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Nitrogen metabolism and milk production in dairy cows fed semi-restricted amounts of ryegrass-legume silage with birdsfoot trefoil (*Lotus corniculatus* L.) or white clover (*Trifolium repens* L.)

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Abstract

In change-over trials, mid-lactation dairy cows were fed concentrate-supplemented, iso-nitrogenous and iso-fibrous perennial ryegrass-legume silage diets that satisfied energy requirements but were sub-optimal with respect to metabolisable protein supply. Legumes were either birdsfoot trefoil with low levels of condensed tannins (typical for hemiboreal conditions), or white clover. Averaged over two experimental years, birdsfoot trefoil-based silage resulted in lower digestibility ($P < 0.001$) of dry matter (50 g kg^{-1}), organic matter (52 g kg^{-1}), neutral detergent fibre (120 g kg^{-1}) and nitrogen (24 g kg^{-1}) and lower rumen total volatile fatty acid concentration (7 m*M*; $P = 0.009$). Milk protein yield was 36 g d^{-1} higher with birdsfoot trefoil silage ($P = 0.002$), while raw milk yield tended to be 0.8 kg d^{-1} higher ($P = 0.06$). Rumen ammonia concentration was similar between diets, but milk urea concentration ($P < 0.001$), urinary urea excretion ($P = 0.002$) and faecal N proportion ($P = 0.001$) were higher with birdsfoot trefoil silage. The results suggest that grass-birdsfoot trefoil silage produced in hemiboreal areas exhibits a protein-sparing effect in dairy rations, despite a low condensed tannin content that is further diluted by companion grasses and ration concentrate proportion.

Introduction

Silage made from grass and legumes, grown in pure stands or mixed leys, is the main forage used for stall-feeding of dairy cows in many cold-temperate regions. During ensiling, protein is partly degraded by plant and microbial enzymes (McDonald *et al*., 1991), often leading to impaired utilisation by ruminants because it releases ammonia too rapidly in the rumen and causes urinary nitrogen losses (Nouisiainen *et al*., 2004). Silage protein degradation can be reduced by silage additives, e.g. formic acid, which 'restrict fermentation' (Jaakkola *et al.*, 2006) or by crop wilting (Merchen and Satter, 1983). Some forage crops contain secondary metabolites that reduce protein degradation, e.g. polyphenole oxidases (Sullivan and Hatfield, 2006) and condensed tannins (CT) (Mueller-Harvey, 2006). The mode of proteolysis inhibition by CT is formation of tannin-protein complexes. In grazing animals, these complexes are formed in the rumen, but with silage, they are formed during ensiling (Salawu *et al.*, 1999). On reaching the acidic abomasum, the pH-labile complexes are dissolved and the protein can be utilised in the small intestine (Jones and Mangan, 1977).

Birdsfoot trefoil (*Lotus corniculatus* L.) is a common CT-containing legume used e.g. in Canada, the United States, South America and New Zealand, mostly as a pasture crop (MacAdam *et al.*, 2006). It can be grown in Scandinavia, but the climate results in low CT concentration as a direct effect and as a cultivar effect from the negative association between winter hardiness and CT concentration (Hedqvist et *al*., 2002). Until recently, the CT concentration in birdsfoot trefoil grown in hemiboreal regions was considered too low to improve ruminant protein utilisation. However, *in vitro* protein degradation of birdsfoot trefoil samples grown in Sweden showed negative correlations to CT concentration (Hedqvist *et al*., 2000), indicating potential for improving ruminant protein supply under Scandinavian conditions. Birdsfoot trefoil silage grown in Wisconsin, in a climate similar to that in Sweden, produced a response on milk yield with positive curvilinearity to tannin concentration (Hymes-Fecht *et al*., 2004).

Crop production experiments suggest that birdsfoot trefoil produced for conserved forage in Scandinavian conditions should be grown in mixed leys with one or more companion grasses (Nilsdotter-Linde, 1999). However, this will further dilute the CT concentration in the end-forage and reduce any potential protein-saving effect. Supplementing silage with a large proportion of concentrate to fulfil the energy requirements of high-yielding dairy cows in Northern Europe (Huhtanen *et al*., 2008) further lowers the CT concentration of the total ration.

The experiments reported here investigated whether birdsfoot trefoil grown in hemiboreal conditions (i.e. with low CT concentration) could substantially improve the protein supply to dairy cows from mixed grass-legume silages supplemented with low-protein concentrate. The emphasis was on N utilisation as an effect of intrinsic crop properties, so a semi-restricted feeding level was used to avoid confounding effects from differing N intake.

Materials and methods

Silage crops

Silage was made from mixed ryegrass-legume swards grown at Rådde, south-west Sweden (57°37´N, 13°15´E; 900 mm annual precipitation). The leys were undersown in oats (cv.

Cilla; seed rate 172 kg ha^{-1}) in spring using the following cultivars and seed rates: BFT silage with birdsfoot trefoil (cv. Oberhaunstaedter; 12 kg ha^{-1}) and late perennial ryegrass (cv. Condesa; 8 kg ha^{-1}); WC silage, with white clover (cv. Lena, 3 kg ha^{-1}) and late perennial ryegrass (cv. Herbie, 20 kg ha^{-1}). The diploid cv. Herbie gives a denser sward than the tetraploid cv. Condesa at the study site, confirming ploidity effects reported by Orr *et al*. (2003). It would therefore be more competitive against white clover, facilitating the production of mixed leys with similar chemical composition from both legumes. As ration crude protein (CP) concentration strongly influences cattle N use efficiency (Huhtanen *et al*., 2008), the silages were designed to provide similar concentrations of CP and neutral detergent fibre (NDF), hence the larger legume proportion in the BFT sward.

In Year 1, leys established in spring were harvested as regrowth by August 24, seven weeks after removal of the annual main crop. The BFT crop contained (DM basis) 310 g kg^{-1} birdsfoot trefoil, while the WC crop contained 170 g kg^{-1} white clover, resulting in identical concentrations of CP and NDF, as intended. In Year 2, different batches were mixed at feeding to achieve higher CT contents and even out CP and NDF concentrations, because that was considered even more important than the possible disadvantage of mixing different growths. WC silage was obtained from the sward established in Year 1 by mixing primary growth harvested in mid-June with a third cut harvested in mid-September in proportions 1:2 to give a white clover content of 480 g kg^{-1}. For BFT silage, a third cut in mid-September from the Year 1 ley was mixed in proportions 3:1 with a pure stand of birdsfoot trefoil established in spring of Year 2 and harvested in mid-October, nine weeks after the previous cut, to give a total birdsfoot trefoil content of 580 g kg^{-1}.

