homeostasis [56]. In cases of imbalance, e.g., bacterial infections, there is greater production of oxidizing compounds (excessive generation of free radicals

or to the detriment of their removal speed) and oxidative stress is established that inhibits tissue remodeling and healing of lesions caused by infectious agents [57].

The antioxidant enzymes SOD and GPx responded to the increase of oxidative reactions; in other words, their activities increased in MG ewes. This may be interpreted as a positive reaction of the organism to the inflammatory process, preventing the establishment of a framework of oxidative stress, because the antioxidant systems were activated, protecting cells against ROS [58]. Atakisi et al. [59] investigated total oxidation and antioxidant capacity in the milk of cows with subclinical mastitis, and found that concentrations of cellular oxidation biomarkers were higher in cows with the disease. These authors suggest that this alteration is a form of protection and reaction of the animal. Ellah [60] notes that mastitis affects the milk production of animals and decreases antioxidant defenses, noting that supplementation with vitamin E, C, beta-carotene and minerals helps in the recovery of animals. A recent study has shown that adding grape flour to sheep diets increases antioxidant levels in milk [61], so these natural additives may be an option in sheep farming.

5. Conclusion

Subclinical mastitis negatively affected udder conformation, production, composition and milk quality in Lacaune sheep. Although the sheep were clinically healthy, subclinical mastitis impaired their health, and they endured inflammatory processes that were intensely activated, reflecting higher energy expenditures as well as greater lipid peroxidation. Bacterial agents were present in the mammary gland of sheep, but did not cause clinical mastitis. The main isolated microbiological agents were those commonly described in ewes with mastitis. Low levels of antimicrobial resistance were detected in this study, possibly as a consequence of the absence of treatment with chemotherapeutic agents in ewes with mastitis. We also concluded that ceftiofur via both routes of administration had low efficacy; however, the intramammary route had 70% grater efficacy than that of the intramuscular route. Antimicrobial residue (ceftiofur) was found in the milk of some sheep within 120 hours after application, exceeding the manufacturer's recommended shelf life for cows. This finding suggests that it is important to use commercial products specific for sheep, in light of the fact that production of dairy sheep has increased in Brazil.

Conflict of interest

The authors declare no conflict of interest.

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Table 1: STAGE I: microorganisms isolated in the 30 dairy sheep; and results of sensitivity testing (S: sensitive; R: resistant) for various antibiotics: AM (amoxicillin + clavulanic acid - 10 μg), CE (ceftiofur - 30 μg), CA (cefalexin - 30 μg), CI (ciprofloxacin - 5 μg), EN (enrofloxacin - 5 μg), ST (streptomycin - 10 μg), GE (gentamicin -10 μg), MA (marbofloxacin - 5 μg), NE (neomycin - 30 μg), OX (oxacillin - 1 μg), PE (penicillin – 10 IU), TE (tetracycline - 30 μg), TR (trimethoprim + sulfamethoxazole - 25 μg).

Sheep[1]	Isolated agent	Antimicrobial												
		AM	CE	CA	CI	EN	ST	GE	MA	NE	OX	PE	TE	TR
M1	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	R	S	S	S
M2	Staphylococcus epidermidis	S	S	S	S	S	S	S	S	S	S	S	S	S
M3	Staphylococcus intermedius	S	S	S	S	S	S	S	S	S	S	S	S	S
M4[3]	Corynebacterium sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
M5	Staphylococcus intermedius	S	S	S	S	S	S	S	S	S	S	S	S	S
M6	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M7	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	R	S	S	S
M8	Staphylococcus epidermidis	S	S	S	S	S	S	S	S	S	S	S	S	S
M9	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M10	Staphylococcus intermedius	S	S	S	S	R	S	S	R	S	I	S	S	S
M11	Staphylococcus intermedius	S	S	S	S	S	S	S	S	S	S	S	R	S
M12[2]	---	-	-	-	-	-	-	-	-	-	-	-	-	-
M13	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M14	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M15	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M16	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M17	Staphylococcus hyicus	S	S	S	S	S	R	S	S	S	S	S	R	S
M18	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
M19	Staphylococcus epidermidis	S	S	S	S	S	S	S	S	S	S	S	S	S
M20	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
C1	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S

C2[2]	---	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S		S
C4	Corynebacterium sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
C5	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	R	S	S	S
C6	Staphylococcus hyicus	S	S	S	S	S	S	S	S	S	S	S	S	S
C7[2]	---	-	-	-	-	-	-	-	-	-	-	-	-	
C8[2]	---	-	-	-	-	-	-	-	-	-	-	-	-	-
C9	Staphylococcus epidermidis	S	S	S	S	S	S	S	S	S	S	S	S	S
C10[2]	---	-	-	-	-	-	-	-	-	-	-	-	-	

Note 1: Animals M1 to M20 belong to the group with mastitis and C1 to C10 to the control group.
Note 2: No etiological agent was isolated (---), resistance test not performed (-).
Note 3: No antibiogram was performed for *Corynebacterium*, because the technique used is only for fast growing bacteria, as *Corynebacterium* has slow growth the result would not be reliable.

Table 2. STAGE I - production, composition and milk quality of sheep diagnosed with subclinical mastitis compared to control (negative in the CMT test).

Variable	**Mastitis group (MG)**	**Control group (CG)**	**P-values**
Production (L)	0.51 (0.2)	0.85 (0.2)	**0.001***
Protein (%)	2.64 (0.9)	3.95 (0.4)	**0.001***
Fat (%)	4.30 (0.9)	5.93 (0.9)	**0.001***
Lactose (%)	4.93 (0.7)	3.90 (0.30)	**0.046***
Total solids (%)	11.91 (0.8)	13.68 (1.0)	**0.008***
CCS ($x10^3$ mL)	3059 (1704)	503.1 (456)	**0.014***
Oxidant/antioxidant status			
ROS (U/DCF mg protein)	0.27 (0.08)	0.20 (0.03)	**0.001***
LPO (µmol CHP/mL milk)	164.3 (96.2)	42.2 (29.1)	**0.001***
NOx (µmol/L)	65.4 (10.5)	81.2 (11.0)	**0.023***
NPSH (SH/g of tissue)	0.80 (0.14)	0.81 (0.12)	**0.956**
SOD (U SOD/mg protein)	1.12 (0.41)	0.62 (0.32)	**0.050***
GPx (U GPx/mg protein)	14.2 (5.4)	9.85 (2.3)	**0.047***

*P <0.05 shows significant difference between groups.

Table 3. STAGE I - Hemogram, serum biochemistry and status of antioxidants and oxidants in serum of sheep diagnosed with subclinical mastitis compared to control (negative California Mastitis Test).

Variable	**Mastitis group (MG)**	**Control group (CG)**	**P-values**
Hemogram			
Erythrocytes ($x10^6$ µL)	7.25 (1.53)	6.22 (1.68)	**0.142**
Hematocrit (%)	35.5 (3.78)	33.10 (5.8)	**0.587**
Hemoglobin (mg/dL)	10.7 (1.78)	10.0 (1.20)	**0.624**
Leukocytes ($x10^3$ µL)	17.1 (5.8)	9.58 (1.9)	**0.001***
Lymphocytes ($x10^3$ µL)	6.36 (2,5)	3.38 (1,6)	**0.001***
Neutrophils ($x10^3$ µL)	10.02 (4.3)	5.68 (2.2)	**0.001***
Monocytes ($x10^3$ µL)	0.72 (0.5)	0.50 (0.27)	**0.534**
Eosinophils ($x10^3$ µL)	0.03 (0.1)	0.0 (0.0)	**0.667**
Serum biochemistries			
Glucose (mg/dL)	62.0 (7.2)	71.8 (9.4)	**0.207**
Cholesterol (mg/dL)	59.0 (11.3)	68.9 (7.4)	**0.159**
Triglycerides (mg/dL)	21.1 (5.4)	23.0 (4.9)	**0.847**
Total protein (g/dL)	8.25 (1.2)	8.0 (0.87)	**0.924**
Albumin (g/dL)	2.92 (0.4)	2.90 (0.40)	**0.947**
Globulin (g/dL)	5.35 (1.23)	5.12 (0.88)	**0.854**
Urea (mg/dL)	45.6 (8.3)	62.3 (11.3)	**0.001***
Oxidant/antioxidant Status			
ROS (U/DCF mg protein)	1.71 (0.35)	1.44 (0.24)	**0.081**
LPO (µmol CHP/mL serum)	226.2 (134)	52.2 (25.5)	**0.001***
NOx (µmol /L)	156.8 (17.0)	129.2 (19.0)	**0.047***
NPSH (SH/g of tissue)	1.51 (0.22)	1.21 (0.10)	**0.034***
SOD (U SOD/mg protein)	0.89 (0.35)	0.41 (0.23)	**0.050***
GPx (U GPx/mg protein)	7.60 (4.2)	7.2 (5.2)	**0.795**

*P <0.05 showns significant difference between groups.

Table 4. STAGE I - Results (PO: positive; NE: negative) of the California Mastitis Test (CMT) after intramuscular treatment (IM) or intramammary treatment (IMM) in sheep compared to the control (CON), as a result of the presence (PR) or absence (AU) of antimicrobial residues in sheep milk 24 and 120 h after final application.

Animal/group	**Organism**	**Racket test (CMT)**		**Residual antibiotic in milk**	
IM group		Day 4 (24 h post-last dose)	Day 9 (5 days post-last dose)	Day 4 (24 h post-last does)	Day 9 (5 days post-last dose)
1	*Staphylococcus hyicus*	PO	NE	PR	PR
2	*Staphylococcus epidermidis*	NE	PO	PR	PR
3	*Staphylococcus intermedius*	PO	NE	AU	AU
4	*Corynebacterium sp.*	NE	NE	AU	AU
5	*Staphylococcus intermedius*	PO	PO	AU	AU
6	*Staphylococcus hyicus*	PO	PO	AU	AU
7	*Staphylococcus hyicus*	PO	PO	AU	AU
8	*Staphylococcus epidermidis*	PO	PO	AU	AU
9	*Staphylococcus hyicus*	NE	NE	PR	PR
10	*Staphylococcus hyicus*	PO	PO	AU	AU
Efficacy		**30% (3/10)**	**40% (4/10)**	**-**	**-**
IMM group					
1	*Staphylococcus intermedius*	NE	PO	PR	AU
2	*Staphylococcus intermedius*	PO	PO	PR	AU
3	---	PO	NE	PR	AU
4	*Staphylococcus hyicus*	NE	NE	PR	AU

5	*Staphylococcus hyicus*	PO	PO	PR	PR
6	*Staphylococcus hyicus*	NE	NE	PR	AU
7	*Staphylococcus hyicus*	PO	NE	PR	AU
8	*Staphylococcus hyicus*	NE	NE	PR	AU
9	*Staphylococcus hyicus*	NE	NE	PR	AU
10	*Staphylococcus epidermidis*	PO	NE	PR	AU
Efficacy		**50% (5/10)**	**70% (7/10)**	-	-
CON					
1	*Staphylococcus hyicus*	NE	NE	AU	AU
2	---	NE	NE	AU	AU
3	*Staphylococcus hyicus*	NE	NE	AU	AU
4	*Corynebacterium sp.*	NE	NE	AU	AU
5	*Staphylococcus hyicus*	NE	NE	AU	AU
6	*Staphylococcus hyicus*	NE	NE	AU	AU
7	---	NE	NE	AU	AU
8	---	NE	NE	AU	AU
9	*Staphylococcus epidermidis*	NE	NE	AU	AU
10	---	NE	NE	AU	AU

Note: Etiologic agent not isolated (---).

Table 5. STAGE II - Milk composition in the sheep of the three experimental groups (intramuscular –IM; intramammary – IMM; control group – CON) on the 9th day of experiment, i.e., 9 days after starting treatment with chemotherapeutic agents.

Variable	**IM**	**IMM**	**CON**	**P-values**
Protein (%)	3.81 (0.3)	3.55 (0.6)	3.97 (0.20)	**0.757**
Fat (%)	5.65 (1.2)	6.87 (1.6)	4.90 (1.5)	**0.052**
Lactose (%)	5.63 (0.5)[a]	5.24 (0.9)[a]	3.85 (0.2)[b]	**0.001***
Total solids (%)	14.79 (1.3)[a]	16.0 (1.9)[a]	12.72 (1.0)[b]	**0.001***
CCS ($x10^3$)	2503 (1463)[a]	1729 (1154)[a]	229.3 (155.2)[b]	**0.001***

P ≤0.05 shows difference between groups, identified by different letters (a, b) on the same line.

Table 6. STAGE II - Hemogram and serum biochemistry in the sheep of the three experimental groups (intramuscular –IM; intramammary – IMM; control group – CON) on the 9th day of the experiment, i.e., 9 days after starting chemotherapy.

Variable	IM	IMM	CON	P-values
Hemogram				
Erythrocytes ($x10^6$ µL)	9.74 (1.4)	10.0 (1,2)	8.45 (1.2)	**0.214**
Hematocrit (%)	32.4 (3.1)	34.4 (4.8)	31.8 (3.2)	**0.567**
Hemoglobin (mg/dL)	9.68 (0.9)	10.3 (1.5)	9.91 (0.9)	**0.814**
Leukocytes ($x10^3$ µL)	18.83 (9.1)	23.14 (13.0)	9.04 (6.3)	**0.093**
Lymphocytes ($x10^3$ µL)	8.89 (6.6)	8.40 (5.5)	4.48 (4.0)	**0.310**
Neutrophils ($x10^3$ µL)	9.30 (3.7)[a]	13.8 (8.7)[a]	3.89 (2.6)[b]	**0.002***
Monocytes ($x10^3$ µL)	0.63 (0.6)	0.04 (0.1)	0.29 (0.3)	**0.064**
Eosinophils ($x10^3$ µL)	0.01 (0.09)	0.04 (0.1)	0.02 (0.07)	**0.798**
Serum biochemistries				
Glucose (mg/dL)	77.2 (16)	72.9 (14)	70.2 (8.2)	**0.745**
Cholesterol (mg/dL)	64.1 (24)	69.5 (19)	74.2 (11)	**0.423**
Triglycerides (mg/dL)	25.5 (12)	27.5 (7.8)	24.7 (8.8)	**0.589**
Total protein (g/dL)	8.15 (1.32)	8.73 (0.85)	7.68 (0.71)	**0.235**
Albumin (g/dL)	3.05 (0.42)	3.01 (0.44)	3.16 (0.47)	**0.806**
Globulin (g/dL)	5.10 (1.01)	5.72 (0.94)	4.52 (0.73)	**0.114**
Urea (mg/dL)	47.9 (8.9)	48.5 (14.1)	43.8 (7.6)	**0.501**

*P <0.05 shows difference between groups, identified by different letters (a, b) on the same line.

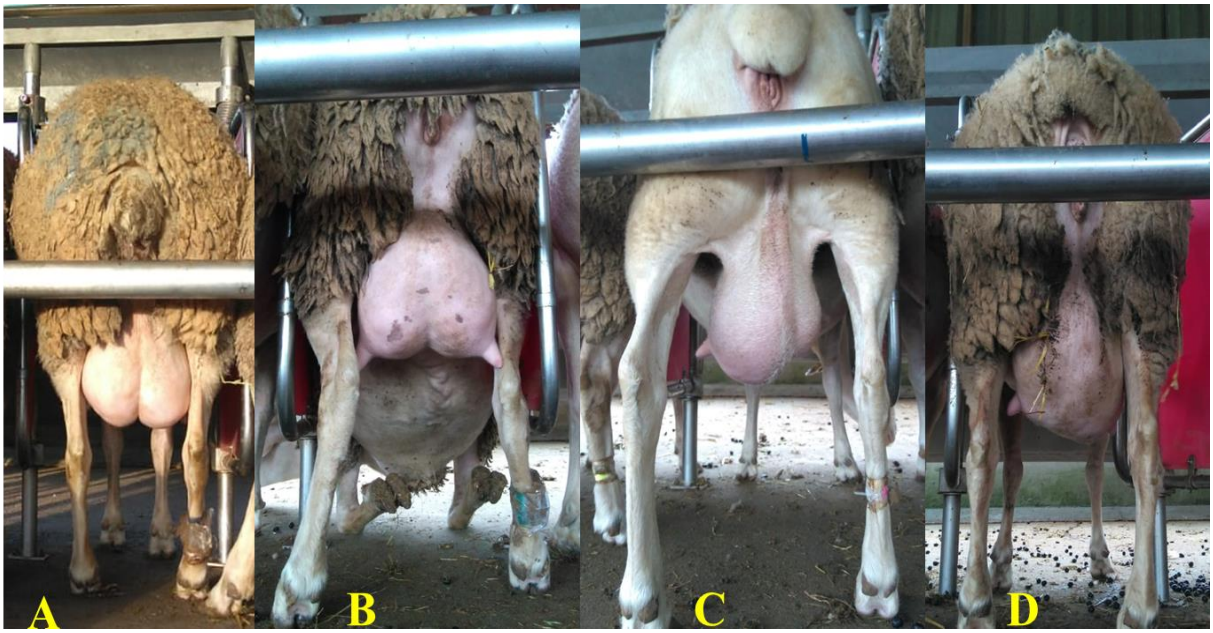

Figure 1. STAGE I - Udder conformation classification of Lacaune sheep diagnosed with subclinical mastitis compared with control (California Mastitis Test-negative). Udders with normal morphology (A and B) and abnormal morphology (C and D).

2.2. ARTIGO II

Use of grape residue flour in lactating dairy sheep in heat stress: effects on health, milk production and milk quality

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Abstract

The objective of this study was to evaluate the effects of grape residue flour (GRF) on antioxidant activities, biochemistry variables, components of the immune system and milk production and quality of Lacaune sheep in heat stress. Twenty-seven multiparous lactating sheep [50 ± 1.8 days (d) milking] were stratified by initial body weight, age, date of lambing and milk production and assigned randomly to 1 of 3 treatments (9 sheep/treatment): no GRF supplementation (control group) or supplementation at 1 % (10 g/kg GRF) or 2 % (20 g/kg GRF) of GRF (bark and seed) in the concentrate (grains and minerals mixture). Each ewe received 0.8 kg/d of concentrate, 3.6 kg/d of corn silage, and 0.25 kg/d of *Cynodon* spp hay. Milk production along with blood and milk samples were collected on d 1, 10 and 15. The 2% GRF sheep had increased serum concentrations of superoxide dismutase and glutathione peroxidase activity on d 15 compared to control sheep. Over time (d 10 to 15), lipid peroxidation was reduced in 2% GRF sheep. Total serum antioxidant capacity was greater in 2% GRF sheep compared to control sheep on d 10 and 15. Superoxide dismutase and glutathione peroxidase activity in milk samples were greater in 2% GRF sheep compared to control sheep. Supplementation with GRF did not affect milk production but GRF sheep were more efficient compared to control sheep. Protein and lactose concentrations were similar between treatments, but total solids and fat concentrations were greater in 2% GRF sheep compared to control sheep on d 15. Somatic cell count was reduced in GRF sheep compared to control sheep. In summary, supplementation with 2% GRF in dairy sheep in heat stress resulted in antioxidant and anti-inflammatory responses, which improved milk quality and reduced somatic cell count and lipid peroxidation.

