Effects of Feeding Formate-Treated Alfalfa Silage or Red Clover Silage on Omasal Nutrient Flow and Microbial Protein Synthesis in Lactating Dairy Cows¹

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ABSTRACT

Eight ruminally cannulated Holstein cows that were part of a larger lactation trial were blocked by days in milk and randomly assigned to replicated 4 × 4 Latin squares to quantify effects of nonprotein N (NPN) content of alfalfa silage (AS) and red clover silage (RCS) on omasal nutrient flows. Diets, fed as total mixed rations, contained 50% dry matter from control AS (CAS), ammonium tetraformate-treated AS (TAS), late maturity RCS (RCS1), or early maturity RCS (RCS2). Silages differed in NPN and acid detergent insoluble N (% of total N): 50 and 4% (CAS); 45 and 3% (TAS); 27 and 8% (RCS1); 29 and 4% (RCS2). The CAS, TAS, and RCS2 diets had 36% high-moisture shelled corn and 3% soybean meal, and the RCS1 diet had 31% high-moisture shelled corn and 9% soybean meal. All diets contained 10% corn silage, 27% neutral detergent fiber, and 17 to 18% crude protein. Compared with RCS, feeding AS increased the supply of rumen-degraded protein and omasal flows of nonammonia N and microbial protein, which may explain the improved milk yield observed in the companion lactation trial. However, omasal flow of rumen-undegraded protein was 34% greater on RCS. Except for Arg, omasal flows of individual AA, branched-chain AA, nonessential AA, essential AA, and total AA did not differ between cows fed AS vs. RCS. Within AS diets, no differences in omasal AA flows were observed. However, omasal flows of Asp, Ser, Glu, Cys, Val, Ile, Tyr, Lys, total nonessential AA, and total AA all were higher in cows fed RCS1 vs. cows fed RCS2.

In this trial, there was no advantage to reducing NPN content of hay-crop silage.

Key words: silage nonprotein nitrogen, omasal flow, microbial protein synthesis, dairy cow

INTRODUCTION

Greater concern regarding environmental N pollution has increased the importance of implementing nutritional strategies to minimize urinary N excretion. There is strong evidence that the high proportion of NPN in hay-crop silages results in depressed formation of microbial protein in the rumen when these are fed as the principal dietary forages (Dewhurst et al., 2000). Protein in alfalfa silage (AS) is extensively degraded to NPN (Muck, 1987). Nagel and Broderick (1992) showed that formic acid treatment of wilted AS decreased NPN formation and substantially improved N utilization when fed to lactating dairy cows. As a result of the polyphenol oxidase system in red clover (Jones et al., 1995), NPN formation is much reduced in red clover silage (RCS; Papadopoulos and McKersie, 1983; Albrecht and Muck, 1991). When RCS replaced dietary AS in 5 previous trials, energy digestibility was greater and there was evidence of improved N efficiency, although yield of milk and milk components was reduced in 2 trials and DMI was depressed in all 5 trials (Broderick et al., 2000, 2001).

We conducted 2 production studies (Broderick et al., 2007) to test the effects of using ammonium tetraformate (ATF) to reduce NPN content in AS and to compare AS (with about 50% of CP present as NPN) to RCS (with about 30% of CP present as NPN). The omasal sampling technique was used in the second of these trials with several objectives: 1) to determine the effects of feeding AS or RCS with differing NPN contents on ruminal digestibility and omasal flow of nutrients and microbial protein in lactating dairy cows; 2) to relate any differences in production and urinary N excretion in the parallel trial (Broderick et al., 2007) to differences in microbial protein synthesis and nutrient digesta flow; and 3) to compare NRC (2001) model predictions

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of RDP, RUP, and microbial NAN flow with in vivo measurements.

MATERIALS AND METHODS

Animals and Diets

Eight ruminally cannulated multiparous Holstein cows, averaging (mean $\pm$ SD) 210 ± 37 DIM and 575 ± 44 kg of BW at the beginning of the study were blocked by DIM and randomly assigned to treatments within 2 replicated 4×4 Latin squares. These animals were part of a larger trial investigating effects of replacing dietary AS with RCS on milk production (Broderick et al., 2007). Each experimental period lasted 28 d, consisting of 14 d for diet adaptation and 14 d for sample collection, except for period 3, where 14 d were allotted for diet adaptation and 7 d for sample collection. This was necessary due to a shortage of one RCS and only 3 treatments were fed in the last period. Dietary sequences within Latin squares were organized to balance carryover effects in succeeding periods (i.e., each treatment followed every other treatment once in each square). Diets were fed as 4 TMR containing (% DM): 50.4% control AS (**CAS**), 9.9% rolled corn silage (**CS**), 36.1% rolled high-moisture shelled corn (**HMSC**), 3.2% solvent soybean meal (**SSBM**), and 17.7% CP; 50.4% ATF-treated AS (**TAS**), 9.9% CS, 36.1% HMSC, 3.2% SSBM, and 18.4% CP; 49.7% late maturity RCS (**RCS1**), 10% CS, 30.6% HMSC, 9.2% SSBM, and 17.2% CP; and 50.6% early maturity RCS (**RCS2**), 9.8% CS, 35.8% HMSC, 3.4% SSBM, and 17.2% CP. Diets averaged 27% NDF. Other details of the trial, including dietary ingredients and composition, are described in the companion report (Broderick et al., 2007). Care and handling of the animals including ruminal cannulation was conducted as outlined in the guidelines of the University of Wisconsin institutional animal care and use committee.

Sampling and Laboratory Analysis

Spot samples of omasal digesta leaving the rumen were obtained from the ruminally cannulated cows using the omasal sampling technique described by Huhtanen et al. (1997), Ahvenjärvi et al. (2000), and Reynal and Broderick (2005). The omasal digesta markers used were indigestible NDF (**INDF**; Huhtanen et al., 1994) for the large particle phase (**LP**), YbCl_3 (modified from Siddons et al., 1985) for the small particle phase (**SP**), and CoEDTA (Udén et al., 1980) for the fluid phase (**FP**). A marker solution containing YbCl_3 , CoEDTA, and $^{15}\text{NH}_4\text{SO}_4$ with 95 atom % excess (**APE**) ^{15}N (Isonics Corporation, Columbia, MD) was prepared as described by Reynal and Broderick (2005). Just before beginning marker infusion, samples of whole ruminal contents

were taken from each cow to determine background abundance of ^{15}N . The marker solution was continuously infused into the rumen for approximately 135 h at a constant rate of 2.66 L/d (providing 2.19 g of Co, 2.76 g of Yb, and 0.21 g of ^{15}N /d) from d 21 to 26 (d 14 to 19 in period 3) using 2 syringe pumps (model no. 33; Harvard Apparatus, Inc., Holliston, MA). Omasal sampling began approximately 64 h after beginning marker infusion. Samples were taken 4 times daily at 2-h intervals over 3 consecutive days to represent the 24 h-feeding cycle: 0000, 0200, 0400, and 0600 h (d 24 in periods 1, 2, and 4; d 17 in period 3), 0800, 1000, 1200, and 1400 h (d 25 in periods 1, 2, and 4; d 18 in period 3), and 1600, 1800, 2000, and 2200 h (d 26 in periods 1, 2, and 4; d 19 in period 3). Before each sampling, it was necessary to confirm the location of the sampling tube and occasionally it had to be repositioned into the omasal canal. Sometimes it also was necessary to unplug the holes in the end of the sampling tube because of the presence of coarse digesta. At each sampling time, 200 mL of omasal digesta was collected, transferred to 3-L containers, and stored at -20°C . Spot samples were pooled over the 3 d to obtain a 2.4-L composite from each cow in each period for later separation into the omasal phases (LP, SP, and FP). At the second and fourth time points each day, 500-mL samples of omasal digesta were taken for isolation of bacteria and protozoa.

