Methane production, nutrient digestion, ruminal fermentation, N balance, and milk production of cows fed timothy silage- or alfalfa silage-based diets

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ABSTRACT

The objective of this study was to investigate the effects of changing forage source in dairy cow diets from timothy silage (TS) to alfalfa silage (AS) on enteric CH₄ emissions, ruminal fermentation characteristics, digestion, milk production, and N balance. Nine ruminally cannulated lactating cows were used in a replicated 3 × 3 Latin square design (32-d period) and fed (ad libitum) a total mixed ration (TMR; forage:concentrate ratio of 60:40, dry matter basis), with the forage portion consisting of either TS (0% AS; 0% AS and 54.4% TS in the TMR), a 50:50 mixture of both silages (50% AS; 27.2% AS and 27.2% TS in the TMR), or AS (100% AS; 54.4% AS and 0% TS in the TMR). Compared with TS, AS contained less (36.9 vs. 52.1%) neutral detergent fiber but more (20.5 vs. 13.6%) crude protein (CP). In sacco 24-h ruminal degradability of organic matter (OM) was higher for AS than for TS (73.5 vs. 66.9%). Replacement of TS with AS in the diet entailed increasing proportions of corn grain and bypass protein supplement at the expense of soybean meal. As the dietary proportion of AS increased, CP and starch concentrations increased, whereas fiber content declined in the TMR. Dry matter intake increased linearly with increasing AS proportions in the diet. Apparent total-tract digestibility of OM and gross energy remained unaffected, whereas CP digestibility increased linearly and that of fiber decreased linearly with increasing inclusion of AS in the diet. The acetate-to-propionate ratio was not affected, whereas ruminal concentration of ammonia (NH₃) and molar proportion of branched-chain VFA increased as the proportion of AS in the diet increased. Daily CH₄ emissions tended to increase (476, 483, and 491 g/d for cows fed 0% AS, 50% AS, and 100% AS, respectively) linearly as cows were fed increasing proportions of AS. Methane production adjusted for dry matter intake (average = 19.8

g/kg) or gross energy intake (average = 5.83%) was not affected by increasing AS inclusion in the diet. When expressed on a fat-corrected milk or energy-corrected milk yield basis, CH₄ production increased linearly with increasing AS dietary proportion. Urinary N excretion (g/d) increased linearly when cows were fed increasing amounts of AS in the diet, suggesting a potential for higher nitrous oxide (N₂O) and NH₃ emissions. Efficiency of dietary N use for milk protein secretion (g of milk N/g of N intake) declined with the inclusion of AS in the diet. Despite marked differences in chemical composition and ruminal degradability, under the conditions of this study, replacing TS with AS in dairy cow diets was not effective in reducing CH₄ energy losses.

Key words: enteric methane, N excretion, alfalfa silage, timothy silage

INTRODUCTION

Methane from enteric fermentation is a major contributor to greenhouse gas emissions from the agricultural sector. It is also a loss in productive energy for dairy cows. Thus, mitigating enteric CH₄ emission would not only contribute to reducing the environmental impact of dairy production, but may also improve efficiency of energy use by the animal, thereby enhancing animal performance and productivity.

The effects of several nutritional practices on enteric CH₄ emissions have been investigated. Dietary mitigation strategies, such as feeding more grain or feed additives, have been extensively examined (Beauchemin et al. 2009; Martin et al., 2010). However, limited information exists on the effects of forage type on enteric CH₄ emissions in dairy cows. Changing forage source in the diet of ruminants from grasses to legumes has been previously suggested as a possible strategy to reduce CH₄ production from ruminants (Benchaar et al., 2001; Beauchemin et al., 2009; Dewhurst, 2013). Legumes are generally characterized by low structural carbohydrates content, rapid physical breakdown, and high rumen fermentation rate compared with grasses (Janssen 2010; Dewhurst, 2013). Thus, feeding legumes

Received February 19, 2014.

Accepted June 14, 2014.

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as dietary forage source may increase DMI and passage rate compared with feeding grasses, which is expected to reduce methanogenic activities in the rumen (Martin et al., 2010; Dewhurst, 2013). Moreover, the higher proportion of NSC in legumes compared with grasses may drive ruminal fermentation toward higher propionate proportion and thus reduce hydrogen availability for methanogenesis (Johnson and Johnson, 1995; Janssen 2010). Also, with the expected increase in DMI when legumes compared with grasses are fed, animal production may increase and, consequently, CH₄ emissions per unit of animal product decline (Martin et al., 2010). Studies comparing dietary effects of grass versus legume silage in the TMR on enteric CH₄ emissions and N balance in dairy cows are scarce. Thus, the objective of this study was to determine the effects of replacing timothy silage (TS) with alfalfa silage (AS) in dairy cow diets on CH₄ production, digestion, ruminal fermentation parameters, N balance, milk production, and milk composition.

MATERIALS AND METHODS

This study was conducted at the Dairy and Swine Research and Development Centre (Sherbrooke, QC, Canada). Animal procedures were conducted under the approval of the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre and were in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Cows, Experimental Design, and Diets

Nine multiparous lactating Holstein cows fitted with rumen cannulas (10 cm; Bar Diamond Inc., Parma, ID) were used in a replicated 3 × 3 Latin square design (32-d period). The cows averaged (mean ± SD) 92 ± 19 DIM at the start of the experiment with an average BW of 696 ± 71 kg and milk yield of 46 ± 4.3 kg/d. The dietary treatments were (1) 0% AS and 54.4% TS in the TMR (0% AS), (2) 27.2% AS and 27.2% TS in the TMR (50% AS), and (3) 54.4% AS and 0% TS in the TMR (100% AS). The chemical composition, ensiling characteristics, and ruminal degradability of the 2 silages are presented in Table 1. In sacco 24-h ruminal degradability of the 2 silages was determined as described in Benchaar et al. (2013). The forage:concentrate ratio of the TMR was 60:40, on a DM basis. Increasing the proportion of AS in the diet at the expense of TS was accompanied by a decrease in the proportions of soybean meal and inert fat, and an increase in the proportions of corn grain and bypass protein supplement (Table 2). Each diet was provided for ad libitum intake (5% orts, on an as-fed basis) and

cows were housed in individual tie-stalls and had free access to water during the experiment.

