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Effect of exchanging *Onobrychis viciifolia* and *Lotus corniculatus* for *Medicago sativa* on ruminal fermentation and nitrogen turnover in dairy cows

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ABSTRACT

The objective of the study was to determine the effect of feeding sainfoin (SF; *Onobrychis viciifolia*) and birdsfoot trefoil (BT; *Lotus corniculatus*), 2 temperate climate forage legumes that contain condensed tannins (CT), on ruminal fermentation and N turnover in dairy cows. Six ruminally cannulated multiparous dairy cows (milk yield = 40 kg/d; 36 d in milk) were used in a replicated 3 × 3 Latin square design. All animals were fed basal diets containing 20% pelleted SF (223 g of CT/kg of dry matter), BT (30.3 g of CT/kg of dry matter), or alfalfa (AL) and concentrate to meet their predicted nutrient requirements. Each experimental period consisted of a 21-d adaptation period in a tiestall, followed by a 7-d collection period in metabolic crates, where feces and urine were collected quantitatively. During the 7-d period, milk yield was recorded daily and milk samples were taken at each milking. Blood, ruminal fluid, and papillae were sampled on d 2 and 5. The relative abundance of selected bacterial strains in ruminal fluid and the gene expression of transporter genes in the papillae were determined with quantitative PCR. Total volatile fatty acids and the abundance of the cellulolytic bacteria *Prevotella* spp. and *Ruminococcus flavefaciens* decreased with SF compared with AL. The relative gene expression of the monocarboxylate transporter 1 was increased with BT compared with AL and SF. Total yields of milk, milk fat, and milk protein were similar among treatments. The proportion of 18:3n-3 in milk fat was greater and those of 22:5n-3 and 22:6n-3 were lower with SF than with BT. The contents of urea N in blood (2.71, 3.45, and 3.90 mmol/L for SF, AL, and BT, respectively), milk (79.8, 100.1, and 110.9 mg/kg for SF, AL, and BT, respectively), and urine were lower with SF than with AL and BT, and a trend toward a lower ruminal ammonia content occurred with SF compared with BT. Intake and excretion of N with milk were similar among treatments, but urine N was lower with SF than with AL. The N excretion to N intake relation showed a shift in a part of urine N (17.5, 20.8, and 19.5% for SF, AL, and BT, respectively) to fecal N (45.2, 41.3, and 38.5% for SF, AL, and BT respectively) with SF compared with AL and BT. In conclusion, SF and BT differed in their effects on fermentation and milk fatty acid profile and SF also showed potential to decrease metabolic and environmental loads. The main reason for the different efficiency was likely a higher CT content of SF compared with BT.
Key words: condensed tannin, ruminal fermentation, microbial community, nitrogen loss

INTRODUCTION

Forage-based production systems for dairy cows are considered economically beneficial when compared with production systems with high-concentrate TMR, and they may have a positive effect on the environment because of the reduced need for fossil energy in feed production and transport (Dillon et al., 2005). However, feeding of forage-dominated diets to dairy cows may also involve risks because these feeds often contain more RDP than can be used by ruminal microbes, which causes excessive NH_3 in the rumen. The absorption and metabolism of this NH_3 then elevates the urea levels in the blood, milk, and urine (Roseler et al., 1993; Spek et al., 2013). Bruinenberg et al. (2002) reported a frequently high excretion of N in the urine due to the imbalance between dietary protein and energy content in herbage-fed cows. This excretion has adverse environmental effects (Dijkstra et al., 2013), and it may also increase the metabolic load, especially during early lactation, because of the extra energy consumption needed for detoxification of excessive ruminal ammonia

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by ureagenesis (Lobley et al., 1995). Furthermore, the reduced flux of (microbial) protein to the duodenum, as a consequence of low dietary supply of fermentable energy, is supposed to be a limiting factor for milk production in cows consuming herbage (Bruinenberg et al., 2002). Therefore, forage-based diets have to be designed in a way that must prevent excessive ruminal protein degradation and increase the amount of ruminal escape protein, thereby increasing the digestive and metabolic N efficiency.

One potential strategy to improve N utilization is to include forages containing condensed tannins (**CT**) into dairy cow diets. These CT, which are polymers of flavanol (flavan-3-ol) units linked by carbon-carbon bonds, are known to form complexes with cellulose, hemicellulose, and pectin (McSweeney et al., 2001), and especially with dietary proteins (Hagerman and Butler, 1981). The complexes protect against ruminal degradation (Waghorn, 2008), resulting in a shift of N excretion from the urinary route to the feces (Carulla et al., 2005). The CT also influence ruminal biohydrogenation (**BH**) of UFA (Khiaosa-Ard et al., 2009). Furthermore, in vitro studies have shown that CT can bind to the cell coat polymers of ruminal bacteria, such as *Butyrivibrio fibrisolvens*, thereby decreasing the bacterial enzyme activity involved in the ruminal BH of dietary UFA (Jones et al., 1994). In addition, experiments in monogastric animals have shown that CT may damage the intestinal mucosa and decrease the absorption of AA (Waghorn, 2008). At present, no study has examined the effect of CT on the ruminal papillae of dairy cows or on the abundance of the transporter genes that are responsible for absorption of many ruminal degradation products.

A great wealth of CT-containing plant species exists in the tropics, whereas the list is far smaller for forage legumes growing in temperate climates. Promising temperate species include sainfoin (**SF**; *Onobrychis viciifolia*) and birdsfoot trefoil (**BT**; *Lotus corniculatus*). Experiments investigating the effect of CT from temperate climate legumes in general, and especially those of SF and BT, are plentiful for sheep (reviewed by Waghorn, 2008) but scarce for dairy cows. Hymes-Fecht et al. (2013) reported that the replacement of alfalfa (**AL**; *Medicago sativa*) silage by BT silage in a TMR improved performance and N utilization in lactating dairy cows. The dairy cows that either grazed a BT pasture (Harris et al., 1998; Turner et al., 2005) or were offered fresh BT in the barn (Woodward et al., 2000) also showed improved performance when compared with cows fed perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). When feeding SF hay to dairy cows as supplement to herbage, Scharenberg et al. (2009) observed a low acceptance even though choice experiments indicated the palatability index was higher for SF than BT in sheep (Scharenberg et al., 2007b) and SF hay was preferred to BT hay in a pretest in nonlactating cows (Scharenberg et al., 2009). These opposing effects could be due to different contents of dietary CT (Hymes-Fecht et al., 2013), the CT structure, or both. The content and structure of CT can vary considerably among species (Mueller-Harvey, 2006) and within species (Azuhnwi et al., 2011) and can change in response to different environmental conditions, such as microclimate and soil quality (Tiemann et al., 2010).