Protozoa were isolated using a modification of the procedure of Hristov et al. (2001). The first of the 500-mL samples was squeezed through 2 layers of cheesecloth and retained solids were washed with 500 mL of McDougall's (1948) buffer, containing 0.5 g of glucose and 50 mg of cysteine-HCl/100 mL, that had been warmed to 39°C . Filtrates were transferred to 1-L separatory funnels and placed in a waterbath at 39°C . After approximately 45 min, separatory funnels were removed from the waterbath and the distinct white layers of sedimented protozoa forming at the bottom of each separatory funnel were carefully drawn off into vials and stored on ice until transported to the laboratory. At the laboratory, 20 mL of sucrose solution (30% wt/vol) was added to 50-mL centrifuge tubes. Protozoal sediments were poured on top of the sucrose solution and tubes were centrifuged at $150 \times g$ for 3 min at 4°C . Supernatants were discarded and the pellets washed 3 times with saline (0.85% wt/vol of NaCl) followed by centrifugation at $1,200 \times g$ for 5 min at 4°C . These pellets were frozen for about 12 h, then transferred to vials and stored at -20°C until freeze-dried. Fluid-associated bacteria (**FAB**) and particle-associated bacteria (**PAB**) were isolated from the second of the daily 500-mL samples obtained from each cow on each sampling day using filtration and differential centrifugation

as described earlier in detail (Reynal and Broderick, 2005). Both the FAB and PAB pellets were stored at -20°C until freeze-drying. After freeze-drying, bacterial and protozoal samples were ground with a mortar and pestle and equal DM from each was pooled for each cow in each period for later analysis.

The 2.4-L pooled omasal composites were thawed at room temperature and separated into the 3 omasal phases (LP, SP, and FP) as described by Reynal et al. (2003), and were stored at -20°C until freeze-dried. After freeze-drying, these samples were ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA) and analyzed for markers. Concentrations of Co, Yb, and INDF in LP and SP, and Co and Yb in FP were determined using the methods detailed earlier (Reynal and Broderick, 2005). Marker concentrations were used to physically recombine DM from the freeze-dried FP, SP, and LP in the correct proportions to reconstitute the omasal true digesta (OTD) flowing out of the rumen using the triple-marker method of France and Siddons (1986). Concentrations of Co, Yb, and INDF were distinctly greater in, respectively, the FP, SP, and LP, thus allowing for successful application of the triple-marker method.

Omasal true digesta samples were analyzed for total N (Leco 2000, Leco Instruments, Inc., St. Joseph, MI), absolute DM (105 $^{\circ}\text{C}$), ash, and OM (AOAC, 1980), sequentially for NDF and ADF using heat stable α -amylase and Na_2SO_3 (Hintz et al., 1995), and for neutral detergent insoluble nitrogen (NDIN) and ADIN. Extracts also were prepared from OTD samples as follows: 10 mL of pH 2.2 buffer (77.5 mM Na citrate) was added to 0.5 g of dry sample and then vortexed. After 30 min at 39°C , extracts were centrifuged ($15,000 \times g$, 15 min, 4°C), and supernatants stored at -20°C for later analysis of ammonia and total free AA using assays (Broderick and Kang, 1980) adapted to flow-injection analysis (Lachat Quik-Chem 8000 FIA; Lachat Instruments, Milwaukee, WI). For determination of individual AA, OTD samples were hydrolyzed for 24 h at 110°C in vials containing 6 N HCl with 0.1% (wt/vol) phenol (Mason et al., 1979) sealed under a N_2 atmosphere. The ratio of sample to acid was about 1 mg of total N per 5 mL of 6 N HCl. After hydrolysis, samples were cooled, HCl removed by evaporation and the residue redissolved in pH 2.2-sample buffer containing norleucine as internal standard. Analysis of individual AA was conducted using ion exchange chromatography with ninhydrin detection (Beckman 6300 Amino Acid Analyzer; Beckman Instruments, Inc., Palo Alto, CA).

Samples of FAB, PAB, protozoa, and OTD were prepared for total NAN and ^{15}N analyses as follows: approximately 100 μg of N from each sample was weighed into tin cups (Elemental Microanalysis Ltd., Okeham-

ton, UK) followed by addition of 50 μL of 72 mM K_2CO_3 . The tin capsules were placed in 96-well microtiter plates and dried in a 60°C oven overnight to volatilize ammonia. Samples of ruminal digesta collected before infusion for determination of background abundance of ^{15}N were freeze-dried and ground to pass both a 1-mm Wiley mill screen and then a 0.5-mm Udy mill screen (Udy cyclone mill; Udy Corp., Fort Collins, CO). These samples also were prepared for NAN and ^{15}N analysis as described above. Nitrogen and ^{15}N were determined using a Carlo-Erba instrument interfaced to an isotope ratio mass spectrometer (University of California-Davis Stable Isotope Facility). Bacteria (FAB and PAB), protozoa, FP, and OTD also were analyzed for absolute DM, ash, and OM (AOAC, 1980).

Calculations

Omasal flow of NAN was determined by difference between total N and ammonia N flows. Total NAN flow at the omasum was assumed to be composed of PAB NAN, FAB NAN, and nonammonia nonmicrobial N (NANMN). The mean ($\pm\text{SD}$) background ^{15}N during trial was $0.36797 (\pm 0.000259)$ atom %. Thus, the ^{15}N APE above background (^{15}N enrichment) for samples from each cow in each period was calculated:

$$\begin{aligned} ^{15}\text{N enrichment } (^{15}\text{N APE}) &= ^{15}\text{N atom \%} \\ &- \text{background } ^{15}\text{N atom \%}. \end{aligned}$$

The FAB and PAB were assumed to be representative of bacteria flowing with, respectively, the FP and the SP plus LP (SP + LP). The particulate phase (PF) fractions used to compute PAB NAN flowing with the SP + LP were obtained by mixing pellets from the $1,000 \times g$ centrifugation step from FAB isolation (Reynal and Broderick, 2005) with the residues retained when the daily 500-mL omasal samples (used in bacterial isolation) were filtered through single layers of 55- μm Dacron mesh. The $1,000 \times g$ pellets were assumed to represent the small particles, and the residues retained on 55- μm Dacron mesh were assumed to represent the large particles. The PF fractions were also freeze-dried, sequentially ground through 1- and 0.5-mm screens, and analyzed for DM, ash, OM, NAN, and ^{15}N as described before. Omasal flows of PAB NAN and FAB NAN were calculated as:

$$\begin{aligned} \text{FAB NAN flow} &= \text{FP DM flow} \times \text{NAN in FP} \\ &\times (\text{FP } ^{15}\text{N APE} / \text{FAB } ^{15}\text{N APE}) \end{aligned}$$

$$\begin{aligned} \text{PAB NAN flow} &= \text{SP + LP DM flow} \times \text{NAN in PF} \\ &\times (\text{PF } ^{15}\text{N APE} / \text{PAB } ^{15}\text{N APE}) \end{aligned}$$

where FP APE was computed using the equations:

$$\text{FP APE} = (^{15}\text{N/g of FP})/(\text{mg of NAN/g of FP});$$

$$^{15}\text{N/g of FP} = (^{15}\text{N/g of OTD})$$

$$- [(\text{SP} + \text{LP DM flow}/\text{total DM flow}) \times (^{15}\text{N/g of PF})];$$

$$\text{mg of NAN/g of FP} = (\text{mg of NAN/g of OTD})$$

$$- [(\text{SP} + \text{LP DM flow}/\text{total DM flow})$$

$$\times (\text{mg of NAN/g of PF})].$$

This was done because centrifugation at $10,000 \times g$ was used to separate FP from SP when the 3 phases were isolated from pooled 2.4-L omasal samples (Reynal et al., 2003). Total microbial NAN, NANMN and RUP flows at the omasal canal, RDP supply, OM truly digested in the rumen (**OMTDR**), and efficiency of microbial NAN synthesis were calculated as follows:

$$\text{Total microbial NAN flow} = \text{FAB NAN flow}$$

$$+ \text{PAB NAN flow};$$

$$\text{NANMN flow} = \text{Total NAN flow}$$

$$- \text{Total microbial NAN flow};$$

$$\text{RUP flow} = \text{Total CP flow}$$

$$- (\text{Total microbial NAN flow} \times 6.25);$$

$$\text{RDP supply} = \text{Total CP intake} - \text{RUP flow};$$

$$\text{FAB or PAB DM flow} = \text{FAB or PAB NAN flow}/$$

$$(\% \text{ NAN FAB or PAB}/100);$$

$$\text{FAB or PAB OM flow} = \text{FAB or PAB DM flow}$$

$$\times (\% \text{ OM FAB or PAB})/100;$$

$$\text{Total microbial OM flow} =$$

$$(\text{FAB OM flow} + \text{PAB OM flow});$$

$$\text{OMTDR} = \text{OM intake}$$

$$- (\text{Omasal OM flow} - \text{Total microbial OM flow});$$

$$\text{Microbial NAN efficiency (g NAN/kg)} =$$

$$\text{Total microbial NAN flow} / \text{OMTDR},$$

where flows and intakes are in grams per day or kilograms per day and NAN concentrations are in grams per gram of OM.

