**KAMILA MACIEL DIAS**

# EFEITO DA SUPLEMENTAÇÃO DIETÉTICA COM EXTRATO TANÍFERO DE *ACACIA MEARNSII* NA PRODUÇÃO, COMPOSIÇÃO QUÍMICA E PERFIL DE ÁCIDOS GRAXOS DO LEITE DE OVELHAS E VACAS EM PASTEJO

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

Orientador: Henrique Mendonça Nunes Ribeiro Filho.

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**Banca Examinadora**

Orientador: _____
Prof. Dr. Henrique Mendonça Nunes Ribeiro Filho
Universidade do Estado de Santa Catarina – UDESC

Membro: _____
Prof. Dr. André Fischer Sbrissia
Universidade do Estado de Santa Catarina – UDESC

Membro: _____
Prof. Dr. André Thaler Neto
Universidade do Estado de Santa Catarina – UDESC

Membro: _____
Prof. Dr. Gilberto Vilmar Kozloski
Universidade Federal de Santa Maria – UFSM

Membro: _____
Dr. Marco Antonio Sundfeld da Gama
EMBRAPA Gado de Leite – CNPGL

**Lages, 21/07/2016.**

Para Família Maciel Dias
e Marcio Zilio

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# RESUMO

DIAS, Kamila Maciel. **Efeito da suplementação dietética com extrato tanífero de *Acacia mearnsii* na produção, composição química e perfil de ácidos graxos do leite de ovelhas e vacas em pastejo.** 2016. 147 f. Tese (Doutorado em Ciência Animal – Área: Produção Animal) – Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência Animal, Lages, 2016.

Os taninos condensados podem reduzir a degradabilidade das proteínas, melhorar a eficiência do uso do nitrogênio (N) e o perfil de ácidos graxos do leite. A suplementação com extrato tanífero de acácia negra (*Acacia mearnsii*) pode aumentar o fluxo de proteína metabolizável (PM), reduzir a excreção urinária de N, o teor de N ureico sanguíneo e emissões de metano. Além disso, o extrato tanífero de *Acacia* spp pode aumentar as concentrações de ácido vacênico e reduzir as concentrações de ácido esteárico no fluido ruminal. No entanto, nenhum trabalho avaliou o efeito do extrato tanífero de acácia negra no perfil de ácidos graxos no leite de ruminantes e a maioria dos trabalhos *in vitro* and *in vivo*, com outras fontes de tanino, normalmente têm mostrado resultados contraditórios. A maioria dos estudos com acácia negra a suplementação foi de forma involuntária, não avaliou o efeito deste tanino no desempenho de animais em lactação, utilizaram animais no início da lactação ou dietas com baixo teor proteíco. O objetivo deste trabalho foi investigar o efeito da suplementação voluntária com tanino de acácia negra na produção, composição e perfil de ácidos graxo (AG) do leite e excreção N de ovelhas e vacas em lactação em pastejo de pastagens com alto teor de proteína. Três experimentos foram conduzidos

entre Janeiro de 2013 à Fevereiro de 2014. O primeiro experimento testou dois níveis de suplementação de tanino de acácia negra (0 e 20 g de extrato tanífero/kg de concentrado; controle e T20) em 24 ovelhas em lactação pastando uma pastagem formada predominantemente por trevo branco (*Trifolium repens*) e suplementadas com silagem de milho (1 kg/dia) e concentrado (600 g/dia). O segundo experimento foi conduzido com dieta similar ao primeiro experimento, porém testou três níveis de acácia negra (0, 30 e 40 g de extrato tanífero/kg de concentrado; controle, T30 e T40) em 24 ovelhas em lactação. O terceiro experimento testou dois níveis de acácia negra (0 e 15 g de extrato tanífero/kg de ração parcialmente misturada-RPM; controle e T15) em 30 vacas em lactação, suplementadas com RPM (600 g/kg de concentrado e 400 g/kg de silagem de milho) e em pastejo de pastagens formadas predominantemente por festuca. O delineamento experimental de todos os experimentos foi completamente casualizados, nos quais os níveis de suplementação do controle, T20, T30, T40 e T15 foram, respectivamente, equivalente a zero, 0,8, 1,2, 1,5 e 1,5% do consumo de matéria seca total (CMST). Amostras individuais de leite para análise de composição química foram coletadas nos dias 1, 3 e 5 do período de avaliação e somente no último dia de cada período para análise e perfil de AG, o qual foi analisado por cromatografia gasosa. O CMST, produção e composição química do leite, N ureico sanguíneo e excreção urinária de N foram similares no primeiro e segundo eperimento, exceto o teor de N fecal do grupo T30 e T40 foram aproximadamente 9% superior ao tratamento controle (P=0.053). Houve uma redução no consumo de silagem de milho e concentrado no grupo T30 mas não houve diferenças na produção de leite e peso vivo. No terceiro experimento, o consumo de pastagem e RPM foram similares entre os tratamentos. A produção e composição química do leite, as concentrações de AGNE, N ureico sanguínero e excreção urinária de N não foram afetados

pelos tratamentos. Houve um aumento nos teores de 6:0, *cis*-12 18:1, *trans*-12 18:1 e *trans*-10, *cis*-12 CLA no tratamento T20 e um aumento linear de 17:0, *cis*-9 17:1, *trans*-10 18:1, *cis*-9, *trans*-11 CLA no leite das ovelhas dos tratamentos T30 e T40. Houve somente um aumento de 18:2 n-6 e 20:0 no leite do tratemento T15. O maior teor de N fecal, quando a proporção de extrato tanífero foi ≥9 g/kg do CMST, sugere um desvio da rota de excreção de N da urina para as fezes, mostrando que a suplementação com extrato tanífero pode ter um amplo impacto no teor fecal de N em dietas ricas em proteína e consequentemente reduzir a contaminação ambiental. A suplementação com 12 e 16 g de extrato tanífero de acácia negra/kg do CMST aumentou os teores de N fecal e aumentou os teores de AG benéficos à saúde humana sem prejudicar o desempenho de ovelhas em lactação.

**Palavras-chave**: Tanino. Proteína. Nitrogênio ureico no leite. Excreção urinária de nitrogênio. Ácidos graxos. Ácido Linolênico Conjugado (CLA).

# ABSTRACT

DIAS, Kamila Maciel. **Effect of dietary supplementation with *Acacia mearnsii* tannin extract on milk yield, composition and milk fatty acid profile of grazing ewes and cows.** 2016. 149 f. Thesis (Doctorate in Animal Science - Area: Animal Production) - Santa Catarina State University. Post Graduate Program in Animal Science, Lages, 2016.

Condensed tannins can reduce ruminal degradability of proteins, improve nitrogen use efficiency and milk fatty acid profile. Supplementation with tannin extracted from black wattle (*Acacia mearnsii*) can increase duodenal flow of metabolisable protein (MP), decrease N urinary excretion, blood urea N content and methane emissions. Besides, tannin extract from *Acacia* spp can increase vacennic acid and reduce stearic acid concentrations in ruminal fluid. However, any study evaluated the effect of black wattle tannin on milk fatty acid (FA) composition and most of *in vitro* and *in vivo* studies, with other sources of condensed tannins, have been showing conflicting results. Most of studies with black wattle tannin provided involuntary supplementation, did not evaluate the effect of this tannin on performance of lactation animals, worked with early lactation cows or low protein diets. The aim of this work was to investigate the effect of voluntary supplementation with black wattle tannin on milk yield, milk composition, milk fatty acid (FA) composition and N excretion of dairy ewes and cows grazing a pasture with high protein content. Three experiments were conducted between January to February of 2013 to 2014. The first experiment tested two levels of black wattle tannin (0 and 20 g of tannin extract/kg of concentrate; control and T20) in 24 lactating ewes grazing a

white clover (*Trifolium repens*) predominant pasture and supplemented with corn silage (1 kg/day) and a concentrate feed (600 g/day). The second experimet had similar diet than first experiment and tested three levels of black wattle tannin (0, 30 and 40 g of tannin extract/kg of concentrate; control, T30 and T40) in 24 lactating ewes. The third experiment tested two levels of black wattle (0 and 15 g tannin extract/kg PMR; control and T15) in 30 lactating cows grazing a fescue predomiant pasture and supplemented with a RPM (600 g/kg of concentrate and 400 g/kg of corn silage). All experiments were a completely randomized experimental design, which the supplementation levels of control, T20, T30, T40 and T15 were, respectively, equivalent to zero, 0.8, 1.2, 1.5 and 1.5% of DMI. Individual milk samples for milk composition were collected in day 1, 3 and 5 of sampling period and for milk FA profile were collected in the last day of sampling period, which were analyzed for FA composition by gas chromatography. Total DMI, milk yield, milk composition, blood urea nitrogen and N urinary excretion were similar in first and second experiment, except N fecal content of T30 and T40 were around 9% higher than treatment control (P=0.053). There was a decrease in corn silage and concentrate intake in T30 treatment but without differences in milk yield and live weight. In third experiment, pasture and PMR intake were similar between treatments. The milk yield, milk composition, concentratios of NEFA, blood urea N and N urea urinary excretion were not affected by treatments. There was higher milk content of 6:0, *cis*-12 18:1, *trans*-12 18:1 and *trans*-10, *cis*-12 CLA in T20 treatment and a linear increase of 17:0, *cis*-9 17:1, *trans*-10 18:1, *cis*-9, *trans*-11 CLA in milk of ewes from T30 and T40 treatments. There only was increase of 18:2 n-6 and 20:0 in milk from T15 treatments. The greater fecal N content when the proportion of tannin extract was ≥9 g/kg of total DMI suggest a diversion of N from urine to feces, showing a strong impact of tannin extract supplementation

mixed with concentrate on N fecal content in N-rich diets and consequently lower environmental pollution. Supplementation with 12 and 16 g/kg of total DMI with Acacia mearnsii tannin, mixed with concentrate, increased N fecal content and improved the level of healthy milk FA without impairing dairy ewes performance.

**Key words**: Taninn. Protein. Milk Urea Nitrogen. N Urinary Excretion. Fatty acid. Conjugated Linolenic Acid (CLA).

## LISTA DE FIGURAS

Figura 1- Estruturas químicas de taninos hidrolisáveis da castanheira portuguesa (*Castanea sativa* Mill.)..... 39
Figura 2- Estruturas químicas de taninos condensados presente em algumas leguminosas forrageiras (a) e em *Acacia mearnsii* (b)..... 40

## LISTA DE TABELAS

Table 1. Chemical composition and energetic value of supplements (corn silage and concentrate). ..... 69
Table 2. Pre and post-grazing pasture characteristics, morphological and chemical composition of white clover dominant pasture grazed by dairy ewes supplemented with corn silage and concentrate with or without inclusion of black wattle tannin extract (First experiment). ..... 73
Table 3. Pre-grazing pasture characteristics and post-grazing sward height of white clover dominant pasture grazed by dairy ewes receiving different levels of black wattle tannin extract (Second experiment). ..... 74
Table 4. Effect of taniferous extract supplementation on DM intake, milk production and composition blood parameters of dairy ewes grazing white clover dominant pasture and supplemented with corn silage + concentrate feed (First experiment). ..... 76
Table 5. Effect of taniferous extract supplementation on DM intake, milk production and composition blood parameters of dairy ewes grazing white clover dominant pasture and supplemented with corn silage + concentrate feed (Second experiment)..... 77
Table 6. Chemical composition and energetic value of supplements (corn silage and concentrate). ..... 93
Table 7. Pre- and post-grazing pasture characteristics grazed by dairy cows with (TAN) or without I tannin extract supplementation. ..... 97
Table 8. Effect of extract tannin supplementation on DM intake, milk yield, milk composition and blood composition parameters of dairy cows. ..... 99

Table 9. Chemical composition of feed and sward characterization in two experiments conducted with dairy ewes. ..... 118
Table 10. Effect of tannin supplementation on supplements intake, milk yield and live weigh of dairy ewes grazing a dominant legume pasture (First and second experiment). ..... 120
Table 11. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (First experiment). ..... 121
Table 12. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (Second experiment)..... 124
Table 13. Chemical composition of feed and sward characterization of dairy cows experiment. ..... 128
Table 14. Effect of tannin supplementation on PMR intake, live weigh and milk yield of dairy cows grazing a dominant grass pasture..... 128
Table 15. Effect of tannin supplementation on milk FA composition of grazing dairy cows supplemented with PMR. ..... 129

**SUMÁRIO**

**1 INTRODUÇÃO** ..... **35**
1.1 REVISÃO BIBLIOGRÁFICA ..... 36
**1.1.1 Efeito dos taninos na proteólise** ..... **42**
**1.1.2 Efeito dos taninos na lipólise** ..... **46**
**1.1.3 Efeito dos taninos no consumo de alimento** ..... **49**
1.2 HIPÓTESES ..... 50
1.3 OBJETIVOS ..... 51
**1.3.1 Objetivos Gerais** ..... **51**
**1.3.2 Objetivos Específicos** ..... **51**
**REFERÊNCIAS** ..... **52**
**3 CAN SUPPLEMENTATION WITH BLACK WATTLE TANNIN EXTRACT MITIGATE N UREA EXCRETION WITHOUT REDUCTIONS ON DAIRY EWES PERFORMANCE?** ..... **65**
3.1 ABSTRACT ..... 65
3.2 INTRODUCTION ..... 66
3.3 MATERIAL AND METHODS ..... 67
**3.3.1 Local, treatments and experimental design** ..... **67**
**3.3.2 Animals** ..... **68**
**3.3.3 Feed and grazing management** ..... **68**
**3.3.4 Feed and sward measurements** ..... **70**
**3.3.5 Animal measurements** ..... **70**
**3.3.6 Chemical Analyses** ..... **71**
**3.3.7 Statistical Analyses** ..... **72**
3.4 RESULTS ..... 73
**3.4.1 Pasture characteristics** ..... **73**
**3.4.2 Animal performance and N excretion** ..... **75**
3.5 DISCUSSION ..... 78
**3.5.1 Effect of tannin extract supplementation on N excretion** ..... **78**
**3.5.2 Effect of tannin extract supplementation on animal performance** ..... **80**

3.6 CONCLUSION ..... 81
**4 PERFORMANCE AND NITROGEN EXCRETION OF GRAZING DAIRY COWS SUPPLEMENTED WITH BLACK WATTLE TANNIN EXTRACT ..... 89**
4.1 ABSTRACT ..... 89
4.2 INTRODUCTION ..... 90
4.3 MATERIALS AND METHODS ..... 92
**4.3.1 Local and experimental design ..... 92**
**4.3.2 Animals and diet ..... 92**
**4.3.3 Feed and sward measurements ..... 94**
**4.3.4 Animal measurements ..... 94**
**4.3.5 Chemical Analyses ..... 95**
**4.3.6 Statistical Analyses ..... 96**
4.4 RESULTS ..... 96
**4.4.1 Pasture characteristics ..... 96**
**4.4.2 Animal performance and milk composition ..... 98**
4.5 DISCUSSION ..... 100
4.6 CONCLUSION ..... 101
**REFERENCES ..... 102**
**5 BLACK WATTLE TANNIN SUPPLEMENTATION AFFECTS MILK FATTY ACID COMPOSITION OF DAIRY EWES ..... 109**
5.1 ABSTRACT ..... 109
5.2 INTRODUCTION ..... 110
5.3 MATERIAL AND METHODS ..... 112
**5.3.1 Local and experimental design ..... 112**
**5.3.2 Animals ..... 113**
**5.3.3 Feed and grazing management ..... 114**
**5.3.4 Feed and sward measurements ..... 114**
**5.3.5 Animal measurements ..... 115**
**5.3.6 Chemical Analyses ..... 116**
**5.3.7 Statistical Analyses ..... 117**
5.4 RESULTS ..... 117
**5.4.1 Dairy ewes experiments ..... 117**
**5.4.2 Dairy cows experiment ..... 127**

5.5 DISCUSSION ..... 132
5.6 CONCLUSION ..... 135
**REFERENCES ..... 136**
**6 CONCLUSÃO GERAL ..... 146**
**AGRADECIMENTOS ..... 147**

# 1 INTRODUÇÃO

A alimentação de animais leiteiros representa de 40 a 60% dos custos da produção de leite e a utilização de pastagem como principal recurso alimentar pode reduzir significativamente o custo da atividade (AROEIRA, 2004). Uma eficiente alternativa na redução dos custos de produção é a intensificação dos sistemas de produção leiteira a pasto, a qual visa obter o maior rendimento possível por unidade de recurso produtivo disponível. Nesse sentido, a utilização de espécies ou cultivares forrageiras de alto valor nutritivo como principal fonte de nutrientes, associado ao uso de práticas racionais de manejo, possibilitam aumentar a produtividade animal e tornar a atividade leiteira mais competitiva.

As forragens de alto valor nutritivo, como as gramíneas e leguminosas de clima temperado, normalmente apresentam altos teores de proteína bruta (WAGHORN et al., 2007). Porém, boa parte da porção proteica dessas forrageiras (56-65%) é rapidamente degradável no rúmen e consequentemente há grande perda ruminal de nitrogênio (N) na forma de amônia (MIN et al., 2000). A amônia absorvida no rúmen é transformada em ureia que pode ser excretada no leite ou urina, as quais são altamente correlacionadas (POWELL et al., 2001). O N excretado na urina é rapidamente hidrolisado em amônia (POWELL et al., 2001) ou transformado em nitrato. O nitrato pode contaminar os lençóis freáticos quando lixiviado ou contribuir para o efeito estufa quando volatilizado na forma de oxido nitroso ($N_2O$) (MONAGHAN et al., 2007). A utilização dessas forragens como principal recurso alimentar normalmente excede a exigência proteica dos animais e por isso há uma menor eficiência de utilização de N com consequências ambientais negativas.

A utilização de alternativas nutricionais para reduzir a degradabilidade das proteínas, como os taninos condensados, pode aumentar o aporte de proteína metabolizável (PM) e

consequentemente melhorar o desempenho animal e reduzir o impacto ambiental gerado pela excreção ambiental de N (ALVES, 2012; CARULLA et al., 2005). Os taninos possuem alto potencial para reduzir a degradabilidade ruminal de alguns componentes da dieta através de pontes de hidrogênio e/ou ligações hidrofóbicas (SILANKOVE et al., 2001; MOLAN et al., 2001), mas atuam principalmente nas proteínas, devido a maior capacidade de interação com esses componentes. Devido a essa característica, os efeitos positivos ou negativos dos taninos no metabolismo animal estão diretamente relacionados ao seu nível de inclusão na dieta animal. Em altas dosagens podem ser tóxicos (taninos hidrolisados), os componentes da dieta podem se tornar indisponíveis para absorção intestinal, a atividade das bactérias ruminais pode ser reduzida à níveis críticos, a atividade de enzimas intestinais pode ficar comprometida (JONES et al., 2000) e consequentemente o desempenho animal pode ser negativamente afetado. Contudo, quando os taninos são utilizados em dosagens baixas a moderadas podem prevenir o timpanismo, aumentar a passagem de proteínas, de alguns aminoácidos essenciais, de ácidos graxos insaturados para o intestino, a produção/composição do leite e reduzir a excreção urinária de N (WAGHORN AND MCNABB, 2003; VASTA et al., 2009b).