After 2 wk of adaptation to experimental diets, feed intake, rumen fermentation characteristics (including protozoa enumeration), apparent total-tract digestibility, N balance, milk performance (production and composition), and CH₄ production were measured. Only 2 air-flow-controlled chambers were available for measuring CH₄ emissions in this study, which limited the number of animals that could be examined at the same time to 2 (i.e., 1 cow per chamber). Consequently, CH₄ determination and all measurements had to be staggered by 4 d within the 18 d of sampling and data collection period to facilitate measurements. Cows were weighed at the beginning and the end of each experimental period on 2 consecutive days before the a.m. feeding and after the a.m. milking.

Intake, Apparent Total-Tract Digestibility, and N Balance

Apparent total-tract digestibility and N outputs were measured over 6 consecutive days as described in Benchaar et al. (2013). Diets were offered in equal amounts twice daily (0900 and 1930 h). Feed consumption was recorded daily by weighing feeds offered to and refused by the cows. Samples of the TMR, feed ingredients, and orts were collected daily and stored at -20°C. Samples were composited by cow within period, freeze-dried, ground to pass a 1-mm screen using a Wiley mill (standard model 4; Arthur M. Thomas Co., Philadelphia, PA) and analyzed for DM, OM, total N, NDF, ADF, starch, ether extract (EE), and gross energy (GE). Samples of TS and AS were also analyzed for lactic acid, VFA, ethanol, NH₃-N, and water-soluble carbohydrates.

Total collection of feces and urine was performed as described by Benchaar et al. (2013), by fitting cows with harnesses and tubes, allowing the collection of feces and urine separately. Feces were analyzed for DM, OM, total N, NDF, ADF, starch, EE, and GE concentrations. Urine was analyzed for total N content. Productive N was calculated by subtracting N excreted in feces and urine from N intake.

Ruminal Fermentation Characteristics and Protozoa Enumeration

Ruminal fluid was collected from cows on 2 successive days before (0 h) and at 1, 2, 4, 6 and 8 h after the a.m. feeding. A total of 250 mL was collected from several locations (anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral) within the rumen using a 50-mL syringe screwed to a stainless

Table 1. Chemical composition of timothy silage and alfalfa silage¹

Item (% of DM, unless otherwise noted)	Experimental silage	
	Timothy	Alfalfa
Chemical composition ²		
DM (%)	35.0 ± 2.93	34.7 ± 2.12
OM	92.1 ± 0.35	90.1 ± 0.10
CP	13.6 ± 0.58	20.5 ± 0.45
NDF	52.1 ± 0.94	36.9 ± 1.29
ADF	35.2 ± 1.39	33.2 ± 0.21
Starch	0.35 ± 0.066	2.67 ± 0.224
Ether extract (% of DM)	3.88 ± 0.164	4.16 ± 0.273
Gross energy (Mcal/kg of DM)	4.56 ± 0.042	4.55 ± 0.042
NFC ³	22.5 ± 0.52	28.5 ± 1.07
Ruminal degradability (%)		
OM	66.9 ± 0.69 ^b	73.5 ± 0.69 ^a
CP	83.5 ± 0.93 ^b	89.1 ± 0.93 ^a
NDF	53.6 ± 1.07 ^a	50.4 ± 1.07 ^b
Ensiling characteristics ²		
pH	4.09 ± 0.121	4.28 ± 0.022
Lactic acid	7.00 ± 0.481	6.85 ± 0.905
Acetic acid	1.15 ± 0.676	1.79 ± 0.233
Propionic acid	1.03 ± 0.874	ND ⁴
Butyric acid	0.03 ± 0.052	ND
Ammonia-N (% of total N)	5.27 ± 1.435	6.95 ± 0.290
Ethanol	0.11 ± 0.122	0.16 ± 0.049
Water-soluble carbohydrates	6.90 ± 0.135	2.58 ± 0.420

^{a,b}Within a row, LSM (±SEM) with different superscript letters differ ($P < 0.05$).

¹Data were analyzed as a randomized complete block (cows) design.

²Mean ± SD of analysis performed on 3 period samples.

³NFC = OM – (CP + NDF + ether extract).

⁴ND = Not detected.

tube ending with a probe covered by a fine metal mesh (RT Rumen Fluid Collection Tube; Bar Diamond Inc.). Ruminal fluid was acidified to pH 2 with 50% sulfuric acid and subsamples (15 mL) were frozen at -20°C for later determination of VFA and NH₃ concentrations.

Ruminal pH was measured continuously for 48 h using a Kahne Bolus system (KB1000; Kahne Ltd., Auckland, New Zealand) as described in Hassanat et al. (2013). The pH data were summarized as mean pH, minimum pH, maximum pH, time spent below pH 6.0, time spent below pH 5.6, time spent below pH 5.2, area (time × pH) when $5.6 < \text{pH} < 6.0$, area when $5.2 < \text{pH} < 5.6$, and area when $\text{pH} < 5.2$ over 24 h (Plaizier et al., 2001; Keunen et al., 2002; Khafipour et al., 2009).

Protozoa were counted from ruminal content collected before (0 h) and 4 h after the a.m. feeding. Ruminal content (approximately 1 L) was strained through 4 layers of cheesecloth and a 5-mL portion of the strained ruminal fluid was preserved using 5 mL of methyl green formalin-saline solution for protozoa enumeration (Ogimoto and Imai, 1981). Protozoa samples were stored at room temperature in darkness until counting. Protozoa were microscopically enumerated using a counting chamber (Neubauer Improved Bright-Line counting cell, 0.1-mm depth; Hausser Scientific, Horsham, PA)

and genera were identified as outlined by Dehority (1993).