Statistical Analysis

Data were analyzed using Proc Mixed in SAS (SAS Institute, 1999–2000) for a replicated 4×4 Latin square design according to the following model:

$$Y_{ijkl} = \mu + S_i + P_j + C_{k(i)} + T_l + ST_{il} + E_{ijkl}$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = effect of square i , P_j = effect of period j , $C_{k(i)}$ = effect of cow k (within square i), T_l = effect of treatment l , ST_{il} = interaction between square i and treatment l , and E_{ijkl} = residual error. All terms were considered fixed, except $C_{k(i)}$ and E_{ijkl} , which were considered random. The interaction term was removed from the model when $P \geq 0.25$. Preplanned, single degree of freedom orthogonal contrasts were constructed to assess effects of ATF treatment of AS (CAS vs. TAS; ATF contrast), RCS maturity (RCS1 vs. RCS2; maturity contrast), and silage source (CAS + TAS vs. RCS1 + RCS2; silage contrast). Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. All reported values are least squares means. When standard errors of the differences of the least squares means were not equal due to unequal replication, the largest standard error was reported.

RESULTS AND DISCUSSION

Omasal Nutrient Flow and Ruminal Digestibility

No differences were observed for DMI, DM flow, and DM apparently digested in the rumen, which averaged, respectively, 21.4, 13.5, and 8.0 kg/d across diets (Table 1). However, a trend ($P = 0.10$) for a greater amount of DM apparently digested in the rumen was detected in cows fed RCS2 vs. RCS1, possibly because of its earlier maturity. Greater DMI was observed in cows fed AS in the parallel production trial conducted with 24 animals (Broderick et al., 2007); lack of significance for DMI was probably due to fewer animals (8 ruminally cannulated cows) being used in this study. When ruminal DM digestibility was expressed as a percentage of DMI, there was a trend ($P = 0.09$) for greater digestibility on CAS vs. TAS, whereas digestibility on RCS1 was lower ($P = 0.02$) than that on RCS2. No significant silage effect was observed; however, apparent total tract digestibility of DM was higher on RCS than AS ($P < 0.01$; Broderick et al., 2007), suggesting greater intestinal DM digestion on the RCS diets. It is important to note that INDF was used as the internal marker for assessing ruminal digestibility whereas indigestible ADF was used to estimate total tract digestibility. Beside the different internal markers, pore size of the indigestible ADF bags was larger (21 μm) than for INDF bags (6 μm). Therefore, differences in marker methodology may account for

Table 1. Effects of feeding dietary forage as alfalfa silage or red clover silage on nutrient intake, flow at the omasal canal, and ruminal digestibility in lactating dairy cows

Item	Diets ¹				SED ²	Contrasts (<i>P</i> -value) ³		
	CAS	TAS	RCS1	RCS2		ATF	Maturity	Silage
DM								
Intake, kg/d	22.5	21.3	20.7	21.2	1.49	0.37	0.72	0.35
Flow, kg/d	13.7	13.8	13.7	12.6	0.78	0.91	0.18	0.21
Apparently digested in the rumen kg/d	8.76	7.49	7.03	8.70	0.97	0.16	0.10	0.69
% of DMI	38.2	33.9	34.0	41.1	2.73	0.09	0.02	0.43
OM								
Intake, kg/d	20.8	19.8	19.0	19.5	1.38	0.40	0.71	0.26
Flow, kg/d	10.8	10.8	10.5	9.56	0.67	0.94	0.19	0.09
Apparently digested in the rumen kg/d	9.99	8.99	8.49	10.0	0.93	0.24	0.12	0.70
% of OM intake	47.3	44.5	44.8	51.5	2.28	0.17	0.01	0.16
Truly digested in the rumen kg/d	14.6	13.6	12.4	14.0	1.00	0.28	0.12	0.18
% of OM intake	70.0	68.5	65.3	71.7	1.61	0.30	<0.01	0.49
NDF								
NDF intake, kg/d	6.03	5.69	5.70	5.71	0.38	0.33	0.97	0.56
NDF flow, kg/d	4.40	3.90	3.58	3.03	0.40	0.18	0.19	<0.01
Apparently digested in the rumen kg/d	1.63	1.79	2.12	2.67	0.25	0.46	0.04	<0.01
% of NDF intake	27.7	30.7	37.8	47.3	4.11	0.42	0.03	<0.01
ADF								
ADF intake, kg/d	4.06	3.79	3.48	3.41	0.25	0.24	0.80	0.01
ADF flow, kg/d	3.02	2.64	2.24	1.77	0.30	0.17	0.14	<0.01
Apparently digested in the rumen kg/d	1.04	1.15	1.24	1.62	0.20	0.56	0.07	0.02
% of ADF intake	26.4	29.6	36.2	47.7	5.69	0.50	0.04	<0.01

¹CAS = control alfalfa silage; TAS = ammonium tetraformate (ATF)-treated alfalfa silage; RCS1 = red clover silage 1; RCS2 = red clover silage 2.

²SED = standard error of the least squares means difference ($n = 8$ observations for CAS, TAS, and RCS1 treatments and $n = 6$ observations for RCS2 treatment).

³ATF = CAS vs. TAS; Maturity = RCS1 vs. RCS2; Silage = CAS + TAS vs. RCS1 + RCS2.

some of these results. Dewhurst et al. (2003) reported that cows fed diets based on RCS or AS had apparent ruminal DM digestibilities of, respectively, 36 and 28%. These authors also showed that ruminal outflow rate was more rapid on AS (6.1%/h) compared with RCS (4.8%/h), which might explain the lower ruminal DM digestibility observed on AS in their trial.

Organic matter intake, omasal OM flow, and OM apparently digested in the rumen did not differ and averaged, respectively, 19.8, 10.4, and 9.4 kg/d across diets (Table 1). However, ruminal OM digestibility as a proportion of intake was 6.7 percentage units greater ($P = 0.01$) on RCS2 vs. the more mature RCS1. Organic matter truly digested in the rumen was not different and averaged, respectively, 14.1 and 13.2 kg/d on the AS and RCS diets (Table 1). As a proportion of intake, OM truly digested in the rumen was 6.4 percentage units higher ($P < 0.01$) on RCS2 vs. RCS1 but did not differ within the 2 AS. The RCS1 was harvested at a later maturity with the intention of producing forage with NDF content similar to AS, as was done in earlier feeding trials (Broderick et al., 2000, 2001). However,

RCS1 contained 2 percentage units more NDF than the 2 AS and RCS2 (Broderick et al., 2007), reflecting the greater maturity of this forage that clearly depressed its ruminal energy digestibility.

Although NDF intake was similar among diets, cows fed AS consumed, on average, 0.48 kg/d more ADF ($P = 0.01$; Table 1) than those fed RCS, partly reflecting the greater ADF content of AS diets (Table 2; Broderick et al., 2007). Omasal flows of both NDF and ADF were higher ($P < 0.01$) on AS than on RCS diets, whereas the opposite was true for ruminal fiber digestibility (Table 1). Dewhurst et al. (2003) showed that a greater proportion of particles <2 mm were present in the rumen of cows fed the AS diet compared with the RCS diet and more small particles might have contributed to the more rapid passage rate observed for AS. This might have accounted for the lower fiber digestibility on AS in the present study. Dewhurst et al. (2003) speculated that thicker ruminal mats on RCS vs. AS resulted in greater entrapment of feed particles, longer ruminal retention times, lower passage rates, and greater fiber digestibility. Presumably, cows derive

Table 2. Effects of feeding dietary forage as alfalfa silage or red clover silage on omasal flow of nitrogen fractions in lactating dairy cows