Keywords: milk quality, lactation, oxidative stress, supplementation, additives.

1. Introduction

In dairy sheep, energy requirements increase with gestation, lambing, and lactation, which leads to an increase in cellular respiration and consequently free radical production and oxidative stress (Mutinati et al., 2013). This occurs due to an imbalance between production of free radicals or reactive metabolites (oxidants) and antioxidants. When antioxidants do not rapidly neutralize free radicals, it leads to the accumulation of oxidants in tissue and consequently damages biomolecules and organs (Durackova, 2009). This effect may be greater when the animal is under heat stress. According to the literature, exposure of sheep to elevated ambient temperature negatively affects biological functions which is reflected in the impairment of production and reproductive traits. Elevated ambient temperatures increase the dissipation of excess body heat in order to negate the excessive heat load. Further, heat stress causes a decrease in feed efficiency and utilization, causes a disturbance in water, protein, energy and mineral balances, and affects enzymatic reactions, hormonal secretions and blood metabolites (Marai et al., 2007).

To maintain the equilibrium between production and elimination of free radicals, the organism utilizes 3 methods of protection: 1) enzymes interfere with free radical formation, 2) antioxidants neutralize molecules with oxidizing action, and 3) repairing the system, by recognizing compromised molecules and removing them (Durackova, 1998). Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase are enzymes with antioxidant action (Durackova, 2009). Further, exogenous substances such as tocopherol (vitamin E) and flavonoids also exhibit antioxidant potential (Durackova, 2009; Silva, 2010).

Antioxidants compounds are capable of donating electrons and neutralizing free radicals, resulting in the prevention of cellular lesions (Saeidnia and Abdollahi, 2013).

Due to the numerous benefits, the search for natural antioxidants in food products, cosmetics and pharmaceuticals has been a primary objective in the last 20 years (Laguerre et al. 2007). Feeds with nutraceutical properties have been sought after by researchers, as recommended by nutritionists, due to their function in the prevention and treatment of disease (Moraes and Colla, 2006). Historically, antioxidants were added to food to avoid lipid peroxidation and rancidification during processing and storage (Salami et al., 2016). According to Salami et al. (2016), antioxidants present in the diet combat oxidative stress in livestock animals and in doing so improves health, welfare, nutritional and organoleptic quality, as well as shelf life of animal products. Resveratrol is a phenolic compound found in grapes (Abe et al., 2007) which possesses antioxidant, antimicrobial, and anti-inflammatory properties (Karami et al., 2018).

Previous research has shown that resveratrol plays a role in the prevention of apolipoprotein-B peroxidation and is associated with low-density lipoproteins (LDL), which ultimately restores glutathione in plasma and tissue (Sahin et al., 2010). Quercetin is a flavonoid found in grapes in the glycosylated form and exhibits antioxidant properties (Behling et al., 2004). Flavonoids are considered effective antioxidants due to their ability to scavenge free radicals and by chelating metal ions (Kandaswami and Middleton, 1994). Antioxidant properties are directly due to the hydroxyl radical ($^{\bullet}OH$) and the superoxide anion ($O2^{-\bullet}$), both of which are highly reactive species involved in the initiation of lipid peroxidation (Behling et al., 2004).

The southern region of Brazil is known for its wine production, producing more than 500 tons of grapes in 2016 (IBGE, 2016). Increased wine production (and other grape derivatives) results in a large volume of waste which is currently not being utilized efficiently. Brenes et al. (2008) reported that the inclusion of grape residues in the diet has positive effects on the welfare of broilers and increased the amount of antioxidants

present in the muscle. Similarly, Ebrahimzadeh et al. (2018) reported that the inclusion of grape residue in the diet of broiler chickens improved the immunological and antioxidant responses. In another recent study, Hamza and El-Shenawy (2017) supplied oral resveratrol to mice exposed to nicotine and observed reduced lipoperoxidation and increased antioxidant enzymes. In dairy cows, the inclusion of grape silage in the diet had positive effects on milk production due to the increase in antioxidant capacity in the group with the greatest addition of grape residue (Santos et al., 2014). Thus, our hypothesis is that inclusion of grape residue flour (GRF) in the diet, will lessen the negative effects associated with heat stress and ultimately improve the quality of milk and animal health.

The GRF is composed of catequin, epicatechin, quercetin, caempferol, and resveratrol; all of which are substances known to improve the antioxidant system (Abe et al., 2007). However, the biggest challenge with providing grape residue in animal feed is its rapid deterioration in natural form. Therefore, the grape residue will be processed into a flour which will then be added to the feed. Our hypothesis is that supplementation with this residue will minimize oxidative stress. Thus, the objective of this study was to evaluate the effects of grape residue flour on antioxidant activities, biochemistry variables, components of the immune system and milk production and quality of Lacaune sheep under heat stress.

2. Material and Methods

2.1. Grape residue flour (GRF)

The GRF used was purchased from a natural products company (Essencial®). Chemical composition was analyzed according to AOAC (2000): dry matter (DM), method 930.15; crude protein (CP), method 976.05; ethereal extract (EE), method 920.39

and ashes, method 942.05. The concentration of neutral detergent fiber (NDFom) and acid (ADFom) was done according to the methodology of Van Soest et al. (1991) without sodium sulfite and amylase.

2.1.1. Resveratrol and quercetin content in GRF

One GRF sample (1 mg mL^{-1}) was analyzed by high performance liquid chromatography coupled with diode array and mass spectrometry detectors (HPLC-DAD-MS/MS) according to Vieira et al. (2001) with minor modifications. High performance liquid chromatography was determined by Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan) equipped with an Auto-Sampler (SIL-20AHT), two Shimadzu LC-20ADT reciprocating pumps connected to the degasser DGU20A3R, integrator CBM20A, UV–VIS detector DAD SPD-M20A, and oven CTO-20A. The HPLC system was coupled to the compact quadrupole time-of-flight (Q-TOF) mass analyzer (Bruker Daltonik GmbH, Bremen, Germany), which was controlled using Ot of Control Software. The parameters for analysis were set using negative ion modes with spectra acquired over a large range from 50 to 1200 m/z. Optimum values for ESI-MS were: capillary voltage of 4500V, drying gas temperature of 215°C, drying gas flow of 10.0 L/min, nebulising gas pressure of 5.0 Bar, collision RF of 150 Vpp, transfer time of 70 s, and a pre-pulse storage of 5 ls. Automatic MS/MS experiments were performed using nitrogen as collision gas and by adjusting the collision energy values as follows: m/z 100, 20 eV; m/z 500, 30 eV; and m/z 1000, 35 eV. The MS data were analyzed using Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany). Analyses were carried out within the C-18 column (4.6 mm x 250 mm, Merck, Germany) packed with 5 µm diameter particles and within the C-18 pre-column (RP 18 5µm, Merck, Germany). The first mobile phase (phase A) was two percent acetic acid at a pH of 4.2. The second mobile phase (phase B) used methanol,

acetic acid, and distilled water at a ratio of 18:1:1, respectively. The gradient elution was 0 min: 20% of B, 0-25 min: 50% of B, 25 min: 20% of B, 30 min: 20% of B (end of run), at a flow rate of 0.8 ml/min. The peaks were identified by comparing the present results with the retention times and mass spectrums from the software library and external standards. The external standards included resveratrol, quercetin and rutin (all standards by Sigma-Aldrich, St Louis, MO, USA) between 1.5 and 24 µg/mL. Sample and standards were tested in triplicate and the results are presented as mean ± standard deviation (SD).

2.1.2. Determination of total phenolic compounds (TPC) and antioxidant activity by elimination of radicals by DPPH

For extraction, 0.5 g of GRF samples were dissolved in 50 ml of distilled water. The mixture was placed in an ultrasonic bath (70 W) for 3 hours and remained in the dark for 3 more hours. The supernatant was filtered with quantitative filter paper ('Whatman' # 40) and stored in a 100 mL volumetric flask wrapped in aluminum foil. After extraction, extracts were stored in Eppendorf tubes and maintained at – 80 ºC until analysis (Larrauri et al., 1997; Bertoletti et al., 2018).

Quantification of TPC was performed by Folin-Ciocalteu colorimetric method modified by Bonoli et al. (2014). An aliquot of each diluted sample was mixed with 0.5 ml of Folin-Ciocalteu reagent and was stirred for 1 minute. Next, 2 ml of sodium carbonate (20 %) was added in the mixture and stirred for 30 s. After 2 h of incubation, the absorbance was read at 750 nm in relation to blank. The standard curve was prepared by solutions of gallic acid in methanol. The concentration of TPC was obtained using an equation derived from the standard curve of gallic acid (expressed in mg of gallic acid

equivalent per g of dry sample; mg EGA/g). Data are presented as the mean ± SD of triplicates.

Evaluation of free radical scavenging activity in the extracts was determined by the antioxidant reduction capacity of DPPH radical according to Brand-Williams et al. (1995). Five dilutions of each extract were prepared in test tubes and 0.3 ml of each diluted extract was added to 2.7 ml of DPPH radical (40 µg/ml). After incubation for 1 h in the dark, the absorbance was read at 515 nm relative to the blank prepared. Free radical scavenging activity was reported as the IC 50 (µg/mL), which is defined as the antioxidant concentration required to eliminate 50% of the DPPH present in the test solution. All tests were performed in triplicate and IC 50 values were reported as means ± SD of triplicates.

2.2. Animals and experimental design

The experiment was conducted at a commercial dairy sheep farm in Chapecó, Santa Catarina, Brazil. Twenty-seven multiparous lactating sheep [50 ± 1.8 days (d) milking] of the Lacaune breed, were stratified by body weight (70.6 ± 2.9 kg), age, date of lambing, and milk production and assigned randomly to 1 of 3 treatments (9 sheep/treatment): no GRF supplementation (control group; CON) or supply of 1 % (1% of GRF) or 2 % (2% of GRF) of GRF (bark and seed) in the concentrate (grains and minerals mixture). Each animal received 0.8 kg/d of concentrate, approximately 3.6 kg/d of corn silage and 0.25 kg/d of *Cynodon* spp hay divided into 2 daily feedings (07:00 h and 17:00 h; Table 1). In order to standardize feed intake, all sheep were fed individually, concentrate was offered first and after consumption (approx. 15 min) silage was offered. The sheep were housed in a covered feedlot with wood shavings on the floor and allocated to 3 pens (1 treatment/pen) located side by side. The experiment occurred over

a15 day period with the first 10 days set as an adaptation period to the experimental diet, protocol similar to that described by Jaguezeski et al. (2018). Concentrate including GRF had less acceptance than control, but after 3 days of adaptation all sheep were consuming the total amount of concentrate offered.

The experiment was carried out in the south of Brazil, in a shed without air conditioning, with lateral openings. The experiment took place during the summer months and temperature was measured inside the building during the day. The minimum and maximum temperature recorded were 27 ºC and 35 ºC, respectively. The hottest temperature occurred around 1430 throughout the experimental period. The maximum and minimum temperature of each day during the experimental period are presented in supplementary Figure 1. These animals showed clinical signs of heat stress in the hottest times of the day, characterized by rapid breathing, intense salivation and open mouth, with exposure of the tongue of many animals.

2.3. Feed analysis

2.3.1. Chemical composition of concentrate, silage and hay

Feed samples were dried in a forced ventilation oven at 55 ºC for 72 h, ground at 1 mm in a Willey mill, and analyzed for chemical composition: DM, CP, NDF, ADF and analyzed as described for GRF. It is important to note that α-amylase was used in the analysis of both concentrate and silage diets (Van Soest et al. 1991).

2.3.2. Total phenolic compounds (TPC) and antioxidant activity in the concentrate

The preparation and extraction of concentrate in the diet was the same as that used for GRF (section 2.1.2) and measurement of TFC and antioxidant activity (IC 50) followed the same methodology previously described for GRF analysis (section 2.1.2).

2.4. Milk measurement

Individual milk production was evaluated twice a day (0600 and 1700) on d 1, 10 and 15 using a meter (True Test®, Aukland, New Zeland) and daily milk production was obtained by determining the sum of both milking events. Productive efficiency (%) was calculated individually based on milk production on days 1, 10 and 15 of the experiment and the increase in milk production from day 1 to 10 and day 1 to 15 for each group. The difference in milk production was assigned a percentage which was then used in the statistical analysis for productive efficiency.

2.5. Blood and milk collection

One milk sample (40 mL) per animal was collected using the equipment WB HI/Pullout (Tru-Test®; which collects a homogeneous sample from each animal for the entire milking) on d 1, 10 and 15. Two mL of milk sample were transferred to microtubes and stored at -20 ºC until further analysis of superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, total antioxidant capacity (ACAP), and lipid peroxidation (LPO) levels.

Blood samples were collected from the jugular vein in blood collection tubes without sodium heparin (for biochemical, oxidant and serum antioxidant analysis) and with EDTA (for haematological analysis) at 0700 before animals were fed. Immediately after collection blood samples were stored on ice. Blood samples without sodium heparin were centrifuged at 5100 g for 10 minutes. Serum was harvested and stored at -20 ºC until further analysis. Blood samples containing EDTA were stored at 4°C and hematological analysis was performed within 2 h after collection.

2.6. Milk analysis

2.6.1. Chemical composition

Concentration of fat, protein, lactose and total dry extract were determined using an infrared analyzer (LactoStar Funke Gerber®) and somatic cell count (SCC) was determined using a digital counter (Ekomilk Scan Somatic Cells Analyzer®).

2.6.2 Analysis of oxidants and antioxidants

Glutathione peroxidase activity was determined according to Wendel (1981) and results were expressed as U GPx/mg of protein. Activity of SOD was determined according to Beutler (1984) and results were expressed as U SOD/mg of protein. The LPO concentrations were determined according to Monserrat et al. (2003) and results were presented as μmol CHP/ml of serum or milk. The ACAP concentrations were determined according to Amado et al. (2009) and results were presented as FU/mg of protein. Protein concentrations in serum were determined by the Coomassie blue method according to Read and Northcote (1981), using bovine serum albumin as a standard.

2.7. Blood analysis

2.7.1. Hematology

Erythrocyte, total leukocyte counts, and hemoglobin (Hb) content were measured using a semi-automated analyzer (Celm® 530). Hematocrit was obtained after capillary centrifugation (11.000 g for 5 min). Leukocyte differential counts were performed in blood smears stained with a commercial dye (Romanowsky method) and viewed with a light microscope at 1000x magnification (Feldman et al., 2000).

2.7.2. Serum biochemistry

Total protein (TP), albumin, urea, triglycerides and cholesterol were measured using a semi-automated analyzer (BioPlus 2000®) with commercial kits (Analisa®, Gold Analisa Diagnóstica, Belo Horizonte, Brazil). Globulin levels were obtained using the following formula: total protein – albumin.

2.7.3. Serum oxidant and antioxidant analysis

The methodology for analyses of LPO, ACAP, GPx and SOD were the same as described in milk analysis.

2.8. Feed intake

Feed intake was measured by weighing the amount of feed offered and orts on d 11 to 15.

2.9. Statistical analysis

Data were submitted to a Shapiro-Wilk normality test. Majority of the data were not normally distributed and were log transformed. Subsequently, statistical analysis was performed using a bilateral, two-way analysis of variance (ANOVA) for independent samples followed by Tukey's post hoc analysis. Tukey's post hoc analysis compared treatment groups (T0, T1 and T2) and time (days 0 to 10; days 0 to 10; and days 10 to 15). Values were considered significant at $P \leq 0.05$.

3. Results

3.1. Resveratrol, quercetin, total phenolic compounds (TPC) and antioxidant activity in GRF

Resveratrol and quercetin concentrations were 52.8 ± 9.4 and 23.4 ± 3.64 mg/g, respectively. The TPC concentrations were 87.4 ± 0.27 mg EAG/g and antioxidant activity against radical DPPH (IC 50) was 111.6 ± 3.45 μg/mL.

3.2. Total phenolic compounds (TPC) and antioxidant activity in diets

The TPC concentration increased with increasing GRF in the diet (Table 2). Similarly, the antioxidant activity (IC50) was greater in the 2% GRF compared to the 1% GRF diet (Table 2).

3.3. Milk production and composition

Milk production (L/sheep/day) did not differ ($P > 0.05$) between treatments throughout the study (Table 3). However, sheep in the GRF groups were more efficient ($P \leq 0.05$) compared to sheep in the CON group (d 1 to 10, and d 1 to 15; Table 3). Protein and lactose concentrations were not different ($P > 0.05$) between groups, but sheep in the 2% GRF group had greater ($P \leq 0.05$; Table 3) lactose concentrations on d 15 compared to d 1. Fat and total solids concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON group on d 15 (Table 3). The SCC was decreased ($P \leq 0.05$) in sheep on the GRF diets compared to the CON on d 15 (Table 3). Over time (d 1 to 15), milk production increased ($P \leq 0.05$) in all treatments as indicated by different letter superscripts between days in Table 3.

3.4. Milk antioxidants and oxidants

Lipid peroxidation concentrations were reduced ($P \leq 0.05$) in 1% GRF and 2% GRF groups compared to CON on d 10 and 15 (Figure 1A). Over time (d 10 to 15), LPO was reduced ($P \leq 0.05$) in the 2% GRF group. Total antioxidant capacity was greater ($P \leq$

0.05) in the 1% GRF and 2% GRF groups compared to CON on d 10 and 15 (Figure 1B). Activity of SOD was increased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15 but there was no difference between the 1% and 2% GRF groups (Figure 1C). Activity of GPx was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 15 (Figure 1D). Total antioxidant capacity, SOD, and GPx activity increased from d 1 to 15 in the 2% GRF group.

3.5. Hematology

Hemoglobin, hematocrit and erythrocytes did not differ ($P \leq 0.05$) between treatment or time (Table 4). Total leukocyte concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and the 1% GRF and 2% GRF groups were decreased ($P \leq 0.05$) compared to CON on d 15 (Table 4). Lymphocyte and neutrophil concentrations ($P \leq 0.05$) were decreased in the 1% GRF and 2% GRF groups compared to CON on d 15 (Table 4). No differences were detected for the main effects of treatment and time ($P > 0.05$) for monocyte and eosinophil concentrations (Table 4). From d 1 to 15, total leukocyte counts were decreased ($P \leq 0.05$) in the 2% GRF group. Similarly, lymphocyte counts were also decreased ($P \leq 0.05$) in both the 1% and 2% GRF groups from d 1 to 15.