In the present study, we assessed the potential for the inclusion of temperate climate CT legumes in diets for dairy cows by testing the following hypotheses: (1) replacing a non-CT legume such as AL by CT-containing legumes would modify ruminal fermentation in a way that would improve both N utilization and milk fatty acid profile, and (2) the level of improvement would differ between legume species (BT vs. SF) due to their differences in CT content and structure. The legumes were offered as pellets because in this form CT legumes are already used as nutraceuticals against digestive parasites in small ruminants (Hoste et al., 2015) and as a well-accepted supplement in dairy cow diets (Girard et al., 2016; Arrigo and Dohme, 2009)

MATERIALS AND METHODS

Experimental Design, Animals, and Diets

Six ruminally cannulated (Bar Diamond Inc., Parma, ID) multiparous Swiss Holstein-Friesian cows were blocked by milk yield and assigned randomly in a monofactorial design to 3 treatments in a replicated 3 × 3 Latin square arrangement. At the beginning of the experiment, the cows were, on average, 36 (±18) DIM, had a BW of 716 (±45.0) kg, and produced 40 (±6) kg of milk per day. Each of the 3 consecutive experimental runs consisted of a 21-d adaptation period, where the cows were individually stalled in a tiestall with rubber mat flooring, and a 7-d data collection period where each cow was kept in a metabolism crate equipped with a slatted floor. Prior to the experiment, the animals were accustomed to the metabolism crates for 1 d. All procedures were conducted in accordance with the Swiss guidelines for animal welfare and were approved (No. 2012_38_FR) by the Animal Care Committee of the Canton Fribourg, Fribourg, Switzerland.

The cows received a basal diet consisting of a partial mixed ration (**PMR**; Table 1) including grass hay, corn silage, and Extrulin 135 (Nutri'Form SA, Hildisrieden, Switzerland; this product contains 60% extruded linseed and 40% wheat bran, and was added to increase the amount of PUFA in the diet). A barley-corn-wheat

Table 1. Ingredients and chemical composition of the diets (means[1] ± SD)

Item	Treatment[2]		
	AL	SF	BT
Ingredient (g/kg of DM)			
Partially mixed ration[3]			
Hay	417 ± 51.7	408 ± 47.5	391 ± 48.1
Corn silage	229 ± 28.4	224 ± 26.1	215 ± 26.5
Extruded linseed[4]	48.6 ± 6.03	47.6 ± 5.54	45.6 ± 5.62
Legume pellets	164 ± 17.5	159 ± 15.4	164 ± 6.7
Cereal mix	108 ± 63.8	122 ± 64.4	143 ± 62.6
Protein concentrate	31.8 ± 20.73	37.5 ± 22.74	39.3 ± 18.42
Analyzed composition[5] (g/kg of DM, unless noted)			
DM[6] (g/kg of wet weight)	770 ± 4.1	773 ± 2.7	774 ± 3.1
OM	928 ± 0.6	932 ± 0.1	929 ± 0.5
CP	142 ± 0.5	139 ± 0.6	149 ± 0.3
NDF	428 ± 7.7	415 ± 4.3	410 ± 5.7
ADF	241 ± 3.3	236 ± 3.1	230 ± 2.2
Total fatty acids	44.1 ± 0.51	44.1 ± 0.52	45.0 ± 0.33
16:0	5.10 ± 0.061	5.16 ± 0.064	5.33 ± 0.053
18:0	0.92 ± 0.010	0.91 ± 0.013	0.88 ± 0.009
18:1n-9	5.56 ± 0.080	5.67 ± 0.103	5.65 ± 0.095
18:2n-6	12.9 ± 0.15	13.1 ± 0.15	13.6 ± 0.13
18:3n-3	14.5 ± 0.49	14.6 ± 0.32	14.8 ± 0.30
Calculated energy and protein supply[7] (per kg of DM)			
NE_L (MJ)	6.34 ± 0.025	6.36 ± 0.012	6.40 ± 0.011
APDE (g)	92.2 ± 0.27	92.4 ± 0.18	95.4 ± 0.15
APDN (g)	91.1 ± 0.45	89.4 ± 0.34	96.1 ± 0.38

[1]Mean over the 3 periods of the Latin square.

[2]AL = alfalfa; SF = sainfoin; and BT = birdsfoot trefoil.

[3]60% hay, 33% corn silage, and 7% linseed.

[4]Extrulin 135 (Nutri'Form SA, Hildisrieden, Switzerland), containing 60% extruded linseed and 40% wheat bran.

[5]Means of 3 determinations per treatment.

[6]Means of 21 determinations per treatment.

[7]Calculated as described by Thanner et al. (2014). APD = absorbable protein at the duodenum when rumen fermentable energy (APDE) or nitrogen (APDN) is limiting microbial protein synthesis in the rumen.

mixture (mixing ratio = 1:1:1) and a soybean cake-based protein concentrate were also offered in amounts to meet each animal's individual predicted nutrient requirements (Agroscope, 2014), which resulted in some, but limited, individual variations in dietary ingredient proportions (Table 1). The cows also were fed 60 g/d of NaCl and 40 g/d of a mineral-vitamin mixture containing (per kg): Ca, 104 g; P, 56 g; Mg, 25 g; Na, 66 g; Zn, 1.3 g; Se, 6.6 mg; I, 20 mg; Co; 3.3 mg, Cu, 160 mg; Mn, 620 mg; vitamin A, 200,000 IU; vitamin D_3, 16,000 IU; vitamin E, 1,300 mg; and biotin, 67 mg.

The experimental variation was generated by offering either AL (*Medicago sativa* L. 'Sanditi'), SF (*Onobrychis viciifolia* L. 'Perly'), or BT (*Lotus corniculatus* L. 'Polom') in amounts representing approximately 20% of the basal diet. The legumes were grown at Posieux, Switzerland (650 m altitude), harvested as the second cut after 49 d of regrowth, at the stage of early flowering, and wilted on the field for 24 h. For the pelleting process, the wilted legumes were chopped to 5 to 8 cm before drying for 4 min at 700°C. Subsequently, the forage was milled, at a temperature of approximately 80°C, to pass a 3-mm sieve and pressed into pellets of 2 cm in length and 8 mm in diameter.

During the adaptation periods, ad libitum intake of the basal diet of each cow was determined and used as a basis for offering a constant amount of the basal diet and concentrate ingredients to the cows during each collection period. Pellets and concentrate ingredients were offered together in 2 equal meals, at 0630 and 1430 h, followed by the PMR at 0700 and 1700 h. Cows had continuous access to tap water.

Data Recording and Sample Collection

Feed intake was recorded daily. Samples of pellets and concentrates were taken every day and samples of hay, corn silage, and linseed were taken every second day of each collection period and pooled over each period for analysis of chemical composition. The PMR was analyzed daily for DM by oven drying at 105°C for 3 h. For chemical analysis, corn silage and feces samples were lyophilized (Christ, Osterode, Germany); all other feed samples were dried at 60°C for 24 h. All samples

were ground to pass a 1-mm screen (Brabender mill, Brabender, Duisburg, Germany).

The BW of the cows was determined twice a day during the adaptation period, after milking in the milking parlor. During the collection periods, cows were milked in their metabolism crates. Milk yields were determined at each milking (0500 and 1600 h) and milk samples were taken from each cow and pooled per day. Daily samples were subdivided. One subsample was preserved in Broad Spectrum Microtabs (Gerber Instruments AG, Effretikon, Switzerland) and stored at 5°C for later infrared analysis of milk gross constituents. A second subsample was stored at –20°C for later analysis of urea. Milk samples were also pooled by cow across the entire collection period and stored at –20°C for later analysis of N content. Daily milk samples were also taken for each cow on d 1, 4, and 7 of each collection period and stored at –20°C for later analysis of fatty acid composition.

Feces and urine were collected quantitatively every day of the collection period. The urine was collected in urinals attached around the vulva with Velcro straps glued to the shaved skin. The urine was acidified directly with 2.5 *M* sulfuric acid to avoid N losses. Approximately 90 g of feces and 150 mL of urine were taken daily, pooled by cow across the entire collection period, and stored continuously at −20°C for later analysis.

