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Red clover silage: an alternative for mitigating the impact of nitrogen excretion in ovine production systems

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ABSTRACT - The objective was to quantify the flow of intestinal nutrients and nitrogen excretion and retention in sheep receiving isoproteic diets. Eight Texel × Lacaune wethers (average body weight = 25±2.5 kg) were fitted with duodenal cannula and housed in metabolic cages. Wethers were assigned to the treatments in a crossover design with two periods of 20 days each, and all feces and urine produced by the wethers were collected. The treatments consisted of two isoproteic (160 g kg^{-1} of crude protein on dry matter basis) diets composed of red clover (RC) or lucerne (LU; Medicago sativa) silages plus corn silage and concentrate feed. The digestible organic matter and metabolizable energy intake did not differ between treatments. The intestinal non-ammonia N (NAN) flow was 5.9 g day^{-1} (37%) higher in RC wethers than in those of the LU treatment. This result was a consequence of both an increase in the efficiency of microbial protein synthesis (12.7% higher) and a decrease in ruminal degradable protein (RDP) content (20% lower) of the diet. However, the increase in the intestinal NAN flow was accompanied by a reduction in intestinal digestibility of N, resulting in similar daily N retention between treatments. The reduction of RDP content was probably the main reason for reductions in N urinary excretion in RC wethers compared with those in the LU treatment, showing that RC silage may be a tool for mitigating the impact of N excretion in ovine production systems, without changes in N retention.

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

Introduction

Concerns regarding environmental nitrogen pollution have increased the importance of nutritional strategies to reduce the ruminal degradable protein (RDP) content of diets and urinary N excretion by ruminants. 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, when in contact with oxygen, catalyzes the oxidation of endogenous phenols to quinones (Lee et al., 2013). Quinones might form complexes with proteins, which reduces their degradability and solubility (Lee, 2014), increasing the duodenal flow of protein and reducing urinary N excretion. Considering that nitrous oxide emitted by the decomposition of urea excreted in the urine is approximately 300 times more polluting than carbon dioxide, a change in the N excretion route from urine to feces is an important strategy for reducing

environmental pollution (Varel et al., 1999). In addition, RC silage has a lower level of soluble protein in the silage mass when compared with several other forages (Sullivan and Hatfield, 2006). Silages with greater amounts of soluble protein result in a lower N use efficiency (Nagel and Broderick, 1992), since ruminal bacteria have a limited capacity for using non-protein nitrogen (NPN) available in the rumen (Van Soest, 1994).

Several studies have measured animal performance or nitrogen balance of diets containing RC silage compared with grass silages (Kuoppala et al., 2009; Moorby et al., 2009; Vanhatalo et al., 2009), but differences in voluntary intake and passage rate between grasses and legumes (Kammes and Allen, 2012) might have biased the results. Moreover, previous studies comparing RC silage with another legume (e.g., lucerne, *Medicago sativa* L.) did not include isoproteic diets (Broderick et al., 2000; Brito et al., 2007), which means there is a risk of misinterpreting a higher N use efficiency in diets containing RC silage.

Accordingly, the objective was to quantify the flow of intestinal nutrients, N excretion, and N retention in sheep receiving isoproteic diets based on either silage RC or Lucerne (LU). The following hypotheses were tested: wethers fed diets based on RC silage should have lower rates of ruminal protein degradation and greater intestinal flow of non-ammonia nitrogen in comparison with those fed diets based on LU silage and the diet containing RC silage should reduce urinary N excretion compared with LU silage.

Material and Methods

Research on wethers was conducted according to the institutional committee on animal use (Protocol number: 1.03.15). The experiment was conducted in 2015 in Lages, Santa Catarina, Brazil (27°47'S, 50°18'W, 960 m asl.).

Eight Texel × Lacaune wethers (average BW = 25±2.5 kg) were assigned to the treatments in a crossover experimental design with two 20-day periods each (13 days for diet adaptation and seven days for data collection). Two months before the experimental period, wethers were surgically fitted with a duodenal cannula. Ten days before the beginning of the experimental period, wethers were dewormed and housed in metabolic cages for acclimation to the experimental conditions.