3.6. Serum biochemistry

Glucose concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON. Glucose concentrations differed from d 1 to 15 for all groups (Table 5). Cholesterol concentrations did not differ ($P > 0.05$) between groups or over time (Table 5). Triglycerides concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15 and concentrations increased over time (d 1 to 15) in 2% GRF

group (Table 5). Total protein and globulin concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 15. Total protein and globulin concentrations were reduced ($P \leq 0.05$) in the 2% GRF group from d 1 to 15 (Table 5). Albumin concentrations did not differ ($P > 0.05$) between groups, but oscillated ($P \leq 0.05$) in the 1% GRF and the 2% GRF groups (Table 5). Urea concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to 1% GRF and CON on d 15. Over time urea concentrations increased ($P \leq 0.05$) in CON and 1% GRF groups (Table 5).

3.7. Serum antioxidants and oxidants

Lipid peroxidation was reduced ($P \leq 0.05$) in the 2% GRF group compared to CON, and from d 10 to 15LPO was reduced ($P \leq 0.05$) in the 2% GRF group (Figure 2A). Total antioxidant capacity was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15, and from d 1 to 15 ACAP increased in the 1% GRF group (Figure 2B). Superoxide dismutase activity was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 15, and increased ($P \leq 0.05$) from d 1 to 10 and d 1 to 15 in the 1% GRF group (Figure 2C). Activity of GPx was increased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15, and increased ($P \leq 0.05$) from d 1 to 10 and d 1 to 15 in the 1% GRF and 2% GRF groups (Figure 2D).

3.8 Feed intake

Feed intake was not different ($P \leq 0.05$) between treatments. Sheep in all three groups consumed more than 95% of the diet provided daily and all concentrate was consumed.

4. Discussion

The increase in TPC and antioxidant activity in the diet with inclusion of GRF explains the elevated antioxidant response in the serum of sheep. Antioxidant activity (IC50) was not detected for the CON diet, suggesting that no chemical reactions were occurring that depend on antioxidants. Increased serum concentrations further support the increase in antioxidant capacity in the milk which has previously been observed in cows supplemented with grape residue silage (Santos et al., 2014). This effect is related to antioxidants, such as quercetin, resveratrol and phenols, that possess strong antioxidant capacity. According to Brower (1998), grape residues have nutraceutical actions when added to the diet because they possess medicinal compounds with beneficial health actions. In this study the inclusion of GRF in the diet minimized the oxidative stress in dairy sheep during heat stress.

Supplementation with GRF did not affect milk production (L), as observed by Santos et al. (2014) who provided grape residue silage for dairy cows. However, this study improved the productive efficiency of the sheep supplemented with GRF. As far as we know, this is the first study to show these effects of grape flour residue on efficiency and milk yield in dairy sheep. In the current study, the increase in productive efficiency with the inclusion of GRF in the diet may be a result of greater control of oxidative stress. Thus, improving the efficiency of nutrient utilization and increasing milk production numerically. Another hypothesis is that the increase in productive efficiency in sheep supplemented with GRF is due to the greater concentration of phenolic compounds and resveratrol which can positively influence immune function (Zunino and Storms, 2009; Cuevas et al., 2013), particularly in sheep with greater genetic potential for milk production.

In the current study, inclusion of GRF in diet (2% GRF) increased milk fat content. These findings disagree with previous results observed in dairy cows fed grape residue silage (Santos et al., 2014). This positive effect is believed to be related to an improvement in mammary gland function by reducing oxidative stress and decreasing amounts of free radicals (Celi 2010). Total antioxidant capacity in milk was greater in sheep supplemented with GRF, which has been supported by previous results in dairy cows (Santos et al., 2014). Furthermore, Sanchez-Alonso (2007) reported that grape residues were able to reduce the oxidation of stored fish meat and showed that oxidation was reduced in the first 90 days when compared to the control group. Therefore, improving the antioxidant activity in the milk of dairy sheep could lead to a reduction in lipid peroxidation. This reduction could potentially increase the shelf life of products such as milk, cheese, and yogurt when animals consume grape residues in the diet. However, shelf life was not evaluated in this study but will be an important variable in future studies performed in this laboratory

It is important to point out the reduction on SCC in milk samples from 2% GRF sheep. It is well understood that SCC includes cells of sanguine origin (leukocytes) and desquamation of the secretory glandular epithelium (Jorge et al., 2005) A reduction in SCC is linked to the systemic anti-inflammatory capacity caused by the components present in the GRF. This anti-inflammatory effect was confirmed by a reduction in the number of lymphocyte and globulin concentrations. These results are similar to those reported in dairy sheep fed a diet supplemented with curcumin (Jaguezeski et al., 2018). While there was a reduction in the number of leukocytes, values remained within the normal range for adult sheep throughout the 15-day study. However, it important to emphasize the need for future studies in order to evaluate the long-term impacts on the immune system, especially in animals suffering from heat stress.

Lactation is a critical period in sheep and cows because they suffer from greater oxidative stress. According to Barbosa et al. (2008), some nutrients (vitamins A, B, C, as well as resveratrol, quercetin, etc.) are beneficial to an animal's health by reducing the degree of oxidative stress. In this regard, addition of GRF for lactating sheep resulted in an increase in total antioxidant capacity which is linked to an upregulation in the enzymes involved in the antioxidant system, including, SOD and GPx. Similarly, addition of grape residues in broiler chicken feed increased the antioxidant levels in the muscle at the same proportion as animals supplemented with vitamin E (Brenes et al., 2008). Suggesting that grape residues can be considered a probable substitute for vitamins currently supplied in feed. The decrease in LPO and increase in SOD enzyme activity in sheep receiving 2% GRF are similar to the results found by Megahed et al. (2008), where the application of vitamin E and selenium (substances with antioxidant action) resulted in a decrease in LPO and nitric oxide concentration and an increase in SOD enzyme activity, which resulted in a reduction of oxidative stress in buffaloes under heat stress.

According to Kang et al. (2005), the presence of oxidative stress signals stimulates Nrf-2 activity, which then stimulates the expression of genes that results in the production of antioxidant enzymes, such as GPx. The increase in GPx activity was more pronounced in animals supplemented with 2 % GRF. Similarly, Janiques et al. (2014) observed an increase on GPx activity in humans supplemented with grape residues. These findings could be explained by the presence of polyphenols in grape residues., Polyphenolic compounds stimulate the activity of Nrf-2 and upregulate the expression of antioxidant genes (Guanin et al., 2011). Thus, the increase in SOD and GPx activity can be related to the stimulation on Nrf-2.

Another factor that may influence the imbalance between antioxidants and oxidants is heat. Sheep undergoing heat stress have an increased respiration rate (Filho et

al., 2011), which was also observed in the current study. Increased respiration rates were observed in the afternoon, when sheep also exhibited decreased physical and alimentary activity, which according to McDowell (1989), can impair food intake and the rumination process, reducing performance and animal production. Variation in seasonal temperatures alters the concentrations of hormones and oxidative markers in buffaloes (Megahed et al., 2008). Authors found that the concentration of estradiol and the enzyme superoxide dismutase (SOD) was decreased in the summer compared to winter while LPO and nitric oxide (ON) were significantly higher in the summer compared to the winter Filho et al. (2011), observed that Santa Inês lambs were in greater thermal comfort at temperatures between 10ºC and 25ºC, reinforcing that sheep were under heat stress during the current experiment, where the maximum temperatures recorded were 27ºC to 35ºC, respectively. Even with the effect of temperature, the 2% GRF group had an increase in total antioxidant capacity in both serum and milk, which is beneficial for the animals.

In this study, serum levels of urea and glucose were reduced while serum triglyceride concentrations were greater in animals supplemented with 2 % GRF. However, when dairy cows were supplemented with grape residue silage, these variables were not altered (Santos et al., 2014). In the current study, digestibility was not evaluated but the study conducted by Santos et al. (2014) revealed that grape residue provided in the diet of dairy cows led to a decrease in the content of nutrients, dry matter, crude protein, ethereal extract and fibers. Similar results were observed in sheep fed a dehydrated wine residue which consisted of different energy sources (Barroso et al., 2006). It is believed that a reduction in digestibility can be related to lower serum levels of glucose and urea and potentially reducing protein and carbohydrate metabolism. The increase in triglycerides could be a consequence of increased amounts of ethereal extract in the 2% GRF diet.

5. Conclusion

Addition of grape residue flour in the diet of dairy sheep increased the levels of total phenolic compounds and antioxidant activity in the concentrate. Increased antioxidants in the diet stimulated the antioxidant response in the sheep in heat stress and reduced oxidative stress. Furthermore, there was an increase in antioxidant capacity in milk which was associated with lower lipid peroxidation. Also, supplementation with 2% grape residue flour improved sheep health and productive efficiency. We concluded that grape residue flour supplementation generates an anti-inflammatory response which has a positive effect on milk quality because it reduces somatic cell count in milk samples. Thus, grape residue supplementation improves health, milk performance and milk quality.

Ethical note

The study was approved by the Committee of Ethics in the Use of Animals of the State University of Santa Catarina (CEUA / UDESC) under number 5184250218.

Conflict of interest

The authors declare no conflict of interest.

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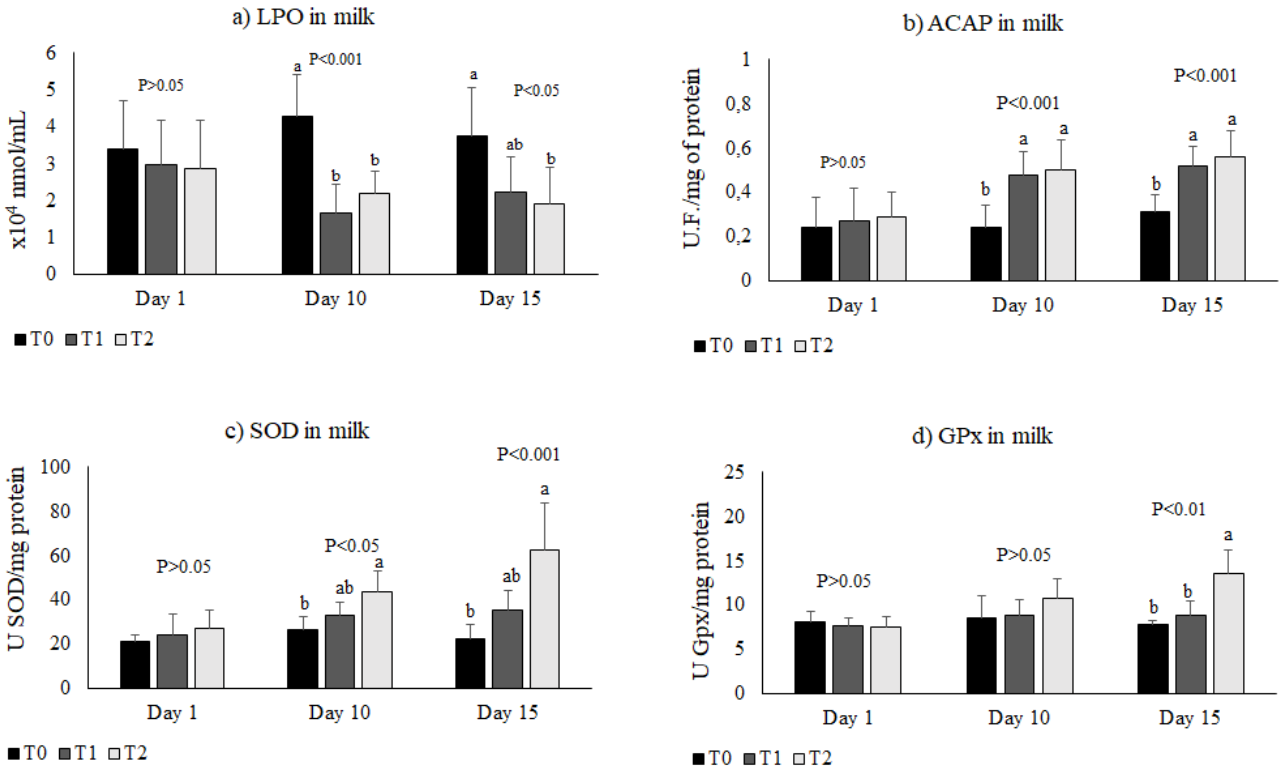

Figure 1. Lipid peroxidation (LPO), total antioxidant capacity (ACAP), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in milk samples of dairy sheep. Treatments: no grape residue flour (GRF) supplementation (control group; CON; T0) or supply of 1 % (1% GRF; T1) or 2 % (2% GRF; T2) of GRF in the concentrate. P-values shows statistical differences between groups (same letters do not differ significantly).

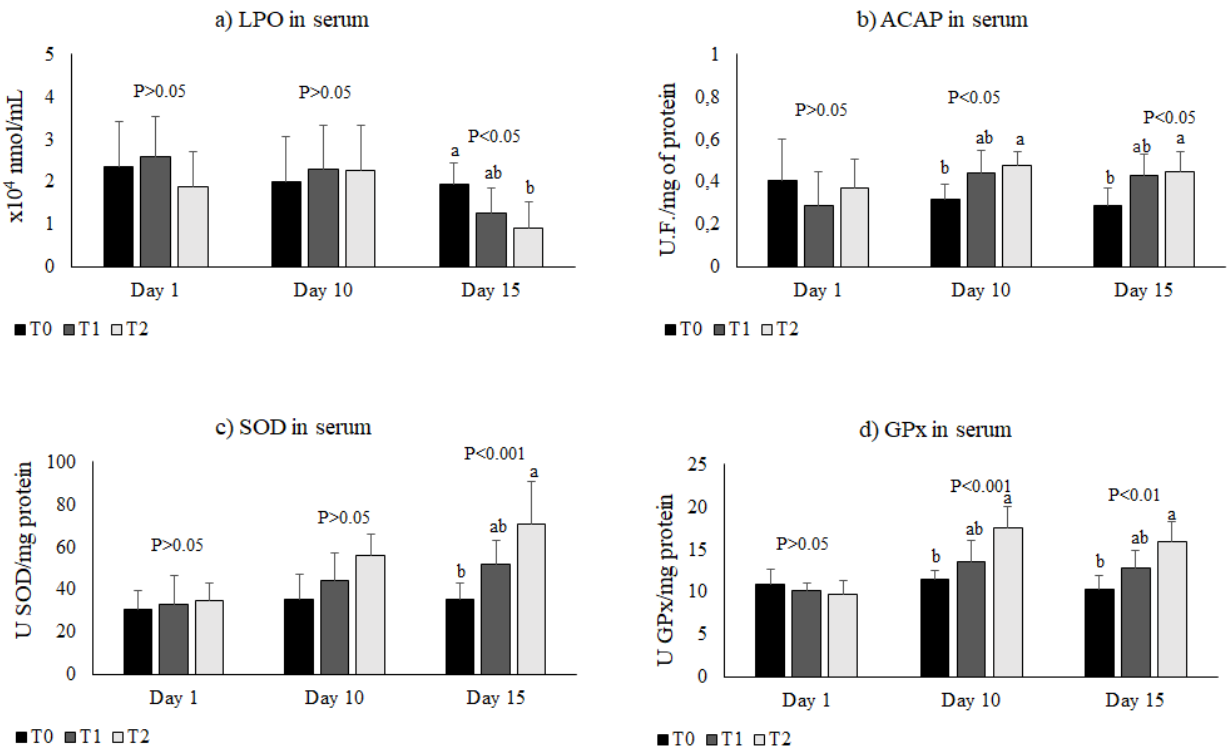

Figure 2. Lipid peroxidation (LPO), total antioxidant capacity (ACAP), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in serum samples of dairy sheep. Treatments: no grape residue flour (GRF) supplementation (control group; CON; T0) or supply of 1 % (1% GRF; T1) or 2 % (2% GRF; T2) of GRF in the concentrate. P-values shows statistical differences between groups (same letters do not differ significantly).

Table 1. Ingredients and chemical composition of ingredients and experimental diets.

Ingredients	**As fed (kg/day)**	**Dry matter (DM; kg/day)**
Corn silage (kg)	3.60	1.17
Concentrate[1] (kg)	0.80	0.71
Hay (kg)	0.25	0.23

Chemical composition[2]	**Grape flour**	**Corn silage**	**Hay**	**Concentrate**		
				CON	**1% GRF**	**2% GRF**
DM, g/kg	934	326	892	889	890	892
Ash, g/kg DM	124	45.0	60.0	87.0	77.0	69.0
CP, g/kg DM	103	72.1	72.0	216	208	205
NDF, g/kg DM	333	401	644	78.0	78.0	85.0
ADF, g/kg DM	217	242	401	28.0	28.0	31.0
EE, g/kg DM	50.2	31.0	16.0	23.0	23.0	26.0

[1.] Ingredients present in 100 kg of concentrate: ground corn (671 g/kg), soybean meal (277 g/kg), calcitic limestone (10 g/kg), sodium bicarbonate (4 g/kg) and 37 g/kg of premix (calcium mim. 180 max. 220g; phosphorus min. 32g; sodium min. 40g; sulfur min. 20g; magnesium min. 20g; cobalt min. 16mg; iodine min. 17mg; manganese min. 420mg; selenium min. 730mg; zinc min. 730mg; fluorine max. 600mg; niacin min. 500mg; vitamin A min. 95000 UI; vitamin D min. 20000 UI; vitamin E min. 350 UI; monensin sodium 1200 mg; *Sacharomyces cerevisae* 2,1 x 10 UFC).

[2.] Note: DM (Dry matter), Ash (Ashes), CP (Crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber) and EE (ethereal extract).

Table 2. Mean and standard deviation (…) of total phenolic compounds (TPC) and antioxidant activity (DPPH assay) of experimental diets.

Treatment/diets[1]	**TPC (mg EAG/100 g of dry mater)**	**Antioxidant activity – DPPH assay - IC_{50} (µg/mL)**
CON	0.04 (0.02)[c]	*
1% GRF	2.87 (0.90)[b]	712 (1.02)[a]
2% GRF	9.69 (0.80)[a]	422 (1.57)[b]

[1]No grape residue flour (GRF) supplementation (control group; CON) or supply of 1 % (1% GRF) or 2 % (2% GRF) of GRF in the concentrate.

*No antioxidant activity (IC_{50}) was detected in the control diet (T0), that is, there was no reaction with DPPH radical, visualized by color change.

Note: When comparing total phenolic compounds (TPC) and IC_{50} levels between treatments, we verified a statistical difference between groups, illustrated by different letters in the same column ($P<0.05$). Analyzes made in triplicate.