## 1.1 REVISÃO BIBLIOGRÁFICA

Nas últimas décadas a população mundial aumentou exponencialmente, alcançando hoje aproximadamente 7 bilhões de pessoas (FAOSTAT, 2015). Esse constante crescimento populacional vem gerando uma maior demanda por alimentos e um dos grandes desafios da agropecuária é conciliar o aumento na produção de alimentos com a preservação do meio ambiente. Afim de vencer esse desafio o nutricionista deve buscar o aumento na capacidade de

conversão de nutrientes de origem vegetal em proteína animal para consumo humano, reduzir os custos na produção e a contaminação ambiental.

O Brasil se destaca por ser o segundo maior produtor de carne bovina e o quarto maior produtor de leite bovino do mundo, produzindo 9,7 milhões de toneladas de carne e 34,5 milhões de toneladas de leite (FAOSTAT, 2015). Apesar dessa alta produção total, os índices de produtividade do país são caracterizados como baixos, onde a produção de leite por vaca, natalidade, mortalidade, taxa de desfrute, idade a primeira cria e idade de abate constituem, respectivamente, 1,7 toneladas/vaca/ano, 60%, 8%, 17%, 3,5 e 4 anos (ANUALPEC, 2009). O sistema brasileiro de bovinocultura de carne e leite é baseado em pastagem (23,5% do território nacional; FAOSTAT, 2015) e a sua utilização de forma intensiva contribui para uma melhor produtividade e preservação dos recursos renováveis.

Uma opção para intensificação da produção de leite à pasto é a utilização de pastagens de melhor valor nutricional, como o trevo branco, azevém e festuca. Essas forragens normalmente possuem altos teores de proteína porém boa parte da proteína é rapidamente degradável no rúmen (WAGHORN et al., 2007). Essa alta concentração de proteína degradável comumente excede a exigência dos microrganismos ruminais, resultando em baixa eficiência na incorporação do N contido na forragem para o leite ou outros tecidos e em grandes quantidades de N excretado no meio ambiente (PACHECO e WAGHORN, 2008). A proteína é um dos nutrientes mais onerosos na dieta e a otimização do uso da proteína da forragem pelo ruminante pode reduzir os custos de produção e o impacto ambiental.

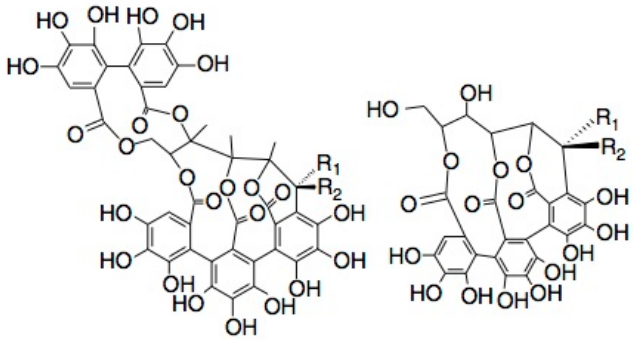
Os taninos são capazes de otimizar o uso do N forrageiro, pois aumentam o fluxo de PM e reduzem a excreção de N através de uma redução na degradabilidade das proteínas (ALVES, 2012; CARULLA et a., 2005). Isso ocorre porque os

taninos tem alta afinidade de ligação com as proteínas devido ao grande número de grupos fenólicos em sua estrutura química que preferencialmente se ligam ao grupo carbonila dos peptídeos (MCLEOD, 1974; HAGERMAN e BUTLER, 1991). A formação desses complexos é específica, tanto em termos de tanino como de proteína utilizada, e o grau de afinidade entre os componentes participantes da dieta está relacionado as características químicas de ambos (ZUCKER, 1983; MANGAN, 1988). Outros fatores que auxiliam na formação dos complexos incluem o alto peso molecular e flexibilidade estrutural (MUELLER-HARVEY e MCALLAN, 1992). As proteínas com maior afinidade aos taninos são normalmente grandes e hidrofóbicas com uma estrutura flexível, aberta e rica em prolina (KUMAR e SINGH, 1984). Os últimos autores sugeriram que os complexos poderiam ser formados através de três tipos de ligações: ligações hidrogenadas (reversível e dependente de pH) entre os radicais hidroxilas dos grupos fenólicos e o oxigênio do grupo amida; interações hidrofóbicas (reversível e dependente de pH) entre o anel aromático dos compostos fenólicos e as regiões hidrofóbicas da proteína; ligações iônicas (reversível) entre o íon fenolato e o sítio catiônico da proteína (exclusivo para os taninos hidrolisáveis); e ligações covalentes (irreversível) através da oxidação dos polifenóis à quinonas e sua consequente condensação com grupos nucleofílicos das proteínas. Durante muitos anos acreditáva-se que a formação dos complexos tannino-proteína eram formados principalmente através de ligações hidrogenadas, porém hoje sabe-se que as interações hidrofóbicas também são importantes.

Os taninos podem ser subdivididos em dois grupos: tanino hidrolisado (TH) e tanino condensado (BRAVO, 1998). Os taninos hidrolisados são constituídos de poliésteres de ácidos fenólicos que possuem uma molécula de açúcar no anel central (Figura 1) e os taninos condensados são formados por polímeros de flavonoides unidos por ligações de carbono

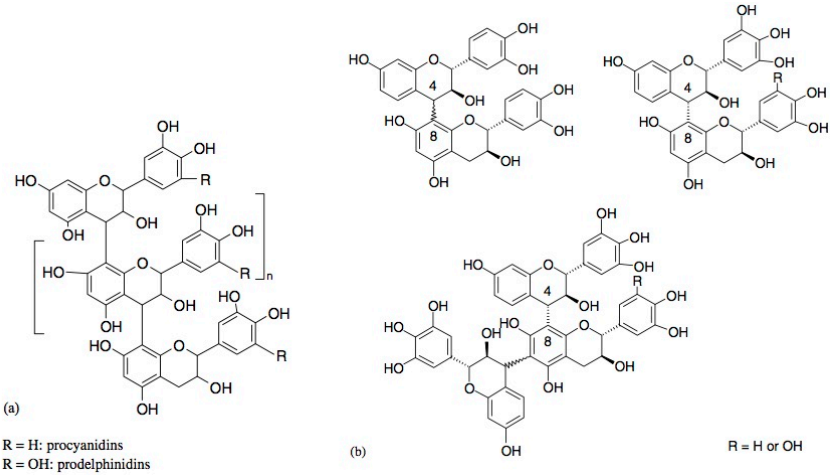
(Figura 2). Devido a diferença na estrutura química entre os grupos, alguns estudos relatam que a hidrólise ruminal pode ser capaz de atuar nos taninos hidrolisados (MUELLER-HARVEY, 2006) e como consequência alguns compostos fenólicos tornam-se aptos à absorção sanguínea. Quando esses compostos fenólicos ultrapassam a capacidade de detoxificação do fígado, podem danificar alguns órgãos como o fígado, rim e baço (GARG et al., 1992), o que aumenta o risco de toxicidade desse grupo quando comparado aos taninos condensados (TERRILL et al., 1994). Alguns autores relatam que as atribuições de que os taninos hidrolisados são mais tóxicos e menos eficientes do que os condensados são simplistas e errôneas (CARRENO et al., 2015). Porém, devido ao risco de toxicidade dos taninos hidrolisados os taninos condensados são mais comumente utilizados na dieta de ruminantes.

Figura 1- Estruturas químicas de taninos hidrolisáveis da castanheira portuguesa (*Castanea sativa* Mill.).




Fonte: MUELLER-HARVEY, 2006

Figura 2- Estruturas químicas de taninos condensados presente em algumas leguminosas forrageiras (a) e em *Acacia mearnsii* (b).




Fonte: MUELLER-HARVEY, 2006.

Os complexos tanino-proteína são formados em pH em torno de 3,5 a 8 e são dissociados em pH<3,5 ou pH>8 (SILANIKOVE et al, 2001). Dessa forma o complexo tanino-proteína pode ser formado no ambiente ruminal (pH≅7) e dissociado no abomaso (pH≅2,5-3) ou duodeno (pH≅8), permitindo a proteção da proteína alimentar da digestão ruminal e o aumento no fluxo intestinal de proteínas. Alguns autores sugerem que os taninos podem impedir a digestão intestinal quando complexos tanino-proteína são formados o início do intestino delgado (pH≅5,5) (MCNABB et al, 1998). Outros autores sugerem que os taninos também podem reduzir a digestão das proteínas quando complexos taninos-proteína são formados com as enzimas digestivas (KUMAR e SINGH, 1984; SILANIKOVE et al, 1994). Entretanto, a maioria desses trabalhos não levaram em conta fatores como a presença de sais biliares que podem evitar que os taninos se liguem às enzimas digestivas.

O mecanismo no qual os taninos reduzem a degradação ruminal de diferentes componentes dietéticos não está bem esclarecido. Os taninos podem interferir na ligação entre os microrganismos ruminais e as paredes celulares das plantas, reduzindo a degradabilidade (MCALLISTER et al, 1994). O crescimento da população microbiana pode ser reduzido quando os carboidratos, proteínas e íons metálicos tornam-se inacessíveis aos microrganismos ruminais (MUELLER-HARVEY e MCALLAN, 1992; SCALBERT, 1991). Os taninos também podem reduzir a degradabilidade dos componentes dietéticos ao interagir com as enzimas microbianas (bacteriana e fúngica; MCSWEENEY et al, 2001). Os taninos condensados inibem mais facilmente as hemicelulases do que as celulases, possivelmente pelo fato de serem enzimas extracelulares e mais sensíveis à ação dos taninos (WAGHORN, 1996). Além disso, os taninos têm efeito direto nos microrganismos ruminais ao alterar permeabilidade de suas membrana (SCALBERT, 1991). Todavia, alguns microrganismos podem tolerar a ação dos taninos (O'DONOVAN e BROOKER, 2001). Todos esses fatores vão depender do tipo de tanino utilizado e o nível de inclusão na dieta (MCALLISTER et al, 1994).

Os efeitos nutricionais dos taninos condensados podem variar de acordo com a estrutura química e peso molecular do tanino utilizado, a quantidade ingerida, a espécie ou categoria animal envolvida e do balanço energético e proteico da dieta (MIN et al, 2003; FRUTOS et al, 2004). Mas de forma geral, em baixas dosagens podem prevenir o timpanismo, aumentar o fluxo duodenal de proteínas, de alguns aminoácidos essenciais e de ácidos graxos insaturados. Já em altas dosagens podem reduzir a digestibilidade da matéria orgânica (MO), o consumo da matéria seca (CMS), a atividade das bactérias ruminais, a digestibilidade intestinal dos nutrientes e afetar o desempenho animal (JONES et al, 2000). Alguns autores aconselham que a inclusão de <50 g de taninos condensado/kg de MS é benéfica

para o desempenho animal (MIN et al., 2003; MCMAHON et al., 2000; HERVÁS et al., 2003). Porém a maior parte dessas recomendações são originárias de ensaios com a espécie *Lotus* e muitas vezes não podem ser aplicadas para outras espécies. Alguns exemplos são as espécies *Hedysarum coronarium* e *O. Viciifolia* as quais tem efeito benéfico em ovelhas com 72 g de tanino condensado/kg e 80 de tanino condensado/kg, respectivamente (ULYATT at al., 1976; STIENEZEN et al., 1996). Em contrapartida, a inclusão de apenas 25 g de tanino condensado/kg de polpa de alfarroba reduziu as taxas de crescimento em cordeiros (PRIOLO et al., 2000). De acordo com essas diferenças, torna-se importante conhecer os efeitos nutricionais dos diversos tipos de taninos disponível para utilização na dieta de ruminantes, assim como a dosagem adequada para alcançar o objetivo proposto.

### 1.1.1 Efeito dos taninos na proteólise

Em uma dieta baseada em forragem de alta qualidade, boa proporção dessas proteínas são rapidamente solúveis no rúmen e consequentemente entre 25 a 35% do N é perdido na forma de amônia no rúmen e excretado como ureia na urina ou leite (MIN et al., 2000). Devido ao alto custo da proteína na dieta animal e a baixa eficiência do uso do N proveniente dessas forragens, algumas pesquisas com taninos foram conduzidas com o intuito de avaliar se o seu uso na dieta de ruminantes melhora a retenção de N, desempenho animal e reduz o impacto ambiental.

Os taninos condensados naturalmente presentes na espécie *Lotus* são capazes de reduzir a proteólise ruminal (AERTS et al., 1999). Apesar de se tratar da mesma espécie forrageira, o tanino proveniente do *Lotus pedunculatus* provocou maior redução na degradação da proteína da forragem do que o tanino proveniente do *Lotus corniculatus* (AERTS et al., 1999; MIN et al., 2005). Aparentemente essa

diferença está relacionada ao maior peso molecular do tanino proveniente do *Lotus pedunculatus* (12300 Da) do que o tanino proveniente do *L. corniculatos* (<5300 Da). Outros trabalhos confirmaram o efeito do tanino presente em *L. corniculatos* na redução da população de bactérias proteolíticas no rúmen e observaram um aumento na produção de leite em vacas (MIN et al., 2002; HYMES-FECHT et al., 2005).

Além dos taninos naturalmente presente em plantas forrageiras, alguns subprodutos como cascas de *Caesalpinia spinosa*, cascas de castanha, cascas de amendoim (*Arachis hypogea* L.) e cascas da semente de tamarindo (*Tamarindus indica* L.), também contém taninos e ao serem incorporados na dieta animal produziram efeitos nutricionais positivos. O tratamento do farelo de soja com 100 g de *Caesalpinia spinosa* reduziu a proteólise ruminal e melhorou o ganho de peso diário, eficiência alimentar e balanço de N em cordeiros (LOWRY e KENNEDY, 1996). Baixas doses de tanino proveniente da castanha reduziu a produção de amônia *in vitro* (sistema Rusitec; SLIWINSKI et al., 2002) e também aumentou o fluxo de N não amoniacal no duodeno em bovinos em crescimento e resultou em maior digestão aparente da proteína intestinal (DECRUYENAERE et al., 1996; tanino à 4% do total de proteína na dieta). Vacas leiteiras com dietas contendo 80-160 g casca amendoim/kg MS (180 g tanino condensado/kg de MS) apresentaram maior consumo de matéria seca (CMS), produção de leite, teor de gordura no leite e menor formação de amônia ruminal e teor de proteína no leite. Neste último trabalho foi concluído que o nível ótimo de casca de amendoim/kg MS é 160 g (WEST et al., 1993). A produção e composição do leite, exceto o teor de N ureico, não foram alteradas com a suplementação de tanino condensado proveniente de *Shinopsis* spp à 3% do CMS (DSCHAAK et al., 2011). Quando uma mistura de tanino proveniente de *Shinopsis* spp e *Castanea vesca* foram incluídos na dieta de vacas leiteiras o teor de proteína no leite aumentou na menor dose

(0,45% do CMS), enquanto na maior dose (1,8% do CMS) o teor de proteína no leite, as concentrações de N uréico do leite (NUL) e amônia no rúmen foram menores (AGUERRE et al., 2010). Entretanto, Benchaar et al. (2008) não encontraram diferenças na composição do leite quando vacas foram suplementadas somente com *Schinopsis balansae* à 0,45% do CMS.

A maior parte do N presente na urina é na forma de ureia (70-80%) e, após ser excretada e em contato com o solo, é rapidamente degradada à amônia, na qual pode ser rapidamente volatilizada nessa forma ou na forma de óxido nitroso. A ureia presente da urina também pode ser transformada à nitrato pelo processo de nitrificação (HAYNES e WILLIAMS, 1993). O nitrato pode ser utilizado pelas plantas ou pode contaminar lençóis freáticos quando lixiviado, principalmente em períodos de alta precipitação pluviométrica. O óxido nitroso é um gases responsáveis pelo aumento da temperatura na superfície da terra e tem um potencial de aquecimento 310 vezes superior ao $CO_2$. Em contrapartida, o N fecal é mais estável do que o N urinário, pois o N fecal é mais lentamente degradado à amônia, resultando em menores produções de gases ou contaminantes ambientais (POWELL et al., 2009). Por essas razões, um aumento na relação N fecal:N urinário torna-se desejável para o ponto de vista ambiental.

A excreção urinária de N tem uma relação linear com o NUL e altas concentração de NUL indica que há um excesso de PB na dieta comparado ao nível de produção animal ou um desbalanço energético-proteico na dieta (CISZUK e GEBREGZIABHER, 1994; JONKER et al., 1998). Dessa forma, menores concentrações de NUL através da suplementação com tanino sugere que a excreção de N urinário pode ser reduzida. Powell et al. (2009) observou uma menor excreção urinária de N e maior excreção fecal de N através do fornecimento de forragem contendo tanino (*Lotus* spp.). BHATTA et al. (2000) observaram que a inclusão de 75 g de

casca de tamarindo/kg MS na dieta de vacas leiteiras reduziu a excreção urinária de N, aumentou a excreção fecal de N e o ganho de peso (cerca de 46%). Também houve redução na excreção de N urinário quando novilhos Jersey foram suplementados com 0,6% do CMS com tanino condensado (*Schinopsis balansae*; BAAH et al., 2007). Uma menor excreção urinária de N pode reduzir a contaminação ambiental através da menor volatilização de amônia, emissões de óxido nitroso e lixiviação de nitrato (PLACE e MITLOEHNER, 2010).

Trabalhos previamente conduzidos testaram o efeito do extrato tanífero proveniente da *Acacia meanrsii* no metabolismo proteico de ruminantes. A suplementação de cordeiros com 2,5% do CMS com esse tanino resultou em uma redução na excreção de N urinário (13,5%), na concentração sanguínea de ureia (5%) e na emissão de metano (13%) quando comparado com os animais que não receberam o tanino (CARULLA et al, 2005). Além disso, com níveis de 1,6% do CMS houve um aumento na oferta de proteína metabolizável (PM) (ALVES, 2012) e níveis acima de 2% a oferta de PM não foi alterada, mas esse nível de suplementação causou um impacto negativo no consumo de energia em cordeiros (KOZLOSKI et al, 2012). Também foi observado uma redução na emissão de metano (14 e 29%) e na excreção de nitrogênio urinário (22 e 26%) em vacas leiteiras suplementadas com 0,9 e 1,5% do CMS, entretanto o baixo teor de PB da dieta (15%) fornecido as vacas no início da lactação resultou na redução na produção de leite e de seus componentes (proteína e gordura) (GRAINGER et al, 2009). Os resultados desses estudos demonstram que a suplementação com esse extrato tanífero em até 1,5% do CMS pode reduzir a degradabilidade ruminal das proteínas, porém o impacto dessa redução na produção, composição química, perfil de ácidos graxos do leite e na concentração de N fecal de ruminantes em pastejo com dietas altamente proteicas deve ser investigado.