Diets were isoproteic total mixture rations (TMR) composed of corn silage + concentrate feed + RC or LU silage (Table 1). The proportion of each ingredient in the diet was calculated according to INRAtion (INRA, 2007). The TMR was individually prepared for each wether and provided twice a day *ad libitum* (20% of orts) at 08.00 and 16.00 h. Water and mineral supplement were available *ad libitum*. Before providing the diet in the morning, all orts from the previous day were collected and weighed. Diet samples were collected between days 14 and 18, and orts between days 15 and 19 of each period.

Table 1 - Formulation and chemical composition of experimental diets

	Lucerne silage	Red clover silage
Formulation (g kg^{-1} DM)		
Corn silage	405	320
Red clover silage	-	585
Lucerne silage	535	-
Soybean meal	60	95
Total dry matter (g kg^{-1})	360	480
Chemical composition (g kg^{-1} DM)		
Organic matter	938	934
Crude protein	154	155
Neutral detergent fiber	462	494
Acid detergent fiber	279	305

Red clover was planted in 2013 and harvested in December 2014, during its vegetative period (weather conditions in December 2014: precipitation = 187 mm; temperature: minimum 14.7 °C, mean 19.5 °C, and maximum 25.3 °C; insolation: 133 hs). Red clover was cut with a motorized harvester approximately 5 cm above the ground level and kept in the field for approximately 5 h, which resulted in partial humidity loss. When the material contained dry matter (DM) of approximately 450 g kg^{-1}, a commercial inoculant containing *Lactobacillus plantarum* at a dosage of 1×10^6 cfu g^{-1} of ensiled material was added, and forage was ensiled in a 200-L barrel, compacted up to an approximate density of 450 kg m^{-3}, and sealed. The LU silage was obtained from a specialized company, and corn silage was produced by the local dairy cattle sector.

The wethers were housed individually in metabolic cages, and all feces and urine were collected. The total amount of feces produced by each wether was weighed daily, and samples were collected from day 15 until 19 of each period. Samples were oven-dried at 60 °C for at least 72 h, ground to pass through a 1-mm sieve, and stored until analysis. Samples were pooled per wether and period for analysis. Total urine was collected with the aid of a urine collector and stored in buckets containing 100 mL of sulfuric acid (3.6 M) to reduce the pH to below 3.0. The total volume was measured daily, and a 1% sample was collected from day 15 until 19. Samples were filtered on gauze and diluted in 100-mL volumetric flasks with distilled water. These samples were pooled per wether in each period and stored at −20 °C for further analysis. To determine the flow of duodenal nutrients, duodenal digesta were collected at 6-h intervals at days 19 and 20. These samples were pooled per wether and period and were stored at −20 °C for further analysis. For analysis, duodenal samples were thawed and homogenized. An aliquot was removed, filtered, and acidified to quantify the NH_3-N (ammonia nitrogen) concentration. The remainder of the sample was lyophilized (BioSan, Model L101) and ground to pass through a 1-mm sieve for analysis.

Dry matter content was determined by drying samples in an oven at 105 °C for 24 h, followed by ash combustion in a muffle furnace at 550 °C for 5 h. The N content was determined by the Kjeldahl method (AOAC, 1995). Neutral detergent fiber was assayed using a heat stable amylase (aNDFom) and acid detergent fiber (ADFom), and both were expressed excluding residual ash contents, following the method described by Van Soest et al. (1991) adapted for 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 subjected to treatment with neutral detergent solution for 1 h.

The NH_3-N concentration was measured using the phenol-hypochlorite method (Weatherburn, 1967). Purines were quantified in the duodenal digesta samples according to the technique proposed by Makkar and Becker (1999). Duodenal and fecal samples were incubated in the rumen of a fistulated animal for 288 h, and subsequently the indigestible aNDFom was used as a flow marker (Krizsan and Huhtanen, 2013).

The total DM intake (DMI) was determined as the difference between the amount of TMR provided and orts.