Table 3. Milk production and milk composition from sheep not supplemented with grape residue flour (GRF; control group; CON) or supplemented with of 1 % (1% GRF) or 2 % (2% GRF) of GRF in the concentrate.

Variable	Day	CON	1% GRF	2% GRF	P-values
Production (L)	1	1.42 ^{B} (0.31)	1.39 ^{B} (0.21)	1.37 ^{B} (0.32)	**0.54**
	10	1.50 ^{A} (0.34)	1.62 ^{A} (0.40)	1.63 ^{A} (0.33)	**0.50**
	15	1.51 ^{A} (0.36)	1.64 ^{A} (0.32)	1.65 ^{A} (0.29)	**0.39**
	p-values	**0.01**	**<0.001**	**<0.001**	
Productive efficiency (%)	1 to 10	8.54 (4.10) ^{b}	19.4 (5.91) ^{a}	25.7 (6.81) ^{a}	**<0.001**
	1 to 15	7.86 (4.31) ^{b}	19.4 (5.43) ^{a}	25.0 (6.54) ^{a}	**<0.001**
Chemical composition					
Protein (g/kg)	1	58.0 (1.10)	58.0 (1.20)	58.0 (2.00)	**0.95**
	10	57.0 (0.90)	59.0 (1.60)	58.0 (2.20)	**0.93**
	15	57.0 (0.80)	57.0 (1.80)	57.0 (1.50)	**0.96**
	p-values	**0.94**	**0.80**	**0.92**	
Fat (g/kg)	1	64.0 (5.90)	63.1 (4.76)	64.0 (5.41)	**0.90**
	10	63.1 (5.42)	59.6 (5.63)	67.6 (7.93)	**0.45**
	15	62.2 (3.82) ^{b}	65.4 (4.20) ^{ab}	69.8 (2.83) ^{a}	**0.03***
	p-values	**0.89**	**0.39**	**0.25**	
Lactose (g/kg)	1	55.0 (2.12)	56.4 (2.51)	55.6 ^{B} (2.60)	**0.93**
	10	57.3 (2.01)	54.6 (3.74)	56.0 ^{AB} (2.93)	**0.62**
	15	57.7 (1.98)	58.0 (2.23)	59.7 ^{A} (1.73)	**0.22**
	p-values	**0.96**	**0.18**	**0.05** $^{+}$	
Total solids (g/kg)	1	178 (5.41)	177 (5.61)	183 (11.2)	**0.90**
	10	178 (7.23)	172 (5.98)	180 (10.8)	**0.88**
	15	176 (4.31) ^{b}	179 (6.22) ^{ab}	185 (5.20) ^{a}	**0.04***
	p-values	**0.914**	**0.920**	**0.824**	
SCC[1] (x10^{3} /mL)	1	1640 (1523)	1977 (1554)	1678 (1534)	**0.62**
	10	2194 (1183)	1475 (1073)	902 (899)	**0.07**
	15	2480 (1420) ^{a}	1117 (949) ^{ab}	607 (593) ^{b}	**0.02***
	p-values	**0.42**	**0.24**	**0.15**	

[1]Somatic cell count.

* $P \leq 0.05$ shows difference between groups (Note: means with the same lowercase letters do not differ);

+ $P \leq 0.05$ shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).

Table 4. Hemogram of sheep not supplemented with grape residue flour (GRF), control group (CON) or supplemented with of 1 % (1% GRF) or 2 % (2% GRF) in the concentrate.

Variable	Day	CON	1% GRF	2% GRF	P-values
Erythrocytes (x10^6 µL)	1	5.14 (1.42)	5.15 (0.90)	5.05 (1.26)	**0.84**
	10	5.54 (1.64)	5.62 (1.41)	4.52 (0.65)	**0.08**
	15	5.80 (1.51)	5.26 (1.32)	4.61 (0.74)	**0.07**
	p-values	**0.35**	**0.41**	**0.74**	
Hematocrit (%)	1	28.0 (6.11)	29.3 (3.60)	30.1 (6.17)	**0.72**
	10	28.0 (5.84)	26.7 (3.41)	25.5 (5.32)	**0.60**
	15	29.1 (5.11)	27.9 (3.12)	29.0 (4.80)	**0.74**
	p-values	**0.84**	**0.78**	**0.65**	
Hemoglobin (mg/dL)	1	8.55 (1.82)	8.54 (1.41)	8.61 (1.60)	**0.90**
	10	8.81 (1.91)	8.22 (1.01)	7.43 (1.07)	**0.12**
	15	8.74 (1.73)	8.30 (0.96)	8.77 (1.02)	**0.41**
	p-values	**0.90**	**0.88**	**0.25**	
Leukocytes (x10^3 µL)	1	6.70 (3.67)	6.61 (1.81)	5.70^{A} (1.51)	**0.79**
	10	8.71 (3.94)^{a}	5.02 (1.40)^{ab}	4.42^{AB} (0.76)^{b}	**0.02**
	15	8.61 (1.71)^{a}	4.91 (0.84)^{b}	3.52^{B} (0.70)^{c}	**<0.001**
	p-values	**0.40**	**0.29**	**0.03**$^{+}$	
Lymphocytes (x10^3 µL)	1	2.01 (1.21)	1.71^{A} (0.60)	1.83^{A} (0.74)	**0.75**
	10	2.42 (1.50)	0.96^{AB} (0.55)	1.04^{AB} (0.45)	**0.07**
	15	2.01 (0.94)^{a}	0.92^{B} (0.17)^{b}	0.70^{B} (0.36)^{b}	**<0.001**
	p-values	**0.81**	**<0.001**	**<0.001**	
Neutrophils (x10^3 µL)	1	4.40 (2.33)	4.11 (1.32)	3.62 (0.91)	**0.55**
	10	5.92 (3.34)	3.90 (1.30)	3.21 (0.74)	**0.06**
	15	5.97 (1.20)^{a}	3.63 (0.81)^{b}	2.52 (0.61)^{b}	**<0.001**
	p-values	**0.46**	**0.35**	**0.12**	
Monocytes (x10^3 µL)	1	0.19 (0.20)	0.07 (0.06)	0.09 (0.08)	**0.65**
	10	0.24 (0.18)	0.15 (0.19)	0.17 (0.11)	**0.70**
	15	0.51 (0.29)	0.28 (0.12)	0.23 (0.11)	**0.21**
	p-values	**0.56**	**0.60**	**0.65**	
Eosinophils (x10^3 µL)	1	0.07 (0.09)	0.12 (0.14)	0.09 (0.09)	**0.80**
	10	0.06 (0.08)	0.04 (0.05)	0.19 (0.36)	**0.52**
	15	0.17 (0.15)	0.03 (0.02)	0.02 (0.05)	**0.33**
	p-values	**0.50**	**0.74**	**0.40**	

* $P \leq 0.05$ shows difference between groups (Note: means with the same lowercase letters do not differ);
+ $P \leq 0.05$ shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).

Table 5. Serum biochemistry of sheep not supplemented with grape residue flour (GRF; control group; CON) or supplemented with of 1 % (1% GRF) or 2 % (2% GRF) of GRF in the concentrate

Variable	Day	CON	1% GRF	2% GRF	P-values
Glucose (mg/dL)	1	64.6^{AB} (11.6)	68.9^{AB} (7.50)	64.2^{B} (8.21)	**0.58**
	10	73.2^{A} (11.5)	80.8^{A} (11.7)	89.3^{A} (17.0)	**0.08**
	15	54.7^{B} (3.10)^{b}	57.0^{B} (7.21)^{ab}	66.6^{B} (7.3)^{a}	**0.03**
	p-values	**<0.001**	**<0.001**	**<0.001**	
Cholesterol (mg/dL)	1	91.8 (34.0)	95.3 (23.4)	127 (27.9)	**0.25**
	10	106 (34.1)	92.0 (20.2)	102 (16.1)	**0.43**
	15	69.9 (14.8)	62.0 (10.0)	78.5 (11.9)	**0.11**
	p-values	**0.20**	**0.12**	**0.22**	
Triglycerides (mg/dL)	1	23.3 (6.90)	27.1 (19.4)	22.1^{B} (5.60)	**0.40**
	10	17.2 (5.80)^{b}	18.0 (5.01)^{b}	31.2^{AB} (8.61)^{a}	**<0.001**
	15	20.1 (3.21)^{b}	26.9 (6.61)^{ab}	36.9^{A} (8.32)^{a}	**<0.001**
	p-values	**0.44**	**0.24**	**<0.001**	
Total protein (g/dL)	1	6.81 (0.95)	7.61 (1.01)	7.81^{AB} (0.61)	**0.15**
	10	8.74 (1.52)	8.62 (0.81)	9.30^{A} (0.82)	**0.69**
	15	7.64 (0.61)^{a}	7.32 (0.70)^{ab}	6.62^{B} (0.33)^{b}	**0.04**
	p-values	**0.26**	**0.25**	**<0.001**	
Albumin (g/dL)	1	2.50 (0.50)	2.71^{B} (0.32)	2.73^{B} (0.31)	**0.89**
	10	3.25 (0.42)	3.46^{A} (0.41)	3.62^{A} (0.62)	**0.62**
	15	2.91 (0.34)	2.82^{B} (0.21)	3.01^{AB} (0.32)	**0.80**
	p-values	**0.06**	**0.01**	**<0.001**	
Globulin (g/dL)	1	4.31 (0.41)	4.94 (0.91)	5.10^{A} (0.84)	**0.20**
	10	5.52 (1.20)	5.26 (0.81)	5.14^{AB} (1.90)	**0.76**
	15	4.76 (0.52)^{a}	4.59 (0.80)^{ab}	3.50^{B} (0.51)^{b}	**0.03**
	p-values	**0.339**	**0.552**	**0.010**	
Urea (mg/dL)	1	24.9^{B} (4.41)	26.7^{B} (8.11)	30.8 (6.60)	**0.18**
	10	42.2^{A} (5.60)	39.9^{AB} (12.1)	51.1 (18.3)	**0.36**
	15	38.0^{A} (3.91)^{a}	37.6^{A} (4.62)^{a}	27.6 (4.50)^{b}	**<0.001**
	p-values	**<0.001**	**<0.001**	**0.36**	

* $P \leq 0.05$ shows difference between groups (Note: means with the same lowercase letters do not differ);
+ $P \leq 0.05$ shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).

2.3. MANUSCRITO I

Biocholine supplementation in pre- and postpartum Lacaune sheep: effects on animal health, milk production and quality

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Vegetable biocholine supplementation in pre- and postpartum Lacaune sheep: effects on animal health, milk production and quality

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Abstract

In the final third of gestation and during early lactation, sheep require a large supply of nutrients to support fetal growth, mammary gland development and milk production. Nevertheless, during this period, the pregnant ewe consumes less food in the context of increased energy expenditure and liver overload. For these reasons, natural produces have been used as supplements to minimize the negative effects of this transition phase. The objective of the present study was to determine whether supplementation with vegetable biocholine (VB) in pregnant and lactating sheep (transition period) would improve productive efficiency, milk quality and overall health. The experiment lasted 65 days, corresponding to the final 20 days of pregnancy and the first 45 days of lactation. A total of 24 Lacaune ewes were separated into two homogeneous groups on day 20 pre-partum: a control group (without supplementation) and a treated group in which the animals received 5 g of VB/animal/day. Blood samples were taken on days 20 and 10 before delivery, the day of delivery and on days 7, 15, 30 and 45 after delivery. Measurements of milk yield, as well as milk collections for composition analysis (fat, protein, lactose and total solids) were performed on the 15^{th}, 30^{th} and 45^{th} postpartum days. The sheep that consumed VB in the diet had higher lactation persistence compared to control ($P<0.05$), although milk production does not differ between groups (Control = 1.94 L/day; Treated = 2.23 L/day – $P=0.10$). Milk composition did not differ significantly between the groups at the three time points ($P>0.05$). We observed greater activity of the enzymes glutathione S-transferase (GST) and glutathione peroxidase (GPx) in milk ($P<0.05$); as well as there was a tendency for lower counting of somatic cells in the milk of the sheep in the group treated on day 45 ($P=0.07$). Higher activity of GPx and GST enzymes also were observed on serum the treated group ($P<0.05$). At 45 days postpartum, sheep in the treated group had lower liver enzyme activity (aspartate aminotransferase and gamma glutamyltransferase) ($P<0.05$); and lower albumin levels at days 15 and 30 ($P<0.05$).

Globulin levels were greater in serum of VB supplemented sheep than in controls (P<0.05). VB intake by sheep reduced serum calcium levels (P<0.05). We conclude that the addition of VB in the diet of sheep had beneficial effects on animal health, especially after the peak of production, where the consumption of VB had hepatoprotective, antioxidant and immunological effects.

Keywords: sheep rearing, pregnancy, phosphatidylcholine, liver function, lactation persistence, milk quality.

1. Introduction

Pregnant ewes have high nutritional requirements during final third of gestation and early lactation periods (Soares et al., 2009). During these periods, ruminants manifest characteristics of low caloric intake associated with increased energy demand. The resulting negative energy balance leads to increased fatty acid oxidation and increased concentration of ketone bodies, both of which predispose the animal to two interrelated diseases, pregnancy toxemia and hepatic steatosis (Berchielli et al., 2011). Pregnancy toxemia is a metabolic disorder that frequently affects sheep in the final third of pregnancy. It is directly related to inadequate nutrition and multiple pregnancies attributable to elevated glucose requirements on the part of the mother and fetus (Sobrinho, 2006). Despite the fact that pregnancy and lactation are physiological (i.e., not pathological), they cause metabolic stress and liver overload in pregnant females (Drackley, 1999). The final third of pregnancy is the most critical phase, because it requires even higher demands for glucose for fetal growth (Sobrinho et al., 1996).

Malnutrition in ewes has negative effects on the fetus, including changes in placental size and reduced fetal growth, as well as decreased fat reserves for newborn use. The female can also be affected, especially with reduced udder development that consequently

affects colostrum supply and interferes with future lactation (Mellor, 1983). Because of the economic and zootechnical impacts caused by these metabolic disorders, prevention using supplementation is recommended (Sobrinho et al., 1996).

An additive used in animal feed is choline. Data indicate that choline has a lipotropic effect preventing the deposition of fat in the liver (Best et al., 1936). In addition, it also plays an important role in the formation and preservation of the metabolism of the cellular structure (Berchielli et al., 2011). A study of Lacaune sheep showed that encapsulated choline chloride in pre-partum supplements decreased blood ketone values as well as the incidence of pregnancy toxemia (Michailoff et al., 2017). The primary source of choline in animal diets is synthetic choline chloride (Fernandes Junior et al., 2010; Pompeu et al., 2011; Khosravinia et al. 2015). In recent years, other sources of choline have been tested in animals, including phosphatidylcholine, a choline of vegetable origin (also known as biocholine) that is extracted from plants such as *Azadirachta indica*, *Citrullus colocynthis*, *Trachyspermum ammi*, and *Achyranthes a*spera (Camacho et al., 2018). Vegetable choline (VB) contains choline in the form of choline conjugates, especially phosphatidylcholine that provides natural resistance against ruminal degradation (Godínez-Cruz et al., 2015).

A recent study demonstrated the ability of VB to express lipotropic effects through mobilization of non-esterified fatty acids (Rodríguez-Guerrero et al., 2018) that decreases stores of liver fat. A study showed that this biocolina has high bioavailability, where the production was similar in the animals that received VB and in those that received encapsulated choline chloride (Crosby et al., 2017).

Given the properties of VB, we believe that if it used as a transition period supplement, it can minimize negative effects associated with this phase in sheep already mentioned in this section. Our hypothesis is that VB, a new source of choline, which has

low ruminal degradation (Godínez-Cruz et al., 2015) will modulate hepatic lipid metabolism (Best et al., 1936; Rodríguez-Guerrero et al., 2018) and antioxidant activity (Baldissera et al. 2019) that will mainly protect the liver, reducing liver overload and production of ketonic bodies in ewes with twin pregnancies avoiding pregnancy toxemia, as well as over time maintaining the persistence of lactation.

Therefore, the objective of the present study was to determine whether VB supplementation in pregnant and later lactating sheep (the transition period) would improve productive efficiency, milk quality and overall animal health.

2. Materials and methods

2.1. Biocholine (phosphatidylcholine)

Biocholine (Biocholine Powder®) was purchased from Nutriquest Technofeed, produced from *Azadirachta indica, Citrullus colocynthis, Trachyspermum ammi,* and *Achyranthes aspera* as described in the commercial product data sheet. The chemical composition of this product was measured prior to the experiment, being detected at the following concentrations: 967 g/kg of dry matter; 853 g/kg of organic material; 47 g/kg protein; 443 g/kg NDF; 380 g/kg FDA; 57.1 g/kg ether extract; 15g/kg calcium; and 3 g/kg phosphorus.

The commercial product (VB) contains guaranteed levels of total phosphatidylcholine (natural choline conjugates) of 16 g/kg VB. A sample of this additive was sent to a specialized laboratory for actual quantification of phosphatidylcholine in VB using high-performance thin-layer chromatography (Kupke and Zeugner 1978); we found that the levels of total phosphatidylcholine were 16.8 g/kg.

2.2. Animals and experimental design

The experiment was carried out at a Lacaune dairy farm located in Lajeado Grande, SC, after approval by the Animal Experimentation Ethics Committee. (CEUA/UDESC) protocol number 2481011018.

We used 24 multiparous ewes (73.4 ± 6.3 kg by body weight) in the final stages of gestation. According to a farmer, the reproductive management used was natural breeding (coitus), with estrus synchronization previously. Data from an ultrasound performed at 60 days of gestation was used to select only animals with twin births for this study. The animals were divided into two groups with 12 animals each, a control group (without VB) and a treated group that received daily supplements of 5 g VB/animal, offered in the morning with the concentrate. The formation of the groups took into account the ewe's age, number of births and milk production at the peak of the previous lactation.

The experiment lasted 65 days, i.e., 20 days before delivery and 45 days after delivery. The animals were fed concentrate (65% wet grain, 30% soybean meal and 5% kernel for lactating sheep), corn silage and free hay twice a day (0700h and 1700h) (Table 1). The ewes were housed in a confined covered shed, separated by group into two 15-m^2 side-by-side collective stalls and pre-calving beds. After delivery, the sheep were housed in another covered shed, with collective stalls and a slatted floor. The lambs remained with their mothers at some times of the day to nurse throughout the experiment.