### 1.1.2 Efeito dos taninos na lipólise

Devido a preocupação dos consumidores com a qualidade dos produtos de origem animal e a crescente demanda por produtos mais saudáveis, pesquisadores e nutricionistas auxiliam na produção de produtos mais seguros e saudáveis. Por isso, nos últimos anos, algumas pesquisas se concentraram na composição dos ácidos graxos presentes nos produtos de origem animal e seus efeitos na saúde humana, especialmente os ácidos graxos polinsaturados (AGP) e o ácido linolênico conjugado (CLA; LOCK e BAUMAN, 2004). Alguns trabalhos observaram que o CLA *cis*-9, *trans*-11 (C18:2) tem efeito na prevenção do câncer (IP et al, 1991) e da aterosclerose em cobaias (BELURY, 2002).

Durante o processo de biohidrogenação (BH) do C18:2 e C18:3 presentes na dieta de ruminantes, isômeros de C18:1 e C18:2 são formados e na última etapa da BH ocorre a formação de C18:0. O isômero CLA *cis*-9, *trans*-11 pode ser formado no rúmen durante a BH, mas é produzido em maior extensão no tecido adiposo e glândula mamária a partir da desaturação do C18:1 *trans*-11 (intermediário da BH) através da ação da enzima delta-9 desaturase. A BH ruminal é realizada principalmente pelas bactérias pertencentes ao gênero *Butyrivibrio*. A bactéria *Butyrivibrio fibrisolvens* tem a capacidade de converter C18:2 *cis*-9, *cis*-12 (ácido linoleico) em C18:2 *cis*-9, *trans*-11 (ácido rumênico) e o ácido rumênico em C18:1 *trans*-11 (ácido vacênico), enquanto a *Butyrivibrio proteoclasticus* (anteriormente classificada como *Clostridium proteoclasticum*) converte C18:1 *trans*-11 em C18:0 (ácido esteárico).

Os taninos tem propriedades antibacterianas e são capazes de interferir na BH ruminal (MCSWEENEY et al., 2001; MUELLER-HARVEY, 2006; VASTA et al., 2009[a]). Algumas pesquisas observaram que o tanino condensado de diferentes leguminosas forrageiras inibem o crescimento

celular e divisão dos microrganismos ruminais, incluindo a *Butyrivibrio fibrisolvens* (JONES et al., 1994; MOLAN et al., 2001) e *B. Proteoclasticus* (MIN et al, 2002b). Como consequência, Khiaosa-Ard et al. (2009) observou uma inibição na última etapa da BH e um acúmulo de ácido vacênico com a suplementação de *Acacia mearnsii* à 7,89% da MS. Quando extratos de *Acacia iteaphylla* (tanino condensado) foram incubados *in vitro* também houve um aumento do ácido vacênico e redução do ácido esteárico (Durmic et al., 2008). Uma redução na BH de C18:3 também foi observado com o tanino proveniente de *Schinopsis* spp. (KRONBERG et al., 2007). Outro estudo *in vitro* utilizando 2 fontes de tanino (castanha portuguesa-tanino hidrolisado ou *Schinopsis balansae*-tanino condensado) em doses de 49 e 82 g/kg MS verificou que os teores dos principais ácidos graxos insaturados das bactérias do líquido ruminal foram afetados pela presença de taninos nas dietas, principalmente o teor de C18:1 *trans*-11, o qual foi significativamente maior, especialmente com o tanino hidrolisado à 49 g/kg MS (BUCCIONI et al, 2011). Vasta et al (2009[a]) estudaram o efeito *in vitro* dos taninos de *Ceratonia siliqua*, *Acacia cyanophylla* e *Schinopsis lorentzii* em 3 concentrações (0, 0,6 e 1,0 mg/ml fluido ruminal de vaca) na BH ruminal e verificaram maior concentração de C18:1 (23%), mas a concentração total de isômeros CLA não foi afetada. Desse modo, as pesquisas *in vitro* mostram que os taninos parecem ser um bom meio para modificar a BH dos lipídios, favorecendo o acúmulo de ácido vacênico e AGP durante a fermentação ruminal.

Com o intuito de verificar os resultados dos experimentos *in vitro*, alguns experimentos *in vivo* foram realizados. Em ovelhas suplementadas com tanino (*Schinopsis* spp.-4% da MS do concentrado) foi observado uma redução na concentração de ácido esteárico (-49%) e um aumento na concentração de ácido vacênico (+97%) no fluido ruminal, o que reduziu as concentrações de AGS no sangue e na carne e

aumentou a concentração de AGP e de ácido rumênico (100%) na carne (VASTA et al, 2009b). O aumento do ácido vacênico no rúmen também foi observado em ovelhas recebendo um concentrado com 6,4% de tanino (*Schinopsis* spp.-9,57% da MS) (VASTA et al, 2010). Nesse mesmo trabalho, a população ruminal de *B. Proteoclasticus* foi menor (30,6%; P<0.1), e a de *B. Fibrisolvens* e protozoários foram maiores (107% e 56,1%, respectivamente; P<0.05) em cordeiros suplementados com tanino do que a dieta controle. Buccioni et al. (2015) utilizando uma mistura de taninos (castanha e *Shinopsis* spp) nas concentrações de 53 g/kg CMS observou um leve aumento na concentração de C18:2, C18:1 e CLA *cis*-9, *trans*-11 e uma redução de ácido esteárico e AGS. DSCHAAK et al. (2011) observaram maiores concentrações totais de C18:1 *trans* e C18:3 com a suplementação com tanino. (3% de tanino condensado de *Shinopsis* spp.).

Esses resultados *in vivo* sugerem que o tanino pode ser útil no acúmulo de ácido vacênico no rúmen através da mudança da população ruminal e favorecer a síntese endógena de ácido rumênico. Porém, esses efeitos não foram observados por Toral et al (2011) que utilizaram ovelhas suplementadas com óleo de girassol e um extrato comercial contendo tanino condensado e hidrolisado (1% da dieta). Nesse experimento, a adição de taninos não afetou a fermentação ruminal, desempenho animal e não teve impacto no perfil de AG. Em vacas suplementadas com 150 g/d de tanino condensando (*Schinopsis balansae*-70% de taninos; 0,45% da CMS) o perfil de AG do leite não foi afetado, porém os resultados revelaram um potencial efeito do tanino em alterar a BH ruminal com a dosagem utilizada (BENCHAAR e CHOUINARD, 2009). Em ovelhas leiteiras suplementadas com uma mistura de extrato tanífero de castanha e *Shinopsis* spp (10 g/kg CMS; TORAL et al., 2011) ou somente *Shinopsis* spp (20 g/kg CMS; TORAL et al., 2013) não houve alteração nas concentrações de ácidos

graxos no leite no sentido de um perfil potencialmente mais saudável.

Alguns experimentos *in vitro* sugeriram que a suplementação com esses compostos fenólicos podem modificar a BH dos AGP presentes na dieta animal e promover o acúmulo de ácido vacênico através da inibição na última etapa da BH (BUCCIONI et al., 2011; KHIAOSA-ARD et al., 2009; VASTA et al, 2009[a]). No entanto, outros trabalhos relataram uma inibição geral na BH ao invés de uma inibição específica na conversão de ácido vacênico à ácido esteárico (KRONBERG et al., 2007; MINIERI et al., 2014). Esse efeito benéfico raramente tem sido observado *in vivo* (VASTA et al., 2009b; KHIAOSA-ARD et al., 2011) e muitos experimentos sugerem efeitos positivos (VASTA et al, 2009b; 2010) ou não significativos (TORAL et al, 2011; BENCHAAR e CHOUINARD, 2009). Devido a grande variação nas características estruturais e reatividade de diferentes taninos (ÁLVAREZ DEL PINO et al., 2005; MUELLER-HARVEY, 2006), todos esses resultados inconsistentes possivelmente estão relacionados ao tipo e/ou concentração dos taninos utilizados. Atualmente, há uma falta de informações sobre o efeito *in vivo* do extrato tanífero provenientes da *Acacia meanrsii* no perfil de ácidos graxos no leite de ruminantes.

### 1.1.3 Efeito dos taninos no consumo de alimento

O efeito dos taninos condensados no CMS em ruminantes são muito variáveis. Alguns trabalhos relataram uma redução no CMS em vacas (DSCHAAK et al., 2011 com 3% de taninos condensado de *Shinopsis* spp.; MCNABB et al., 1996 com 5,5% de tanino condensado de *Lotus pedunculats*; PRIOLO et al., 2000 com 2,5% de tanino condensado de polpa de alfarroba) e em ovelhas (BARRY AND MCNABB, 1999 com 7,5 a 10% de tanino condensado de *Lotus pedunculatus*) enquanto outros trabalhos observaram aumento no CMS

(CARULLA et al., 2005 com 2,5% de tanino condensado de *Acacia mearnsii*) ou não observaram efeito no CMS (BAAH et al., 2007 com 0,6% de tanino condensado de *Schinopsis balansae*; BENCHAAR et al., 2008 com 0,45% de tanino condensado de *Schinopsis balansae*). De acordo com alguns trabalhos, a suplementação com taninos condensados pode reduzir o CMS devido a redução na digestibilidade ruminal da fibra (PRIOLO et al., 2000), do carboidrato (BARRY e MCNABB, 1999), da proteína (MCNABB et al., 1996) ou da redução da atividade microbiana (LANDAU et al, 2000). Os taninos também podem se ligar às proteínas salivares ou diretamente nos receptores gustativos e produzir uma sensação de adstringência quando ingerido e inibir o consumo do alimento (DSCHAAK et al., 2011; LANDAU et al., 2000). Esses resultados sugerem que a suplementação com altas concentrações de taninos condensados tem efeitos negativos no CMS de ruminantes e esses efeitos podem variar com a fonte de tanino utilizada.

## 1.2 HIPÓTESES

De acordo com os efeitos positivos na partição nitrogenada com a suplementação com *Acacia meanrsii* observados em trabalhos prévios e aos efeitos contraditórios na BH *in vitro* e *in vivo* entre diversos tipos de tanino, as hipóteses deste estudo são:

(i) A suplementação com o extrato tanífero de *Acacia mearnsii* na dieta de bovinos e ovinos em lactação melhora a eficiência de uso do nitrogênio alimentar com redução na excreção urinária de nitrogênio, aumento na concentração fecal de nitrogênio e nos teores ou produção de proteína no leite.

(ii) A suplementação com o extrato tanífero de *Acacia mearnsii* aumenta as concentrações de ácidos graxos

poliinsaturados, C18:1 *trans*-11 e CLA *cis*-9, *trans*-11 no leite de bovinos e ovinos.

## 1.3 OBJETIVOS

### 1.3.1 Objetivos Gerais

O presente trabalho teve como objetivo avaliar o efeito da suplementação com o extrato tanífero de *Acacia mearnsii* na produção, composição química, perfil de ácidos graxos do leite, no consumo de matéria seca e na excreção de N de bovinos e ovinos em pastagens temperadas.

### 1.3.2 Objetivos Específicos

(i) Avaliar a produção de leite, os teores de proteína, caseína, uréia, gordura e perfil de ácidos graxos no leite de bovinos e ovinos suplementados com extrato tanífero de *Acacia mearnsii*;

(ii) Avaliar o consumo de matéria seca, excreção urinária de N, concentração fecal de N, composição química e morfológica dos alimentos consumidos por bovinos e ovinos em pastejo suplementados com extrato tanífero de *Acacia mearnsii*;

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# 3 CAN SUPPLEMENTATION WITH BLACK WATTLE TANNIN EXTRACT MITIGATE N UREA EXCRETION WITHOUT REDUCTIONS ON DAIRY EWES PERFORMANCE?

## 3.1 ABSTRACT

Condensed tannins can reduce ruminal degradability of proteins and improve nitrogen use efficiency. The aim of this work was to investigate the effect of tannin extract supplementation on N excretion, milk yield and milk composition in dairy ewes grazing a pasture with high protein content. The tannin was extracted from *Acacia mearnsii* (black wattle). Two experiments tested four levels of black wattle in twenty-four lactating ewes grazing a white clover (*Trifolium repens*) predominant pasture and supplemented with fresh corn silage (1 kg/day) and a concentrate feed (600 g/day). The first experiment tested two levels of black wattle (control and 20 g of tannin extract/kg of concentrate) and the second experiment tested three level (control, 30 and 40 g of tannin extract/kg of concentrate). Total DMI, milk yield, milk composition, blood urea nitrogen and N urinary excretion were similar between treatments in both experiments, except N fecal content of T30 and T40 were around 9% higher than treatment control (P=0.053). The greater fecal N content when the proportion of tannin extract was ≥9 g/kg of total DMI suggest a diversion of N from urine to feces, showing a strong impact of tannin extract supplementation mixed with concentrate on N fecal excretion in N-rich diets. Black wattle supplementation levels between 9 and 14 g/kg of total DMI showed a potential to increase N fecal excretion without a negative effect on milk yield and milk composition of ewes.

**Keywords:** Lacaune, fecal nitrogen, *Acacia mearnsii*, black wattle, milk composition, nitrogen excretion.

## 3.2 INTRODUCTION

Diets based on temperate grass or legume pastures are rich in proteins readily soluble in rumen, which result in low nitrogen use efficiency (NE) due to ruminal nitrogen (N) loss. Improving NE of dairy ewes, by supplementation with a source of ruminal undegradable protein (RUP), helps to maximize profitability and minimize N losses on farm. The ruminal N absorbed as ammonia is transformed to urea and excreted as urea N in milk and urine. Most of N urinary is quickly hidrolisate to ammonia (Powell et al., 2011) or transformed to nitrate, which can contaminate water or contribute to greenhouse (Monaghan et al., 2007). On the other hand, the N excreted in feces is relatively stable because is slower degraded than N urinary (Rotz, 2004). Additionally, it has been shown that supplementation with RUP to dairy ewes may increase milk protein content (Purroy and Jaime, 1995) and milk yield (Penning et al., 1988).

The use of alternatives that reduce ruminal degradability of proteins, such as condenseded tannins, can improve NE by increasing duodenal flow of metabolisable protein (MP), improving animal performance and decreasing environmental impact (Waghorn and McNabb, 2003). In the specific case of black wattle (*Acacia mearnsii*) tannin extract, supplementation levels up to 2.5% of total DM intake (DMI) increase duodenal flow of MP in steers (Alves, 2012) and decrease N urinary excretion, blood urea N content and methane emissions in lambs (Carulla et al., 2005). However, supplementation levels higher than 2% of DMI can also reduce OM digestibility and metabolizable energy intake (Kozloski et al., 2012), with reductions on animal performance when the crude protein (CP) content of pasture was near to 160 g kg $DM^{-1}$ (Grainger et al., 2009).

According to these results the aim of this study was to investigate the effect of supplementation, up to 1.5% of DMI,

with black wattle tannin extract on N excretion, milk yield and milk composition in dairy ewes grazing a pasture with high protein content.

## 3.3 MATERIAL AND METHODS

This study was undertaken in accordance with Ethics Committee for Animal Experimentation (CETEA; protocol number: 01.19.14), from Agroveterinary Science Center (CAV) at the Santa Catarina State University (UDESC), according to current legislation and ethical principles published by the Brazilian College of Animal Experimentation (COBEA).

### 3.3.1 Local, treatments and experimental design

The study was performed at a commercial farm in Bom Retiro city (Santa Catarina state, Brazil; 27°45'34S, 49°38'25.3"W, 890 m of altitude). The experiment was conducted from January to May 2013. The clime is Cfb (humid subtropical) with mean temperature and annual rainfall of 19ºC and 1400 mm, respectively. Two experiments were conducted with lactating ewes grazing a white clover (*Trifolium repens*) predominant pasture and supplemented with corn silage and concentrated feed. In the first experiment two levels of black wattle tannin extract were tested: zero (control) and 20 g $kg^{-1}$ of concentrate (T20). The tannin extract was mixed with the concentrate to be equivalent to 0.8% of DMI, which was estimated by the relationship between energetic requirements of animals and energetic value of concentrate feed, corn silage and pasture, using equations proposed by INRA (2007). In the second experiment three levels of tannin extracted from black wattle were tested: zero (control), 30 (T30) e 40 (T40) g $kg^{-1}$ of concentrate feed. The tannin extract was mixed with the concentrate to be equivalent to 1.2 and 1.5% of total DMI. In

both experiments treatments were compared according to a completely randomized design repeated twice in the time. Each experimental period lasted 21 days, with the last five days for measurements.

### 3.3.2 Animals

Twenty-four lactating ewes were used in each experiment. In the first experiment 12 Lacaune and 12 Milchschaf ewes were selected and separated into two homogeneous groups according to racial group (Lacaune × Milchschaf), milk production (1.3 ± 0.3 kg $day^{-1}$), lactation stage (82.6 ± 8.3 days), parity (2.4 ± 0.5) and live weight (62.8 ± 6 kg). In the second experiment, twenty-four Lacaune ewes were selected and separated into three homogeneous groups according to milk production (1.3 ± 0.4 kg $day^{-1}$), lactation stage (78.6 ± 9.8 days), parity (2.5 ± 0.6) and live weight (52.4 ± 5.3 kg).

### 3.3.3 Feed and grazing management

Each ewe received 300 g of a concentrate feed after each milking and 1 kg of fresh corn silage after afternoon milking. The tannin extract was mixed with the concentrate, according supplementation levels of each treatment. The concentrate composition was: 470 g kg of corn $ground^{-1}$, 200 g kg of soybean $meal^{-1}$, 280 g kg of soybean $hulls^{-1}$ and 50 g kg of mineral $mixed^{-1}$. Silage and concentrate supplementation was balanced to avoid any ruminal energy restriction (Table 1; INRA, 2007).

Table 1. Chemical composition and energetic value of supplements (corn silage and concentrate).

| Item | Supplement | |
|---|---|---|
| | Corn Silage | Concentrate[1] |
| **First experiment** | | |
| Dry matter (g/kg) | 341 | 902 |
| Chemical composition (g/kg DM) | | |
| Organic matter | 956 | 916 |
| Crude protein | 108 | 199 |
| Neutral detergent fiber | 443 | 357 |
| Acid detergent fiber | 213 | 148 |
| Lignin | 139 | 41 |
| Crude fat | 164 | 151 |
| Energetic value | | |
| $NE_L$ (MJ/kg DM)[2] | 7.4 | 7.6 |
| **Second experiment** | | |
| Dry matter (g/kg) | 337 | 900 |
| Chemical composition (g/kg DM) | | |
| Organic matter | 963 | 920 |
| Crude protein | 79 | 219 |
| Neutral detergent fiber | 410 | 337 |
| Acid detergent fiber | 205 | 144 |
| Lignin | 60 | 39 |
| Crude fat | 386 | 178 |
| Energetic value | | |
| $NE_L$ (MJ/kg DM)[2] | 8.2 | 7.7 |

[1]470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed.
[2]Net energy for lactation estimated according to Weiss et al. (1992).
Fonte: produção do próprio autor, 2016.

White clover predominant pastures, were managed under rotational grazing. In both experiments the ewes had access to pasture during 8 h $day^{-1}$, between morning and afternoon milking. After afternoon milking ewes were closed to protect them against natural predators. Pastures had similar pre-grazing sward height and the target to change to a new paddock was a grazing down level of 50% of pre-grazing sward pastures heights. During measurements periods (5 days)

each experimental group had access to separated paddocks, however both groups were allocated to a same paddock between measurements periods (16 days).