Item ¹	Diets ²				SED ³	Contrasts (<i>P</i> -value) ⁴		
	CAS	TAS	RCS1	RCS2		ATF	Maturity	Silage
N intake, g/d	648	627	580	583	40	0.56	0.94	0.05
RDP supply								
g/d	3,330	3,045	2,378	2,595	224	0.19	0.68	<0.01
% of DMI	14.7	14.2	11.5	12.0	0.45	0.26	0.29	<0.01
Flow at omasal canal								
Total N, g/d	532	567	555	522	28	0.20	0.25	0.61
Total N, % of N intake	84.0	92.0	96.0	89.2	4.45	0.06	0.15	0.14
Ammonia N, g/d	5.84	5.14	3.67	4.03	0.86	0.38	0.68	0.01
Total free AA N, g/d	22.2	24.5	16.1	14.6	2.66	0.37	0.59	<0.01
NDIN, g/d	42.1	36.0	47.1	44.0	4.24	0.12	0.47	0.03
ADIN, g/d	12.8	11.8	8.82	7.82	1.16	0.34	0.40	<0.01
Fraction B ₃ , g/d	29.3	24.2	38.3	36.3	3.49	0.12	0.57	<0.01
NAN, g/d	526	560	552	518	28	0.19	0.25	0.67
NAN, % of N intake	83.1	91.1	95.3	88.5	4.38	0.05	0.14	0.12
NANMN, ⁴ g/d	109	134	196	174	17	0.12	0.22	<0.01
NANMN, % of NAN	21.0	23.9	35.6	33.3	2.48	0.21	0.35	<0.01
NANMN, % of N intake	17.4	22.0	33.9	29.7	2.49	0.05	0.11	<0.01
RUP								
g/d	717	871	1,246	1,111	104	0.13	0.22	<0.01
% of DMI	3.31	4.22	6.07	5.21	0.46	0.04	0.08	<0.01
FAB NAN								
g/d	121	122	135	151	15	0.93	0.29	0.04
% of microbial NAN	29.0	28.5	38.1	43.2	3.62	0.88	0.17	<0.01
PAB NAN								
g/d	296	304	221	200	23	0.71	0.36	<0.01
% of microbial NAN	71.0	71.5	61.9	56.8	3.62	0.88	0.17	<0.01
Total microbial NAN								
g/d	417	426	356	350	23	0.66	0.80	<0.01
% of total NAN	79.0	76.1	64.4	66.7	2.48	0.20	0.35	<0.01
Microbial N flow, ⁵ g/d	261	254	212	248	30	0.80	0.25	0.19
Microbial N flow, ⁶ g/d	275	270	248	266	31	0.87	0.58	0.48
Microbial efficiency								
g of NAN/kg of OMTDR	29.2	32.4	29.0	24.9	2.34	0.14	0.10	0.03

¹NDIN = neutral detergent insoluble N; Fraction B₃ = NDIN – ADIN; FAB NAN = fluid-associated bacteria NAN; PAB NAN = particle-associated bacteria NAN; NANMN = nonammonia nonmicrobial N; OMTDR = organic matter truly digested in the rumen.

²CAS = control alfalfa silage; TAS = ammonium tetraformate (ATF)-treated alfalfa silage; RCS1 = red clover silage 1; RCS2 = red clover silage 2.

³SED = standard error of the least squares means difference (n = 8 observations for CAS, TAS, and RCS1 treatments and n = 6 observations for RCS2 treatment).

⁴ATF = CAS vs. TAS; Maturity = RCS1 vs. RCS2; Silage = CAS + TAS vs. RCS1 + RCS2.

⁵Estimated from urinary allantoin excretion according to Vagnoni et al. (1997).

⁶Estimated from urinary excretion of total purine derivatives according to Vagnoni et al. (1997).

more energy with greater fiber digestion and, thus, should produce more milk. However, cows yielded less milk and milk components on RCS (Broderick et al., 2007), despite greater ruminal and total tract digestibility of fiber on RCS diets. This is somewhat difficult to explain but may be related to differences in energy partitioning between the 2 silage sources. It can be speculated that energy derived from this greater fiber digestibility was channeled to fat deposition rather than production, which is supported by the greater BW gains ($P < 0.01$; Broderick et al., 2007) in cows fed RCS instead of AS. As was observed for DM and OM, apparent ruminal digestibility of NDF and ADF as a propor-

tion of intake also was lower on RCS1 than on the less mature RCS2 (Table 1).

Microbial Protein Synthesis and Omasal Nitrogen Flow

Despite the greater N intake ($P = 0.05$; Table 2) in cows fed AS, omasal flows of total N and NAN did not differ, averaging, respectively, 544 and 539 g/d across diets, and do not explain the greater production of cows fed AS diets (Broderick et al., 2007). However, NAN entering the omasal canal derives from microbial protein, RUP, and endogenous CP, each of which has a

different intestinal digestibility with distinct AA profiles. Omasal flows of total N and NAN, expressed as percentages of N intake, also did not differ between silage pairs but tended to be greater ($P = 0.06$), or were greater ($P = 0.05$), respectively, on TAS than on CAS. Omasal flow of NANMN, representing the sum of RUP plus endogenous CP, averaged 64 g/d greater ($P < 0.01$) on RCS than on AS; 23 and 35% of the total NAN flow derived from NANMN on AS and RCS diets, respectively ($P < 0.01$; Table 2). As a percentage of N intake, omasal NANMN flow also was higher ($P < 0.01$) on RCS compared with AS and was greater ($P = 0.05$) on TAS than on CAS. Similarly, RUP flow was increased by 385 g/d ($P < 0.01$) on RCS diets, which can be explained by reduced proteolysis during ensiling due to the action of polyphenol oxidase in the red clover plant (Jones et al., 1995). During wilting, polyphenol oxidase forms o-quinones, which quickly react with red clover proteins to protect them against breakdown in the silo (Hatfield and Muck, 1999). These quinone-protein complexes might also be resistant to ruminal degradation, resulting in increased RUP. In fact, the RCS (particularly RCS1) contained more protein fraction B₃ (NDIN – ADIN) as well as more ADIN than the AS (Broderick et al., 2007), probably reflecting this interaction of o-quinones with red clover proteins.

Flow of FAB NAN was higher ($P = 0.04$) on RCS diets than on AS diets, whereas the opposite was true for PAB NAN flow ($P < 0.01$; Table 2). The FAB NAN averaged 29 (AS diets) and 41% (RCS diets) and PAB NAN 71% (AS diets) and 59% (RCS diets) of the total microbial NAN flow, which differed significantly between silage pairs (Table 2). Reynal and Broderick (2005) also observed that total microbial NAN flow was contributed mainly from PAB. Craig et al. (1987), using ¹⁵N as the microbial marker to distinguish between FAB and PAB, observed that 70 to 80% of microbial OM in whole ruminal contents was associated with the particulate phase. However, Hristov and Broderick (1996) found similar contributions from PAB and FAB to the total microbial NAN flow, possibly because the outflow rate of the fluid phase was 3 times greater than that of the particulate phase, which may also explain the greater contribution from FAB than PAB in the studies of Ahvenjärvi et al. (2002) and Olmos Colmenero and Broderick (2006). Discrepant results can also be associated with digesta flow measurements, methodology used to isolate bacteria, bacterial marker, and diet composition.

Omasal flow of total microbial NAN was, on average, 69 g/d greater ($P < 0.01$) in cows fed AS compared with those fed RCS, and 78 and 66% of the total NAN flow was contributed by microbial NAN on AS and RCS diets ($P < 0.01$), respectively (Table 2). However, no difference was observed in total microbial NAN flow within AS

and RCS. However, Dewhurst et al. (2003) found that cows fed diets containing RCS (either RCS alone or 50:50 mixtures of RCS plus white clover) yielded numerically greater microbial NAN flows than cows fed AS diets. Greater microbial protein synthesis on AS in the present trial may have been due to greater RDP supply on CAS and TAS diets (Table 2). Supply of RDP averaged 3,188 and 2,487 g/d, respectively, for AS and RCS diets ($P < 0.01$). Santos et al. (1998) reported that, in 29 comparisons from 15 metabolism trials, microbial N flow to the duodenum was significantly decreased by feeding high RUP sources in 10 comparisons and was numerically higher for soybean meal in 25 comparisons, indicating that replacing soybean meal with supplements rich in RUP might limit microbial protein synthesis due to inadequate RDP supply. Ipharraguerre and Clark (2005), using a meta-analysis approach, found an overall 7% depression ($P < 0.05$) in microbial NAN flow to the small intestine in response to RUP supplementation of dairy cows. Reynal and Broderick (2005) showed that reducing the RDP supply from 3,076 to 2,403 g/d, by replacing SSBM with expeller soybean meal, decreased the omasal flow of microbial NAN from 470 to 384 g/d. Olmos Colmenero and Broderick (2006) observed a linear increase in microbial NAN flow from 425 to 480 g/d when dietary RDP increased from 1,979 to 3,028 g/d.