2.3. Chemical food analysis

Chemical analysis of the diets for silage and concentrate (pre- and post-partum) are displayed in Table 1. The analyses of samples were pre-dried for 72 h at 55ºC, milled (Willey mill, 1mm) and subjected to analysis for determination of dry matter content (DM, 930.15; AOAC, 1990), crude protein (CP, 984.13; AOAC, 1990) was obtained by multiplying the result by 6.25 and the result was expressed in g/kg DM, ash (MM,

942.05; AOAC, 1990) g/kg DM, and ethereal extract (EE) g/kg DM, according to Silva and Queiroz (2002). The NDF (NDFom, method number 2002.04) and ADF (ADFom, method number 973.18) contents were analyzed according to Van Soest (1994) expressed exclusive of residual ash and without amylase.

2.4. Quantitation of milk

The milking was mechanized (Ordemilk, Treze Tílias, Santa Catarina, Brazil), performed twice a day (0600h and 1700h). Individual volumetric verification of milk was performed on the 15^{th}, 30^{th} and 45^{th} postpartum days. The volumes produced in the morning and afternoon milking were measured using a digital scale (RKMIX, Assis, São Paulo, Brazil), and the daily value was the sum total of the two milking.

Then, lactation persistence (LP) was calculated using the formula: LP = [1- ((PMP - LMP) x (30 / IDMM) / PMP)] x 100; [where: PMP = previous milk production; LMP = later milk production; IDMM = Interval in days between milk measurement].

2.5. Collection of blood and milk samples

Milk samples (40 mL) from the complete milking of the animal were collected for composition and quality analysis on days 15, 30 and 45 of the experiment. Two milliliters of milk from each sample were transferred to microtubes for evaluation of oxidant and antioxidant status biomarkers.

The animals were manually restrained for blood collection. Blood samples were collected by jugular vein puncture into vacuum tubes on day 20 and 10 before delivery, and on days 7, 15, 30 and 45 postpartum. A total of 4 mL were stored in tubes containing EDTA (ethylenediamine tetraacetic acid) for complete blood counts, and another 4 mL

were placed in tubes without anticoagulant centrifuged at 5100 *g* for 10 min to obtain serum for biochemical analysis and levels of oxidants and antioxidants.

2.6. Milk analysis

2.6.1. Chemical composition

Milk composition (fat, protein, lactose and total dry extract) was determined using an infrared analyzer (LactoStar Funke Gerber®) and somatic cell count (SCC) was performed using a digital counter (Ekomilk Scan Somatic Cells Analyzer®).

2.6.2 Analysis of oxidant/antioxidant status in serum and milk

We measured in the activity of the enzyme glutathione peroxidase (GPx) serum and milk, using tert-butyl hydroperoxide as substrate (Wendel, 1981). Enzyme activity was determined by monitoring the disappearance of NADPH at 340 nm in a medium containing potassium phosphate buffer (100 mM) + ethylenediamine tetraacetic acid (1 mM), pH 7.7. Results were expressed as U mg protein^{-1}. Lipid peroxidation in the homogenates was obtained using the technique described by Buege and Aust (1978) by measuring thiobarbituric acid reactive substances (TBARS).

2.7. Blood analyses

2.7.1. Hemogram

Total erythrocyte and leukocyte counts as well as hemoglobin concentration were measured using a semi-automatic cell counter (CELM model CC530). Hematocrit was obtained using microhematocrit capillary tubes by centrifuging for 5 min at 5100 *g*.

2.7.2. Serum biochemistry

Tubes containing blood were kept refrigerated in a thermal box until arrival at the laboratory (10 ºC), then centrifuged (5100 *g* for 10 minutes) for serum separation. Supernatants were transferred to Eppendorf tubes, identified and stored at -20 °C until the time of analysis of: aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), calcium, glucose, total protein (TP), albumin, triglycerides, cholesterol and urea using a semi-automatic analyzer (Bio-2000 BioPlus®) and specific commercial kits (Analisa®). Globulin values were calculated as the difference between total protein and albumin levels.

2.7.3. Serum oxidant and antioxidant status

The methodology for evaluating TBARS, GPx and SOD were the same as for milk described in section 2.6.2. GPx activity were measured according to the methodology described by Wendel (1981) and Beutler (1984), respectively. TBARS levels were measured using the methodology described by Buege and Aust (1978)

2.7.4. Measurement of serum ketone bodies

Blood collected with anticoagulant was used for this analysis. For the determination of ketone bodies in the blood, the commercial Ketovet® equipment was used, with readings up to 1 min after blood collection.

2.8. Statistical analysis

Animal was considered the experimental unit for all analyses. All dependent variables were tested for normality using Univariate procedure of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4) and log transformed when needed. Then, all data were analyzed using MIXED procedure of SAS, with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. Persistence of lactation

was tested for fixed effect of treatment using animal(treatment) as random effects. All others variables of the study were analyzed as repeated measures and tested for fixed effects of treatment, day, and treatment × day, using animal(treatment) as random variables and animal(treatment) as subject. All results obtained on d 20 prepartum for each variable were included as covariates in each respective analysis, but were removed from the model when $P > 0.10$. The first order autoregressive covariance structure was selected for milk production and compound symmetric covariance structure was selected for all others variables. The covariance structures were selected according to the lowest Akaike information criterion. Means were separated using PDIFF and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$, and tendency when $P > 0.05$ and ≤ 0.10.

3. Results

During the experimental period, two pathological disorders were observed in two sheep in the control group. 1) one sheep was diagnosed with toxemia on the day of farrowing, animals that had elevated ketone bodies (3.13 mmol/L), as well as farrowing had to be assisted and the two lambs were born dead; 2) another sheep had a clinical mastitis at 38 days of lactation, which stopped milk production (data and samples of this sheep were not collected at 45 days).

3.1. Production, composition and quality of milk

Effects of treatment, but not treatment × day ($P = 0.36$) tended to be detected ($P = 0.10$) for milk production, and treated sheep tended to have greater production, compared to control sheep (Table 2). Effects of treatment were detected for persistence of lactation ($P \leq 0.04$), and treated sheep had grater values, compared to control sheep (Table 2).

Effects of treatment × day and treatment were not detected ($P \geq 0.25$) for milk concentration of protein, fat, lactose, total dry extract and TBARS (Table 2). Effects of treatment × day, but not treatment ($P = 0.17$) tended to be detected ($P = 0.09$) for milk SCC, and treated sheep had lower count on d 45 postpartum, compared to control sheep (Table 2). Effects of treatment were detected ($P = 0.05$), and effects of treatment × day tended to be detected ($P = 0.09$) for milk concentration of GPx, and treated sheep had greater values on d 30 postpartum, compared to control sheep (Table 2). Effects of treatment were detected ($P = 0.02$), and effects of treatment × day tended to be detected ($P = 0.09$) for milk concentration of GST, and treated sheep had greater values on d 30 and 45 postpartum, compared to control sheep (Table 2).

3.2. Hemogram and clinical biochemistry

Effects of treatment × day and treatment were not detected ($P \geq 0.18$) for blood concentration of erythrocytes, hematocrit, hemoglobin, leukocytes and ketone bodies (Table 3). Effects of treatment × day was detected ($P = 0.04$) and treatment tended to be detected ($P = 0.07$) for AST, and treated sheep had greater concentration on d 45 postpartum, compared to control sheep (Table 3). Effects of treatment × day, but not treatment ($P = 40$), were detected ($P = 0.05$) for GGT, and treated sheep had greater concentration on 45 postpartum, compared to control sheep (Table 3).

Effects of treatment × day and treatment were not detected ($P \geq 0.17$) for serum concentration of total protein, cholesterol, triglycerides and urea (Table 4). Effects of treatment × day were detected ($P = 0.03$) and treatment tended to be detected ($P = 0.08$) for serum concentration of albumin, and treated sheep had greater value on d 30 and 45 postpartum, compared to control animals (Table 4). Effects of treatment × day, but not treatment ($P = 0.32$) tended to be detected ($P = 0.08$) for serum concentration of globulin,

and treated sheep had greater value on d 15 and 45 postpartum, compared to control sheep (Table 4). Effects of treatment × day, but not treatment (P = 0.64) tended to be detected (P = 0.09) for serum concentration of glucose, and treated sheep had greater concentration in the day of delivery, compared to control sheep (Table 4).

Effects of treatment × day and treatment were detected ($P \leq 0.05$) for serum concentration of total calcium, and treated sheep, had lower values from d 10 prepartum until d 30 postpartum, compared to control sheep (Figure 1).

3.3. Serum oxidant and antioxidant status

Effects of treatment, but not treatment × day (P = 0.55), tended to be detected (P = 0.10) for serum concentration of TBARS, and treated sheep had lower value, compared to control sheep (Table 5). Effects of treatment × day, but not treatment (P = 0.88) tended to be detected (P = 0.07) for serum concentration of GPx, and treated sheep had greater concentration on d 45 postpartum, compared to control sheep (Table 5). Effects of treatment × day, but not treatment (P = 0.34) were detected (P = 0.03) for serum concentration of GST, and treated sheep had greater concentration on d 45 postpartum, compared to control sheep (Table 5).

4. Discussion

The data suggest that our main hypothesis was accepted, as we found that supplemented sheep diets with VB to improve liver function and consequent milk production. We found no difference in hepatic enzyme levels (AST and GGT) during the transition period. During this period, especially after delivery, there is a large mobilization of body fat for milk production generating negative energy balance (Bell, 1995; Raoof et al., 2013); nevertheless, VB supplementation had no effect on enzymes

used as markers of liver function up to day 30 postpartum. However, on day 45 postpartum, the group receiving VB supplementation had significantly lower serum AST and GGT levels. This finding justifies future studies, because it is important to determine whether there was a change in the amount of liver fat stored, because the literature suggests that choline has lipotropic action (Best and Huntsman, 1935; Blanch, 2016).

We did not observe any difference in levels, cholesterol and triglycerides in our study; this was different from results reported Rodríguez-Guerrero et al. (2018), who observed increased glucose, cholesterol and non-esterified fatty acid levels in the serum of lambs supplemented with VB. The authors suggested that the product stimulates lipid mobilization. Michailoff *et al.* (2017) observed decreased ketone body levels in sheep supplemented with encapsulated choline in the pre-partum period, in animals supplemented with 25 grams of choline/animal/day. In our study, the dose of VB did not differ blood ketones between groups during of all experiment. Therefore, our hypotheses that VB would have an effect (reduce) on serum ketones bodies cannot be evaluated, as there was no significant increase in serum levels of ketones bodies during the pre-calving period in most sheep (despite twin gestation), as we only verified body levels ketonic > 1 mmol/L on the day of partum in three sheep (2 controls; 1 treated).

The demand for choline during pregnancy is higher because of fetal development, the fetus uses choline and derivatives for cell formation and nervous system development. In humans, daily intake of choline for pregnant women of approximately 450 mg is recommended, and even higher for breastfeeding women (550 mg) (Zeisel and Costa, 2009). Zeisel *et al.* (1980) found that newborn humans and rats have higher serum choline concentrations than do adult individuals. This further reinforces the idea that pregnant women should consume choline-containing foods during pregnancy and lactation. Studies have found that rats' milk is choline-rich, and rat pups that did not receive milk had

significantly lower serum choline concentrations than did breastfed pups (Zeisel and Wurtman, 1981). In the present study, we did not evaluate levels of choline and derivatives in newborn lambs, only factors related to sheep (mother); nevertheless, we did not find differences between groups in terms of liver function and antioxidant levels during the transition period. Zeisel (2013) stresses that pregnant women and lactating women should ingest choline, because the fetus requires choline and lactation depletes maternal choline reserves; it is essential for the birth of healthy children and the mother's liver health. Guedes *et al.* (2015) pointed out that maternal nutrition during pregnancy affects both fetal development, health and performance of lambs after birth. This experiment took place lasted less than 3 months; however, the farmer continued to use 5 g of VB in the pre-birth diet of the animals, which, according to the producer, reduced considerably (± 80%) of the problems related to toxemia of pregnancy and in babies born dead or week, a common occurrence on the farm before the use of biocolina (personal communication).

The addition of 4 grams of choline chloride to cow diets resulted in increased daily milk production by 3.7 liters (Erdman *et al.,* 1984). The addition of vegetable choline (5 grams) in sheep diets here had no significant effect on milk production; however, the production remained constant longer in the group that received dietary VB. This may be related to the liver's better efficiency in metabolizing fatty acids (De Veth et al, 2016). Yao and Vance (1988) explain that the release of very-low-density lipoproteins (VLDL) by the liver is closely related to the availability of choline and phosphatidylcholine. Liver-produced VLDLs are rich in triglycerides that are hydrolyzed in the bloodstream, releasing fatty acids (Xavier et al., 2013). This suggests that phosphatidylcholine supplementation may have favored hepatic VLDL production,

increasing the availability of serum triglycerides, and enabling the maintenance of milk production by the mammary gland.

The way mammals obtain choline is related to food, as well as the hepatic pathway, where phosphatidylethanolamine methylation by the enzyme N-methyltransferase occurs, generating phosphatidylcholine that corresponds to approximately 95% of choline-containing compounds in the liver (Li *et al.,* 2005). In ruminants, choline chloride is not absorbed well in the intestine, and most choline is degraded in the rumen (Sharma e Erdman, 1989). This can be overcome by encapsulating the choline, increasing its availability for intestinal absorption (Baldi and Pinotti, 2006). Crosby *et al. (*2017) observed that the addition of VB and encapsulated choline chloride in sheep diets one month before calving improved the weight of the mothers on the day of delivery and 30 days postpartum, as well as increasing the weight of lambs at birth, promoting higher milk production and improving the weight gain of lambs during the first 30 days of life. Phosphatidylcholine supplementation in beef cattle concentrate also increased daily weight gain by 18% compared to the control group (Fernandes *et al.,* 2008). Martínez-Aispuro et al. (2019) also found improvements in the weight gain of lambs that received increasing doses of VB in the diet; the data from our study reinforce the idea that VB is resistant to ruminal degradation, allowing its addition to ruminant diets in its natural form.

Studies of VB in ruminant diets are relatively recent; published data focused mainly on the productive effects of birds as described below for contextual purposes. Calderano *et al.* (2015) used various concentrations of vegetable choline in broiler diets and observed that the animals performed similarly to those receiving synthetic choline chloride. Blanch (2016) also conducted a study with synthetic and natural choline sources in broiler chickens and observed similar weight gain in poultry as well as lower weight of

animal livers because it contains less fat than the synthetic choline group. Bustamante (2018) added phosphatidylcholine at various doses to laying hen diets (doses between 160 and 320 g per ton) and found that this additive altered egg yield and production but did not affect egg quality at the intermediate dose. Gangane *et al.* (2010) used choline in the diet of broilers and found that it decreased levels of triglycerides and serum cholesterol in birds.

Importantly, the adequate formulation of the diet provided to the animals in this study (corn silage, concentrate and mineral mixture for lactating sheep) may reduce the chances of pregnant ewes presenting health problems such as pregnancy toxemia and liver steatosis. This is because 30% of the concentrate provided to the sheep was soybean meal, also a natural choline source (McDowell 2000). Soybean meal contains 2.916 ppm choline in dry matter. Another researcher observed that corn and soybean meal have 1,000 and 2,700 mg/kg choline, respectively (Bertechini 2006), with bioavailability ranging from 60% to 75%. Choline levels were not measured in the diet provided; nevertheless, we believe that the dietary levels of both groups were sufficient to meet the needs of the sheep during the transition period.

The addition of vegetal choline did not alter blood count, clinical biochemistry or liver markers in a way that would suggest toxicity. Nevertheless, we found antioxidant activity, with GPx and GST activities increased in milk. GPx activity was also greater in serum and TBARS levels were lower in serum of sheep supplemented with VB on day 45 postpartum; both of these are positive results in terms of health of the sheep. In tilapia, Baldissera *et al*. (2019) also found similar results. The authors observed greater total antioxidant capacity and lower lipid peroxidation in the livers of fish supplemented with VB. This suggests an antioxidant effect of plant choline. Koujalagi et al. (2018), in a study on supplementation of cows in the pre- and postpartum transition period with plant

choline (20 grams per 100 kg body weight) and another herbal product with liver function (Livoliv®), found that supplementation minimized the effects of negative energy balance and metabolic disturbances during the transition period.

There was a reduction in the levels of calcium during the study for the animals that received the biocolina, since it surprised us, because we expected that the product helps in the balance of this mineral during the transition period. We believe that the data differed little between groups because the dose of choline contained in the biocolina is low, and of the 5 g of biocolina supplied, it generated little amount of choline available to the sheep's organism.

There are still no concrete data indicating whether there is a difference between the bioavailability of VB and choline chloride. Assessing the doses of choline used in other studies, we believe that the dose of VB needed to improve the health and performance of sheep in transition is higher than used. Furthermore, this study had some limitations, because there were ewes of different ages in both groups (second to the sixth calving); lactating lambs may affect the behavior of ewes during milking (not all milk produced was milked, because females stocked milk to feed lamb after milking). This could explain the lower milk production in the period (30 to 45 days postpartum) that is considered the time of peak production for sheep. Due to these limitations, a future study focusing on productive efficiency will be carried out, using a standardized group of sheep at the beginning of lactation with higher doses of VB.

5. Conclusion

We found that, over time, VB supplementation maintained lactation persistence in the treated group. VB supplementation had no effect on milk composition and quality, except for higher activity of glutathione enzymes (GPx and GST) in milk at the end of the

experiment. During the transition period, hematological and biochemical variables were not influenced by VB supplementation. Nevertheless, after the transition period, as well as at the peak of production, consumption of VB generated hepatoprotective, antioxidant and immunological effects, all of which are desirable for animal health.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Ingredients and chemical composition of ingredients and experimental diets.

Ingredients prepartum	**As fed (kg/day)**	**Dry matter (kg/day)**
Corn silage (kg)	2.50	0.98
Concentrate[1] (kg)	0.80	0.64
Tifton hay (kg)	0.30	0.25
Ingredients postpartum		
Corn silage (kg)	3.50	1.37
Concentrate (kg)	1.50	1.19
Tifton hay (kg)	0.50	0.42

Chemical composition	**Corn silage**	**Hay**	**Concentrate**	
			Prepartum	**Postpartum**
DM (g/kg)	392	844	806	796
Ash (g/kg DM)	47.0	72.0	61.7	43.6
CP (g/kg DM)	71.9	118	149	204
NDF (g/kg DM)	256	583	83.0	181
ADF (g/kg DM)	135	331	41.8	47.5
EE (g/kg DM)	40.8	37.5	12.5	12.3

Ingredients present in 100 kg of concentrate[1]: wet grain corn (65%), soy meal (30%) and buffering lactation nucleus (5%). Buffer core composition: Calcium (min. 170 g and max 190 g); Phosphorus (min) 30 g; Sulfur (min) 10 g; Magnesium (min) 30 g; Sodium (min) 60 g; Potassium (min) 14 g; Cobalt (min) 25 mg; Chromium (min) 5 mg; Iron (min) 590 mg; Iodine (min) 25 mg; Manganese (min) 600 mg; Selenium (min) 9 mg; Zinc (min) 1,150 mg; Fluoride (max) 700 mg; Vitamin A (min) 110,000 IU; Vitamin D3 (min) 24,000 IU; Vitamin E (min) 300 IU; Sodium monensin (min) 500 mg; Saccharomyces cerevisiae 2.5 x 10^{10} CFU.