### 3.3.4 Feed and sward measurements

Daily samples of supplements were collected in the last five days of each experimental period and the weight of supplement refusals was recorded at each milking. The pre and post-grazing pasture height were measured with a rising plate meter (100 measures; Farmworks®, F200 model, New Zealand) and the pre-grazing pasture mass was estimated from a relationship between pre-grazing sward height and the pasture mass at ground level into six representatives areas of 0.1 m2 in each period. Pre- and post-grazing, twenty handfuls (approximately 500 g fresh) of each paddock, were randomly cut at ground level and used for botanical classification (legumes, fescue, other species and dead tissues). The pasture samples for chemical composition was collected on first, third and fifth day of sampling period, by hand plucked technique. All feed samples were oven-dried at 60°C for at least 72 h and only feed for chemical analyses was ground to pass a 1 mm diameter sieve.

### 3.3.5 Animal measurements

Ewes were milked twice a day (0600 and 1530 h) and individual milk yield was recorded daily during two days before starting each experiment (day zero) and on sampling period. The milk fat, protein and milk urea N concentrations were measured every other day during sampling period (on days 17, 19 and 21th) by infrared spectrophotometry (International IDF Standard 141C:2000). The live weight was measured once a week after morning milking. Fecal samples were individually collected after each milking, during all days

of second sampling period and a homogeneous fecal sample per ewe was obtained for N analyses.

The pasture intake was estimated by the relationship between metabolic energy (ME) requirements (for lactation and maintenance) and ME supplied by supplements and pasture. The ME for maintenance (Mem) was calculated from live weight and ME for lactation (Mel) was calculated from milk production and milk composition, according to INRA (2007). The energetic value of pasture, corn silage and concentrate were estimated as proposed by Weiss et al (1992). The pasture intake was calculated as a difference between ME requirements and ME consumption from supplements, as proposed by Baker (2004).

Blood samples were collected from each ewe after morning milking at last day of each experimental period from jugular vein using 5 mL Vacuntainer tubes without EDTA. Blood samples were immediately placed on ice before centrifugation (3500 x g, 20 minutes, and -4ºC) and plasma obtained was stored at -80ºC for non-esterified FA (NEFA) and blood urea N analysis.

### 3.3.6 Chemical Analyses

Dry matter concentration was determined by drying at 105°C for 24 hours. The ash content was determined by combustion in a muffle furnace at 550°C for 4 hours, and the organic matter (OM) content was determined by mass difference. The total N was determined using the Kjeldahl method (Method 984.13; AOAC 1997). Neutral detergent fiber (NDF) analyses was performed according to Mertens (2002), except that the samples were weighed into filter bags and treated with neutral detergent in ANKOM equipment (ANKOM Technology, Macedon NY, USA). This analysis included alpha-amylase but did not include sodium sulphite. The concentration of acid detergent fiber (ADF) and sulphuric

acid detergent lignin (ADL) were analyzed according to Method 973.18 of AOAC (AOAC 1997). The ether extract (EE) was determined in a reflux system with ethyl ether at 180º C over 4 h (Oil and fat extractor MA491, Marconi, Brazil).

NEFA analyses were performed using commercial kits (Wako NEFA-HR, Wako Chemicals EUA ®, Richmond, EUA) according to Ballou et al (2009). As blood urea N is positively related with urea urinary N (Kohn et al., 2002), N urinary excretion was estimated according to Kohn (2007), where: N urinary excretion (g/d) = 0.013 × live weight (kg) × BUN (mg $dL^{-1}$).

### 3.3.7 Statistical Analyses

*Akaike's* Information Criterion was used to choose the variance-covariance matrix (Wolfinger et al., 1993). Variables were analyzed using period as repeated measurements and taking into account the random effect of animal and fixed effect of treatments. Analyses were performed using the PROC MIXED of SAS (Statistical Analysis System – Littell et al., 1998) and the results of day zero were considered covariates in statistical model. Day zero was the results from the day of animals selection, before start the experiment and the new diet.

In the first experiment, the differences between means were determined by the probability of difference (PDIFF) method using Student t-test at a 5% significance level and the means were estimated with the LSMEANS procedure. In the second experiment the linear and quadratic effects of supplementation level were tested using polynomial orthogonal contrasts, in which the quadratic component was equivalent to the lack of fit sum of squares for linearity. Each F value was a ratio of the contrast mean square to the residual (experimental error) mean square.

## 3.4 RESULTS

### 3.4.1 Pasture characteristics

The pre-grazing sward characteristics were similar between treatments on experiment 1 (Table 2). Pre-grazing herbage mass and pre-grazing sward height averaged 4720 kg $ha^{-1}$ and 13 cm, respectively. The CP and NDF content were 300 and 456 g $kg^{-1}$, respectively. The energetic value of pasture was 6.7 MJ NEL kg $DM^{-1}$ and the proportion of legumes averaged 65% of total DM. The grazing down level was on average 43% of pre-grazing sward height.

Table 2. Pre and post-grazing pasture characteristics, morphological and chemical composition of white clover dominant pasture grazed by dairy ewes supplemented with corn silage and concentrate with or without inclusion of black wattle tannin extract (First experiment).

| Item | Treatments[1] | | SEM | |
|---|---|---|---|---|
| | C | T20 | (n=4) | *P*-value |
| Pre-grazing pasture mass (kg DM/ha) | 4998 | 4439 | 191.9 | 0.109 |
| Pre-grazing sward height (cm) | 12.8 | 13.2 | 0.90 | 0.854 |
| Post-grazing sward height (cm) | 7.2 | 7.5 | 1.21 | 0.856 |
| Chemical composition (g/kg DM)[2]: | | | | |
| Dry matter | 167 | 168 | 11.4 | 0.953 |
| Organic matter | 917 | 918 | 4.7 | 0.888 |
| Crude protein | 289 | 306 | 6.2 | 0.117 |
| Neutral detergent fiber | 462 | 449 | 33.9 | 0.799 |
| Acid detergent fiber | 222 | 220 | 11.1 | 0.905 |
| Lignin | 164 | 214 | 89 | 0.016 |
| Morphologic composition (% of DM): | | | | |
| Leguminous | 61.0 | 70.5 | 6.20 | 0.343 |
| Fescue | 14.0 | 13.0 | 2.91 | 0.820 |
| Others sp. | 14.5 | 7.5 | 6.25 | 0.473 |
| Dead material | 10.5 | 9.0 | 1.35 | 0.475 |
| Nutritive value | | | | |
| $NE_L$, MJ/kg DM[3] | 7.1 | 7.1 | 0.11 | 0.962 |

[1]Treatments: C= control (without supplementation with tanniferous extract); T20= supplemented with tanniferous extract as a proportion of 20 g/kg of concentrate.
[2]Sample collected by hand plucked method.
[3]Net energy for lactation estimated according to Weiss et al. (1992).
Fonte: produção do próprio autor, 2016.

In the second experiment, there was a quadratic effect of pre-grazing herbage mass, which was 16.2% higher in the treatment control when compared with the other treatments (Table 3). There was a positive linear effect of post-grazing sward height and a negative linear effect of OM, CP and proportion os leguminous. The grazing down levels were always lower than 50% for all treatments. In the same way, CP content was higher than 260 g kg $DM^{-1}$ and NDF content lower than 500 g kg $DM^{-1}$, independently of treatment.

Table 3. Pre-grazing pasture characteristics and post-grazing sward height of white clover dominant pasture grazed by dairy ewes receiving different levels of black wattle tannin extract (Second experiment).

| Item | Treatments[1] | | | SEM | *P*-value | |
|---|---|---|---|---|---|---|
| | C | T30 | T40 | (n=6) | Linear | Quad |
| Pre-grazing pasture mass (kg DM/ha) | 3943 | 3377 | 3226 | 37.6 | 0.005 | 0.046 |
| Pre-grazing sward height (cm) | 9.0 | 8.3 | 9.2 | 1.22 | 0.911 | 0.622 |
| Post-grazing sward height (cm) | 4.6 | 5.0 | 5.6 | 0.22 | 0.015 | 0.698 |
| Chemical composition (g/kg DM): | | | | | | |
| Dry matter | 169 | 174 | 181 | 2.9 | 0.092 | 0.916 |
| Organic matter | 921 | 919 | 916 | 0.5 | 0.019 | 0.244 |
| Crude protein | 316 | 269 | 263 | 8.6 | 0.049 | 0.194 |
| Neutral detergent fiber | 362 | 445 | 488 | 2.1 | 0.054 | 0.533 |
| Acid detergent fiber | 193 | 239 | 226 | 1.4 | 0.238 | 0.235 |
| Lignin | 106 | 122 | 159 | 1.59 | 0.140 | 0.651 |

Table 3. Pre-grazing pasture characteristics and post-grazing sward height of white clover dominant pasture grazed by dairy ewes receiving different levels of black wattle tannin extract (Second experiment).

| Item | Treatments[1] | | | SEM | *P*-value | |
|---|---|---|---|---|---|---|
| | C | T30 | T40 | (n=6) | Linear | Quad |
| Morphologic Composition (%): | | | | | | |
| Leguminous | 65 | 61.5 | 51 | 1.88 | 0.034 | 0.267 |
| Fescue | 10.7 | 15.2 | 30.0 | 5.22 | 0.120 | 0.504 |
| Others sp. | 4.1 | 9.3 | 5.6 | 1.06 | 0.414 | 0.074 |
| Dead Material | 20.2 | 13.9 | 13.3 | 2.71 | 0.214 | 0.482 |
| *Energetic value* | | | | | | |
| $NE_L$, MJ/kg DM[2] | 7.0 | 6.8 | 6.6 | 0.08 | 0.062 | 0.691 |

[1]Treatments: C= control (without supplementation with taniferous extract); T30 = supplemented with a taniferous extract as a proportion of 30 g/kg of concentrate; T40 = supplemented with a taniferous extract as a proportion of 40 g/kg of concentrate.
[2]Net energy for lactation estimated according to Weiss et al. (1992).
Fonte: produção do próprio autor, 2016.

### 3.4.2 Animal performance and N excretion

Milk yield, milk composition, NEFA, blood urea N, N urinary excretion and N fecal content were similar between treatments in the first experiment (Table 4). The proportion of taniferous extract on DMI was around 0.7% of total DMI.

Table 4. Effect of taniferous extract supplementation on DM intake, milk production and composition blood parameters of dairy ewes grazing white clover dominant pasture and supplemented with corn silage + concentrate feed (First experiment).

| Parameter | Treatments[1] | | SEM | |
|---|---|---|---|---|
| | CL | T20 | (n=24) | *P*-value |
| DM intake (kg/ewe/day): | | | | |
| Pasture | 0.63 | 0.70 | 0.06 | 0.349 |
| Corn silage | 0.34 | 0.34 | 0.002 | 0.694 |
| Concentrate | 0.54 | 0.53 | 0.004 | 0.596 |
| Total | 1.51 | 1.58 | 0.06 | 0.415 |
| Crude protein (g/ewe/day) | 326 | 359 | 17.15 | 0.186 |
| Tannin extract (% DMI) | 0 | 0.7 | - | - |
| Milk yield (kg/day) | 1.29 | 1.23 | 0.11 | 0.262 |
| 6.5% FCM yield (kg/day) | 1.29 | 1.24 | 0.07 | 0.498 |
| Milk fat concentration (%) | 6.53 | 6.74 | 0.37 | 0.453 |
| Milk protein concentration (%) | 5.37 | 5.46 | 0.13 | 0.512 |
| Milk urea concentration (mg/dL) | 26.6 | 26.2 | 2.08 | 0.697 |
| Milk fat yield (g/day) | 83.6 | 81.5 | 4.73 | 0.679 |
| Milk protein yield (g/day) | 69.7 | 66.8 | 4.27 | 0.406 |
| Live weight (kg) | 60.4 | 63.8 | 1.81 | 0.176 |
| NEFA[2] (mg/dL) | 5.73 | 6.32 | 0.38 | 0.296 |
| $NE_l$ balance[3] | 0.97 | 0.97 | 0.002 | 0.940 |
| BUN[4] (mg/dL) | 49.2 | 49.1 | 1.21 | 0.968 |
| NUE (g/d)[5] | 41.4 | 43.7 | 1.76 | 0.358 |
| N fecal (%) | 3.14 | 3.03 | 0.05 | 0.246 |

[1]Treatments: C= control (without supplementation with taniferous extract); T20 = supplemented with a taniferous extract as a proportion of 20 g/kg of concentrate.
[2]Non esterified FA.
[3]Calculed as: $NE_l$ total intake ($NE_m + NE_l$)
[4]Blood Urea Nitrogen.

[5]N Urinary excretion, estimated by: 0.0259 × LW (kg) × MUN (mg $dL^{-1}$), where: LW = live weight, (Kauffman and St-Pierre, 2001).
Fonte: produção do próprio autor, 2016.

In the second experiment, the total DMI, milk yield, milk composition, blood urea N and N urinary excretion were similar between treatments (Table 5). There was a quadratic effect of corn silage, concentrate intake and NEFA content with tannin supplementation. The corn silage and concentrate intake was lower, whereas NEFA blood concentration increased in ewes of T30 compared with other treatments. The proportion of taniferous extract was around 1.0 and 1.6% of total DMI in the treatments T30 and T40, respectively. The N urinary excretion was similar between treatments, but N fecal content was around 9% higher in treatments T30 and T40 when compared with treatment control.

Table 5. Effect of taniferous extract supplementation on DM intake, milk production and composition blood parameters of dairy ewes grazing white clover dominant pasture and supplemented with corn silage + concentrate feed (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| Parameter | C | T30 | T40 | SEM | Linear | Quad |
| DM intake (kg/day): | | | | | | |
| Pasture | 0.54 | 0.64 | 0.58 | 0.08 | 0.755 | 0.440 |
| Corn silage | 0.30 | 0.24 | 0.29 | 0.01 | 0.651 | <0.001 |
| Concentrate | 0.52 | 0.38 | 0.47 | 0.02 | 0.153 | <0.001 |
| Total | 1.35 | 1.26 | 1.34 | 0.09 | 0.948 | 0.407 |
| Crude protein (g/ewe/day) | 307 | 279 | 282 | 27.04 | 0.398 | 0.537 |
| Tannin extract (%DMI) | 0 | 1.0 | 1.6 | - | - | - |
| Milk yield (kg/day) | 1.27 | 1.06 | 1.20 | 0.08 | 0.531 | 0.075 |
| 6.5% FCM yield (kg/day) | 1.21 | 1.03 | 1.12 | 0.08 | 0.402 | 0.176 |
| Milk fat concentration (%) | 5.93 | 5.83 | 5.75 | 0.27 | 0.658 | 0.970 |

Table 5. Effect of taniferous extract supplementation on DM intake, milk production and composition blood parameters of dairy ewes grazing white clover dominant pasture and supplemented with corn silage + concentrate feed (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| Parameter | C | T30 | T40 | SEM | Linear | Quad |
| Milk protein concentration (%) | 5.27 | 5.21 | 5.12 | 0.16 | 0.539 | 0.955 |
| Milk urea concentration (mg/dL) | 29.2 | 26.9 | 26.5 | 2.04 | 0.358 | 0.715 |
| Milk fat yield (g/day) | 76.2 | 65.1 | 68.8 | 5.53 | 0.354 | 0.292 |
| Milk protein yield (g/day) | 63.6 | 56.1 | 60.9 | 4.43 | 0.678 | 0.266 |
| Live weight (kg) | 53.8 | 53.1 | 53.5 | 0.56 | 0.747 | 0.485 |
| NEFA[2] (mg/dL) | 5.87 | 7.01 | 5.19 | 0.32 | 0.337 | 0.020 |
| $NE_l$ balance | 0.97 | 0.97 | 0.97 | 0.002 | 0.790 | 0.562 |
| BUN[3] (mg/dL) | 48.1 | 53.6 | 46.4 | 2.10 | 0.707 | 0.108 |
| NUE[4] (g/d) | 40.3 | 38.4 | 37.0 | 2.52 | 0.350 | 0.938 |
| N fecal (%) | 2.83 | 3.14 | 3.04 | 0.05 | 0.092 | 0.053 |

[1]Treatments: C= control (without supplementation with taniferous extract); T30 = supplemented with a taniferous extract as a proportion of 30 g/kg of concentrate; T40 = supplemented with a taniferous extract as a proportion of 40 g/kg of concentrate.
[2]Non esterified FA.
[3]Calculed as: $NE_l$ total intake ($NE_m + NE_l$)
[4]Blood Urea Nitrogen.
[5]N Urinary excretion, estimated by: 0.0259 × LW (kg) × MUN (mg $dL^{-1}$), where: LW = live weight, (Kauffman and St-Pierre, 2001).
Fonte: produção do próprio autor, 2016.

## 3.5 DISCUSSION

### 3.5.1 Effect of tannin extract supplementation on N excretion

The greater N fecal content of second experiment suggest a diversion of N from urine to feces and consequently lower

contribution to greenhouse effect only when the proportion of tannin extract was ≥10 g kg of total $DMI^{-1}$. This result could be a consequence of a reduction on N digestibility, because reductions on N digestibility have been observed when the proportion of tannin extract supplementation was higher than 10 g kg of total $DMI^{-1}$ (Grainger et al., 2009; Kozloski et al., 2012). The impact of tannin extract supplementation on increase of N fecal excretion is related to supplementation level, diet CP content and supplementation method. Griffiths et al. (2009) observed that N fecal excretion increased only 4.5% when black wattle tannin extract was offered as a proportion of 16 g kg of total $DMI^{-1}$ in diets with a CP content of 240 g kg $DM^{-1}$. Similar supplementation levels (11 g kg of total $DMI^{-1}$) in diets with lower CP content (160 g kg $DM^{-1}$) increased N fecal excretion by 19% (Grainger et al. 2009). However, when Griffiths et al. (2009) tested similar proportions of black wattle tannin extract (12 g kg of total $DMI^{-1}$) mixed with pellets, there was no difference in milk yield or milk urea nitrogen (MUN) as when tannin extract was offered as a drench by the same authors and also by Grainger et al (2009). In our study the CP content of diets was always higher than 260 g kg $DM^{-1}$ and the N fecal content increased 9.2% when the tannin extract supplementation was ≥10 g kg $DMI^{-1}$, showing a strong impact of tannin extract supplementation mixed with concentrate on N fecal excretion in N rich diets.

The similarity of blood urea N, N urinary excretion and MUN content between treatments in both experiments may be, at least partially, explained by expected milk yield of ewes and the CP content of diets higher than 250 g kg $DM^{-1}$. In the current study milk yield averaged 1.3 kg $day^{-1}$ in the beginning of two experiments, which may be considered a medium level of production. On the other hand, has been shown that blood urea N concentration of dairy ewes increased linearly when CP content of diets increase from 140 to 212 g kg $DM^{-1}$ (Cannas et al., 1998). In this situation, probably the metabolisable protein

(MP) from pasture already exceeds the amino acids requirements of animals and these components are deaminated and excreted through urine and milk as urea (Reynolds and Kristensen, 2008). Thus, in the present study *Acacia mearnsii* tannin extract supplementation probably increased MP, as showed by other authors (Alves, 2012), reducing N digestibility of concentrate, which was excreted in feces.