Reduced RDP supply on RCS may have depressed microbial protein synthesis via lower ruminal concentrations of ammonia and total free AA on those diets (Broderick et al., 2007). Satter and Slyter (1974) reported that mixed populations of ruminal bacteria reach maximum yields of cell protein if the ruminal fluid has a minimum concentration of 5 mg of ammonia N/dL. Kang-Meznarich and Broderick (1980) found that microbial protein synthesis in the rumen was maximal at 8.5 mg ammonia N/dL. Ruminal ammonia N on both AS diets was never below 5 mg/dL and was greater than 8.5 mg/dL at all times except at prefeeding and 24 h postfeeding on CAS, and at prefeeding, 18, and 24 h postfeeding on TAS (Figure 1). However, ruminal ammonia N was lower than 8.5 mg/dL at prefeeding and from 4 to 24 h postfeeding on RCS1 and at prefeeding and from 8 to 24 h postfeeding on RCS2 (Figure 1). Ammonia is often the main N source for microbial protein synthesis (Nolan, 1975; Aharoni et al., 1991) and is essential for the growth of several species of ruminal bacteria (Blackburn, 1965; Allison, 1970; Bryant, 1973); however, preformed AA and peptides stimulated bacterial growth and yield in vivo (Chikunya et al., 1996) and in vitro (Argyle and Baldwin, 1989; Carro and Miller, 1999). In the present trial, greater ($P < 0.01$) omasal flow of total free AA N was observed on AS diets, which averaged 23 g/d vs. 15 g/d on the RCS diets

Figure 1. Effects of dietary forage source on ruminal ammonia concentrations after feeding (refer to Broderick et al., 2007, for further details). CAS = control alfalfa silage; TAS = ammonium tetraformate-treated alfalfa silage; RCS1 = red clover silage 1 (late maturity); RCS2 = red clover silage 2 (early maturity). Error bars = ± 1 SEM. A significant time $\times$ diet interaction ($P < 0.01$) was observed. Significant differences among treatments, which are indicated in the figure (*), occurred at: 1 h and 2 h (CAS and TAS were greatest, RCS2 was intermediate, and RCS1 was lowest); at 4 h (CAS was greatest, TAS was similar to CAS and RCS2 but different from RCS1, and RCS2 was different from RCS1); and at 8, 12, and 18 h (CAS and TAS were greater and RCS1 and RCS2 were lower).

(Table 2). Greater ruminal concentrations of total free AA N also were found on AS vs. RCS diets (Broderick et al., 2007). Thus, these data strongly suggest that increased RDP supply contributed more ruminal ammonia and free AA, and possibly peptides, for microbial protein synthesis.

Microbial efficiency was higher ($P = 0.03$) on AS than on RCS diets (Table 2). Although OM truly digested in the rumen was similar across diets, greater microbial efficiency on AS diets indicated that RDP rather than fermentable energy limited ruminal bacteria growth. The principal energy source was processed HMSC, which would be highly fermentable in the rumen. Ground shelled corn is not as rapidly digested (NRC, 2001); ruminal fermentable energy may have been more limiting had ground shelled corn replaced HMSC in the present study. Reynal and Broderick (2005) reported a linear increase in microbial efficiency as RDP was increased in 4 diets that did not differ in OM truly digested in the rumen, also suggesting that N supply was limiting. Olmos Colmenero and Broderick (2006) observed a linear increase in bacterial efficiency when RDP was increased from 1,979 to 3,028 g/d in diets that also did not differ in ruminal OM true digestibility. Even though AS diets improved microbial efficiency in the present study, this increase was not associated with improvement in overall N utilization. Cows fed AS excreted more N in urine and tended to be less efficient

Table 3. Comparisons between measured and NRC (2001)-predicted flows of nitrogen fractions at the omasum

Item	Diets ¹			
	CAS	TAS	RCS1	RCS2
Microbial flow, g/d				
Measured (using ¹⁵ N)	417	426	356	350
Estimated ² (using PD)	275	270	248	266
Predicted	328	325	310	306
RDP supply, g/d				
Measured	3,330	3,045	2,378	2,595
Predicted	3,292	3,360	2,842	2,884
RUP flow, g/d				
Measured	717	871	1,246	1,111
Predicted	1,128	1,134	1,171	1,031
Lys flow, g/d				
Measured	130	132	145	126
Predicted	191	189	190	180
Met flow, g/d				
Measured	48.0	48.4	49.2	45.3
Predicted	56.0	55.0	53.0	52.0
Lys:Met ratio				
Measured	2.74	2.77	2.99	2.83
Predicted	3.44	3.43	3.58	3.47
Metabolizable Lys flow, g/d				
Calculated ³	104	104	118	104
Predicted	152	150	155	144
Metabolizable Met flow, g/d				
Calculated ³	39.0	39.0	40.0	38.0
Predicted	45.0	44.0	43.0	42.0
Metabolizable Lys:Met ratio				
Calculated ³	2.71	2.72	2.98	2.79
Predicted	3.40	3.37	3.57	3.46

¹CAS = Control alfalfa silage; TAS = ammonium tetraformate (ATF)-treated alfalfa silage; RCS1 = late maturity red clover silage; RCS2 = early maturity red clover silage.

²Estimated from urinary excretion of total purine derivatives (PD) according to Vagnoni et al. (1997).

³Calculated using in vivo omasal flow of Lys and Met and intestinal digestibility coefficients of the NRC (2001) model.

converting feed N into milk N (Broderick et al., 2007). This suggested that any limitations in MP supply for milk protein were small and the extra microbial protein in cows fed AS diets was largely catabolized for energy and to urea N, rather than being captured in milk protein. The greater urinary N excretion also was driven by the greater N intake on AS than on RCS diets (Broderick et al., 2007).

Microbial N synthesis estimated using the NRC (2001) model averaged 327 and 308 g/d, respectively, for the AS and RCS diets, a mean overall underprediction of 18% compared with microbial yields measured using ¹⁵N (Table 3). Although the NRC (2001) model predicted a reduction of only 19 g/d of microbial NAN flow on RCS, the actual decrease observed was 69 g/d. Reynal et al. (2003) also reported that the NRC (2001) model underpredicted microbial protein synthesis by 50 to 89% when total purines were used as the internal microbial marker. Similarly, Reynal and Broderick (2005) found that microbial N flows predicted by the

NRC (2001) model were lower than in vivo measurements using ^{15}N as the bacterial marker. These apparently low estimates of microbial protein synthesis by the NRC (2001) model might be related to the bacterial marker used in its measurement. Most of the data used to develop the NRC (2001) prediction for microbial protein synthesis derived from studies in which microbial protein was quantified with the Zinn and Owens (1986) purine assay. Obispo and Dehority (1999) and Makkar and Becker (1999) both reported low recoveries of total purines using the Zinn and Owens (1986) method. Disproportionate recoveries of purines between bacterial and duodenal samples could lead to underestimates of microbial flow according to Reynal et al. (2003) who modified the Zinn and Owens (1986) assay to improve purine recovery in omasal samples. In addition, Reynal et al. (2005) reported less reliable estimates of microbial protein flow when total purines rather than ^{15}N were used as marker. Furthermore, many factors influencing microbial growth in the rumen, such as source and amount of RDP, ruminal pH and dilution rate, source and amount of dietary carbohydrate, and excessive levels of dietary fat, are not considered by the NRC (2001) model, which may lead to discrepancies between predicted and observed microbial protein flows.

Microbial N flows estimated from urinary excretion of either allantoin or total purine derivatives (PD) did not differ significantly across diets (Table 2), although an analysis of urinary allantoin excretion from all cows in the lactation study indicated that microbial protein synthesis was greater ($P = 0.02$) with the feeding of AS (Broderick et al., 2007). Urinary PD excretion also underpredicted microbial N flows by 32% compared with using ^{15}N as the microbial marker (Table 3). Perez et al. (1996) reported that microbial N flow in sheep fed alfalfa supplemented with incremental amounts of rolled barley grain was, on average, 29% lower using allantoin than ^{15}N . Similarly, Reynal et al. (2005) observed an average underestimation of 51% comparing PD to ^{15}N in lactating dairy cows. Despite underestimating microbial N flow, Perez et al. (1997) found that regression of PD on ^{15}N measurements indicated a good relationship between methods. Although both allantoin and PD underestimated microbial N flow in all of these studies, it was possible to detect significant treatment differences using urinary PD excretion, which support their usefulness as indirect estimators of microbial protein synthesis in the rumen.