The leys were cut with a mower/conditioner, wilted and ensiled in big bales with a bacterial inoculant (*Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici* and *Lactococcus lactis* at a total concentration of 2×10^5 CFU g^{-1} FM) containing cellulase and sodium benzoate (Lactisil 200 NB, Medipharm, Kågeröd, Sweden), applied at the recommended dosage of 4 L water solution per tonne FM.

Animals and experimental design

All animal handling was approved by the Uppsala Local Ethics Committee. Mid-lactating Swedish Red and White dairy cows were used in change-over feeding experiments, with three 28 d periods, where the first 23 d were used for adaptation and days 24-28 for sampling. The experiments utilised 12 and 14 cows in Years 1 and 2, respectively, of which six individuals participated in both years. Four cows in each year were primiparous, the average parity being 2.3 (± 1.3) in both years. At experimental onset in Years 1 and 2, the cows were 72 (± 16) and 60 days in milk (DIM) (± 28), weighed 630 (± 56) and 598 (± 82) kg and yielded 32 (± 5) and 35 (± 5) kg energy-corrected milk (ECM) (mean, with SD in brackets). Nitrogen balance was assessed in eight cows per year (two individuals were utilised in both years) and rumen studies were carried out on four rumen-cannulated cows (three individuals utilised in both years). Because of late calving in Year 2, two rumen-cannulated cows entered the experiment at the beginning of Period 2.

The cows were housed in individual tie-stalls with rubber mats and sawdust bedding, and milked in their stalls at 06.30 and 15.30 h. Silage and concentrates were fed individually in separate troughs, with automatic feeders used during adaptation periods, but manual feeding during sampling periods. Silage was fed in three equal meals (05.45, 12.00 and 17.00 h) and

concentrate in four equal meals (06.00, 09.00, 12.15 and 17.15 h). Orts were weighed daily at 11.45 h.

Silage comprised 650 and 700 g kg^{-1} of ration total DM in Year 1 and 2, respectively. The Year 1 silages were supplemented with specially manufactured pelleted concentrate comprising (per kg as fed): barley (472 g), oats (200 g), rapeseed cake (120 g), peas (180 g) and a mineral-vitamin premixture (28 g). In Year 2, the silages were supplemented with rolled barley. A mineral supplement (Kvarnby VM 0.9, Kvarnbyfoder, Staffanstorp, Sweden) was fed at 150 g d^{-1}.

The aim was to maintain a similar N intake in both diets, so feed was not provided *ad libitum* during the experiments but individually set to a DM level of the least energy-dense diet (BFT) calculated to cover energy demands (Spörndly, 2003) at experimental onset and kept throughout each experiment. However, the actual production level and concentrate proportion resulted in a feed allowance that usually gave small amounts of refusals.

Sampling and laboratory analyses

Fifteen samples of birdsfoot trefoil swards were collected immediately before harvest and birdsfoot trefoil plants were separated, pooled to a general sample, and promptly frozen. The birdsfoot trefoil material was later lyophilised and analysed in quadruplicate for extractable tannins by the radial diffusion assay (Hedqvist *et al.*, 2000), but with tannic acid (BDH Ltd, Poole, England) as standard. However, direct-frozen samples were not obtained from one cut in Year 2, so only the pure stand constituting 43% of the birdsfoot trefoil portion in BFT silage in Year 2 was analysed by radial diffusion assay.

Experimental protocol and analytical methods were as described by Eriksson (2010), with N balance performed on eight cows and rumen studies on four cows. The cows were weighed at 10.00 h on days 20 and 21 of each period. Milk yield was recorded from afternoon milking on d 23 to morning milking on d 25 and the milk was analysed for fat, protein and lactose by infrared spectroscopy (FT 120, Foss, Hillerød, Denmark), somatic cell count (SCC) (Fossomatic 5000; Foss, Hillerød, Denmark), and urea by flow injection analysis (Ramsing *et al.*, 1980).

Feed samples from each meal during the sampling periods were stored at -30°C and subsequently pooled within feed type and period. Concentrates were dried at 60°C, whereas silages were lyophilised. The silage weight proportion remaining after lyophilisation is reported as DM and all other results are expressed on that basis (Dewhurst *et al.*, 2003). Samples were milled through a 1-mm screen on a hammer mill (Kamas, Malmö, Sweden) and BFT silage samples were analysed for extractable, protein-bound and fibre-bound tannin fractions, as described by Lorenz *et al.* (2010). These are reported as proportional distribution rather than absolute amounts, because the method was semi-quantitative at best (I. Mueller-Harvey, personal communication, 2007). All feeds were analysed by standard procedures (Eriksson *et al.*, 2004) for DM, ash, ether extract, Kjeldahl-N, crude fibre and non-structural carbohydrates, with the results expressed as water-soluble carbohydrates (WSC). Minerals were analysed by inductively coupled plasma atomic emission spectroscopy (Spectroflame, Spectro GmbH, Kleve, Germany). Acid-insoluble ash was analysed according to Van Keulen and Young (1977). Ash-free NDF (aNDFOM) was analysed by adapting the oven method of Chai and Udén (1998) to the Mertens (2002) standard. Organic matter digestibility (OMD) of silage was determined by the standard Swedish 96 h VOS *in vitro* method (Åkerlind *et al.*, 2011) and also with addition of 100 mg polyethylene glycol (PEG, molar weight 4000 g) to

each 500 mg *in vitro* sample (OMD_{PEG}). *In sacco* degradation rates of CP and NDF were determined by 0-96 h time-series incubations (Åkerlind *et al.*, 2011) and indigestible NDF (iNDF) proportion by 288-h *in sacco* incubation (Åkerlind *et al.*, 2011). Nitrogen solubility was analysed in borate-phosphate buffer (Åkerlind *et al.*, 2011), with soluble true protein defined as the CP fraction precipitable by trichloroacetic acid (Hedqvist and Udén, 2006). Silage juice from hydraulic pressing was used for determination of ammonia-N and α-amino-N (Broderick and Kang, 1980), pH analyses and analysis of fermentation products by HPLC (Andersson and Hedlund, 1983). Metabolisable energy, metabolisable protein expressed as amino acids absorbed in the digestive tract (AAT) and protein balance in the rumen (PBV) of the feeds were calculated from analyses according to Madsen (1985) and Spörndly (2003). Tabulated effective degradability values (Spörndly, 2003) were used for concentrates but actual *in sacco* values for silages.