### 3.5.2 Effect of tannin extract supplementation on animal performance

The lack of response on milk production and composition may be also explained by the relationship between supply and requirement of MP and energy of animals. The relationship between supply and requirement of MP was discussed above, evidencing that neither deficiency in amino acids could be expected in this experiment. In the same way, the similarity of DMI between treatments explains, at least partially, the maintaining of milk production in ewes supplemented with tannin extract when compared with ones that was not supplemented. These results are in agreement with other authors (Orlandi et al., 2015; Alves, 2012), which used similar supplementation levels of black wattle and did not observe effects on OM digestibility and DMI. In contrast, Grainger et al (2009) observed reductions on milk yield (-9.7 kg $day^{-1}$) and DMI (-2.3 kg $day^{-1}$), when dairy cows receiving black wattle tannin extract as a proportion of 19 g kg of $DMI^{-1}$ were compared with dairy cows without extract supplementation.

The quadratic effect of concentrate and corn silage intake in the second experiment was unexpected. This result may be a consequence of lower tannin acceptability of some animals in T30 treatment (visual observations), what did not happen with animals in the treatment T40. The tannin extract acceptability seems to be strongly affected by individual variations and is associated with higher astringency resulting from tannin-

glycoprotein complex formation in mouth and saliva of animals (Reed, 1995). In consequence, the higher blood NEFA concentrations in ewes of T30 treatment can be explained by lower OM digestible intake in this treatment, but this value does not indicate negative energy balance (Caldeira, 2005). Therefore, supplementations with black wattle tannin extract mixed with a concentrate in proportions higher than 30 g $kg^{-1}$ may reduce DMI and have an impact on body score and reproductive performance.

The maintaining of milk yield in animals supplemented with black wattle tannin extract, even with reductions on DM concentrate and corn silage intake, can be also explained by pre-grazing features of pastures (pre-grazing pasture mass and nutritive value) and grazing management. It is well known that pre-grazing pasture mass higher than 3000 kg $ha^{-1}$, with a level of depletion lower than 50% of pre-grazing sward height do not limit pasture DM intake (Amaral et al, 2013; Fonseca et al., 2012; Zanini et al., 2012). Moreover, pastures with CP content higher than 200 g $kg^{-1}$ DM and NDF lower than 500 g kg $DM^{-1}$, as observed in our experiment, can be classified as good quality (INRA, 2007).

## 3.6 CONCLUSION

Dairy ewes supplemented with black wattle tannin extract as a proportion between 10 and 16 g kg of total $DMI^{-1}$ showed a potential to increase N fecal concentration without losses on milk yield and milk composition. When the black wattle tannin extract was mixed with a concentrate as a proportion of 30 g $kg^{-1}$, it has shown a potential risk to reduce DMI and increase body reserve mobilization. Further studies should investigate the effect of similar doses of tannin extract in dairy ewes with higher milk yield potential, mixed in concentrate with different CP contents and/or using diets with lower N content than those used in this experiment.

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# 4 PERFORMANCE AND NITROGEN EXCRETION OF GRAZING DAIRY COWS SUPPLEMENTED WITH BLACK WATTLE TANNIN EXTRACT

## 4.1 ABSTRACT

Condensed tannins can reduce ruminal degradability of proteins, improve nitrogen use efficiency and milk fatty acid profile. Black wattle (*Acacia mearnsii*) supplementation levels up to 25 g/kg of total DM intake (DMI) can increase duodenal flow of MP, decrease N urinary excretion, blood urea N content and methane emissions, but supplementation levels higher than 20 g/kg of DMI can also reduce OM digestibility and metabolizable energy intake. The aim of this work was to investigate the effect of black wattle supplementation included in a partial mixed ration (PMR) on N excretion, milk yield and milk composition of dairy cows grazing a fescue predominant pasture. Two levels of black wattle were tested (control and 15 g tannin extract/kg PMR; C and T15) in 30 lactating cows, in a completely randomized experimental design, which were, respectively, equivalent to zero and 1.5% of total DMI. The cows were grazing a fescue predomiant pasture and supplemented with a RPM (600 g/kg of concentrate and 400 g/kg of corn silage). Pasture and PMR intake were similar between treatments. The milk yield, milk composition, NEFA, blood urea N concentrations and N urea excretion were not affected by treatments. Supplementation with 1.6% DMI of *Acacia mearnsii* did not reduce environmental impact or improve milk yield and composition in mid-lactation Jersey cows grazing fescue pasture.

**Key words**: Taninn. Protein. Milk Urea Nitrogen. N Urinary Excretion. Fatty acid. Conjugated Linolenic Acid (CLA).

## 4.2 INTRODUCTION

Diets based on temperate forages with high nutritive value are rich in proteins readily soluble in rumen, which result in low nitrogen use efficiency (NE) due to ruminal N loss and energetic cost for urea excretion in milk or urine. Milk urea N (MUN) does not contribute to cheese manufacturing and also can affect consistence (Bonanno et al., 2013; Girardi et al., 2015), ripening process (Martin et al., 1997) and coagulation time (Vintila and Marcu, 2011). Additionally, most of N urinary is quickly hidrolisate into ammonia or transformed to nitrate, which can contaminate water resources when is leached or contribute to greenhouse effect when is volatilized to nitrous oxide ($N_2O$) (Monaghan et al., 2007). Thus, the use of alternatives that reduce ruminal degradability of proteins, such as condensed tannins, can increase duodenal flow of metabolisable protein (MP), improving milk yield (Faverdin et al., 2003), milk composition (Girardi et al. 2015) and NE, helping to maximize farm profitability.

Condensed tannins are plant phenolic compound able to bind to dietary proteins and microbial enzymes, reducing ruminal degradability of proteins (Min et al., 2003). Generally, moderate doses of tannin (20-45 g/kg of pasture DM) can prevent bloat, increase duodenal flow of MP (Alves, 2012) and animal performance (Waghorn and McNabb, 2003), but doses higher than this threshold can impair animal performance due to reductions on dry matter intake (DMI), ruminal bacterial activity and intestinal digestibility of nutrients (Jones et al., 2000). However, the nutritional effects of tannins do not only depend of tannin supplementation level, but also of tannin plant source and diet protein level.

In the specific case of black wattle (*Acacia mearnsii*) tannin extract, supplementation levels up to 25 g/kg of total DM intake (DMI) increase duodenal flow of MP in steers (Alves, 2012) and decrease N urinary excretion, blood urea N

content and methane emissions in lambs (Carulla et al., 2005). In contrast, supplementation levels higher than 20 g/kg of DMI can also reduce OM digestibility and metabolizable energy intake (Kozloski et al., 2012). Some studies with dairy cows showed that the same tannin extract used in the current study decreased milk urea N (MUN) and N urinary excretion, but also decreased milk yield, milk fat, milk protein yield and DMI, especially in pastures with lower crude protein (CP) content (Grainger et al., 2009; Griffiths et al., 2013). Additionally, two experiments of our group observed a increase of N fecal content without changes in milk yield or milk composition when ≥30 g of tannin extract/kg of concentrate (≥10 g/kg of total DMI) was supplied for dairy ewes grazing legumes predominant pastures (Dias et al., unpublished data). However, the concentrate intake was reduced and the dietary CP was too high for the moderate supplementation level, exceeding 20 g/kg of DM, which might contributed to reach a plateau value of MUN and reduce the sensitivity of calculate N urea excretion using MUN (Reynolds and Kristensen, 2008).

According to these results we hypothesized that moderate black wattle tannin extract supplementation level (37 g/kg of concentrate or 15 g/kg of total DMI), offered as a partial mixed ration (PMR) to dairy cows grazing pastures with lower CP content (<20 g of CP/kg of DM), can reduce N excretion of pasture based systems rich in ruminal degradable protein, and improve milk yield and composition. The current research aimed to investigate the effect of supplementation with black wattle tannin extract included in a PMR on N excretion, milk yield and milk composition of dairy cows grazing a fescue predominant pasture.

## 4.3 MATERIALS AND METHODS

This study was undertaken in accordance with Ethics Committee for Animal Experimentation (CETEA; protocol number: 01.19.14), from Agroveterinary Science Center (CAV) at the Santa Catarina State University (UDESC), according to current legislation and ethical principles published by the Brazilian College of Animal Experimentation (COBEA).

### 4.3.1 Local and experimental design

The study was performed at a commercial farm in Bom Retiro city (Santa Catarina state, Brazil; 27°45'S, 49°38'W). The experiment was conducted from January to March 2014. The altitude is 890 m and the clime is Cfb (humid subtropical) with mean temperature and annual rainfall of 19ºC and 1400 mm, respectively. The experiment was conducted with dairy cows grazing a fescue predominant pasture and supplemented with a PMR. The treatments were two levels of tannin extract from black wattle (*Acacia meanrsii*): zero (control) and 37g of tannin extract/kg of fresh concentrate (TAN). The tannin extract was first mixed with the concentrate feed, then mixed with corn silage to avoid feed rejection and offered as a PMR to be equivalent to 15 g/kg of DMI (estimated by INRA, 2007). The treatments were compared according to a completely randomized design repeated twice in the time. Each experimental period lasted 21 days, with the last five days for measurements.

### 4.3.2 Animals and diet

Thirty lactating Jersey cows were separated into two homogeneous groups according milk yield (16.3 ± 3.1 kg/day) and LW (386 ± 39 kg). Cows received, before afternoon

milking, 10 kg/day of fresh PMR constituted of 4 kg of concentrate feed and 6 kg of corn silage. The concentrate composition was: 470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed. Silage and concentrate supplementation was balanced to avoid any ruminal energy restriction (Table 6; INRA, 2007).

Table 6. Chemical composition and energetic value of supplements (corn silage and concentrate).

| Item | Supplement | |
|---|---|---|
| | Corn Silage | Concentrate[1] |
| Dry matter (g/kg) | 340 | 924 |
| *Chemical composition (g/kg DM):* | | |
| Organic matter | 966 | 921 |
| Crude protein | 81 | 209 |
| Neutral detergent fiber | 355 | 280 |
| Acid detergent fiber | 186 | 133 |
| Lignin | 76 | 15 |
| *Energetic Value:* | | |
| $NE_L$ (MJ/kg DM)[2] | 8.3 | 8.3 |

[1]470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed.
[2]Net energy for lactation estimated according to Weiss et al. (1992).
Fonte: produção do próprio autor, 2016.

Cows remained in the paddoks after each milking and had, at least, eight daylight hours per day of pasture access, which was predominant constituted of fescue. The cows remained in the same paddock, except during sampling period that each group remained in separate paddocks. The paddocks chosen had similar canopy height, morphological and botanical composition. The grazing method was rotational grazing,

setting a post-grazing herbage height not lower than 50% of pre-grazing herbage height.

### 4.3.3 Feed and sward measurements

Daily samples of supplements were collected in the last five days of each experimental period and the weight of supplement refusals was recorded at each milking. The pre and post-grazing pasture height were measured with a sward stick (100 measures/paddock) and the pre-grazing pasture mass was estimated from a relationship between pre-grazing sward height and the pasture mass at ground level into eight representatives areas of 0.1 $m^2$ in each period. Twenty handfuls (approximately 500 g fresh) of each paddock, during pre- and post-grazing, were randomly cut at ground level and used for botanical classification (fescue, legumes, other species and dead tissues). The pasture samples for chemical composition was collected on first, third and fifth day of sampling periods, by two trained persons, twice per day and by hand plucked technique. All feed samples were oven-dried at 60°C for at least 72 h and only feed for chemical analyses was ground to pass a 1 mm diameter sieve.

### 4.3.4 Animal measurements

Cows were milked twice a day (0600 and 1530 h) and individual milk yield was recorded daily during two days before starting each experiment (day zero) and on sampling period. The milk fat, protein and milk urea N concentrations were measured every other day during sampling periods (on days 17, 19 and 21$^{th}$) by infrared spectrophotometry (International IDF Standard 141C:2000). Theses samples were stored in vials with bronopol at refrigerator before send to SARLE laboratory (Dairy Herds Analysis Service) at the University of Passo Fundo (UPF, Brazil).

The live weight (LW) was measured once a week after morning milking and was used to estimate the pasture intake. The pasture intake was estimated by the relationship between Net energy lactation ($NE_L$) requirements and $NE_L$ supply. The $NE_L$ requirements for maintenance were calculated from LW and $NE_L$ requirements for lactation was calculated from milk yield and milk composition, using the equations from the INRA (2007). It was assumed that animals did not lose or gain weight during sampling period. The $NE_L$ of pasture and PMR were estimated as proposed by Weiss et al (1992). The pasture intake was calculated accounting the $NE_L$ supply calculated from the PMR intake and the $NE_L$ concentrations of selected pasture, as proposed by Baker (2004).

Blood samples from each cow were collected after morning milking at last day of each experimental period from coccygeal vein using 5 mL Vacuntainer tubes without EDTA. As blood urea N is positively related with urea urinary excretion (Kohn et al., 2002), N urinary excretion was estimated according to Kohn (2007), where: N urinary excretion (g/d) = 0.013 × live weight (kg) × BUN (mg/dL).

### 4.3.5 Chemical Analyses

DM concentration was determined by drying at 105°C for 24 hours. The ash content was determined by combustion in a muffle furnace at 550°C for 4 hours, and the organic matter (OM) content was determined by mass difference. The total N was determined using the Kjeldahl method (Method 984.13; AOAC 1997). Neutral detergent fiber (NDF) analyses was performed according to Mertens (2002), except that the samples were weighed into filter bags and treated with neutral detergent at ANKOM equipment (ANKOM Technology, Macedon NY, USA). This analysis included alpha-amylase but did not include sodium sulphite. The concentration of acid detergent fiber (ADF) and sulphuric acid detergent lignin

(ADL) were analyzed according to Method 973.18 of AOAC (AOAC 1997). The ether extract (EE) was determined in a reflux system with ethyl ether at 180º C over 4 h (Oil and fat extractor MA491, Marconi, Brazil).

Blood samples were immediately placed on ice before centrifugation (3500 × g, 20 minutes, and -4ºC) and plasma obtained was stored at -80ºC for non-esterified fatty acids (NEFA) and blood urea N analysis at Federal University of Pelotas (UFPEL, Brazil). NEFA analyses was performed using commercial kits (Wako NEFA-HR, Wako Chemicals EUA ®, Richmond, EUA) according to Ballou et al (2009).

### 4.3.6 Statistical Analyses

Variables were analyzed using period as repeated measurements and taking into account the random effect of animal and fixed effect of treatments. *Akaike's* Information Criterion was used to choose the variance-covariance matrix (Wolfinger, 1993). Analyses were performed using the PROC MIXED of SAS (Statistical Analysis System – Littell et al., 1998) and the results of day zero (milk yield or milk composition) were considered covariates in statistical model. The means were estimated with the LSMEANS procedure and the differences between means were determined by the probability of difference (PDIFF) method using Student t-test at a 5% significance level.

## 4.4 RESULTS

### 4.4.1 Pasture characteristics

The pre-grazing sward characteristics were similar between treatments (Table 7). The pasture mass, herbage allowance and pre-grazing pasture height averaged 2989 kg DM/ha, 19.6 kg DM/cow/day e 15.1 cm, respectively. Organic

matter, CP, NDF and ADF average content of pasture were 890, 191, 511 e 263 g/kg DM, respectively. The grazing down level was on average 53.5% of pre-grazing sward height and a reduction in the proportion of white clover was observed when pre- and post-grazing botanical composition were compared (Table 7).

Table 7. Pre- and post-grazing pasture characteristics grazed by dairy cows with (TAN) or without I tannin extract supplementation.

| Item | Treatments[1] | | SEM (n=4) | *P*-value Treatment |
|---|---|---|---|---|
| | C | TAN | | |
| Pre-grazing pasture mass (kg DM/ha) | 2943 | 3035 | 223.8 | 0.785 |
| Herbage allowance (kg DM/cow/d) | 19.29 | 19.98 | 1.83 | 0.803 |
| Pre-grazing height (cm) | 15.6 | 14.7 | 1.36 | 0.687 |
| Post-grazing height (cm) | 6.7 | 7.4 | 0.76 | 0.587 |
| Dry matter (%) | 20.6 | 21.2 | 4.61 | 0.421 |
| *Chemical composition (g/kg DM):* | | | | |
| Organic matter | 882 | 898 | 4.69 | 0.077 |
| Crude protein | 186 | 196 | 9.40 | 0.521 |
| Neutral detergent fiber | 508 | 515 | 19.20 | 0.828 |
| Acid detergent fiber | 261 | 265 | 13.55 | 0.870 |
| Lignin | 280 | 246 | 5.72 | 0.415 |
| *Pre-grazing botanical composition (% DM):* | | | | |
| Fescue | 37.9 | 38.7 | 1.77 | 0.765 |
| White clover | 28.8 | 25.0 | 8.87 | 0.775 |
| Others sp. | 21.8 | 28.1 | 6.77 | 0.550 |
| Dead Material | 11.5 | 8.2 | 1.62 | 0.232 |
| *Post-grazing botanical composition (% DM):* | | | | |
| Fescue | 39.3 | 36.7 | 3.82 | 0.657 |
| White clover | 5.8 | 7.0 | 1.37 | 0.571 |
| Others sp. | 27.2 | 29.3 | 11.43 | 0.902 |

Table 7. Pre- and post-grazing pasture characteristics grazed by dairy cows with (TAN) or without I tannin extract supplementation.

| | Treatments[1] | | SEM | *P*-value |
|---|---|---|---|---|
| Item | C | TAN | (n=4) | Treatment |
| Dead Material | 27.7 | 27.0 | 8.15 | 0.952 |
| *Nutritive value:* | | | | |
| $NE_L$, MJ/kg DM[2] | 6.4 | 6.8 | 0.28 | 0.441 |

[1]Treatments: C= control, PMR without tannin; TAN= PMR with 1,5% of tannin/DMI/day, which corresponding to 150 g/day;
[2]Net energy for lactation estimated according to Weiss et al. (1992).
Fonte: produção do próprio autor, 2016.

### 4.4.2 Animal performance and milk composition

Pasture and PMR intake were similar between treatments (Table 8). Considering that there were no refusals of PMR, the proportion of tannin extract on DMI was around 1.6% of total DM intake. The milk production, milk composition, NEFA, blood urea N concentrations and N urea excretion were not affected by treatments. Milk yield and 4% fat-correct milk (FCM) were, respectively, 13.2 kg/d e 13.9 kg/d. Milk protein, casein and urea content were, respectively, 3.5%, 2.8% e 14.1 mg/dL.