[2.] Note: DM (Dry matter), Ash, CP (Crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber) and EE (ethereal extract).

Table 2. Production, composition and milk quality of sheep supplemented with vegetable choline during the transition period (20 days before delivery to 45 days of lactation).

Variables[1]	Treatments[2]		SEM	P-values	
	Control	Treated		Treatment	Treatment × day
Daily milk production (L)	1.94	2.23	0.11	0.10	0.36
Persistence of lactation (%)					
15 to 30 d postpartum	88.6	96.4	3.6	0.04	-
15 to 45 d postpartum	62.0	80.3	8.7	0.01	-
Milk analyses					
Protein (%)	3.93	3.84	0.19	0.74	0.25
Fat (%)	5.70	5.47	0.24	0.47	0.83
Lactose (%)	5.35	5.26	0.22	0.76	0.73
Total dry extract (%)	14.7	14.3	0.40	0.50	0.73
SCC ($x10^6$ / mL)				0.17	0.07
d 15 postpartum	2.49	1.37	0.40		
d 30 postpartum	1.02	1.09	0.40		
d 45 postpartum	1.43[a]	0.64[b]	0.42		
TBARS(nmol MDA/mL)	7.94	8.47	0.80	0.61	0.34
GPx (U/mg protein)				0.05	0.09
d 15 postpartum	3.76	3.72	0.93		
d 30 postpartum	2.96[b]	7.42[a]	0.97		
d 45 postpartum	3.47	5.15	0.98		
GST (U/mg protein)				0.02	0.04
d 15 postpartum	5.59	6.55	1.07		
d 30 postpartum	5.89[b]	9.49[a]	1.06		
d 45 postpartum	3.66[b]	7.89[a]	1.14		

[1]SCC, somatic cell count, GPx, glutathione peroxidase, GST, glutathione S-transferase.

[2]Treatments: control, without supplementation, treated, 5 g of vegetable choline/animal/day.

[a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

Table 3. Blood count, ketone bodies and liver enzymes of sheep supplemented with vegetable choline during the transition period (20 days before delivery to 45 days of lactation).

Variables [1]	Treatments[2]		SEM	P-values	
	Control	Treated		Treatment	Treatment × day
Erythrocytes (x10^6µL)	9.26	10.5	0.73	0.23	0.18
Hematocrit (%)	32.3	29.6	1.60	0.25	0.62
Hemoglobin (g/dL)	10.1	11.5	0.86	0.35	0.45
Leukocytes (x10^3 µL)	18.2	23.0	2.53	0.18	0.84
Ketone bodies (mmol/L)	0.59	0.67	0.15	0.24	0.59
AST (U/L)				0.05	0.04
d 20 prepartum	62.0	58.0	9.4		
d 10 prepartum	64.9	67.8	10.7		
d of delivery	72.3	70.3	11.8		
d 17 postpartum	125	115	10.3		
d 15 postpartum	123	117	10.2		
d 30 postpartum	143	104	10.2		
d 45 postpartum	129[a]	83.6[b]	10.7		
GGT (U/L)				0.04	0.05
d 20 prepartum	65.9	54.2	10.5		
d 10 prepartum	76.8	71.3	11.5		
d of delivery	75.0	73.1	12.0		
d 17 postpartum	109	99.1	10.9		
d 15 postpartum	134	157	10.95		
d 30 postpartum	150	124	10.95		
d 45 postpartum	171[a]	128[b]	11.22		

[1]AST, aspartate aminotransferase, GGT, gamma glutamyltransferase.

[2]Treatments: control, without supplementation, treated, 5 g of vegetable choline/animal/day.

[a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

Table 4. Serum biochemistry of sheep supplemented with plant choline during the transition period (20 days before delivery to 45 days of lactation).

Variables	Treatments[1]		SEM	P-values	
	Control	Treated		Treatment	Treatment × day
Total protein (g/dL)	7.53	7.60	0.13	0.76	0.80
Albumin (g/dL)				0.08	0.03
d 20 prepartum	3.31	3.26	0.10		
d 10 prepartum	2.73	2.88	0.11		
d of delivery	2.83	2.73	0.13		
d 7 postpartum	2.83	2.85	0.11		
d 15 postpartum	4.03[a]	3.41[b]	0.11		
d 30 postpartum	3.85[a]	3.40[b]	0.11		
d 45 postpartum	3.54	3.56	0.13		
Globulin (g/dL)				0.32	0.04
d 20 prepartum	5.13	4.91	0.16		
d 10 prepartum	4.97	5.12	0.19		
d of delivery	4.84	4.74	0.17		
d 7 postpartum	5.09	5.15	0.17		
d 15 postpartum	3.09[b]	3.85[a]	0.17		
d 30 postpartum	3.01	3.33	0.17		
d 45 postpartum	3.50[b]	4.32[a]	0.17		
Glucose (mg/dL)				0.64	0.09
d 20 prepartum	75.3	81.1	6.05		
d 10 prepartum	55.7	55.9	6.89		
d of delivery	75.4[b]	105[a]	7.25		
d 7 postpartum	69.4	53.7	6.30		
d 15 postpartum	80.5	77.4	6.30		
d 30 postpartum	72.5	71.6	6.30		
d 45 postpartum	70.0	67.9	7.26		
Cholesterol (mg/dL)	74.6	72.5	3.61	0.67	0.87
Triglycerides (mg/dL)	33.0	31.5	1.53	0.47	0.17
Urea (mg/dL)	26.9	27.3	1.36	0.84	0.84

[1]Treatments: control, without supplementation, treated, 5 g of vegetable choline/animal/day.

[a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

Table 5. Lipid peroxidation (TBARS) and activity of glutathione peroxidase (GPx) and glutathione S-transferase (GST) enzymes in serum.

Variables	Treatments[1]		SEM	P-value	
	Control	Treated		Treatment	Treatment × day
TBARS (nmol MDA/mL)	4.80	4.50	0.11	0.10	0.55
GPx (U/mg protein)				0.88	0.05
d 20 prepartum	0.65	0.56	0.08		
d 10 prepartum	0.30	0.31	0.08		
d of delivery	0.39	0.29	0.09		
d 7 postpartum	0.39	0.26	0.08		
d 15 postpartum	0.46	0.46	0.09		
d 30 postpartum	0.32	0.46	0.08		
d 45 postpartum	0.19[b]	0.42[a]	0.08		
GST (U/mg protein)				0.34	0.03
d 20 prepartum	12.2	11.5	0.70		
d 10 prepartum	6.96	7.21	0.70		
d of delivery	3.91	4.77	0.85		
d 7 postpartum	3.76	5.01	0.73		
d 15 postpartum	7.29	6.81	0.73		
d 30 postpartum	5.99	6.20	0.76		
d 45 postpartum	4.16[b]	7.78[a]	0.80		

[1]Treatments: control, without supplementation, treated, 5 g of vegetable choline/animal/day.

[a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

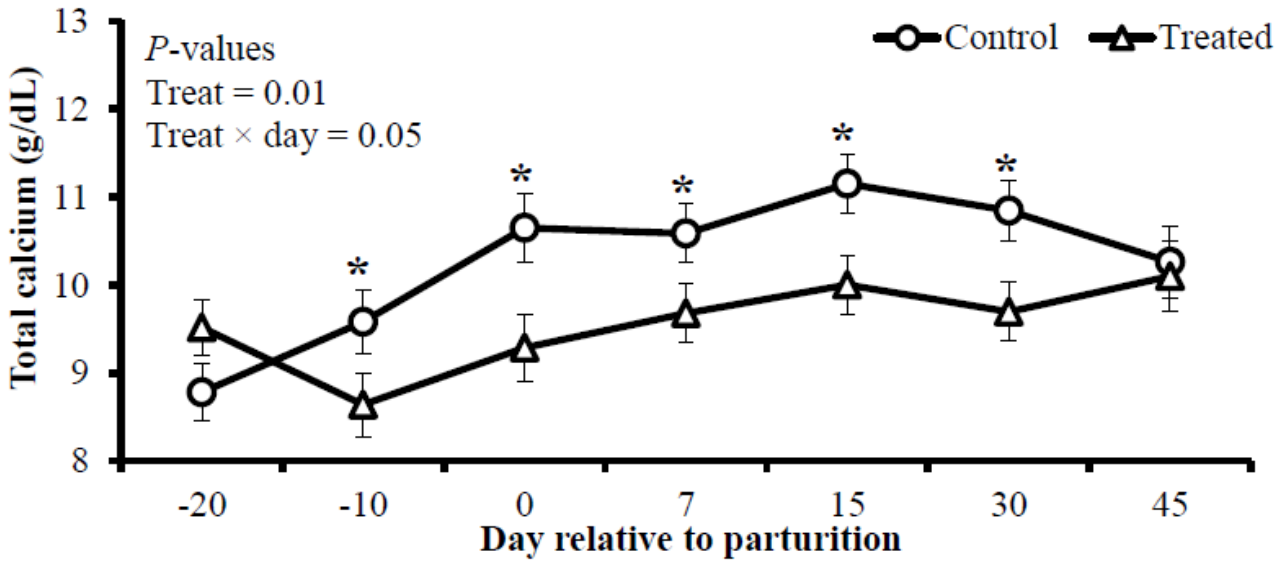

Figure 1: Serum calcium levels in sheep supplemented with vegetable biocholine (VB) during the transition period (20 days before delivery to 45 days of lactation). Treatments: control, without supplementation, treated, 5 g of VB/animal/day. * Represent significate differences between treatments each day ($P < 0.05$).

2.4. MANUSCRITO II

Vegetable biocholine supplementation for lactating ewes under heat stress improves milk quality, productive efficiency and stimulates the antioxidant system

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Abstract

Choline supplements in animal diets are usually provided as choline chloride, a synthetic form; nevertheless, recent studies have illustrated the benefits of a natural substitute based on plant extracts, i.e., vegetable biocholine (VB). In the present study, we determined whether VB supplements in the diets of Lacaune ewes at peak lactation during summer would minimize the negative effects of heat on health, production and milk quality. We also aimed to determine the characteristic effects of VB as a food additive. We used Lacaune ewes at 30 days of lactation, divided into three groups: T0 (control, without VB), T5 (5 g of VB/animal/day) and T10 (10 g of VB/animal/day). The experimental period lasted 20 days. Blood and milk samples were collected at various time points. Groups T5 and T10 had significant higher production and better productive efficiency on day 20 than did group T0. Group T5 consumed more food, and consequently had higher feed efficiency. Milk composition (fat, protein, lactose) did not significantly differ between treatments; however, somatic cell count (SCC) in milk was significantly lower in ewes that consumed VB. Supplementation significantly reduced lipoperoxidation (LPO) and levels of reactive oxygen species (ROS) in sheep's milk, associated with increased levels of non-enzymatic antioxidants (non-protein thiols). In the serum of sheep that consumed VB (T10) we found significantly higher levels of globulins than in T0. Furthermore, in serum, we found that animals in T5 and T10 had significantly greater total antioxidant capacity levels associated with reduced LPO and ROS. These data suggest that VB supplementation in sheep diets stimulates antioxidant responses and increases the concentration of globulins in a manner beneficial to sheep health, as well as generating improvements in the productive efficiency and quality of milk.

Keywords: Supplements, Biocholine, Production, Lactation, Health animal.

1. Introduction

In 2017, the size of the Brazilian sheep herd was estimated at over 17 million animals, making it the fourth largest in Brazil (IBGE, 2018), a country with vast territorial area and substantial climate variability throughout the year. Dairy sheep activity is increasing in the market because of its nutritional superiority to cow's milk (i.e., higher total solids content) (Pavic et al., 2002), despite being produced in smaller amounts. Currently, several industries are seeking to increase production and improve the quality of animal products, as well as to achieve sustainability and maximize animal welfare in order to meet an increasingly demanding consumer market. Maintaining sheep in thermal comfort during the summer is a substantial challenge for several reasons, not least because of the fact that the length or coloring of wool or fur affects thermoregulation (McManus et al., 2009; Leitão et al., 2013), even in animals that are shorn during the summer.

Sheep milk can be produced in high yields even for small and medium producers (EPAGRI, 2019) because of its high protein and fat content, both of which are desirable in dairy products such as cheese and yogurt (FAO, 2013). Sheep health influences milk composition and quality (Alba et al., 2019b); therefore, we seek improvements in production and nutrition systems, with emphasis on investments in the selection of appropriate breeds. Such dairy sheep breeds as East Friesian and Lacaune are most common in Brazil (Ticiani et al., 2013). Milk nutritional requirements increase substantially from late gestation to peak production, and especially during the peripartum period there are lower levels of endogenous antioxidants (Bernabucci et al., 2005); this condition often stresses the capacity of the liver, an organ responsible for functions such as detoxification (Adler, 1970; Baird, 1977; Rukkwamsuk et al. 1999, Kokkonen et al., 2005). Feed additives with functional properties and antioxidant capacities such as grape

flour and acai oil have shown high antioxidant capacities in dairy sheep diets (Alba et al., 2019a; Santos et al., 2019). We believe that hepatoprotective feed additives may be beneficial to animal health and may improve production. Among the supplements often used as food supplements in farm animals is choline, a nutrient that is part of animal and plant cells (Vieira et al., 2001). Choline is an essential nutrient for animals because it has lipotropic effects and participates in processes fundamental to metabolism, including the construction and maintenance of cell structure and formation of acetylcholine (Berchielli et al., 2011). The primary form of choline on the market is synthetic choline chloride; however, a new formulation, known as vegetable biocholine (VB), is being marketed in the form of phosphatidylcholine (Souza et al., 2020). Supplementation with VB has shown potential to improve livestock production: 25% increase in weight gain in Nile tilapia (Baldissera et al., 2019); up to 18% increase in average daily weight gain in beef cattle (Fernandes et al. 2008); increased colostrum production in pre- and postpartum dairy cows (Valencia Narváez 2019); and improvement in hepatic metabolism in sheep and rams, reflected in greater serum levels of non-esterified fatty acids, glucose and cholesterol available to meet animal demands (Rodriguez-Guerrero et al. 2018).

We hypothesized that VB supplementation would modulate liver metabolism, as well as stimulate antioxidant responses and consequently improve the health of ewes exposed to intense summer heat. The aim of this study was to determine whether VB supplementation for Lacaune ewes at peak lactation during the summer would have positive effects on animal health, milk production and quality.

2. Material and Methods

2.1. Choline

Biocholine (Biocholine Powder®) was purchased from Nutriquest Technofeed Company, produced from *Azadirachta indica*, *Citrullus colocynthis*, *Trachyspermum ammi*, *Achyranthes aspera* plants as described in the commercial product feed tag. The chemical composition of the product is displayed in Table 1.

The commercial product is known as Biocholine Powder® contains guaranteed levels of total phosphatidylcholine (natural choline conjugates) of 16 g/kg VB. A sample of this additive was sent to a specialized laboratory for actual quantification of phosphatidylcholine in VB using high-performance thin-layer chromatography (Kupke and Zeugner 1978); we found that the levels of total phosphatidylcholine were 16.8 g/kg.

2.2. Animals and experimental design

The experiment was conducted at a commercial dairy sheep farm in Chapecó, Santa Catarina, Brazil. Thirty multiparous lactating sheep [30 ± 3 days (d) milking] of the Lacaune breed were stratified by body weight (65.6 ± 3.5 kg), age, date of lambing, and milk production and were assigned randomly to 1 of 3 treatments (10 sheep/treatment): no choline supplementation (control group; T0); 5 g VB/animal/day (T5) or 10 g VB/animal/day (T10) added in the concentrate (grain and mineral mixture). We used the feed available on the farm in proportions determined by the owners; we only added the biocholine to the concentrate. Each animal received 1.2 kg/d of concentrate, approximately 4.0 kg/d of corn silage (green matter) divided into 2 daily feedings (0700h and 1700h; Table 1). To standardize feed intake, all sheep were fed individually. Concentrate was offered first and subsequently (approximately 15 min. later), silage was offered. The sheep were housed in a covered feedlot with wood shavings on the floor and

were allocated to 3 pens (1 treatment/pen) located side-by-side. The trial occurred over 20 days with first 14 days set as the adaptation period to the experimental diet.

The experiment was carried out in the south of Brazil, in a shed without air conditioning, with lateral openings. The experiment took place during the summer months and the temperature was measured inside the building during the day. The minimum and maximum temperatures recorded were 14.4 ºC and 38.2 ºC, respectively. The maximum and minimum temperatures, as well as the temperature-humidity index (THI; Mader et al., 2006) during the experimental period are presented in Figure 1.

2.3. Feed Analysis

2.3.1. Chemical composition of concentrate, and silage

Feed samples were dried in a forced ventilation oven at 55 ºC for 72 h, ground to 1-mm size in a Wiley mill, and analyzed for chemical composition: dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF). It is important to note that α-amylase was used in the analysis of both concentrate and silage diets (Van Soest et al. 1991).

2.3.2. Determination of total phenolic content (TPC) and determination of antioxidant activity by elimination of radicals by DPPH in vegetable choline and diets

For extraction, we used 0.5 grams of samples, and added 50 mL of distilled water. The mixture was placed in an ultrasonic bath (70 W) for 3 hours and was set in the dark for an additional three hours. The supernatants were filtered using quantitative filter paper (Whatman No. 40) and stored in 100-ml volumetric flasks wrapped in aluminum foil. Immediately after extraction, the extracts were stored in Eppendorf tubes and kept frozen

at –80 °C, if necessary and for no longer than 24 hours, until use (Dacoreggio et al., 2019).

The TPC quantification was performed using the Folin-Ciocalteau colorimetric method. An aliquot of each diluted sample was mixed with 0.5 mL Folin-Ciocalteau reagent, and stirred for 1 minute. We then added 2 mL of sodium carbonate (20%) and stirred for 30 s. After incubation for 2 h in the dark, the absorbance at 750 nm was read against the prepared blank. The standard curve was prepared using solutions of gallic acid in methanol. The concentration of total phenolic compounds in the extracts in gallic acid equivalents was calculated using an equation obtained from the standard gallic acid graph and was expressed as mg of gallic acid equivalent per 100 g of dry sample (mg EGA/g). Data were expressed as the mean analysis ± SD of triplicates (Bonoli et al., 2004; Shi et al. 2011).