On d 2 and 5 of the collection period, at 0700 and 1700 h, before offering the PMR, blood was sampled from the jugular vein using serum tubes (Greiner Bio-One, Kremsmuenster, Austria). After sampling, the tubes were stored for 1 h at room temperature and then centrifuged for 15 and 2 min at 3,000 and 4,000 × *g*, respectively, at room temperature. The retrieved serum was stored at –20°C for later analysis of urea. On the same days and at the same time points, ruminal fluid, particulate matter content, and papillae were also taken. The ruminal fluid was collected from the ventral rumen using a tube equipped with a terminal cone fitted with a 1-mm pore size sieve. Samples were cooled on ice directly after sampling. For later analysis of VFA and NH_3 contents, 10 mL of ruminal fluid were mixed with 0.2 mL of 50% (vol/vol) sulfuric acid or with 0.2 mL of 50% (vol/vol) trichloroacetic acid for analysis of VFA and NH_3, respectively, and stored at −20°C. For the later determination of protozoal counts, 0.5 mL of ruminal fluid was mixed with 0.5 mL of 6% (vol/vol) formalin. Particulate matter was obtained by taking approximately 150 g of material by hand from the ventral rumen and squeezing slightly to release ruminal fluid. The remainder was immediately frozen at −20°C. Ruminal papillae were taken through the rumen cannula from the area of the *atrium ruminis* in the ventral rumen. Every biopsy consisted of 3 to 5 papillae that were removed using only the fingers while wearing gloves. The papillae were washed in PBS solution and then stored in RNA*later* TissueProtect solution (Qiagen, Venlo, the Netherlands) at 4°C.

General Laboratory Analysis

The DM and OM contents of feeds and feces were determined gravimetrically by oven-drying at 105°C and ashing at 550°C for 3 and 4 h, respectively. Contents of ADF (AOAC International, 1995; procedure 973.18) and NDF (AOAC International, 1995; procedure 2002.4) were analyzed using the Ankom fiber analyzer (Ankom Technology Corporation, Fairport, NT), where NDF was analyzed with heat-stable amylase and sodium sulfite. Both ADF and NDF were expressed without residual ash, determined by 1 h of incineration. The total N contents of feeds, feces, urine, and milk were analyzed by the Kjeldahl method (AOAC International, 1995; procedure no. 988.05) and multiplied by 6.25 for the feed, to calculate the CP content. The dietary contents of NE_L and absorbable protein in the duodenum were calculated as described by Thanner et al. (2014). The total fatty acid contents were extracted with toluene and 5% HCl dissolved in methanol for 3 h at 70°C, with methyl nonadecanoate as an internal standard. After extraction, the samples were purified with a solid phase extraction cartridge (Merck, Darmstadt, Germany) and fatty acid composition was determined by GC on a 15-m × 0.1-mm column (Supelcowax, Sigma-Aldrich, St. Louis, MO) and flame ionization detection (Agilent 6850, Agilent Technologies, Santa Clara, CA). The contents of total, soluble, protein-bound, and fiber-bound CT of the SF and BT pellets were analyzed using the HCl-butanol method described by Terrill et al. (1992). The CT standards were prepared in advance from fresh SF and BT by extraction with acetone and water (7:3, vol/vol) and purification on a Sephadex column (Sigma-Aldrich). Total phenols were analyzed by quantification of the phenolic hydroxyl groups with the Folin-Ciocalteau assay, according to Salminen and Karonen (2011), and were expressed as tannic acid equivalents.

Milk samples were analyzed for fat, protein, and lactose content using Fourier transformed infrared spectrometry (Milkoscan FT 6000, Foss, Hillerød, Denmark). The MUN content was determined using the UreaFil test kit (MEA 549 EC Milk Urease, Eurochem, Moscow, Russia). The fatty acid composition was determined by dissolving the milk fat in hexane, followed by transesterification and subsequent separation of the fatty acids on a capillary column (CP-SIL 88, 100 m, 0.25-mm i.d., 0.20-μm film thickness, P.H. Stehelin & Cie AG, Basel, Switzerland) by GC (Agilent 6890, Agi-

lent Technologies), following the procedure described by Ampuero-Kragten et al. (2014).

The ruminal VFA profile was analyzed by HPLC (Dionex, Dionex Corporation, Sunnyvale, CA) with a Shodex detector (Showa Denko K.K, Minato, Japan). Ruminal NH_3 was determined colorimetrically with a commercial test kit (S 180, BioMerieux, Geneva, Switzerland). Ruminal ciliates were counted with a 0.1-mm Bürker counting chamber (LO Laboroptic Ltd., Lancing, UK) and were characterized as entodiniomorphid and holotrich cells. Urinary and serum urea contents were determined by enzymatic treatment with urease (EC 3.5.1.5) and glutamate dehydrogenase (EC 1.4.1.2) using a commercial test kit (No. 61974 BioMerieux, Marcy l'Etoile, France).

DNA Extraction and PCR Analysis of Ruminal Samples

The genomic DNA from ruminal microbes was extracted from particulate ruminal content using a DNA Stool Mini Kit (Qiagen). The extractions were carried out following the manufacturer's protocol (version 06/2012, Qiagen) with minor modifications. Briefly, 1.4 mL of lysis buffer was added to 100 mg of lyophilized material. The samples were then incubated at 95°C for 15 min. After centrifugation (2,000 × *g* for 3 min at room temperature), the samples were incubated again at 65°C for 10 min in presence of ribonuclease A (EC 3.1.27.5) and proteinase K (EC 3.4.21.64), followed by purification on QIAamp spin columns (Qiagen). The DNA was eluted by applying 100 μL of the elution buffer to the top of the columns. The DNA content was determined using a NanoDrop spectrometer (ND-1000, Witec AG, Luzern, Switzerland), and the samples were then diluted with nuclease-free water to 4 ng/μL. Primers for the bacteria and for the transporter genes were purchased in desalted quality (Eurogentec, Seraing, Belgium, and Microsynth, Balgach, Switzerland). Primers not taken from the literature were generated with the National Center for Biotechnology Information's Primer-BLAST software tool (Ye et al., 2012). Generally, the primers were designed based on the gene coding for the 16S ribosomal RNA of a certain species, while performing a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against all bacterial genomes available. The target and housekeeping gene primers are shown in Appendix Table A1. All reactions regarding the bacteria were carried out in duplicate within an Eco Illumina Real-Time PCR System (Illumina Inc., San Diego, CA) in a reaction volume of 20 μL. The amount of 16S ribosomal DNA of the bacteria was measured with SYBR green technology with the Kapa SYBR Fast Universal 2× quantitative PCR Master Mix (Kapa Biosystems, Woburn, MA). The thermal profile consisted of 5 min of activation at 95°C, followed by a 40-cycle, 3-step PCR with 10 s of denaturation at 95°C, 20 s of annealing at 60°C, and 20 s of extension at 72°C. The assay specificity was ensured by generating a melting curve at the very end by slow denaturation through heating from 55 to 95°C. The amplification efficiency (E) for each primer pair was calculated using a mixture of DNA derived from 6 different random fibrous samples; this mixture was diluted at least 5-, 10-, and 50-fold, as well as 100- and 500-fold for more-abundant bacteria, and amplified as described above. The efficiency was calculated automatically by the associated EcoStudy software (Illumina Inc.), where the quantification cycle (Cq) values and amount of template are plotted against each other, a linear regression was calculated, and the efficiency was determined according to the equation: $E = 10^{(-1/\text{slope})}$. For the 16S ribosomal DNA (target gene, T) of the selected species, the percentage (P) in relation to the total bacterial 16S ribosomal DNA (reference gene, R) was calculated using the following formula:

$$P = \frac{E_R^{Cq(R)}}{E_T^{Cq(T)}}. \quad [1]$$

The expression of the transporter genes was determined by extracting the total RNA from the papillae using the Nucleospin RNA XS kit (Macherey-Nagel, Dueren, Germany), following the manufacturer's procedure. Briefly, 3 to 5 papillae were thawed and homogenized in a MiniLys (Bertin, CNIM Group, Paris, France) using a CK14 Precellys Lysing Kit (Precellys 24, CNIM Group) in 0.3 mL of lysis buffer. The total RNA was further extracted and the RNA content was determined using a NanoDrop Spectrophotometer (ND-1000, Witec AG). A 250-ng sample of RNA was used for reverse transcription with the Quantitect Reverse Transcription kit (Qiagen). The resulting reaction product was diluted 1:1 with water free of RNA-degrading enzymes before further analysis. The expression of the genes for the monocarboxylate co-transporter isoform 1 (*MCT1*), the sodium hydrogen exchanger isoforms 1 and 3 (*NHE1* and *NHE3*, respectively), and the urea transporter B (*UTB1*) in the ruminal papillae was determined with a quantitative real-time PCR, using a KAPA Sybr Fast quantitative PCR Kit (Kapa Biosystems) with an Eco Ilumina-Real Time PCR System (Illumina Inc.). The program consisted of a preincubation step at 95°C for 5 min, followed by 40 cycles of 5 s at 95°C, and 20 s at 40°C. The expression of each targeted gene was evaluated using the ΔCt (cycle threshold) method and normalized using *GAPDH* as housekeeping gene. All

the calculations were performed using the EcoStudy software (Illumina Inc.).

Statistical Analysis

Data collected over several days were averaged per cow per period before statistical analysis. The results are presented in the tables as least squares means and standard error of the mean. Data were subjected to ANOVA using the MIXED procedure of the SAS 9.2 software (SAS Institute Inc., Cary, NC), according to the following model:

$$Y_{ijkl} = \mu + t_j + s_i + p_k + c_l + e_{ijkl}, \quad [2]$$

where Y_{ijkl} represents the dependent variable; μ represents the least squares mean; t_j represents the fixed effect of the treatment (j = Al, SF, BT); s_i represents the fixed effect of the sequence in the Latin square (i = 1, 2, 3); p_k represents the fixed effect of the Latin square period (k = 1, 2, 3); c_l represents the random effect of the cow; and e_{ijkl} represents the random error. When samples were taken at different time points during the sampling period, they were treated as repeated measurements and the statement was included in the model reading:

$$Y_{ijkl} = \mu + t_i + r_j + (tr)_{ij} + p_k + c_l + e_{ijkl}, \quad [3]$$

where, in addition to the elements explained above, r_j represents the time point (j = 1,2) and $(tr)_{ij}$ represents the effect of the interaction between treatment i and time j. The least squares means of the treatment groups, but not of the time points, are reported in the tables, because if an effect of time point (morning and evening) occurred, it represented the known diurnal variation. No interactions between treatment groups and time points were found; therefore, P-values were not reported. Degrees of freedom for the first model were determined using the Kenward-Roger option. Multiple comparisons among means were calculated with both models using Tukey's procedure. Significance was declared at $P < 0.05$ and trends were discussed at $P < 0.10$.

RESULTS

The chemical composition varied among the 3 legume pellet types with respect to contents of CP, total fatty acids, and 18:2n-6; these were highest in BT, followed by AL and SF (Table 2). The content of 18:3n-3 was highest with BT and lowest with AL, whereas SF took an intermediate position. The AL pellets had the highest and SF the lowest NDF and ADF content, whereas the BT pellets took an intermediate position. The contents of total CT and of all CT fractions were higher in SF than in BT, and we assumed that AL contained

Table 2. Chemical composition of the *Medicago sativa*, *Onobrychis viciifolia*, and *Lotus corniculatus* pellets (means[1] ± SD)

	Pellets[2]		
Item	AL	SF	BT
Analyzed composition (g/kg of DM)			
OM	873 ± 5.4	895 ± 2.1	881 ± 1.4
CP	182 ± 3.6	153 ± 1.7	208 ± 2.3
NDF	421 ± 21.9	367 ± 3.8	380 ± 4.7
ADF	327 ± 1.2	302 ± 0.5	306 ± 0.1
Total fatty acids	27.4 ± 1.95	26.7 ± 0.58	33.9 ± 0.84
16:0	4.11 ± 0.183	4.23 ± 0.042	5.09 ± 0.123
18:0	0.65 ± 0.031	0.57 ± 0.023	0.48 ± 0.020
18:1n-9	0.79 ± 0.227	0.99 ± 0.050	0.96 ± 0.076
18:2n-6	4.60 ± 0.269	4.33 ± 0.101	5.87 ± 0.138
18:3n-3	9.74 ± 1.506	12.02 ± 0.294	15.91 ± 0.526
Condensed tannins			
Total	NA[3]	223 ± 29.0	30.3 ± 2.93
Soluble	NA	141.8 ± 6.31	11.5 ± 1.79
Protein-bound	NA	65.0 ± 26.83	14.4 ± 1.61
Fiber-bound	NA	16.5 ± 1.82	4.4 ± 0.59
Total phenols[4]	9.70 ± 0.217	32.41 ± 9.752	16.33 ± 1.931

[1]3 determinations per legume species.

[2]AL = alfalfa; SF = sainfoin; BT = birdsfoot trefoil.

[3]Not analyzed.

[4]Total polyphenols are expressed as grams of tannic acid equivalent per kilogram of DM.

Table 3. Effects of the type of legume pellets on feed and nutrient intake, and total-tract digestibility

Item	Treatment[1]			SEM	*P*-value
	AL	SF	BT		
Daily intake per cow (kg, unless noted)					
Total DM	21.5	21.2	21.2	1.31	0.82
Partial mixed ration	14.9	14.3	13.8	1.10	0.38
Legume pellets	3.53	3.43	3.48	0.158	0.18
Concentrate[2]	3.11	3.49	3.87	0.947	0.36
OM	20.0	19.7	19.7	1.23	0.88
ADF	5.18	4.98	4.88	0.304	0.28
NDF	9.22	8.76	8.72	0.533	0.26
Total fatty acids (g)	952	934	956	55.9	0.75
16:0	111	110	113	7.2	0.56
18:0	20.2	19.5	19.0	1.10	0.27
18:1n-9	121	121	121	7.9	0.99
18:2n-6	279	279	288	22.2	0.67
18:3n-3	312	308	315	21.1	0.85
Condensed tannins (g)	NA[3]	754[a]	107[b]	31.4	<0.001
Digestibility (%)					
OM	68.6	70.1	71.8	2.40	0.18
NDF	64.9	67.1	68.4	3.21	0.19
ADF	57.3[y]	60.4[xy]	64.3[x]	4.11	0.07
N	58.7[xy]	54.8[y]	61.5[x]	2.86	0.09

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[x,y]Means within a row with different superscripts tend to differ ($P < 0.10$).