Quantitative urine collection into 1.8 M sulphuric acid was performed for 72 h, starting at 06.00 h on d 25 (Eriksson, 2010). Collection containers were replaced every 12^{th}, the amount of urine was weighed and a subsample of 50 mL kg^{-1} urine was taken and kept refrigerated until the end of the 72 h period, when subsamples were pooled to a period sample per cow and frozen at -30°. The samples were later thawed and analysed for Kjeldahl-N with the same methodology as for the feeds, and for urea, creatinine and allantoin with the AutoAnalyzer applications used by Eriksson *et al.* (2004). Faecal samples of approx. 500 g were obtained from spontaneous defecations twice daily (05.30-08.00 and 15.00-17.00 h) on days 25-28 and immediately frozen at -30°C. The samples were subsequently thawed, pooled within cow and weighed into Petri dishes for lyophilisation and analysis of DM, ash, acid-insoluble ash, Kjeldahl-N and aNDFOM using the same methods as for the feeds. The weight proportion remaining after lyophilisation is reported as DM, as for silage.

Ruminal liquid samples from the four cannulated cows were obtained on 18 different hours during days 25-28 (Eriksson *et al.*, 2004), promptly strained through a tea strainer, pH was measured (Mettler Toledo MP 125, Mettler-Toledo AG, Schwerzenbach, Switzerland) and subsamples were frozen at -30°C in Eppendorf tubes for subsequent analysis. All samples were analysed for ammonia-N and α-amino-N (Broderick and Kang, 1980), and VFA was determined by HPLC (Andersson and Hedlund, 1983) in samples pooled within cow and period. Total rumen evacuations were performed at 11.00 h on days 25 and 27, according to procedures described by Eriksson (2010). Lyophilised samples were analysed for DM, ash, Kjeldahl-N and aNDFOM as described for feeds. Again, the weight proportion remaining after lyophilisation is reported as DM and used in calculations.

Calculation and statistical analysis

Digestibility was calculated from analyses of acid-insoluble ash in feeds and faeces. ECM was calculated as (Sjaunja *et al.*, 1991):
ECM = milk (kg) × [383 × (fat%) + 242 × (protein%) + 165 × (lactose%) + 20.7] / 3140

Results from both years were analysed together as one experiment. The statistical model included fixed effects of the class variables Cannula (yes/no), Lactation Class (primiparous or multiparous), Year (1 or 2), Period (1, 2 or 3), and Diet (BFT or WC) and as a covariate DIM at the start of Period 1 (estimated for two cows entering the experiment in Period 2 in Year 2).

In formal terms, the model was:

$$y_{ijktmn} = \mu + \alpha_i + c_{ij} + \lambda_k + \theta_t + (c\theta)_{ijt} + \pi_m + \gamma_n + (\alpha\lambda)_{ik} + (\alpha\theta)_{it} + (\alpha\pi)_{im} + (\alpha\gamma)_{in}$$
$$+ (\lambda\theta)_{kt} + (\lambda\pi)_{km} + (\lambda\gamma)_{kn} + (\theta\pi)_{tm} + (\theta\gamma)_{tn} + (\pi\gamma)_{mn} + (\alpha\lambda\pi)_{ikm} + (\alpha\lambda\gamma)_{ikn} + (\alpha\theta\gamma)_{itn}$$
$$+ (\alpha\pi\gamma)_{imn} + (\lambda\theta\gamma)_{ktn} + (\lambda\pi\gamma)_{kmn} + (\theta\pi\gamma)_{tmn} + \beta x_{ijkt} + e_{ijktmn}$$

where α_ι, λ_κ, θ_τ, π_μ, and γ_ν are the effects of cannula, lactation class, year, period and diet, respectively. The overall and yearly random effects $\chi_{\iota\varphi}$ and $(\chi\theta)_{\iota\varphi\tau}$ of cows and residuals $\varepsilon_{\iota\varphi\kappa\tau\mu\nu}$ were assumed to be independent and normally distributed with expectations 0 and variances σ_c^2, $\sigma_{c\theta}^2$, and σ_e^2, respectively.

The fixed effects of the model were tested in separate steps for three-factor interactions, two-factor interactions and main effects, applying Holm's (1979) adjustment for sequential rejections in each step. Possible differences in carry-over effects between diets were tested by the special contrast $(\pi\gamma)_{32}$ - $(\pi\gamma)_{12}$ - $(\pi\gamma)_{31}$ + $(\pi\gamma)_{11}$, as the cow effect vanishes for this linear combination. Because diet comparison was the experimental objective, diet main effects were always kept in the final model for calculating the least square means. Values for SCC were log-transformed before analysis and the least-square means were re-transformed by anti-logs and adjusted for the non-linearity of the transformation.

For time-series data on rumen pH, ammonia-N and the logarithm of α-amino-N (transformation required for normal distribution), no interactions were significant and the model was simplified to:

$$y_{ijktmns} = \mu + c_j + \lambda_k + \theta_t + (c\theta)_{ijt} + \pi_m + \gamma_n + \beta x_{ijkt} + e_{ijktmn} + \tau_s + w_{ijktmns}$$

where the effects have the same meaning as for period observations except for τ_s, which corresponds to hour of the day, and the random error $w_{ijktmns}$, which is assumed to follow an autoregressive dependence according to the physical time spacing used in the experiment.

Time-series data were also subjected to curve fitting. For rumen pH values, which increased from 24.00 to 06.00 h, decreased until 18.00 h and then slightly increased, τ_s was modelled by two segments of quadratic functions, mathematically formulated as:

$$\tau_s = \eta_{11} s + \eta_{12} s^2 + \eta_{21} (s - 6)_+ + \eta_{22} (s - 6)_+^2$$

where $(s - 6)_+ = \max(s - 6, 0)$ and η_{11}, η_{12}, η_{21}, and η_{22} are the parameters to be estimated.