Milk Production and Milk Composition

Cows were milked twice daily at 0700 and 1900 h in their stalls and milk production was recorded at each milking. During digestibility measurements, milk samples were collected from each cow at each milking, stored at $+4^{\circ}\text{C}$ with a preservative (2-bromo-2-nitropropan-1,3-diol), and then sent to a commercial laboratory (Valacta Dairy Production Center of Expertise Quebec-Atlantic, Sainte-Anne-de-Bellevue, QC, Canada) for analyses of fat, protein, lactose, MUN, and SCC.

Enteric CH₄ Production

Enteric CH₄ emissions were determined as described in Benchaar et al. (2013) using 2 air-flow-controlled chambers for measurement of CH₄ production. Briefly, the chambers measured 4.09 m length × 2.95 m width × 2.84 m height. When the chamber doors were closed, air entered the chamber through a ventilation duct and

Table 2. Ingredient and chemical composition of the experimental diets

Item	Treatment ¹		
	0% AS	50% AS	100% AS
Ingredient (% of DM)			
Alfalfa silage	—	27.2	54.4
Timothy silage	54.4	27.2	—
Corn grain, ground	24.8	28.5	32.1
Soybean meal	11.6	5.8	—
Timothy hay	5.4	5.4	5.4
Rumen inert fat ²	1.9	1.5	1.3
Bypass protein supplement ³	—	2.4	4.9
Mineral and vitamin supplement ⁴	1.3	1.3	1.3
Dicalcium phosphate	0.6	0.6	0.6
Chemical composition (% of DM, unless otherwise noted)			
OM	92.7	92.5	92.2
CP	15.8	16.5	17.2
NDF	37.4	33.5	29.5
ADF	24.3	23.6	22.2
Starch	13.5	15.6	18.6
Ether extract	4.63	4.86	4.97
NFC ⁵	34.9	37.6	40.5
Starch:CP	0.86	0.96	1.10
NFC:CP	2.23	2.28	2.36
Gross energy (Mcal/kg of DM)	4.51	4.50	4.49

¹AS = alfalfa silage.²Energy Booster 100 (Milk Specialties Global Animal Nutrition, Carpentersville, IL).³AminoPlus (Ag Processing Inc., Hastings, NE).⁴Contained 12.48% Ca, 6.80% P, 6.81% S, 7.72% Na, 1.97% K, 96 mg of I/kg, 2,877 mg of Fe/kg, 620 mg of Cu/kg, 2520 mg of Mn/kg, 3,777 mg of Zn/kg, 83 mg of Co/kg, 628,000 IU of vitamin A/kg, 81,000 IU of vitamin D/kg, 3,739 IU of vitamin E/kg, and 27.8 mg of Se/kg.⁵NFC = OM – (CP + NDF + ether extract).

exited through an exhaust. Air temperature within the chamber was maintained at $18 \pm 1^\circ\text{C}$. Air flow into and out of the chamber was measured using Inline Mass Flowmeters (FT2; Fox Thermal Instruments Inc., Marina, CA) and maintained at $180 \pm 10 \text{ m}^3/\text{h}$. Methane concentration was continuously measured at the air entrance and exhaust ducts using CH_4 analyzers (MGA3000; ADC Gas Analysis Ltd., Hoddesdon, UK). The amount of CH_4 (entering and leaving the chamber) was calculated by multiplying the concentration of CH_4 by the airflow (at the entrance and at the exhaust). The difference between the incoming and outgoing mass of CH_4 corresponded to the amount of enteric CH_4 emitted in each chamber by the animal. Methane was recorded every minute over a period of 3 consecutive days and fluxes were averaged to derive 24-h CH_4 emissions. Cows were preconditioned to the environmental chambers before the beginning of the experiment. Cows entered the chambers 18 h before starting CH_4 measurements. Cows in the chambers had free access to water and were fed twice daily for ad libitum intake (5% orts on an as-fed basis). Offered feed and orts were weighed daily to determine feed consumption. Samples of feed offered and orts were collected, pooled, and kept frozen for later determination of DM and GE concentrations.

Chemical Analyses

Dry matter content was determined by drying samples in a vacuum oven at 100°C overnight (AOAC, 1990; method 934.01). Silage DM content was adjusted for volatile compounds lost in drying as in Haigh (1995). Ash content was determined by incineration at 550°C overnight in a muffle furnace (AOAC, 1990; method 942.05) and the OM content was calculated as the difference between 100 and the percentage of ash. Crude protein ($\text{N} \times 6.25$) was determined using the macro-Kjeldahl procedure (AOAC, 1990; method 990.03). The concentration of NDF was determined as described by Van Soest et al. (1991) without the use of sodium sulfite and with the inclusion of heat-stable α -amylase. The ADF content was determined according to the method of the Association of Official Analytical Chemists (AOAC, 1990; method 973.18). The NDF and ADF procedures were adapted for use in an Ankom200 Fiber Analyzer (Ankom Technology Corp., Fairport, NY). The concentration of starch was determined colorimetrically according to the procedure of Hall et al. (2001). Ether extract content was determined using the Soxtec 2047 SoxCap hydrolysis unit in combination with Soxtec extraction systems (Foss, Eden Prairie, MN) according to the method of the

Association of Official Analytical Chemists (AOAC, 1990; method 920.39). Gross energy was determined using an oxygen bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL). The concentration of N in acidified urine samples was determined by micro-Kjeldahl analysis (AOAC, 1990). Water-soluble silage extracts were analyzed for water-soluble carbohydrates (DuBois et al., 1956), VFA, lactic acid (Taylor, 1996) and ethanol [gas-liquid chromatograph (GLC) model 6850; Agilent Technologies Inc., Mississauga, ON, Canada]. Analysis of VFA was performed using a gas chromatograph equipped with a flame ionization detector and autoinjector (7683 series; Agilent Technologies Inc.) and fitted with a DB-FFAP column (30 m × 0.250 mm, 0.25-μm film thickness; Agilent Technologies Inc.) as described in Benchaar et al. (2014). Ammonia N concentration was determined as in Weatherburn (1967). Protein, fat, lactose, urea N, and SCC in milk samples were analyzed by infrared spectroscopy (MilkoScan FT 6000; Foss Electric A/S, Hillerød, Denmark). Milk composition was corrected for differences in milk yield between a.m. and p.m. milkings.