The apparent digestibility of DM and its constituents was calculated as follows:

$$[\text{DMI (g day}^{-1}) - \text{fecal DM (g day}^{-1})] / \text{DMI (g day}^{-1}) \text{ (Van Soest, 1994)}$$

The true organic matter digestibility was estimated assuming that the neutral detergent soluble fractions of feces are from endogenous origin, and that only the aNDFom fraction of feces originates from feed, as follows:

$$[(\text{OM intake (g day}^{-1}) - \text{fecal aNDfom (g day}^{-1})] / \text{OM intake (g day}^{-1}) \text{ (Van Soest, 1994)}$$

The true N digestibility was calculated as follows:

$$[(\text{N intake (g day}^{-1}) - \text{fecal neutral detergent insoluble N (g day}^{-1})] / \text{N intake (g day}^{-1})$$

The metabolizable energy (ME; MJ day^{-1}) was calculated according to AFRC (1993) as follows:

$$[0.0157 \times \text{digestible OM intake (g day}^{-1})]$$

The intestinal flow of DM (g day^{-1}) was calculated on the basis of indigestible aNDFom (iaNDFom) concentration in duodenal digesta and feces as follows:

[(fecal iaNDFom (g kg^{-1} DM) × fecal DM (g day^{-1})] /duodenal iaNDFom (g kg^{-1} DM) (Huhtanen et al., 1994; Krizsan and Huhtanen, 2013)

The intestinal flow of each nutrient (g day^{-1}) was calculated by multiplying their concentration in the duodenal digesta by duodenal flow of DM (g day^{-1}) (Huhtanen et al., 1994).

The intestinal flow of non-ammonia N (NAN) (g day^{-1}) was calculated as the difference between N flow and NH_3-N flow as follows:

[N flow (g day^{-1}) – NH_3-N flow (g day^{-1})] (Owens and Goetsch, 1988)

The microbial N flow was calculated by considering the DM flow and the amount of purines in duodenal digesta.

The ruminal degradability of dietary N was calculated as follows:

1 – [(duodenal N (g day^{-1}) – microbial N (g day^{-1}) – NH_3-N flow (g day^{-1})]/ N intake (g day^{-1}) (Owens and Goetsch, 1988)

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

[microbial N (g day^{-1})/OM truly digestible intake (kg day^{-1})] (Fox et al., 2003)

The nitrogen ruminal utilization was calculated as follows:

[(microbial N (g day^{-1})/rumen degradable N intake (g day^{-1})] (Fox et al., 2003)

The N retention was calculated as follows:

[N intake (g day^{-1}) – N excreted in feces (g day^{-1}) – N excreted in urine (g day^{-1})]

(Van Soest, 1994)

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

{[(intake (g day^{-1}) – duodenal flow (g day^{-1})] / [(intake (g day^{-1}) – excretion (g day^{-1})]}

(Van Soest, 1994)

The intestinal digestibility of N was calculated as follows:

[(NAN flow (g day^{-1}) – fecal neutral detergent insoluble N (g day^{-1})/ NAN flow (g day^{-1})]

(Van Soest, 1994)

The ruminal undegradable protein was calculated as follows:

Non-ammonia non-microbial N flow (g day^{-1}) / N intake (g day^{-1}) (Owens and Goetsch, 1988)

The variables were subjected to analysis of variance (ANOVA) using the MIXED procedure of SAS (Statistical Analysis System, version 9.2), according to the following model:

$$Yijk = \mu + Ai + Pj + Tk + eijk,$$

in which Yijk = dependent variable, μ = average of observations, Ai = random effect of wether i, Pj = random effect of period j, Tk = fixed effect of treatment k, and eijk = residual error. The data were presented as adjusted means (LSMEANS). Values of $P<0.05$ were considered significant, and values between $P>0.05$ and $P<0.10$ were considered trend. All variables analyzed had a normal distribution (Shapiro-Wilk test, $P>0.05$) and homogeneity of variance (Bartlett's test; $P>0.05$).

Results

The OM, digestible OM, and ME intake did not differ between treatments (P>0.05). However, aNDFom and ADFom intake were greater (P>0.05) in RC wethers compared with LU wethers (Table 2). The apparent digestibility of DM was lower (P>0.05), whereas the apparent and true OM digestibility tended be lower in wethers in the RC treatment compared with LU ones. Ruminal digestibility of DM, OM, aNDFom, and ADFom did not differ (P>0.05) in both treatments. The intestinal flow of OM tended to be greater (P = 0.055) in RC wethers compared with those in the LU treatment, and duodenal flow of aNDFom and ADFom were similar in both treatments.