Table 8. Effect of extract tannin supplementation on DM intake, milk yield, milk composition and blood composition parameters of dairy cows.

| | Treatments[1] | | | *P*-value |
|---|---|---|---|---|
| Parameter | C | TAN | SEM | Treatment |
| *DM intake (kg/day):* | | | | |
| Pasture | 3.69 | 3.50 | 0.292 | 0.656 |
| PMR | 5.80 | 5.70 | 0.022 | 0.114 |
| Total | 9.49 | 9.20 | 0.295 | 0.511 |
| Tannin extract (% DMI) | 0 | 1.6 | - | - |
| Milk yield (kg/day) | 13.4 | 13.1 | 0.35 | 0.658 |
| 4% FCM production (kg/day) | 13.7 | 14.0 | 0.38 | 0.606 |
| Milk fat concentration (%) | 4.05 | 4.21 | 0.109 | 0.264 |
| Milk protein concentration (%) | 3.51 | 3.58 | 0.051 | 0.328 |
| Milk casein concentration (%) | 2.76 | 2.82 | 0.047 | 0.332 |
| Milk urea concentration (mg/dL) | 13.9 | 14.3 | 0.32 | 0.425 |
| Total solids (%) | 12.8 | 12.8 | 0.131 | 0.813 |
| Milk fat production (kg/day) | 0.55 | 0.57 | 0.013 | 0.434 |
| Milk protein production (kg/day) | 0.47 | 0.48 | 0.016 | 0.659 |
| Milk total solids production (kg/day) | 1.79 | 1.75 | 0.058 | 0.611 |
| Live weight (kg) | 400 | 387 | 6.87 | 0.163 |
| NEFA[2] (mg/dL) | 7.99 | 7.59 | 0.355 | 0.435 |
| $NEL_l$ balance | 0.97 | 0.80 | 0.03 | <0.001 |
| BUN[3] (mg/dL) | 27.2 | 25.0 | 1.17 | 0.210 |
| NUE[4] (g/d) | 142 | 131 | 6.50 | 0.234 |

[1]Treatments: C= control, PMR without tannin; TAN= fresh PMR with 1,5% of tannin/day, which corresponding to 150 g/day.
[2]Non esterified fatty acid.
[3]Calculed as: $NE_l$ total intake ($NE_m + NE_l$)
[4]Blood Urea Nitrogen.
[5]N Urinary excretion, estimated by: 0.0259 × LW (kg) × MUN (mg $dL^{-1}$), where: LW = live weight, (Kauffman and St-Pierre, 2001).
Fonte: produção do próprio autor, 2016.

## 4.5 DISCUSSION

The lack of tannin extract supplementation effect on milk composition and N urinary excretion was unexpected, because similar levels of supplementation with black wattle reduced milk urea content and increased fecal N content in dairy cows, suggesting a diversion of N from urine when tannin extract was used in a range of 1.1 to 1.4% of total DMI (Grainger et al., 2009; Griffiths et al., 2013). As the results of Grainger et al (2009) study were conducted using lower CP content diets (≈160 g/kg DM) provided to early lactation cows, probably the difference between Griffiths et al (2013) and our study is associated with supplementation method and pasture protein levels. Griffiths et al (2013) offered similar levels of tannin extract as pellets (1.2% DMI) or drench (1.4% DMI) for cows grazing 240 g CP/kg of pasture DM and only observed difference in MUN, N fecal excretion and DMI when tannin extract was supplemented as drench. Our hypothesis is that when tannin extract supplementation is offered as drench there is a higher effect on rumen microbial population than when is offered mixed to feed, which can reduce ruminal proteolysis of pasture more effectively and consequently reduce N fecal excretion, MUN and N urea excretion. When tannin extract is mixed to feed (concentrate, pellets or PMR) it seems that tannin extract has greater effect to protect the protein of the feed supplement.

Decreases in N urinary excretion were observed with higher levels of *Acacia mearnsii* in high CP diets, but this result was associated with lower OM digestibility and DMI (Kozloski et al., 2012; Carulla et al., 2005). Besides these negative effects, higher level of tannin supplementation can reduce feed acceptability (Basha et al., 2012) because the sensation of astringency when tannins bind to salivary glycoproteins (Reed, 1990). When tannin extract was pelleted with barley (Griffiths et al., 2013) or mixed with concentrate

(Dias et al., unpublished data) and provided about 1.2% of total DM, the pellet and concentrate acceptability was reduced. In our work, when tannin extract was mixed with PMR and provided as a proportion of 1.6% of total DM, PMR acceptability was not affected.

The supplementation method seems to influence tannins nutritional effect and supplementation with *Acacia mearnsii* tannin extract as a proportion of 1.6% of total DMI did not reduce environmental impact or improve milk yield and composition in mid-lactation Jersey cows grazing fescue pasture. When tannin extract was mixed with concentrate and corn silage, did not reduce PMR acceptability by cows. As higher supplementation levels can cause negative impact on DMI and OM digestibility, the effect of similar doses this tannin extract in different supplementation methods or other tannin extract source on nitrogen excretion should be tested in grazing cows.

## 4.6 CONCLUSION

Diet supplementation with 1.6% DMI of *Acacia meanrsii* tannin extract did not reduce environmental impact, improve milk yield or milk composition in mid-lactation Jerseys grazing fescue pasture. The supplementation level included in a PMR was well accepted by dairy cows and did not reduce PMR intake. Further studies should evaluate the effect of different supplementation methods with the same tannin source or test another tannin source on nitrogen excretion and animal performance of grazing cows.

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# 5 BLACK WATTLE TANNIN SUPPLEMENTATION AFFECTS MILK FATTY ACID COMPOSITION OF DAIRY EWES

## 5.1 ABSTRACT

Some sources of condensed tannins can increase milk fat rumenic acid content, but *in vitro* and *in vivo* studies have been show conflicting results. Tannin extract from *Acacia* spp can increase vacennic acid and reduce stearic acid in ruminal fluid, however no study evaluated the effect of *Acacia mearnsii* tannin (black wattle) on milk fatty acid (FA) composition of ruminants. The aim of this work was to evaluate the effect of moderate levels of black wattle supplementation on milk FA composition of cows and ewes grazing temperate forages. Four tannins supplementation levels were tested on dairy ewes (control, 20, 30 and 40g of tannin/kg of fresh concentrate; C, T20, T30 and T40) and two on dairy cows (control and 15g of tannin/kg fresh PMR; C and T15) in a completely randomized experimental design, which were, respectively, equivalent to 0.8, 1.2, 1.5 and 1.5% of dry matter intake (DMI). Individual milk samples were collected in the last day of sampling period and analyzed for FA composition by gas chromatography. There was higher milk content of 6:0, *cis*-12 18:1, *trans*-12 18:1 and *trans*-10, *cis*-12 CLA in T20 treatment (first experiment) and a linear increase of 17:0, *cis*-9 17:1, *trans*-10 18:1, *cis*-9, *trans*-11 CLA in milk from T30 and T40 treatments (second experiment). There only was increase of 18:2 n-6 and 20:0 in milk from T15 treatments. Probably there was a change in rumen mibrobiota with tannin supplementation that inhibited *B. fibrisolvens* and led an increase in linileic acid intermediates, as rumenic acid, trans-10, cis-12 CLA and its intermediates. There was a decrease in corn silage and concentrate intake in T30 treatment but without differences in milk yield and live weight. Supplementation with 1.2% and

1.6% of estimated DMI with *Acacia mearnsii* TAN, mixed with concentrate, was possible under farm conditions and improved the level of healthy milk FA in dairy ewes without impairing animal performance.

**Keywords:** conjugated linoleic acid, methyl ester, lipids, rumenic acid, lacaune ewe.

## 5.2 INTRODUCTION

During the last decade several researches studies have been conducted to improve the level of healthy fatty acid (FA) in food deriving from ruminants, which includes lower saturated FA content (SFA) and higher content of polyunsaturated FA (PUFA), n-3 and conjugated linoleic acid (CLA) (Patel et al., 2013; Mapiye et al., 2013). This fat composition should be aimed for dietary recommendations because milk fat of ruminants is a natural source of beneficial FA such *cis*-9, *trans*-11 CLA (RA), *trans*-10, *cis*-12 CLA, *trans*-11 C18:1 (VA: Vaccenic acid), n-3 FA (especially C18:3 n-3: Linolenic acid), *cis*-9 C18:1, *trans*-9 C16:1 and odd- and branched-chain FA (OBCFA; Kratz et al., 2013; Santaren et al., 2014). Rumenic, vaccenic acid and *trans*-10, *cis*-12 CLA has been reported to have a wide range of beneficial effects including anticarcinogenic (Parodi, 1994), antiobesity activities (Pariza et al., 1996) and the ability to stimulate immune function (Miller et al., 1994) while saturated odd-chain FA and *trans*-9 C16:1 were associated to lower type 2 diabetes (Mozaffarian et al., 2013; Santaren et al., 2014). Even so, current dietary recommendations have been restricting fat intake from animal origin because the increased risk of cardiovascular diseases are associated to SFA, especially C12:0, C14:0 and C16:0 (Givens and Shingfield, 2004; Kliem and Givens, 2011). However, several researches have been reporting that milk fat intake is associated to lower obesity,

cardiovascular and type 2 diabetes risk (Kratz et al., 2013; Mozaffarian, 2014; Astrup, 2014; Yakoob et al., 2014) based on the fact that increased cholesterol levels from SFA intake is due to higher HDL (known as "good cholesterol") and large LDL particles instead of small and dense LDL particles (Siri-Tarino et al., 2010).

Higher levels of healthy milk FA can be achieved increasing animal intake of fresh grass (Morales-Almaráz et al., 2010; Elgersma et al., 2004), legume (Wiking et al., 2010), grass or legume hay (Bernardini et al., 2010; Kalač and Samková, 2010), supplementing animal diets with plant oil (Dhiman et al., 2000), marine oil (Abu-Ghazaleh et al., 2003), monensin (Fellner et al., 1997) or tannin (Vasta et al., 2010). Condensed tannins are phenolic compounds able to bind to microbial enzymes, dietary proteins and carbohydrates, which can reduce ruminal biohydrogenation (BH) of linolenic and linoleic (Linoleic acid: C18:2 n-6) acids. The lower BH of linolenic and linoleic acid can result in higher ruminal content of vaccenic and rumenic acids respectively. However, the majority milk rumenic acid content is formed by stearoyl-CoA desaturase (Δ9-desaturase) activity on vaccenic acid in mammary gland (endogenous synthesis; Bauman et al., 2003).

Some studies observed that quebracho and chestnut tannins can increase milk rumenic acid content of ewes supplemented with sunflower (Toral et al., 2013) or soybean oil (Buccioni et al., 2015). However, *in vitro* and *in vivo* studies evaluating the effect of tannin on the accumulation of BH intermediates in the rumen and on the animal performance have been shown conflicting results (Durmic et al., 2008; Buccioni et al, 2011; Vasta et al., 2009a, 2009b; Toral et al., 2011, 2013). This is probably due to differences in tannin source and supplementation levels as nutritional effects depend on chemical composition, molecular structure and are dose-dependent. Some studies observed that *Acacia* spp tannin can change growth of rumen microbial population, increase

vaccenic acid and reduce stearic acid (C18:0) in ruminal fluid (Durmic et al., 2008; Carulla et al., 2005; O'Donovan and Brooker 2001; Brooker et al. 1994) but no study evaluated the effect of *Acacia mearnsii* tannin on milk FA composition. As linolenic and linoleic acids are the main PUFA in forages, we hypothesized that tannin supplementation with *Acacia mearnsii* extract (black wattle) can reduce BH and increase milk vaccenic acid, rumenic acid and PUFA contents. The aim of this work was to evaluate the effect of moderate levels of black wattle supplementation on milk FA composition, especially vaccenic acid, rumenic acid and PUFA, of cows and ewes fed fresh forage-based diets.

## 5.3 MATERIAL AND METHODS

The experiments were conducted in accordance with Ethics Committee for Animal Experimentation (CETEA; protocol number: 01.19.14), of Agroveterinary Science Center (CAV) at the Santa Catarina State University (UDESC), according to current legislation and ethical principles published by the Brazilian College of Animal Experimentation (COBEA).

### 5.3.1 Local and experimental design

The study was performed at a commercial farm in Bom Retiro city (Santa Catarina state, Brazil; 27°45'34.6"S 49°38'25.3"W). Two experiments were conducted with dairy ewes between January to May 2013 and a third experiment with dairy cows between January to February 2014. The altitude is 890 m and the clime is Cfb (humid subtropical) with mean temperature and annual rainfall of 19ºC and 1400 mm, respectively. In the first two experiments dairy ewes grazed a legume (*Trifolium repens*) predominant pasture and received a supplementation with corn silage and concentrated feed. In the

first experiment two levels of tannin extracted from black wattle (*Acacia mearnsii*) were tested: zero (control, C) and 20g tannin extract/kg of fresh concentrate (T20). The tannin was mixed with the concentrate to be equivalent to 0.8% of dry matter intake (DMI), which was estimated according to INRA (2007). After previous results of first experiment, a second experiment was conducted in order to test three levels of tannin extracted from black wattle: zero (control), 30g (T30) and 40g (T40) of tannin extract/kg of fresh concentrate, which were mixed with the concentrate to be equivalent to 1.2 and 1.5% of DMI (INRA, 2007). The third experiment was conducted with dairy cows grazing a fescue predominant pasture and supplemented with a partial mixed ration (PMR). The treatments were two levels of tannin extract from black wattle: zero (control) and 15g (T15) of tannin extract/kg fresh PMR. The tannin was mixed with the PMR to be equivalent to 1.5% of DMI (INRA, 2007). In both experiments treatments were compared according to a completely randomized design. The experimental period lasted 21 days, with the last five days for measurements (sampling period).

### 5.3.2 Animals

In the first two experiments 18 to 24 dairy ewes were selected. In the first experiment 9 Lacaune and 9 Milchschaf ewes were selected and separated into two homogeneous groups according to racial group (Lacaune or Milchschaf), milk yield (1.3 ± 0.3 kg/day), lactation stage (82.6 ± 8.3 days), parity (2.4 ± 0.5) and live weight (LW; 62.8 ± 6 kg). In the second experiment, 24 Lacaune ewes were selected and separated into three homogeneous groups according to milk yield (1.3 ± 0.4 kg/day), lactation stage (78.6 ± 9.8 days), parity (2.5 ± 0.6) and LW (52.4 ± 5.3 kg). In the third experiment, 18 Jersey cows were separated into two groups

according milk yield (17,4 ± 3,0 kg/day) and LW (390,7 ± 34,1 kg).

### 5.3.3 Feed and grazing management

Ewes individually received 300 g of fresh concentrate after each milking and 1 kg of fresh corn silage after afternoon milking. Cows individually received 10 kg of fresh PMR constituted of 60% of fresh corn silage and 40% of fresh concentrate. The concentrate composition from both experiments was: 470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed. In order to test our hypothesis the diets were balanced allowing excess of ruminal degradable N, without restrict ruminal energetic supply (INRA, 2007). Paddocks with white clover and fescue predominant pastures, under rotational grazing, were available for the ewes and cows, respectively. During sampling period each experimental group had access to separated paddocks, however both groups were allocated to a same paddock during interval periods. Paddocks had similar pre-grazing herbage heights, morphological and botanical composition and grazing down levels up to 50% of pre-grazing height was allowed. Daily time at pasture was around 8 hours, after morning milking. Water and mineral salt were always available to animals.

### 5.3.4 Feed and sward measurements

Daily samples of supplements were collected in sampling period and the weight of individual supplements refusals was recorded at each milking for chemical composition analyses and DMI measurement, respectively. The pre- and post-grazing pasture height were measured with a rising plate meter (100 measures; Farmworks®, F200 model, New Zealand) or a sward stick and the pre-grazing pasture mass was estimated from six

or eight representatives areas (0.1 $m^2$) at ground level in ewes or cows experiment, respectively. Twenty handfuls (approximately 500 g fresh) of each paddock, in pre- and post-grazing, were randomly cut at ground level and used for botanical classification (legumes, fescue, other species and dead tissues). The pasture samples for chemical composition was collected on first, third and fifth day of sampling period, by hand plucked technique. Two trained persons collected these samples twice per day. All feed samples were oven-dried at 60°C for at least 72 h and only feed for chemical analyses was ground to pass a 1 mm diameter sieve.

### 5.3.5 Animal measurements

Animals were milking twice a day (0600 and 1530h) and individual milk yield was recorded daily during two days before starting each experiment (day zero) and on sampling period. Milk samples, composed from morning and afternoon, of each animal were collected in the last day of sampling period and frozen without bronopol. The LW was measured once a week after morning milking.

The pasture intake was estimated by the relationship between metabolic energy (ME) requirements (for lactation and maintenance) and ME supplied by supplements and pasture. The ME for maintenance ($ME_m$) was calculated from LW and ME for lactation ($ME_l$) was calculated from milk production and milk composition, according to INRA (2007) or NRC (2001) for dairy ewes and cows, respectively. The energetic value of pasture, corn silage and concentrate were estimated as proposed by Weiss et al (1992). The pasture intake was calculated as a difference between ME requirements and ME consumption from supplements, as proposed by Baker (2004).

### 5.3.6 Chemical Analyses

DM concentration was determined by drying at 105°C for 24 hours. The ash content was determined by combustion in a muffle furnace at 550°C for 4 hours, and the organic matter (OM) content was determined by mass difference. The total N was determined using the Kjeldahl method (Method 984.13; AOAC 1997). Neutral detergent fiber (NDF) analyses was performed according to Mertens (2002), except that the samples were weighed into filter bags and treated with neutral detergent in ANKOM equipment (ANKOM Technology, Macedon NY, USA). This analysis included alpha-amylase but did not include sodium sulphite. The concentration of acid detergent fiber (ADF) and sulphuric acid detergent lignin (ADL) were analyzed according to Method 973.18 of AOAC (AOAC 1997). The ether extract (EE) was determined in a reflux system with ethyl ether at 180º C over 4 h (Fat and oils extractor MA491, Marconi, Brazil).

Milk samples were thawed at room temperature and a volume of 1 mL was used for lipid extraction using a mixture of diethylether and hexane according to a reference procedure (AOAC Official Method 989.05). The organic phase containing the milk fat (~20 mg) was evaporated to dryness at 40°C under oxygen-free nitrogen. FA methyl esters (FAME) were obtained by base-catalyzed transmethylation using a freshly prepared sodium methoxide solution as described in detail elsewhere (Baldin et al., 2013). FAMEs were quantified by a gas chromatograph (model 7820-A, Agilent Technologies) fitted with a flame-ionization detector and equipped with a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm × 0.2 μm film thickness; Varian Inc). Operating conditions were the same described by Cruz-Hernandez et al. (2007). The FAMEs were identified by comparison of retention times with commercial FAME standards, and minor *trans*/*cis*-C18:1 isomers were identified according to their order of elution

reported under the same GC conditions (Cruz-Hernandez et al., 2007). Milk FA composition was expressed as a weight percentage of total FA using theoretical relative response factors (Wolff et al., 1995). Stearoyl-CoA desaturase (SCD) indices were calculated for four pairs of FA by expressing each product as a proportion of the precursor plus product (i.e. $SCD_{14}$ = *cis*-9 14:1/14:0 + *cis*-9 14:1; $SCD_{16}$ = *cis*-9 16:1/16:0 + *cis*-9 16:1; $SCD_{18}$ = *cis*-9 18:1/18:0 + *cis*-9 18:1; and $SCD_{RA}$= RA/VA + RA) (Kesley et al., 2003).

### 5.3.7 Statistical Analyses

Variables were analyzed taking into account the random effect of animal and the fixed effect of treatments, using PROC GLM (Statistical Analysis System, SAS). *Akaike's Information Criterion was used to choose the variance-covariance matrix* (Wolfinger et al., 1993).