The NRC (2001) model predicted a mean RDP supply that was 8.3% higher than that measured in vivo across all diets (Table 3); however, RDP supply was underpredicted by only 1.1% for CAS but overpredicted by 9.4, 16, and 10% for, respectively, TAS, RCS1, and RCS2. The NRC (2001) model overpredicted RUP flow by 36

and 23% for CAS and TAS and underpredicted RUP flow by 6.0 and 7.2% for RCS1 and RCS2 (Table 3). In the present trial, RUP flow was calculated by subtracting omasal microbial NAN flow from total N flow. Therefore, any inaccuracy in determination of microbial NAN flow would result in inaccurate RUP measurement and could have contributed to these differences. Moreover, RUP flows in the NRC (2001) model were computed from in situ data. Because of differences in methodologies, discrepancies between RUP flow estimated in situ vs. omasal RUP flow measured in vivo are to be expected and comparisons should be made cautiously.

AA Flow

Except for Arg, omasal flow of individual AA as well as omasal flow of branched-chain AA (BCAA), nonessential AA (NEAA), essential AA (EAA), and total AA did not differ significantly between silage pairs (Table 4). Omasal flow of Glu ($P = 0.10$) and His ($P = 0.08$) tended to be greater on cows fed RCS compared with AS diets. Based on these findings, the greater yields of 1.5 kg/d more milk and 95 g/d more protein observed when AS replaced RCS (Broderick et al., 2007) cannot be explained by differences in AA supply. The AA determined in the OTD were contributed by microbial protein, feed protein escaping ruminal degradation, and, to a lesser extent, endogenous protein. The lack of a significant silage effect on omasal AA flows probably occurred because greater NANMN supply (feed RUP plus endogenous protein) on RCS was counterbalanced by greater microbial NAN supply on AS (Table 2). Replacing dietary soybean meal with RUP increased the intestinal supply of NANMN but decreased that of microbial NAN (Ipharraguerre and Clark, 2005). Differences in yields of milk and milk protein observed in the companion lactation trial (Broderick et al., 2007) might have occurred because the profile of absorbed AA was closer to the EAA requirements of lactating cows (NRC, 2001) on AS diets. Microbial protein has an AA profile more closely resembling that of milk protein (Santos et al., 1998) and microbial NAN flow was greater when AS replaced RCS in the diet (Table 2). According to Clark et al. (1992), the CP source modulates the intestinal AA supply by altering flows of RUP and microbial N to the lower gastrointestinal tract. In addition, the contribution of RUP to total protein passage, and RUP AA composition, influence the pattern of AA absorbed at the small intestine (Rulquin and Verite, 1993; NRC, 2001). Therefore, it is also possible that the AA profile of RUP in cows fed AS was more complementary to microbial protein, thus improving milk production. However, differences in milk yield between silages

Table 4. Effects of feeding dietary forage as alfalfa silage or red clover silage on omasal flow of individual AA, branched-chain AA, essential AA, nonessential AA, and total AA

Amino acid, g/d	Diets ¹				SED ²	Contrasts (<i>P</i> -value) ³		
	CAS	TAS	RCS1	RCS2		ATF	Maturity	Silage
Asp	309	314	344	298	18	0.76	0.02	0.43
Thr	136	138	148	132	8.12	0.77	0.07	0.62
Ser	130	130	147	129	7.8	0.93	0.03	0.15
Glu	399	406	464	396	23	0.72	<0.01	0.10
Pro	119	117	130	119	8.2	0.81	0.20	0.27
Gly	148	149	157	142	8.1	0.87	0.08	0.87
Ala	199	182	186	168	20	0.34	0.35	0.30
Cys	28.4	34.5	24.6	40.3	6.95	0.34	0.04	0.83
Val	149	148	161	149	10	0.97	0.05	0.79
Met	48.0	48.4	49.2	45.4	3.30	0.88	0.25	0.68
Ile	122	122	132	113	9.0	1.00	0.05	0.92
Leu	224	227	253	227	15	0.83	0.09	0.16
Tyr	132	132	145	128	8.4	0.97	0.05	0.42
Phe	178	166	182	160	14	0.36	0.13	0.90
His	46.5	46.1	52.6	47.7	3.16	0.88	0.14	0.08
Lys	130	132	145	126	9.3	0.81	0.05	0.50
Arg	115	119	134	121	7.9	0.58	0.11	0.05
Lys:Met ratio	2.74	2.77	2.99	2.83	0.13	0.78	0.23	0.08
BCAA ⁴	494	497	546	480	33	0.93	0.06	0.46
EAA ⁵	1,148	1,147	1,257	1,112	74	0.99	0.06	0.48
NEAA ⁶	1,464	1,464	1,597	1,418	87	1.00	0.05	0.48
Total AA	2,612	2,611	2,854	2,529	159	0.99	0.05	0.48

¹CAS = control alfalfa silage; TAS = ammonium tetraformate (ATF)-treated alfalfa silage; RCS1 = red clover silage 1; RCS2 = red clover silage 2.

²SED = standard error of the least squares means difference (n = 8 observations for CAS, TAS, and RCS1 treatments and n = 6 observations for RCS2 treatment).

³ATF = CAS vs. TAS; Maturity = RCS1 vs. RCS2; Silage = CAS + TAS vs. RCS1 + RCS2.

⁴BCAA = branched-chain AA (Val, Ile, and Leu).

⁵EAA = essential AA (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val).

⁶NEAA = nonessential AA (Ala, Asp, Cys, Glu, Gly, Pro, Ser, and Tyr).

sources may also be explained by greater energy intake: cows fed CAS and TAS ingested on average 1.4 kg/d more DM and 2.1 Mcal/d more NE_L than those fed RCS1 and RCS2 (Broderick et al., 2007).

No significant differences in omasal flows of individual AA, BCAA, NEAA, EAA, and total AA were observed within AS diets (Table 4). However, omasal flows of Asp, Ser, Glu, Val, Ile, Tyr, Lys, NEAA, and total AA were significantly greater in cows fed RCS1 vs. RCS2 (Table 4); Lys flow was increased by 13%. Furthermore, omasal flows of Thr, Gly, Leu, BCAA, and EAA all tended to be greater when RCS1 replaced RCS2 in the diet. The RCS1 had greater proportions of NDIN, ADIN, and fraction B₃ (NDIN – ADIN; Broderick et al., 2007) and feeding RCS1 tended (*P* = 0.08) to increase RUP flow when expressed as percentage of DMI (Table 2). Because microbial NAN flow did not differ between the 2 RCS diets, the greater AA omasal flows likely were contributed by RUP from RCS1. Despite these differences in AA passing the omasal canal, production did not differ between the 2 RCS diets. Acid detergent insoluble nitrogen, fraction C of the Cornell Net Carbohydrate and Protein System, is partly composed of heat-

damaged proteins such as the Maillard products that are nutritionally unavailable (Sniffen et al., 1992); ADIN accounted for 7.5% of total N in RCS1 and 4.4% of total N in RCS2 (Broderick et al., 2007). Thus, it is possible that a substantial proportion of the AA, including Lys, entering the intestines on the RCS1 diet could not be absorbed and utilized.

