GABRIELA CRISTINA GUZATTI

EFEITO DA ENSILAGEM DO TREVO VERMELHO SOBRE A UTILIZAÇÃO DO NITROGÊNIO E O PERFIL DE ÁCIDOS GRAXOS NO LEITE DE OVINOS

Tese apresentada ao Programa de Pós-graduação em Ciência Animal, da Universidade do Estado de Santa Catarina, como requisito parcial à obtenção do título de Doutor em Ciência Animal, Área de Concentração: Produção Animal.

Orientador: Henrique M.N.R. Filho

Coorientador: Vincent Niderkorn

LAGES

2017

Ficha catalográfica elaborada pelo(a) autor(a),
com auxílio do programa de geração automática
da Biblioteca Setorial do CAV/UDESC

Guzatti, Gabriela Cristina
Efeito da ensilagem do trevo vermelho sobre a utilização do nitrogênio e o perfil de ácidos graxos no leite de ovinos / Gabriela Cristina Guzatti. - Lages , 2017.
100 p.

Orientador: Henrique Mendonça Nunes Ribeiro-Filho
Co-orientador: Vincent Niderkorn
Tese (Doutorado) - Universidade do Estado de Santa Catarina, Centro de Ciências Agroveterinárias, Programa de Pós-Graduação , Lages, 2017.

1. polifenol oxidase. 2. degradação da proteína. 3. bio-hidrogenação ruminal. 4. partição nitrogenada. I. Ribeiro-Filho, Henrique Mendonça Nunes. II. Niderkorn, Vincent. , .III. Universidade do Estado de Santa Catarina, Centro de Ciências Agroveterinárias, Programa de Pós-Graduação . IV. Título.

GABRIELA CRISTINA GUZATTI

EFEITO DA ENSILAGEM DO TREVO VERMELHO SOBRE A UTILIZAÇÃO DO NITROGÊNIO E O PERFIL DE ÁCIDOS GRAXOS NO LEITE DE OVINOS.

Tese apresentada ao Programa de Pós-graduação em Ciência Animal, da Universidade do Estado de Santa Catarina, como requisito parcial à obtenção do título de Doutor em Ciência Animal, Área de Concentração: Produção Animal.

Banca examinadora:

Orientador: _____
Professor Dr. Henrique M. N. Ribeiro Filho
Universidade do Estado de Santa Catarina - UDESC

Membro: _____
Professor Dr. Luiz Gustavo Nussio
Universidade de São Paulo – USP

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

Membro: _____
Professor Dr. Dimas Estrasulas de Oliveira
Universidade do Estado de Santa Catarina – UDESC

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

Lages, 10 de julho de 2017

Dedico esta tese aos meus pais Wilson e Zenir e ao meu noivo Paulo por todo amor e compreensão!

AGRADECIMENTOS

Agradeço à Deus por me dar a cada dia força e saúde para realização dos meus projetos! A toda minha família, em especial aos meus Pais Wilson e Zenir pelo apoio e carinho sempre incondicionais, essenciais nesta caminhada. Ao Paulo, meu noivo, amigo e companheiro por todo amor, compreensão e paciência, estando sempre ao meu lado em todas as etapas percorridas durante o mestrado e doutorado. Ao professor Henrique M.N.R. Filho pela orientação, amizade e exemplo, seus ensinamentos e colaboração foram imensos! Ao professor André F. Sbrissia pela amizade e ajuda sempre valiosos e a todos os demais professores do Departamento. A Universidade do Estado de Santa Catarina pela oportunidade e estrutura disponível para realização deste Doutorado. Ao professor Gilberto Kozloski e a Universidade Federal de Santa Maria pela contribuição e parceria na realização dos experimentos. A fazenda Estrela da Serra pela acolhida, ajuda e oportunidade de realização de um experimento. A Embrapa Gado de Leite e ao pesquisador Marco Antônio S. da Gama pela parceria nas análises do leite. Ao pesquisador Vincent Niderkorn pela coorientação. A FAPESC/CAPES pela concessão da bolsa de Doutorado. Aos colegas que auxiliaram na condução dos experimentos. A todos os meus amigos pelos momentos de alegria e descontração. Enfim, meus sinceros agradecimentos a todos que de um modo ou de outro sempre estiveram comigo e torcendo por mim!

“Pensar é o trabalho mais difícil que existe, e esta é provavelmente a razão por que tão poucos se dedicam a ele.”
—Henry Ford

RESUMO

GUZATTI, Gabriela Cristina. **Efeito da ensilagem do trevo vermelho sobre a utilização do nitrogênio e o perfil de ácidos graxos no leite de ovinos.** 2017. 100 p. Tese (Doutorado em Ciência Animal – Área: Produção Animal). Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência Animal. Lages, 2017.

O emurchecimento do trevo vermelho (TV, *Trifolium pratense*) é um processo que permite a formação de *quinonas*, as quais podem diminuir a degradabilidade ruminal da proteína e a bio-hidrogenação (BH) ruminal dos ácidos graxos poli-insaturados (AGPI). O objetivo geral desta tese foi avaliar o potencial do uso da silagem de trevo vermelho em melhorar a eficiência de uso do nitrogênio (N) e o perfil de ácidos graxos produzidos no leite de ovinos. Para isso, foram conduzidos três experimentos. O primeiro ensaio avaliou os efeitos associativos de misturas de TV e uma gramínea de clima tropical (capim-quicuio, *Pennisetum clandestinum*) em diferentes proporções (0:1000, 250:750, 500:500, 750:250 e 1000:0 g/kg de MS) sobre a proteólise do material ensilado e a degradabilidade *in vitro* da proteína e da matéria orgânica (MO). O segundo ensaio avaliou o efeito de duas rações isoproteicas totalmente misturadas - compostas de silagem de trevo vermelho, silagem de milho e alimento concentrado (RC) ou silagem de alfafa *(Medicago sativa*), silagem de milho e alimento concentrado (LU) - sobre o fluxo intestinal de nutrientes, a excreção e a retenção nitrogenada em oito ovinos dotados de cânula duodenal, alojados em gaiolas metabólicas, e distribuídos num delineamento experimental de reversão simples. O terceiro ensaio avaliou o efeito das mesmas dietas do ensaio 2 sobre a produção de leite, a composição química e perfil de ácidos graxos do leite em 16 ovelhas alojadas em baias individuais e distribuídas em um delineamento experimental de blocos ao acaso. No primeiro ensaio a quantidade de N amoniacal na silagem, a degradabilidade *in vitro* e a taxa de degradação da proteína diminuíram linearmente com o aumento da proporção de TV, enquanto a produção cumulativa de gases em 24 horas de incubação aumentou quadraticamente com incrementos até 500 g/kg de TV. No segundo ensaio a digestibilidade *in vivo* da MO tendeu a diminuir no tratamento RC em comparação ao LU, mas o consumo de MO digestível foi semelhante para ambas as dietas. O fluxo intestinal de N não amoniacal foi superior enquanto a degradabilidade ruminal da proteína, a digestibilidade intestinal do N e a excreção urinária de N diminuíram na dieta RC quando comparada à LU. No ensaio 3, o consumo de MS, a produção e a composição química do leite foram semelhantes para ambas as dietas. A proporção de (AGPI) e a relação AGPI/ácidos graxos saturados (AGS) aumentaram, enquanto a relação *n6/n3* de ácidos graxos tendeu a diminuir no tratamento RC em comparação ao LU. O ácido α-linolênico (18:3 *n-3*) e o ácido linoleico (18:2 *n-6*) foram, respectivamente, 31 e 22% superiores no leite dos animais que receberam a dieta RC em comparação aos animais que receberam a dieta LU. Em conclusão, a redução na degradabilidade ruminal da proteína *in vitro* e *in vivo* associadas a evidências de menor BH ruminal dos AGPI mostram que o uso do TV na forma de silagem pode ser uma ferramenta para mitigar o impacto da excreção nitrogenada em sistemas de produção e melhorar o perfil de ácidos graxos produzidos no leite de ovinos. Ainda, efeitos sinérgicos sobre o padrão fermentativo da silagem e a produção de gás *in vitro* indicam potencial de melhoria do valor energético da dieta quando o TV é ensilado com uma gramínea de clima tropical na mesma proporção da MS total.

Palavras chave*:* degradação da proteína; bio-hidrogenação ruminal; partição nitrogenada; polifenol oxidase

ABSTRACT

GUZATTI, Gabriela Cristina. **Effect of red clover ensilage on the nitrogen use and the fatty acid profile in ewes' milk** 2017. 100 p. Thesis (Doctorate in Animal Science – Area: Animal Production. Santa Catarina State University. Post Graduate Program in Animal Science. Lages, 2017.

The wilting of red clover (*Trifolium pretense*) is a process that allows formation of quinones, which may decreases the ruminal degradable protein and ruminal biohydrogenation (BH) of polyunsaturated fatty acids (PUFA). The aim of this thesis was to evaluate the potential of red clover silage on improving the N use efficiency and fatty acids profile in dairy ewes' milk. Three experiments were conducted. The first experiment evaluated the associative effects between red clover and a tropical grass (kikuyu grass - *Pennisetum cladestinum*) mixed in different proportions (0:1000, 250:750, 500:500, 750:250, and 1000:0 g/kg of dry matter (DM)) on the proteolysis of ensiled material and *in vitro* degradation of protein and organic matter (OM). The second experiment assessed the effect of two isoproteic total mixed rations - composed by red clover silage + corn silage + concentrate feed (RC) or lucerne silage (*Medicago sativa*) + corn silage + concentrate feed (LU) - on the intestinal nutrients flow and N excretion and retention in ovine. Eight wethers (Texel×Lacaune) fitted with duodenal cannula, housed in metabolic cages and assigned to the treatments in a cross over design were used. The third experiment evaluated the effect of same diets from the second experiment on milk yield, milk composition and milk fatty acids (FA) profile, using sixteen dairy ewes housed in individual stall and distributed in a complete block design. In the first assay the ammoniacal N in the silage, *in vitro* degradation and degradation rate of protein decreased linearly with increases in red clover proportion, while the cumulative gas production at 24 hours of incubation increased quadratically when red clover was increased to 500 g/kg DM. In the second experiment the *in vivo* OM digestibility tended to decrease in RC when compared to LU treatment, however, the digestible OM intake did not differ between treatments. The intestinal non-ammoniacal N flow was greater while the ruminal degradable protein, intestinal digestibility of N and N urinary excretion were lesser in RC when compared to LU treatment. In the third experiment, DM intake, milk yield and milk composition were similar for both treatments. The total PUFA and PUFA/saturated fatty acid (SFA) ratio increased, while the *n-6/n-3* FA ratio tended to decrease in RC when compared to LU treatment. The α-linolenic (18:3 *n-3*) and linoleic (18:2 *n-6*) acids were, respectively, 31 and 22% greater in the milk of ewes receiving RC when compared those receiving LU treatment. In conclusion, the decrease in *in vitro* and *in vivo* ruminal degradability of protein associated with an evidence of decreases in ruminal BH of PUFA have showed that red clover silage may be a tool to mitigate the impact of nitrogen excretion in production systems and improves fatty acids profile in ewes' milk. Moreover, synergetic effects on silage fermentative pattern and *in vitro* gas production indicated a potential of red clover improves the energetic value of diets when ensiled with a tropical grass at the same proportion of total DM.

Key words*:* protein degradation; ruminal biohydrogenation; nitrogen partition; polyphenol oxidase

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO

O crescimento mundial da população tem exigido cada vez mais do setor agropecuário aumentos em produção para atender a demanda por alimentos. Nesse sentido, o uso em larga escala de fertilizantes e ração animal com alta concentração proteica tem gerado impactos negativos ao meio ambiente. Portanto, junto com os aumentos em produção tem crescido a preocupação com a conservação ambiental e segurança alimentar. Nesse cenário, o uso de forragens que melhorem o desempenho animal e diminuam o *input* de fertilizantes no sistema como as leguminosas tem se destacado.

As leguminosas forrageiras são capazes de realizar fixação biológica de nitrogênio (FBN) atmosférico, o que, além de reduzir os gastos com a utilização de adubos nitrogenados (HOWIESON et al., 2008) reduzem perdas importantes de nitrogênio (N) por meio de lixiviação e volatilização de adubos químicos aplicados ao solo. Entretanto, as leguminosas geralmente se caracterizam por apresentarem altos teores de proteína rapidamente degradável no rúmen. Sendo assim, quando os ruminantes consomem dietas com altos teores deste tipo de proteína, a quantidade de N livre no rúmen é maior que a capacidade de utilização pelos micro-organismos ruminais para a síntese de proteína microbiana. Esse excesso de N no rúmen pode resultar em perdas de N na ordem de 25-30% na forma de amônia, a qual é convertida em ureia no fígado e eliminada principalmente através da urina dos animais (VAN SOEST, 1994), sendo essa, uma importante fonte de poluição ambiental em sistemas intensivos de produção animal (WOODWARD et al., 2009).

No intuito de amenizar este problema, trabalhos têm investigado o uso de leguminosas que possuem compostos secundários/bioativos capazes de reduzir a excreção de N pelos ruminantes. Isto normalmente ocorre em função da menor degradabilidade ruminal desta fração (BRITO et al., 2007; BRODERICK et al., 2004), o que possivelmente elevaria o fluxo intestinal de N de origem alimentar e melhoraria a eficiência do uso do N pelos animais. Dentre os compostos secundários conhecidos os taninos condensados são conhecidos por diminuir a degradação ruminal da proteína devido sua capacidade em se complexar com proteínas dietéticas, retardando a digestão dessa fração (MC SWEENY et al., 2001). Outro composto que impede a quebra das proteínas no rúmen, são as *quinonas* formadas a partir da reação de compostos secundários, presentes no vacúolo celular, e o oxigênio atmosférico, a partir da ação da enzima polifenol oxidase (PPO), presente no cloroplasto de algumas forrageiras (LEE, 2014).

O trevo vermelho (*Trifolium pratense*) é uma planta forrageira que possui a enzima PPO (WINTERS et al., 2008), e quando utilizado na forma de silagem ou pré-secado é capaz de formar *quinonas*. As *quinonas*, são capazes de se complexarem com as proteínas e reduzir a proteólise tanto do material ensilado (JONES et al., 1995; LEE et al., 2008) quanto, posteriormente, do material no ambiente ruminal (BRODERICK et al., 2004; MERRY et al., 2006). Assim, seu uso pode melhorar o desempenho animal como resposta ao maior fluxo de N para o duodeno dos animais, além de reduzir o impacto ambiental proveniente das elevadas excreções de ureia pela urina dos mesmos.

Além das questões produtivas e ambientais, é crescente a preocupação com a qualidade dos alimentos de origem animal produzidos. Nesse sentido, a produção de alimentos de origem animal tem encontrado grandes desafios por apresentarem quantidades elevadas de ácidos graxos saturados (AGS), os quais podem ser associados a uma dieta de menor qualidade e risco elevado de doenças cardiovasculares (KATO et al., 1973; LIMA et al., 2000). Isso, é resultado do processo de bio-hidrogenação ruminal que transforma os ácidos graxos poli-insaturados, presentes em grandes quantidades na dieta animal, em saturados, de maneira que o produto final contenha altos níveis de AGS (CHILLIARD et al., 2000). Entretanto, o consumo de uma dieta com relação *n6/n3* de ácidos graxos abaixo de 5:1, além do consumo de ácido linoleico conjugado (CLA) *cis 9 trans 11* presente em produtos de origem animal, são benéficos a saúde humana (SIMOMPOULOS, 2002). Assim, a busca por alternativas que melhorem o perfil dos ácidos graxos (AG) presentes nos produtos de origem animal, ou seja, elevem a quantidade de ácidos graxos poli-insaturados e dos seus intermediários no processo de bio-hidrogenação, tem sido foco de estudos nos últimos anos.

Diversos estudos têm encontrado maiores proporções de ácido α-linolênico (18:3 *n-3*) e ácido linoleico (18:2 *n-6*) no leite/carne produzido por vacas consumindo silagem de trevo vermelho em comparação a outras dietas (ADLER et al., 2013; AL MABRUCK et al., 2004; LEE et al., 2009a,b; VANHATALO et al., 2007). Resultados neste sentido têm sido justificados em função da menor bio-hidrogenação ruminal dos ácidos graxos poli-insaturados, uma vez que as *quinonas* impedem a lipólise inicial desses ácidos graxos, que não se tornam livres para serem bio-hidrogenados (BUCCIONI et al., 2012; CABIDDU et al., 2014; LEE; 2014). Contudo, avaliações em ovinos são menos frequentes.

Assim, o objetivo geral desta tese foi avaliar o potencial da silagem de trevo vermelho em melhorar a eficiência de uso do N e o perfil de ácidos graxos produzidos no leite de ovinos. Para isso, foram realizados três experimentos. O primeiro avaliou em laboratório os efeitos associativos de misturas de trevo vermelho e uma gramínea de clima tropical sobre a proteólise

do material ensilado e a degradabilidade *in vitro* da proteína e da matéria orgânica. O segundo ensaio objetivou quantificar o fluxo intestinal de nutrientes, bem como a excreção e retenção nitrogenada de ovinos recebendo dietas isoproteicas, contendo silagem de trevo vermelho ou alfafa (*Medicago sativa*; leguminosa sem presença de compostos secundários). O terceiro ensaio avaliou a produção de leite, a composição química e o perfil de ácidos graxos do leite produzido por ovelhas consumindo as mesmas dietas do segundo ensaio.

2 REVISÃO DE LITERATURA

2.1 O TREVO VERMELHO E A POLIFENOL OXIDASE

O trevo vermelho é uma leguminosa forrageira com hábito de crescimento ereto e raízes profundas. Suas folhas e caule possuem pilosidade característica, sendo as folhas marcadas com um "V" de cor branca (HANNAWAY, 2004) e as inflorescências aglomerados de flores roxas e tubulares. Além disso, apresenta grande relevância em regiões de clima temperado e subtropical de altitude, sendo considerado uma forrageira altamente produtiva, com elevado valor nutritivo e boa capacidade de fixação biológica de nitrogênio (N). Seu crescimento máximo ocorre em temperaturas entre 18 e 25°C podendo ser utilizada para pastejo ou colhida e conservada como feno e silagem.

O trevo vermelho é uma forrageira que se caracteriza por possuir polifenol-oxidase (PPO), uma enzima presente no cloroplasto celular que catalisa a oxidação de compostos secundários em *quinonas*. Contudo, a grande maioria desta enzima se encontra na planta em sua forma latente (90%) (LEE et al., 2013), sendo necessário que a massa de forragem sofra algum tipo de dano (mastigação, corte, murcha, entrada de patógenos) para que ocorra sua ativação, contato com oxigênio atmosférico e posterior formação das *quinonas*. Assim, durante o processo de ensilagem do material são atendidas as condições necessárias a sua formação. Isso porque, durante o corte ou murcha do material o conteúdo celular (compostos secundários) é exposto ao oxigênio atmosférico e sendo essa reação catalisada pela enzima PPO formam-se as *quinonas*. Este composto bioativo é caracterizado por reduzir a degradação da proteína durante a ensilagem e no ambiente ruminal e a lipólise dos ácidos graxos poli-insaturados no rúmen (LEE et al., 2004, 2011, 2013; LEE, 2014; VANHATALO et al, 2009), sendo portanto, seu uso na forma de silagem, uma alternativa em sistemas de produção animal.

Na planta, a formação de *quinonas* ocorre como mecanismo de defesa contra a ação de fitopatógenos. Embora não pareçam essenciais - porque aparentemente não possuem relação direta com metabólicos de crescimento, respiração e fotossíntese - os compostos secundários do trevo vermelho e seus produtos (compostos fenólicos) apresentam vantagens para defesa e perpetuação da espécie (SANTOS, 2004). Isso ocorre pelo fato de serem substâncias fungitóxicas, antibacterianas e antiviróticas (LO & NICHOLSON et al., 2008). Durante o

processo de infecção da planta, o qual ocorre pela entrada de fitopatógenos, a descompartimentalização celular permite a exposição do conteúdo presente no vacúolo celular e elevação da atividade da enzima PPO (AGRIOS, 1997). Assim, quando o tecido da planta é infectado ocorre exposição do conteúdo celular, entrada de O_2 e ativação da PPO, o que permite a formação de *quinonas*. Estas, se complexam com N celular reduzindo sua disponibilidade para o patógeno, e o processo de infecção cessa nesses tecidos. Portanto, a planta gasta energia para fazer e armazenar compostos secundários como um mecanismo de defesa aprimorado ao longo do processo evolutivo.

2.2 O PROCESSO DE ENSILAGEM E A AÇÃO DA POLIFENOL OXIDASE

A produção de forragem é caracterizada por uma distribuição desuniforme ao longo dos meses com períodos de máxima produtividade e outros de estacionalidade produtiva. Assim, conservar a forragem produzida em excesso durante os meses de maior taxa de acúmulo torna-se uma alternativa para alimentação dos animais nos meses em que a produção forrageira é escassa. A produção de uma silagem de qualidade irá depender de fatores como o teor de umidade, quantidade de carboidratos solúveis e capacidade tampão do material. Por exemplo, materiais com alta capacidade tampão, dificultam abaixamento do pH da massa ensilada e a produção de microrganismos benéficos são dificultados (PEREIRA et al., 2008).