Statistical Analyses

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The statistical model included treatment and period as fixed effects and square and cow within square as random effects. Ruminal fermentation characteristics (pH, VFA, NH₃, and protozoa) data were analyzed as repeated measures using the same model with the addition of the fixed effects of day, sampling time (i.e., hour) and all interactions. The CH₄ data were also analyzed as repeated measures with the inclusion of the fixed effects of day and day × treatment interaction. The fixed effect of the chamber was initially included in the model but was removed because it was not significant. The first-order autoregressive was used as the covariance structure and cow within treatment × period as subject for the repeated measures. Orthogonal polynomial contrasts (linear and quadratic) were used to examine treatment effect on response variables. Significant effect of treatment on least squares means was declared at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$.

RESULTS

Silage and Diet Composition

Chemical composition and ensiling characteristics of TS and AS are presented in Table 1. Both silages were harvested in early-development stages; first-cut timothy silage was harvested at the vegetative-early heading

stage, whereas second-cut alfalfa silage was harvested at the budding stage. Alfalfa silage contained 7 percentage units more CP, 6 percentage units more NFC, but 15 percentage units less NDF than TS. The low pH value, the high lactic acid content and low NH₃-N and butyric acid concentrations are an indication of good preservation of the 2 silages. Organic matter and CP 24-h ruminal degradability of AS was higher than that of TS (73.5 vs. 66.9% and 89.1 vs. 83.5% for OM and CP, respectively), whereas NDF degradability was lower for AS than for TS (50.4 vs. 53.6%, respectively).

Increasing the proportion of AS in the diet (to replace TS) involved increasing corn grain and bypass protein supplement contribution to the diet and decreasing soybean meal and inert fat proportions. Still, the chemical composition of the diets reflected the differences in chemical composition between the 2 silages. Indeed, the concentrations of CP and NFC increased (up to 1.4 and 5.6 percentage units, respectively), whereas NDF concentration decreased (by up to 7.9 percentage units) as the proportion of AS increased in the diet (Table 2). Gross energy concentration was similar between diets and averaged 4.50 Mcal/kg of DM.

Nutrient Intake and Digestibility

The effect of experimental treatments on nutrient intakes is presented in Table 3. Increasing the proportion of AS in the diet caused a linear increase ($P \leq 0.01$) in DMI (kg/d or % of BW). Consequently, OM and GE intakes increased linearly ($P = 0.02$) as the dietary proportion of AS increased. Intake of CP, starch, and EE also increased linearly ($P < 0.01$) with increasing inclusion level of AS in the diet. A linear decline ($P < 0.01$) in NDF intake occurred when the AS dietary proportion increased, whereas ADF intake averaged 5.4 kg/d and was not affected by experimental treatments.

Apparent total-tract digestibility of DM, OM, and GE was not affected by increasing AS inclusion in the diet (Table 3), averaging 70.4, 71.8, and 69.2%, respectively. Increasing the AS proportion in the diet caused a linear decline ($P < 0.01$) in NDF and ADF digestibility (up to 8 percentage units for the 100% AS diet). Crude protein and starch digestibility increased slightly but significantly ($P \leq 0.03$) as the proportion of AS in the diet increased.

Ruminal Fermentation Characteristics and Protozoa

Interactions between sampling day or sampling time and treatment were not significant for any of the rumen fermentation parameters. Therefore, only averages of the 2-d measurements and the different sampling times measurement were reported. Continuous monitoring of

Table 3. Intake and apparent total-tract digestibility of nutrients lactating cows fed increasing proportions of alfalfa silage (AS) in the diet

Item	Treatment ¹			SEM	P-value	
	0% AS	50% AS	100% AS		Linear	Quadratic
Intake (kg/d, unless otherwise noted)						
DM	22.5	23.7	24.6	0.99	<0.01	0.81
DM (% of BW)	3.27	3.46	3.54	0.13	0.01	0.48
OM	20.9	21.8	22.5	0.89	0.02	0.84
CP	3.58	3.91	4.20	0.162	<0.01	0.82
NDF	8.36	7.83	7.17	0.349	<0.01	0.84
ADF	5.43	5.51	5.38	0.233	0.85	0.59
Starch	3.08	3.75	4.59	0.137	<0.01	0.44
Ether extract	1.05	1.15	1.22	0.068	<0.01	0.57
Gross energy (Mcal/d)	102	106	110	4.62	0.02	0.83
Digestibility (%)						
DM	70.3	70.7	70.3	0.40	0.94	0.35
OM	71.6	72.1	71.8	0.42	0.64	0.44
CP	66.3	68.0	68.3	0.60	0.03	0.36
NDF	62.0	58.7	53.4	1.15	<0.01	0.50
ADF	62.7	59.1	54.7	1.24	<0.01	0.81
Starch	95.9	97.0	97.3	0.58	<0.01	0.28
Ether extract	51.1	53.1	52.9	1.42	0.37	0.52
Gross energy	68.8	69.5	69.2	0.45	0.48	0.29

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

ruminal pH revealed no effects of treatments on ruminal pH parameters, with a daily mean ruminal pH of 6.36 and a minimum ruminal pH averaging 5.82 (Table 4).