Duodenal flows of Lys and Met predicted by the NRC (2001) model averaged 188 and 54 g/d among diets indicating an overall difference of 29 and 12% between in vivo and predicted data, respectively (Table 3). According to the NRC (2001) model, the optimal use of MP for both maintenance and milk protein production requires concentrations of Lys and Met in MP that approximate 7.2 and 2.4%, respectively, for an optimum MP Lys:Met ratio of 3:1. The measured Lys:Met ratio averaged 2.8:1, whereas that predicted by the NRC (2001) model was 3.5:1 across diets (Table 3), suggesting that Lys may have been marginally limiting on this trial. However, it should be noted that the observed Lys:Met ratios were closer to the optimum of 3:1 than those predicted by the NRC (2001) model. Although the Lys:Met ratio tended (*P* = 0.08; Table 4) to be greater

Table 5. Effects of feeding dietary forage as alfalfa silage or red clover silage on composition and isotopic enrichment of isolated ruminal microorganisms

Item	Diets ¹				SED ²	Contrasts (<i>P</i> -value) ³		
	CAS	TAS	RCS1	RCS2		ATF	Maturity	Silage
Fluid-associated bacteria								
OM, % of DM	82.3	82.0	80.5	81.8	0.92	0.66	0.17	0.13
NAN, % of DM	8.00	8.07	7.62	7.36	0.16	0.63	0.13	<0.01
¹⁵ N atom % excess	0.037	0.037	0.052	0.052	0.002	0.86	0.76	<0.01
Particle-associated bacteria								
OM, % of DM	86.6	86.3	85.5	86.1	0.35	0.31	0.08	0.01
NAN, % of DM	7.56	7.68	7.72	7.42	0.08	0.14	<0.01	0.36
¹⁵ N atom % excess	0.030	0.030	0.043	0.043	0.02	0.74	0.79	<0.01
Protozoa								
OM, % of DM	93.6	96.0	96.2	95.4	0.90	<0.01	0.38	0.13
NAN, % of DM	3.07	3.00	2.56	2.63	0.51	0.89	0.90	0.21
¹⁵ N atom % excess	0.031	0.031	0.043	0.041	0.003	0.84	0.43	<0.01

¹CAS = control alfalfa silage; TAS = ammonium tetraformate (ATF)-treated alfalfa silage; RCS1 = red clover silage 1; RCS2 = red clover silage 2.

²SED = standard error of the least squares means difference (n = 8 observations for CAS, TAS, and RCS1 treatments and n = 6 observations for RCS2 treatment).

³ATF = CAS vs. TAS; Maturity = RCS1 vs. RCS2; Silage = CAS + TAS vs. RCS1 + RCS2.

and was closer to the optimum on RCS, lower production on those diets also suggested that some Lys may have been unavailable for synthesis of milk and milk protein at the mammary gland.

Microbial Composition

No differences due to forage source were observed for the OM content of FAB, which averaged 82% across diets (Table 5). However, OM content of PAB was significantly higher ($P = 0.01$) for AS than RCS diets. In addition, a trend ($P = 0.08$) for greater OM content in PAB was found for RCS2 compared with RCS1. Organic matter content of PAB was numerically higher than that of FAB, which was in agreement with previous reports (Martin et al., 1994; Rodriguez et al., 2000). The NAN content of FAB was greater ($P < 0.01$) in cows fed AS rather than RCS (Table 5). Conversely, feeding either AS or RCS diets did not affect the NAN content of PAB. Within silages, RCS1 gave rise to greater ($P < 0.01$) PAB NAN content than RCS2, whereas no difference was found between CAS and TAS. The ¹⁵N enrichment of both FAB and PAB were lower ($P < 0.01$) on AS than on RCS diets, which may reflect greater dilution from the larger ammonia N pool when AS was fed (Broderick et al., 2007). On average, ¹⁵N enrichment of FAB was 18% higher than PAB (Table 5). This numerical difference could be attributed to differential utilization by PAB and FAB of free ammonia N in the FP. Microbial species that are associated primarily with particulate matter may also utilize greater amounts of AA and peptides as N sources, thus diluting the ¹⁵N-ammonia. Other workers (Hristov and Broderick, 1996; Ahvenjärvi et al., 2002; Reynal and Broderick, 2005)

have also observed greater ¹⁵N enrichment of FAB; therefore, differential isotopic enrichments of FAB and PAB must be accounted for when estimating microbial NAN flows from the rumen.

Organic matter and NAN contents of protozoa did not differ comparing AS vs. RCS diets and averaged, respectively, 95 and 2.82% across diets (Table 5). However, NAN content of protozoa was much lower than that of bacteria in the current study, which may have been due to contamination with feed particles plus dilution from glycogen accumulation due to the use of glucose to aid protozoa sedimentation. Using microscopic examination, Ahvenjärvi et al. (2002) found that protozoal pellets were always contaminated with fine feed particles; therefore, these workers used saline to wash protozoal pellets through a polyester filter. With this method, they found similar N contents in FAB and protozoa but greater N contents in PAB. Reynal et al. (2005) observed an average 3.1% NAN in protozoal samples isolated using the same method as the present study. According to Reynal et al. (2005), contamination with feed N probably did not account for low protozoal NAN because ¹⁵N enrichment of protozoa was only slightly lower than FAB and PAB and contamination likely was from nonnitrogenous sources. Sylvester et al. (2005) reported that the N content of sedimented protozoa (3.82% N) was 45% lower than that of filtrated protozoa (6.95% N), suggesting extensive contamination with plant material without filtering. Isotopic enrichment of protozoa was greater ($P < 0.01$) on RCS than on AS diets (Table 5). Differences were expected because of the greater ¹⁵N enrichment of both PAB and FAB on RCS and because protozoa become enriched indirectly through bacterial predation. Literature re-

ports (Firkins et al., 1987; Hristov and Broderick, 1996; Ahvenjärvi et al., 2002) have shown that ^{15}N enrichment of protozoa usually is somewhat lower than that of bacteria because direct utilization of unlabeled feed protein N dilutes protozoal ^{15}N below that of bacteria. In the present trial, mean protozoal:FAB and mean protozoal:PAB ^{15}N enrichment ratios were, respectively, 0.82 and 1.0. Greater protozoal:bacterial ratios in the current study compared with literature data was possibly caused by bacterial contamination of the protozoal pellets. Sylvester et al. (2005) reported a 33-fold reduction in bacterial contamination of the protozoa pellet using filtration rather than a sedimentation technique to isolate protozoal cells.

CONCLUSIONS

Replacing dietary AS with RCS resulted in greater ruminal fiber digestibility as well as reduced RDP and increased RUP supplies. Reducing NPN content of AS by ATF treatment did not appear to alter dietary RDP or RUP supply. Microbial NAN flow was greater on AS, suggesting that reduced NPN or increased RUP contents of RCS may have depressed microbial protein synthesis in the rumen. Generally, there were no differences in omasal AA flow when AS and RCS were fed, suggesting that greater RUP flow was compensated for by reduced microbial protein formation in cows fed RCS. Greater omasal flow of RUP and most AA on the more mature RCS may have resulted from its greater content of NDIN, ADIN, and fraction B₃. Increased yield of milk and milk protein on AS (Broderick et al., 2007) may have been the result of greater supply of nutritionally available AA from high quality microbial protein. The NRC (2001) model and urinary PD excretion underestimated microbial protein synthesis by 18 and 32%, respectively, compared with that measured in vivo using ^{15}N . Moreover, significant differences in microbial protein synthesis between AS and RCS were not detected using PD excretion.

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REFERENCES

- Aharoni, Y., H. Tagari, and R. C. Boston. 1991. A new approach to the quantitative estimation on nitrogen metabolic pathways in the rumen. *Br. J. Nutr.* 66:407–422.