Rumen ammonia-N concentration peaked two hours after feeding and returned to the baseline two hours later. This was modelled as $\tau_s = \eta(1 - (s - s_p)^2 / 4)$, $s_p - 2 \leq s \leq s_p + 2$ for the peak hours s_p = 8, 14, 19, where the parameter η to be estimated is the maximum level after feeding. The logarithm of rumen α-amino-N concentration, which peaked one hour after feeding (s_p = 7, 13, 18) and returned to the baseline two hours later, was modelled by $\tau_s = \eta[\mathrm{I}(s = s_p) + (1 / 2)\mathrm{I}(s = s_p + 1)]$, where $\mathrm{I}(c) = 1$ if the condition c is true and $\mathrm{I}(c) = 0$ otherwise.
Results are presented with effects of diet, year and year × diet. Effects with $P < 0.05$ were considered significant and effects with $P < 0.10$ as tendencies. The numerical evaluations

were performed using the Mixed procedure of SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina, USA).

Energy and protein supply and digestibility were further assessed by modelling the experimental diets in the semi-mechanistic model NorFor (Volden, 2011), which evaluates the entire ration and calculates feed value parameters for the actual feeding situation. Diets were modelled per year × treatment × lactation class (primiparous or multiparous) and weighted together to treatment means according to the respective number of animals.

Results

Feed composition

In terms of chemical composition and nutritive value, the concentrations of CP and aNDFOM in silages were almost identical between diets in Year 1, whereas the CP concentration was higher in BFT silage in Year 2 (Table 1). Digestible proportion of aNDFOM (PDNDF), fibre digestion rate and OMD were lowest with BFT silage. The numerical response to PEG addition at *in vitro* OMD determination was <10 g kg^{-1} OM. Buffer-soluble N proportion was slightly lower in the BFT silage and potentially degradable N correspondingly higher. The fermentation profile of the silage was similar between the two silage types, with significant amounts of lactic acid despite high DM content. Tannin concentration (not tabulated) measured by radial diffusion assay on the pure birdsfoot trefoil crop was 16 g kg^{-1} DM in Year 1 and 18 g kg^{-1} DM in the one cut analysed in Year 2. Tannin fractionation on the mixed silage (Table 2) indicated that about half the tannin content was extractable and that the protein-bound fraction was about twice the fibre-bound fraction.

Intake of DM and nutrients

The semi-restricted feed allowance resulted in small orts and similar intakes of silage and total DM between diets, although there were period × year interactions, with ranges 12.7-14.2 and 20.2-21.0 kg d^{-1} for silage and DM intake, respectively (Table 3). Energy supply was lowest with the BFT diet, both when ME was estimated from OMD only and for the NE_L supply calculated by the NorFor model from animal data together with the detailed chemical analysis and degradation properties in Table 1. However, for both diets energy balance was still positive. The supply of metabolisable protein (AAT) and also of rumen undegraded feed CP (RUP) was slightly higher with the BFT diet than with the WC diet. The intake of AAT was sub-optimal for both diets when calculated by the NorFor model, but in balance with requirements according to the system of Madsen (1985). The concentration of rumen-degradable protein (RDP) per kg DM and the RDP surplus (PBV) relative to ruminally available energy were both lowest with the BFT diet.

Milk yield and composition

Dietary differences in milk yield and milk composition were moderate (Table 4). A tendency for higher milk yield ($P = 0.06$) and somewhat higher milk protein concentration resulted in higher protein yield ($P = 0.002$) with the BFT diet, although ECM yield did not differ significantly ($P = 0.20$) between the diets. The higher protein yield with the BFT diet also gave a tendency for increased recovery of feed N as milk N. There was a curvilinear period effect for milk N : feed N with the WC diet in multiparous cows, with Period 2 being highest. The values ranged from 0.318 (multiparous cows, BFT diet, Period 1) to 0.254 (primiparous cows, WC diet, Period 3). Milk urea concentration was consistently higher ($P < 0.001$) with the BFT diet.

N balance

Urinary (P = 0.002) and faecal (P < 0.001) N proportions were both highest with the BFT diet, while recovery of feed N as milk N did not differ between diets for the 8 cows subjected to N measurements (Table 5). This resulted in a much higher unknown proportion (N balance) with the WC diet than the BFT diet. Urea-N proportion of total urinary N was similar between the diets, so the BFT diet also gave greater daily urinary urea-N excretion. Allantoin showed a fairly strong relationship to digested OM (direct correlation 0.47), explained by strong correlations of underlying cow and yearly cow effects. Treatment means for daily urinary output did not differ, although there was a strong diet × year interaction with 26 and 30 kg for BFT and WC diets, respectively, in Year 1 but 23 and 19 kg in Year 2.

Digestibility and rumen fermentation

The digestibility of DM, OM, aNDFOM, PDNDF and CP (expressed as its counterpart faecal-N proportion) was considerably higher with the WC diet than the BFT diet (Table 5). The dietary difference in OM and aNDFOM *in vivo* digestibility agreed reasonably well with predictions from the NorFor model and with the OMD from *in vitro* determinations. The magnitude of diet DM digestibility difference not accounted for by aNDFOM and CP was about 0.01.

Ruminal amounts of fresh matter, DM and OM were similar for both diets, but the BFT diet had higher aNDFOM content and lower CP content than the WC diet (Table 6). The NorFor model predicted ruminally digested aNDFOM to be 3500 g d^{-1} with the BFT diet but 4100 g d^{-1} with the WC diet. The WC diet had higher total VFA concentration, lower ruminal pH, less acetate but more propionate and butyrate than the BFT diet. The time-series model for pH is illustrated in Figure 1a with different diet levels but parallel curves based on the quadratic model for τ_h estimated as:

$$\hat{\tau}_h = 0.0807h - 0.00191h^2 - 0.151(h-6)_+ + 0.00547(h-6)_+^2$$

Analytically, the estimated curve had its minimum at h = 19. Treatment means for ruminal concentrations of ammonia-N and α-amino-N were not significantly different for the diets. The maximum increase in ammonia-N from the baseline was estimated at $\hat{\eta} = 15.1$ mg NH_3-N 100 mL^{-1} (Figure 1b). The maximum increase 1 hour after feeding of the logarithm of α-amino-N (not shown) was estimated at $\hat{\eta} = 1.027$ corresponding to a 2.79-fold increase in α-amino-N compared with the baseline.