A quadratic response ($P = 0.01$) of total VFA concentration to increasing dietary AS proportion was observed (Table 5), with the highest concentration (129 mM) observed for cows fed the 50% AS diet compared with cows fed the 0% or the 100% AS diets (average of 119 mM). Acetate and propionate molar proportions averaged 67.2 and 19.3%, respectively, and were not affected by treatment. Thus, the acetate:propionate ratio averaged 3.57 and was not changed by experimental treatments. Molar proportions of butyrate, valerate, and branched-chain VFA and NH_3 concentrations in-

creased linearly ($P \leq 0.03$) with increasing AS inclusion in the diet. The population of total protozoa and entodiniomorphs increased linearly ($P \leq 0.03$) with increasing proportion of AS in the diet, whereas the holotrich population declined linearly ($P < 0.01$) as the level of AS increased in the diet.

Milk Production and Milk Composition

With the exception of milk protein content and MUN concentration, which increased linearly ($P \leq 0.02$) as the proportion of AS increased in the diet, no changes were observed for other milk performance variables (Table 6). Feed efficiency, expressed as kilograms of

Table 4. Ruminal pH parameters of lactating dairy cows fed increasing proportions of alfalfa silage (AS) in the diet

Item	Treatment ¹			SEM	P-value	
	0% AS	50% AS	100% AS		Linear	Quadratic
pH						
Mean	6.39	6.33	6.37	0.039	0.70	0.30
Minimum	5.80	5.82	5.83	0.078	0.76	0.95
Maximum	6.86	6.79	6.84	0.054	0.72	0.36
Time pH < 6.0 (min)	97	117	87	46.1	0.86	0.61
Time pH < 5.6 (min)	21	19	7	8.1	0.18	0.59
Time pH < 5.2 (min)	0	0	0	0	—	—
Area 5.6 < pH < 6.0 (pH × min/d)	8.72	10.74	7.84	4.259	0.87	0.59
Area 5.2 < pH < 5.6 (pH × min/d)	1.82	1.62	0.64	0.696	0.18	0.59
Area pH < 5.2 (pH × min/d)	0	0	0	0	—	—

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

Table 5. Total VFA concentration, VFA molar proportions, NH₃ concentration, and protozoa numbers in ruminal fluid of lactating dairy cows fed increasing proportions of alfalfa silage (AS) in the diet

Item	Treatment ¹			SEM	P-value	
	0% AS	50% AS	100% AS		Linear	Quadratic
Total VFA (mM)	118	129	119	3.5	0.82	0.01
VFA profile (mol/100 mol)						
Acetate (A)	67.8	67.3	66.6	0.70	0.12	0.90
Propionate (P)	19.5	19.2	19.3	0.72	0.71	0.67
Butyrate	10.2	10.8	11.0	0.30	0.03	0.58
Valerate	0.80	0.87	0.96	0.040	<0.01	0.93
Branched-chain VFA ²	1.58	1.85	2.18	0.099	<0.01	0.73
A:P ratio	3.56	3.62	3.54	0.184	0.90	0.59
NH ₃ (mM)	5.29	6.34	7.27	0.446	<0.01	0.89
Protozoa						
Total ($\times 10^5$ /mL)	4.96	5.20	5.66	0.382	0.03	0.68
Entodiniomorphs ($\times 10^5$ /mL)	4.85	5.10	5.63	0.376	0.01	0.60
Holotrichs ³ ($\times 10^3$ /mL)	11.39	9.68	3.82	1.590	<0.01	0.26

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

²Isobutyrate + isovalerate.

³Holotrichs = isotrichs and dasytrichs.

milk, FCM, or ECM produced, decreased linearly ($P < 0.01$) with increasing inclusion levels of AS in the diet.

Enteric CH₄ Production

Interactions between day and treatment for CH₄ production were not significant and, therefore, only averages over the 3-d measurements are presented (Table 7). Daily enteric CH₄ emissions tended ($P = 0.10$) to

increase linearly with increasing AS inclusion in the diet (476, 483, and 491 g/d for cows fed 0% AS, 50% AS, and 100% AS, respectively). When adjusted to DMI, CH₄ production averaged 19.8 g/kg and was not affected by increasing dietary proportion of AS. Similarly, CH₄ energy losses expressed as a proportion of GE or digestible energy intakes averaged 5.8 and 8.4%, respectively, and were not affected by experimental treatments. The amount of CH₄ emitted per kilogram

Table 6. Milk production and milk composition of lactating cows fed increasing proportions of alfalfa silage (AS) in the diet

Item	Treatment ¹			SEM	P-value	
	0% AS	50% AS	100% AS		Linear	Quadratic
Production (kg/d)						
Milk	38.5	38.0	38.0	1.30	0.65	0.80
FCM ²	37.9	37.5	37.0	1.03	0.40	0.99
ECM ³	40.6	40.1	39.9	1.18	0.55	0.90
Composition (%)						
Fat	3.91	3.94	3.85	0.132	0.30	0.28
Protein	3.11	3.13	3.17	0.070	<0.01	0.54
Lactose	4.63	4.63	4.63	0.041	0.80	0.89
SCC ($\times 10^3$ /mL)	124	187	211	68.5	0.15	0.70
MUN (mg/dL)	10.0	11.5	11.5	0.69	0.02	0.18
Yield (kg/d)						
Fat	1.50	1.48	1.46	0.040	0.27	0.80
Protein	1.20	1.18	1.20	0.046	0.84	0.57
Feed efficiency						
Milk/DMI	1.72	1.60	1.55	0.047	<0.01	0.16
FCM/DMI	1.69	1.58	1.51	0.033	<0.01	0.41
ECM/DMI	1.81	1.70	1.63	0.036	<0.01	0.28

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

²4% FCM (Tyrrell and Reid, 1965) = $0.4 \times \text{milk yield (kg/d)} + 15 \times \text{fat yield (kg/d)}$.