The evaluation of free radical scavenging activity of the extracts was based on the measurement of antioxidant reduction capacity by the DPPH radical. Five dilutions of each extract were prepared in test tubes. Reaction mixtures were prepared from diluted extracts. In a dark environment, 0.3 mL of each extract was diluted in 2.7 mL of DPPH radical (40 µg/mL). After incubation for 1 h in the dark, the absorbance at 515 nm was read against the prepared blank. Activity was expressed as the IC_{50} value (µg/mL), defined as the concentration of antioxidant required to eliminate 50% of the DPPH present in the test solution. All tests were performed in triplicate and IC_{50} values were reported as means ± SD of triplicates (Brand-Williams et al., 1995, Shi et al. 2011).

The TPC and IC_{50} of BV and concentrates are displayed in Table 1. We found that the concentrations of TPC were low in VB, and not-different of concentrate (T0, T5 and T10), suggesting that VB supplementation did not change TPC levels. Although IC_{50}

activity was high in VB, it was not sufficient to increase the IC_{50} in the concentrate (T0, T5 and T10).

2.4. Milk measurement

Individual milk production was evaluated twice a day (0600h and 1700h) on days 1, 7, 14, 17 and 20 using a meter (True Test®, Auckland, New Zealand). Milk production was measured by determining the sum of both milking events. Productive efficiency (%) was calculated individually based on milk production on days 1, 14 and 20 of the experiment and the increase in milk production from days 1 to 14 and from days 1 to 20 in each group. The difference in milk production was assigned a percentage that was then used in the statistical analysis of productive efficiency. The ratio between liters of milk produced per kg of concentrate consumed (green matter) was also calculated, as was the relationship between the quantity of food consumed (green matter) and the volume of milk produced.

2.5. Blood and milk collection

One milk sample (40 mL) per animal was collected using WB HI/Pullout equipment (Tru-Test®) that collects a homogeneous sample from each animal for the entire milking) on days 1, 14 and 20. Two milliliters of milk sample were transferred to microtubes and stored at –20 ºC until further analysis.

Blood samples were collected from the jugular vein in blood collection tubes without anticoagulant (for biochemical, oxidant and serum antioxidant analysis) at 0700h before animals were fed. Immediately after collection, blood samples were stored on ice. Blood samples without heparin were centrifuged at 5100 *g* for 10 minutes. Serum was harvested and stored at –20 ºC until further analysis.

2.6. Milk analysis

2.6.1. Chemical composition

Concentrations of fat, protein, lactose and total dry extract were determined using an infrared analyzer (LactoStar Funke Gerber®). Somatic cell count (SCC) was determined using a digital counter (Ekomilk Scan Somatic Cells Analyzer®).

2.6.2 Analysis of oxidants and antioxidants

The activity of the superoxide dismutase (SOD) was determined according to the pyrogallol self-oxidation principle (inhibition in the presence of SOD). The variation in optical density was determined kinetically for two minutes at 420 nm at ten second intervals according to the methodology described by Beutler (1984). Activity was expressed as U mg/protein. Antioxidant capacity against peroxyl radicals (ACAP) concentrations were determined according to Amado et al. (2009) and results were presented as FU/mg of protein.

Levels of reactive oxygen species (ROS) in milk were analyzed using the method described by Ali et al. (1992). Aliquots of 10 μL of serum were incubated with 12 μL of dichlorofluorescein per 1 mm at 37 °C for 1 h in the dark. Fluorescence was determined using 488 nm for excitation and 520 nm for emission and the results were expressed as U DCF/mL. Levels of lipid peroxidation (LPO) were measured using the methodology proposed by Monserrat et al. (2003). Results were expressed nmol CHP/mL.

2.7. Serum analysis

2.7.1. Biochemistry

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), total protein (TP), albumin, urea, triglycerides and cholesterol were measured using a semi-automated analyzer (BioPlus 2000®) with commercial kits (Analisa®, Gold Analisa Diagnóstica, Belo Horizonte, Brazil). Globulin levels were calculated using the following formula: total protein level – albumin level.

2.7.2. Serum oxidant and antioxidant analysis

Determination of superoxide dismutase (SOD) enzyme levels were performed according to Beutler's (1984) methodology. Non-protein thiols (NPSH) were measured following the methodology of Sedlak and Lindsay (1968). Reactive oxygen species (ROS) were quantified according to Ali et al. (1992). Lipid peroxidation (LPO) was obtained following the method of Monserrat et al. (2003).

2.7.3. Enzymes of energetic metabolism

Serum creatine kinase (CK) activity was assayed based on the colorimetric method established by Hughes (1962), estimating creatine levels at a wavelength of 540 nm, and the results were expressed as nmol creatine formed/min/mg of protein. Pyruvate kinase (PK) activity was assayed according to protocol established by Leong et al. (1981) and activity was expressed as nmol pyruvate formed/min/mg of protein.

2.8. Feed intake

On days 15, 16, 17, 18 and 19, feed intake was measured by subtracted orts weight from the amount of feed offered daily.

2.9. Statistical analysis

An individual ewe was considered the experimental unit for all analyses. All dependent variables were tested for normality using univariate procedure of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4) and were log transformed when needed. Then, all data were analyzed using the MIXED procedure of SAS, with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. Persistence of lactation, feed intake and feed conversion were tested for fixed effect of treatment using animal (treatment) as random effects. All others variables of the study were analyzed as repeated measures and tested for fixed effects of treatment, day, and treatment × day, using animal (treatment) as random variables and animal (treatment) as subjects. All results obtained on d 0 for each variable were included as covariates in each respective analysis, but were removed from the model when $P > 0.10$. The first-order autoregressive covariance structure was selected for serum concentration of ROS and SOD and milk concentration of ROS, and compound symmetric covariance structure was selected for all others variables. The covariance structures were selected according to the lowest Akaike information criterion. Means were separated using PDIFF and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$, and tendency when $P > 0.05$ and ≤ 0.10.

3. Results

3.1. Milk production, composition and quality

Effects of treatment × day, but not treatment ($P = 0.27$) were detected ($P = 0.01$) for milk production, and T10 ewes had grater production on day 20, compared to T0 and T5 ewes (Table 2). The T5 ewes had greater ($P = 0.01$) productive efficiency from day 0 to 15 and T5 and T10 ewes had greater ($P = 0.01$) productive efficiency from day 0 to d 20, compared to T0 ewes, respectively (Table 2). The T5 ewes had greater ($P = 0.01$) feed intake, compared to T0 and T10 ewes (Table 2). The T5 ewes had higher that the T0, and T10 the lower feed intake compared to T0 ($P = 0.01$; Table 2). Effects of treatment × day and treatment were not detected ($P \geq 0.14$) for milk concentration of protein, fat and lactose (Table 2). Effects of treatment × day, but not treatment ($P = 0.45$) were detected ($P = 0.05$) for total solids, and T10 ewes had greater concentration on day 20, compared to T0 and T5 ewes (Table 2). Effects of treatment × day and treatment ($P < 0.0001$) for SCC, and T5 and T10 had lower concentration on day 15 and 20, compared to T0 ewes (Table 2).

3.2. Serum biochemistries

Effects of treatment × day and treatment were not detected ($P \geq 0.11$) for serum concentration of glucose, total protein, albumin, cholesterol, triglycerides, urea, AST, ALT, GGT, CK and PK (Table 3). Effects of treatment × day tended to be detected ($P = 0.09$) and treatment were detected for serum concentration of globulin and T5 and T10 ewes had greater concentration on day 15, and T10 had greater concentration on day 20, compared to T0 ewes (Table 3).

3.3. Oxidants and antioxidants in sheep serum

Effects of treatment × day and treatment were detected ($P < 0.01$) for serum levels of LPO and ROS, and T5 and T10 ewes, had lower concentration on day 15 and 20, compared to T0 ewes (Figure 2). Effects of treatment × day, but not treatment ($P = 0.29$) were detected ($P < 0.01$) for serum concentration of NPSH, and T5 and T10 ewes, had greater concentration on day 20, compared to T0 ewes (Figure 2). Effects of treatment × day and treatment were not detected ($P \geq 0.38$) for serum concentration of SOD (Figure 2).

3.4. Oxidants and antioxidants in sheep's milk

Effects of treatment × day, but not treatment ($P = 0.59$) were detected ($P = 0.05$) for milk levels of LPO, and T5 and T10 ewes, had lower levels on day 20, compared to T0 ewes (Figure 3). Effects of treatment × day and treatment were detected ($P \leq 0.03$) for milk concentration of ROS, and T5 and T10 ewes had lower concentration on day 20, compared to T0 ewes (Figure 3). Effects of treatment × day and treatment were detected ($P < 0.01$) for milk ACAP, and T5 and T10 ewes, had greater values on day 20, compared to T0 ewes (Figure 3). Effects treatment × day but not treatment ($P = 0.96$) were detected ($P < 0.01$) for milk concentration of SOD, and T10 ewes had greater concentration on day 20, compared to T0 and T5 ewes (Figure 3).

4. Discussion

Milk production is directly associated with the amount of feed available to sheep, as well as to the quality and availability of nutrients in the diet. In a study encapsulated choline chloride added to cow feed, there was an increase in milk production in supplemented animals, without changing milk fat and protein levels, as well as plasma

glucose and cholesterol levels (Pinotti et al., 2003); these findings were similar to those observed in the present study. In our study, we found higher levels of total solids in the milk of sheep that ingested the highest dose of VB, a positive effect mainly because milk is mainly intended for the production of processed foods; nevertheless, the reasons that led to increased total solids need to be investigated.

Various concentrations of encapsulated choline supplements in multiparous cows promoted increased milk production in animals receiving 30 g per day, also without changing the milk composition (Xu et al., 2005), as was the case in our study. According to the authors, choline supplementation in the diets of high-yield dairy cows is essential to maintain milk yield and quality, because choline deficiency may be a limiting factor in production (Pinotti et al., 2002; Baldi and Pinotti, 2006). In our study, sheep at peak production in winter increased production when supplemented with VB.

In the present study, serum hepatic biochemical variables did not respond to supplementation, as previously described in another study (Michailoff et al., 2013). In one study, the addition of encapsulated choline in Holstein cow feed decreased blood ketone concentrations (Michailoff et al., 2013). Supplementation of protected choline to pregnant sheep reduced serum ketone levels, in addition to decreasing clinical and subclinical cases of ketosis (Michailoff et al., 2017). The lower susceptibility to ketosis in early lactating dairy cows supplemented with encapsulated choline may occur because of improvements in hepatic fat export (Zom et al., 2011). Although not reported in ruminants, hepatoprotective effects were observed by directly analyzing the livers of tilapia supplemented with VB (Baldissera et al. 2019); therefore, we cannot rule out liver protection provided by VB, because we used only indirect variables (i.e., liver enzyme levels).

According to the literature, choline chloride is the preferred source of choline used in animal diets; however, this molecule is hygroscopic, and is also considered a stressor for other nutrients (vitamins and minerals) present in the composition of concentrates (Baker, 1995) that might limit its use in animal diets. The VB used in our study includes choline conjugates, mainly in the form of phosphatidylcholine that displays natural resistance to ruminal degradation (Godínez-Cruz et al., 2015). A study in sheep showed that this biocholine has high bioavailability, as the productive results of animals supplemented with VB were similar to those receiving encapsulated choline chloride (Crosby et al., 2017). In our study, ewes receiving VB showed greater milk production, possibly related to greater passage of phosphatidylcholine through the rumen and its absorption in the intestine. Deuchler et al. (1998) found that the concentration of choline in milk was related to the amount of choline supplied in the diet, and that the quantification of choline or milk derivatives is a method that can be used to verify whether nutrient absorption has occurred. In our study, we did not evaluate choline or phosphatidylcholine levels in blood and milk; however, knowing the antioxidant activity of VB (Baldissera et al., 2019), we believe that VB was absorbed because there was a reduction in lipid peroxidation and free radicals in this milk. Pinotti et al. (2003) reported that supplementation with 20 g/animal/day of choline in cow diets increased the concentration of choline secreted in milk, i.e., 6% of the intake of choline was found in milk. In future studies, it would be interesting to measure the amount of biocholine present in sheep serum and milk.

Another interesting finding from our study was the decrease in SCC in animals supplemented with the highest dose of VB. Low SCC values mean better mammary gland health that is directly related to better quality milk produced. Animals with mastitis produce lower volumes of milk with higher cellularity as well as higher levels of oxidants

(Alba et al., 2019b), factors that may affect the productive chain (microbiological quality and shelf life of milk product) (Coelho et al., 2014). Because choline participates in cell formation, cell signaling and tissue integrity (Zeisel and Blusztajn, 1994; Zeisel, 1998; Pompeu et al., 2011), we believe that animals receiving VB may have stronger cell structures, with consequent reduction in desquamation of the udder secretory epithelium, thereby reducing SCCs. Studies suggest that lack of choline stimulates apoptosis in nerve cells (Holmes-McNary et al., 1997; Yen et al., 2001) and hepatocytes (Albright et al., 1996) and causes DNA damage and lymphocyte apoptosis (Costa et al., 2006). Because of this, we believe that supplementation increased the availability of choline, reducing apoptosis of mammary gland cells, thereby reducing SCC in milk. Importantly, in dairy cows, lower SCC is an indication of milk quality, allowing higher producer compensation when SCC is low (Bozo et al., 2013), as well as penalizing producers when SCC values exceed limits delineated in regulations. This encourages the search for preventive measures for producers to control SCC, which also improves the quality of milk and dairy products. Even though dairy farming has been rarely explored in Brazil, products and managements that decrease SCC are important for the productive system and for food quality.

In mammals, the main fate of choline is conversion to phosphatidylcholine, a major cell membrane compound and a precursor of signaling molecules. In the liver, it participates in the secretion of bile and very low-density lipoprotein (VLDL) (Li and Vance, 2008). In ruminants, the addition of choline chloride has low intestinal absorption, as most choline is degraded in the rumen; this can be prevented by encapsulating choline, increasing its availability for intestinal absorption (Baldi and Pinotti, 2006). Supplementation with choline (biocholine) sources that show resistance to ruminal degradation may improve health and, consequently, production, because the increased

availability of choline or phosphatidylcholine improves cell function and antioxidant status. Pinotti et al. (2002) suggested that choline may be a production constraint in high-production animals, if not supplied in sufficient quantity to meet the animal's requirements. Mohsen et al. (2011) added encapsulated choline to the feed of lactating cows and observed greater nutrient digestibility that increased yield and feed conversion in an economically efficient manner.

The bioavailability of choline and derivatives is important for maintaining cellular integrity, because, according to the literature, choline deficiency increases levels of reactive oxygen species and lipid peroxidation that in turn causes necrosis of the convoluted renal tubules (Ossani et al., 2007). Repetto et al. (2010) also found increased lipid peroxidation and reduced antioxidant levels in the serum of choline-deficient rats, while in the brain, lipid peroxidation levels increased by more than 300%, and therefore the animals developed hepatic steatosis, tubular and glomerular necrosis in addition to inflammation and necrosis in the heart after 7 days of deficiency. In our study, supplementation with VB promoted increases in NPSH and ACAP levels, as well as reduced ROS and LPO levels. Supplementation of Nile tilapia (800 and 1200 mg/kg diet) showed that the product has antioxidant potential mediated by decreases in hepatic LPO and increased ACAP levels, as well as stimulating hepatic energy metabolism, improving the zootechnical performance of the fish (Baldissera et al., 2019); we did not observe this in our study at the serum; the CK and PK activities did not change. Another study showed that aflatoxin-challenged and VB-supplemented Nile tilapia showed liver protection, i.e., minimization of negative effects caused by the mycotoxin (Souza et al., 2020). Overall, we believe that VB supplementation in sheep feed improved health, helping to prepare the animals for the natural challenge of heat stress.

Heat stress occurs when any combination of environmental conditions causes the effective ambient temperature to be higher than the thermoneutral zone. According to the literature, this leads to reduce milk production, as reported in cows (Armstrong, 1994). Cows exposed to heat stress in summer had higher activity of the enzymes superoxide dismutase and glutathione peroxidase, as well as higher levels of lipid peroxidation markers (TBARS) compared to cows in periods where the ambient temperature was lower, suggesting that heat causes oxidative stress (Bernabucci et al., 2002). In our study, VB balanced the antioxidant status of animals, reflected in higher milk yield. The same occurred in heat-stressed sheep supplemented with grape residues (Alba et al., 2019a) and açai (*Euterpe oleracea*) oil (Santos et al., 2019), where the animals also displayed antioxidant status balance, as well as increased milk yields. Chauhan et al. (2014) observed that heat stress negatively affected the oxidative state of sheep; this damage was reduced by supplementing higher amounts of antioxidants in the diet. Taken together, the data suggest that dietary additives/supplements such as biocholine reduces the damage caused during periods of heat stress, maintaining the health and performance of animals.

5. Conclusion

Vegetable biocholine supplementation in the diets of sheep at peak lactation increased antioxidant levels in serum as well as in in milk, reducing oxidant levels and lipid peroxidation. The addition of 10 g of VB to feed improved animal production and feed efficiency, and decreased somatic cell counts in milk. We conclude that VB supplementation in the diets of heat-stressed sheep improves animal health, milk production and quality.

Ethics approval

The study was approved by the Committee of Ethics in the Use of Animals of the State University of Santa Catarina (CEUA / UDESC) under number 6601130319.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Ingredients and chemical composition of ingredients and experimental diets.

Ingredients	**As fed (kg/day)**		**Dry matter (kg/day)**		
Corn silage (kg)	4.00		1.31		
Concentrate (kg)	1.20		0.96		
Chemical composition	**Corn silage**	**Biocholine**	**Concentrate (T0)**	**Concentrate (T5)**	**Concentrate (T10)**
DM, g/kg	329	964	879	883	885
Ash, g/kg DM	42.1	18.0	65.2	69.3	67.7
CP, g/kg DM	80.1	47.0	156	148	152
NDF, g/kg DM	331	445	106	119	100
ADF, g/kg DM	179	384.0	45.0	51.0	46.0
EE, g/kg DM	43.9	57.0	34.3	36.7	34.7
TPC (mg EGA/100 g DM)	-	28.2	9.74	9.85	9.89
IC_{50} (µg/mL)	-	0.30	5.52	5.37	5.11

Ingredients present in 100 kg of concentrate: corn (70%), soybean meal (25%) and buffering lactation nucleus (5%), i.e., ground corn (671 g/kg), soybean meal (277 g/kg), calcitic limestone (10 g/kg), sodium bicarbonate (4 g/kg) and 37 g/kg of premix (calcium min. 180 max. 220 g; phosphorus min. 32 g; sodium min. 40 g; sulfur min. 20 g; magnesium min. 20 g; cobalt min. 16 mg; iodine min. 17 mg; manganese min. 420 mg; selenium min. 730 mg; zinc min. 730 mg; fluorine max. 600 mg; niacin min. 500 mg; vitamin A min. 95000 IU; vitamin D min. 20000 IU; vitamin E min. 350 IU; monensin sodium 1200 mg; *Saccharomyces cerevisae* 2.1 x 10^{10} UFC).