The N intake was greater (P>0.05) in wethers in the RC treatment, but N apparent digestibility, N true digestibility, N true intestinal digestibility, and RDP content were lower (P<0.01; Table 3). The ruminal undegradable protein and intestinal flow of total N, NAN, microbial N, and non-ammonia and non-microbial N (NANMN) were greater (P>0.05) in RC wethers, whereas NH_3-N flow did not differ between treatments. The efficiency of rumen microbial protein synthesis and efficiency of N ruminal utilization were greater (P>0.05) in wethers in the RC treatment. The urinary N excretion was lower, but fecal N excretion was greater in wethers in the RC treatment, compared with those in the LU treatment. However, the proportion of total N excreted in urine was 15% greater in wethers in the LU treatment when compared with those in the RC treatment, whereas daily N retention did not differ between treatments.

Table 2 - Intake, digestibility, and intestinal flow of dry matter, organic matter, and fiber content in sheep fed total mixed rations containing lucerne or red clover silages

	Lucerne	Red clover	SEM	P-value
		Intake		
Dry mater (g day^{-1})	807.8	877.9	36.04	0.100
Organic matter (g day^{-1})	753.8	815.4	33.46	0.115
Organic matter (g kg^{-1} BW$^{0.75}$)	67.3	72.3	2.75	0.160
aNDFom (g day^{-1})	359.1	416.7	18.23	0.019
ADFom (g day^{-1})	218.2	257.2	11.16	0.013
Digestible OM (g day^{-1})	486.7	506.2	18.36	0.247
ME (MJ day^{-1})	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^{-1})		
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; BW - body weight; ME - metabolizable energy; OMTD - organic matter true digestibility; SEM - standard error of the means.

Table 3 - Intake, digestibility, nitrogen balance, and intestinal flow of nitrogen compounds in sheep fed total mixed rations containing lucerne or red clover silages

	Lucerne	Red clover	SEM	P-value
Intake (g day^{-1})	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^{-1})	8.63	8.11	0.134	0.011
Urinary excretion (g g N intake^{-1})	0.42	0.35	0.011	0.002
Fecal NDIN excretion (g day^{-1})	1.56	2.74	0.143	0.001
Fecal excretion (g day^{-1})	7.10	9.80	0.590	0.006
Fecal excretion (g g N intake^{-1})	0.34	0.42	0.009	<0.001
N retention (g day^{-1})	5.00	5.50	0.381	0.246
N retention (g g N intake^{-1})	0.24	0.23	0.008	0.322
(N urinary excreted N total^{-1})	54.6	46.3	1.355	<0.001
Intestinal flow (g day^{-1})				
Total N	16.1	22.1	1.34	0.004
Microbial N	11.3	13.5	0.74	0.028
Non-amonia 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^{-1}); NRU - nitrogen ruminal utilization (g microbial N rumen degradable N intake^{-1}); SEM - standard error of the means.

Discussion

The similar digestible OM and ME intake between treatments, even though OM digestibility tended to be lower in the RC treatment when compared with the LU treatment, may be explained, at least partially, by the fact that rumen fill was not the preponderant factor affecting the regulation of intake. As such, both treatments resulted in a ME intake (average = 7.7 MJ day^{-1}) greater than the daily ME requirements of experimental wethers (7.2 MJ day^{-1}; AFRC, 1993). The NDF intake were higher in the RC treatment 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 it is the case for dairy cows receiving TMR (Mertens, 1994).

Despite the lower apparent digestibility of DM and OM in the RC diet, the ruminal digestibility of these components (as a proportion of total digestibility), as well as the apparent and ruminal digestibility of fiber, did not differ between the treatments. Conversely, previous studies evaluating the inclusion of condensed tannins (as a modulator of ruminal fermentation) in animal diets reported a reduction in OM and fiber digestibility (Naczk et al., 1994; Ávila et al., 2015). This result evidenced that condensed tannins may also form complexes with bacterial enzymes and/or with polysaccharides such as cellulose and hemicellulose (Priolo et al., 2000), thus reducing fiber degradation. In the present study, fiber digestibility did not differ between treatments, suggesting that, unlike condensed tannins, quinones do not form complexes with polysaccharides in the rumen, instead exerting a greater effect on nitrogen compounds.