³ECM (Tyrrell and Reid, 1965) = $0.327 \times \text{milk yield (kg/d)} + 12.95 \times \text{fat yield (kg/d)} + 7.2 \times \text{protein yield (kg/d)}$.

Table 7. Methane production of lactating cows fed increasing proportions of alfalfa silage (AS) in the diet

CH ₄ production	Treatment ¹				P-value	
	0% AS	50% AS	100% AS	SEM	Linear	Quadratic
g/d	476	483	491	13.9	0.10	0.87
g/kg of DMI	19.7	20.1	19.7	0.51	0.79	0.19
% of GE intake ²	5.73	5.90	5.85	0.158	0.23	0.21
% of DE intake ³	8.34	8.48	8.46	0.232	0.49	0.58
g/kg of milk ⁴	12.5	12.8	13.0	0.48	0.14	0.72
g/kg of FCM ⁴	12.6	12.9	13.3	0.36	0.03	0.90
g/kg of ECM ⁴	11.8	12.1	12.4	0.37	0.05	0.99
g/kg of milk fat ⁴	318	326	345	9.4	0.01	0.66
g/kg of milk protein ⁴	401	410	411	17.3	0.38	0.72

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

²GE = gross energy.

³DE = digestible energy (estimated from energy digestibility measured over the 6-d collection period).

⁴Yields of milk, FCM, ECM, milk fat, and milk protein measured over 6 consecutive days.

of milk or milk protein produced remained unaffected by treatment. However, CH₄ concentration, expressed per kilogram of FCM, ECM, or milk fat produced, increased linearly ($P \leq 0.05$) with increasing proportion of AS in the diet.

N Balance

A linear increase ($P < 0.01$) in N intake was observed when AS replaced TS in the diet, due to increases in DMI and dietary CP concentration (Table 8). The amounts (g/d) of N excreted daily in feces and urine increased linearly ($P \leq 0.01$) with increasing AS proportion in the diet. However, when expressed as a pro-

portion of N intake, fecal N excretion decreased linearly ($P = 0.03$), whereas urinary N remained unaffected by increasing inclusion of AS in the diet. Efficiency of N utilization for milk production (N in milk/N intake) declined linearly ($P < 0.01$) as the proportion of AS in the diet increased. Productive N (g/d) increased linearly ($P < 0.01$) with increasing AS proportion in the diet, but no significant effect of treatment was observed when productive N was expressed on N intake basis.

DISCUSSION

Replacing TS silage with AS in the diet of dairy cows caused an increase in DMI. The lower NDF concen-

Table 8. Nitrogen balance in lactating cows fed diets containing increasing proportions of alfalfa silage (AS) in the diet

Item	Treatment ¹				P-value	
	0% AS	50% AS	100% AS	SEM	Linear	Quadratic
Intake N (g/d)	574	626	673	25.9	<0.01	0.82
Fecal N						
g/d	192	200	213	9.1	0.01	0.66
% of N intake	33.7	32.0	31.7	0.60	0.03	0.36
Urinary N						
g/d	177	203	209	12.3	<0.01	0.22
% of N intake	30.7	32.3	31.2	1.74	0.77	0.36
Total N excretion						
g/d	369	403	422	18.2	<0.01	0.43
% of N intake	64.4	64.3	62.9	1.66	0.39	0.64
Milk N						
g/d	188	186	189	7.2	0.84	0.57
% of N intake	33.2	29.7	28.2	0.88	<0.01	0.11
Productive N ²						
g/d	204	223	251	14.9	<0.01	0.72
% of N intake	35.6	35.7	37.1	1.66	0.39	0.64

¹The diet (60:40 forage:concentrate ratio; DM basis) contained 0% AS (54.4% timothy silage), 50% AS (27.2% timothy silage and 27% alfalfa silage), or 100% AS (54.4% alfalfa silage), on a DM basis.

²N intake – total N excretion.

tration (36.9 vs. 52.1%) and higher OM 24-h ruminal degradability (73.5 vs. 66.9%) of AS compared with TS possibly reduced physical fill in the rumen and allowed greater DMI when cows were fed diets with increasing proportions of AS. Lower NDF concentration and higher ruminal degradability in alfalfa compared with timothy has been previously reported (Hoffman et al., 1993; Yu et al., 2003, 2004). A recent meta-analysis study showed that DMI of dairy cows decreases with increasing forage NDF concentration in the diet (Ferraretto et al., 2013). Further, it is well recognized that ruminants fed legume forage-based diets have higher DMI than those fed grass forage-based diets. This has been observed in dairy cows fed alfalfa silage-based diets versus grass silage-based diets (Dewhurst et al., 2003a), red clover silage-based diets compared with ryegrass silage-based diets (Moorby et al., 2009), and alfalfa silage-based diets versus orchardgrass silage-based diets (Kammes and Allen, 2012). In a review of 20 studies, Steinshamn (2010) concluded that cows offered legume silage-based diets consume 2 kg of DM/d more than cows offered grass silage-based diets, which is in agreement with our findings. Dewhurst et al. (2003a) and Kammes and Allen (2012) observed smaller rumen pool size, higher rumen turnover rate, and higher passage rate when cows were fed legume forages compared with grass forages.

Several review and modeling studies suggested lower CH₄ emissions per kilogram of DMI or as a proportion of GE intake when legumes replace grasses in ruminant diets (Benchaar et al., 2001; Beauchemin et al., 2009; Dewhurst, 2013). Differences in CH₄ emissions between legumes and grasses have been attributed to the higher NSC content, lower fiber concentration, and the brittle cell wall structure of legumes compared with grasses. These changes would increase ruminal passage rate, reduce extent of ruminal fermentation, and shift ruminal VFA pattern toward less acetate and more propionate production. All these factors are known to cause a decrease in CH₄ energy losses.