[1]AL = alfalfa; SF = sainfoin; BT = birdsfoot trefoil.

[2]Consists of cereal mix and protein concentrate.

[3]Not analyzed.

a negligible concentration of CT. Total phenol content was highest in SF followed by BT and AL.

Daily total DMI did not differ among treatments (Table 3). The same was true for the daily intake of OM, ADF, and NDF, as well as total and individual fatty acids. The daily intake of CT was higher ($P < 0.01$) for SF than for BT. The total-tract digestibility of OM and NDF did not differ among treatments, but the ADF digestibility showed a trend to being higher ($P < 0.10$) for BT than for AL, whereas SF took an intermediate position. The apparent total-tract N digestibility showed a trend to being higher ($P < 0.10$) for BT than for SF, whereas AL took an intermediate position.

Cows in the different treatment groups produced similar amounts of milk, with similar percentages of fat and lactose, and consequently secreted similar amounts of fat and lactose per day (Table 4). Milk protein percentage was greater ($P < 0.05$) for cows fed BT than for cows fed SF, whereas cows fed AL took an intermediate position. However, the numerically lower milk yield of BT compared with SF cows made this difference insignificant when protein was expressed as a daily yield. The milk of cows fed SF had a lower content of MUN ($P < 0.01$) when compared with the MUN of cows fed BT, whereas cows fed AL took an intermediate position.

The fatty acid composition of the milk fat was similar among treatments, with a few exceptions. One was that the proportion of 18:1n-9 in milk fat was greater ($P < 0.05$) with SF than with AL, whereas BT took an intermediate position. The proportion of 18:3n-3 also tended to be higher ($P < 0.10$) with SF than with BT, and AL took an intermediate position. In contrast, the proportions of 20:4n-6 tended to be high ($P < 0.10$) with AL and low with BT, and SF took an intermediate position. The proportions of 22:6n-3 and 22:5n-3 ($P < 0.10$) tended to be lowest with SF and highest with BT, and AL again took an intermediate position. The transfer rate of 18:3n-3 was also numerically higher ($P = 0.29$) with SF (5.87%) than with AL (4.96%) and BT (4.81%).

The BUN levels were lower in SF-fed cows ($P < 0.01$) than in cows fed AL and BT (Table 5). Concomitantly, ruminal ammonia N content tended to be lower ($P < 0.10$) in cows fed SF than in those fed BT, with intermediate values seen in AL cows. Total VFA content was lower ($P < 0.05$) for SF cows than in AL cows, but not in BT cows. No treatment differences were observed for the ruminal VFA profile, except for the proportion of valerate, which was lower ($P < 0.05$) for SF cows than for BT cows but not for AL cows. Counts of total ciliate protozoa and entodiniomorphids were greater ($P < 0.05$) for SF cows than for BT cows, whereas AL cows

Table 4. Effect of the type of legume pellets on milk yield and composition

Item	Treatment[1]			SEM	*P*-value
	AL	SF	BT		
Daily milk yield (kg)					
Total	38.2	38.4	37.3	2.54	0.65
Fat	1.49	1.47	1.47	0.071	0.96
Protein	1.14	1.12	1.16	0.102	0.59
Lactose	1.95	1.97	1.87	0.129	0.41
Milk composition (%, unless noted)					
Fat	3.95	3.87	4.00	0.238	0.32
Protein	2.96^{ab}	2.89^{b}	3.11^{a}	0.089	0.02
Lactose	5.09	5.12	5.01	0.064	0.15
MUN (mg/kg)	100.1^{ab}	79.8^{b}	110.9^{a}	6.96	0.01
Milk fat composition (g/100 g of fatty acids)					
16:0	27.2	26.4	27.4	1.17	0.59
16:1	1.31	1.27	1.39	0.149	0.75
18:0	12.4	13.1	11.9	0.80	0.38
18:1n-9	17.5^{b}	18.6^{a}	17.8^{ab}	0.68	0.03
trans-11 18:1	1.79	1.85	1.71	0.115	0.70
Σ18:1 *trans*	4.06	4.11	3.72	0.276	0.56
18:2n-6	1.61	1.76	1.70	0.129	0.18
cis-9,*trans*-11 18:2	0.47	0.46	0.46	0.034	0.96
ΣCLA[2]	0.62	0.60	0.60	0.041	0.94
18:3n-3	1.03^{yz}	1.18^{y}	1.01^{z}	0.052	0.06
20:0	0.150	0.166	0.172	0.0013	0.31
20:3n-6	0.055	0.042	0.050	0.0041	0.14
20:4n-6	0.082^{y}	0.069^{yz}	0.068^{z}	0.0043	0.07
20:5n-3	0.079	0.081	0.081	0.0039	0.89
22:5n-3	0.077^{yz}	0.069^{z}	0.081^{y}	0.0054	0.10
22:6n-3	0.019^{yz}	0.018^{z}	0.021^{y}	0.0008	0.06
Σ saturated	69.6	68.5	69.7	0.91	0.18
Σ unsaturated	30.4	31.6	30.3	0.91	0.16

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[y,z]Means within a row with different superscripts tend to differ ($P < 0.10$).

[1]AL = alfalfa; SF = sainfoin; BT = birdsfoot trefoil.

[2]Σ CLA = 18:2 *trans*-12,*trans*-14 + 18:2 *trans*-11,*trans*-13 + 18:2 *trans*-10,*trans*-12 + 18:2 *trans*-9,*trans*-11 + 18:2 *trans*-8,*trans*-10 + 18:2 *trans*-7,*trans*-9 + 18:2 *trans*-6,*trans*-8 + 18:2 *cis*-12,*trans*-14 + 18:2 *trans*-11,*cis*-13 + 18:2 *cis*-11,*trans*-13 + 18:2 *trans*-10,*cis*-12 + 18:2 *cis*-9,*trans*-11 + 18:2 *trans*-8,*cis*-10 + 18:2 *trans*-7,*cis*-9.

took an intermediate position. Holotrich counts were similar among treatments. The abundance of *Prevotella* spp. relative to total bacterial 16S rDNA was lower ($P < 0.05$) and that of *Ruminococcus flavefaciens* tended ($P < 0.10$) to be lower in SF cows when compared with AL cows, whereas BT cows took an intermediate position. The relative abundance of the other bacterial species was similar among treatments.

The relative abundance of the *MCT1* gene in the ruminal papillae was higher ($P < 0.05$) in BT compared with SF and AL cows (Figure 1). The relative abundance of the *NHE1*, *NHE3*, and *UTB1* genes did not differ among treatments.

Intake and fecal excretion of N were similar among treatments (Table 6). However, when expressed as a percentage of N intake, the fecal N tended ($P < 0.01$) to be higher in SF than in BT cows, but not in AL cows. The absolute urinary N excretion ($P < 0.05$) and its percentage of N intake were lower ($P < 0.05$) in SF than in AL cows, but not in BT cows. This shift was accompanied by concomitant changes in urinary urea N excretion ($P < 0.05$) and the proportion of urea N in total urinary N ($P < 0.01$). The sum of fecal and urinary N excretion, both in absolute amounts or expressed as a percentage of N intake, did not differ among treatments. Milk N excretion was similar among treatments, both when expressed in absolute amounts or as a percentage of N intake. Body N retention was positive for all treatments, but tended ($P < 0.01$) to be lower in SF than in BT cows, whereas AL cows took an intermediate position.