The lower RDP content (20%) in the RC diet compared with the LU diet may be related to PPO enzyme activity in RC producing quinones, which might form complexes with proteins, thus reducing their ruminal degradation (Albrecht and Broderick, 1992; Broderick et al., 2004; Merry et al., 2006).

Moreover, the effect of PPO may occur during the ensilage process (Jones et al., 1995; Sullivan and Hatfield, 2006; Lee et al., 2008), decreasing the proportion of soluble N in the total N of the silage.

The NAN flow in wethers fed RC diet was 37% higher than in those fed LU diet, as a consequence of increased flow of both microbial and NANMN, which may be clearly associated with RDP reduction and increased ruminal microbial growth. Usually, the supply of diets with decreased RDP content results in a higher flow of NAN and NANMN, but a lower microbial flow of N (Ipharraguerre and Clark, 2005; Reynal and Broderick, 2005; Olmos Colmenero and Broderick, 2006). 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 wethers was a consequence of both an increase in the efficiency of rumen microbial protein synthesis (+12.7%) and an increase in the efficiency of N ruminal utilization (+31.0%) of this diet. 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.

The replacement of LU silage by RC silage increased N duodenal flow, without increasing N retention. This result may be a consequence of a reduction in the true intestinal digestibility of nitrogen (−3.0%), and a greater NDIN excretion by RC wethers. This may indicate that quinone-protein complexes resulted in the formation of insoluble complexes inside the gastrointestinal tract (Reed, 1995).

Wethers fed RC diet excreted less N in their urine, but more N in their feces compared with LU wethers. Diets containing secondary compounds have been associated with increased secretion of endogenous proteins, probably because of the 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% lower for wethers in the RC treatment compared with those in the 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 (Makkar, 2003; Broderick et al., 2007; Theodoridou et al., 2010). The route diversion of N excretion in RC wethers from urine to feces has environmental advantages, because the N present in ruminant feces is generally in the organic form, which reduces the substrate available for nitrification and nitrous oxide formation, and does not result in the emission of greenhouse gases (Varel et al., 1999; Alves, 2016). On the other hand, the majority of urinary N is in urea form, which is hydrolyzed within one or two days, and nitrous oxide is approximately 300 times more polluting than CO_2 (IPCC, 1995). Therefore, the use of RC silage may be an alternative for reducing the environmental impact of livestock systems, as has been recommended by other authors, based on trials focusing on dairy cows (Misselbrook et al., 2005).

Conclusions

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

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: G.C. Guzatti, V. Niderkorn and H.M.N. Ribeiro-Filho. Data curation: G.C. Guzatti, G.V. Kozloski, V. Niderkorn and H.M.N. Ribeiro-Filho. Formal analysis: G.C. Guzatti, G.V. Kozloski and H.M.N. Ribeiro Filho. Funding acquisition: H.M.N. Ribeiro-Filho. Investigation: G.C. Guzatti and P.G. Duchini. Methodology: G.C. Guzatti, P.G. Duchini and H.M.N. Ribeiro-Filho. Project administration: G.C. Guzatti and H.M.N. Ribeiro-Filho. Supervision: G.C. Guzatti and H.M.N. Ribeiro-Filho. Writing-original draft: G.C. Guzatti, P.G. Duchini, G.V. Kozloski, V. Niderkorn and H.M.N. Ribeiro-Filho.

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References

AFRC - Agricultural and Food Research Council. 1993. Energy and protein requirements of ruminants. CAB International, Wallingford.

Albrecht, K. A. and Broderick, G. A. 1992. Ruminal *in vitro* degradation of protein from different legume species. p.92-94. In: Research summaries. US Dairy Forage Research Centre, Madison, WI.

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. Tese (D.Sc.). Universidade do Estado de Santa Catarina, Lages.

AOAC - Association of Official Analytical Chemists. 1995. Official methods of analysis. 16th ed. AOAC, Washington, DC.