Após o fechamento de um silo três fases distintas ocorrem até a sua completa estabilização. Na primeira, denominada fase aeróbica, ocorre consumo do oxigênio que não foi expulso pela compactação. Quando o oxigênio no material ensilado se esgota inicia-se a segunda fase, chamada anaeróbica, onde os micro-organismos anaeróbicos começam a crescer formando ácido acético + etanol + ácido lático + CO_2 decorrentes da fermentação das hexoses (glicose e frutose) e pentoses (xilose e ribose), baixando o pH do ambiente. Após a queda do pH se inicia a terceira fase, a fase de estabilização. Nesta fase, os baixos valores de pH inibem o crescimento da população de bactérias indesejáveis, interrompendo assim os processos de fermentação e iniciando a fase de estabilidade que se prolongará até que o silo seja aberto. Quanto mais rápido se completar o processo fermentativo, mais nutrientes (peptídeos e aminoácidos) serão preservados, melhorando o valor nutritivo da silagem (PEREIRA et al., 2008). Contudo, mesmo atendendo as exigências para um bom processo fermentativo algumas perdas são inevitáveis. Durante o processo de ensilagem a degradação proteica por enzimas das

plantas e a ação de bactérias transformam proteína verdadeira a nitrogênio não proteico (NNP) (RODRIGUEZ et al., 1998). Esta transformação resulta em uma silagem com grande parte de seu N na forma de amônia. Além disso, a produção de grandes quantidades de N-solúvel altera o curso da fermentação da silagem, uma vez que os aminoácidos básicos, aminas e N-amoniacal, produtos da hidrólise proteica, podem retardar a queda do pH e interferir na qualidade da silagem produzida.

Autores que estudaram a transformação de proteína a NNP durante a ensilagem, observaram que no trevo vermelho a proteólise da massa ensilada era menor que nos demais tipos de forragens (PAPADOPOULOS e McKERSIE, 1983). Estudos posteriores que buscaram entender os motivos pelo qual a proteólise diminuía com a presença do trevo vermelho associaram–na à presença da enzima PPO e formação das *quinonas* que reagiam rapidamente com as proteases e substratos de proteína reduzindo a proteólise do material ensilado (HATFIELD e MUCK, 1999; JONES et al., 1995; LEE et al., 2008) (Tabela 1).

É necessário considerar que o trevo vermelho caracteriza-se por ser uma planta de difícil ensilagem visto que é uma leguminosa com elevada capacidade de tamponamento. Assim, para o sucesso da silagem de trevo vermelho processos como a pré-secagem (murchamento) do material e o uso de inoculante apropriado são extremamente importantes por favorecerem a fermentação e a qualidade da silagem (DARDNI, 2010).

Trabalhos que estudaram o momento de corte do trevo vermelho sobre a qualidade do material produzido ainda não são conclusivos. Segundo Wiersma et al. (1998) menor quantidade de proteína bruta (PB) e maior quantidade de fibra em detergente neutro (FDN) ocorre quando o material é colhido na primavera em comparação a cortes realizados no verão. De outra forma, Grabber (2009) encontrou valores de PB inferiores no corte feito no verão quando comparado ao corte realizado na primavera.

Estudos que determinaram o tempo e as condições de pré-secagem até o momento da ensilagem mostram que uma pré-secagem excessiva pode levar a uma grande perda de nutrientes, bem como a quebra e perda de folhas muito secas durante o processo de ensilagem. Já a condição de pré-secagem: secagem 100% na sombra ou totalmente ao sol, não influenciou na qualidade do material (OWENs et al.,1998). Ademais, o processo de pré-secagem antes da ensilagem aumenta atividade da enzima PPO no trevo vermelho (VAN RANST et al., 2010). Lee et al. (2008) estudando o efeito do uso de inoculantes, aplicados durante a ensilagem, sob a atividade da enzima PPO em forragem de trevo vermelho, não encontraram diferenças em sua ação, entretanto, os materiais inoculados apresentaram pH inferior e melhor qualidade quando comparado ao material sem inoculante.

Por fim, considerando que a ativação da enzima PPO é dependente de danos mecânicos e da presença de oxigênio e que esses pressupostos são facilmente alcançados durante a ensilagem estudos avaliaram se o uso do trevo vermelho em pastejo (considerando tempo de mastigação e deglutição até a chegada no ambiente anaeróbico do rúmen para formação das *quinonas*) reduziria a degradação proteica no rúmen (LEE et al., 2009c; LEE et al., 2011). Os autores concluíram que o processo de mastigação é suficiente para ativação desta enzima, mas que o tempo de exposição da enzima ao oxigênio foi considerado limitante para completa formação de *quinonas*, restringindo assim sua atuação na redução das perdas de nitrogênio pelo animal. Desta forma, estudos que buscam compreender os efeitos da PPO (*quinonas*) fazem uso do material na forma de silagem.

Tabela 1 - Comparação do trevo vermelho *versus* outra forragem na redução da proteólise durante a ensilagem e sua associação com a enzima polifenol oxidase (PPO).

	Forragem	Proteólise	PPO
Papadopoulos e McKersie, 1983	Alfafa	↓	
Owens et al., 1998	Alfafa	↓	*
Al-brecht e Muck, 1991	Alfafa	↓	
Jones et al., 1995	Alfafa	↓	*
Sullivan e Hatfield, 2006	Alfafa	↓	*
Lee et al., 2008	Trevo vermelho ↓*PPO*	↓	*
Krawutscke et al., 2013	Azevém	↓	*

↓ Redução na proteólise do material ensilado com uso do trevo vermelho; * associação entre a PPO presente no trevo vermelho e a redução na proteólise do material.

2.3 UTILIZAÇÃO DAS PROTEÍNAS E EFEITO DA POLIFENOL OXIDASE

As proteínas chegam ao rúmen como compostos de elevado peso molecular que precisam ser degradados para que possam ser utilizadas pelos micro-organismos do rúmen e posteriormente pelo animal. A degradação inicial envolve a hidrólise das moléculas proteicas a oligopeptídeos, dipeptídeos e aminoácidos, através da ação de enzimas (proteases, peptidases e desaminases) secretadas pelas plantas ou pelos micro-organismos ruminais (KOZLOSKI, 2009). As menores frações (aminoácidos e oligopeptídeos com até cinco resíduos) podem entrar na célula bacteriana e então serem incorporados em proteínas ou serão desaminados e metabolizados a ácidos graxos voláteis.

A proteína que chega ao rúmen pode ser dividida em duas frações, a proteína degradável no rúmen (PDR) e proteína não degradável no rúmen (PNDR). A PDR é composta de proteína verdadeira e nitrogênio não proteico (NNP). A proteína verdadeira pode ser degradada em peptídeos e aminoácidos que podem ser desaminados ou incorporados à proteína microbiana. O destino dos aminoácidos livres na célula depende de fatores como a disponibilidade de substratos energéticos, espécie bacteriana, taxa de crescimento e perfil dos aminoácidos disponíveis. O NNP é rapidamente e totalmente degradado no rúmen, e é composto de ácidos nucléicos, amônia (NH_3), aminoácidos, e pequenos peptídeos. Os peptídeos, aminoácidos e NH_3 são utilizados como fonte de N pelos micro-organismos do rúmen (BACH et al., 2005), sendo a incorporação destes compostos altamente variável. Como exemplo, de 40 a 95% de proteína microbiana pode vir da incorporação de amônia e de 5 a 60% de aminoácidos e peptídeos, sendo que a relação volumoso:concentrado pode influenciar diretamente a proporção desta incorporação (KOZLOSKI, 2009). A PNDR é oriunda da proteína verdadeira de origem alimentar que por algum motivo não foi degradada no rúmen e pode significar uma segunda fonte de aminoácidos que chega ao intestino delgado e é disponibilizada para os ruminantes (SANTOS, 2006).

Após a saída do rúmen as proteínas de origem microbiana e dietética têm seu processo de digestão química no abomaso iniciado. Isto ocorre pela ação de diferentes enzimas, as quais hidrolisam componentes de parede celular das bactérias auxiliando a pepsina na digestão das proteínas. Os compostos nitrogenados que chegam ao intestino de um ruminante são provenientes da proteína de origem microbiana (originada da PDR, entre 55 a 80% do N disponível no intestino) e da proteína de origem alimentar que não foi degradada no rúmen (PNDR, entre 15 a 40%). Estas duas frações compõem a proteína metabolizável (PM) ou proteína digestível no intestino que será utilizado pelo animal.

2.3.1 Fracionamento proteico

A proteína bruta (PB) dos alimentos pode ser fracionada de acordo com sua degradabilidade ruminal em fração A, fração B_1, fração B_2, fração B_3 e fração C. A fração A é solúvel em tampão borato-fosfato e é rapidamente e completamente degradada no rúmen, sendo composta principalmente por nitrogênio não proteico. Na fração B da proteína estão inseridas as denominadas proteínas verdadeiras, sendo divididas em três subfrações (B_1, B_2 e B_3) de acordo com sua taxa de degradabilidade ruminal. A subfração B_1 é composta de proteínas verdadeiras de alta degradabilidade ruminal, por isso também é solúvel em tampão borato-

fosfato e rapidamente degradada no rúmen, porém precipita em contato com o ácido tricloroacético (TCA). As proteínas que compõem a subfração B_2 são fermentadas no rúmen, mas uma parte é degradada e outra passa para o intestino delgado, dependendo de suas taxas de degradação e de passagem. A subfração B_3 é composta por proteínas que são insolúveis em detergente neutro, mas são solúveis em detergente ácido. Estas proteínas são lentamente degradáveis no rúmen por estarem associadas às paredes celulares. Por fim, fazem parte da fração C da proteína alimentar as proteínas que são insolúveis em detergente ácido. Estas proteínas são consideradas indisponíveis porque elas estão associadas com lignina, complexadas com taninos ou ainda como produto da reação de Maillard, o que as tornam muito resistentes à ação dos micro-organismos ruminais e de enzimas secretadas por mamíferos. Por este motivo, as proteínas da fração C não fornecem aminoácidos pós-rúmen, de tal forma que não podem ser utilizadas pelos ruminantes (SNIFFEN et al., 1992).

2.3.2 Excesso de PDR

Conhecer os níveis ideais de PDR e PNDR exigida para cada categoria animal e a degradação proteica dos alimentos oferecidos permite o fornecimento de dietas balanceadas. Os níveis ideais de PDR são aqueles que suprem as exigências das bactérias permitindo um crescimento microbiano ótimo. Após o adequado suprimento de PDR, é necessário identificar as quantidades exigidas de PNDR para o atendimento das exigências nutricionais dos ruminantes para obtenção de melhores desempenhos (NRC, 2001).

O fornecimento de dietas que contenham alta concentração de proteína solúvel e rapidamente degradável (basicamente fração A e B1 da proteína), podem resultar em elevada perda de N, principalmente através da urina dos animais, e consequentemente elevar a poluição ambiental em sistemas de produção (DEWHURST et al. 2010). Isso ocorre porque as bactérias ruminais possuem capacidade limitada de uso da amônia que é liberada pela degradação das proteínas na luz ruminal, sendo ainda altamente dependente da quantidade de energia disponível. Assim, quando a liberação de amônia na luz ruminal é alta, uma parte não conseguirá ser utilizada pelas bactérias ruminais sendo absorvida pelo epitélio ruminal e levada ao fígado através da circulação portal. No fígado, a amônia é transformada em ureia que será eliminada pela urina dos animais ou irá retornar ao trato gastrointestinal via saliva ou transepitelial. Contudo, a quantidade de amônia carreada para o fígado pode, em alguns casos, superar a capacidade deste órgão em metabolizá-la acarretando na elevação de sua concentração na circulação geral onde é tóxica em níveis superiores a 2 mg/dL. Assim, o uso de dietas

contendo altas concentrações de proteína com elevada taxa de degradação no rúmen são um problema tanto do ponto de vista econômico como ambiental. Assim, a redução da PDR em excesso e elevação da PNDR pode ser uma alternativa para elevar os índices zootécnicos.

2.3.3 Alternativas que elevam a PNDR

O estudo de técnicas capazes de reduzir a degradação ruminal da proteína em excesso, elevando a quantidade de PNDR e o aporte de aminoácidos para o intestino delgado vem se destacando na área de nutrição de ruminantes. Diversas são as maneiras de modificar a degradação da proteína no ambiente ruminal. Por exemplo, o tratamento térmico é uma alternativa bem conhecida e visa reduzir a solubilidade da proteína (KAMALAK et al., 2005), porém quando o tratamento térmico é realizado com aquecimento ou tempo excessivos, provocam a formação de complexos proteicos (reação de Maillard), que não podem mais ser utilizados pelo animal (McNIVEN et al., 2002). Outra alternativa, se baseia em aumentar a taxa de passagem do alimento pelo rúmen o que reduziria a quantidade de proteína degradada como resposta ao menor tempo de fermentação ruminal. Outra possibilidade são os chamados compostos fenólicos/bioativos, os quais estão naturalmente presentes em algumas plantas e são capazes de aumentar o fluxo de PNDR para o intestino por reduzirem a degradação ruminal da proteína.

Neste sentido, os taninos são compostos fenólicos que agem dificultando a aderência dos microrganismos às partículas de alimento, mas sua habilidade em se complexar com proteínas dietéticas é a principal causa do retardamento na digestão ruminal dessa fração (FRUTOS et al., 2004; Mc SWEENY et al., 2001; WAGHORN e MCNABB, 2003). Outro composto conhecido por se complexar com proteínas e diminuir sua degradação ruminal são as *quinonas* (LEE et al., 2003a; VANHATALO et al., 2006; 2009). As *quinonas* são formadas em plantas que se caracterizam por possuírem a enzima PPO que na presença de oxigênio transforma o-fenóis (presentes no vacúolo celular) em o-*quinonas* (composto bioativo). O trevo vermelho é uma leguminosa forrageira que possui esta enzima e portanto, pode ser capaz de reduzir a PDR quando fornecido como silagem.

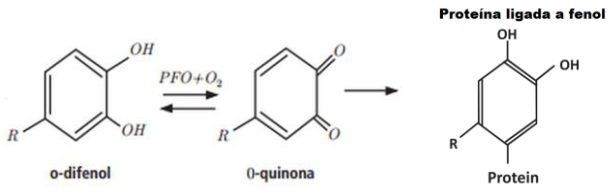
2.3.4 O efeito da polifenol oxidase sob a degradabilidade proteica em ruminantes

As dietas fornecidas aos ruminantes geralmente possuem elevados níveis de PDR. Isso, geralmente resulta em N disponível no rúmen acima da capacidade de utilização pelos micro-

organismos ruminais, o que além de, gerar perdas deste nutriente para o meio ambiente pode levar a uma redução no desempenho animal devido ao comprometimento da oferta de proteína metabolizável. Nessa lógica, estudos mostram que a enzima PPO pode reduzir a excreção de compostos nitrogenados pelos animais minimizando parte dos impactos ambientais causados pelo sistema de produção, além de representar uma alternativa para reduzir a PDR e melhorar a utilização de N pelos ruminantes.

A enzima PPO pertence ao grupo das oxirredutases contendo o cobre como grupo prostético e está envolvida nas reações de escurecimento das plantas quando estas são cortadas ou esmagadas e expostas ao oxigênio. Esta enzima encontra-se no cloroplasto das células e, na presença de oxigênio, catalisa a reação de oxidação de fenóis endógenos, localizados no vacúolo celular, para *quinonas* (MACHEIX et al., 1991). As *quinonas* são moléculas eletrofílicas altamente reativas, que modificam de forma covalente uma variedade de constituintes celulares nucleofílicos, tais como proteínas, aminas e amidas, os quais conduzem à formação dos pigmentos de melanina (BROWN, 1983) (proteínas ligada a fenol) (Figura 1). A redução na proteólise ruminal ocorre através da complexação das *quinonas* com proteínas foliares reduzindo sua solubilidade e degradabilidade (WINTERS e MINCHIN, 2001) ou pela inibição da ação das proteases. Contudo, de maneira geral, devido à grande quantidade de proteases no rúmen, acredita-se que a redução da proteólise aconteça mais pela complexação com proteínas foliares do que pela desnaturação das proteases *per se* (LEE, 2014).

Figura 1. Esquema enzimático da polifenol oxidase e via de auto-oxidação para *o –quinona* e posterior transição para proteína ligada a fenol.



Fonte: (Adaptado de LEE et al., 2013).

Trabalhos que avaliaram a silagem de trevo vermelho *versus* silagem de alfafa encontraram, de forma geral, que a quantidade de PNDR se eleva e há uma tendência de maior eficiência de uso do N nas dietas com silagem de trevo vermelho. Contudo, a maioria dos trabalhos não forneceram dietas isoproteicas, e apenas um deles determinou o fluxo intestinal de nutrientes (Tabela 2). Broderick et al. (2001) trabalhando com vacas em lactação e

fornecendo silagem de alfafa ou trevo vermelho não encontraram diferença na produção de leite embora sugiram maior eficiência de uso do N nos animais que consumiam silagem de trevo vermelho. Mais tarde, Broderick et al. (2007) conduziram experimento comparando silagem de alfafa controle ou tratada com ácido fórmico *versus* a silagem de trevo vermelho e encontraram redução na excreção urinária de N para o meio ambiente e tendência de melhoria na eficiência de utilização do N com uso da silagem de trevo vermelho, embora, não tenham detectado diferença quando a eficiência de utilização do N foi expressa em quilogramas (kg) de leite produzido por kg de N total excretado. Em trabalho conjunto com Broderick et al. (2007), Brito et al. (2007) mediram o fluxo omasal de nutrientes e encontraram aumento na quantidade de proteína que não foi degradada no rúmen e no fluxo de N não amoniacal não microbiano (NNANM) nos animais que receberam a silagem de trevo vermelho.

Tabela 2. Trevo vermelho *versus* alfafa sobre o consumo de MS (CMS), quantidade de proteína não degradável no rúmen (PNDR), fluxo de nitrogênio (N) não amoniacal não microbiano (NNANM), produção de leite (PL), N excretado na urina (NEU) e eficiência de uso do N (EUN g N ingerido/g N no leite).

	CMS	PNDR	NNANM	PL	NEU	EUN
Brito et al., 2007	=	↑	↑	-	-	-
Broderick et al., 2007	↓	↑	-	=	↓	↑
Broderick et al., 2001	↓	-	-	=	-	↑
Broderick et al., 2000	↓	-	-	↓	-	-
Hoffman et al., 1997	=	-	-	=	-	-

↑ Superior no trevo vermelho; ↓Redução no trevo vermelho; = sem diferença entre as espécies; - variável não medida.

A maior parte dos trabalhos que estudaram a silagem de trevo vermelho à compararam a uma silagem de gramínea. Em geral, o fluxo de NNANM e a PNDR se elevaram com uso da silagem de trevo vermelho. Já os valores da eficiência de síntese de proteína microbiana bem como de produção de leite são ainda divergentes (Tabela 3). Vanhatalo et al. (2009) forneceram, para vacas em lactação, dietas contendo silagem de festuca (*Festuca pratensis*) ou trevo vermelho e encontraram elevação na PNDR e no fluxo intestinal de NNANM, nos animais que receberam silagem de trevo vermelho, porém com a mesma produção de leite. Assim, considerando que houve tendência de uma menor ingestão de MS na dieta de trevo vermelho, mas não houve diminuição na produção de leite, os autores sugerem uma maior eficiência no uso dos nutrientes nesta dieta. É importante destacar que nos trabalhos que compararam silagem de trevo vermelho *versus* silagem de gramíneas vários outros fatores resultado da interação entre as espécies podem ter influenciado nos resultados. Por exemplo, sabe-se que as

leguminosas favorecem o consumo voluntário e elevam a taxa de passagem ruminal em relação as gramíneas (KAMMES E ALLEN, 2012; THORNTON E MINSON, 1973), o que pode ter favorecido as elevações no fluxo de NNANM e redução na PNDR ao invés de efeitos resultantes da ação da enzima PPO.

Tabela 3. Consumo de MS (CMS), quantidade de proteína não degradável no rúmen (PNDR), fluxo de N não amoniacal não microbiano (NNANM), produção de leite (PL) e eficiência de síntese de proteína microbiana (ESPM) da silagem de trevo vermelho em comparação com gramíneas de clima temperado.

	Forragem	CMS	PNDR	NNANM	PL	ESPM
Merry et al., 2006	Azevém	↑	↑	↑	-	↓
Vanhatalo et al., 2009	Festuca	↓	↑	↑	=	↑
Moorby et al., 2009	Azevém	↑	-	-	↑	-
Bertilsson e Murphy, 2003	Azevém	↑	-	-	↑	-
Dewhurst et al., 2003	Azevém	↑	-	-	↑	-
Halmemies-Beauchet-Filleau et al., 2014	Festuca	↑	↑	↑	=	↓

↑ Superior no trevo vermelho; ↓ Inferior no trevo vermelho; = sem diferença entre as espécies; - variável não medida.