DISCUSSION

The CT content of the SF in the present study was extraordinarily high when compared with values reported in other studies that used the same cultivar and applied the HCl-butanol method for the analysis of CT (Azuhnwi et al., 2011; Theodoridou et al., 2012). However, the growing period of our SF was characterized

by high temperatures (average 22.5°C), which can cause an increase in CT content (Ushio and Adams, 2011). Azuhnwi et al. (2011) also showed that CT content in SF increases in the second compared with the first harvest. The climatic conditions had apparently less effect on the CT production in BT; the method used for CT analysis also has to be treated with caution. The most serious issue in the HCl-butanol method is the use of the standard, as every CT structure gives a different color intensity (Mueller-Harvey, 2006). We counteracted this by producing each standard from the plant itself by extracting with acetone and water (7:3, vol/vol) and purification on a Sephadex column (Sigma-Aldrich). The purification step separates CT, but does not remove other polyphenols and sugars, which will be present in the standard. We performed the purification step twice to reduce the amount of compounds other than CT, but still faced an overestimation of the CT content. Nevertheless, no other reliable method is available to differentiate soluble, protein-bound, and fiber-bound CT.

When we took a closer look at the CT in SF and BT we observed effects due not only to the large difference in CT content but to differences in the CT structure. We noted a difference in the proportions of CT bound to protein and fiber (37 and 62% for SF and BT, respectively), and in the proportion of soluble CT (63 and 38% for SF and BT, respectively). Additionally, the structure of CT (i.e., the proportions of prodelphinidins and procyanidins) is known to differ between SF and BT. The prodelphinidins occur most frequently in SF, whereas the procyanidins predominate in BT (Mueller-Harvey, 2006). Barry and McNabb (1999) stated that CT, up to a concentration of 90 g/kg of DM in plants, bind with the protein originating from the plant itself, but CT above this concentration will act as free CT and may interact with other nutrients in the feed mixture or with ruminal microbes. This might also explain the different effects of SF and BT, because the CT content of SF was higher than this threshold, whereas that of BT was below it.

The forage quality achieved with the 2 CT legumes can be considered equivalent to that of the non-CT legume, AL. This is obvious from the similar performance of the cows when fed a substantial proportion of these legumes in the form of small pellets. For that reason, the effects found in several traits can be associated with the specific properties of these plants, and especially their CT.

Effects on Ruminal Fiber Fermentation and Total-Tract Digestion

Supplementation of CT can decrease carbohydrate fermentation (Carulla et al., 2005) due to the formation of complexes between CT and cellulose, hemicellulose,

Table 5. Effect of the type of legume pellets on BUN content and various ruminal fluid variables, counted ciliate protozoa and bacterial species

	Treatment[1]				*P*-value	
Item	AL	SF	BT	SEM	Treatment	Time
BUN (mmol/L)	3.45^{a}	2.71^{b}	3.90^{a}	0.170	<0.001	0.04
Ammonia N (mmol/L)	3.80^{xy}	3.01^{y}	4.51^{x}	0.475	0.10	0.002
VFA (mmol/L)	122^{a}	107^{b}	119^{ab}	4.2	0.03	<0.001
VFA (molar percent)						
Acetate	64.4	64.4	62.6	0.81	0.22	<0.001
Propionate	20.4	20.0	21.4	0.77	0.44	<0.001
n-Butyrate	12.4	12.9	13.0	0.30	0.37	0.13
Isobutyrate	0.55	0.58	0.55	0.025	0.66	<0.001
n-Valerate	1.34^{ab}	1.18^{b}	1.55^{a}	0.099	0.04	0.06
Isovalerate	0.87	0.87	0.91	0.076	0.89	0.05
Ciliate protozoa counts (per mL)						
Total ($\times 10^5$)	1.32^{ab}	1.71^{a}	1.22^{b}	0.127	0.02	0.45
Entodiniomorphids ($\times 10^5$)	1.24^{ab}	1.65^{a}	1.14^{b}	0.122	0.01	0.35
Holotrichs ($\times 10^3$)	7.69	6.20	8.06	1.481	0.65	0.24
Bacterial species (% of total 16S rDNA)[2]						
Prevotella spp.	54.9^{a}	43.7^{b}	46.5^{ab}	2.66	0.02	0.72
Ruminococcus flavefaciens	1.73^{y}	1.34^{z}	1.45^{yz}	0.118	0.07	0.61
Ruminococcus albus	0.01	0.01	0.01	0.001	0.13	0.58
Fibrobacter succinogenes	2.40	2.87	2.18	0.267	0.20	0.58
Butyrivibrio fibrisolvens	0.07	0.06	0.06	0.006	0.60	0.59

[a,b]Means within a row with different superscripts differ ($P < 0.5$).

[x,y]Means within a row with different superscripts tend to differ ($P < 0.10$).

[1]AL = alfalfa; SF = sainfoin; BT = birdsfoot trefoil.

[2]Relative abundance of bacterial 16S rDNA.

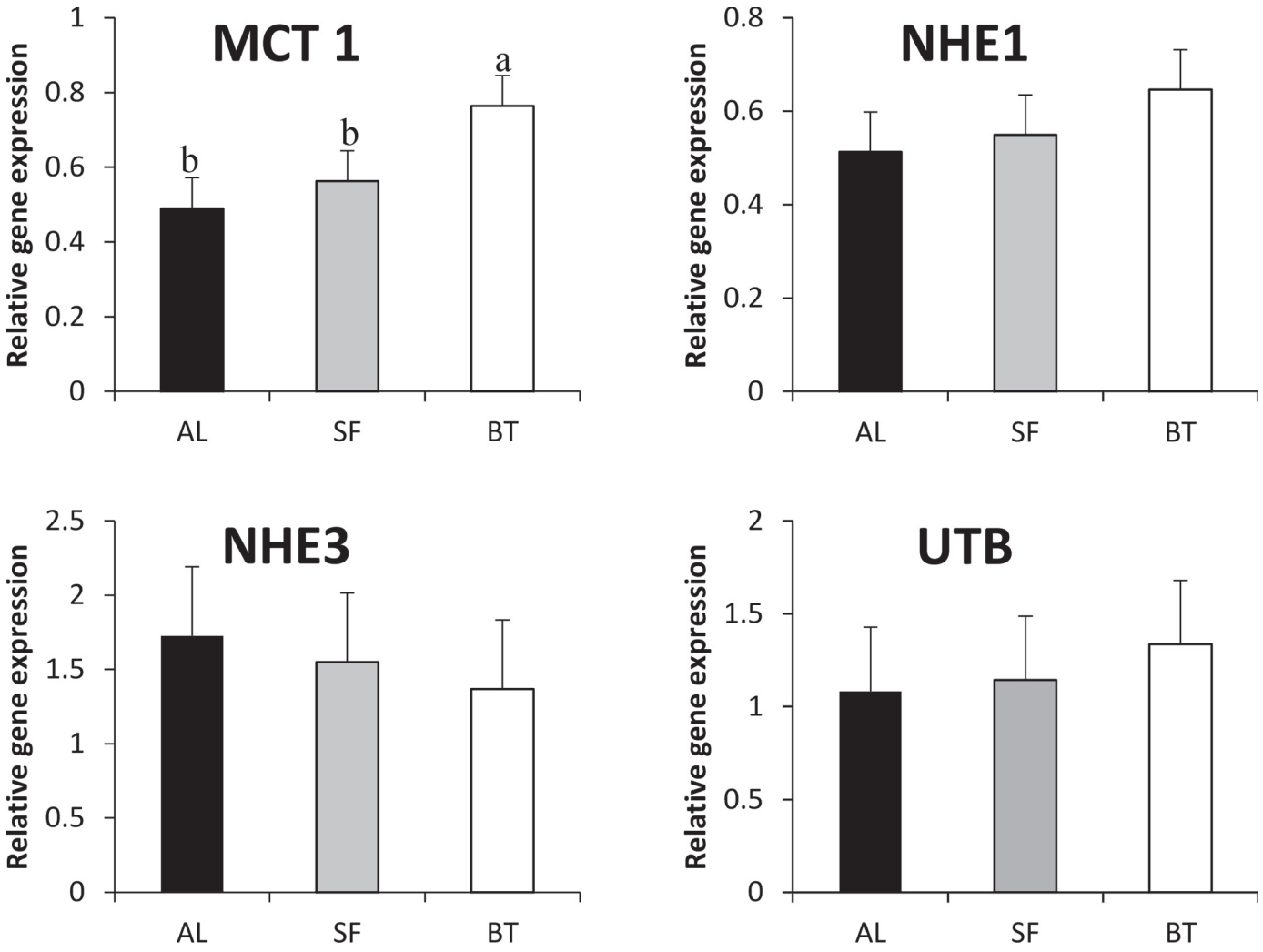

Figure 1. Effect of feeding alfalfa (AL), sainfoin (SF), or birdsfoot trefoil (BT) as pellets on the relative gene expression in the ruminal papillae of monocarboxylate co-transporter isoform (MCT) 1 (treatment: $P = 0.01$; time: $P = 0.04$; SEM = 0.08), sodium hydrogen exchanger isoform (NHE) 1 (treatment: $P = 0.29$; time: $P = 0.17$; SEM = 0.08), NHE3 (treatment: $P = 0.75$; time: $P = 0.22$; SEM = 0.47), and urea transporter B (UTB; treatment: $P = 0.75$; time: $P = 0.29$; SEM = 0.34). Least squares means with different letters (a,b) differ ($P < 0.05$).

and pectin (McSweeney et al., 2001). Accordingly, decreases in fiber digestibility are likely when feeding CT-containing legumes (Scharenberg et al., 2007a) or supplementing with CT extracts (Carulla et al., 2005). Concomitantly, the pattern of ruminal VFA often changes, reflecting a decrease in the proportion of acetate, when CT extracts are supplemented to the feed (Carulla et al., 2005; Beauchemin et al., 2007). The ruminal VFA pattern in the present study remained almost unchanged, whereas the content of total ruminal VFA decreased, after SF feeding. However, total-tract fiber digestibility showed no negative effects, which suggested a shift of fiber fermentation to the hindgut. The exact mechanism by which CT decrease ruminal degradation of carbohydrates remains unclear. On the one hand, CT form complexes with feed particles, thereby preventing microbial attachment (Scalbert, 1991; McSweeney et al., 2001); on the other hand, CT bind to the cell walls of the microbes and inhibit cell-associated enzyme activity (Jones et al., 1994). Tannins are also known to bind to metal ions, which could limit mineral ion availability to the microbes (Scalbert, 1991). Nevertheless, some microbes seem to have greater tolerance to CT than others. We observed a decrease in the prevalence of the carbohydrate-degrading bacteria, *Prevotella* spp. and *Ruminococcus flavefaciens*, in SF-fed cows compared with AL-fed cows, whereas the other bacterial strains were not affected. Scalbert (1991) pointed out that some microorganisms may secrete polymers with high affinity to tannins as a protection mechanism, and Jones et al. (1994) stated that the cellulolytic (and the proteolytic) activity of microbes can continue when CT

Table 6. Effect of the type of legume pellets on the N balance

Item	Treatment[1]			SEM	*P*-value
	AL	SF	BT		
N balance (g/d)					
Intake	479	459	493	28.7	0.21
Fecal N	199	207	192	28.8	0.28
Urinary N	98.3^{a}	79.4^{b}	93.5^{ab}	8.04	0.04
Urinary urea N	66.1^{a}	41.2^{b}	74.7^{a}	8.08	0.01
Fecal N and urinary N	297	286	285	26.1	0.29
Milk N	173	173	179	15.9	0.69
Body N retention	9^{yz}	1^{z}	29^{y}	0.4	0.06
N balance (% of N intake)					
Fecal N	41.3^{yz}	45.2^{y}	38.5^{z}	2.86	0.09
Urinary N	20.8^{a}	17.5^{b}	19.5^{ab}	1.64	0.04
Fecal N and urinary N	62.1	62.7	57.9	2.53	0.15
Milk N	35.8	37.4	36.1	1.35	0.39
Urinary urea N (% of total urinary N)	66.3^{ab}	51.8^{b}	79.0^{a}	4.25	0.005
Urinary N (% of fecal N and urinary N)	33.6^{y}	28.5^{z}	33.5^{y}	2.80	0.08

^{a,b}Means within a row with different superscripts differ ($P < 0.5$).

^{y,z}Means within a row with different superscripts tend to differ ($P < 0.10$).

[1]AL = alfalfa; SF = sainfoin; BT = birdsfoot trefoil.

levels are low. This indicates that the CT content of BT might be too low to obtain the same effects seen with SF.

Effects on Indicators of Nutrient Absorption Through the Ruminal Wall

The absorption of nutrients from the rumen via the blood vessels and into the cells is assisted by nutrient transporters present in the ruminal papillae. We therefore examined the gene expression of the *MCT1* transporter, which regulates the diffusion of monocarboxylates (e.g., acetate, lactate, and butyrate), as well as the ketone bodies, together with protons, in the ruminal epithelium (Kirat et al., 2006). A higher content of VFA in the ruminal fluid (which would decrease the rumen pH) was reported to upregulate the expression the *MCT1* gene in the epithelium (Yan et al., 2014). Consistent with this, we observed that the relative abundance of the *MCT1* gene was greatest in BT cows, where VFA content was numerically the highest. Furthermore, the average pH and the minimum pH were numerically the lowest in the BT cows, and the time when the pH was below 5.8 was numerically the longest in the BT cows (data not shown). Whether this effect is directly related to the CT, or whether it reflects other effects, such as location and enumeration of the transporter in the different epithelium layers (Kirat et al., 2006), is not clear. None of the other transporters investigated showed changes in gene expression, showing that neither CT plant had any adverse effect on the function of the ruminal wall transporter system.

Effects on Milk Fatty Acid Composition

We observed an increase in the proportion of 18:3n-3 in milk fat by SF, which resulted in a numerically higher transfer rate. Accordingly, SF appears to have protected a part of the 18:3n-3 from ruminal BH. By contrast, an unknown mechanism appeared to promote chain elongation and desaturation of n-3 PUFA, because the proportions of 22:5n-3 and 22:6n-3 showed a trend toward an increase in the milk of the BT cows. Conversely, the numerically greater 18:2n-6 and lower 20:4n-6 levels in milk fat of the SF and BT groups suggest that elongation and desaturation rate of n-6 PUFA is decelerated in the SF and BT cows, compared with the AL cows.