In the first and third experiment, the differences between means were determined by the MEANS procedure using Student`s t-test at a 5% significance level. In the second experiment the linear and quadratic effects of supplementation level were tested using polynomial orthogonal contrasts, in which the quadratic component was equivalent to the lack of fit sum of squares for linearity. Each *F* value was a ratio of the contrast mean square to the residual (experimental error) mean square.

## 5.4 RESULTS

### 5.4.1 Dairy ewes experiments

Feed composition of two experiments with dairy ewes was similar, with OM, CP, NDF and ADF contents of corn silage and concentrate averaging 960 and 918, 94 and 209, 427 and 347, 209 and 146 g/kg DM (Table 9). Paddocks had high

proportion of leguminous, especially white clover, and consequently high CP content (≈273 g/kg DM). Pasture depletion was 36% and 33,5% in the first and second experiment, respectively.

Table 9. Chemical composition of feed and sward characterization in two experiments conducted with dairy ewes.

| Item | First experiment | | | Second experiment | | |
|---|---|---|---|---|---|---|
| | P[1] | CS[2] | C[3] | P[1] | CS[2] | C[3] |
| *Chemical composition (g/kg DM)* | | | | | | |
| DM | 152 | 341 | 902 | 191 | 337 | 900 |
| OM | 924 | 956 | 916 | 916 | 963 | 920 |
| CP | 289 | 108 | 199 | 258 | 79 | 219 |
| NDF | 503 | 443 | 357 | 454 | 410 | 337 |
| ADF | 237 | 213 | 148 | 223 | 205 | 144 |
| *Sward characterization* | | | | | | |
| Pre-grazing sward height (cm) | 28.2 | | | 15.5 | | |
| Post-grazing sward height (cm) | 18.0 | | | 10.3 | | |
| Leguminous (% DM) | 58.0 | | | 57.0 | | |
| Fescue (% DM) | 17.5 | | | 18.4 | | |

[1]Pasture.
[2]Corn Silage.
[3]Concentrate: 470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed.
Fonte: produção do próprio autor, 2016.

There was no difference in corn silage and concentrate intake, milk yield or LW when low tannin supplementation level was tested in first experiment (Table 10). When higher levels of tannin supplementation were offered (TAN30) in second experiment there was a decrease in corn silage and

concentrate intake but without differences in milk yield and LW. However, there was no difference in supplements intake and animal performance between the highest level of tannin supplementation (TAN40) and C treatment. According to concentrate intake, the proportion of tanin supplementation was around 0.7, 1.0 and 1.6% of total DMI in the treatments T20, T30 and T40, respectively.

There were only slight changes in milk FA profile with tannin supplementation in first experiment which were a decrease in *trans*-9 16:1, *trans*-6 to *trans*-9 18:1, *trans*-11 18:1, 20:2 n-6, 23:0 and n-6:n-3 ratio (Table 11). However, higher content of 6:0, *cis*-12 18:1, *trans*-12 18:1 and *trans*-10, *cis*-12 CLA (undetectable in control treatment) was observed in T20 treatment.

Table 10. Effect of tannin supplementation on supplements intake, milk yield and live weigh of dairy ewes grazing a dominant legume pasture (First and second experiment).

| | Treatments[1] | | | | Treatments[1] | | | | | *P-value* |
|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | C | T20 | SEM | *P*-value | C | T30 | T40 | SEM | Linear | Quadratic |
| | First experiment | | | | Second experiment | | | | | |
| DM intake (kg/day) | | | | | | | | | | |
| Corn silage | 0.34 | 0.34 | 0.001 | 0.3322 | 0.31[a] | 0.26[b] | 0.31[a] | 0.008 | 0.7998 | 0.0119 |
| Concentrate | 0.5 | 0.5 | 0.003 | 0.8440 | 0.5[a] | 0.4[b] | 0.5[a] | 0.015 | 0.1860 | 0.0006 |
| Milk yield (kg/day) | 1.2 | 1.2 | 0.058 | 0.7350 | 1.1 | 0.9 | 1.1 | 0.091 | 0.7734 | 0.2546 |
| Live weight (kg) | 61.6 | 64.5 | 1.269 | 0.3373 | 54.4 | 53.2 | 54.0 | 1.107 | 0.9064 | 0.6807 |

[a,b]Means within a row with different superscripts differ ($P<0.05$);
[1]Treatments: C = ewes without supplementation with taniferous extract; T20, T30 and T40 = ewes supplemented with 20, 30 and 40 g tannin extract/kg of concentrate, respectively (0.8, 1.2 and 1.6% of estimated DMI).
Fonte: produção do próprio autor

Table 11. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (First experiment).

| FA | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T20 | SEM | *P*-value |
| 4:0 | 3.85 | 3.96 | 0.095 | 0.5876 |
| 6:0 | 2.81[b] | 3.04[a] | 0.050 | 0.0329 |
| 8:0 | 2.53 | 2.77 | 0.087 | 0.1926 |
| 10:0 | 7.80 | 8.30 | 0.388 | 0.5276 |
| 10:1c9 | 0.23 | 0.25 | 0.011 | 0.4121 |
| 12:0 | 4.41 | 4.42 | 0.269 | 0.9886 |
| *cis*-9 12:1[2] | 0.16 | 0.16 | 0.009 | 0.9532 |
| 14:0 | 11.20 | 10.91 | 0.322 | 0.6585 |
| 14:0 iso | 0.11 | 0.10 | 0.005 | 0.4467 |
| *cis*-9 14:1 | 0.19 | 0.20 | 0.010 | 0.5468 |
| 15:0 | 1.10 | 1.05 | 0.027 | 0.3446 |
| 15:0 iso | 0.26 | 0.24 | 0.008 | 0.2006 |
| 15:0 anteiso | 0.48 | 0.44 | 0.013 | 0.2084 |
| 16:0 | 25.28 | 24.10 | 0.408 | 0.1663 |
| 16:0 iso | 0.21 | 0.19 | 0.005 | 0.171 |
| *cis*-9 16:1[3] | 1.16 | 1.13 | 0.039 | 0.7573 |
| *trans*-9 16:1[4] | 0.55[a] | 0.49[b] | 0.011 | 0.0175 |
| 17:0 | 0.56 | 0.53 | 0.016 | 0.3526 |
| *cis*-9 17:1 | 0.19 | 0.18 | 0.006 | 0.1589 |
| 18:0 | 8.22 | 8.65 | 0.348 | 0.5501 |
| *cis*-9 18:1 | 15.01 | 14.92 | 0.497 | 0.9281 |
| *cis*-11 18:1 | 0.49 | 0.50 | 0.024 | 0.8749 |
| *cis*-12 18:1 | 0.24[y] | 0.32[x] | 0.021 | 0.0755 |
| *cis*-13 18:1 | 0.06 | 0.07 | 0.005 | 0.1953 |
| *trans*-6-8 18:1 | 0.16[a] | 0.13[b] | 0.007 | 0.0317 |

Table 11. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (First experiment).

| FA | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T20 | SEM | *P*-value |
| *trans*-9 18:1 | 0.22[a] | 0.18[b] | 0.007 | 0.0265 |
| *trans*-10 18:1 | 0.33 | 0.29 | 0.021 | 0.4088 |
| *trans*-11 18:1 | 1.90[x] | 1.60[y] | 0.078 | 0.0728 |
| *trans*-12 18:1 | 0.38[y] | 0.45[x] | 0.019 | 0.0626 |
| *trans*-13-14 18:1 | 0.44 | 0.50 | 0.019 | 0.1083 |
| *trans*-9, *trans*-12 18:2 | 0.03 | 0.03 | 0.002 | 0.5089 |
| *cis*-9, *trans*-12 18:2 | 0.07 | 0.07 | 0.005 | 0.4548 |
| *trans*-9, *cis*-12 18:2 | 0.05 | 0.04 | 0.003 | 0.6646 |
| *trans*-11 *cis*-13 18:2 | 0.04 | 0.03 | 0.003 | 0.1141 |
| 18:2 n-6 | 1.61 | 1.68 | 0.003 | 0.6534 |
| *cis*-9, *trans*-11 CLA | 1.05 | 0.89 | 0.046 | 0.1067 |
| *trans*-10, *cis*-12 CLA | 0.00[b] | 0.01[a] | 0.001 | 0.0023 |
| *trans*-9, *cis*-11 CLA | 0.02 | 0.03 | 0.002 | 0.189 |
| 18:3 n-3 | 0.67 | 0.86 | 0.061 | 0.1497 |
| 20:0 | 0.16 | 0.14 | 0.007 | 0.2261 |
| *cis*-11 20:1 | 0.05 | 0.05 | 0.002 | 1.00 |
| 20:2 n-6 | 0.02[a] | 0.01[b] | 0.001 | 0.0483 |
| 20:4 n-6 | 0.13 | 0.12 | 0.005 | 0.2815 |
| 20:5 n-3 | 0.04 | 0.05 | 0.003 | 0.4011 |
| 21:0 | 0.05 | 0.04 | 0.003 | 0.2102 |
| 22:5 n-3 | 0.08 | 0.08 | 0.002 | 0.3091 |
| 23:0 | 0.03[a] | 0.02[b] | 0.002 | 0.0078 |
| 24:0 | 0.03 | 0.03 | 0.001 | 0.3464 |
| Σ unidentified | 4.00 | 4.42 | 0.160 | 0.2037 |

*Summation by origin*

Table 11. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (First experiment).

| FA | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T20 | SEM | *P*-value |
| De novo (4:0-15:0) | 35.32 | 36.02 | 0.958 | 0.7211 |
| Mixed (16:0+16:1) | 27.51 | 26.22 | 0.423 | 0.1456 |
| Preformed (≥18:0) | 32.41 | 32.64 | 0.869 | 0.8998 |
| *Summation by saturation* | | | | |
| SFA[5] | 69.34 | 69.17 | 0.735 | 0.9112 |
| MUFA[6] | 22.61 | 22.28 | 0.572 | 0.7759 |
| PUFA[7] | 3.86 | 3.95 | 0.138 | 0.7433 |
| ΣOBCFA[8] | 3.24 | 3.05 | 0.061 | 0.1245 |
| n-6:n-3 ratio[10] | 2.52[x] | 2.11[y] | 0.100 | 0.0500 |
| SCD index[11] | | | | |
| $SCD_{14}$ | 0.02 | 0.02 | 0.001 | 0.3531 |
| $SCD_{16}$ | 0.04 | 0.04 | 0.001 | 0.7296 |
| $SCD_{18}$ | 0.65 | 0.63 | 0.007 | 0.3783 |
| $SCD_{RA}$ | 0.35 | 0.35 | 0.007 | 0.9871 |

[a,b]Means within a row with different superscripts differ ($P<0.05$); [x,y]Means within a row with different superscripts differ ($P<0.10$);[1]Treatments: C = ewes without supplementation with TAN; T20 = ewes supplemented with 20 g tannin extract/kg of concentrate, respectively (0.8% of estimated DMI); [2]Co-eluted with 13:0; [3]Co-eluted with 17:0 anteiso; [4]Co-eluted with 17:0 iso; [5]Saturated FA; [6]Monounsaturated FA; [7]Polyunsaturated FA; [8]Odd- and branched-chain FA with co-elutions; [9]Ratio of SFA to Unsaturated FA (UFA); [10]Calculated as (18:2 n-6 + 18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6) ÷ (C18:3 n-3 + C20:5 n-3 + C22:5 n-3); [11]Calculated according to Kesley et al. (2003).

Fonte: produção do próprio autor, 2016.

There were more differences in milk FA profile, especially in some branched-chain FA (BCFA) and long chain FA (LCFA), with higher tannin supplementation in second than first experiment (Table 12). However, most of these changes in milk FA composition were observed in the intermediary level of tannin supplementation (T30). There was a linear increase in 17:0, *cis*-9 17:1, *trans*-10 18:1, *cis*-9, *trans*-11 CLA.

Table 12. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| FA | C | T30 | T40 | SEM | Linear | Quad. |
| 4:0 | 3.82 | 3.88 | 3.81 | 0.046 | 0.9411 | 0.4833 |
| 6:0 | 3.12 | 3.11 | 2.98 | 0.042 | 0.2069 | 0.5097 |
| 8:0 | 3.03 | 2.94 | 2.78 | 0.057 | 0.1003 | 0.7961 |
| 10:0 | $9.75^{x}$ | $9.28^{xy}$ | $8.93^{y}$ | 0.165 | 0.0591 | 0.8717 |
| *cis*-9 10:1 | 0.30 | 0.28 | 0.31 | 0.008 | 0.3329 | 0.2165 |
| 12:0 | $5.60^{x}$ | $5.20^{xy}$ | $5.04^{y}$ | 0.125 | 0.0828 | 0.6482 |
| *cis*-9 12:1[2] | $13.19^{b}$ | $14.71^{a}$ | $13.30^{b}$ | 0.004 | 0.8475 | 0.0071 |
| 14:0 | 11.74 | 11.18 | 11.57 | 0.202 | 0.7363 | 0.2711 |
| 14:0 iso | $0.09^{b}$ | $0.11^{a}$ | $0.11^{a}$ | 0.003 | 0.0015 | 0.0054 |
| *cis*-9 14:1 | 0.22 | 0.18 | 0.22 | 0.011 | 0.8987 | 0.1093 |
| 15:0 | $1.25^{a}$ | $1.09^{b}$ | $1.25^{a}$ | 0.025 | 0.9793 | 0.008 |
| 15:0 iso | $0.21^{b}$ | $0.26^{a}$ | $0.22^{b}$ | 0.005 | 0.3711 | 0.0003 |
| 15:0 anteiso | 0.45 | 0.48 | 0.46 | 0.012 | 0.6583 | 0.3346 |
| 16:0 | 23.72 | 22.83 | 24.88 | 0.457 | 0.3172 | 0.1415 |
| 16 iso | $0.17^{y}$ | $0.20^{x}$ | $0.19^{x}$ | 0.005 | 0.0844 | 0.0525 |
| *cis*-9 16:1[3] | 1.11 | 1.03 | 1.15 | 0.029 | 0.5744 | 0.1276 |
| *trans*-9 16:1[4] | $0.46^{b}$ | $0.53^{a}$ | $0.49^{a}$ | 0.010 | 0.1746 | 0.0172 |
| 17:0 | $0.47^{y}$ | $0.51^{x}$ | $0.50^{x}$ | 0.008 | 0.0990 | 0.2133 |

Table 12. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| FA | C | T30 | T40 | SEM | Linear | Quad. |
| *cis*-9 17:1 | 0.17$^{b}$ | 0.17$^{b}$ | 0.20$^{a}$ | 0.004 | 0.0207 | 0.1177 |
| 18:0 | 6.29$^{b}$ | 7.86$^{a}$ | 6.33$^{b}$ | 0.234 | 0.9437 | 0.0052 |
| *cis*-9 18:1 | 13.19$^{b}$ | 14.71$^{a}$ | 13.30$^{b}$ | 0.232 | 0.8475 | 0.0071 |
| *cis*-11 18:1 | 0.59$^{a}$ | 0.57$^{ab}$ | 0.52$^{b}$ | 0.012 | 0.0325 | 0.5282 |
| *cis*-12 18:1 | 0.30$^{a}$ | 0.25$^{b}$ | 0.22$^{b}$ | 0.009 | 0.0019 | 0.6897 |
| *cis*-13 18:1 | 0.08$^{a}$ | 0.05$^{b}$ | 0.06$^{b}$ | 0.002 | 0.0051 | 0.0012 |
| *trans*-6-8 18:1 | 0.13 | 0.11 | 0.16 | 0.012 | 0.2471 | 0.1571 |
| *trans*-9 18:1 | 0.20$^{xy}$ | 0.18$^{y}$ | 0.22$^{x}$ | 0.007 | 0.3778 | 0.0863 |
| *trans*-10 18:1 | 0.22$^{b}$ | 0.30$^{a}$ | 0.31$^{a}$ | 0.014 | 0.0284 | 0.3541 |
| *trans*-11 18:1 | 1.76 | 1.84 | 1.96 | 0.052 | 0.1346 | 0.8374 |
| *trans*-12 18:1 | 0.39 | 0.38 | 0.35 | 0.009 | 0.1271 | 0.7476 |
| *trans*-13+14 18:1 | 0.46 | 0.47 | 0.51 | 0.021 | 0.3070 | 0.7872 |
| *trans*-9, *trans*-12 18:2 | 0.03$^{b}$ | 0.03$^{b}$ | 0.04$^{a}$ | 0.002 | 0.0993 | 0.0017 |
| *cis*-9, *trans*-12 18:2 | 0.11$^{a}$ | 0.08$^{b}$ | 0.07$^{b}$ | 0.004 | 0.0008 | 0.1450 |
| *trans*-9, *cis*-12 18:2 | 0.05 | 0.05 | 0.04 | 0.002 | 0.1061 | 0.9606 |
| *trans*-11, *cis*-13 18:2 | 0.04 | 0.04 | 0.05 | 0.002 | 0.2804 | 0.7231 |
| 18:2 n-6 | 1.74 | 1.68 | 1.58 | 0.050 | 0.1994 | 0.827 |
| *cis*-9, *trans*-11 CLA | 1.07$^{b}$ | 1.17$^{ab}$ | 1.30$^{a}$ | 0.034 | 0.0137 | 0.8212 |
| *trans*-10, *cis*-12 CLA | 0.00 | 0.00 | 0.01 | 0.001 | 0.5069 | 0.4808 |
| *trans*-9, *cis*-11 CLA | 0.03 | 0.03 | 0.03 | 0.001 | 0.8993 | 0.5518 |
| 18:3 n-3 | 0.80 | 0.79 | 0.75 | 0.034 | 0.4900 | 0.8666 |

Table 12. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| FA | C | T30 | T40 | SEM | Linear | Quad. |
| 20:0 | 0.18 | 0.18 | 0.18 | 0.003 | 0.9012 | 0.8070 |
| *cis*-11 20:1 | 0.04 | 0.05 | 0.04 | 0.002 | 0.8061 | 0.1447 |
| 20:2 n-6 | 0.02[a] | 0.01[b] | 0.02[ab] | 0.002 | 0.2739 | 0.0243 |
| 20:4 n-6 | 0.14 | 0.13 | 0.13 | 0.005 | 0.6762 | 0.9927 |
| 20:5 n-3 | 0.06 | 0.12 | 0.06 | 0.019 | 0.9731 | 0.1529 |
| 21:0 | 0.05[a] | 0.04[b] | 0.05[a] | 0.002 | 0.1157 | 0.0034 |
| 22:5 n-3 | 0.07 | 0.15 | 0.08 | 0.024 | 0.9411 | 0.1517 |
| 23:0 | 0.03 | 0.03 | 0.03 | 0.001 | 0.3485 | 0.5770 |
| 24:0 | 0.04 | 0.04 | 0.04 | 0.002 | 0.5381 | 0.5444 |
| Σ unidentified | 4.32 | 3.87 | 4.82 | 0.273 | 0.8179 | 0.9110 |
| *Summation by origin* | | | | | | |
| De novo (4:0-15:0) | 40.22 | 38.47 | 38.22 | 0.469 | 0.1017 | 0.4533 |
| Mixed (16:0+16:1) | 25.77 | 24.92 | 27.03 | 0.416 | 0.6535 | 0.4882 |
| Preformed (>17:0) | 29.04 | 32.06 | 29.23 | 0.567 | 0.8078 | 0.7782 |
| *Summation by saturation* | | | | | | |
| SFA[5] | 70.47 | 69.54 | 69.73 | 0.423 | 0.4906 | 0.5374 |
| MUFA[6] | 20.78 | 22.09 | 21.07 | 0.289 | 0.6076 | 0.7738 |
| PUFA[7] | 4.27 | 4.33 | 4.21 | 0.103 | 0.8206 | 0.6640 |
| ΣOBCFA[8] | 3.34 | 3.22 | 3.38 | 0.047 | 0.7429 | 0.1694 |
| n-6:n-3 ratio[9] | 2.30 | 2.19 | 2.22 | 0.116 | 0.6760 | 0.6921 |
| SCD index[10] | | | | | | |
| $SCD_{14}$ | 0.02[xy] | 0.02[y] | 0.02[x] | 0.001 | 0.7329 | 0.0504 |

Table 12. Effect of tannin supplementation on milk FA composition of dairy ewes grazing a dominant legume pasture and supplemented with corn silage and concentrate (Second experiment).

| | Treatments[1] | | | | *P*-value | |
|---|---|---|---|---|---|---|
| FA | C | T30 | T40 | SEM | Linear | Quad. |
| $SCD_{16}$ | 0.04 | 0.04 | 0.04 | 0.001 | 0.9347 | 0.5316 |
| $SCD_{18}$ | $0.68^{x}$ | $0.65^{y}$ | $0.68^{x}$ | 0.006 | 0.9677 | 0.0664 |
| $SCD_{RA}$ | 0.38 | 0.39 | 0.4 | 0.007 | 0.1738 | 0.8608 |

[a,b]Means within a row with different superscripts differ ($P<0.05$); [x,y]Means within a row with different superscripts differ ($P<0.10$);[1]Treatments: C = ewes without supplementation with TAN; T30 and T40 = ewes supplemented with 30 and 40 g tannin extract/kg of concentrate, respectively (1.2 and 1.6% of estimated DMI); [2]Co-eluted with 13:0; [3]Co-eluted with 17:0 anteiso; [4]Co-eluted with 17:0 iso; [5]Saturated FA; [6]Monounsaturated FA; [7]Polyunsaturated FA; [8]Odd- and branched-chain FA with co-elutions; [9]Ratio of SFA to Unsaturated FA (UFA); [10]Calculated as (18:2 n-6 + 18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-6) ÷ (C18:3 n-3 + C20:5 n-3 + C22:5 n-3); [11]Calculated according to Kesley et al. (2003).
Fonte: produção do próprio autor, 2016.