2.4 UTILIZAÇÃO DOS LIPÍDEOS E EFEITO DA POLIFENOLOXIDASE

Os lipídios são substâncias insolúveis em água, mas solúveis em solventes orgânicos. São formados por ésteres de glicerol (um álcool que possui 3 carbonos) e ácidos graxos. Os principais lipídios encontrados nos alimentos são os triglicerídeos (sementes), galactolipídeos e fosfolipídeos (folhas) (Mc DONALD et al., 2010). Após a ingestão pelos animais, os lipídeos são altamente degradados por lipases associadas a membrana celular das bactérias, liberando glicerol, galactose e ácidos graxos de cadeia longa (saturados e insaturados). O glicerol e a galactose entram na célula bacteriana e são metabolizados a ácidos graxos voláteis, enquanto os ácidos graxos de cadeia longa insaturados estão sujeitos ao processo de bio-hidrogenação. O processo de bio-hidrogenação ocorre pela ação de isomerases e redutases, as quais hidrogenam ácidos graxos insaturados, como α-linolênico (C18:3) e linoleico (C18:2), a esteárico (C18:0). Durante a bio-hidrogenação os ácidos graxos que se encontram na sua forma insaturada recebem hidrogênio livre do ambiente ruminal e saturam suas ligações ficando apenas com ligações simples, ou seja, tornando-se ácidos graxos saturados. Este processo ocorre

provavelmente porque os ácidos graxos poli-insaturados são tóxicos para as bactérias ruminais e/ou como forma de drenar equivalentes de redução do ambiente ruminal.

Estudos mostram que diferentes alimentos e dietas podem alterar a bio-hidrogenação ruminal e seus intermediários (ADLER et al., 2013; HALMEMIES-BEAUCHET-FILLEAU et al., 2013; LEE et al., 2006, 2007, 2009a; LOOR et al., 2003; LOURENÇO et al., 2008). Por exemplo, o aumento da proporção de concentrado na dieta diminui a lipólise e a bio-hidrogenação aumentando a proporção de ácidos graxos insaturados do grupo *trans-10* devido a mudanças do valor de pH ruminal e das espécies de bactérias que constituem a massa microbiana. O uso de sais de cálcio insolúvel podem prevenir a bio-hidrogenação ruminal, isto porque, para ser bio-hidrogenado o grupo carboxila do ácido graxo necessita estar livre e os sais de cálcio se ligam ao grupo carboxila e impedem a lipólise desta gordura (KOZLOSKI, 2009). Além disso, a presença de compostos secundários/bioativos de plantas, como os taninos e a *quinona*, também podem proteger os lipídios contra a lipólise e posterior bio-hidrogenação ruminal e elevar as concentrações de ácidos graxos insaturados no leite e na carne (LEE et al., 2009a; ADLER et al., 2013).

Considerando o consumo de uma dieta adequada em relação ao seu perfil em ácidos graxos, é preciso notar que a ingestão de ácidos graxos essenciais é indispensável, sendo os ácidos graxos ômega 6 e ômega 3 considerados essenciais. A relação ômega 6/ ômega 3 (*n6/n3)* da dieta consumida é mais importante que a quantidade individual de cada um deles (RUSSO, 2009). A relação média das dietas normalmente consumidas pelos seres humanos é de aproximadamente 15:1 (SIMOPOULOS, 2002), o que pode ser considerada muito elevada. De outra forma, relações abaixo de 5:1 são consideradas saudáveis (INSTITUTE OF MEDICINE).

O consumo de uma dieta com elevada relação *n6/n3* de ácidos graxos é associado a diversas doenças, incluindo doenças cardiovasculares, autoimune e câncer (SIMOPOULUS, 2006). A elevação no consumo de ômega 3, bem como a melhor relação *n6/n3* da dieta resulta em efeitos supressivos destas doenças (SIMOPOULUS e CLELAND, 2003). Os efeitos benéficos ou deletérios associados ao consumo desses ácidos graxos decorre do fato de que ambos são capazes de modificarem a expressão gênica de enzimas associadas a estas doenças. Além do consumo equilibrado de *n6/n3* o consumo de ácido linoleico conjugado (CLA) *cis 9 trans 11* tem sido apontado como anti-carninogênico e redutor do acúmulo de gordura corporal como resposta a sua atuação na enzima lipase do tecido adiposo (WILLIAMS, 2000).

2.4.1 Lipólise e bio-hidrogenação ruminal sob ação da polifenol oxidase

Estudos recentes tem encontrado redução da bio-hidrogenação ruminal em dietas contendo silagem de trevo vermelho em comparação a outras dietas (ADLER et al., 2013; BUCCIONI et al., 2012; CABIDDU et al., 2014; VAN RANST et al., 2011). A menor bio-hidrogenação resulta em uma maior proporção de ácidos graxos poli-insaturados e intermediários da bio-hidrogenação e menores proporções de ácidos graxos saturados no produto final. Trabalhos que estudaram a silagem de trevo vermelho encontraram de maneira geral maiores quantidades de ácidos graxos C18:2 e C18:3 chegando ao duodeno, no leite e carne dos animais alimentados com silagem de trevo vermelho (Tabela 4).

A ação da enzima PPO inicia com a formação do composto *quinona*, o qual, age na proteção lipídica dos ácidos graxos poli-insaturados no rúmen. De acordo com Lee et al. (2003b) a proteção acontece pela desativação da enzima lipolítica e formação de complexos entre os fenóis e os lipídeos. Al Mabruk et al. (2004) trabalhando com diferentes tipos de forragem ensilada (trevo vermelho, trevo branco, alfafa) encontraram maiores proporções de ácidos graxos poli-insaturados no leite de vacas recebendo dietas a base de trevo vermelho. Contudo, encontraram elevação na deterioração oxidativa deste leite como resultado da elevação na proporção de C18:3, porém, este problema é minimizado com a suplementação extra de vitamina E aos animais.

Tabela 4. Silagem de trevo vermelho em comparação com outra forragem sobre os valores de $C_{18:2}$ e $C_{18:3}$ no leite, carne e fluxo pós rúmen.

	Forragem	$C_{18:2}$	$C_{18:3}$
Leite de vacas			
Dewhurst et al., 2003	Azevém	↑	↑
Dewhurst et al., 2003	Trevo branco	=	=
Vanhatalo et al., 2007	Festuca	↑	↑
Bio-hidrogenação in vitro			
Lee et al., 2007	Trevo vermelho↓PPO	↑	↑
Carne de bovinos			
Lee et al., 2009a	Azevém	=	↑
Fluxo pós rúmen			
Loor et al., 2003	Dáctilis	↑	↑
Lee et al., 2003b	Trevo branco	↓	↓
Lee et al., 2003b	Gramínea	↑	↑

↑ Superior no trevo vermelho; ↓ Inferior no trevo vermelho; = sem diferença entre as espécies.

Dewhurst et al. (2003) trabalhando com silagem de trevo vermelho ou azevém encontraram elevação nos níveis do ácido graxo poli-insaturado no leite de vacas alimentadas com trevo vermelho. Da mesma forma, Lee et al. (2003b) trabalhando com touros encontraram redução da bio-hidrogenação ruminal dos ácidos graxos C18:3 e C18:2 quando foram alimentados com silagem de trevo vermelho em relação à gramínea. Outros trabalhos demostraram elevação na concentração de fitoestrógenos do grupo das isoflavonas em dietas contendo silagem de trevo vermelho (STEINSHAMN et al., 2008; ANDERSEN et al., 2009; MUSTONEN et al., 2009), sendo o aumento da concentração do fitoestrógeno Equol benéfico a saúde humana (LUND et al., 2004). Estes resultados sugerem que ruminantes alimentados com dietas que contenham a enzima PPO, na forma de silagem, podem melhorar a qualidade do produto final oferecido ao consumidor.

2.5 REFERÊNCIAS

ADLER, S. A. et al. Effect of silage botanical composition on ruminal biohydrogenation and transfer of fatty acids to milk in dairy cows. **Journal of Dairy Science,** p.1135-1147, 2013.

AGRIOS, G. N. **Plant pathology**. San Diego: Academic Press, p. 635, 1997.

ALBRECHT, K. A.; MUCK. R. E. Proteolysis in ensiled forage legumes that vary in tannin concentration. **Crop Science**, p. 464–469, 1991.

AL MABRUK R.M.; BECK N.F.G.; DEWHURST R.J. Effects of silage species and supplemental vitamin E on the oxidative stability of milk. **Journal of Dairy Science**, p. 406-412, 2004.

ANDERSEN C. et al. Effect of forage on the content of phyto-oestrogens in bovine milk. **Animal,** p. 617-622, 2009.

BACH, A.; CALSAMIGLIA, S.; STEREN, M. D. Nitrogen metabolism in the rumen. **Journal of Dairy Science**, p. E9-E21, 2005.

BERTILSSON, J.; MURPHY, M. Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. **Grass and Forage Science**, p. 309-322, 2003.

BRITO, A. F.; BRODERICK, G. A.; REYNAL, S. M. Effects of different protein supplements on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. **Journal of Dairy Science**, p. 1828–1841, 2007.

BROWN, C. R. Banana polyphenol oxidase. **School Science Review**, p. 690–695, 1983.

BRODERICK, G. A., WALGENBCAH, R. P.,STERRENBURG, E. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. **Journal of Dairy Science,** 83, 1543–1551, 2000.

BRODERICK, G. A.; WALGENBACH,R. P.; MAIGNAN, S. Production of Lactating Dairy Cows Fed Alfalfa or Red Clover Silage at Equal Dry Matter or Crude Protein Contents in the Diet. **Journal of Dairy Science**, p. 1728–173, 2001.

BRODERICK, G. A. et al. Genetic variation in red clover for rumen protein degradability. **Animal Feed Science and Technology**, p. 157-167, 2004.

BRODERICK, G. A. et al. Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on the Production of Lactating Dairy Cows. **Journal of Dairy Science**, p. 1378–1391, 2007.

BUCCIONI, A. et al. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. **Animal Feed Science and Technology**, p. 1-25, 2012.

CABIDDU, A. et al. Characterization of polyphenol oxidase activity in a range of forage ecotypes with different phenol substrates. A new insight for PPO and protein-bound phenol evaluation. **Grass and Forage Science,** p.678-692, 2014.

CHILLIARD, Y. et al. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. **Annales de Zootechnie** p. 181–205, 2000.

DARDNI. **Red clover**. Department agriculture Rural Development North Ireland. 2010.

DEWHURST, R. J. et al. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. **Journal of Dairy Science**, p. 2598–2611, 2003.

DEWHURST, R. J. et al. Effects of mixtures of red clover and maize silages on the partitioning of dietary nitrogen between milk and urine by dairy cows. **Animal,** p. 732-738, 2010.

FRUTOS, P. et al. Review. Tannins and ruminant nutrition. **Spanish Journal of Agricultural Research**, p. 191-202, 2004.

GRABBER, J. H. Forage management effects on protein and fiber fractions, protein degradability, and dry matter yield of red clover conserved as silage. **Animal Feed Science and Technology**, p. 284–291, 2009.

HALMEMIES-BEAUCHET-FILLEAU, A. et al. Effect of replacing grass silage with red clover silage on ruminal lipid metabolism in lactating cows fed diets containing a 60: 40 forage to concentrate ratio. **Journal of Dairy Science,** p. 5882-5900, 2013.

HALMEMIES-BEAUCHET-FILLEAU, A. et al. Effect of replacing grass silage with red clover silage on nutrient digestion, nitrogen metabolism and milk fatty acid composition in lactating cows fed diets containing 60:40 forage-to-concentrate ratio. **Journal of Dairy Science** 97, 3761–3776, 2014.

HATFIELD, R. D.; R. E. MUCK. Characterizing proteolytic inhibition in red clover silage. Page 149–150 **in Proc. XIIth Int. Silage Conf**. Swedish University of Agricultural Sciences, Upp- sala, Sweden. 1999.

HANNAWAY, D. B. **Red Clover** (*Trifolium pretense* L.) Oregon State University. Species Selection Information System. 2004.

HOFFMAN, P. C. et al. Performance of lactating dairy cows fed red clover or alfalfa silage. **Journal of Dairy Science,** 80, 3308–3315, 1997.

HOWIESON, J. G.et al. **Prospects for the future use of legumes**. In: Dilworth, M. J. et al. Nitrogen-fixing Leguminoses Symbioses, p. 363-393, 2008.

INSTITUTE OF MEDICINE. 2002. Dietary Reference Intakes (DRIs) for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. Part 1. Washington (DC): National Academy Press.

JONES, B. A.; MUCK, R. E.; HATFIELD, R .D. Red clover extracts inhibit legume proteolysis. **Journal of the Science of Food and Agriculture**, p. 329–333, 1995.

KAMALAK, A. et al. Protected protein and amino acids in ruminant nutrition. KSU. **Journal of Science and Engineering**, p. 84-86, 2005.

KAMMES, K. L.; ALLEN, M. S. Rates of particle size reduction and passage are faster for legume compared with cool-season grass, resulting in lower rumen fill and less effective fiber. **Journal of Dairy Science**, p. 3288-3297, 2012.

KATO, H. J. et al. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California. **American Journal of Epidemiology**, 97-372–385, 1973.

KOZLOSKI, G. V. **Bioquímica dos ruminantes**. 2. ed. Santa Maria: Ed. UFSM, 2009, 216p.

KRAWUTSCHKE, M. et al. Effects of inoculants and wilting on silage fermentation and nutritive characteristics of red clover–grass mixtures. **Grass and Forage Science**, p. 326-338, 2013.

LEE, M. R. F.; MARTINEZ, E. M.; SCOLLAN, N. D. Plant enzyme mediated lipolysis of Lolium perenne and Trifolium pratense in an *in vitro* simulated rumen environment. **Aspects of Applied Biology.** p. 115–120, 2003a.

LEE, M. R. F. et al. The effect of clover silages on long chain fatty acid rumen transformations and digestion in beef steers. **Animal Science**, p. 491–501, 2003b.

LEE, M. R. F. et al. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. **Journal of the Science of Food and Agriculture**, p. 1639–1645, 2004.

LEE, M.R.F. et al. Polyphenol oxidase activity in grass and its effect on plant-mediated lipolysis and proteolysis of *Dactylis glomerata* (cocksfoot) in a simulated rumen environment. **Journal of the Science of Food and Agriculture,** p. 1503–1511, 2006.

LEE, M. R. F. et al. Lipolysis in red clover with different polyphenol oxidase activities in the presence and absence of rumen fluid. **Journal of the Science of Food and Agriculture**, p. 1308–1314, 2007.

LEE, M. R. F. et al. The effect of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (Lactobacillus plantarum L54). **Animal Feed Science and Technology**, p. 125–136, 2008.

LEE, M. R. F. et al. A comparison between red clover silage and grass silage feeding on fatty acid composition, meat stability and sensory quality of the M. *Longissimus* muscle of dairy cull cows. **Meat Science,** p. 738-744, 2009a.

LEE, M. R. F. et al. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. **Journal of Dairy Science,** p, 1136-1147, 2009b.

LEE, M. R. F. et al. Red clover polyphenol oxidase: activation, activity and efficacy under grazing. **Animal Feed Science and Technology**, p. 250-264, 2009c.

LEE, M. R. F. et al. *In vitro* rumen simulated (RUSITEC) metabolism of freshly cut or wilted grasses with contrasting polyphenol oxidase activities. **Grass and Forage Science**, p. 196-205, 2011.

LEE, M. R. F. et al. Oxidation of ortho-diphenols in red clover with and without polyphenol oxidase (PPO) activity and their role in PPO activation and inactivation. **Grass and Forage Science**, p. 83-92, 2013.

LEE, M. R. Forage polyphenol oxidase and ruminant livestock nutrition. **Frontiers in Plant Science,** 2014.

LIMA, F. E.L. et al. Ácidos graxos e doenças cardiovasculares: uma revisão. **Revista de Nutrição**., Campinas, p. 73-80, 2000.

LO, S. C. C.; NICHOLSON, R. L. **Compostos fenólicos e a importância nas doenças em plantas.** PASCHOLATI, S. F. Interação planta patógeno: fisiologia, bioquímica e biologia molecular. Piracicaba: FEALQ, 2008.

LOOR, J. J. et al. Biohydrogenation of unsaturated fatty acids in continuous culture fermenters during digestion of orchardgrass or red clover with three levels of ground corn supplementation. **Journal of Animal Science**, p. 1611–1627, 2003.

LOURENÇO, M. et al. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. **Animal Feed Science and Technology**, p. 418-437, 2008.

LUND, T. D. et al. Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback. **Biololgy of Reproduction**, p. 1188-1195, 2004.

MACHEIX, J. J. et al. Phenolic compounds and polyphenoloxidase in relation to browning in grapes and wines. **Critical Reviews in Food Science & Nutrition**, p. 441-486, 1991.

MCDONALD, P.et al. Lipids. In: MCDONALD, P. et al. **Animal Nutrition**. 7 ed. 2010, 712p.

MCNIVEN, M. A. et al. Laboratory procedure to determine protein digestibility of heat-treated feedstuffs for dairy cattle. **Animal Feed Science and Technology**, p. 1-13, 2002.

MCSWEENEY, C. S. et al. Microbial interactions with tannins nutritional consequences for ruminants. **Animal Feed Science and Technology,** p. 83-93, 2001.

MERRY, R. J.; et al. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. *In vitro* and in vivo studies of nitrogen utilization. **Journal of Animal Science**., p. 3049-3060, 2006.

MOORBY, J. M. et al. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. **Journal of Dairy Science,** p. 1148–1160, 2009

MUSTONEN, E. A. et al. Equol in milk of dairy cows is derived from forage legumes such as red clover**. British Journal of Nutrition**, p. 1552-1556, 2009.

NATIONAL RESEARCH COUNCIL. **Nutrient Requirements of Dairy Cattle** . 7.ed. Washington, DC: National Academy Press, 2001, 381 p.

OWENS, V. N.; ALBRECHT, K. A.; MUCK, R. E. Protein degradation and ensiling characteristics of red clover and alfalfa wilted under varying levels of shade. **Canadian Journal of plant science**, p. 209-222, 1998.

PAPADOPOULOS, Y. A.; MCKERSIE, D. A comparison of protein degradation during wilting and ensiling of six forage species. **Canadian Journal of Plant Science**, p. 903–912, 1983.

PEREIRA, R. G. DE A. et al. **Processos de ensilagem e plantas a ensilar.** Embrapa Rondônia, 2008. 13 p.

RODRIGUEZ, N.M. et al. Silagens de sorgo de porte alto com diferentes teores de tanino e de umidade no colmo. III – Quebra de compostos nitrogenados. **Arquivos Brasileiros de Medicina Veterinária e Zootecnia**. p.161-165, 1998.

RUSSO, G. L. Dietary n− 6 and n− 3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. **Biochemical pharmacology**, p. 937-946, 2009.

SANTOS, F. P. Metabolismo da proteína. In: **Nutrição de ruminantes**. BERCHIELLI, T. T.; PIREZ, A.V.; OLIVEIRA, S. G. Jaboticabal: Funep, 2006, 583 p.

SANTOS, R. I. Metabolismo básico e origem dos metabólitos secundários. In: SIMÕES, et al. **Farmacognisia: da planta ao medicamento**. 5 ed. Porto Alegre, UFRGS. Florianopolis, UFSC, 2004. p. 403-434.

SIMOPOULOS, A. P. The importance of the ratio of omega-6/ omega-3 essential fatty acids. **Biomed Pharmacother** p. 365–379, 2002.

SIMOPOULOS, A. P. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. **Biomedicine & pharmacotherapy**, p. 502-507, 2006.

SIMOPOULOS, A. P.; CLELAND, L. G. Omega-6/omega-3 essential fatty acid ratio: the scientific evidence **World rev nutr diet**, vol. 92. Basel:Karger; 2003

SNIFFEN, C. J. et al. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. **Journal of Animal science**, p.3562-3577, 1992.

STEINSHAMN, H. et al. Effects of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. **Journal of Dairy Science**, p. 2715-2725, 2008.

SULLIVAN, M. L; HATFIELD, R. D. Polyphenol Oxidase and-Diphenols Inhibit Postharvest Proteolysis in Red Clover and Alfalfa. **Crop Science**, p. 662-670, 2006.

THORNTON, R. F.; MINSON, D. J. The relationship between apparent retention time in the rumen, voluntary intake, and apparent digestibility of legume and grass diets in sheep. **Crop and Pasture Science**, p. 889-898, 1973.

VAN DORLAND, H. A. et al. Comparative potential of white and red clover to modify the milk fatty acid profile of cows fed ryegrass-based diets from zero-grazing and silage systems. **Journal of the Science of Food and Agriculture,** p. 77-85, 2008.

VANHATALO, A.; GÄDDNÄS, T.; HEIKKILÄ, T. Microbial protein synthesis, digestion and lactation responses of cows to grass or grass-red clover silage diet supplemented with barley or oats. **Agricultural Food Science**., p. 252–267, 2006.

VANHATALO A et al.Effects of forage species and stage of maturity on bovine milk fatty acid composition. **European Journal of Lipid Science and Technology** 109, 856–867, 2007.