Studies examining the effects of different forage types on enteric CH₄ emissions in dairy cows are scarce. Results of the current study show a small increase in daily CH₄ emissions (g/d) with increasing AS proportion in the diet, which could be linked to the increase in DMI. However, CH₄ production adjusted to DM, GE, or DE intake was not affected by increasing the proportion of AS in the diet. In agreement with our findings, van Dorland et al. (2007) replaced 40% of ryegrass silage with white clover silage in the diet of dairy cows and reported tendencies for an increase in daily CH₄ emissions (g/d) associated with a numerical increase in DMI. However, in that study, CH₄ production adjusted for OM digested was similar between cows fed ryegrass

silage-based diets and those fed white clover silage-based diets. Enteric CH₄ emissions (adjusted for DMI) were not different between sheep fed fresh ryegrass versus fresh white clover (Hammond et al., 2013), fresh ryegrass versus fresh (75:25) alfalfa-ryegrass (Niderkorn et al., 2011), or ryegrass hay versus alfalfa-ryegrass (50:50) hay (Carulla et al., 2005). Archimède et al. (2011) concluded in a meta-analysis study that CH₄ emissions adjusted to OM or DM intake are not influenced by feeding legumes compared with C3 grasses (such as timothy).

In the current study, the lack of major changes in ruminal fermentation characteristics (pH and molar proportions of acetate and propionate) supports the absence of an alteration of ruminal methanogenesis when cows were fed increasing amounts of AS in the diet. Bannink et al. (2010) suggested that at a pH of approximately 6.3 (the average value observed in this study), fermentation of starch in the rumen results in less propionate production than at lower pH, which may contribute to explain the lack of changes in CH₄ production observed in our study. Carulla et al. (2005), Keli et al. (2013), and Hammond et al. (2013) also reported that replacing partially or completely ryegrass with alfalfa (fresh or hay) in sheep diets had no significant effect on ruminal pH, total VFA concentration, or molar proportion of acetate or propionate. Other studies observed an increase (Kammes and Allen, 2012) or a decrease (Dewhurst et al. 2003a; Vanhatalo et al. 2009) in molar proportion of propionate in the rumen when legumes replaced grasses in dairy cow or sheep diets. Propionate is known to be an important hydrogen sink in the rumen, whereas acetate production releases hydrogen that is used by methanogens for CH₄ synthesis (Beauchemin et al., 2009; Martin et al., 2010; Dijkstra et al., 2011). The increase in protozoal population (important hydrogen producer) with increasing AS proportion in the diet may explain the slight increase in daily CH₄ emissions and could be related to an increase in DMI, as previously observed (Aoki et al. 2010; Benchaar et al., 2012, 2014). Protozoa are butyrate producers (Morgavi et al., 2012) and, therefore, the increase in protozoa numbers in this study is consistent with the higher butyrate molar proportion as cows were fed increasing AS proportions in the diet. The increase in starch concentration in the diet with increasing AS proportion could also explain the increase in protozoal population, as protozoal populations respond positively to increasing starch concentration in forage-based diets (Dijkstra, 1994).

As the proportion of AS in the diet increased, ruminal NH₃ concentration and branched-chain VFA (isobutyrate and isovalerate) molar proportion increased. This response can be explained by the changes that occurred

in diet composition by shifting the forage source from TS to AS. First, the concentration of CP was higher in AS diet than in TS diet and DMI increased as the proportion of AS in the diet increased. As a consequence, CP intake increased with increasing inclusion of AS in the diet. Second, the increase in the proportion of AS in the diet was associated with a concomitant reduction of the dietary contribution of TS and the main protein source in the diet, namely soybean meal. Alfalfa silage contains more soluble and degradable CP than TS and soybean meal (NRC, 2001). These changes had likely contributed to increase soluble and degradable CP supply in the rumen, and consequently to increase rumen NH_3 concentration and branched-chain VFA molar proportion. Several studies observed higher ruminal NH_3 and branched-chain VFA levels when legumes replaced grasses in the diet of dairy cows (Dewhurst et al., 2003a; Vanhatalo et al., 2009; Kammes and Allen, 2012).

Carbohydrate availability strongly influences the utilization of NH_3 by rumen microbes (Russell et al., 1983). In our study, the ratio of starch:CP was 0.86, 0.96, and 1.10 for the 0% AS, 50% AS, and 100% AS diet, respectively. The ratio of NFC:CP was 2.23, 2.28, and 2.36 for the 0% AS, 50% AS, and 100% AS diet, respectively (Table 2). Enhancing the supply of fermentable carbohydrates (e.g., starch) in the rumen can decrease NH_3 in the rumen by reducing the deamination process or enhancing the capture of released AA by ruminal microbes, or it can increase microbial capture of released NH_3 in the rumen. Several studies (Hristov et al., 2005; Hassanat et al., 2013; Benchaar et al., 2014) observed lower ruminal NH_3 concentration when the starch supply increased in the diet. In the current study, despite higher starch supply (via increasing dietary proportion of corn grain) when cows were fed more AS, ruminal NH_3 concentration increased, suggesting that the utilization of NH_3 by ruminal microbes was not enhanced by greater availability of readily fermentable carbohydrates. According to Dewhurst et al. (2003b) and Dijkstra et al. (2011), the imbalance energy-N supply to the rumen in alfalfa silage-based diets (due to excessive N supply) is the reason behind the inability of ruminal microbes to capture NH_3 for protein synthesis. The increase in protozoal population with increasing dietary AS proportion may have also contributed to enhancing protein degradation in the rumen and accumulation of NH_3 . Protozoa are responsible for degrading dietary and microbial protein, reducing microbial synthesis, and increasing NH_3 production in the rumen (Ivan et al., 1991; Koenig et al., 2000; Oldick and Firkins, 2000).