### 5.4.2 Dairy cows experiment

Pasture depletion was around 34.5%, however paddocks had lower proportion of leguminous and consequently lower CP content (Table 13). Supplementation with tannin did not affect PMR intake, milk yield or LW of cows (Table 14).

Table 13. Chemical composition of feed and sward characterization of dairy cows experiment.

| | Third Experiment | | |
|---|---|---|---|
| Item | Pasture | Corn Silage | Concentrate[1] |
| *Chemical composition (g/kg DM)* | | | |
| DM | 212 | 338 | 912 |
| OM | 888 | 966 | 922 |
| CP | 170 | 84 | 204 |
| NDF | 539 | 359 | 286 |
| ADF | 282 | 187 | 134 |
| *Sward characterization* | | | |
| Pre-grazing sward height (cm) | 13.6 | | |
| Post-grazing sward height (cm) | 8.9 | | |
| Leguminous (% DM) | 12.7 | | |
| Fescue (% DM) | 38.4 | | |

[1]470 g/kg of corn ground, 200 g/kg of soybean meal, 280 g/kg of soybean hulls and 50 g/kg of mineral mixed.
Fonte: produção do próprio autor, 2016.

Table 14. Effect of tannin supplementation on PMR intake, live weigh and milk yield of dairy cows grazing a dominant grass pasture.

| | Treatments[1] | | | |
|---|---|---|---|---|
| Parameter | C | T15 | SEM | *P*-value |
| PMR intake (kg DM/day) | 5.8 | 5.8 | 0.001 | 0.998 |
| Milk yield (kg/day) | 14.2 | 13.1 | 0.317 | 0.329 |
| Live weight (kg) | 403.7 | 403.3 | 4.761 | 0.980 |

[1]Treatments: C = cows without supplementation with tannin extract; T15 = cows supplemented with 15 g tannin/kg of fresh PMR (1.5% of estimate DMI).
Fonte: produção do próprio autor

There were only minor changes in milk FA composition with tannin supplementation in third experiment (Table 15). These changes were restricted to a increase of 18:2 n-6 and 20:0 and a decrease of 20:2 and 24:0 FA.

Table 15. Effect of tannin supplementation on milk FA composition of grazing dairy cows supplemented with PMR.

| | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T15 | SEM | *P*-value |
| 4:0 | 4.23 | 4.26 | 0.104 | 0.9034 |
| 6:0 | 2.56 | 2.53 | 0.062 | 0.7698 |
| 8:0 | 1.42 | 1.40 | 0.034 | 0.8603 |
| 10:0 | 2.96 | 2.93 | 0.075 | 0.8727 |
| *cis*-9 10:1 | 0.33 | 0.32 | 0.009 | 0.5071 |
| 12:0 | 3.15 | 3.20 | 0.079 | 0.7566 |
| *cis*-9 12:1[2] | 0.15 | 0.16 | 0.004 | 0.6938 |
| 14:0 | 10.59 | 10.46 | 0.133 | 0.6279 |
| 14:0 iso | 0.14 | 0.14 | 0.004 | 0.6857 |
| *cis*-9 14:1 | 0.97 | 0.93 | 0.036 | 0.6117 |
| 15:0 | 0.99 | 1.04 | 0.020 | 0.1944 |
| 15:0 iso | 0.31 | 0.33 | 0.008 | 0.2140 |
| 15:0 anteiso | 0.51 | 0.52 | 0.014 | 0.7820 |
| 16:0 | 27.95 | 26.71 | 0.474 | 0.2079 |
| 16:0 iso | 0.16 | 0.20 | 0.026 | 0.5723 |
| *cis*-9 16:1[3] | 1.49 | 1.50 | 0.033 | 0.8539 |
| *trans*-9 16:1[4] | 0.47 | 0.49 | 0.009 | 0.2408 |
| 17:0 | 0.57 | 0.59 | 0.008 | 0.2147 |

Table 15. Effect of tannin supplementation on milk FA composition of grazing dairy cows supplemented with PMR.

| | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T15 | SEM | *P*-value |
| *cis*-9 17:1 | 0.20 | 0.20 | 0.005 | 0.8366 |
| 18:0 | 10.36 | 10.65 | 0.151 | 0.3502 |
| *cis*-9 18:1 | 17.04 | 17.45 | 0.375 | 0.5853 |
| *cis*-11 18:1 | 0.57 | 0.60 | 0.015 | 0.2587 |
| *cis*-12 18:1 | 0.21 | 0.22 | 0.007 | 0.3402 |
| *cis*-13 18:1 | 0.05 | 0.06 | 0.002 | 0.2837 |
| *trans*-6-8 18:1 | 0.25 | 0.25 | 0.007 | 0.9352 |
| *trans*-9 18:1 | 0.23 | 0.23 | 0.006 | 0.7241 |
| *trans*-10 18:1 | 0.34 | 0.32 | 0.012 | 0.4302 |
| *trans*-11 18:1 | 2.38 | 2.49 | 0.068 | 0.4543 |
| *trans*-12 18:1 | 0.32 | 0.33 | 0.010 | 0.6639 |
| *trans*-13-14 18:1 | 0.43 | 0.49 | 0.025 | 0.2427 |
| *trans*-9, *trans*-12 18:2 | 0.01 | 0.01 | 0.001 | 0.6239 |
| *cis*-9, *trans*-12 18:2 | 0.03 | 0.03 | 0.001 | 0.4354 |
| *trans*-9, *cis*-12 18:2 | 0.03 | 0.03 | 0.001 | 0.6239 |
| *trans*-11, *cis*-13 18:2 | 0.05 | 0.05 | 0.003 | 0.7209 |
| 18:2 n-6 | 1.38[y] | 1.56[x] | 0.042 | 0.0507 |
| *cis*-9, *trans*-11 CLA | 1.05 | 1.08 | 0.040 | 0.6323 |
| *trans*-10, *cis*-12 CLA | 0.01 | 0.01 | 0.001 | 1.000 |
| *trans*-9, *cis*-11 CLA | 0.02 | 0.02 | 0.001 | 1.000 |
| 18:3 n-3 | 0.55 | 0.58 | 0.011 | 0.1753 |
| 20:0 | 0.13[b] | 0.15[a] | 0.003 | 0.0138 |
| *cis*-11 20:1 | 0.05 | 0.05 | 0.003 | 0.5145 |
| 20:2 n-6 | 0.03[a] | 0.02[b] | 0.001 | 0.0136 |
| 20:4 n-6 | 0.13 | 0.13 | 0.004 | 0.5646 |
| 20:5 n-3 | 0.05 | 0.06 | 0.002 | 0.2114 |

Table 15. Effect of tannin supplementation on milk FA composition of grazing dairy cows supplemented with PMR.

| | Treatments[1] | | | |
|---|---|---|---|---|
| | C | T15 | SEM | *P*-value |
| 21:0 | 0.03 | 0.04 | 0.002 | 0.3256 |
| 22:5 n-3 | 0.07 | 0.08 | 0.003 | 0.1397 |
| 23:0 | 0.06 | 0.06 | 0.004 | 0.8786 |
| 24:0 | 0.024[x] | 0.020[y] | 0.001 | 0.0851 |
| Σ unidentified | 3.96 | 3.97 | 0.099 | 0.9427 |
| *Summation by origin* | | | | |
| De novo (4:0-15:0) | 28.39 | 28.30 | 0.370 | 0.9083 |
| Mixed origin (16:0+16:1) | 30.28 | 29.10 | 0.473 | 0.2322 |
| Preformed (>17:0) | 36.60 | 37.83 | 0.576 | 0.3020 |
| *Summation by saturation* | | | | |
| SFA[5] | 66.29 | 65.37 | 0.518 | 0.3857 |
| MUFA[6] | 25.66 | 26.31 | 0.448 | 0.4824 |
| PUFA[7] | 3.46 | 3.72 | 0.082 | 0.1287 |
| ΣOBCFA[8] | 3.12 | 3.26 | 0.058 | 0.2314 |
| n-6:n-3 ratio[9] | 2.59 | 2.66 | 0.061 | 0.5739 |
| SCD index[10] | | | | |
| $SCD_{14}$ | 0.08 | 0.08 | 0.003 | 0.7188 |
| $SCD_{16}$ | 0.05 | 0.05 | 0.001 | 0.2247 |
| $SCD_{18}$ | 0.62 | 0.62 | 0.005 | 0.9058 |
| $SCD_{RA}$ | 0.30 | 0.30 | 0.005 | 0.8829 |

[a,b]Means within a row with different superscripts differ ($P<0.05$); [x,y]Means within a row with different superscripts differ ($P<0.10$); [1]Treatments: C = control (without supplementation with tannin extract); T15 = cows supplemented with 1.5% of estimate DMI with tannin extract (15 g/kg of PMR); [2]Co-eluted with 13:0; [3]Co-eluted with 17:0 anteiso; [4]Co-eluted with 17:0 iso; [5]Saturated FA; [6]Monounsaturated FA; [7]Polyunsaturated FA; [8]Odd- and branched-chain FA with co-elutions; [9]Ratio of SFA to Unsaturated FA (UFA); [10]Calculated as (18:2 n-6 + 18:3 n-6 + 20:2 n-6 + 20:3 n-6 + 20:4 n-

6) ÷ (C18:3 n-3 + C20:5 n-3 + C22:5 n-3); [11]Calculated according to Kesley et al. (2003).
Fonte: produção do próprio autor, 2016.

## 5.5 DISCUSSION

The major PUFA of grazing ruminant diets is linolenic acid, which is the precursor of vaccenic and rumenic acid. Nevertheless, most of linolenic acid is BH to stearic acid (18:0) and to increase milk rumenic and vaccenic acids contents usually result to an increase in feeding costs because of the use of plant or marine oils. *In vitro* studies showed that tannin can reduce ruminal BH (Khiaosa-Ard et al., 2009; Vasta et al., 2009a) and increase muscle Δ9-desaturase protein expression in sheep (Vasta et al., 2009b). However, results from *in vitro* and *in vivo* are contradictory. While some *in vitro* studies observed positive effects on rumen vaccenic acid accumulation (Khiaosa-Ard et al., 2009; Vasta et al., 2009a), the *in vivo* studies did not observe significant and in some cases observed negative effects (Benchaar and Chouinard, 2009; Cabiddu et al., 2009; Vasta et al., 2009b).

According to milk FA results, there was a change in BH with tannin supplementation that led an increase in linoleic acid intermediates, as rumenic acid, *trans*-10, *cis*-12 CLA and its subproducts. As soybean and corn are sources of linileic acid (Zambom et al., 2012) and tannin was mixed with the concentrate, tannin probably inhibited *B. fibrisolvens* and consequently reduced BH of linoleic acid. *B. fibrisolvens* is the major bacteria in the BH of FA and can use rumenic acid, linoleic acid and *trans*-10, *cis*-12 18:2 as substrates to produce, respectively, vaccenic acid, rumenic acid or *trans*-10, *cis*-12 18:2 and *cis*-12 18:1 or *trans*-10 18:1 (McKain et al., 2010; Maia et al., 2007; Wallace et al., 2007). Furthermore, it was observed that tannin from *Acacia mearnsii* declined protozoa counts (74%) and increased bacterial counts (2.6 times; Khiaosa-Ard et al., 2009). As protozoa are important predators

of bacteria, the imbalance of bacterial population and consequently in BH can occur as a consequence of protozoa population decrease with tannin supplementation.

Buccioni et al (2015) observed a *B. fibrisolvens* increase (about 3 to 5 times) and *B. proteoclasticus* decrease (about 5 to 15 times) in ruminal population, which increased milk rumenic and vaccenic acids contents when dairy ewes were supplemented with quebracho or chestnut tannin. However, Durmic et al (2007) observed that the minimum inhibitory concentrations (MIC) of *B. proteoclasticum* was lower (<1 mg/mL) than *Butyrivibrio fibrisolvens* (10 mg/mL) with *Acacia mearnsii* tannin. Most of these differences between studies are probably due to the great diversity in the chemical structural of tannin and, consequently, the reactivity of these secondary compounds (Mueller-Harvey, 2006). As endogenous source is responsible for 85% of milk rumenic acid synthesis (Bauman et al., 1999), which manly substrate is vaccenic acid that is formed by ruminal biohydrogenation of linolenic acid (Griinari and Bauman, 1999), this supported their findings of an ruminal accumulation of vaccenic acid and formation of milk rumenic acid with *Acacia mearnsii* tannin supplementation. Nevertheless, there was no increase in milk vaccenic acid in our study and the tannin was mixed with the concentrate, indicating that higher milk rumenic acid content was because higher ruminal rumenic acid bypass produced from lower BH of linoleic acid and not from accumulation of linolenic acid intermediates.

Previous *in vitro* and *in vivo* studies reported that tannin did not stimulate alternative rumen BH pathway of linoleic acid and, therefore, no accumulation of *trans*-10 18:1 has been reported (Vasta et al., 2009a; Buccioni et al., 2011 and 2015; Toral et al., 2011; Vasta and Luciano, 2011). However, as in our study, Toral et al. (2013) observed an increase of *trans*-10 18:1 content in milk fat over time, suggesting that the effects of tannin should be evaluated in long-term experiments. The

inconsistent *trans*-10 18:1 results were attributed not only to the type and dose of tannin but also to the basal diet composition (Vasta et al., 2009b). Marine lipid supplementation shift toward to *trans*-10 18:1 formation at the expense of vaccenic acid and often resulted in milk fat depression (Shingfield et al., 2010; Toral et al., 2010). However, most of the experiments resulted in vaccenic acid concentrations always being much higher than those of *trans*-10 18:1 and no effects on milk fat content. Given the low concentration of *trans*-10, *cis*-12 18:2 and *trans*-10 18:1, no reduction in milk fat content was observed in this experiment.

When the highest tannin supplementation level of second experiment (T40) was mixed as PMR there was no feed rejection by cows. However, there were only slight changes in milk FA composition. As it was used the same tannin source in both experiments, probably the different of tannin effect on BH between both experiments was due to variation in the susceptibility of microbial populations and supplementation method (PMR or concentrate). Frutos et al (2004) observed variations in the susceptibility of microbial populations to effects of tannin (quebracho) in different ruminants species, then divergent results in milk FA composition from different ruminant species also can be expected. Furthermore, Griffiths et al. (2013) observed MUN decrease when *Acacia meanrsii* tannin was supplied to cows through drench, but they did no observe MUN difference when similar tannin supplementation levels was supplied with pellets. Despite of this study did not evaluate milk FA composition, they chose cows with similar days in milk and similar diet which suggest that supplementation method also can influence tannin nutritional effect.

## 5.6 CONCLUSION

Supplementation with 1.2% and 1.6% of estimated DMI with *Acacia mearnsii* tannin extract improved the level of healthy milk FA (*cis*-9 18:1 and some OBCFA) in dairy ewes without impairing animal performance. However, supplementation with 1.2% of estimated DMI had a negative effect on concentrate and corn silage intake which may impact the DMI and live weight. There were not large changes in milk FA composition or DMI when similar tannin level was included in a PMR for dairy cows, then it seems that dairy cows are less susceptible than ewes or supplementation method (concentrate vs PMR) can influence tannin effect.

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# 6 CONCLUSÃO GERAL

A suplementação com extrato tanífero de acácia negra (*Acacia meanrsii*) na proporção de 1 a 1.6% do CMST aumentou as concentrações de ácidos graxos benéficos à saúde humana (ácido rumêmico, 18:1 *cis*-9 e AGCIR) e aumentou a concentração de N fecal sem afetar o desepenho de ovelhas leiteiras. Quando o extrato tanífero foi misturado ao concentrado na proporção de 30g/kg, foi observado um menor consumo de concentrado e silagem de milho, o que pode afetar o CMST e desempenho animal. Já a adição do tanino em uma RPM não afetou o consumo alimentar e não foi capaz de reduzir a excreção nitrogenada, melhorar a produção ou composição química do leite de vacas Jersey. Aparentemente as vacas são menos susceptíveis ao tanino do que as ovelhas ou o método de fornecimento pode influenciar o efeito nutricional do tanino.

Futuros trabalhos deveriam investigar o efeito de doses similares deste tanino em animais de maior potencial produtivo, a inclusão deste tanino em concentrado com diferentes teores de proteína e o efeito de diferentes métodos de suplementação no desempenho animal e excreção nitrogenada.

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