VANHATALO, A. et al. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. **Journal of Dairy Science,** p. 5620-5633, 2009.

VAN RANST, G. et al. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. **Animal**, p. 1528–1540, 2010.

VAN RANST, G., LEE, M. R., & FIEVEZ, V. Red clover polyphenol oxidase and lipid metabolism. **Animal,** p. 512-521, 2011.

VAN SOEST, P. J. **Nutritional ecology of the ruminant**. 2nd ed. New York: Cornell University Press , 1994, 476 p.

WAGHORN, G. C.; MCNABB, W. C. Consequences of plant phenolic compounds for productivity and health of ruminants. **Proceedings of the Nutrition Society**, p. 383- 392, 2003.

WIERSMA, D. W. et al. Harvest management effects on red clover forage yield, quality, and persistence. **Journal of Production Agricultural**., p. 309–313, 1998.

WILLIAMS, Christine M. Dietary fatty acids and human health. In: **Annales de Zootechnie.** EDP Sciences, 2000. p. 165-180.

WINTERS, A. L.; MINCHIN, F. R.; Red clover and the future for pasture legumes as an alternative protein source for ruminants. **IGER Innovations**, p. 30-33, 2001.

WINTERS, A. L. et al. Latent and active polyphenol oxidase (PPO) in red clover (Trifolium pratense) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. **Journal of Agricultural and Food Chemistry,** 56(8), 2817-2824, 2008.

WOODWARD, S. et al. Feeding birdsfoot trefoil (Lotus corniculatus) reduces the environmental impacts of dairy farming. In: **Proceedings of the New Zealand Society of Animal Production**. p. 179-183, 2009.

3 HIPÓTESES

3.1 GERAL

A silagem de trevo vermelho contribui para a melhoria da eficiência de uso do nitrogênio e do perfil de ácidos graxos no leite de ovinos.

3.2 ESPECÍFICAS

- A inclusão de trevo vermelho em silos de gramínea de clima tropical reduz a proteólise do material ensilado e degradabilidade *in vitro* da proteína.
- O uso de silagem de trevo vermelho para alimentação animal é capaz de aumentar o fluxo intestinal de proteína verdadeira e reduzir o teor de ureia excretada na urina e no leite de ovinos.
- O fornecimento de dietas contendo silagem de trevo vermelho para ovelhas em lactação aumenta a concentração de ácidos graxos poli-insaturados no leite.

4 ASSOCIATIVE EFFECTS BETWEEN RED CLOVER AND KIKUYU GRASS SILAGE: PROTEOLYSIS REDUCTION AND SYNERGY DURING *IN VITRO* ORGANIC MATTER DEGRADATION

4.1 ABSTRACT

Red clover (RC; *Trifolium pratense*) is a forage legume that contains polyphenol oxidase (PPO), an enzyme capable of transforming phenolic compounds into quinones, which reduce forage protein degradation during ensiling and rumen fermentation. The aim of this study was to evaluate the effects of the association between RC and a tropical grass (kikuyu grass) on the proteolysis of ensiled material and the *in vitro* degradation of protein and organic matter. RC and kikuyu grass were ensiled in the following proportions: 0:1000, 250:750, 500:500, 750:250, and 1000:0 g/kg of dry matter (DM). Samples of ensiled material were freeze-dried and incubated using the *in vitro* gas technique. Protein degradation in the silo and rumen was assessed by measuring the concentration of ammonia nitrogen (NH3-N) in fluid extracted from the silage and incubation medium, respectively. Increasing the proportion of RC to 500:500 g/kg reduced the pH of the silo ($P < 0.001$). The fraction of rapidly degradable protein and the NH3-N content decreased linearly ($P < 0.001$) with the increase in the proportion of RC in the ensiled material: 520 to 348 g/kg of crude protein and 103 to 47.6 g/kg of total N, respectively. *In vitro* protein degradation and the degradation rate decreased linearly ($P < 0.001$) with the increasing proportion of RC. Cumulative gas production after 24 hours of incubation showed a positive quadratic effect when RC was increased to 500 g/kg ($P < 0.001$). The silages with the highest RC content reduced proteolysis more effectively during ensiling and ruminal fermentation, demonstrating their potential to reduce ruminal nitrogen (N) losses. Inter-species synergistic effects positively affected in vitro gas production, which was optimal when RC and kikuyu grass were ensiled in the same proportions as that of total DM.

Keywords: associative effects, in vitro rumen fermentation, legume–grass mixture, polyphenol-oxidase

Guzatti, G. C., Duchini, P. G., Kozloski, G. V., Niderkorn, V., & Ribeiro-Filho, H. M. N. (2017). Associative effects between red clover and kikuyu grass silage: proteolysis reduction and synergy during in vitro organic matter degradation. *Animal Feed Science and Technology*, v. 231, p. 107-110

4.2 INTRODUCTION

Ensiling is one way of avoiding the disadvantages arising from forage seasonality; however, it usually reduces the quality of the plant material owing to the transformation of true protein into non-protein nitrogen (NPN) (Aufrère et al., 2000; Fairbairn et al., 1988; Repetto et al., 2005). This can be ameliorated by using certain legumes containing bioactive compounds capable of decreasing proteolysis in the silo (Jones et al., 1995; Sulivan and Hatfield, 2006), and the ruminal degradation of forage protein. The ingestion of such legumes can reduce the urinary excretion of nitrogen (N) and minimize its associated environmental impact (Hymes-Fecht et al., 2005; Dewhurst et al., 2010).

The legume red clover (RC; *Trifolium pratense*) particularly as silage, may improve the efficiency of N metabolism in ruminants because it contains polyphenol oxidase (PPO). In the presence of oxygen, this enzyme can oxidize the phenolic compounds released from plant vacuoles into quinones, which are in turn capable of forming complexes with proteins and decreasing proteolysis in both the silo (Jones et al., 1995; Lee et al., 2008) and the rumen (Albrecht and Brodercik, 1992; Broderick et al., 2004; Merry et al., 2006). A recent study demonstrated that mixing RC and temperate-climate grass silages reduces urinary N excretion and increases the digestible organic matter (OM) intake and N retention in sheep (Niderkorn et al., 2015). Similarly, the association between a tanniferous legume and a temperate-climate grass resulted in a synergistic effect on protein degradability in the rumen, indicating that legume tannins can interfere with legume proteins and reduce protein degradation of the companion grass (Niderkorn et al., 2011, 2012). The effects of ensiling RC with a tropical grass, such as kikuyu (*Pennisetum clandestinum*), also deserves investigation owing to the potential for broader geographical coverage.

This study tested the following hypotheses: i) that an increase in the proportion of RC in the silage mixture synergistically reduces proteolysis during ensiling and in vitro rumen fermentation; and ii) that mixing RC and kikuyu grass leads to better in vitro OM degradation than fermenting the same species individually.

4.3 MATERIAL AND METHODS

4.3.1 Forage and silage preparation

The plant material used for silage production was obtained from six 40 m^2 paddocks (field repetitions). Three contained kikuyu grass (10 year perennial pasture) and three contained RC (sown in April 2013). They were located at Lages, Santa Catarina (SC), Brazil (27°47′S, 50°18′W, 960 m alt.). The soil at the experimental site is a clay Inceptisol (Halumbrept) (Cambissolo Húmico Alumínico – EMBRAPA, 2006) and the fertiliser was applied according to the Manual de Adubação e Calagem para os Estados do Rio Grande do Sul e Santa Catarina, Brazil (Comissão de Química e fertilidade do solo - RS/SC, 2004). The pastures (monocultures of either kikuyu grass or red clover with no weeds) were standardized in January 2014 by cutting them 5 cm above the soil level. In April 2014, during the vegetative development stage of the pastures, the silage materials were collected using a motorized harvester at ~10 cm above soil level to avoid collecting dead material and to minimize the stem and petiole content. The collected material was chopped into ~5 cm lengths and kept in the shade for 28 hours, which resulted in partial humidity loss. Subsequently, the forages were ensiled in 3.8-L plastic buckets using the following proportions of RC and kikuyu grass (based on DM): 0:1000, 250:750, 500:500, 750:250, and 1000:0 g/kg (n = 15 silos, 3 paddocks × 5 proportions). At the time of ensiling, a commercial inoculant containing *Lactobacillus plantarum* at a dosage of 1×10^6 colony-forming units (CFU)/g of ensiled material was added. The silos were compacted to an approximate density of 500 kg/m^3 and sealed. A sample of fresh (not wilted) RC was collected, frozen, and lyophilized for later *in vitro* rumen fermentation. Protein degradation in the fresh RC was compared to that in ensiled RC, thereby facilitating the detection of PPO enzyme activity.

4.3.2 Silage sampling and *in vitro* rumen fermentation

After 100 days of ensiling, the silos were opened and two representative samples of ensiled material were collected per micro silo. One sample (~400 g) was compacted using a hydraulic press to collect the fluid and characterize the fermentative pattern of the silos. The fluid was filtered using paper (20 µm, quick filtration), the pH was immediately measured, and the fluid was frozen prior to the analysis of ammonia nitrogen (NH3-N). Another sample (~200

g) was lyophilized (Labconco, Model FreeZone12), ground through a 1-mm porosity sieve, and stored until chemical analysis and *in vitro* rumen fermentation.

The rumen inoculum was obtained from a castrated bull with a rumen cannula grazing a *Cynodon dactylon*-predominant pasture, and had been supplied with ryegrass (*Lolium multiflorum*) hay and a mineral supplement (Bovipasto, Tortuga, Brazil) *ad libitum*. After collection, the rumen inoculum was filtered using two layers of gauze under continuous CO_2 injection. Grounded samples from each micro silo (0.5 g) and fresh RC were incubated in duplicate in 160-mL bottles with 50 mL of buffered rumen inoculum at 39°C, placed in a water bath, and stirred slowly. The inoculum was a mixture of buffer/ruminal fluid in a ratio 4:1 v/v (anaerobic phosphate: carbonate buffer solution, as described by Goering and Van Soest (1970)). Bottles without plant substrate were also incubated. Three *in vitro* runs were carried out, generating a total of 108 observations (3 paddocks × 6 treatments (5 proportions of ensiled material + fresh RC) × 2 replicates × 3 runs). For each treatment values were averaged per replicate and paddock within each run, which was considered the experimental unit, remaining 18 observations (3 per treatment).

The gas production in the bottles was measured at the following time-points: 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 60, 72, and 96 hours after incubation. The measurement was based on liquid displacement (i.e., water and ethanol mixture, 9:1 v/v) in a graduated pipette. The lower orifice of the pipette was connected via a silicone tube to a tank used to regulate the level of the liquid in the pipette, and the upper orifice was connected via a silicone tube to a three-way tap, with a needle inserted in the bottle caps. The gas production in each bottle was corrected to the gas production in the bottles containing no sample.

To estimate the ruminal protein degradation rate, 500 µL of incubation medium was collected from each bottle at the following time-points: 0, 12, 24, 48, and 60 hours after incubation, with the aid of a 1-mL syringe with a needle. The collected content was transferred to flasks containing 4.5 mL of 2% H2SO4 (v/v), and kept at -20°C until analysis. The ammonia concentration at each time-point was corrected the concentration of ammonia in the bottles containing no sample at time 0.

4.3.3 Chemical analysis

DM content was determined in an oven at 105°C for 24 hours, and the ash content was determined by combustion in a muffle furnace at 550°C for 5 hours. The N content was determined by the Kjeldahl method and ether extract (EE) was determined using ether

extraction during 4 hours in Soxhlet extractor (Marconi MA-491, Brazil) system (AOAC, 1995). Neutral detergent fibre assayed with a heat stable amylase (aNDF) and acid detergent fibre (ADF), both expressed inclusive of residual ash content, were investigated according to the method described by Van Soest et al. (1991) adapted to a Fiber Analyzer (ANKOM Technology, Macedon NY, USA). The NPN + true soluble protein (A) and indigestible N (C) fractions of crude protein (CP) were determined after the sample had been submitted to treatment with borate-phosphate buffer solution or acid detergent solution for 1 hour, respectively (Licitra et al., 1996). The insoluble protein fraction (B) was determined by subtracting fraction A from fraction C. The analysis of NH3-N concentration in the fluid extracted from the silos and *in vitro* incubation medium was carried out using the phenol-hypochlorite colorimetric method (Weatherburn, 1967).

4.3.4 Calculations and statistical analysis

The curves of gas production throughout the incubation time were adjusted using the unicompartmental logistic model of Schofield et al. (1994) to estimate the gas production rate (kd). The non-fibrous carbohydrate (NFC) content was calculated using the equation: OM – ((aNDF – (NIDN × 6.25)) + CP + EE), where NIDN is N insoluble in neutral detergent, CP is crude protein (N × 6.25). The *in vitro* N degradation (IVND) at each incubation time was calculated using the equation: IVND = (([NH3-N] - [NH3-N Br]) × volume (mL)) / incubated N (mg), were [NH3-N] = concentration (mg/mL) of N-ammonia measured in the bottle containing the sample; [NH3-N Br] = concentration of N-ammonia in the sample collected from blanks at time 0. The fractional rate of protein degradation was estimated as the regression coefficient between the natural logarithm values of the non-degradable fraction (Ln (1 – IVND), y) vs the incubation time (x), according to Broderick (1987).

The quality parameters of the ensiled material were submitted to analysis of variance using the SAS PROC GLM program (SAS Institute, Cary, NC, USA), considering the treatment as fixed effect. The *in vitro* incubation parameter data were analysed using the SAS PROC MIXED program using a model that included the fixed effect of the treatment and the random effect of the run. The effect of increased RC proportion over all evaluated parameters was tested by orthogonal polynomial contrast considering the linear and quadratic effects, and differences were considered significant at $P < 0.05$.

4.4 RESULTS

4.4.1 Chemical composition and quality of silages

The ADF and NFC content increased and the aNDF content decreased linearly ($P < 0.01$, Table 5) at increased proportion of RC. The OM and CP content, and the B fraction of protein, were similar in all treatments. However, the A fraction of protein decreased and the C fraction of protein increased linearly with increasing the RC proportion in the silage ($P < 0.001$). The pH values decreased with increased RC proportion in the ensiled material, both linear and quadratic effects being significant ($P < 0.001$), whereas the NH3-N in the fluid extracted from the silage decreased linearly with increased RC proportion ($P < 0.001$).

Table 5. Chemical composition (g/kg DM), pH and NH3-N (g/kg of total N) of the ensiled material containing different proportions of red clover and kikuyu grass.

	Red clover(g/kg of total DM)					*RSD*	*P-value*	*Contrasts*	
	0	*250*	*500*	*750*	*1000*			*L*	*Q*
Composition (g/kg DM):									
DM	349	367	395	417	459	28.6	0.0063	<0.001	0.4921
OM	903	897	893	898	896	4.9	0.2677	-	-
NDF	585	545	511	477	435	13.7	<0.001	<0.001	0.8696
ADF	278	288	293	305	316	11.7	0.0190	0.0013	0.7683
NFC	131	159	179	215	252	16.5	<0.001	<0.001	0.3540
CP	200	200	202	201	200	6.4	0.9959	-	-
Protein fractions (g/ kg CP)									
A	520	508	483	410	348	31.9	<0.001	<0.001	0.0569
B	428	423	422	462	486	38.1	0.2291	-	-
C	52	69	95	128	166	13.7	<0.001	<0.001	0.1220
Silage fermentation									
pH	5.48	5.28	5.01	5.00	4.90	0.021	<0.001	<0.001	<0.001
$N\text{-}NH_3$	103.0	88.8	76.3	70.5	47.6	0.578	<0.0001	<0.0001	0.4021

(DM) dry matter; (OM) organic matter; (aNDF) neutral detergent fiber; (ADF) acid detergent fiber; (NCF) non-fiber carbohydrate; (CP) crude protein. RSD= residual standard deviation. (L) linear and (Q) quadratic contrasts.
Fonte: próprio autor.

4.4.2 Protein ruminal degradation and kinetics of gas production

Protein degradation at the different incubation times and protein degradation rate both decreased linearly (P < 0.001) with increased RC proportion in the silage (Table 6). Protein degradation in the fresh RC was higher than in the ensiled RC after 24 hours of incubation (Figure 2). The ratio of released NH3-N/g of incubated soluble N in the incubation medium did not decrease as the RC proportion increased (Figure 3).

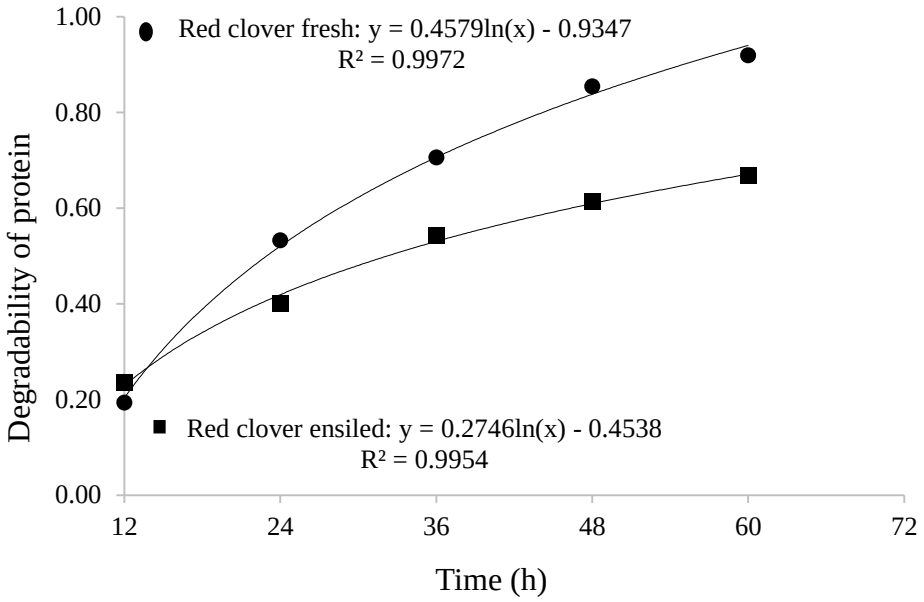
The impact of the treatments on the cumulative production of gas varied throughout the incubation period. At early time-points (i.e., 6 and 12 hours) the production of gas increased linearly and quadratically (P < 0.01) with increased RC proportion in the silage (Table 7). At 24 hours of incubation, cumulated gas production varied quadratically with increasing RC proportion (P < 0.01), whereas from 48 to 96 hours, it decreased linearly and quadratically (P < 0.01). The gas production rate showed positive linear and quadratic effects in response to increased RC proportion in the silage.

Table 6. Protein ruminal degradation *in vitro* and protein ruminal degradation rate (/h) in the ensiled material containing different proportions of red clover and kikuyu grass.

	Red clover (g/kg of total DM)					*RSD*	*P-value*	*Contrasts*	
Hours	*0*	*250*	*500*	*750*	*1000*			*L*	*Q*
Ruminal degradability of protein (g/kg CP)									
12	396	328	310	268	235	19.6	<0.001	<0.001	0.301
24	530	500	460	430	400	18.5	<0.001	<0.001	0.851
48	690	710	660	600	610	19.3	<0.001	<0.001	0.554
60	760	740	700	670	670	18.1	<0.001	<0.001	0.435
Protein degradation rate (/h))									
	0.023	0.023	0.020	0.018	0.019	0.0009	<0.001	<0.001	0.498

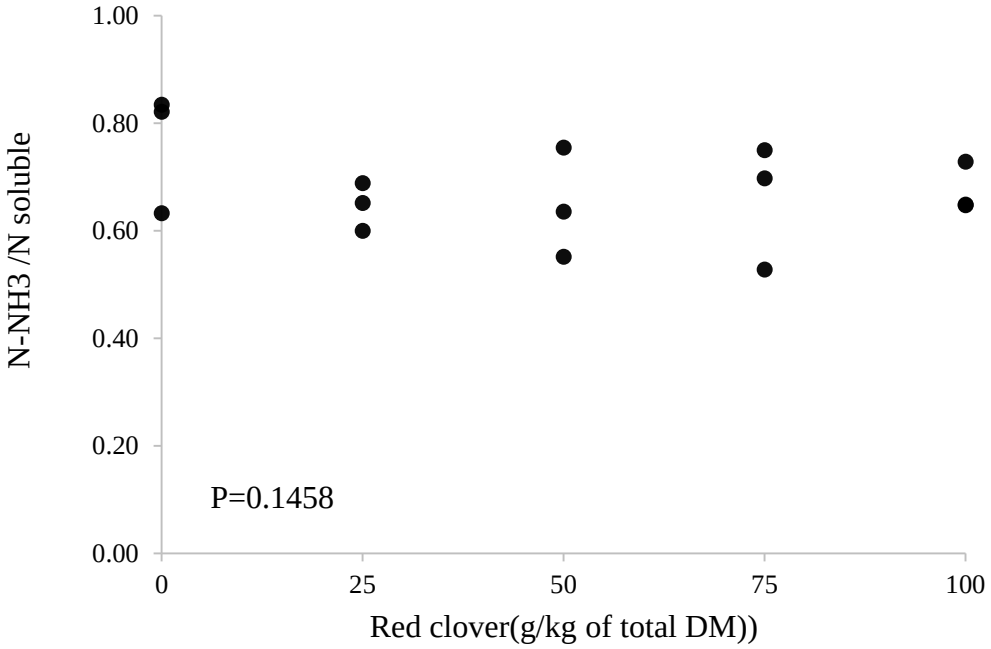
RSD= residual standard deviation. (L) linear and (Q) quadratic contrasts. Fonte: próprio autor.