Discussion

Tannin content

The tannin concentration (16-18 g kg^{-1} DM by radial diffusion assay) in the pure, direct-frozen birdsfoot trefoil samples was much higher than the 3-10 g kg^{-1} DM previously reported by Hedqvist *et al.* (2000) for birdsfoot trefoil grown in Sweden. However, in recent Swedish variety trials (Halling, 2008), cv. Oberhaunstaedter consistently had a CT content in the range 11-20 g kg^{-1} DM by radial diffusion assay. The fractionation of tannins in silage by the HCl/butanol method resulted in a relatively high extractable proportion and a relatively low protein-bound proportion, resembling that reported by Scharenberg *et al.* (2007) for fresh rather than ensiled birdsfoot trefoil. Lorenz *et al.* (2010) found a protein-bound tannin proportion of 0.70 in sainfoin silage, twice that in the BFT silage fed in our experiment, with identical analytical methodology. The pH range of 4.5-5.0 in the BFT silage most likely promoted the persistence of tannin-protein complexes (Jones and Mangan, 1977; Lorenz and

Udén, 2011), so the low protein-bound proportion may be caused by failure to create bonds rather than dissociation of complexes.

Nutrient supply and production

The positive energy balance for both diets simply reflects the decline in milk yield over the course of time from experimental onset when feed allowance was fixed until the experimental mean yield was reached. This fulfilment of energy requirements may explain why the BFT diet was not hampered by the lower digestibility of OM and aNDFOM and hence lower supply of ME and net energy. Instead, it is most likely that metabolisable protein (AAT) supply limited milk protein in the experiments reported here, so that the moderate dietary differences in that respect affected production. The static system of Madsen (1985) estimated that AAT supply was in balance with requirements for both diets, the 48 g d^{-1} higher intake with the BFT diet corresponding to the difference in milk protein yield. The corresponding AAT balance from the NorFor model shows the supply relative to the requirement for maximum milk protein yield rather than relative to a minimum requirement, but was clearly below the recommended limit of 95% (Volden, 2011). The relative surplus of rumen-degradable protein (PBV) for both diets was at a level that should guarantee N supply for rumen microbes (Madsen, 1985). Furthermore, when expressed as rumen-degradable protein (RDP) from the NorFor model, rumen N supply was above the recommendations of NRC (2001), while ruminally undegraded feed CP (RUP) was deficient.

Even if protein supply in terms of metabolisable protein or RUP was sub-optimal, the overall CP concentration of 150 g kg^{-1} DM could be expected to give less response to improved protein supply than that which may occur at even lower ration CP concentrations (Colmenero and Broderick, 2006). There were numerically positive responses for production variables to the BFT diet that reached significance for protein yield with 36 g d^{-1} and a tendency for raw milk production with 0.8 kg d^{-1}, but effects on dairy cows reported in the literature are often of a larger magnitude. Hymes-Fecht *et al.* (2004) found an FCM yield increase of 2.5 kg d^{-1} on exchanging a low tannin birdsfoot trefoil with a medium tannin variety in 50% silage diets. Woodward *et al.* (2009) demonstrated an apparently linear milk response from 14.3 to 18.5 kg milk d^{-1} for incremental birdsfoot trefoil proportions in forage-only diets. Both these experiments used diets where the birdsfoot trefoil constituted a larger proportion of total ration than in our experiment because of pure stands (Hymes-Fecht *et al.*, 2004) or no concentrate supplementation (Woodward *et al*., 2009). For instance, the range of CT that Woodward *et al.* (2009) provided was from 0 to 19 g CT kg^{-1} ration DM, while CT concentration in our experiments would have been about 3 g kg^{-1} ration DM in Year 1 and 7 g kg^{-1} ration DM in Year 2. That would correspond to an expected CT effect across years of 44 g milk protein and 1.1 kg milk d^{-1}, if regression slopes for treatment means versus CT concentration from the experiment of Woodward *et al*. (2009) are directly applied. That does not take into account the possible mechanisms for the effect or the multitude of methodological differences between the experiments.

Since protein rather than energy supply seems to have limited production in the experiments reported here, the lower OM digestibility with the BFT diet was probably of minor importance. The situation may have been different if RUP had been in excess or if a true *ad libitum* forage allowance had resulted in large intake differences between the diets.

Rations with CT could be expected to reduce intake because of slower digestion rate and hereby slower clearance from the rumen (Waghorn, 2008). To our knowledge, there are no

published reports where *ad libitum* intakes of silage made from either white clover or birdsfoot trefoil are directly compared in dairy cattle. However, intake of fresh white clover and birdsfoot trefoil did not differ in stall-fed heifers (Nilsdotter-Linde *et al.*, 2004) or in grazing growing cattle and sheep (Molle *et al.*, 2008).

N metabolism

Even if there was an effect on milk protein production that seemed to be linked to improved protein supply, other effects on N metabolism were the opposite to those expected. Rumen ammonia concentration, which could be expected to decrease with less ruminal protein degradation (Waghorn, 2008), was similar between diets. However, the somewhat more extensive fermentation with the WC diet should result in the utilisation of more ruminally degraded protein for microbial synthesis, but this was not supported by daily allantoin excretion, which was actually numerically higher (P = 0.16) with the BFT diet. Excretion of milk urea and urinary urea were in agreement, with 13-14% higher excretion of both items with the BFT diet rather than the expected lower excretion (Waghorn, 2008). Milk urea dietary differences, regarding both concentration and total excretion, were more prominent at morning milking than at afternoon milking (data not shown). There was a numerically higher rumen ammonia concentration (Figure 1) for the BFT diet at 06.00 and 07.00 h, but since there is a lag of up to 2 h between rumen ammonia peak and plasma urea peak (Gustafsson and Palmquist, 1993), it is unlikely that milk urea concentration would be increased by morning feeding. Faecal N excretion was indeed higher with the BFT diet, as could be expected from the total N digestibility reduction caused by CT (Waghorn, 2008).