Despite increased DM and GE intakes with increasing inclusion of AS in the diet, milk production remained unchanged. As a result, feed efficiency, expressed as ki-

lograms of milk, FCM, or ECM produced per kilogram of DMI, decreased as the proportion of AS in the diet increased. In agreement with our findings, Dewhurst et al. (2003a) observed similar milk yields in cows fed ryegrass silage-based diets and cows fed alfalfa silage-based diets. van Dorland et al. (2007) and Kammes and Allen (2012) also reported no change in milk yield but lower feed efficiency (kg of milk/kg of DMI) in cows fed legumes versus grasses silage. In our study, a slight but significant ($P = 0.03$) increase occurred in the BW of cows consuming diets that contained AS (data not shown). Thus, the increase in DMI with diets containing AS contributed more to BW gain rather than milk production, which explains the decline in feed efficiency.

In the present study, feeding cows increasing proportions of AS in the diet did not affect the concentration and yield of milk fat, which agrees with the lack of change in ruminal acetate molar proportion. Milk protein concentration increased as dietary proportion of AS increased, but milk protein yield was not affected by treatment. Little or no effect of silage source on milk component concentration or yield was observed when alfalfa replaced ryegrass (Dewhurst et al. 2003a,b) or orchardgrass (Kammes and Allen 2012) in the diet of dairy cows. Vanhatalo et al. (2009) reported a significant increase in MUN concentration when silages of legumes replaced grasses in the diet of dairy cows, whereas only numerical differences were reported by Kammes and Allen (2012). Milk urea N is an indicator of blood urea N. If not captured by rumen microbes for protein synthesis, NH_3 accumulates in the rumen, absorbed via the ruminal wall, and is transferred to the liver where it is converted to urea and released into the bloodstream (Van Soest, 1994). Higher MUN concentration as AS inclusion increased in the diet is consistent with increases in ruminal NH_3 concentration. The increase in MUN concentration when AS was incorporated into the diet may also have resulted from deamination of absorbed AA. Higher AA supply to the animal, as shown by increased CP intake and digestibility, may have occurred when AS replaced TS in the diet. The observed decline in N use for milk protein production when the AS proportion in the diet increased indicates that an excess of N intake was not used to enhance milk protein synthesis. This decrease in milk N efficiency is linked to the decrease in rumen N efficiency. In agreement, other studies have reported a reduction in N use for milk production when legumes replaced grasses in the diet of dairy cows (Dewhurst et al., 2003b; Moorby et al., 2009; Vanhatalo et al., 2009).

Because milk production and yield of milk protein were not affected by experimental treatments, CH_4 emissions adjusted to milk or milk protein yield remained unchanged by increasing AS dietary proportion. Like-

wise, van Dorland et al. (2007) also reported no change in the amount of CH₄ produced per kilogram of milk or milk protein when white or red clover replaced ryegrass in dairy cow diets. On the other hand, CH₄ emissions adjusted to FCM, ECM, or milk fat yield increased with increasing AS dietary proportion. This occurred because the increase in DMI prompted an increase in daily CH₄ production, but did not promote a parallel increase in FCM, ECM, or milk fat yield.

Methane emission is not the only pollutant of concern in dairy production. Nitrous oxide (N₂O) and NH₃ emissions are also of concern because they are related to N excretion in the manure. Therefore, any diet manipulation aiming to reduce enteric CH₄ emissions from dairy cows should also take into account possible effects on N₂O and NH₃ emissions. This study shows that the increase in N intake when AS replaces TS in the diet increased fecal and urinary N outputs (g/d) by up to 11 and 18%, respectively. Several studies reported increases in fecal and urinary N outputs when legume forages replaced grasses in diets of dairy cows (van Dorland et al., 2007; Moorby et al., 2009; Vanhatalo et al., 2009). Nitrogen excretion responds positively to increasing N intake, with a larger response in urinary N excretion than fecal N excretion (Dijkstra et al., 2011). The higher urinary N excretion when cows were fed increasing AS proportions in the diet is a reflection of a decrease in efficiency of N utilization in the rumen (i.e., higher ruminal NH₃ concentration and branched-chain VFA proportion) as AS proportion increased in the diet.

CONCLUSIONS

Replacing TS with AS in the diet of dairy cows had no significant effects on CH₄ energy losses (i.e., CH₄ as a proportion of GE intake), which was consistent with the lack of effects of increasing AS proportion in the diet on VFA pattern (i.e., molar proportions of acetate and propionate). Ruminal NH₃ concentration increased with increasing inclusion of AS in the diet and this change was associated with an increase in urinary N concentration. Higher N excretion in urine has the potential to enhance N₂O and NH₃ emissions. Under the conditions of this study, replacing TS with AS in the diet does not represent an opportunity to reduce CH₄ energy losses from dairy cows.

ACKNOWLEDGMENTS

The authors are thankful to L. Croteau and P. Warburton (Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada) for their technical assistance, F. Tremblay (Agriculture and Agri-Food Canada) for

his help with methane measurements, and S. Méthot (Agriculture and Agri-Food Canada) for his help with statistical analyses. We also appreciate the work of the barn crew of the Dairy and Swine Research Centre (Sherbrooke, QC, Canada) for care of the animals. Recognition is also extended to Y. Leboeuf (Université Laval, Quebec, QC, Canada) for technical support. Funding of the study through a Dairy Research Cluster grant from the Dairy Farmers of Canada (Ottawa, ON, Canada), Agriculture and Agri-Food Canada (Ottawa, ON, Canada), and the Canadian Dairy Commission (Ottawa, ON, Canada) is greatly appreciated.

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