Figure 2. *In vitro* protein degradation of red clover fresh or ensiled.



Fonte: próprio autor.

Figure 3. Relationship between the ratio NH3-N/soluble N in the incubation medium and the proportion of red clover in the ensiled material.



Fonte: próprio autor.

Table 7. Cumulative gas production at various incubation times and gas production rate during ruminal fermentation of the ensiled material containing different proportions of red clover and kikuyu grass.

	Red clover (g/kg of total DM)					*RSD*	*P-value*	*Contrasts*	
Hours	*0*	*250*	*500*	*750*	*1000*			*L*	*Q*
In vitro cumulative gas production (ml/g OM)									
6	16	23	26	26	29	1.31	<0.001	<0.001	0.004
12	55	66	70	71	74	1.65	<0.001	<0.001	<0.001
24	104	113	112	109	108	1.5	<0.001	0.262	<0.001
48	145	149	145	138	132	2.2	<0.001	<0.001	<0.001
96	160	161	157	149	141	2.2	<0.001	<0.001	0.001
Gas production rate (/h)									
	0.034	0.035	0.035	0.037	0.042	0.0004	<0.001	<0.001	<0.001

RSD= residual standard deviation. (L) linear and (Q) quadratic contrasts. Fonte: próprio autor.

4.5 DISCUSSION

4.5.1 The RC and fermentative pattern of silage

The linear increase in total DM and ADF content and the reduction in aNDF content with increasing RC proportion were the direct consequences of the different chemical compositions of the two ensiled plants. However, NH3-N content lower than 80 g/kg of total N in the silos with higher RC proportions (500–1000 g/kg DM) is an important qualitative indicator of silage, because silages with such characteristics can be considered to be of optimum quality (Huhtanen, 2013; Silveira, 1975). Lower proportions of RC (0–250 g/kg DM) resulted in higher NH3-N content indicating high degradation of the true proteins into non-protein nitrogen (Albrecht and Muck, 1991; Evangelista et al., 2005). One possible explanation for the reduction of NH3-N content with increased RC proportion in the silage could be the activity of the PPO enzyme present in RC. It is well known that RC is rich in this enzyme (Jones et al., 1995; Winters et al., 2008). When the PPO is released from plant chloroplasts and mixed with phenols present in plant vacuoles in the presence of atmospheric oxygen, for instance during cell breakdown after chopping and wilting, o-diphenols are converted into o-quinones, which are highly reactive and form complexes with protein, leading to a reduction of proteolysis in the silos (Grabber, 2009; Grabber and Coblentz, 2009; Sullivan and Hatfield, 2006).

The decrease in pH values with the increase in the RC proportion can be partly explained by the reduced concentration of NH3-N in the silos. NH3-N acts as a weak base, capturing H+ in solutions with low pH values. Furthermore, the higher NFC content with higher RC proportion may also have contributed to a reduction in the pH, because water-soluble carbohydrates are an important constituent of NFC, and can be very rapidly used by lactic acid bacteria to acidify the forage (McDonald et al., 1991). Despite the presence of RC, which contributed to the reduction in the pH of the silage from 5.5 to 4.9, the pH was still above the threshold of 4.2, which is considered the limit for satisfactory quality (Vilela, 1998). However, it should be noted that ensiled materials with high DM content, such as wilted forage, have a stable pH above 4.2 owing to the lower concentration of organic acids (Jobim et al., 2007).

4.5.2 Effect of RC silage on *in vitro* protein degradation

The lower *in vitro* protein degradation and degradation rate associated with a greater proportion of RC can be attributed to the quinones that form during the ensiling process. Quinones are capable of reducing ruminal proteolysis (Broderick et al., 2004; Merry et al., 2006) by inhibiting the action of proteases or by forming complexes with foliar proteins, thereby reducing their solubility and degradability (Lee, 2014). However, owing to the large quantity of proteases in the rumen, it is thought that the most efficient mechanism by which degradable proteins are decreased involves PPO “protection” through the formation of complexes between quinones and foliar proteins (Winters and Minchin, 2001).

It is important to note that *in vitro* protein degradation in the fresh RC was higher than in the ensiled RC, at and after 24 hours of incubation (Figure 2). Moreover, the proportion of released NH3-N/g of incubated soluble N in the incubation medium at 12 hours did not decrease, whereas the legume proportion increased (Figure 3). This indicates that the presence of quinones resulted in a decrease in the degradation rate of the insoluble fraction of protein, instead of the soluble fraction of protein. In other words, the lower degradation up to the first 12 hours in materials with a higher legume proportion directly reflected the lower amount of fraction A incubated. However, it is worth noting that the lower amount of the A fraction in materials with higher RC proportion is resulted of lower proteolysis during the ensiling of this material

In this study, the linear decrease *in vitro* protein degradation that occurred concomitantly with the increase in the proportion of RC indicates that there was no synergy between the quinones generated in RC and kikuyu grass proteins. In contrast, when examining

the association between a temperate-climate grass and a tanniferous legume, Niderkorn et al. (2012) found a synergistic effect on rumen protein degradation due legume tannins over the grass protein. This was demonstrated by the presence of a negative quadratic effect on the concentration of NH3-N present in the incubation medium. Therefore, the results of our study indicate that the quinones present in RC form complexes mainly with proteins present in the RC itself, with little effect on protease activity present in the ruminal environment.

From the perspective of protein fractionation, diets with a high concentration of rapidly degradable protein (basically protein A and B1 fraction) might result in large N urinary losses and environmental pollution in animal production systems. According to Grabber and Coblentz (2009), the quinones present in RC convert part of the proteins of the A fraction into B2 and B3, which are more slowly degraded in the rumen, which might increase the N flow in the duodenum, and ultimately improving N use efficiency. In the present study, increasing the proportion of RC decreased both the protein fraction A and the degradation rate of protein fraction B. However, increased RC proportions produced silages with higher proportions of protein fraction C, possibly as a result of the association of protein with lignin (present in relatively high quantities in legumes) or protein–quinone complexes (Krishnamoorthy et al., 1983). Anyway, the concentration of N-NH3 in the incubation medium was always higher than 5 mg N-NH3/100 mL of ruminal liquid, which is considered the minimum ammonia concentration which does not o limit microbial growth (Roffler and Satter, 1975).

4.5.3 Organic matter degradation and *in vitro* gas production

The mixture of kikuyu and RC in the same proportions of total DM has a synergistic effect on *in vitro* gas production and consequently on OM degradation. This effect has been found as a result of first derivate from second degree equation, which have shown the maximum point in the proportion of 500:500 g/kg DM. The effects of the synergy between kikuyu and RC on total gas production at 24 hours after incubation can be attributed to improved ruminal microbiota when grasses and legumes are used in combination, compared with when these plants are used alone (Niderkorn et al., 2011).. In the first hours of incubation, the higher values of gas production with the highest proportions of RC might be explained by the greater amount of rapidly fermentable NFC and the lower quantities of fibrous carbohydrates in this material, because large quantities of fibre are inversely related to organic matter degradation, and consequently gas production. In the last hours of incubation, the lower gas production with substrates containing the highest proportions of RC might reflect the higher quantity of slowly

digestible components, because the ADF content in NDF was 47% and 72% in the pure grass and pure RC, respectively.

Moreover, according to Waghorn (2008), secondary compounds in forages can decrease OM ruminal degradation, which results in lower volumes of gas produced *in vitro*. According to Khazaal et al. (1993), the accumulation of anti-nutritional factors (phenolic compounds) in the incubation medium inhibits the microbial growth responsible for material degradation and gas generation throughout the incubation period. Therefore, high proportions of RC (+ 750 g/kg of DM) could affect fermentation 48 hours after incubation, although this is a specific feature of *in vitro* results.

4.6 CONCLUSION

The proteolysis during ensiling and *in vitro* protein degradation reduces linearly increasing RC proportion. However, a mixture of kikuyu and RC in the same proportions of total DM (500:500 g/kg) can improve the fermentative pattern of the silo, showing a synergistic effect on *in vitro* gas production and organic matter degradation.

4.7 REFERENCES

Albrecht, K.A., Broderick, G.A., 1992. Ruminal *in vitro* degradation of protein from different legume species, in Research Summaries. US Dairy Forage Research Centre, Madison, WI, pp 92–94.

Albrecht, K.A., Muck. R.E., 1991. Proteolysis in ensiled forage legumes that vary in tannin concentration. Crop Sci. 31, 464–469.

AOAC, 1995. Official Methods of Analysis of the Association of Official Analytical Chemists, 15th ed. Association of Official Analytical Chemists Inc., Arlington, Virginia, USA.

Aufrère, J., Graviou, D., Baumont, R., Detour, A., Demarquilly, C., 2000. Degradation in the rumen of protein from fresh lucerne forage in various stages of growth and conserved as silage or hay. Ann. Zootech. 49, 461–474.

Broderick, G.A., 1987. Determination of protein degradation rates using a ruminal *in vitro* system containing inhibitors of microbial nitrogen metabolism. Br. J. Nutr. 58, 463- 475.

Broderick, G.A., Albrecht, K.A., Owens, V.N., Smith, R.R., 2004. Genetic variation in red clover for rumen protein degradability. Anim. Feed Sci. Technol. 113, 157-167.

Comissão de química e fertilidade do solo - RS/ SC., 2004. Manual de adubação e calagem para os Estados do Rio Grande do Sul e Santa Catarina (Liming and fertilizers handbook for Rio Grande do Sul and Santa Catarina States, Brazil). Brasil: Porto Alegre.

Empresa Brasileira de Pesquisa Agropecuária– EMBRAPA, 2006. Sistema brasileiro de classificação de solos (Brazilian system of soil classification) 2nd edn. Rio de Janeiro, Brasil: Embrapa Solos.

Dewhurst, R. J., Davies, L. J., Kim, E. J. 2010. Effects of mixtures of red clover and maize silages on the partitioning of dietary nitrogen between milk and urine by dairy cows. Animal. 05, 732–738.

Evangelista, A.R., Abreu, J.G., Amaral, P.N.C., Pereira, R.C., Salvador, F.M., Lopes, J., Soares, L.Q., 2005. Composição bromatológica de silagens de sorgo (*Sorghum bicolor* (L.) MOENCH) aditivadas com forragem de leucena (*Leucaena leucocephala* (LAM.) DEWIT). Ciênc. Agrotec. 29, 429-435.

Fairbairn, R., Inteaz, A., Baker, B.E., 1988. Proteolysis associated with the ensiling of chopped alfalfa. J. Dairy Sci. 71, 152–158.

Goering, H.K., Van Soest, P.J., 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). In: Agriculture Handbook No. 379. ARS-USDA, Washington, DC.

Grabber, J. H., 2009. Forage management effects on protein and fiber fractions, protein degradability, and dry matter yield of red clover conserved as silage. Anim. Feed Sci. Technol. 154, 284–291.

Grabber, J.H., Coblentz, W.K., 2009. Polyphenol, conditioning, and conservation effects on protein fractions and degradability in forage legumes. Crop Sci. 49, 1511-1522.

Huhtanen, P. An overview on manipulation of fermentation in silages effects on nutritive value. In: Daniel, J.L.P.; Santos, M. C.; Nussio, L.G. (Ed.). III International symposium on forage quality and conservation, v.1., São Pedro, 2013.Proceedings…Piracicaba: FEALQ, 2013. p.183-195.

Hymes-Fecht, U.C., Broderick, G.A., Muck, R.E., 2005. Effects of feeding legume silage with differing tannin levels on lactating dairy cattle. Page 163 in Proc XIV Intl. Silage Conf, a satellite workshop of the XXt h Intl. Grassl. Congr., Belfast, UK.

Jobim, C. C., Nussio, L. G., Reis, R. A., Schmidt, P., 2007. Avanços metodológicos na avaliação da qualidade da forragem conservada. R. Bras. Zootec. 101-119.

Jones, B.A., Muck, R.E., Hatfield, R.D., 1995. Red clover extracts inhibit legume proteolysis. J. Sci. Food Agric. 67, 329–333.

Khazaal, K., Markantonatos, X., Nastis, A., Orskov, E.R., 1993. Changes with maturity in fibre composition and levels of extractable polyphenols in greek browse: efects on *in vitro* gas production and *in sacco* dry matter degradation. J. Sci. Food Agric. 63, 237-244.

Krishnamoorthy, U.C.; Sniffen, C.J.; Stern, M.D.; Van Soest, P.J., 1983. Evaluation of a mathematical model of digesta and *in-vitro* simulation of rumen proteolysis to estimate the rumen undegraded nitrogen content of feedstuffs. Br. J. Nutr. 50, 555-568.

Lee, M.R.F., Scott, M.B., Tweed, J.K.S., Minchin, F.R., Daviesavi, D.R., 2008. The effect of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (*Lactobacillus plantarum* L54). Anim. Feed Sci. Technol. 144, 125–136.

Lee, M. R., 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Front. Plant Sci. 5, doi: 10.3389/fpls.2014.00694.

Licitra, G., Hernadez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347-358.

McDonald, P., Henderson, A.R., Heron, S.J.E., 1991. The Biochemistry of Silage. Marlow: Chalcombe Publications.

Merry, R.J., Lee, M.R.F., Davies, D.R., Dewhurst, R.J., Moorby, J.M., Scollan, N.D., Theodorou, M.K., 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. *In vitro* and *in vivo* studies of nitrogen utilization. J. Anim. Sci. 84, 3049-3060.

Niderkorn, V., Baumont, R., 2009. Associative effects between forages on feed intake and digestion in ruminants. Animal. 3, 951–960.

Niderkorn, V., Baumont, R., LeMorvan, A., Macheboeuf. D., 2011. Occurrence of associative effects between grasses and legumes in binary mixtures on *in vitro* rumen fermentation characteristics. J. Anim. Sci. 89, 1138–1145.

Niderkorn, V., Mueller-Harvey, I., Le Morvan, A., Aufrère, J., 2012. Synergistic effects of mixing cocksfoot and sainfoin on *in vitro* rumen fermentation. Role of condensed tannins. Anim. Feed Sci. Technol. 178, 48-56.

Niderkorn, V., Martin, C., Rochette, Y., Julien, S., Baumont, R., 2015. Associative effects between orchardgrass and red clover silages on voluntary intake and digestion in sheep: Evidence of a synergy on digestible dry matter intake. J. Anim. Sci. 93, 4967–4976.

Repetto, J.L., Cajarville, C., D'Alessandro, J., Curbelo, A., Soto, C., Garín, D., 2005. Effect of wilting and ensiling on ruminal degradability of temperate grass and legume mixtures. Anim. Res. 54, 73–80.

Roffler, R.E., Satter, L.D., 1975. Relationship between ruminal ammonia and nonprotein nitrogen utilization by ruminants. I. Development of a model for preddicting nonprotein nitrogen utlilization by cattle. J. Dairy Sci. 58, 1880-1888.

Schofield, P., Pitt, R.E., Pell, A.N., 1994. Kinetics of fiber digestion from *in vitro* gas production. J. Anim Sci. 72, 2980-2991.

Silveira, A.C., 1975. Técnicas para produção de silagens. In: Simpósio sobre manejo da pastagem, 2., Piracicaba. Anais... Piracicaba: ESALQ, p. 156-186.

Sullivan, M.L., Hatfield, R.D., 2006. Polyphenol Oxidase and-Diphenols Inhibit Postharvest Proteolysis in Red Clover and Alfalfa. Crop Sci. 46, 662-670.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vilela, D., 1998. Aditivos para silagem de plantas de clima tropical. Reunião Anual da Sociedade Brasileira de Zootecnia, 35, 73-108.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production. Progress and challenges. Anim. Feed Sci. Technol. 147, 116–139.

Weatherburn, M.W., 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry, Washington D.C., 39, 971-974.

Winters, A.L., Minchin, F.R., Michaelson-Yeates, T.P., Lee, M.R., Morris, P., 2008. Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. J. Agric. Food Chem. 6, 2817-2824.

Winters, A.L., Minchin, F.R., 2001. Red clover and the future for pasture legumes as an alternative protein source for ruminants. IGER Innov. 5, 30-33.

5 RED CLOVER SILAGE: AN ALTERNATIVE TO MITIGATE THE IMPACT OF NITROGEN EXCRETION IN OVINE PRODUCTION SYSTEMS

5.1 ABSTRACT

Red clover (*Trifolium pratense*) is a legume containing polyphenol oxidase enzyme, which could form quinones that shows potential to decrease the protein degradation during ensiling and in the ruminal environment. Thus, the aim of this study was to quantify the intestinal nutrients flow and the N excretion and retention in sheep receiving isoproteic diets containing red clover or lucerne (Medicago sativa L) silages. Eight Texel × Lacaune wethers (average 25 ± 2.5 kg live weight), fitted with duodenal cannula and housed in metabolic cages with total collect of feces and urine, were assigned to the treatments in a cross over design with two periods of 20 days each (13 to adaptation and 7 to data collection). The treatments were two isoproteic (16% of CP on dry matter (DM) basis) diets composed by red clover (RC) or lucerne (LU) silages + corn silage and concentrate feed. The DM decreased and organic matter (OM) digestibility tended to decrease in RC when compared to LU treatment, however, the digestible OM and metabolizable energy intake did not differ between treatments. The intestinal non-ammoniacal N (NAN) flow increased 5.9 g/day in RC compared to LU treatment (37% higher). This result was a consequence of both increase in the efficiency of microbial protein synthesis (12.7% higher) and decrease on ruminal degradable protein (RDP) content (20% lower) of diet. However, the increases in intestinal NAN flow was accompanied by a reduction in intestinal digestibility of N, resulting in similar daily N retention between treatments. The reduction on RDP content was probably the main reason to reductions on N urinary excretion on RC compared to LU treatment, showing that RC silage may be a tool to mitigate the impact of nitrogen excretion in ovine production systems without impairing their performance.

Key words: bioactive compounds, duodenal flow, polyphenol-oxidase, quinones, urinary nitrogen excretion

5.2 INTRODUCTION

Concerns regarding environmental nitrogen (N) pollution have increased the importance of nutritional strategies to reduce ruminal degradable protein (RDP) content of diets and N urinary excretion by ruminants. The polyphenol-oxidase (PPO) is an enzyme present in chloroplasts of some forage plants such as red clover (RC, *Trifolium pratense* L.) (Winters et al., 2008), which in contact with oxygen, catalyzes the oxidation of endogenous phenols to quinones (Lee et al., 2013). Quinones might to complex with proteins and reduce their degradability and solubility (Albrecht and Brodercik, 1992; Broderick et al., 2004; Lee, 2014; Merry et al., 2006) increasing the duodenal flow of protein and reducing the urinary N excretion. Considering that nitrous oxide emitted by the decomposition of urea excreted by the urine is around 300 times more polluting than carbon dioxide, a change in the route of excretion of N from urine to feces is an important strategy to reduce environmental pollutions (Varel et al., 1999). Besides that, the RC silage has lower level of soluble protein in the silage mass when compared to several other forages (Jones et al., 1995; Sullivan and Hatfield, 2006), probably due a lower degradation of true protein to non-protein nitrogen (NPN) during ensiling. Silages with greater amounts of soluble protein present lesser N use efficiency (Nagel and Broderick, 1992), since ruminal bacteria have a limited capacity of using NPN use available in the rumen (Van Soest, 1994).

Several studies have measured animal performance or nitrogen balance of diets containing red clover silage compared to grasses silages (Bertilsson and Murphy, 2003; Kuoppala et al., 2009; Merry et al., 2006; Moorby et al., 2009; Vanhtalo et al., 2006, 2009), but differences in voluntary intake and passage rate between grasses and legumes (Kammes and Allen, 2012) maight have biased the results. Moreover, preview studies comparing red clover silage with another legume (lucerne) did not present isoproteic diets (Brito et al., 2007; Broderick et al., 2000; Hoffman et al., 1997) with the risk to misinterpret a higher N use efficiency in diets containing red clover silage.

Thus, the aims of this study were to quantify the intestinal nutrients flow, N excretion and N retention in sheep receiving isoproteic diets based on either silage red clover or lucerne (*Medicago sativa* L.). The following hypotheses were tested: i) animals receiving diets based on red clover silage should present lower ruminal protein degradation and greater intestinal flow of non-ammoniacal nitrogen in comparison to those receiving diets based on lucerne

silage; ii) the diet containing red clover silage should decreases the N urinary excretion compared to lucerne silage.