[2.] Note: DM (Dry matter), Ash (Ashes), CP (Crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), EE (ethereal extract), TPC (total phenolic content) and IC_{50} (determination of antioxidant activity by elimination of radicals by DPPH).

Table 2. Milk production and composition of Lacaune ewes at peak lactation supplemented with dietary vegetal biocholine (VB)

Variables[1]	Treatments[2]			SEM	P-value	
	T0	T5	T10		Treatment	Treatment × day
Production (L)					0.27	0.01
day 0	1.96	1.79	2.01	0.10		
day 7	1.98	1.87	2.13	0.09		
day 15	2.17	2.05	2.31	0.11		
day 20	2.11^{b}	2.06^{b}	2.44^{a}	0.09		
Productive efficiency (%)						
day 0 to 15	8.67^{b}	14.5^{a}	12.9^{ab}	1.81	0.01	
day 0 to 20	7.60^{b}	15.0^{a}	19.4^{a}	2.11	0.01	
Feed intake						
day 15 to 20	87.2^{b}	93.3^{a}	84.2^{b}	13.3	0.01	
Feed conversion						
day 15 to 20	2.14^{b}	2.37^{a}	1.91^{c}	0.10	0.01	
Milk composition						
Protein (g/kg)	3.72	3.52	3.67	0.09	0.85	0.44
Fat (g/kg)	4.45	4.33	4.35	0.15	0.85	0.42
Lactose (g/kg)	5.32	5.21	5.43	0.14	0.59	0.14
Total solids (g/kg)					0.45	0.05
day 0	13.2	13.2	12.8			
day 15	14.0	12.6	12.7			
day 20	13.1^{b}	13.1^{b}	15.6^{a}			
SCC[1] (x10^{3}/mL)					<0.0001	<0.0001
day 0	364	368	564	16.9		
day 15	398^{a}	281^{b}	98.0^{b}	17.0		
day 20	436^{a}	219.9^{b}	79.1^{b}	17.1		

[1]Somatic cell count.
[2]T0, T5 and T10 represents 0, 5 and 10 g of biocholine/animal/day.
[a-c]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

Table 3. Serum biochemistry of peak lactation sheep Lacaune supplemented with dietary vegetal biocholine (VB): T0 (0 g/day), T5 (5 g/day) and T10 (10 g/day).

Variables[1]	Treatments[2]			SEM	P-value	
	T0	T5	T10		Treatment	Treatment × day
Glucose (mg/dL)	44.3	42.3	42.5	1.01	0.31	0.95
Total Protein (g/dL)	6.62	6.65	6.59	0.13	0.95	0.97
Albumin (g/dL)	2.81	2.93	2.86	0.05	0.33	0.84
Globulin (g/dL)					0.02	0.09
day 0	3.79	3.77	3.79	0.06		
day 15	3.76[b]	4.14[a]	4.22[a]	0.06		
day 20	3.37[b]	3.37[b]	3.71[a]	0.06		
Cholesterol (mg/dL)	56.3	61.2	58.2	2.27	0.29	0.35
Triglycerides (mg/dL)	16.5	16.5	16.3	1.51	0.98	0.11
Urea (mg/dL)	34.5	36.1	32.6	1.85	0.40	0.17
AST (U/L)	108	109	102	4.37	0.45	0.61
ALT (U/L)	15.0	15.5	16.0	0.39	0.18	0.35
GGT (U/L)	104	103	97.7	3.19	0.31	0.77
CK (pmol creatine formed/min/mg of protein)	1.67	1.60	1.72	0.05	0.26	0.48
PK (pmol pyruvate formed/min/mg protein	8.71	7.96	7.96	0.36	0.26	0.13

[1]Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), creatine kinase (CK), and pyruvate kinase (PK)

[2]T0, T5 and T10 represents 0, 5 and 10 g of biocholine/animal/day.

[a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

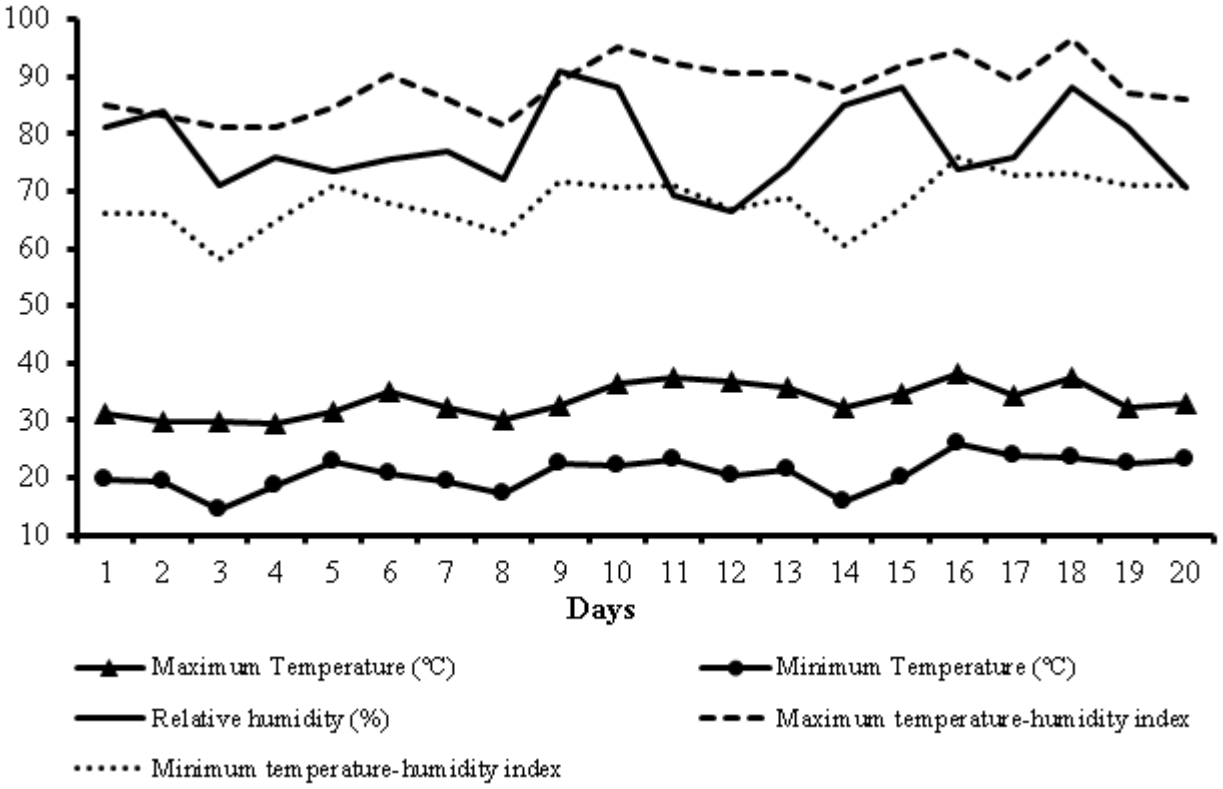

Figure 1: Temperature (ºC), relative humidity (%) and temperature-humidity index (THI = 0.8 × ambient temperature + [(% relative humidity ÷ 100) × (ambient temperature − 14.4)] + 46.4; Mader et al., 2006) collected during the study.

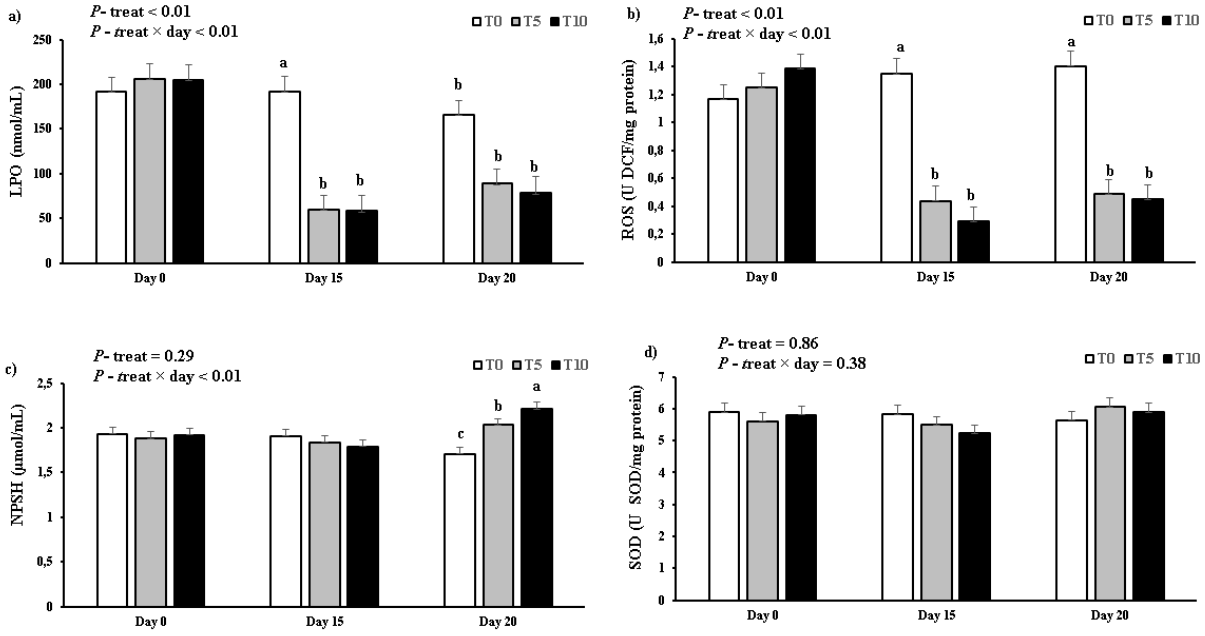

Figure 2. Serum levels of oxidants (lipid peroxidation [LPO] and reactive oxygen species [ROS]) and antioxidants (non-protein thiols [NPSH] and superoxide enzyme dismutase [SOD]) in lactating peak lactose supplemented with dietary biocholine. T0, T5 and T10 represents 0, 5 and 10 g of biocholine/animal/day. [a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

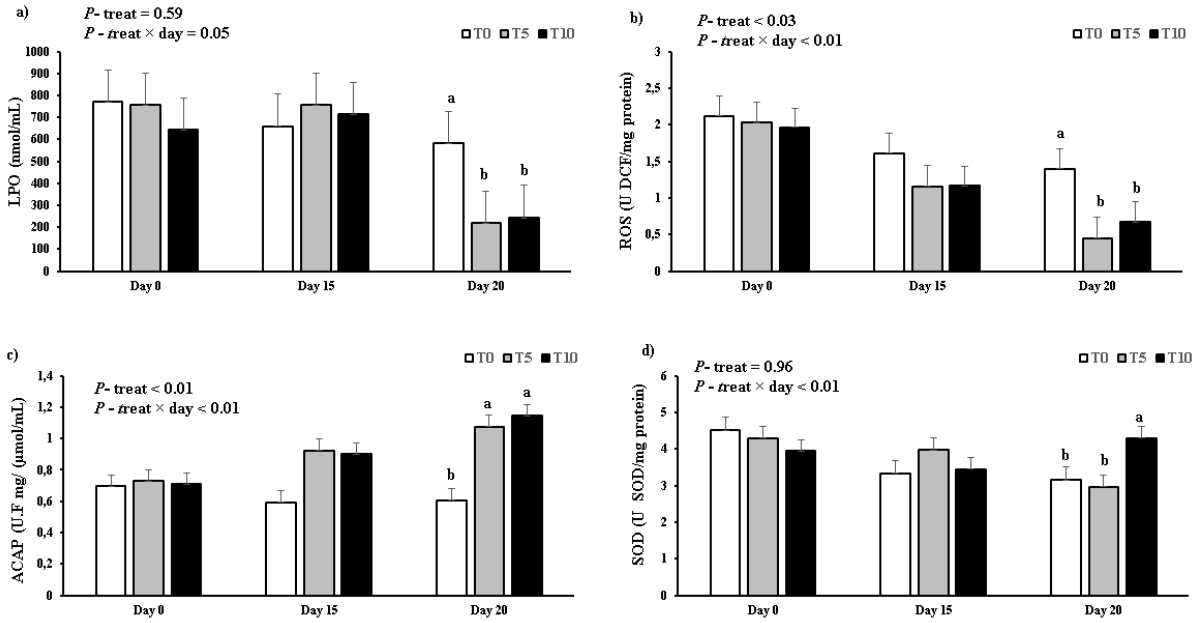

Figure 3. Milk levels of oxidants (lipid peroxidation [LPO] and reactive oxygen species [ROS]) and antioxidants (non-protein thiols [NPSH] and superoxide enzyme dismutase [SOD]) in lactating peak lactose supplemented with dietary biocholine. T0, T5 and T10 represents 0, 5 and 10 g of biocholine/animal/day. [a-b]Differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) between treatments each respective day.

3. CONSIDERAÇOES FINAIS

Com a realização desses estudos é possível concluir que a mastite é uma patologia importante em ovinos leiteiros, pois afeta significativamente a produção, qualidade do leite e a saúde animal, visto que o processo inflamatório afetou em um todo as ovelhas, devido ao aumento da peroxidação lipídica no soro e no leite. Mesmo que os agentes isolados tiverem baixos níveis de resistência, os tratamentos não tiveram uma eficácia clínica relevante, isso dificulta o tratamento e controle da mastite no rebanho, o que pode resultar em descarte de número significativo de matrizes. Além disso, ressalta-se a necessidade de produção de medicamentos para o tratamento de mestites destinados a ovinos para obter melhor eficácia, bem como um período de retenção no leite conhecido, para permitir o uso pelos produtores com segurança.

A utilização da FRU na alimentação das ovelhas mostrou que a capacidade antioxidante presente na uva foi absorvida pelos animais, melhorando a saúde, produção e qualidade do leite. O fato de o resíduo estar na forma de farinha pode ter colaborado para uma melhor absorção das substâncias bioativas devido a maior área de contato com o epitélio intestinal, evidenciando que esse resíduo pode ser utilizado na alimentação animal não apenas como um alimento, mas sim como uma fonte de antioxidantes.

A adição de colina vegetal (5 g animal/dia) durante o período de transição não foi eficaz na melhora da função hepática e perfil oxidativo, no entanto, passado esse período melhorou o sistema antioxidante dos animais e manteve a produção de leite. Já no pico de produção a colina vegetal (10 g animal/dia) melhorou a produção de leite e estatus antioxidante no soro e no leite, o que é muito importante para as ovelhas, que nesse momento possuem um metabolismo energético elevado devido a significativa produção de leite, melhorando o funcionamento do organismo.

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UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação da qualidade do leite, isolamento e perfil de sensibilidade a antimicrobianos de bactérias causadoras de mastite em ovelhas mantidas em sistema confinado não submetidas à antibioticoterapia", protocolada sob o CEUA nº 8278020918 (ID 000715), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 19/09/2018.

We certify that the proposal "Evaluation of milk quality, isolation and antimicrobial susceptibility profile of bacteria causing mastitis in ewes kept in a confined system not submitted to antibiotic therapy", utilizing 30 Ovines (30 females), protocol number CEUA 8278020918 (ID 000715), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 09/19/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 09/2018 a 07/2019 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Ovinos	sexo:	Fêmeas	idade:	2 a 4 anos	N:	30
Linhagem:	Lacaune			Peso:	50 a 70 kg		

Local do experimento: O experimento será realizado em uma cabanha localizada no município de Chapecó-SC.

Lages, 23 de outubro de 2019

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

em aberto
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Suplementação de ovelhas leiteiras com farinha de casca e semente de uva: investigação dos benefícios sobre produção e qualidade do leite, parâmetros bioquímicos e hematológicos, resposta imune e perfil oxidativo", protocolada sob o CEUA nº 5184250218 (ID 000533), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 07/03/2018.

We certify that the proposal "Supplementation of dairy sheep with bark meal and grape seed: investigation of the benefits on milk production and quality, biochemical and hematological parameters, immune response and oxidative profile.", utilizing 27 Ovines (27 females), protocol number CEUA 5184250218 (ID 000533), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 03/07/2018.

Finalidade da Proposta: Pesquisa (Comercial)

Vigência da Proposta: de 04/2018 a 12/2018 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Ovinos	sexo:	Fêmeas	idade:	15 a 20 meses	N:	27
Linhagem:	Lacaune			Peso:	50 a 60 kg		

Local do experimento: O experimento será realizado na cabanha Chapecó, localizada no município de Chapecó-SC. Udesc Oeste tem convenio com a fazenda.

Lages, 23 de outubro de 2019

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

em aberto
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Inclusão de colina vegetal e sais aniônicos da dieta de ovelhas leiteiras: efeitos sobre saúde animal e eficiência produtiva", protocolada sob o CEUA nº 2481011018 (ID 000747), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 10/10/2018.

We certify that the proposal "Inclusion of vegetable choline and anionic salts of dairy sheep diet: effects on animal health and productive efficiency", utilizing 40 Ovines (40 females), protocol number CEUA 2481011018 (ID 000747), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 10/10/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 10/2018 a 04/2019 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Ovinos	sexo:	Fêmeas	idade:	2 a 4 anos	N:	40
Linhagem:	lacaune			Peso:	50 a 70 kg		

Local do experimento: O projeto será realizado na Cabanha Três Leite onde desenvolve-se a criação de ovinos de corte e leite da raça Lacaune, localizada na cidade de Lajeado Grande, região Oeste de Santa Catarina.

Lages, 23 de outubro de 2019

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

em aberto
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Inclusão de fosfatidilcolina na dieta de ovelhas leiteiras: efeitos sobre saúde animal e eficiência produtiva ", protocolada sob o CEUA nº 6601130319 (ID 000843), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 15/03/2019.

We certify that the proposal "Inclusion of phosphatidylcholine in dairy sheep diet: effects on animal health and productive efficiency", utilizing 30 Ovines (30 females), protocol number CEUA 6601130319 (ID 000843), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 03/15/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2019 a 12/2019 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Ovinos	sexo:	Fêmeas	idade:	2 a 3 anos	N:	30
Linhagem:	lacaune			Peso:	50 a 70 kg		

Local do experimento: Fazenda Cabanha Chapecó - convêncio com udesc oeste

Lages, 23 de outubro de 2019

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

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Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina