Effects of the Method of Conservation of Timothy on Nitrogen Metabolism in Lactating Dairy Cows

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ABSTRACT

Six ruminally and duodenally cannulated lactating primiparous Holstein cows were used to study the effects of different methods of conservation of timothy on N metabolism. Cows were assigned randomly to 2 replicated 3 × 3 Latin squares (35-d periods). Because of missing data from 2 cows, data were analyzed as a 3 × 4 Youden square. Diets contained a similar concentrate (44% of total ration on a dry matter basis) plus first-cut timothy conserved as hay, or as restrictively (formic) or extensively fermented silage (inoc). Crude protein contents were 10.4, 13.6, and 14.8% for hay, formic, and inoc, respectively. Hay and formic had a high soluble carbohydrate content (≥8.0% of dry matter) and formic and inoc had a high soluble protein content (≥8.0% of dry matter). Haying and restricting fermentation resulted in increased efficiency of partition to milk N (30.9, 28.2, 24.7% of N intake for hay, formic, and inoc, respectively). Despite a 14% lower N intake with hay, no effects of treatments were detected on microbial protein synthesis and apparent intestinal digestion of essential AA. Haying reduced feed protein degradation in the rumen, whereas this effect was not observed when restricting fermentation in the silage. Haying and restricting fermentation induced a lipogenic fermentation pattern in the rumen (4.55, 4.23, and 3.78 ratio of acetate to propionate for hay, formic, and inoc), but no effects on milk fat yield and plasma glucose were observed. Whole-body protein metabolism was unaffected by treatments.

Key words: dairy cow, metabolism, timothy, nitrogen

INTRODUCTION

Prior to harvest, more than 75% of the total N in plants is true protein, whereas the remainder is NPN. The proportion of NPN may increase to 40% when

the plant is ready to be baled or chopped for silage, depending on the ambient temperature and drying rate. There are generally very small further increases in NPN during storage with hay; however, more proteolysis occurs during ensiling, which may in turn increase NPN up to 60 to 70% of total N when silage is fed to the animals (Kung and Muck, 2006).

Proteolysis in silage can be reduced by using additives such as formic acid, which artificially acidifies the forage mass, or products that enhance the production of lactic acid (e.g., inoculants, enzymes). Upon application, formic acid induces a rapid drop in pH, whereas silage inoculants bring about a more variable rate of acidification. Their ability to reduce proteolysis therefore differs. Another advantage of silage additives is the decreased catabolism of water-soluble carbohydrates (WSC), which are degraded to support acid synthesis by bacteria (McDonald et al., 1991). Hence, this effect is not as good with silage inoculants because WSC are the main substrate for lactic acid bacteria.

Efficiency of microbial protein synthesis was improved by restricting fermentation during ensiling (Jaakkola et al., 1993; Huhtanen et al., 1997; Verbič et al., 1999). By reducing NPN or increasing capture of N in the rumen, the use of additives can decrease ammonia absorption with subsequent hepatic urea production and reduce N losses to the environment. The metabolic fate of body N is generally studied using N balance. However, N balance gives only the net result of much larger opposite dynamic flows, namely, whole-body (WB) protein synthesis and protein breakdown. Those flows are controlled by endocrine, physiological, and nutritional factors such as protein (Lapierre et al., 2002; Raggio et al., 2006) and energy (Raggio et al., 2006) supplies, which can both be affected by the method of conservation of the forage.

We hypothesized that increasing the WSC content and decreasing N solubility of the forage would improve microbial capture of N in the rumen, increase milk protein secretion, and decrease urinary N losses by increasing WB protein synthesis. Therefore, the objective of this experiment was to compare the effects of feeding timothy conserved either as hay or as re-

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strictively or extensively fermented silage on ruminal metabolism, microbial protein synthesis, intestinal flow of nutrients, N utilization, Leu kinetics, and milk production in lactating dairy cows.

MATERIALS AND METHODS

Animals were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993), and all procedures involving animals were previously approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre.

Forage Production

Three forages (2 silages and 1 hay) were prepared from the same first-cut timothy (*Phleum pratense* L. cv. Champ) sward mowed on June 18, 2001, from 0900 to 1430 h. The first forage (**inoc**) was harvested after 20 h of wilting (1 tedding) and ensiled with an inoculant containing *Lactobacillus plantarum* LPH-1 and *Pediococcus cerevisiae* PCH-3 (final application rate of 1.25×10^5 cfu/g of fresh forage). The second forage (**formic**) was harvested after 24 h of wilting (no tedding) and ensiled with the application of 85% formic acid at a rate of 6 L/t of fresh forage. Both silages were conserved in plastic-tube silos. The third forage (hay) was harvested as small-bale hay. It was tedded 24 and 48 h after the cut and was raked and baled on June 20, 2001. The drying process was completed in the barn with a hay dryer during 10 d. Weather conditions were good during forage harvesting. Silos were opened after 214 d.

Cows and Diets

Six primiparous Holstein cows cannulated in the rumen (10 cm; Bar Diamond Inc., Parma, ID) and duodenum (closed T-shaped cannula made of Teflon-coated stainless steel) were used in 2 replicated 3×3 Latin squares with 35-d periods. Cows averaged 576 ± 77 kg of BW and 112 ± 23 DIM at the start of the experiment. Treatments were hay, inoc, or formic supplemented with a concentrate pelleted in a single batch at a local feed mill. The forage was offered at approximately 56% of the ration DM. Table 1 shows the ingredients and chemical composition of the diets used in the experiment. During the experiment, the ration was restricted at 95% of ad libitum DMI, as determined during the first week of period 1, to maintain steady-state conditions and minimize variation in DMI within and between experimental periods. Table 2 summarizes the chemical composition of dietary

Table 1. Ingredients and chemical composition of the diets

Composition	Diet		
	Hay	Formic	Inoc
Ingredient, % of DM			
Hay (unchopped) ¹	—	5.1	5.3
Hay (chopped)	56.2	—	—
Restrictively fermented silage	—	51.8	—
Extensively fermented silage	—	—	48.9
Concentrate ²	43.8	43.1	45.8
Chemical			
DM, %	91.4	54.5	55.8
OM, % of DM	94.2	93.3	93.0
CP, % of DM	12.5	14.6	15.2
NPN, % soluble N	83.4	85.7	86.1
Soluble protein, % of DM	3.7	5.5	5.9
Neutral detergent insoluble CP, % of DM	2.1	2.2	2.1
Acid detergent insoluble CP, % of DM	0.7	1.0	1.1
NDF, % of DM	41.6	38.9	35.4
ADF, % of DM	25.6	24.4	22.7

¹Chemical composition: 91.4% DM, and (% of DM) 90.5% OM; 16.1% CP; 57.6% NDF; 35.7% ADF.

²Ingredients in the concentrate (% DM basis) = 76.1% ground shelled corn; 13.2% soybean meal; 4.72% Megalac; 2.23% mineral and vitamin premix; 1.52% dicalcium phosphate; 1.31% molasses; 0.51% Lignosol; 0.22% MgO; 0.20% Cr₂O₃. The mineral and vitamin premix contained (DM basis) 20.2% Na; 7.8% Cl; 6.0% Ca; 0.3% P; 4.6% Mg; 0.24% K; 1.6% S; 58.4 mg/kg of Co; 5,192 mg/kg of Mn; 7,788 mg/kg of Zn; 31.2 mg/kg of Se; 280 mg/kg of Fe; 700,940 IU of vitamin A/kg; 155,760 IU of vitamin D/kg; 2,336 IU of vitamin E/kg.

ingredients offered during the experiment. Animals were housed in individual tie stalls equipped with rubber mats and had free access to water throughout the trial. Adaptation to experimental treatments was from d 1 to 16, milk yield and sampling and total collection of feces and urine were from d 17 to 21, Leu kinetics were on d 26 and 27, and rumen and blood sampling were on d 34 and 35.

Feed Intake

All diets were fed as TMR and distributed every other hour in 12 equal meals per day with automated feeders (Ankom Technology, Fairport, NY). The hay was coarsely chopped [2 to 3 in. long 5 to 8 cm] to facilitate delivery through the automated feeders. In both the formic and inoc diets, 1 kg (as-is basis) of unchopped hay was fed to prevent ruminal disturbances. Feed consumption was recorded daily by weighing feeds offered to and refused by the cows. Samples of TMR, feed ingredients, and orts were collected daily and kept frozen. Samples were composited by period, freeze-dried, ground through a 1-mm screen Wiley mill (standard model 4; Arthur H. Thomas, Philadelphia, PA), and analyzed for DM, OM, total N, NPN, soluble protein, CP insoluble in neutral or acid detergent, NDF, ADF, and AA concentrations. Fresh silage extracts were analyzed for pH with an Accumet

Table 2. Chemical composition and fermentation characteristics of dietary ingredients

Item	Hay	Restrictively fermented silage	Extensively fermented silage	Concentrate
Chemical composition				
DM, %	91.9	39.9	39.6	90.7
OM, % of DM	95.3	94.3	93.5	92.7
CP, % of DM	10.4	13.6	14.8	15.0
NPN, % soluble N	88.4	89.2	92.4	74.3
Soluble protein, % of DM	4.6	8.0	8.2	2.0
Neutral detergent insoluble CP, % of DM	3.4	3.4	3.3	0.5
Acid detergent insoluble CP, % of DM	0.8	1.5	1.5	0.5
NDF, % of DM	68.2	63.7	59.2	7.8
ADF, % of DM	43.5	41.7	39.0	2.9
WSC, ¹ % of DM	8.0	9.3	4.3	—
AA, % of CP				
Arg	4.2	3.7	2.7	5.7
His	1.7	1.8	1.6	2.5
Ile	3.8	4.2	4.1	3.9
Leu	6.5	7.0	6.9	9.1
Lys	4.1	4.7	4.5	4.3
Met	1.6	1.6	1.5	1.9
Phe	4.3	4.6	4.3	4.6
Thr	4.1	4.3	4.1	3.8
Trp	2.0	1.6	1.3	1.5
Val	5.1	5.5	5.6	4.9
Ala	5.7	6.4	6.3	5.7
Asx	9.3	9.5	8.8	9.2
Cys	1.1	0.7	0.6	1.9
Glx	9.1	9.0	7.4	17.9
Gly	4.6	4.7	4.4	4.1
Pro	6.4	5.2	4.7	7.0
Ser	4.0	4.0	3.1	4.9
Essential AA	37.3	38.8	36.8	42.0
Nonessential AA	40.1	39.4	35.4	50.6
Branched-chain AA	15.3	16.7	16.7	17.8
Total AA	77.4	78.2	72.2	92.6
Fermentation characteristics				
pH	—	4.2	4.4	—
NH ₃ -N, % of total N	—	4.5	6.7	—
Lactic acid, % of DM	—	3.8	9.0	—
Acetic acid, % of DM	—	0.7	1.5	—
Propionic acid, % of DM	—	0.02	<0.01	—
Butyric acid, % of DM	—	0.03	<0.01	—

¹WSC = water-soluble carbohydrates (Dubois et al., 1956).

pH meter (model 815 MP; Fisher Scientific, Fairlawn, NJ), and frozen at -20°C for later determination of WSC, NH₃-N, lactic acid, and VFA concentrations.

Ruminal Fermentation Characteristics and Duodenal Sampling

Ruminal digesta was sampled at 0900, 1100, 1300, and 1500 h on d 34 and at 0800, 1000, 1200, and 1400 h on d 35. Ruminal samples were strained through 2 layers of cheesecloth. Ruminal pH was measured immediately after sampling (Piccolo pH meter; Hanna Instruments, Woonsocket, RI), and subsamples were acidified to pH 2 with sulfuric acid (50% vol/vol; 1:17 wt/wt acid:rumen fluid) and frozen at -20°C for later determination of VFA, lactic acid, and NH₃-N concentrations. The same collection schedule was used for

duodenal samples. Ruminal and duodenal subsamples were formolized (75 mL of formol saline added to 300 mL of digesta) and stored at -20°C until analyses. Chromium sesquioxide (Cr₂O₃) was used as an indigestible marker. It was mixed with ground shelled corn (7.5/80.0, wt/wt as-is basis) and 21.5 kg of this premix was included with the other ingredients of the concentrate before pelleting. Calculations of flows through the duodenum were based on the amounts of Cr excreted during the total collection of feces.

Milk Production and Milk Composition

Cows were milked twice daily in their stalls at approximately 0800 and 1600 h, and milk yield was recorded at each milking. Milk samples were collected from each cow at each milking from d 17 (p.m.) to

d 22 (a.m.). Milk subsamples were kept frozen until analyzed for fat, protein, and lactose content. Milk subsamples were pooled on a yield basis and kept frozen until analyzed for urea N and noncasein N content. Averages were then calculated for each cow at each period.

Apparent Total Tract Digestibility and N balance

Urine was collected in stainless-steel containers via a Gooch tube (BF Goodrich Co., Kitchener, ON, Canada) and acidified with sulfuric acid (50% vol/vol; 125 mL at 0800, 1400, and 2000 h) to maintain pH <3.0. A representative sample (2%) was taken and kept frozen at -20°C for analyses of purine derivatives (PD), creatinine, total N, $\text{NH}_3\text{-N}$, and urea N concentrations. Feces were collected in preweighed plastic-lined plywood boxes and mixed daily. A representative sample (2%) was taken, stored at -20°C , and subsequently thawed, freeze-dried, and ground through a 1-mm screen (Wiley mill) for chemical analysis.

Ruminal Microbial N Outflow

Preliminary results showed a lack of concordance between estimations of duodenal microbial nitrogen (MN) flow based on PD and purine bases (Martineau et al., 2005). This effect possibly resulted from unreliable purine:N ratios in reference bacteria isolated from the thawed, formalized duodenal digesta. Therefore, the ruminal MN outflow was determined from urinary excretion of PD according to the equations of Chen and Gomes (1992):

$$Y \text{ (mmol/d)} = (0.85 \times X) + (0.385 \times \text{BW}^{0.75}),$$

$$\text{MN (g/d)} = [70/(0.116 \times 0.83 \times 1000)] \times X = 0.727 \times X,$$

where X and Y are the absorption and the excretion of PD (mmol/d), respectively. Those equations assume that the daily excretion of PD in milk is 1 mmol/kg of milk produced, the N content of purines is 70 mg/mmol, the ratio of purine N to total N in mixed rumen microbes is 0.116, the endogenous contribution to urinary PD excretion is $0.385 \times \text{BW}^{0.75}$ mmol/d, the true intestinal digestibility of microbial purines is 83%, and the recovery of absorbed purines as urinary PD is 85%.

WB Protein Metabolism

A polyvinyl catheter was inserted in both jugular veins on d 25 of each period. On d 26, the CO_2 entry rate (CER) was measured using a 6-h (from 0900 to

1500 h) primed (1.4 mmol) continuous jugular infusion of $\text{NaH}^{13}\text{CO}_3$ (1.0 mmol/h; 99 atom %; Cambridge Isotope Laboratories, Andover, MA). Blood samples from the contralateral jugular vein were collected into heparinized syringes in the hour before the infusion to determine ^{13}C natural abundance in CO_2 , followed by 6 hourly samples, starting 1 h after the initiation of the infusion. Immediately after collection, two 1-mL blood aliquots were injected into evacuated Vacutainers (Becton Dickinson, Franklin Lakes, NJ) containing 1 mL of frozen lactic acid, snap-frozen in liquid N (Read et al., 1984), and kept frozen at -20°C until analyzed for determination of the isotopic enrichment (IE).

On d 27 of each period, the Leu irreversible loss rate (ILR) was used to estimate WB protein metabolism or protein turnover using a 6-h (from 0900 to 1500 h) primed (2.4 mmol) continuous jugular infusion of L- $[1\text{-}^{13}\text{C}]\text{Leu}$ (2.4 mmol/h; 99 atom %; Cambridge Isotope Laboratories). Blood samples from the contralateral jugular vein were collected into heparinized syringes in the hour before the infusion to determine ^{13}C natural abundance in Leu, 4-methyl 2-oxopentanoate (MOP) and CO_2 , followed by 6 hourly samples, starting 1 h after the initiation of the infusion. Immediately after collection, two 1-mL blood aliquots were treated and stored as previously described. The remaining blood was centrifuged and plasma was stored at -20°C for the determination of ^{13}C IE of Leu and MOP.

In all equations of protein turnover, WB ILR and infusion rates are expressed in millimoles per hour and IE in atom percent excess. Tracer refers to $^{13}\text{C}]\text{Leu}$ or $^{13}\text{C}]\text{bicarbonate}$ and tracee is the unlabeled Leu and bicarbonate. Whole-body Leu ILR or CER was calculated as follows:

$$\text{WB-ILR}_{\text{tracee}} \text{ or } \text{CER}_{\text{tracee}} = [(\text{IE}_{\text{inf}}/\text{IE}_{\text{p}}) - 1] \times \text{Inf},$$

where IE_{inf} and IE_{p} are the ^{13}C IE of the infusate and the mean ^{13}C IE of venous plasma CO_2 , MOP, or Leu, respectively, and Inf is the infusion rate of the labeled bicarbonate (d 26) or Leu (d 27). The mean enrichment was the arithmetic mean of the 6 hourly samples taken under plateau conditions during the infusion. Leucine tracer oxidation (mmol/h) was calculated as:

$$\text{Leu}_{\text{tracer}} \text{ oxidation} = \text{CER} \times \text{IE}_{\text{CO}_2\text{-Leu}}/100,$$

where CER is the CO_2 entry rate (d 26), $\text{IE}_{\text{CO}_2\text{-Leu}}$ is the mean venous plasma ^{13}C IE of CO_2 measured during the infusion of labeled Leu (d 27), assuming that CER production on d 26 is the same as d 27.

The fractional rate of oxidation (**FO**) of Leu (tracer + tracee) was calculated as:

$$FO_{\text{tracer} + \text{tracee}} = \text{Leu}_{\text{tracer}} \text{ oxidation} / (\text{Inf}_{\text{Leu}} \times \text{IE}_{\text{inf}}),$$

where Inf_{Leu} is the infusion rate of labeled Leu and IE_{inf} is the [^{13}C] IE of the infusate. It was assumed that tracee and tracer were metabolized similarly and that the equivalent of the infused dose was in excess and was oxidized (Lobley et al., 2003); therefore, $\text{Leu}_{\text{tracee}}$ oxidation (**LO**) was calculated as:

$$LO = FO_{\text{tracer} + \text{tracee}} \times (\text{WB-ILR}_{\text{tracee}} + \text{Inf}_{\text{Leu}}) - \text{Inf}_{\text{Leu}},$$

where Inf_{Leu} is the infusion rate of Leu (d 27). The FO of Leu (tracee) was calculated as:

$$FO_{\text{tracee}} = LO / \text{WB-ILR}_{\text{tracee}}$$

Leucine used for protein synthesis was calculated as the difference between $\text{WB-ILR}_{\text{tracee}}$ of Leu and LO, using MOP as representative of the precursor pool. Leucine used for protein synthesis was further partitioned into Leu secreted in milk protein or used for nonmilk protein. Leucine secreted in milk or retained (mmol/h) was determined during the total collection of feces and urine, assuming constant fractions of 98 g of Leu/kg of milk protein secreted (Swaigood, 1995) and 63 g of Leu/kg of synthesized tissue protein (Lobley et al., 1980). The same constant fractions were used to estimate protein synthesis on a kilogram per day basis. Leucine returning to the compartment from protein breakdown was calculated as the difference between Leu used for protein synthesis minus Leu in milk and retained in tissues. Leucine absorbed was calculated by the difference between WB ILR and WB protein breakdown.

Blood Metabolites

Six heparinized blood samples were taken hourly (from 1000 to 1500 h) on d 27 to determine plasma urea N, glucose, and AA concentrations. For AA analyses, 0.2 g of an internal standard solution was added to 1 g of plasma and samples were kept frozen at -80°C until analyses. The internal standard solution was prepared with labeled AA diluted in water to the following concentrations (μM): DL-His- α - ^{15}N (155), L-Ile- ^{15}N (733), L-Leu-1- ^{13}C (876), DL-Lys- α - ^{15}N -2HCl (313), DL-Met-1- ^{13}C (106), L-Phe-1- ^{13}C (238), L-Thr- ^{15}N (475), L-Trp-1- ^{13}C (84), L-Val- ^{15}N (832), L-Ala-1- ^{13}C (1,082), L-Gln-1- ^{13}C (905), L-Glu-1- ^{13}C (292), Gly-1- ^{13}C (1,240), L-Ser-1- ^{13}C (478), and L-Tyr- ^{15}N (260). Labeled AA (95 to 99 atom %) were supplied by CDN Isotopes

Inc. (Montreal, QC, Canada) for His, Leu, Lys, Met, and Phe; by Cambridge Isotope Laboratories for Ile, Thr, Trp, Ala, Gln, Glu, Gly, Ser, and Tyr; and by Isotec Inc. (Miamisburg, OH) for Val.

Chemical Analyses

Analytical DM content was determined by oven-drying at 135°C for 2 h. Subsamples were ashed at 550°C for 12 h in a muffle furnace. The total N content was determined by thermal conductivity (Leco model FP-428 Nitrogen Determinator; Leco, St. Joseph, MI) and CP was calculated as $\text{N} \times 6.25$ (or 6.38 for milk). Soluble protein and NPN were determined after solubility in a borate-phosphate buffer at 39°C during 1 h and precipitation of soluble protein supernatant with TCA (0.8 M; Roe et al., 1990). The concentration of NDF was determined as described by van Soest et al. (1991) using sodium sulfite and heat-stable α -amylase. The ADF content was determined as described by Robertson and Van Soest (1981). The NDF and ADF procedures were done using an Ankom²⁰⁰ fiber analyzer (Ankom Technology). Soluble carbohydrates were assayed by the phenol-sulfuric acid method (Dubois et al., 1956). Concentrations of AA were measured with an AA analyzer (Biochrom 20; Amersham Pharmacia Biotech, Piscataway, NJ) after a 24-h acid hydrolysis with 6 N phenol-HCl at 110°C . A performic acid oxidation step was done for Met and Cys prior to acid hydrolysis (method 994.12; AOAC, 2000). Concentration of $\text{NH}_3\text{-N}$ was analyzed using the indophenol-blue method (Novozamsky et al., 1974). Concentration of VFA was determined with an HPLC Gold System (Beckman Instruments, San Ramon, CA). Chromium was extracted (Siddons et al., 1985) and measured by atomic absorption with an air-acetylene flame.

The concentrations of N in acidified urine samples, in NPN filtrate, and in NDF and ADF residues were determined by micro-Kjeldahl analysis (method 955.04; AOAC, 1990). Purine derivatives and creatinine were determined according to the procedure of Balcells et al. (1992) with a Beckman System Gold chromatograph (Beckman Instruments, Fullerton, CA).

Milk fat concentration was measured according to the Röse-Gottlieb procedure (method 905.02; AOAC, 1990). Contents of DM and ash were measured with a thermogravimetric analyzer (model TGA-601; Leco Corp.). Milk lactose was calculated by the difference between the amount of OM and CP plus fat. Casein N was calculated by the difference after precipitation of milk proteins at pH 4.6 with 10% acetic acid. Urea N was determined colorimetrically on a Technicon autoanalyzer (Technicon Instruments, Tarrytown, NY).

The blood samples frozen on lactic acid were thawed immediately prior to analysis and reacted at room temperature. The liberated CO₂ was analyzed for [¹³C] IE as mass-to-charge ions 44, 45, 46 on a triple collector isotopic ratio mass spectrometer (Sira 12; VG Masslab, Manchester, UK). Results were pooled by cow. The plasma samples for determination of the IE free Leu and its oxo-acid (MOP) were deproteinized with sulfosalicylic acid and desalted on AG-50 H⁺ resin. The freeze-dried eluate was derivatized with *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide:acetonitrile (1:1) to form the *N*-(tert-butyldimethyl) AA derivative. The IE was determined with mass-to-charge ions 302, 303 for Leu and 259, 260 for MOP by GC-MS (model GC6890-MS5973; Agilent Technologies, Wilmington, DE), as described by Calder and Smith (1988). Isotopic enrichments for Leu, MOP, and CO₂ were corrected for background abundance, expressed as atom percent excess, and pooled by cow.

Plasma AA on hourly samples were determined by isotopic dilution (model GC6890-MS5973; Agilent Technologies; Calder et al., 1999) and pooled by cow. Plasma glucose on pooled hourly samples was measured by the Roche Hitachi glucose oxidase method (Roche Diagnostics, Mannheim, Germany).

Statistical Analyses

Because of the loss of 2 cows due to problems unrelated to the treatments, the statistical analysis was performed as a 3 × 4 Youden square (Cochran and Cox, 1957). The period 1 data from another cow could not be used because the cow refused to eat the chopped hay. Data were analyzed using the MIXED procedure of SAS (SAS Institute, 2000) according to the model

$$Y_{ijk} = \mu + a_i + \beta_j + \tau_k + e_{ijk},$$

where Y_{ijk} is the response variable, μ is the overall mean, a_i is the random effect of cow i (1, . . . , 4), β_j is the effect of period j (1, 2, 3), τ_k is the effect of treatment k (1, 2, 3), and e_{ijk} is the residual error. For the statistical analysis of ruminal fermentation characteristics and plasma urea N, sampling time and sampling time × treatment were added to the model and analyzed as repeated measures using PROC MIXED. Autoregressive order 1 and compound symmetry (homogeneous and heterogeneous) were tested as covariance structures and the covariance structure with the least Akaike's information criterion was retained in the final model. Sums of squares for treatment were separated into single degree of freedom preplanned orthogonal contrasts: hay vs. silages, and formic vs. inoc.

Results are reported as least squares means ± standard errors of the means. Significance was declared at $P \leq 0.05$ and a trend at $P \leq 0.10$.

RESULTS AND DISCUSSION

Composition of Forages

The forages had contrasting contents of soluble protein and WSC (Table 2). The preservation of WSC is a prominent feature of hays and of restrictively fermented silages as opposed to extensively fermented silages in which WSC are fermented into lactic acid and VFA (McDonald et al., 1991). Significant losses of Arg and Trp were apparent in the extensively fermented silage, as previously reported by Anderson (1983). The extensively fermented silage contained twice the amount of fermentation acids and more ammonia N than the restrictively fermented silage, thus reflecting a greater extent of fermentation and proteolysis (McDonald et al., 1991).

Ruminal Fermentation Characteristics

Compared with formic, inoc reduced ($P < 0.03$; Table 3) ruminal pH. This effect was related to the greater VFA content, because pH and total VFA were negatively correlated ($r = -0.82$; $P < 0.01$). Ruminal NH₃-N paralleled the concentration of the rapidly degradable protein fraction (fraction a, % of CP), as determined in a parallel in situ study using the same forages (Martineau et al., 2006). The lower ($P < 0.01$) ruminal NH₃-N observed with hay than with silages is in agreement with the findings of Shingfield et al. (2002), whereas the reduced ($P = 0.04$) ruminal NH₃-N with restrictively fermented silage, compared with extensively fermented silage, is in accordance with the findings of Keady et al. (1995). Results suggest that haying and restricting fermentation in silage decreased the degradation of N in the rumen or increased the uptake of degraded N by ruminal microbes, because silage VFA (Chamberlain, 1987) and lactic acid (Jaakkola and Huhtanen, 1992) are poor ATP substrates for ruminal microbes compared with WSC.

The molar proportion of propionate was lower for hay than silages ($P = 0.03$) and for formic vs. inoc ($P = 0.07$). However, the molar proportion of acetate and the ratio of acetate to propionate were greater for hay than silages ($P = 0.02$) and for formic vs. inoc ($P = 0.04$). Ruminal fermentation patterns with relatively high proportions of lipogenic short-chain fatty acids and small proportions of propionate are typical of diets based on hay compared with silage (Vagnoni and Broderick, 1997; Shingfield et al., 2002), or based on restrictively fermented silage in comparison with extensively

Table 3. Effects of the method of conservation of timothy on ruminal fermentation characteristics, milk production, and milk composition of dairy cows

Item	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
pH	6.3	6.5	6.3	0.1	0.43	0.03
NH ₃ -N, mg/dL	8.0	9.6	11.0	0.7	<0.01	0.04
Total VFA, mM	123.1	120.3	129.3	3.5	0.68	0.08
VFA, mol/100 mol						
Acetate (A)	69.4	68.8	66.8	0.3	<0.01	<0.01
Propionate (P)	15.3	16.3	17.7	0.5	0.03	0.07
Butyrate	10.2	9.9	10.1	0.5	0.73	0.73
A:P	4.55	4.23	3.79	0.14	0.02	0.04
Milk production, kg/d	23.9	24.4	22.4	1.9	0.71	0.21
4% FCM, ³ kg/d	23.1	23.0	22.8	2.6	0.81	0.85
Milk composition, %						
Fat	3.77	3.59	4.00	0.27	0.80	0.02
Protein	2.92	2.90	2.97	0.10	0.93	0.61
Lactose	4.79	4.89	4.83	0.09	0.50	0.53
CN N, % of total N	89.2	89.1	89.3	1.2	0.95	0.65
MUN, mg/dL	7.20	6.69	8.94	0.64	0.21	0.01
Milk yield, kg/d						
Fat	0.91	0.88	0.92	0.13	0.89	0.32
Protein	0.69	0.71	0.66	0.07	0.90	0.17
Lactose	1.15	1.19	1.10	0.11	0.93	0.33

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

³4% FCM = 0.4 × (milk, kg/d) + 15.0 × (fat, kg/d).

fermented silage (van Vuuren et al., 1995; Huhtanen, 1998). Those changes in the ruminal fermentation pattern were previously linked to differences in WSC (van Vuuren et al., 1995) and lactic acid (Martin et al., 1994; van Vuuren et al., 1995) concentrations in the forage.

Milk Production and Milk Composition

Production of milk and 4% FCM averaged 23.4 and 23.0 kg/d, respectively, and was not affected by the method of conservation of timothy (Table 3). Milk fat concentration was lower (*P* = 0.02) with formic than with inoc, but milk fat yield was unaffected. van Vuuren et al. (1995) reported that feeding silages of restricted fermentation, as compared with high-lactate silages, consistently increased the milk fat content, likely because silages rich in WSC favor a rumen fermentation pattern rich in acetate, butyrate, or both, thereby changing the lipogenic:glucogenic VFA ratio. In our study, the milk fat content was lower with formic than with inoc possibly due to a dilution effect, because milk production was numerically greater. The method of conservation of timothy did not affect milk protein concentration and yield.

Digestion of OM and NDF

The recovery rates of Cr were not statistically different among treatments (Table 4) and the duodenal DM

flows were based on mean individual fecal excretion of Cr during the total collection of feces. Intakes of DM averaged 17.1 kg/d and were unaffected by treatment. The disappearance of digestible true OM before the small intestine tended (*P* = 0.09) to be lower with hay than with silages, whereas the apparent total tract digestibility was unaffected by treatments, suggesting a greater extent of OM digestion in the intestine with hay than with silages.

Intake of NDF was higher (*P* = 0.04) with hay than with silages without affecting the apparent total tract digestibility, because the fecal flow of NDF was concomitantly increased (*P* = 0.02). The disappearance of digestible NDF before the small intestine was higher (*P* = 0.02) with formic compared with inoc, suggesting that the hydrolysis of hemicellulose during ensiling was less severe, thereby increasing the digestibility of remaining NDF (McDonald et al., 1991).

Digestion of N and Efficiency of Microbial Synthesis

The lower CP (or N) percentage in hay resulted in lower N intake because DMI (17.1 kg/d) were not different across treatments (Table 5). Expressed as a proportion of N intake, total tract apparent digestibility of N was lower (63.8 vs. 68.2%; *P* = 0.02) with hay than with silages because the lower N intake was not followed by a reduced fecal N output. Total tract ap-

Table 4. Effects of the method of conservation of timothy on OM and NDF digestion in dairy cows

Item	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
Chromium recovery rate, %	90.3	84.7	84.7	2.5	0.15	0.99
DMI, kg/d	17.3	17.0	17.0	1.6	0.45	0.91
OM flows, kg/d						
In feed	16.3	15.9	15.8	1.5	0.28	0.81
At duodenum	9.2	8.3	8.5	0.8	0.29	0.71
In feces	4.6	4.4	4.1	0.4	0.13	0.15
OM apparent digestibility, %						
In the rumen	44.8	47.6	46.5	2.9	0.53	0.77
In the rumen, true ³	54.5	61.5	59.0	3.5	0.11	0.42
In the intestine ⁴	49.8	47.1	51.8	2.2	0.87	0.12
In the total tract	72.6	72.4	74.3	1.0	0.54	0.21
OM disappearance before small intestine, ⁵ %						
Apparent	61.7	65.7	62.4	3.5	0.57	0.47
True, ³ %	75.5	84.9	79.3	4.2	0.09	0.13
NDF flows, kg/d						
In feed	7.2	6.4	6.0	0.6	0.04	0.26
At duodenum	2.4	2.3	2.4	0.2	0.53	0.50
In feces	2.7	2.5	2.2	0.2	0.02	0.02
NDF digestibility, %						
In the rumen	66.9	64.8	61.1	2.1	0.21	0.26
In the total tract	63.3	60.6	64.3	1.8	0.70	0.19
NDF disappearance before small intestine, ⁵ %	1.06	1.07	0.95	0.03	0.14	0.02

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

³Assuming that ruminal bacteria contain 10% N (Van Soest, 1994) and 10% ash on a DM basis.

⁴Duodenal flow minus fecal flow divided by duodenal flow.

⁵Amount digested before the small intestine, divided by the amount digested in the total tract.

parent N digestibility is often reduced at low N intake compared with higher N intake because of similar endogenous N secretions in feces. Secretion of milk N (as a percentage of N intake) was greater ($P \leq 0.04$) with hay compared with silages, and with formic compared with inoc. Comparison between hay and silages should take into account a lower CP intake for cows fed hay. Nitrogen excreted in urine was lower ($P = 0.01$) with hay than with silages and with formic compared with inoc. However, as a proportion of N intake, only N excreted in urine tended ($P = 0.09$) to be lower with formic than with inoc. Approximately 59% of urinary N losses were in the form of urea N, and this proportion tended (57.3 vs. 61.1%; SEM = 1.3; $P = 0.07$) to be lower with formic than with inoc. Overall, N balance results suggest that cows fed the silages were producing milk at maximal capacity, and that the excess dietary N was either retained or excreted to urine. By contrast, even though N intake was lower with the hay diet, the available absorbed N appeared sufficient, so the cows did not need to store "excess" N and urine excretion was normal.

Despite the 14% lower N intake when hay was fed compared with silages, duodenal N flows were not different; therefore, a difference in milk protein yield would not be expected. The net amount of N recycled

prior to the small intestine was higher ($P = 0.02$) and, conversely, the apparent digestibility of N in the rumen was lower ($P = 0.01$) with hay compared with silages, suggesting that more N was captured as MN or more feed N was undegraded when hay was fed. The disparity between the values of the apparent and the true digestibility in the rumen reflected the large net recycling of N to the rumen in our study. The lower proteolysis of hay during haying meant less soluble N. In consequence, more insoluble N was present to pass through the rumen. On the other hand, the true digestibility of N in the rumen was similar between silages, suggesting that N degradability in the rumen was not affected by the method of conservation.

Efficiencies of MN synthesis were not affected by treatments (Table 5). In contrast to our results, Huh-tanen et al. (1997) reported a 12% increase in efficiency of MN synthesis on restrictively fermented silage compared with extensively fermented silage. In their study, WSC contents of both silages were higher than the presently reported values, and WSC concentration of the restrictively fermented silage was nearly 3-fold greater than that of the extensively fermented silage (17.7 vs. 6.8% of DM), and therefore contrasted more than in our study.

Table 5. Effects of the method of conservation of timothy on N digestion and microbial synthesis in dairy cows

Item	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
N intake, g/d	348	398	414	37	0.02	0.28
Feces	127	127	133	13	0.59	0.39
Milk	108	111	104	11	0.90	0.17
Urine	93	101	116	9	0.01	0.01
N retained	21	59	62	9	0.02	0.80
% of N intake						
Feces	36.2	31.9	31.7	0.9	0.02	0.81
Milk	30.9	28.2	24.7	0.8	0.02	0.04
Urine	27.0	25.4	28.4	1.1	0.96	0.09
N retained	5.9	14.4	15.2	1.9	0.03	0.74
Duodenal flow, g/d						
Total N	403	387	400	42	0.51	0.41
NH ₃ -N	10	12	13	1	0.08	0.60
Microbial N	187	248	230	50	0.16	0.57
Feed N ³	171	93	124	29	0.10	0.34
Net N recycling before small intestine, ⁴ g/d	54	-12	-15	17	0.02	0.85
N apparent digestibility, %						
In the rumen	-14.6	3.3	4.4	3.7	0.01	0.80
In the rumen, true ⁵	51.7	75.7	68.5	8.6	0.06	0.40
In the intestine ⁶	68.7	66.7	66.7	1.3	0.06	0.93
Efficiency of microbial synthesis						
Microbial N/OMADR, ⁷ g/kg	27.2	32.7	30.5	5.1	0.50	0.74
Microbial N/OMTDR, ⁷ g/kg	21.8	25.2	23.4	3.1	0.53	0.68
Microbial N/N intake, g/g	0.54	0.61	0.53	0.08	0.65	0.30

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

³Total N - (NH₃-N + microbial N + endogenous N), where endogenous N (g/d) = 1.9 × DMI (kg/d) (NRC, 2001).

⁴Total N flow at duodenum minus intake of N.

⁵Intake of N minus feed N flow at duodenum divided by intake of N.

⁶Duodenal flow minus fecal flow divided by duodenal flow.

⁷OM apparently (OMADR) or truly (OMTDR) digested in the rumen.

WB Protein Metabolism

Leucine kinetics were unaffected by treatments except for estimation of absorbed Leu, which was higher (*P* = 0.03) with formic than with inoc (Table 6). The CER averaged 14.0 mol/h and was unaffected by treatments (SEM = 1.5; data not shown). Whole-body ILR of Leu was similar to values reported earlier in dairy cows by Bequette et al. (1996), Lapierre et al. (2002), and Raggio et al. (2006). Using MOP IE rather than Leu IE as representative of the precursor pool increased ILR by 31%. Because MOP is formed intracellularly from deamination of Leu arising from plasma as well as from protein breakdown, MOP released in the plasma has a lower IE than plasma Leu.

The FO averaged 10.9%, being in the low range of previous reports (Lapierre et al., 2002; Raggio et al., 2006), which was probably linked to the low protein supply in the present study. Leucine used for protein synthesis yielded an estimated WB protein synthesis of 4.3 kg/d (SEM = 0.7; *P* = 0.65), of which 687 g/d was

secreted as milk protein, indicating the high protein turnover in lactating dairy cows. Estimated Leu absorbed was 116, 136, and 114 g/d or 76, 109, and 87% of Leu apparently digested for hay, formic, and inoc, respectively (Table 7).

AA Flows and Digestion

Intakes of essential AA (**EAA**; except Arg because of its greater content with hay) and branched-chain AA (**BCAA**) were, on average, 17% lower (*P* ≤ 0.05; data not shown) with hay than with silages. However, duodenal flow of AA was unaffected by treatments (data not shown). The duodenal profile of EAA closely matched that predicted by NRC (2001), except for His, which was 2-fold greater (7.8% of EAA) than the flow predicted by NRC (2001). Such discrepancies are difficult to explain because microbial (approximately 4% of EAA; NRC, 2001) and endogenous proteins (5.2% of EAA in ruminal epithelial cells; Larsen et al., 2000)

Table 6. Effects of the method of conservation of timothy on whole-body Leu kinetics in dairy cows

Leucine, mmol/h	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
ILR MOP ³	102.2	111.1	102.1	15.9	0.51	0.23
ILR Leu ³	84.3	78.6	78.2	6.5	0.12	0.90
Fractional rate of oxidation, ⁴ %	13.0	11.5	8.2	2.0	0.19	0.19
Oxidation ⁴	13.4	13.8	8.0	3.6	0.45	0.15
Used for protein synthesis ⁴	89.6	97.3	94.2	13.9	0.44	0.69
Secreted in milk protein	21.5	22.1	20.6	2.1	0.90	0.17
Used in nonmilk protein	68.1	75.3	73.5	12.1	0.41	0.81
From protein breakdown ⁴	65.6	67.9	65.8	11.8	0.84	0.76
Absorbed ⁴	36.9	43.2	36.3	5.8	0.20	0.03

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

³Irreversible loss rate (ILR_{tracee}) calculated with the jugular enrichment of 4-methyl 2-oxopentanoate (MOP) or Leu as representative of the precursor pool.

⁴Estimated using the isotopic enrichment of MOP as representative of the precursor pool.

are not particularly rich in His. Fecal flow of EAA was unaffected by treatments; however, the proportion of fecal total AA as EAA was lower (48.7 vs. 49.2%; SEM = 0.2; *P* = 0.01) with hay compared with silages. The apparent intestinal digestibilities of EAA, non-EAA, BCAA, and total AA averaged 75, 72, and 74% for hay, formic, and inoc, respectively, and tended (*P* ≤ 0.08) to be higher for hay compared with silages and lower with formic than with inoc. The apparent intestinal digestibilities ranged from 67% for Met to 78% for His.

The flows of digestible EAA to the small intestine calculated using NRC (2001) were 637, 718, and 750 g/d for hay, formic, and inoc, respectively, whereas the measured amounts of apparently digested EAA were 749, 660, and 699 for hay, formic, and inoc, respectively (Table 7). The discrepancy between the NRC (2001) prediction and our result for hay is likely because NRC (2001) does not account for recycled N captured as MN when the RDP supply is deficient, thereby leading to MP being underestimated.

Table 7. Effects of the method of conservation of timothy on AA apparently digested in the intestine of dairy cows

AA, g/d	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
Arg	75	71	74	8	0.46	0.51
His	64	55	56	8	0.30	0.83
Ile	71	68	73	9	0.59	0.01
Leu	153	125	131	15	0.13	0.66
Lys	108	96	103	12	0.23	0.27
Met	28	24	26	3	0.06	0.15
Phe	82	73	78	9	0.21	0.37
Thr	79	70	75	9	0.29	0.50
Val	91	78	83	9	0.17	0.45
Ala	102	93	94	11	0.17	0.81
Asx	167	151	160	18	0.25	0.37
Cys	24	20	21	3	0.19	0.68
Glx	228	198	207	25	0.15	0.55
Gly	89	98	93	7	0.22	0.37
Pro	74	64	67	8	0.22	0.64
Ser	80	70	71	8	0.21	0.84
Essential AA	749	660	699	81	0.19	0.44
Nonessential AA	764	694	714	76	0.23	0.66
Branched-chain AA	314	271	287	33	0.14	0.44
Total AA	1,513	1,354	1,413	156	0.21	0.53

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

Table 8. Effects of the method of conservation of timothy on concentrations of urea N, glucose, and AA in plasma of dairy cows

Item	Conservation method ¹			SEM	Contrast, ² <i>P</i> -value	
	Hay	Formic	Inoc		H vs. F + I	F vs. I
Urea N, mg/dL	6.8	7.6	8.5	0.9	0.28	0.40
Glucose, mM	3.2	3.2	3.3	0.1	0.78	0.79
AA, μ M						
His	25	20	19	4	0.15	0.72
Ile	112	110	104	6	0.27	0.18
Leu	141	134	129	6	0.12	0.34
Lys	71	69	71	6	0.81	0.79
Met	19	19	17	1	0.16	0.03
Phe	40	36	36	1	0.11	0.93
Thr	81	84	76	5	0.81	0.06
Trp	50	54	48	3	0.73	0.08
Val	198	190	168	7	0.11	0.09
Ala	240	268	255	11	0.14	0.33
Gln	235	252	272	6	0.01	0.03
Glu	60	63	59	4	0.64	0.22
Gly	259	274	303	15	0.16	0.18
Ser	73	72	74	4	0.98	0.71
Tyr	42	42	40	2	0.74	0.63
Essential AA (EAA)	733	717	668	28	0.10	0.07
Nonessential AA	906	971	1,004	16	0.02	0.20
Branched-chain AA	448	434	401	18	0.11	0.10
Total AA (TAA)	1,647	1,689	1,672	22	0.09	0.33
EAA, % of TAA	45	42	40	1	0.06	0.12

¹Hay = haying; formic = restrictively fermented silage; inoc = extensively fermented silage; least squares means with SEM given for *n* = 3.

²Contrast: H vs. F + I = hay vs. silages; F vs. I = formic vs. inoc.

Blood Metabolites

Plasma urea N was unaffected by treatments (Table 8), but was correlated with ruminal $\text{NH}_3\text{-N}$ ($r = 0.66$; $P = 0.03$) and MUN ($r = 0.71$; $P = 0.01$). The proportion of plasma total AA as EAA tended ($P = 0.06$) to be greater with hay compared with silages because of a tendency for an increase ($P = 0.10$) in plasma EAA and a decrease ($P = 0.02$) in plasma non-EAA. Concentrations of all EAA, except Trp, were numerically greater with hay than with silages, whereas the concentration of Gln was lower ($P = 0.01$) and concentrations of Ala, Glu, and Gly were numerically lower with hay compared with silages. When comparing silage types, concentrations of EAA, BCAA, Met, Thr, Trp, and Val tended ($P \leq 0.10$) to be higher with formic than with inoc, whereas the opposite was true for Gln ($P = 0.03$).

Miettinen and Huhtanen (1997) reported lower plasma glucose concentrations and markedly higher ratios of lipogenic to glucogenic ruminal VFA concentrations (Huhtanen et al., 1997) in cows fed a restrictively fermented silage compared with an extensively fermented silage. In our study, the ratio of ruminal acetate to propionate was increased by restricting silage fermentation (Table 3), but without a concomitant effect on plasma glucose concentrations (Table 7). The

ratios of ruminal acetate to propionate were negatively correlated with plasma concentrations of Gln ($r = -0.83$; $P < 0.01$) and Gly ($r = -0.79$; $P < 0.01$), suggesting a possible use of Gln and Gly for gluconeogenesis. However, other glucogenic sources (e.g., glycerol, lactate, pyruvate) have not been assessed in this study.

CONCLUSIONS

In the conditions of the present study, both formic and inoc methods produced silages with good fermentation quality. Haying reduced feed protein degradation in the rumen, whereas this effect was not observed when restricting fermentation in the silage. Haying and restricting fermentation in silages resulted in a better N utilization, as indicated by lower ruminal ammonia, reduced urinary excretion of N, and an increased efficiency of milk protein secretion (as a proportion of N intake). Haying and restricting fermentation in silages induced a lipogenic fermentation pattern in the rumen but did not affect the yield of milk components and concentration of plasma glucose.

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