5.3. MATERIAL AND METHODS

5.3.1 Experimental design, diet and treatments

Eight Texel × Lacaune wethers (average 25 ± 2.5 kg live weight (LW)) were assigned to the treatments in a cross over experimental design with two periods of 20 days each (13 days for diet adaptation and 7 days for data collection). Two months before the experimental period, the animals were surgical fitted with a duodenal cannula. The experimental protocol was approved by the Ethics Committee for Animal Experimentation (Protocol: 1.03.15) from the Santa Catarina State University (UDESC). Ten days before beginning of the experimental period, the animals were dewormed and housed in the metabolic cages for adaptation to the experimental conditions.

Diets were isoproteic total mixture rations (TMR) composed by red clover (RC) or lucerne (LU) silage + corn silage + concentrate feed. The proportion of each ingredient in the diet was calculated according to the INRAtion (2007). Experimental diets are described in Table 8. The TMR was prepared individually by animal and provided twice a day *ad libitum* (20% of orts) at 0800 and 1600h. The water and mineral supplement were available *ad libitum*. Before providing the diet in the morning all orts from the previous day were removed and weighed. Diet samples were collected between the 14^{th}-18^{th} days and orts between the 15^{th}-19^{th} days of each period.

The red clover was implanted in 2013 in Lages, Santa Catarina (SC), Brazil (27°47′S, 50°18′W, 960 m alt.) and harvested in December 2014 (vegetative period) cut with a motorized harvester at ~5 cm above ground level and kept in the field for approximately five hours, which resulted in partial humidity loss. When the material present approximately 45% of dry matter (DM) a commercial inoculant containing *Lactobacillus plantarum* at a dosage of 1×10^6 colony-forming units (CFU)/g of ensiled material was added, and forage was ensiled in 200 liters barrel, compacted up to an approximate density of 450 kg/m^3 and sealed. The lucerne silage was obtained from specialized industry and the corn silage was produced by the Dairy Cattle Sector of the Santa Catarina State University.

Table 8. Formulation and chemical composition of experimental diets containing Lucerne (LU) or red clover (RC) silage.

	LU	*RC*
Formulation (g/kg DM)		
Corn Silage	405	320
Red clover silage	-	585
Lucerne silage	535	-
Soybean meal	60	95
Total dry matter (g/kg)	360	480
Chemical composition (g/kg DM)		
Organic matter	938	934
Crude protein	154	155
Neutral detergent fiber	462	494
Acid detergent fiber	279	305

Fonte: próprio autor

5.3.2 Animal measurements

The wethers were housed individually in metabolic cages with total collect of feces and urine. The total amount of feces produced by each animal was weighed daily and sampled from the 15th to the 19th day of each period. Samples were oven-dried at 60°C for at least 72 h, ground to pass a 1 mm sieve and stored until analysis. Samples were pooled per animal and period for analysis. Total urine was collected with the aid of a urine collector and stored in buckets containing 100 mL of sulphuric acid (3.6 M) to reduce the pH below 3.0. The total volume was measured daily and a sample of 1% was collected from the 15th to the 19th day, filtered on gauze and diluted in 100 ml volumetric flasks with distilled water. These samples were pooled per animal in each period and stored at -20 ° C for further analysis. To determine the duodenal nutrients flow, duodenal digesta was collected at 6 hours interval from the 19th to the 20th days. These samples were pooled by animal and period and were stored at -20 °C for further analysis. For analysis, duodenal samples were thawed and homogenized, removing an aliquot which was filtered and acidified to quantify the NH3-N concentration. The remainder sample was lyophilized (BioSan, Model L101) ground to pass 1mm sieve for analysis.

5.3.3 Chemical analysis

Dry matter content was determined in an oven at 105°C for 24 hours and the ash by combustion in a muffle furnace at 550°C for 5 hours. The N content was determined by the Kjeldahl method (AOAC, 1995). Neutral detergent fiber assayed with a heat stable amylase (aNDFom) and acid detergent fiber (ADFom), both expressed exclusive of residual ash contents, were determined according to the method described by Van Soest et al. (1991) adapted to a Fiber analyzer (ANKOM Technology, Macedon NY, USA). The neutral detergent insoluble N (NDIN) was determined by the Kjeldahl method after the sample had been submitted to a treatment with neutral detergent solution for 1 hour.

The NH3-N concentration was performed using the phenol-hypochlorite method (Weatherburn, 1967). The purines were quantified in the duodenal digesta samples according to the technique proposed by Makkar and Becker (1999). Duodenal and feces samples were incubated in the rumen of a fistulated animal for 288 hours and the indigestible aNDFom was used as a flow marker (Krizsan and Huhtanen, 2013).

5.3.4 Calculations

The total DM intake was determined by the difference between the amount of TMR provided and orts.

The apparent digestibly of DM and their constituents were calculated as follows:

[DM intake (g/day) – fecal DM (g/day)] / DM intake (g/day).

The true OM digestibility (TOMD) was estimated assuming that neutral detergent soluble fractions of feces are from endogenous origin and only aNDFom fraction of feces is originated from feed as follows:

[(OM intake (g/day) – fecal aNDfom (g/day)] / OM intake (g/day)

The true N digestibility was calculated as follows:

[(N intake (g/day) –fecal neutral detergent insoluble N (g/day)] / N intake (g/day)

The metabolizable energy (ME (MJ/day) was calculated according to AFRC (1993) as follows:

[0.0157 × digestible OM intake (g/day)]

The intestinal flow of DM (g/day) was calculated on the basis of indigestible aNDFom (iaNDFom) concentration in duodenal digesta and the feces as follows:

[(fecal iaNDFom (g/kg DM) × fecal DM (g /day)] /duodenal iaNDFom (g/kg DM)

The intestinal flow of each nutrient (g/day) was calculated multiplying their concentration in the duodenal digesta by duodenal flow of DM (g/day).

The intestinal flow of non-ammoniacal N (NAN) (g/day) was calculated by the difference between N flow and NH_3-N flow as follows:

[N flow (g/day) – NH_3-N flow (g/day)]

The microbial N flow was calculated taking into account the DM flow and the amount of purines in duodenal digesta.

The ruminal degradability of dietary N (RDN) was calculated as follows:

1 – [(duodenal N (g/day) – microbial N (g/day) - NH_3-N flow (g/day) / N intake (g/day)

The efficiency of rumen microbial protein synthesis (EMPS) was calculated as follows:

[microbial N (g/day)/OM truly digestible intake (kg/day)]

The nitrogen ruminal utilization (NRU) was calculated as follows:

[(microbial N (g/day)/rumen degradable N intake (g/day)]

The N retention was calculated as follows:

[N intake (g/day) - N excreted in feces (g/day) - N excreted in urine (g/day)]

The ruminal digestibility of each nutrient (proportion of total apparent digestibility) was calculated by the relationship between intake, duodenal flow and fecal excretion for each specific nutrient as follows:

{[(intake (g/day) – duodenal flow (g/day)] / [(intake (g/day) – excretion (g/day)]}

The intestinal digestibility of N was calculated as follows:

[(NAN flow (g/day) - fecal neutral detergent insoluble N (g/day)/ NAN flow (g/day)]

5.3.5 Statistical Analysis

The variables were submitted to the analysis of variance using PROC MIXED of SAS (SAS Institute, Cary, NC, USA), according to the model: $Yijk = \mu + Ai + Pj + Tk + eijk$ Where Yij = dependent variable; μ = average of observations; Ai = random effect of animal i; Pj = random effect of period j; Tk = fixed effect of treatment k; eijk = residual error . The data were presented as adjusted means (LSMEANS) and values of $P<0.05$ were considered significant and between $P>0.05$ and $P<0.10$ were considered trend. All variables analyzed showed normal distribution (Shapiro-Wilk test, $P> 0.05$) and homogeneity of variance (Bartlett's test, $P> 0.05$)

5.4 RESULTS

The OM, digestible OM and ME intake did not differ between treatments, however aNDFom and ADFom intake were greater ($P < 0.05$) in RC compared to LU treatment (Table 9). The apparent digestibility of DM was lower ($P < 0.05$), whereas the apparent and true OM digestibility tended be lower in RC compared to LU. Ruminal digestibility of DM, OM, aNDFom and ADFom were similar in both treatments. The intestinal flow of OM tended to be greater in RC compared to LU and duodenal flow of aNDFom and ADFom were similar in both treatments.

The N intake was greater ($P < 0.05$), but N apparent digestibility, N true digestibility, N true intestinal digestibility and RDP content were lower ($P < 0.01$) in RC treatment (Table 10). The ruminal undegradable protein (RUP) and intestinal flow of total N, NAN, microbial N, and non-ammonia and non-microbial N (NANMN) were greater ($P < 0.05$) in RC treatment, whereas NH3-N flow did not differ between treatments. The EMPS and efficiency of N ruminal utilization were greater ($P < 0.05$) in RC treatment. The N urinary excretion was lower, but N fecal excretion was greater in RC treatment compared to LU treatment. However, the proportion of total N excreted by urine was 15 % greater in LU treatment when compared to RC, whereas daily N retention did not differ between treatments.

Table 9. Intake, digestibility, and intestinal flow of dry matter, organic matter and fiber contents in sheep fed total mixed rations containing lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-value*
		Intake		
Dry mater (g/day)	807.8	877.9	36.04	0.100
Organic matter (g/day)	753.8	815.4	33.46	0.115
Organic matter (g/kg $BW^{0.75}$)	67.3	72.3	2.75	0.160
aNDFom (g/day)	359.1	416.7	18.23	0.019
ADFom (g/day)	218.2	257.2	11.16	0.013
Digestible OM (g/day)	486.7	506.2	18.36	0.247
ME (MJ/day)	7.6	7.9	0.239	0.245
		Total apparent digestibility		
Dry matter	0.63	0.60	0.010	0.013
Organic matter	0.65	0.62	0.009	0.058
aNDFom	0.51	0.50	0.017	0.639
ADFom	0.45	0.46	0.019	0.752
OMTD	0.77	0.75	0.008	0.071
		Ruminal digestibility (proportion of total apparent digestibility)		
Dry matter	0.71	0.68	0.038	0.378
Organic matter	0.81	0.78	0.029	0.299
aNDFom	1.03	1.04	0.027	0.641
ADFom	1.04	1.10	0.031	0.102
		Intestinal flow (g/day)		
Dry matter	441.4	523.8	32.75	0.045
Organic matter	357.8	421.8	26.92	0.055
aNDFom	173.4	197.9	13.71	0.124
ADFom	116.7	127.5	8.61	0.259

aNDFom, neutral detergent fiber; ADFom, acid detergent fiber; ME, metabolizable energy; OMTD, organic matter true digestibility; SEM, standard error of the means; Statistical significance: NS, not significant; † $P < 0.1$; ∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. Fonte: próprio autor.

Table 10. Intake, digestibility, nitrogen balance and intestinal flow of nitrogen compounds in sheep fed total mixed rations containing lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-value*
Intake (g/day)	20.7	23.1	0.93	0.043
Total apparent digestibility	0.66	0.58	0.008	<0.001
Total true digestibility	0.92	0.88	0.003	<0.001
True intestinal digestibility	0.90	0.87	0.007	0.003
Urinary excretion (g/day)	8.63	8.11	0.134	0.011
Urinary excretion (g/g N intake)	0.42	0.35	0.011	0.002
Fecal NDIN excretion (g/day)	1.56	2.74	0.143	0.001
Fecal excretion (g/day)	7.10	9.80	0.590	0.006
Fecal excretion (g/g N intake)	0.34	0.42	0.009	<0.001
N retention (g/day)	5.00	5.50	0.381	0.246
N retention (g/g N intake)	0.24	0.23	0.008	0.322
(N urinary excreted/ N total)	54.6	46.3	1.355	<0.001
Intestinal flow (g/day)				
Total N	16.1	22.1	1.34	0.004
Microbial N	11.3	13.5	0.74	0.028
Non-amoniacal N	15.8	21.7	1.30	0.004
NANMN	4.5	8.2	0.71	0.002
NH_3-N	0.34	0.38	0.043	0.336
RDP	0.78	0.65	0.024	0.001
RUP	0.22	0.35	0.024	0.001
EMPS	19.6	22.1	0.99	0.047
NRU	0.71	0.93	0.055	0.007

NIDN, neutral detergent insoluble N; NANMN, non-ammonia and non-microbial N; RDP, ruminal degradable protein; RUP, ruminal undegradable protein; NH_3-N, amoniacal nitrogen; EMPS, efficiency of rumen microbial protein synthesis (g microbial N /OM truly digestible intake); NRU, nitrogen ruminal utilization (g microbial N/rumen degradable N intake); SEM, standard error of the means; Statistical significance: NS, not significant; † $P < 0.1$; ∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. Fonte: próprio autor.

5.5 DISCUSSION

5.5.1 Effect of red clover ensiling on OM digestibility and energy intake

The similar digestible OM and ME intake between treatments, even with tended lower OM digestibility of RC when compared to LU treatment, may be explained, at least partially, because rumen fill was not the preponderant factor on the regulation of intake. This way, both treatments showed a ME intake (average = 7.7 MJ/day) greater than daily ME requirements of

experimental animals (7.2 MJ/day – AFRC, 1993). Increases on NDF intake was higher in RC as a consequence of NDF content (Huhtanen et al., 2007). This result indicates that daily NDF intake did not act as a preponderant factor on feed intake regulation, as recommended to dairy cows receiving TMR (Mertens, 1994).

Despite lower apparent digestibility of DM and OM in RC diet, the ruminal digestibility of these components (as proportion of total digestibility), as well as the apparent and ruminal digestibility of fiber did not change among the treatments. Instead, studies evaluating the inclusion of condensed tannins (as a modulator of ruminal fermentation) in animal diets found a reduction in OM and fiber digestibility (Avila et al., 2015; Naczk et al., 1994). This results evidenced that condensed tannins may also complex with bacterial enzymes and / or with polysaccharides such as cellulose and hemicellulose (Priolo et al., 2000), reducing fiber degradation. In the present study fiber digestibility did not change among treatments, suggesting that, unlike condensed tannins, quinones do not complex with polysaccharides in the rumen, acting with greater importance on nitrogen compounds.

5.5.2 Effect of red clover ensiling on ruminal degradability and duodenal flow of nitrogen fractions

The lower RDP content (20 %) in RC compared to LU diet may be associated to PPO enzyme activity in RC producing quinones, which might to complex with proteins reducing their ruminal degradation (Albrecht and Brodercik, 1992; Broderick et al., 2004; Merry et al., 2006). Moreover, the effect of PPO may have occurred during the ensilage process (Jones et al., 1995; Lee et al., 2008; Sullivan and Hatfield, 2006) decreasing the proportion of soluble N in total N of silage.

The NAN flow in animals receiving RC was 37% higher than in those receiving LU treatment, as a consequence of increased flow of both microbial and NANMN, which may be clearly associated with RDP reduction and greater ruminal microbial growth. Usually, the supply of diets with lower RDP contents results in a higher flow of NAN and NANMN, but with lower microbial N flow (Ipharraguerre and Clark, 2005; Olmos Colmenero and Broderick, 2006; Reynal and Broderick, 2005). This is attributed to factors such as the lower availability of peptides, amino acids or ammonia in the rumen. (Clark et al., 1992). However, in the present study, the higher microbial N flow in the animals ingesting RC was a consequence of both increase on efficiency of rumen microbial protein synthesis (+ 12.7%) and efficiency of N ruminal utilization (+ 31.0%) for this treatment. The efficiency of rumen microbial protein

synthesis may be an indicator of energy use, while the efficiency of N ruminal utilization may be an indicator of N use in the rumen (Bach et al., 2005). These results indicate that the amount of proteins bound to quinones did not limit the substrates for microbial growth in the ruminal environment.

5.5.3 Effect of red clover ensiling on the retention and excretion of nitrogen compounds

The replacement of lucerne silage by red clover silage increased N duodenal flow, without increasing N retention. This result may be a consequence of reductions on true intestinal digestibility of nitrogen (-3.0%), and greater neutral detergent insoluble N (NDIN) excretion by animals receiving red clover, which may indicate that quinone-protein complex resulted in a formation of insoluble complexes inside gastrointestinal tract (Reed, 1995).

Animals receiving RC showed lesser N excretion through the urine, but greater N excretion through the feces compared to animals receiving LU treatment. Diets containing secondary compounds have been associated to increased secretion of endogenous proteins, probably associated to increased desquamation of intestinal cells (Waghorn, 1996). Nevertheless, in the present study, the ratio between endogenous N and total N excreted (Nfecal – NDINfecal / Nfecal) was 7.7% lesser for RC when compared to LU treatment.

The use of secondary compounds in diets for ruminants has already been reported as a modifier of the route of N excretion from urine to feces (Broderick et al., 2007, Makkar 2003, Theodoridou et al. 2010). The redirection of the route of N excretion in RC treatment from urine to feces shows environmental advantages. This occurs because the N present in ruminant feces is generally in the organic form reducing the substrate for nitrification and nitrous oxide formation and practically does not emit greenhouse gases (Alves, 2016; Varel et al., 1999). On the other hand, the majority of urinary N is in the form of urea, which is hydrolyzed within 1 or 2 days being the nitrous oxide around 300 times more polluting than CO_2. Therefore, the use of RC silage may be an alternative to reduce environmental impact in livestock systems, which has been recommended by other authors from trials focusing on dairy cows (Misselbrook et al., 2005).

5.6 CONCLUSION

Red clover silage may be a tool to reduce N ruminal degradability, N urinary excretion and mitigate the impact of N excretion in ovine production systems without impairing animal performances.

5.7 REFERENCES

Agricultural and Food Reaserch Council – AFRC, 1993. Energy and protein requirements of ruminants. Wallingford: CAB International, pp.159.

Albrecht, K.A., Broderick G.A., 1992. Ruminal in vitro degradation of protein from different legume species. IN: Research Summaries. US Dairy Forage Research Centre, Madison, WI, pp. 92–94.

Alves, T.P., 2016. Emissão de gases de efeito estufa e uso do extrato tanífero de acacia mearnsii para vacas leiteiras em pastos de inverno e verão. Ph.D. Thesis, Universidade do Estado de Santa Catarina, Lages.

Association of Official Analytical Chemists., 1995.Officia lMethods of Analysis. 16th ed. AOAC, Washington, DC

Avila, S. C., Kozloski, G.V., Orlandi, T., Mezzomo, M.P., Stefanello, S., 2015. Impact of a tannin extract on digestibility, ruminal fermentation and duodenal flow of amino acids in steers fed maize silage and concentrate containing soybean meal or canola meal as protein source. J. Agr. Sci. 153, 943 –953.

Bach, A., Calsamiglia, S., Stern, D.M., 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88, 9–21.

Bertilsson, J., Murphy, M., 2003. Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. Grass Forage Sci. 58, 309–322.

Brito, A.F., Broderick, G.A., Olmos Colmenero, J., Reynal, S.M., 2007. Effects of feeding formate-treated alfalfa silage or red clover silage on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy Sci. 90, 1392–1404.

Broderick, G.A., Brito, A.F., Olmos Colmenero, J., 2007. Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on the Production of Lactating Dairy Cows. J. Dairy Sci. 90, 1378–1391.

Broderick, G.A., Albrecht, K.A., Owens, V.N., Smith, R. R., 2004. Genetic variation in red clover for rumen protein degradability. Anim. Feed Sci. Technol. 113, 157–167.

Broderick G.A., Walgenbach R.P., Sterrenburg, E., 2000. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. J. Dairy Sci. 83, 1543–1551.

Clark, J.H., Klusmeyer, T.H., Cameron, M.R., 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy, Sci. 75, 2304–2323.

Hoffman, P.C., Combs, D.K., Brehm, N.M., Welch, D.A., 1997. Performance of lactating dairy cows fed red clover or alfalfa silage. J. Dairy Sci. 80, 3308–3315.

Huhtanen, P., Asikainen, U., Arkkila, M., Jaakkola, S., 2007. Cell wall digestion and passage kinetics estimated by marker and in situ methods or by rumen evacuations in cattle fed hay 2 or 18 times daily. Anim. Feed Sci. Technol. 133, 206–227.

Ipharraguerre, I. R., Clark, J.H., 2005. Impacts of the source and amount of crude protein on the intestinal supply of nitrogen and performance of lactating dairy cows. J. Dairy Sci. 88, 22–37.

Jones, B.A., Muck, R.E., Hatfield, R.D., 1995. Red clover extracts inhibit legume proteolysis. J. Sci. Food Agr. 67, 329–333.

Kammes, K.L., Allen, M.S., 2012. Rates of particle size reduction and passage are faster for legume compared with cool-season grass, resulting in lower rumen fill and less effective fiber. J. Dairy Sci. 95, 3288-3297.

Kriszan, S.J., Huhtanen, P., 2013. Effect of diet composition and incubation time on feed indigestible neutral detergent fiber concentration in dairy cows. J. Dairy Sci. 96, 1715–1726.

Kuoppala, K., Ahvenjarvi, S., Rinne, M., Vanhatalo, A., 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 2. Dry matter intake and cell wall digestion kinetics. J. Dairy Sci. 92, 5634–5644.

Lee, M.R., 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Front. Plant Sci., 5(694), 1–9.