Overall N use efficiency in the experiment, expressed as recovery of feed N in milk, was typical for North European silage diets at the actual level of milk yield (Huhtanen *et al*., 2008). However, as is often the case in studies on dairy cows (Spanghero and Kowalski, 1997), N balance was generally higher than could be accounted for by biologically reasonable tissue incorporation and was highest with the WC diet. Previous studies indicate that white clover silage may be especially prone to result in unreasonably high N balances, for unclear reasons (Auldist *et al.*, 1999; Bertilsson and Murphy, 2003). It is unlikely that urinary urea losses in collection or analysis caused the high N balance for white clover in our experiments, because in that case milk urea concentration would probably have been higher with the WC diet. Furthermore, there were no between-diet differences in recovery of urinary Kjeldahl-N by Dumas analysis when a sub-set of samples was analysed in parallel by the two methods (data not shown) to test for possible failure of the Kjeldahl method to detect all N compounds. The absence of year × diet effects on the urinary volume marker creatinine does not suggest failures in urinary collection. The year × diet interaction for urinary output is most likely an effect of the different K levels (Nennich *et al.*, 2006), because year × diet means were correlated to K intake (r = 0.99).

Rumen digestion

Ruminal total VFA concentration difference simply reflected dietary differences in amount of digested OM, with values for the BFT diet being 0.94-0.95 of the corresponding WC diet values. Ruminal pH, in turn, was strongly correlated to VFA concentration (r = -0.89 for the entire dataset with no dietary differences), suggesting that differences in rumen environment were mainly the result of ration digestibility. The modest shift in VFA proportions, with acetate in the BFT diet increasing at the expense of propionate and butyrate, matches the response reported from *in vitro* experiments on *Lotus pedunculatus* Cav. (Tavendale *et al*., 2005).

Condensed tannins are reported to reduce fibre, CP and OM digestibility because of their binding properties, inhibition of rumen microbes and also because CT themselves are ruminally indigestible (Waghorn, 2008). In this study, a substantial decrease in aNDFOM digestibility was observed when birdsfoot trefoil replaced white clover as the legume component in silage. The difference in OM digestibility between diets actually corresponded well to (dietary CT content + aNDFOM digestibility difference + CP digestibility difference). The decrease in total aNDFOM digestibility was caused both by a lower proportion of PDNDF in the BFT silage and by lower digestibility of this PDNDF, with the latter in relative agreement with digestion rate differences determined *in sacco*.

Actual degradation *in vivo* is the end-result of competition between degradation and passage. When passage kinetics of the diets fed in Year 2 were estimated from intake and rumen pool of iNDF in a meta-analysis project (Krizsan *et al*., 2010), passage rate was similar between the diets (0.023 for BFT and 0.025 h^{-1} for WC; $P = 0.16$). The NorFor model also estimated similar ruminal passage rates for forage aNDFOM of both diets, 0.016 h^{-1}.

The effects of CT may be negated by addition of polyethylene glycol (PEG) to samples or as a drench to feeds (Waghorn, 2008). Miller and Ehlke (1994) made pair-wise comparisons against untreated controls and reported increased *in vitro* DM digestibility of 26.6 g kg^{-1} DM for PEG addition to birdsfoot trefoil samples with CT concentration 85.3 g kg^{-1} DM. Applying this approach to the BFT silage studied here resulted in a tendency ($P < 0.10$, n = 6) for increased OM digestibility of 8.4 g kg^{-1} silage OM with PEG addition. However, this would only represent a minor part of the *in vivo* dietary digestibility difference, so there may also be differences in fibre degradation properties not related to CT. Indirect effects from the companion grasses themselves or how they influenced the properties of the legumes may have contributed.

Conclusions

The results suggest that under the hemiboreal conditions typical of Scandinavia, birdsfoot trefoil as the legume component in grass-legume silage for dairy cows can improve protein supply compared with white clover. This is in spite of a climate-specific low condensed tannin concentration that is further diluted by companion grasses and ration concentrate. Under these conditions, birdsfoot trefoil has a potential to increase milk protein yield in rations that satisfy energy requirements but are limited in metabolisable protein. The lower digestibility of dry matter, organic matter and fibre, and hence lower volatile fatty acid production with birdsfoot trefoil, indicates a lower energy supply compared with white clover, which is consistent with *in vitro* and *in situ* results.

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Table 1 Chemical composition, degradation properties and nutritive value of birdsfoot trefoil-ryegrass (BFT) silage, white clover-ryegrass (WC) silage and supplementary concentrates fed to dairy cows at a restricted level. Mean and standard deviation for 3 determinations.

	BFT silage Year 1	WC silage Year 1	Concentrate Year 1	BFT silage Year 2	WC silage Year 2	Barley Year 2
Dry matter, g/kg	484 (12)	435 (23)	905 (12)	462 (24)	548 (46)	872 (1)
Chemical composition, g/kg DM						
Ash	85 (2)	96 (1)	63 (0)	90 (4)	88 (12)	23 (0)
Crude protein	161 (3)	162 (2)	152 (2)	167 (6)	155 (2)	123 (1)
aNDFOM	410 (8)	401 (5)	154 (4)	358 (3)	374 (4)	132 (10)
Crude fibre	-	-	61 (3)	-	-	46 (4)
Ether extract	-	-	51 (0)	-	-	28 (0)
Water-soluble carbohydrates	35 (1)	34 (4)	32 (2)	89 (23)	118 (10)	26 (6)
Starch	-	-	429 (9)	-	-	615 (5)
Minerals						
Ca	7.9 (0.2)	8.3 (0.6)	8.7 (0.2)	9.2 (0.8)	8.0 (0.6)	0.4 (0.1)
P	3.8 (0.1)	4.3 (0.1)	4.7 (0.1)	3.4 (0.2)	3.2 (0.2)	3.6 (0.3)
Mg	1.9 (0.1)	1.8 (0.1)	5.0 (0.2)	2.1 (0.1)	1.8 (0.1)	1.1 (0.1)
K	27.4 (0.9)	33.7 (0.9)	6.5 (0.2)	25.7 (2.0)	21.4 (0.3)	4.8 (0.3)
Na	1.6 (0.1)	1.1 (0.0)	2.7 (0.1)	1.5 (0.2)	1.1 (0.2)	0.1 (0.0)
S	2.3 (0.0)	2.6 (0.1)	2.0 (0.2)	2.7 (0.1)	2.1 (0.2)	1.3 (0.1)
N fractionation, g/kg N						
Total buffer-soluble N	527 (16)	617 (13)	385 (17)	485 (40)	531 (47)	237 (1)
Soluble true protein-N	6 (14)	26 (10)	279 (19)	33 (8)	31 (13)	151 (6)
α-amino-N	228 (34)	276 (12)	-	173 (23)	185 (33)	-
Ammonia-N	44 (5)	56 (7)	-	55 (9)	66 (10)	-
Degradation properties						
Pot deg N, g/kg N	381	338	-	451	406	-
kD pot deg N g/kg N/h	82	79	-	65	71	-
Pot deg NDF, g/kg NDF	740 (11)	856 (2)	756	757 (17)	842 (15)	662
kD pot deg NDF g/kg NDF/h	39	46	-	38	45	-