If ruminal BH cannot be avoided, its deceleration or interruption at certain steps may still be advantageous, because some intermediates can also have benefits. As previously shown, CT may lead to an accumulation of *trans*-11 18:1 (Khiaosa-Ard et al., 2009) and decrease the formation of the terminal product of BH, 18:0 (Vasta et al., 2009). We observed no effect on the conversion of 18:3n-3 and 18:2n-6 into *trans*-11 18:1 and 18:0 in the present study. The same result was reported by Khiaosa-Ard et al. (2009) when SF as a whole plant was incubated, whereas this differed with CT extracts from acacia and quebracho (Khiaosa-Ard et al., 2009; Vasta et al., 2009). Nevertheless, the proportion of the 18:1n-9 in milk fat, which can be produced in the mammary gland from 18:0, was highest in the SF cows in the present study.

The BH of PUFA to SFA is mainly done by microbes of the *Butyrivibrio* genus (Vasta et al., 2010), with *Bu-*

tyrivibrio fibrisolvens being responsible for converting PUFA into *trans*-11 18:1 (Vasta et al., 2010). However, the abundance of *B. fibrisolvens* was not affected in the present study, which is in line with the lack of changes observed in the BH intermediates in the milk fat. Protozoa, or bacteria associated with them, are also postulated to play a role in BH (Khiaosa-Ard et al., 2009). The numbers of ciliate protozoa were greater in the SF and in the BT cows in the present study, which contradicts the findings of Vasta et al. (2010), who reported no effects on ciliate protozoa populations when feeding quebracho tannin extracts. These opposing results reflect the present lack of knowledge needed to explain how CT affect ciliate protozoa. The changes in the ciliate protozoa population caused by the CT plants in the present study were obviously too small to generate differences in ruminal BH.

Effects on N Turnover

The use of CT is one way of efficiently reducing ruminal protein degradation by binding up the protein (e.g., Hagerman and Butler, 1981) under ruminal pH conditions; this can therefore counteract an excess of RDP as the microbes are not able to degrade CT (McSweeney et al., 2001) or CT-protein complexes. In the present study, we observed that SF, but not BT, fulfilled this purpose, as evident from the changes determined in ruminal ammonia, BUN, and MUN. Lower ruminal ammonia and BUN levels have been repeatedly observed when feeding SF (e.g., Scharenberg et al., 2007a; Arrigo and Dohme, 2009; Azuhnwi et al., 2013). The declining content in ruminal ammonia in SF cows was also associated with a lower proportion of valerate, a VFA originating from protein degradation. This is in line with a decrease in the relative abundance of the protein-degrading bacteria, *Prevotella* spp., in the SF cows. By contrast, a simultaneous decrease in urea in BUN, MUN, and urine of dairy cows fed BT was only shown by Hymes-Fecht et al. (2013). The lack of effect from BT in the present study must have resulted from the low CT content of the BT used.

Complexes with CT are released below pH 4 (Mangan, 1988), as is the case in the abomasum, so that the protein protected from ruminal degradation can ideally be digested in the intestine (Waghorn, 2008), thereby contributing to the metabolic protein supply. However, in reality, with CT this phenomenon rarely results in a substantially better N utilization, likely due to an incomplete separation of the CT-protein complexes (Beauchemin et al., 2007). We also observed a decrease in the apparent total-tract N digestibility in the present study, and feeding the high-CT plant (SF) did not elevate N retention in the milk or the body. The BT cows showed a slight increase in body N retention at the same N excretion and a slightly higher N intake. This suggests that these animals might have received additional protein for maintenance or gaining BW, but this appears not to be a CT content-related effect considering the huge difference in CT levels between the 2 legumes.

For that reason, a full compensation occurs in the lowered N excretion in the urine, in the form of fecal N, as has been observed repeatedly when feeding SF to sheep (e.g., Scharenberg et al., 2007a; Azuhnwi et al., 2013). This is of great relevance for an environmentally friendly animal husbandry, as less gaseous (nitrous oxide and ammonia emissions) and liquid (nitrate leaching) N is emitted (Dijkstra et al., 2013). In addition, Dijkstra et al. (2013) showed that decreasing the urea N proportion of total urinary N, as in the present study with SF, has a great potential for reducing the N emission potential because other compounds present in urine are less prone to generate emissions.

CONCLUSIONS

Replacing AL with SF at 20% of the basal diet resulted in some effects on protein turnover, with changes also taking place in the ruminal microbial community. This reduced the N emission potential of the manure. Although the effect on the milk fatty acid profile was minor, the proportion of 18:3n-3 was slightly increased with SF, possibly due to a numerically higher transfer from the feed to the milk. By contrast, the effects of the same amount of BT were limited. Feeding BT seemed to enhance the amount of protein arriving in the intestine, and thereby protein utilization, especially for body protein retention. The structure and activity of the CT present in the 2 tanniferous forage legumes differed, but the different effects were likely due to the large differences in the CT contents. The SF cultivar and growing conditions favored a very high CT content (a proportion in the total diet of 3.6%), so that CT effects could be expected. With BT, the resulting 0.5% CT in the diet was probably too low to have much effect. Further studies should concentrate on setting maximal dietary proportions of these legumes and dietary CT content, to maximize the effects on desired traits without adversely affecting performance. Comparisons of the 2 tanniferous forage legumes in terms of effects of their CT structures will require future experiments using diets with similar CT content.

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APPENDIX

Table A1. Primers used for the quantitative PCR

Item	Forward primer (5′-3′)	Reverse primer (3′-5′)	Product size (bp)	Amplification efficiency
Total bacteria	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	145	0.98
Ruminococcus albus	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA	176	0.98
Ruminococcus flavefaciens	TGTCCCAGTTCAGATTGCAG	GGCGTCCTCATTGCTGTTAG	171	0.92
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121	0.95
Butyrivibrio fibrisolvens	ACCGCATAAGCGCACGGA	CGGGTCCATCTTGTACCGATAAAT	65	0.95
Prevotella genus	CCAGCCAAGTAGCGTGCA	TGGACCTTCCGTATTACCGC	152	0.92
Streptococcus bovis group	GACTTTAGCTTGCTAAAGTTGG	GTGAAGCAATTGCTCCTTTC	154	NA[1]
GAPDH	GGG TCA TCA TCT CTG CAC CT	GGT CAT AAG TCC CTC CAC GA	176	0.83
NHE1[2]	TCC CAC ACG ACC ATC AAG TA	CCC GTA GGC AAT GAT GAA CT	243	1.17
NHE3[3]	GTC ATC CTG CAG ACC TGG AT	ATG ATG GTG GTG CTG ACA AA	173	0.94
MCT1[4]	TTA ATG CCA CCA CCA GTG AA	GAA GGA AGC AGC AAT CAA GC	164	0.98
UTB[5]	CCA GGA CAG GTC CGC CAT TA	GGC ACT CGA GAA GAC TGG ACA	157	0.96

[1]No meaningful efficiency calculation possible due to low abundance of *S. bovis* species. For quantification, efficiency was set to one.

[2]*NHE1* = sodium hydrogen exchanger isoform 1.

[3]*NHE3* = sodium hydrogen exchanger isoform 3.

[4]*MCT1* = monocarboxylate transporter 1.

[5]*UTB* = urea transporter B.