Lee, M.R.F., Tweed, J.K.S., Sullivan, M.L., 2013. Oxidation of ortho-diphenols in red clover with and without polyphenol oxidase (PPO) activity and their role in PPO activation and inactivation. Grass Forage Sci. 68, 83–92.

Lee, M.R.F., Scott, M.B., Tweed, J.K.S., Minchin, F.R., Davies, D.R., 2008. The effect of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (Lactobacillus plantarum L54). Anim. Feed Sci. Technol. 144, 125–136.

Makkar, H.P.S., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin. Res. 49, 241–256.

Makkar, H.P.S., Becker, K., 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. Br. J. Nutr. 81, 107–112.

Merry, R.J., Lee, M.R.F., Davies, D.R., Dewhurst, R.J., Moorby, J.M., Scollan, N.D., Theodorou, M.K., 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. J. Anim. Sci. 84, 3049–3060.

Mertens, D.R., 1994. Regulation of forage intake. in: Fahey, G.C. (ed), Forage quality, evaluation and utilization. WI: American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, pp. 450–493.

Misselbrook, T.H., Powell, J.M., Broderick, G.A., Grabber, J.H., 2005. Dietary manipulation in dairy cattle: laboratory experiments to assess the influence on ammonia emissions. J. Dairy Sci. 88, 1765–1777.

Moorby, J.M., Lee, M.R.F., Davies, D.R., Kim, E.J., Nute, G.R., Ellis, N.M., Scollan. N.D., 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. J. Dairy Sci. 92, 1148–1160.

Naczk, M., Nichols, T., Pink, D., Sosulski, F., 1994. Condensed tannins in Canola hulls. J. Agric. Food Chem. 42, 2196–2200.

Nagel, S.A., Broderick, G.A., 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. J. Dairy Sci. 75, 140–154.

Olmos Colmenero, J.J., Broderick, G.A., 2006. Effect of dietary crude protein concentration on ruminal nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 89, 1694–1703.

Priolo, A., Waghorn, G.C., Lanza, M., Biondi, L. Pennisi, P., 2000. Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: effects on lamb growth performance and meat quality. J. Anim. Sci., 78:810–816.

Reed, J.D., 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73, 1516–1528.

Reynal, S.M., Broderick, G.A., 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88, 4045–4064.

Sullivan, M.L., Hatfield, R.D., 2006. Polyphenol Oxidase and-Diphenols Inhibit Postharvest Proteolysis in Red Clover and Alfalfa. Crop Sci. 46, 662–670.

Theodoridou, M.K., Aufrère, J., Andueza, D., Pourrat, J., Le Morvan, A., Stringano, E., Mueller-Harvey, I., Baumont, R., 2010. Effects of condensed tannins in fresh sainfoin (Onobrychis viciifolia) on in vivo and in situ digestion in sheep. Anim. Feed Sci. Technol. 160, 23–38.

Vanhatalo, A., Kuoppala, K., Ahvenjärvi, S., Rinne, M., 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. J. Dairy Sci. 92, 5620-5633.

Vanhatalo, A., Gäddnäs, T., Heikkilä, T., 2006. Microbial protein synthesis, digestion and lactation responses of cows to grass or grass-red clover silage diet supplemented with barley or oats. Agric. Food Sci. 15, 252–267.

Van Soest, P.J., 1994. Nutritional ecology of the ruminant. 2nd ed. New York: Cornell University Press, pp, 476.

Van Soest, P.J. Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Varel, V.H., Nienaber, J.A., Freetly, H.C., 1999. Conservation of nitrogen in cattle feedlot waste with urease inhibitors. J. Anim. Sci., 77, 1162–1168.

Waghorn, G.C., 1996. Condensed tannins and nutrient ab- sorption from the small intestine. Proc of the 1996 Ca- nadian Society of Animal Science Annual Meeting, Leth- bridge, Canada (Rode L.M., ed.). pp. 175-194.

Weatherburn, M.W., 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry, Washington D.C., 39, 971-974.

Winters, A.L., Minchin, F.R., Michaelson-Yeates, T.P., Lee, M.R., Morris, P., 2008. Latent and active polyphenol oxidase (PPO) in red clover (Trifolium pratense) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. J. Agric. Food Chem. 6, 2817-2824.

6 RED CLOVER SILAGE IMPROVES THE MILK FATTY ACID COMPOSITION IN DAIRY EWES

6.1 ABSTRACT

Polyphenol oxidase (PPO) activity in red clover (*Trifolium pratense*) has been shown to reduce both proteolysis and lipolysis in the rumen, improving nitrogen (N) use efficiency and increasing milk fat polyunsaturated fatty acid (PUFA) content . The aim of this study was to evaluate the effects of two isoproteic total mixed rations (TMR) containing either red clover or lucerne (*Medicago sativa*) silage as the major forage sources on milk yield, milk composition, and milk fatty acid (FA) profile in dairy ewes. Sixteen dairy ewes received either a LU diet composed of lucerne silage, corn silage, and concentrate or a RC diet composed of red clover silage, corn silage, and concentrate with an experimental period of 13 days, with the last five days used for sample collection and measurements. The animals were housed individually and milked twice a day (0600 and 1500h). Total dry matter (DM) intake was similar between treatments, but intakes of neutral detergent fiber and acid detergent fiber tended to increase in ewes fed the RC diet. Milk yield, milk composition and N use efficiency were unaffected by treatments. The *n-6/n-3* FA ratio tended to decrease in milk fat from ewes fed the RC diet, while total PUFA and PUFA/Saturated fatty acid (SFA) ratio increased in RC when compared to LU treatment. The contents of α-linolenic (18:3 *n-3*) and linoleic (18:2 *n-6*) acids in milk fat were 31 and 22% higher, respectively, in ewes fed the RC diet compared to LU diet. Compared to LU diet, the red clover diet had no effect on milk yield and composition, but improved the milk fatty acid profile of dairy ewes due to an increased proportion of PUFA (in particular C18:3 *n-3* and, to a lesser extent, C18:2 *n-6*) and a decreased *n-6/n-3* FA ratio.

Keywords: human health; legumes; milk fat quality; nitrogen use efficiency; polyphenol oxidase; secondary compounds

6.2 INTRODUCTION

A number of studies have been conducted to evaluate the effect of feeding practices on milk and meat fatty acid (FA) composition of livestock. Achieving a more balanced *n-6/n-3* FA ratio as well as increased contents of monounsaturated and polyunsatured FA (PUFA) such as *cis*-9 C18:1, *cis-9 trans-11* CLA, and *n-3* FA are common targets in most studies since higher intakes of those FA have been associated with beneficial health effects (Pariza et al., 1996; Simopoulos, 2002). As dairy products are a major source of saturated FA in the human diet, and given that some of these FA increase the plasma levels of total cholesterol and LDL-cholesterol, reducing their contents in milk fat have also been of particular interest from a nutritional point of view.

Greater PUFA proportions, mainly α-linolenic acid (18:3 *n-3*) and linoleic acid (18:2 *n-6*), have been found in milk fat of cows fed red clover silage compared to those fed grass silage (Dewhurst et al., 2003, Al Mabruck et al., 2004; Vanhatalo et al., 2007; Lee et al., 2009a). This effect may be associated with an inhibition of ruminal biohydrogenation (BH) of dietary PUFA as a result of polyphenol oxidase (PPO) activity in red clover. The PPO enzyme, in contact with oxygen, transforms phenolic compounds into quinones, which protects plant lipids from lipolysis and subsequent BH in the rumen (Buccioni et al., 2012; Adler et al., 2013; Cabiddu et al., 2014; Lee, 2014).

Furthermore, studies comparing diets containing red clover or grass silage reported greater milk yield in cows consuming red clover (Bertilsson and Murphy, 2003; Dewhurst et al., 2003; Moorby et al., 2009). However, when red clover was compared with other legumes, no changes in milk yield were found (Hoffman et al., 1997; Broderick et al., 2001, 2007), although an improved N use efficiency has been observed in cows fed red clover silage (Broderick et al, 2001, 2007). The greater N use efficiency has been attributed to reductions in rumen protein degradation due to leaf proteins complexation with quinones, which may be formed by the action of PPO enzyme during the ensiling process (Albrecht and Brodercik, 1992; Broderick et al., 2004; Merry et al., 2006).

Previous studies evaluating the effects of red clover silage on milk yield, milk composition and milk FA profile have been performed using non-isoprotein diets and/or having grass silage as the control treatment. Moreover, most of the studies have been conducted with dairy cows, while the effects of red clover silage on dairy ewes' performance and milk composition remains unknown. Thus, the aim of this study was to evaluate the effects of two

isoproteic total mixed rations (TMR) containing either red clover silage or lucerne silage as the major forage sources on milk yield, milk composition and milk FA profile in dairy ewes.

6.3 MATERIAL AND METHODS

6.3.1 Local, experimental design and treatments

The experimental protocol was approved by the Ethics Committee for Animal Experimentation (Protocol: 1.03.15) from the University of Santa Catarina State (UDESC). The experiment was performed at Estrela da Serra Farm in Bom Retiro, SC, Brazil (27°45'34.6"S 49°38'25.3"W), in April 2016. The experimental period lasted 13 days, with the last five days for measurements and sample collection. Sixteen dairy ewes (8 in each treatment) were assigned to one of two dietary treatments (TMRs based on either red clover silage or lucerne silage) in a randomized block design. Prior to the experimental period, ewes were separated into two homogeneous groups according to milk yield (1.6 ± 0.4 kg/day) and breed (Lacaune or Milchschaf).

The treatments were two isoproteic total mixed rations (TMR) either composed of red clover silage plus corn silage plus concentrate (RC diet), or lucerne silage plus corn silage plus concentrate (LU diet). The proportions of each ingredient in the diets were calculated according to the INRAtion (2007). Ingredients and chemical composition of experimental diets are presented in Table 11.

The red clover was grown on an area implanted in 2013 in Lages, SC, Brazil (27º47′ S, 50º18′ W, 960 m alt.) The material was harvested in December 2014 (vegetation period) with the aid of a motorized harvester at ~5 cm above soil level and kept in the field for five hours, which resulted in partial humidity loss. A commercial inoculant containing *Lactobacillus plantarum* at a dosage of 1×10^6 CFU/g of ensiled material was added when the material reached 45% DM. Subsequently, forage was ensiled in 200 liters barrels, compacted to a density of approximately 450 kg/m^3, and sealed. The lucerne silage and soybean meal were obtained from specialized industry, and the corn silage was produced on the same farm where the experiment was carried out.

6.3.2 Animal measurements

During the experimental period, animals were housed individually in closed pens with concrete floor covered by sawdust. The TMR was prepared for each animal and provided twice a day *ad libitum* (20% of orts) at 0800 and 1600h, after each milking. Samples of TMR were collected from the 8th to the 12th day of the experimental period. In order to adjust the amount of TMR to be provided in the morning, refusals from the previous day were removed, weighed, and samples were collected from the 9th to the 13th day. Samples (TMR and orts) were oven-dried at 60°C for at least 72 h and ground to pass a 1 mm diameter sieve. Then, the DM intake was determined by the difference between the amount provided and orts Water and a mineral supplement were available *ad libitum*.

Animals were milked twice a day (0700 and 1500h) and individual milk yield recorded daily. Milk composition (fat, protein, lactose, casein, and urea contents) was determined from milk samples (a.m. and p.m. milkings (average weighted according the production)) collected from the 9th to the 13th day of the experimental period. The milk samples were stored at 6^{o}C in vials with bronopol and sent to Laboratório Estadual de Qualidade do Leite da Universidade do Contestado, Concórdia-SC, Brazil to analysis. Another set of composite milk samples (a.m. plus p.m. milking) was collected on the last day of the experiment and frozen (-20^{o}C) without bronopol until the analysis of milk FA composition.

Table 11. Formulation and chemical composition of experimental diets containing lucerne (LU) or red clover (RC) silage.

	LU	*RC*
Formulation (g/kg DM)		
Corn Silage	440	380
Red clover silage	-	525
Lucerne silage	490	-
Soybean meal	70	95
Total dry matter (g/kg)	343	460
Chemical composition (g/kg DM)		
Organic matter	936	925
Crude protein	184	180
Neutral detergent fiber	432	457
Acid detergent fiber	257	267

Fonte: próprio autor

6.3.3 Chemical analysis

DM content was determined in an oven at 105°C for 24 hours and the ashes content by combustion in a muffle furnace at 550°C for 5 hours. The N content was determined by the Kjeldahl method (AOAC, 1995). Neutral detergent fiber assayed with a heat stable amylase (aNDF) and acid detergent fiber (ADF), both expressed inclusive of residual ash contents, were determined according to the method described by Van Soest et al. (1991) adapted to a Fiber analyzer (ANKOM Technology, Macedon NY, USA).

For FA profile analysis, milk samples were thawed at room temperature and a volume of 1 mL was used for lipid extraction using a mixture of diethyl ether and hexane according to 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). FAME were separated and quantified by a gas chromatograph (model 7820-A, Agilent Technologies) fitted with a flame-ionization detector and equipped with a CPSil 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 FAME were identified by comparison of retention times with reference FAME standards, and minor *trans/cis*-C18:1 isomers were identified according to their order of elution reported under the same GC analytical 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). Indices of Δ-9 desaturase activity in mammary gland were calculated according to Kelsey et al. (2003).

6.3.4 Statistical Analysis

The variables were submitted to the analysis of variance using PROC GLM of SAS (SAS Institute, Cary, NC, USA), according to the model: $Yijkl = \mu + Ti + Bj + Rk + eijkl$. Where: Yijkl = dependent variable; µ = average of observations; Ti = treatment effect i; Bj = block effect j; Rk = breed effect k; eijk = residual error. The data were presented as adjusted means and values of $P<0.05$ were considered significant and values between $P>0.05$ and $P<0.10$ were considered trend.

6.4 RESULTS

6.4.1 Feed intake, animal performance and milk composition

For all variables analyzed, there are no effects of block or breed. Total DM, OM and N intake were similar between treatments, but intakes of NDF and ADF tended to increase in RC treatment (Table 12). Milk yield, milk composition and N use efficiency (g N excreted in milk/ g N ingested) were unaffected by treatments (Table 13).

Table 12. Intake (g/kg of body weight (BW)) of dairy ewes fed total mixed rations containing lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-*
Dry matter	28.2	34.1	1.91	NS
Organic matter	26.4	31.5	1.75	NS
Nitrogen	0.88	1.05	0.05	NS
Neutral detergent fiber	11.4	14.8	0.88	†
Acid detergent fiber	6.60	8.50	0.500	†

SEM, standard error of the means; Statistical significance: NS, not significant; † P < 0.1; ∗ P < 0.05; ∗∗ P < 0.01; ∗∗∗ P < 0.001. Fonte: próprio autor

Table 13. Milk yield, milk composition and nitrogen (N) use efficiency of dairy ewes fed total mixed rations containing lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-*
Milk yield *(g/day)*	937	960	87.7	NS
Feed efficiency *(g milk/kg DM intake)*	556	476	45.4	NS
Milk composition (%)				
Casein	3.98	4.21	0.10	NS
Fat	6.30	6.23	0.22	NS
Protein	4.64	4.85	0.10	NS
Lactose	4.57	4.52	0.05	NS
Total solids	16.5	16.6	0.28	NS
Somatic cell count	71	59	8.37	NS
Dry extract defatted	10.19	10.39	0.083	NS
Urea *(mg/dl)*	18.6	19.0	1.15	NS
N use efficiency (*N milk/g N ingested)*	0.131	0.118	0.016	NS

SEM, standard error of the means; Statistical significance: NS, not significant; † P < 0.1; ∗ P < 0.05; ∗∗ P < 0.01; ∗∗∗ P < 0.001. Fonte: próprio autor

6.4.2 Milk fatty acid composition

The concentrations of *iso* 15:0, trans-12 16:1, 18:0, trans-4 18:1, 18:2 n-6, *trans- 9, cis-11* CLA, 18:3 *n-3*, 20:0, 21:0, 23:0, and 24:0 increased in milk fat from ewes fed the RC diet (Table 14). However, milk fat 16:0 and *trans-9, cis- 12* C18:2 contents decreased in ewes receiving the RC diet, while the concentrations of others FA were unaffected by treatments.

The *n-6/n-3* FA ratio tended to decrease and mixed FA decreased in milk fat from ewes fed the RC diet. Total PUFA, Σ *n-3* and *n-6* FA, PUFA/SFA ratio increased and preformed FA tended to increase in milk fat from ewes fed the RC diet (Table 15). Fatty acids originated from *de novo* synthesis, total saturated fatty acid (SFA), odd- and branched-chain fatty acid (OBCFA) and mono-unsaturated fatty acids (MUFA) were similar between treatments. The indices of Δ-9 desaturase activity in mammary gland were unaffected by treatment.

Table 14**.** Fatty acid composition of the milk (g/100g of total fatty acid) from dairy ewes fed the total mixture ration containing lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-value*
4:0	4.14	4.21	0.114	NS
6:0	2.65	2.68	0.103	NS
8:0	2.26	2.23	0.110	NS
10:0	6.70	6.19	0.379	NS
12:0	3.90	3.61	0.195	NS
iso 14:0	0.11	0.11	0.005	NS
14:0	11.6	11.5	0.242	NS
iso 15:0	0.25	0.29	0.009	*
anteiso 15:0	0.38	0.41	0.014	NS
cis-9 14:1	0.16	0.16	0.006	NS
15:0	0.99	1.00	0.012	NS
iso 16:0	0.26	0.27	0.024	NS
16:0	31.9	28.3	0.789	*
trans-9 16:1+ *iso* 17:0	0.36	0.37	0.009	NS
trans-12 16:1	0.22	0.28	0.010	**
cis-9 16:1+ *anteiso* 17:0	1.12	1.15	0.051	NS
17:0	0.70	0.74	0.012	NS
*iso 1*8:0	0.06	0.05	0.003	NS
cis-9 17:1	0.21	0.22	0.009	NS
18:0	6.66	7.99	0.298	*
trans-4 18:1	0.01	0.02	0.0006	**
trans-5 18:1	0.01	0.02	0.003	NS
trans-6+8 18:1	0.12	0.14	0.006	†
trans-9 18:1	0.13	0.15	0.005	NS
trans-10 18:1	0.19	0.20	0.007	NS

trans-11 18:1	0.79	0.76	0.030	NS
trans-12 18:1	0.22	0.23	0.009	NS
trans-13+14 18:1	0.19	0.21	0.011	NS
trans-16 18:1	0.26	0.27	0.012	NS
cis-9 18:1	14.9	16.6	0.680	NS
cis-11 18:1	0.53	0.59	0.006	NS
cis-12 18:1	0.21	0.23	0.021	NS
cis-13 18:1	0.07	0.07	0.003	NS
cis-15 18:1 + 19:0	0.16	0.17	0.005	NS
trans- 9 trans-12 18:2	0.01	0.01	0.0005	NS
cis- 9 trans-12 18: 2	0.06	0.06	0.002	NS
trans- 9 cis-12 18:2	0.05	0.03	0.002	**
18:2 *n*-6	1.30	1.59	0.053	*
cis- 9 trans-11 CLA	0.57	0.52	0.026	NS
trans- 9 cis-11 CLA	0.019	0.024	0.001	*
trans- 10 cis-12 CLA	0.004	0.004	0.0003	NS
18:3 *n*-6	0.04	0.04	0.003	NS
18:3 *n-3 + cis 11* 20:1	0.70	0.92	0.036	**
20:0	0.23	0.52	0.038	***
20:2*n*-6	0.01	0.01	0.0009	NS
20:3*n*-6	0.05	0.05	0.003	NS
20: 4*n*-6	0.18	0.18	0.010	NS
20:5*n*-3	0.05	0.05	0.002	NS
21:0	0.06	0.08	0.004	***
22:0	0.05	0.03	0.005	NS
22:5*n*-3	0.09	0.09	0.003	NS
23:0	0.04	0.07	0.004	***
24:0	0.05	0.07	0.003	**

CLA, conjugated linoleic acid; SEM, standard error of the means; Statistical significance: NS, not significant; † $P < 0.1$; ∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. Fonte: próprio autor

Table 15. Groups of fatty acids (g/100g of fatty acid) and indications of Δ-9 desaturase activity in milk from dairy ewes fed the total mixture ration containing Lucerne (LU) or red clover (RC) silages.

	LU	*RC*	*SEM*	*P-value*
Fatty acids group				
n-3	0.84	1.08	0.040	∗∗
n-6	1.59	1.88	0.056	∗
n-6/*n*-3	1.91	1.73	0.056	†
De novo (4:0-15:0)	33.0	32.1	0.552	NS
Mixed (16:0+16:1)	33.6	30.1	0.415	*
Preformed (≥18:0)	28.1	32.0	0.472	†
SFA	69.1	66.3	0.816	NS
MUFA	18.3	20.2	0.739	NS
PUFA	3.14	3.59	0.090	∗
PUFA/SFA	0.046	0.054	0.0018	∗
Σ OBCFA	4.17	4.36	0.082	NS
Indices of Δ*-9 desaturase activity*				
14:1/14:0+14:1	0.014	0.014	0.004	NS
16:1/16:0+16:1	0.03	0.04	0.006	NS
18:1*c*9/18:0+18:*11c9*	0.69	0.67	0.003	NS
18:2 *c*9*t*11/C18:1*t*1118:2 *c*9*t*11	0.41	0.41	0.003	NS

MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acid; ΣOBCFA, odd- and branched-chain fatty acid (*iso* 14:0 + *iso* 15:0 + *anteiso* 15:0 + 15:0 + *iso* 16:0 + *iso* 17:0 + *anteiso* 17:0 + 17:0); SEM = standard error of the means; Statistical significance: NS, not significant; † P < 0.1; ∗ P < 0.05; ∗∗ P < 0.01; ∗∗∗ P < 0.001.Fonte: próprio autor.