Ávila, S. C.; Kozloski, G. V.; Orlandi, T.; Mezzomo, M. P. and 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. Journal of Agriculture Science 153:943-953. https://doi.org/10.1017/S0021859615000064

Bach, A.; Calsamiglia, S. and Stern, M. D. 2005. Nitrogen metabolism in the rumen. Journal of Dairy Science 88(Suppl 1):E9-E21. https://doi.org/10.3168/jds.S0022-0302(05)73133-7

Brito, A. F.; Broderick, G. A.; Olmos Colmenero, J. J. and 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. Journal of Dairy Science 90:1392-1404. https://doi.org/10.3168/jds.S0022-0302(07)71625-9

Broderick, G. A.; Albrecht, K. A.; Owens, V. N. and Smith, R. R. 2004. Genetic variation in red clover for rumen protein degradability. Animal Feed Science and Technology 113:157-167. https://doi.org/10.1016/j.anifeedsci.2003.12.004

Broderick, G. A.; Brito, A. F. and Olmos Colmenero, J. J. 2007. Effects of feeding formate-treated alfalfa silage or red clover silage on the production of lactating dairy cows. Journal of Dairy Science 90:1378-1391. https://doi.org/10.3168/jds.S0022-0302(07)71624-7

Broderick, G. A.; Walgenbach, R. P. and Sterrenburg, E. 2000. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. Journal of Dairy Science 83:1543-1551. https://doi.org/10.3168/jds.S0022-0302(00)75026-0

Clark, J. H.; Klusmeyer, T. H. and Cameron, M. R. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. Journal of Dairy Science 75:2304-2323. https://doi.org/10.3168/jds.S0022-0302(92)77992-2

Fox, D. G.; Tylutki, T. P.; Tedeschi, L. O.; Van Amburgh, M. E.; Chase, L. E.; Pell, A. N; Overton, T. R. and Russell, J. B. 2003. The Net Carbohydrate and Protein System for Evaluating Herd Nutrition and Nutrient Excretion. Cornell University, Ithaca.

Huhtanen, P.; Kaustell, K. and Jaakkola, S. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Animal Feed Science and Technology 48:211-227. https://doi.org/10.1016/0377-8401(94)90173-2

Huhtanen, P.; Asikainen, U.; Arkkila, M. and 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. Animal Feed Science and Technology 133:206-227. https://doi.org/10.1016/j.anifeedsci.2006.05.004

INRA - Institut National de la Recherche Agronomique. 2007. Alimentation des bovins, ovins et caprins: besoins des animaux - valeurs des aliments: Tables Inra 2007. Editions Quae, Versailles. 330p.

IPCC - Intergovernmental Panel on Climate Change. 1995. Climate Change 1994: Radioactive forcing of climate change and an evaluation of the IPCC IS92 emission scenarios. University Press, Cambridge.

Ipharraguerre, I. R. and Clark, J. H. 2005. Impacts of the source and amount of crude protein on the intestinal supply of nitrogen fractions and performance of dairy cows. Journal of Dairy Science 88(Suppl 1):E22-E37. https://doi.org/10.3168/jds.S0022-0302(05)73134-9

Jones, B. A.; Muck, R. E. and Hatfield, R. D. 1995. Red clover extracts inhibit legume proteolysis. Journal of the Science of Food and Agriculture 67:329-333. https://doi.org/10.1002/jsfa.2740670309

Kammes, K. L. and 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. Journal of Dairy Science 95:3288-3297. https://doi.org/10.3168/jds.2011-5022

Krizsan, S. J. and Huhtanen, P. 2013. Effect of diet composition and incubation time on feed indigestible neutral detergent fiber concentration in dairy cows. Journal of Dairy Science 96:1715-1726. https://doi.org/10.3168/jds.2012-5752

Kuoppala, K.; Ahvenjarvi, S.; Rinne, M. and 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. Journal of Dairy Science 92:5634-5644. https://doi.org/10.3168/jds.2009-2250

Lee, M. R. F. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. Frontiers in Plant Science 5:694. https://doi.org/10.3389/fpls.2014.00694

Lee, M. R. F.; Scott, M. B.; Tweed, J. K. S.; Minchin, F. R. and Davies, D. R. 2008. 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 144:125-136. https://doi.org/10.1016/j.anifeedsci.2007.09.035

Lee, M. R. F.; Tweed, J. K. S. and 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 and Forage Science 68:83-92. https://doi.org/10.1111/j.1365-2494.2012.00873.x