OMD, g/kg OM	743 (6)	788 (6)	-	746 (10)	775 (4)	-
OMD_{PEG}, g/kg OM	754 (13)	790 (3)	-	748 (14)	774 (8)	-
Nutritional value						
MJ ME	10.0 (0.0)	10.4 (0.1)	13.2 (0.0)	10.1 (0.2)	10.3 (0.3)	13.7 (0.1)
AAT	75 (0)	71 (0)	86 (0)	79 (0)	76 (1)	93 (1)
PBV	29 (2)	39 (2)	8 (2)	29 (6)	23 (2)	-34 (1)

aNDFOM, amylase-treated neutral detergent fibre expressed on an ash-free basis; soluble true protein-N, trichloroacetic acid-precipitable, buffer-soluble N; pot deg N, 'b' fraction from *in sacco* determination corrected for particle loss by buffer-soluble N; pot deg NDF, (1000-indigestible NDF) after 288 *in sacco* incubation; OMD, organic matter digestibility from 96-h *in vitro* incubation; OMD_{PEG}, OMD from 96-h *in vitro* incubation with addition of polyethylene glycol; AAT, amino acids absorbable in the digestive tract; PBV, protein balance in the rumen.

Table 2 Fermentation products, pH and tannin fractions in birdsfoot trefoil-ryegrass (BFT) silage and white clover-ryegrass (WC) silage fed to dairy cows at a semi-restricted level. Mean and standard deviation for 3 determinations.

Product, g/kg DM	BFT silage Year 1	WC silage Year 1	BFT silage Year 2	WC silage Year 2
Succinic acid	3.6 (0.6)	5.0 (0.6)	3.8 (0.1)	3.4 (0.6)
Lactic acid	53.5 (9.7)	72.1 (5.4)	25.9 (7.4)	35.7 (16.9)
Acetic acid	6.1 (1.2)	9.5 (1.8)	5.6 (1.6)	4.8 (1.4)
Propionic acid	<0.8	<0.8	<0.6	<0.6
2,3 butanediol	0.7 (0.07)	1.1 (0.32)	1.2 (0.33)	1.0 (0.34)
Ethanol	2.4 (0.6)	3.3 (0.2)	4.4 (2.8)	4.1 (1.6)
Butyric acid	<0.4	0.9 (0.75)	0.3 (0.00)	0.4 (0.15)
pH	4.5 (0.08)	4.4 (0.04)	5.0 (0.14)	4.8 (0.28)
Tannin fractions, proportional distribution				
Extractable tannins	0.51 (0.01)	-	0.40 (0.05)	-
Protein-bound tannins	0.34 (0.03)	-	0.39 (0.05)	-
Fibre-bound tannins	0.16 (0.03)	-	0.22 (0.05)	-

Table 3 Intake and orts for dairy cows fed a semi-restricted level of either birdsfoot trefoil-ryegrass (BFT) silage or white clover-ryegrass (WC) silage in change-over experiments during 2 consecutive years (N = 76).

	LS Means		*P*		
	BFT	WC	Diet	Year	Year×Diet
Total DM, kg/d	20.4	20.4	ns	ns	ns
Silage DM, kg/d	13.37	13.43	ns	**	ns
Concentrate DM, kg/d	6.67	6.61	ns	***	ns
Silage orts, kg DM/d	0.46	0.62	ns	ns	ns
Concentrate orts, kg DM/d	0.16	0.20	ns	†	ns
OM, kg/d	18.9	18.8	ns	ns	ns
aNDFOM, g/d	6081	6132	ns	**	***
Crude protein, g/d	3106	3032	***	*	***
Soluble CP, g/d	1414	1515	***	***	***
Soluble true protein, g/d	260	276	***	***	ns
WSC, g/d	1066	1242	***	***	ns
Starch, g/d	3308	3269	ns	ns	ns
MJ ME	222	227	***	ns	ns
AAT, g/d	1653	1605	***	ns	ns
PBV, g/d	336	357	*	***	***
NorFor model predictions					
RDP, g/kg DM	*116*	*120*			
RUP, g/kg DM	*40*	*33*			
Net energy balance, %	*102*	*110*			
AAT balance, %	*88*	*92*			

LS Means, Least square means obtained with significant main effects and interactions; ns, non-significant; aNDFOM, amylase-treated neutral detergent fibre expressed on an ash-free basis; soluble true protein-N, trichloroacetic acid–precipitable, buffer-soluble N; WSC, water-soluble carbohydrates; AAT, amino acids absorbable in the digestive tract; PBV, protein balance in the rumen; RDP, rumen-degradable protein; RUP, ruminally undegraded feed CP. RUP and RDP are arithmetic means and do not exactly add up to LS Means of CP.
†, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$.

Table 4 Milk yield and milk composition for dairy cows fed a semi-restricted level of either birdsfoot trefoil-ryegrass (BFT) silage or white clover-ryegrass (WC) silage in change-over experiments during 2 consecutive years (N = 76).