6.5 DISCUSSION

6.5.1 Effect of red clover diet on milk yield and milk composition

The lack of difference in milk yield may be explained by the similar DM intake between treatments, since this is the main determinant of milk yield (Waldo and Jorgensen, 1981), while the trend (P<0.10) for increased fiber intake in RC diet was probably a consequence of differences in NDF content between the diets. Changes in fiber intake in animals consuming similar amounts of DM have been reported in the literature (Huhtanen et al, 2007), and would be indicative of situations where rumen fill does not play a preponderant role in the regulation of voluntary intake.

In contrast to previous reports (Broderick et al., 2000; Dewhurst et al., 2003; Broderick et al., 2007), daily N use efficiency (g N excreted in milk/ g N ingested) was unaffected by

treatments. This may be explained, at least in part, by a surplus of circulating amino acids in relation to the animal's requirement, or due to changes in the profile of amino acids available for absorption in the small intestine. Improvements in N use efficiency in diets containing RC silage are usually attributed to the presence of PPO enzyme, which transforms phenolic compounds into quinones in the presence of oxygen. Quinones complex with food proteins reducing their degradability and solubility (Lee, 2014), and consequently the amount of dietary N lost as ammonia in the rumen. The smaller ruminal degradability of protein increases its flow from food to duodenum (Brito et al., 2007), but the amino acids profile absorbed in the small intestine may also be changed. Besides, amino acids that contain sulphur can serve as a binding site for quinone, which could reduce the intestinal digestibility of proteins (Lee et al., 2009a; Vanhatalo et al., 2009). This effect has been observed by our group in a previous study (G. Guzatti, unpublished data) where sheep were fed diets based on RC silage as compared to alfalfa silage, showing that RC reduces both ruminal degradability and intestinal digestibility of N when compared to alfalfa

6.5.2 Effect of red clover diet on milk fatty acid composition

Increased PUFA contents in milk fat from ewes fed the RC diet is likely to be a consequence of an inhibition of ruminal BH of dietary PUFA due to the presence of secondary/bioactive compounds in RC (Lee et al., 2009b; Van Ranst et al., 2011). Secondary and/or bioactive compounds found in some plants have been reported to affect ruminal BH (Buccioni et al., 2012; Cabiddu et al., 2014). In red clover, the PPO enzyme can form quinones in the presence of oxygen. The quinones, in turn, have been shown to inhibit ruminal lipolysis of PUFA present in forages (Lee et al., 2009b; Van Ranst et al., 2011; Buccioni et al., 2012; Adler et al., 2013; Cabiddu et al., 2014;), resulting in a lower ruminal BH. This occurs because rumen bacteria can only perform BH from free PUFA (i.e. PUFA must be first released by hydrolysis to undergo BH) (Buccioni et al., 2012). Thereby, increases in milk fat PUFA content, as well as the higher PUFA/SFA ratio observed in animals fed the RC diet in our study may be explained, at least partially, by the lower ruminal BH of PUFA (Lee et al., 2004). However, the mechanism by which quinones inhibit PUFA lipolysis in the rumen has not yet been fully understood. Deactivation of lipolytic enzymes (Van Ranst et al., 2009) and/or formation of complexes between lipid-protein-phenols (Lee et al., 2003, 2011) have been suggested as putative mechanisms.

On the other hand, the amount and type of FA present in the diet may also influence the FA composition of the final product, i.e., diets rich in PUFA usually result in higher concentrations of PUFA in milk fat from cows and goats (Bernard et al., 2009; Halmemies-Beauchet-Filleau et al., 2011). However, even in diets containing high PUFA contents (e.g. fresh green forages), factors such as forage species can modify the extension of rumen BH as a result of fiber content and passage rate of the diets. For example, feeding legumes usually increases the PUFA content in the final product when compared to grasses due to higher passage rates (Mosely et al., 1984), which reduces the fermentation time and/or the extension of ruminal BH. In the present work, both experimental diets were based on forage legumes, and differences in milk fatty acid profile seems to be associated with differences in ruminal BH.

These results are in agreement with several previous studies comparing RC silage with grass silage, in which greater amounts of PUFA were found in milk and meat from animals receiving RC silage due to decreased ruminal BH, as a function of quinones present in this legume (Dewhurst et al., 2003; Lee et al., 2009a; Moorby et al., 2009). Otherwise, in a study were bulls were fed on red clover or grass silage, Huws et al. (2010) also found lower ruminal BH in red clover silage, but these authors associated this effect with the lower population of Anaerovibrio lipolytica responsible for PUFA release in the rumen. In the present study, a legume containing no secondary or bioactive compounds (Lucerne) was used as the control, which reinforces the hypothesis of inhibitory mechanisms on ruminal BH of PUFA in the RC-containing diet.

Another factor associated with lower ruminal BH is a greater DM intake, which may be a consequence of faster passage rate and reductions in fermentation time of feeds (Mertens, 1994). In this regard, it should be noted that, despite the similar DM intake between treatments in our study, fiber intake tended to be higher in the RC diet , which may have increased the retention time of digesta (Lourenço et al., 2009), favoring the growth of cellulolytic bacteria and, therefore, ruminal BH. This observation further reinforces our hypothesis that the greater PUFA content in milk fat from ewes fed the RC diet is a result of bioactive/secondary compounds present in RC.

6.5.3 Implications

Besides of a diet rich in PUFA, the higher conjugated linoleic acid intake (CLA) and a desirable *n-6/n-3* FA ratio are characteristics of diets considered human healthier (Patel et al., 2013). In this way, PUFA content increased and *n-6/n-3* FA ratio trended to decrease in RC

diet. According to Lee et al. (2009a), the improvement in *n-6/n-3* FA ratio may be associated a greater protection of α-linolenic acid (18:3 *n-3*) than linoleic acid (18:2 *n-6*). This is a consequence its preferential incorporation into photosynthetic structures such as chloroplasts which contain the PPO enzyme in RC. In the present study the α-linolenic acid was31 % higher while the linoleic acid was 22% in milk from animals receiving RC diet. The n-*6/n-3* FA ratio founded in the present work is similar another works that used diets containing red clover silage (Van Dorland et al., 2008; Campidonico et al., 2016). Although RC diet improves the *n-6/n-3* FA ratio, is important to note that both treatments had healthier ratios, with values lesser than 5:1 (Institute of Medicine, 2002). Otherwise, CLA content was the same for both treatments. These results were probably a consequence of similar Δ-9 desaturase activity in the mammary gland, verified by similar ratio of C18:2*c9t11*/C18:1 *t11*, reinforced once more that modifications in milk FA profile was a consequence of modifications in ruminal BH.

6.6 CONCLUSION

Replacement of alfalfa silage with red clover silage in ewes' diet has no effect on animal performance and N use efficiency, but increases the proportion of PUFA and decreases the *n-6/n-3* FA ratio in milk.

6.7 REFERENCES

Adler, S. A., S. K. Jensen, E. Thuen, A. M. Gustavsson, O. M. Harstad, and H. Steinshamn. 2013. Effect of silage botanical composition on ruminal biohydrogenation and transfer of fatty acids to milk in dairy cows. J. Dairy Sci. 96:1135-1147.

Albrecht, K. A., and G. A. Broderick. 1992. Ruminal in vitro degradation of protein from different legume species. IN: Research Summaries. US Dairy Forage Research Centre, Madison, WI, pp 92–94.

Al Mabruk R.M.. N. F. G. Beck, and J. Dewhurst. 2004. Effects of silage species and supplemental vitamin E on the oxidative stability of milk. J. Dairy Sci. 87:406-412.

Association of Official Analytical Chemists. 1995.Officia lMethods of Analysis. 16th ed. AOAC, Washington, DC

Baldin, M., M. A. S. Gama, R. Dresch, K. J. Harvatine, and D. E. Oliveira. 2013. A rumen unprotected conjugated linoleic acid supplement inhibits milk fat synthesis and improves energy balance in lactating goats. J. Anim. Sci. 91:3305-3314.

Bernard, L., K. J. Shingfield, J. Rouel, A. Ferlay, and Y. Chilliard. 2009. Effect of plant oils in the diet on performance and milk fatty acid composition in goats fed diets based on grass hay or maize silage. Br. J. Nutr.101:213-224.

Bertilsson, J., and M. Murphy. 2003. Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. Grass Forage. Sci. 58: 309-322.

Brito, A. F., G. A. Broderick, and S. M. Reynal. 2007. Effects of different protein supplements on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy Sci. 90: 1828–1841.

Broderick, G. A., K. A. Albrecht, V. N. Owens, and R. R. Smith. 2004. Genetic variation in red clover for rumen protein degradability. Anim. Feed Sci. Technol. 113:157-167.

Broderick, G. A., A. F. Brito, and J. J. Olmos Colmenero. 2007. Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on the Production of Lactating Dairy Cows. J. Dairy Sci. 90:1378–1391.

Broderick, G. A., R. P. Walgenbach, and S. Maignan. 2001. Production of Lactating Dairy Cows Fed Alfalfa or Red Clover Silage at Equal Dry Matter or Crude Protein Contents in the Diet. J. Dairy Sci. 84:1728–1737.

Broderick, G. A., R. P. Walgenbach, and E. Sterrenburg. 2000. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. J. Dairy Sci. 83:1543-1551.

Buccioni, A., M. Decandia, S. Minieri, G. Molle, and A. Cabiddu. 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. Anim. Feed Sci. Technol. 174: 1-25.

Cabiddu, A., M. R. F. Lee, M. Decandia, G. Molle, L. Salis, M. Vargiu, and A. L. Winters. 2014. Characterization of polyphenol oxidase activity in a range of forage ecotypes with different phenol substrates. A new insight for PPO and protein-bound phenol evaluation. Grass Forage Sci. 69:678-692.

Campidonico, L., P. G. Toral, A. Priolo, G. Luciano, B. Valenti, G. Hervás, P. Frutos, G. Copani, C. Ginane, and V. Niderkorn. 2016. Fatty acid composition of ruminal digesta and longissimus muscle from lambs fed silage mixtures including red clover, sainfoin, and timothy. J. Anim. Sci. 94:1550–1560.

Cruz-Hernandez, C., J. K. G. Kramer, J. J. Kennelly, D. R. Glimm, B. M. Sorensen, E. K. Okine, L. A. Goonewardene, and R. J. Weselake. 2007. Evaluating the conjugated linoleic acid and trans 18:1 isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil. J. Dairy Sci. 90:3786-3801.

Dewhurst, R. J., W. J. Fisher. J. K. S. Tweed, and R. J. Wilkins. 2003. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. J Dairy Sci. 86:2598–2611.

Halmemies-Beauchet-Filleau, A., T. Kokkonen, A. M. Lampi, V. Toivonen, K. J. Shingfield, and A. Vanhatalo. 2011. Effect of plant oils and camelina expeller on milk fatty acid composition in lactating cows fed diets based on red clover silage. J. Dairy Sci. 94:4413–4430.

Hoffman P.C., D. K. Combs, N. M. Brehm, and D. A. Welch. 1997. Performance of lactating dairy cows fed red clover or alfalfa silage. J. Dairy Sci. 80:3308-3315.

Huhtanen, P., U. Asikainen, M. Arkkila, and S. Jaakkola. 2007. Cell wall digestion and passage kinetics estimated by marker and in situ methods or by rumen evacuations in cattle fed hay 2 or 18 times daily. Anim. Feed Sci. Technol. 133:206-227.

Huws, S. A., M. R. F. Lee, S. B. Muetzel, M. B. Scott, R. J. Wallace, and N. D. Scollan. 2010. Forage type and fish oil cause shifts in rumen bacterial diversity. FEMS Microbiol. Ecol. 73:396–407.

Institute of Medicine. 2002. Dietary Reference Intakes (DRIs) for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. Part 1. Washington (DC): National Academy Press.

Kelsey, J. A., B. A. Corl, R. J. Collier; and D. E. Bauman. 2003. The effect of breed, parity, and stage of lactation on conjugated linoleic acid (CLA) in milk fat from dairy cows J. Dairy Sci., 86:2588–2597.

Lee, M. R. F., A. Cabiddu, F. Hou, V. Niderkorn, E. J. Kim, R. Fychan, and N. D. Scollan. 2011. In vitro rumen simulated (RUSITEC) metabolism of freshly cut or wilted grasses with contrasting polyphenol oxidase activities. Grass Forage Sci. 66:196–205.

Lee, M. R. F., P. R. Evans, G. R. Nute, R. I. Richardson, and N. D. Scollan. 2009a. A comparison between red clover silage and grass silage feeding on fatty acid composition, meat stability and sensory quality of the M. Longissimus muscle of dairy cull cows. Meat Sci. 81:738–744.

Lee, M. R. F., E. M. Martinez, and N. D. Scollan. 2003. Plant enzyme mediated lipolysis of Lolium perenne and Trifolium pratense in an in vitro simulated rumen environment. Asp Appl Biol. 70:115–120.

Lee, M. R. F., V. J. Theobald, J. K. S. Tweed, A. L. Winters, and N. D. Scollan. 2009b. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. J. Dairy Sci. 92:1136–1147.

Lee, M. R. F., A. L. Winters, N. D. Scollan, R. J. Dewhurst, M. K. Theodorou, and F. R. Minchin. 2004. Plant mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. J. Sci. Food Agric. 84:1639–1645.

Lee, M. R. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Front. Plant Sci. 5. Article 694.

Lourenço, M., G. Van Ranst, B. Vlaeminck, S. Smet, and V. Fievez. 2009. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. Anim. Feed Sci. Technol. 145:418–437.

Merry, R. J., M. R. F. Lee, D. R. Davies, R. J. Dewhurst, J. M. Moorby, N. D. Scollan, and M. K. Theodorou. 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. J. Anim. Sci. 84:3049-3060.

Mertens, D.R. 1994. Regulation of forage intake. In: Fahey Jr., G.C., (Ed.) Forage quality, evaluation and utilization. Madison: American Society of Agronomy, p.450-493.

Moorby, J. M., M. R. F. Lee, D. R. Davies, E. J. Kim, G. R. Nute, N. M. Ellis, and N. D. Scollan. 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. J. Dairy Sci. 92:1148–1160.

Mosely, G., and J. R. Jones. 1984. The physical digestion of perennial ryegrass (Lolium perenne) and white clover (Trifolium repens) in the foregut of sheep. Br. J. Nutr. 52:381–390.

Pariza, M.W., Y. Park, M. Cook, K. Albright, and W. Liu. 1996. Conjugated linoleic acid (CLA) reduces body fat. FASEB J. 10:3227 (Abstr.)

Patel, M., E. Wredle, and J. Bertilsson. 2013. Effect of dietary proportion of grass silage on milk fat with emphasis on odd- and branched-chain fatty acids in dairy cows. J. Dairy Sci. 96:390-397.

Simopoulos, A. P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother. 56:365–379.

Van Dorland, H. A., M. Kreuzer, H. Leuenberger, and H. R. Wettstein. 2008. Comparative potential of white and red clover to modify the milk fatty acid profile of cows fed ryegrass-based diets from zero-grazing and silage systems. J. Sci. Food Agric. 88:77-85.

Vanhatalo, A., K. Kuoppala, S. Ahvenjärvi, and M. Rinne. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. J. Dairy Sci. 92:5620-5633.

Vanhatalo, A., K. Kuoppala, V. Toivonen, and K. J. Shingfield. 2007. Effects of forage species and stage of maturity on bovine milk fatty acid composition. Eur J Lipid Sci Technol. 109:856-867.

Van Ranst, G., V. Fievez, M. Vandewalle, J. Riek, and E. Van Bockstaele. 2009. In vitro study of red clover polyphenol oxidase activity, activation, and effect on measured lipase activity and lipolysis. J. Agric. Food Chem. 57:6611–6617.

Van Ranst, G., M. R. F. Lee, and V. Fievez. 2011. Red clover polyphenol oxidase and lipid metabolism. Animal. 5:512-521.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Waldo, D. R., and N. A. Jorgensen. 1981. Forages for high animal production: Nutritional factors and effects of conservation. J. Dairy Sci. 64:1207-1229.

Wolff, R. L., C. C. Bayard, and R. J. Fabien. 1995. Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on trans-18:1 acids. Application to the study of seasonal variations in French butters. J. Am. Oil Chem. Soc. 72:1471-1483.

7 CONSIDERAÇÕES FINAIS

Essa tese foi desenvolvida com o objetivo de compreender os impactos do uso da silagem de trevo vermelho sobre a eficiência de uso do nitrogênio e o perfil de ácidos graxos produzido no leite de ovinos, uma vez que o processo de ensilagem do trevo vermelho permite a formação de *quinona*, um composto bioativo capaz de reduzir a proteólise durante o processo de ensilagem e degradação ruminal, bem como a lipólise e posterior bio-hidrogenação ruminal dos ácidos graxos poli-insaturados.

Com base nos resultados obtidos, fica evidente que a silagem de trevo vermelho é uma ferramenta capaz de diminuir a degradabilidade ruminal da proteína *in vitro*, *in vivo* e a excreção urinária de nitrogênio, mitigando os impactos da excreção nitrogenada em sistemas intensivos de produção animal. Além disso, o fornecimento de dietas contendo silagem de trevo vermelho pode melhorar o perfil dos ácidos graxos produzido no leite de ovinos, elevando a concentração de ácidos graxos poli-insaturados em comparação à silagem de alfafa.

De maneira mais específica, a redução na digestibilidade ruminal da proteína proporcionada pela dieta contendo silagem de trevo vermelho permitiu elevar o fluxo intestinal de nitrogênio não amoniacal em 37%, o que poderia resultar em uma maior retenção nitrogenada pelos animais recebendo essa dieta. Embora, a retenção nitrogenada tenha sido a mesma para os animais recebendo ambas as dietas, a redução na digestibilidade intestinal do nitrogênio foi de apenas 3%, de modo que a variabilidade dos resultados e o potencial produtivo dos animais utilizados podem ter influenciado para que as retenções nitrogenadas fossem semelhantes. Neste sentido, o maior fluxo intestinal de nitrogênio na dieta contendo silagem de trevo vermelho parece ter excedido as exigências em aminoácidos dos animais utilizados. Além disso, a utilização de uma dieta controle possivelmente capaz de suprir as exigências em proteína dos ovinos pode ter contribuído para a semelhança na retenção nitrogenada neste estudo. Assim, o uso de animais com maior exigência nutricional e/ou de dietas com menor teor de proteína poderia ser objeto de futuros estudos quando o objetivo for aumentar a retenção nitrogenada e o desempenho animal. Isto se justifica pelo fato que a silagem de trevo vermelho é capaz de reduzir perdas ruminais de N, além de aumentar a síntese de proteína microbiana em comparação à silagem de alfafa.

De qualquer modo, o uso da silagem de trevo vermelho resultou em menor proporção de nitrogênio excretado via urina em comparação às fezes (-15%), proporcionando benefícios ambientais sem prejudicar a retenção nitrogenada e o desempenho animal. Entretanto, o

consumo da silagem de trevo vermelho aumentou a proporção de ácidos graxos poli-insaturados e melhorou a relação entre os ácidos graxos *n6/n3*. Contudo, é possível que a utilização de animais com maior capacidade produtiva resultasse em melhoria de rendimento leiteiro nos animais recebendo silagem de trevo vermelho, como resposta ao maior fluxo intestinal de nitrogênio decorrente do consumo dessa dieta. Ademais, a ocorrência de efeitos sinérgicos sobre o valor energético da dieta (*in vitro*) quando o trevo vermelho é ensilado com uma gramínea de clima tropical na mesma proporção da MS total parecem estar mais associados as interações digestivas entre leguminosas e gramíneas do que pela presença das *quinonas per se*.

Por fim, embora os resultados obtidos nesses experimentos sugiram a atuação das *quinonas*, sua quantificação permitiria conclusões mais incisivas. Além disso, a silagem produzida no primeiro experimento teve apenas valores de pH e nitrogênio amoniacal mensurados, sendo que a determinação de outros parâmetros como capacidade tampão, aminas biogênicas, contagem de *Clostridium* e ácidos orgânicos permitiria a elaboração de uma discussão mais consistente sobre a qualidade da silagem produzida. Dessa forma, sugere-se a realização de trabalhos futuros para estudar com maior detalhe a qualidade de silagens produzidas com trevo vermelho em associação com gramíneas de clima tropical. Ainda, estudos futuros devem ser conduzidos para avaliar os efeitos sinérgicos da ensilagem de trevo vermelho e capim quicuio em experimentos *in vivo*.