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 Ruminant Research 49:241-256. https://doi.org/10.1016/S0921-4488(03)00142-1

Makkar, H. P. S. and Becker, K. 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. British Journal of Nutrition 81:107-112. https://doi.org/10.1017/S0007114599000227

Merry, R. J.; Lee, M. R. F.; Davies, D. R.; Dewhurst, R. J.; Moorby, J. M.; Scollan, N. D. and 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. Journal of Animal Science 84:3049-3060. https://doi.org/10.2527/jas.2005-735

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

Misselbrook, T. H.; Powell, J. M.; Broderick, G. A. and Grabber, J. H. 2005. Dietary manipulation in dairy cattle: laboratory experiments to assess the influence on ammonia emissions. Journal of Dairy Science 88:1765-1777. https://doi.org/10.3168/jds.S0022-0302(05)72851-4

Moorby, J. M.; Lee, M. R. F.; Davies, D. R.; Kim, E. J.; Nute, G. R.; Ellis, N. M. and Scollan, N. D. 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. Journal of Dairy Science 92:1148-1160. https://doi.org/10.3168/jds.2008-1771

Naczk, M.; Nichols, T.; Pink, D. and Sosulski, F. 1994. Condensed tannins in canola hulls. Journal of Agricultural and Food Chemistry 42:2196-2200. https://doi.org/10.1021/jf00046a022

Nagel, S. A. and Broderick, G. A. 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. Journal of Dairy Science 75:140-154. https://doi.org/10.3168/jds.S0022-0302(92)77748-0

Olmos Colmenero, J. J. and Broderick, G. A. 2006. Effect of dietary crude protein concentration on ruminal nitrogen metabolism in lactating dairy cows. Journal of Dairy Science 89:1694-1703. https://doi.org/10.3168/jds.S0022-0302(06)72237-8

Owens, F. N. and Goetsch, A. L. 1988. Ruminal fermentation. p.146-171. In: The ruminant animal: digestive physiology and nutrition. Church, D. C., ed. Prentice Hall, Englewood Cliffs, NJ.

Priolo, A.; Waghorn, G. C.; Lanza, M.; Biondi, L. and 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. Journal of Animal Science 78:810-816. https://doi.org/10.2527/2000.784810x

Reed, J. D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. Journal of Animal Science 73:1516-1528. https://doi.org/10.2527/1995.7351516x

Reynal, S. M. and Broderick, G. A. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. Journal of Dairy Science 88:4045-4064. https://doi.org/10.3168/jds.S0022-0302(05)73090-3

Sullivan, M. L. and Hatfield, R. D. 2006. Polyphenol oxidase and *o*-diphenols inhibit postharvest proteolysis in red clover and alfalfa. Crop Science 46:662-670. https://doi.org/10.2135/cropsci2005.06-0132

Theodoridou, K.; Aufrère, J.; Andueza, D.; Pourrat, J.; Le Morvan, A.; Stringano, E.; Mueller-Harvey, I. and Baumont, R. 2010. Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. Animal Feed Science and Technology 160:23-38. https://doi.org/10.1016/j.anifeedsci.2010.06.007

Vanhatalo, A.; Kuoppala, K.; Ahvenjärvi, S. and 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. Journal of Dairy Science 92:5620-5633. https://doi.org/10.3168/jds.2009-2249

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

Van Soest, P. J.; Robertson, J. B. and Lewis, B. A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74:3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Varel, V. H.; Nienaber, J. A. and Freetly, H. C. 1999. Conservation of nitrogen in cattle feedlot waste with urease inhibitors. Journal of Animal Science 77:1162-1168. https://doi.org/10.2527/1999.7751162x

Waghorn, G. C. 1996. Condensed tannins and nutrient absorption from the small intestine. p.175-194. In: Proceedings of the Canadian Society of Animal Science Annual Meeting, Canada.

Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry 39:971-974. https://doi.org/10.1021/ac60252a045

Winters, A. L.; Minchin, F. R.; Michaelson-Yeates, T. P. T.; Lee, M. R. F. and 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. Journal of Agricultural and Food Chemistry 56:2817-2824. https://doi.org/10.1021/jf0726177