	LS Means		*P*		
	BFT	WC	Diet	Year	Year×Diet
Milk, kg/d	26.1	25.3	†	**	ns
ECM, kg/d	27.7	26.4	ns	*	ns
Protein, g/kg	34.0	33.5	**	ns	ns
Fat, g/kg	43.3	43.9	ns	**	ns
Lactose, g/kg	46.1	46.4	**	ns	ns
SCC, 1000/mL	79.8	65.5	ns	ns	ns
Fat, g/d	1105	1092	ns	*	ns
Lactose, g/d	1193	1169	ns	*	ns
Protein, g/d	892	856	**	†	ns
Milk N : Feed N	0.284	0.277	†	ns	ns
Milk urea m*M*	4.53	4.10	***	*	ns

LS Means, Least square means obtained with significant main effects and interactions; ns, non-significant; SCC, somatic cell count.
†, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$.

Table 5 Digestibility and nitrogen balance data for dairy cows fed a semi-restricted level of either birdsfoot trefoil-ryegrass (BFT) silage or white clover-ryegrass (WC) silage in change-over experiments during 2 consecutive years (N = 48).

	LS Means		*P*		
	BFT	WC	Diet	Year	Year×Diet
Digestibility					
Dry matter	0.705	0.755	***	*	*
Organic matter	0.720	0.772	***	*	†
aNDFOM	0.602	0.722	***	*	ns
PDNDF	0.812	0.868	***	**	*
NorFor model predicted digestibility					
Organic matter	*0.750*	*0.787*			
aNDFOM	*0.599*	*0.687*			
N balance					
N intake, g/d	505	492	*	ns	†
Milk N proportion	0.285	0.280	ns	ns	***
Urinary N proportion	0.298	0.283	*	ns	*
Faecal N proportion	0.328	0.304	***	***	†
Balance	0.088	0.133	***	**	*
Urinary N fractions					
Total urinary N, g/d	151	140	**	**	**
Urea N, g/d	102	90	**	*	ns
Allantoin N, g/d	16.7	15.9	ns	ns	ns
Creatinine N, g/d	5.68	5.59	ns	ns	ns
Voided urine, kg/d	23.3	23.0	ns	ns	***

LS Means, Least square means obtained with significant main effects and interactions; ns, non-significant; aNDFOM, amylase-treated neutral detergent fibre expressed on an ash-free basis; PDNDF, potentially digestible NDF determined by 288-h *in sacco* incubation.
†, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$.

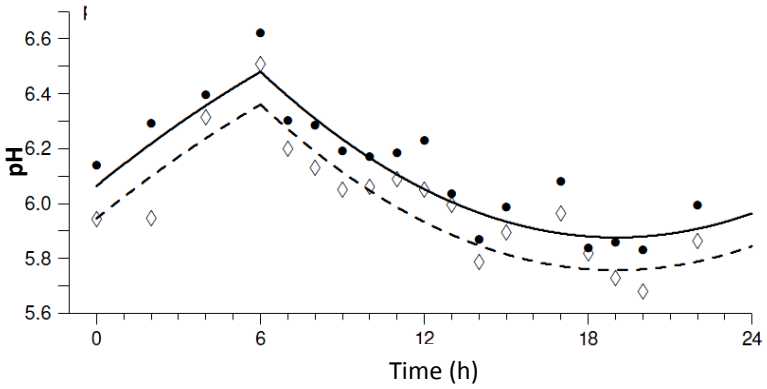
Table 6 Rumen characteristics for dairy cows fed a restricted level of either birdsfoot trefoil-ryegrass (BFT) silage or white clover-ryegrass (WC) silage in change-over experiments during 2 consecutive years. (N = 22).

	LS Means		*P*		
	BFT	WC	Diet	Year	Year×Diet
Rumen contents					
Fresh weight, kg	97.6	99.2	ns	*	ns
DM, kg	12.1	12.0	ns	**	ns
OM, kg	11.0	10.8	ns	**	ns
aNDFOM, g	5818	5277	*	*	ns
CP, g	2139	2271	*	***	ns
Total VFA, m*M*	136	143	**	ns	ns
Molar proportion of VFA					
Acetate	0.662	0.650	**	ns	ns
Propionate	0.190	0.200	*	ns	ns
Butyrate	0.107	0.111	*	ns	ns
Valerate	0.015	0.016	ns	ns	ns
Iso-acids	0.022	0.021	ns	ns	ns
NH_3-N, mg 100 mL^{-1}	12.80	12.53	ns	ns	ns
α-amino-N, mg 100 mL^{-1}	2.91	3.04	ns	ns	ns
pH	6.10	5.98	*	ns	ns

LS Means = Least square means obtained with significant main effects as there were no significant interactions. ns, non-significant; aNDFOM, amylase-treated neutral detergent fibre expressed on an ash-free basis; VFA, volatile fatty acids; iso-acids, (iso-butyrate + iso-valerate).

†, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$.

a)



b)

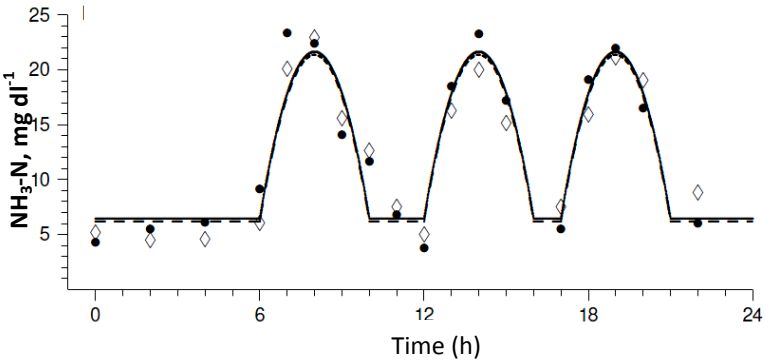


Figure 1 Diurnal variation in ruminal variables for dairy cows fed a semi-restricted level of grass-legume silage [treatment BFT (●), solid curve, birdsfoot trefoil as the legume; treatment WC (◊), dashed curve, white clover as the legume] together with concentrate constituting 300-350 g kg^{1} ration DM. Panel a) Least square means of ruminal pH and estimated effects according to a segmented quadratic function. Panel b) Least square means of ammonia-N (mg 100 mL^{-1}) and estimated effects according to parabolas with maximum 2 hours after feeding.