- Ahvenjärvi, S., A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or rapeseed meal to dairy cows fed grass red-clover silage: I. Rumen degradability and microbial flow. *J. Anim. Sci.* 80:2176–2187.
- Ahvenjärvi, S., A. Vanhatalo, P. Huhtanen, and T. Varvikko. 2000. Determination of reticulo-rumen and whole-stomach digestion in lactating cows by omasal canal or duodenal sampling. *Br. J. Nutr.* 83:67–77.
- Albrecht, K. A., and R. E. Muck. 1991. Proteolysis in ensiled forage legumes that vary in tannin concentration. *Crop Sci.* 31:464–469.
- Allison, M. J. 1970. Nitrogen metabolism of ruminal micro-organisms. Pages 456–472 in *Physiology of Digestion and Metabolism in the Ruminant*. A. T. Phillipson, ed. Oriel Press, Newcastle upon Tyne, UK.
- AOAC. 1980. *Official Methods of Analysis*. 13th ed. Association of Official Analytical Chemists, Washington, DC.
- Argyle, J. L., and R. L. Baldwin. 1989. Effects of amino acids and peptides on rumen microbial growth yields. *J. Dairy Sci.* 72:2017–2027.
- Blackburn, T. H. 1965. Nitrogen metabolism in the rumen. Pages 322–334 in *Physiology of Digestion in the Ruminant*. R. W. Dougherty, R. S. Allen, W. Burroughs, N. L. Jacobson, and A. D. McGilliard, ed. Oriel Press, Newcastle upon Tyne, UK.
- Broderick, G. A., A. F. Brito, and J. J. Olmos Colmenero. 2007. Effects of feeding formate-treated alfalfa silage or red clover silage on the production of lactating dairy cows. *J. Dairy Sci.* 90:1378–1391.
- Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *J. Dairy Sci.* 63:64–75.
- Broderick, G. A., R. P. Walgenbach, and S. Maignan. 2001. Production of lactating dairy cows fed alfalfa or red clover silage at equal dry matter or crude protein contents in the diet. *J. Dairy Sci.* 84:1728–1737.
- Broderick, G. A., R. P. Walgenbach, and E. Sterrenburg. 2000. Performance of lactating dairy cows fed alfalfa or red clover silage as the sole forage. *J. Dairy Sci.* 83:1543–1551.
- Bryant, M. P. 1973. Nutritional requirements of the predominant rumen cellulolytic bacteria. *Fed. Proc.* 32:1809–1813.
- Carro, M. D., and E. L. Miller. 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). *Br. J. Nutr.* 82:149–157.
- Chikunya, S., C. J. Newbold, L. Rode, X. B. Chen, and R. J. Wallace. 1996. Influence of dietary rumen-degradable protein on bacterial growth in the rumen of sheep receiving different energy sources. *Anim. Feed Sci. Technol.* 63:333–340.
- Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Symposium: Nitrogen metabolism and amino acid nutrition in dairy cattle: Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *J. Dairy Sci.* 75:2304–2323.
- Craig, W. M., G. A. Broderick, and B. D. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J. Nutr.* 117:56–62.
- Dewhurst, R. J., D. R. Davies, and R. J. Merry. 2000. Microbial protein supply from the rumen. *Anim. Feed Sci. Technol.* 85:1–21.
- Dewhurst, R. J., R. T. Evans, N. D. Scollan, J. M. Moorby, R. J. Merry, and R. J. Wilkins. 2003. Comparison of grass and legume silages for milk production. 2. In vivo and in sacco evaluations of rumen function. *J. Dairy Sci.* 86:2612–2621.
- Firkins, J. L., S. M. Lewis, L. Montgomery, L. L. Berger, N. R. Merchen, and G. C. Fahey, Jr. 1987. Effects of feed intake and dietary urea concentration on ruminal dilution rate and efficiency of bacterial growth in steers. *J. Dairy Sci.* 70:2312–2321.
- France, J., and R. C. Siddons. 1986. Determination of digesta flow by continuous marker infusion. *J. Theor. Biol.* 121:105–120.
- Hatfield, R. D., and R. E. Muck. 1999. Characterizing proteolytic inhibition in red clover silage. Pages 149–150 in *Proc. XIIth Int. Silage Conf.* Swedish University of Agricultural Sciences, Uppsala, Sweden.
- Hintz, R. W., D. R. Mertens, and K. A. Albrecht. 1995. Effects of sodium sulfite on recovery and composition of detergent fiber and lignin. *J. AOAC* 78:16–22.

- Hristov, A. N., and G. A. Broderick. 1996. Synthesis of microbial protein in ruminally cannulated cows fed alfalfa silage, alfalfa hay, or corn silage. *J. Dairy Sci.* 79:1627–1637.
- Hristov, A. N., P. Huhtanen, L. M. Rode, S. N. Acharya, and T. A. McAllister. 2001. Comparison of the ruminal metabolism of nitrogen from ^{15}N -labeled alfalfa preserved as hay or as silage. *J. Dairy Sci.* 84:2738–2750.
- Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers for assessing fermentative digestion and duodenal flow of nutrients in cattle given six different diets. *Anim. Feed Sci. Technol.* 48:211–227.
- Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. *J. Anim. Sci.* 75:1380–1392.
- Ipharraguerre, I. R., and J. H. Clark. 2005. Impacts of the source and amount of crude protein on the intestinal supply of nitrogen fractions and performance of dairy cows. *J. Dairy Sci.* 88(E Suppl.):E22–E37.
- Jones, B. A., R. E. Muck, and R. D. Hatfield. 1995. Red clover extracts inhibit legume proteolysis. *J. Sci. Food Agric.* 67:329–333.
- Kang-Meznarich, J. H., and G. A. Broderick. 1980. Effects of incremental urea supplementation on ruminal ammonia concentration and bacterial protein formation. *J. Anim. Sci.* 51:422–431.
- Makkar, H. P. S., and K. Becker. 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. *Br. J. Nutr.* 8:107–112.
- Mason, V. C., S. Bech-Andersen, and M. Rudemo. 1979. Hydrolysate preparation for amino-acid determinations in feed constituents. 1. Stability of bound amino-acids to oxidation with performic acid hydrogen peroxide reagents. *Z. Tierphysiol. Tierer.* 41:226–235.
- Martin, C., A. G. Williams, and D. B. Michalet. 1994. Isolation and characteristics of the protozoal and bacterial fractions from bovine ruminal contents. *J. Anim. Sci.* 72:2962–2968.
- McDougall, E. I. 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochem. J.* 43:99–109.
- Muck, R. E. 1987. Dry matter level effects on alfalfa silage quality I. Nitrogen transformations. *Trans. ASAE* 30:7–14.
- Nagel, S. A., and G. A. Broderick. 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. *J. Dairy Sci.* 75:140–154.
- National Research Council. 2001. *Nutrient Requirements of Dairy Cattle*. 6th rev. ed. Natl. Acad. Sci., Washington, DC.
- Nolan, J. 1975. Quantitative models of nitrogen metabolism in sheep. Pages 416–431 in *Digestion and Metabolism in the Ruminant*. I. W. McDonald and A. C. I. Warner, ed. University of New England Publishing Unit, Armidale, NSW, Australia.
- Obispo, N. E., and B. A. Dehority. 1999. Feasibility of using total purines as a marker for ruminal bacteria. *J. Anim. Sci.* 77:3084–3095.
- Olmos Colmenero, J. J., and G. A. Broderick. 2006. Effect of dietary crude protein concentration on ruminal nitrogen metabolism in lactating dairy cows. *J. Dairy Sci.* 89:1694–1703.
- Papadopoulos, Y. A., and B. D. McKersie. 1983. A comparison of protein degradation during wilting and ensiling of six forage species. *Can. J. Plant Sci.* 63:903–912.
- Perez, J. F., J. Balcells, J. A. Guada, and C. Castrillo. 1996. Determination of rumen microbial-nitrogen production in sheep: A comparison of urinary purine excretion with methods using ^{15}N and purine bases as markers of microbial-nitrogen entering the duodenum. *Br. J. Nutr.* 75:699–709.
- Perez, J. F., J. Balcells, J. A. Guada, and C. Castrillo. 1997. Rumen microbial production estimated either from urinary purine derivative excretion or from direct measurements of ^{15}N and purine bases as microbial markers: Effect of protein source and rumen bacteria isolates. *Anim. Sci.* 65:225–236.
- Reynal, S. M., and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. *J. Dairy Sci.* 88:4045–4064.
- Reynal, S. M., G. A. Broderick, S. Ahvenjärvi, and P. Huhtanen. 2003. Effect of feeding protein supplements of differing degradability on omasal flow of microbial and undegraded protein. *J. Dairy Sci.* 86:1292–1305.
- Reynal, S. M., G. A. Broderick, and C. Bearzi. 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. *J. Dairy Sci.* 88:4065–4082.
- Rodriguez, C. A., J. Gonzalez, M. R. Alvir, J. L. Repetto, C. Centeno, and F. Lamrani. 2000. Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. *Br. J. Nutr.* 84:369–376.
- Rulquin, H., and R. Verite. 1993. Amino acid nutrition in dairy cows: Production effects and animal requirements. Pages 55–77 in *Recent Advances in Animal Nutrition*. P. C. Garnsworthy and D. J. A. Cole, ed. Nottingham University Press, Nottingham, UK.
- Santos, F. A. P., J. E. P. Santos, C. B. Theurer, and J. T. Huber. 1998. Effects of rumen-undegradable protein on dairy cow performance: A 12-year literature review. *J. Dairy Sci.* 81:3182–3213.
- SAS Institute. 1999–2000. *SAS/STAT User's Guide*. Release 8.1. SAS Institute, Inc., Cary, NC.
- Satter, L. D., and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. *Br. J. Nutr.* 32:199–208.
- Siddons, R. C., J. Paradine, D. E. Beever, and P. R. Cornell. 1985. Ytterbium acetate as a particulate-phase digesta-flow marker. *Br. J. Nutr.* 54:509–520.
- Sniffen, C. J., J. D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *J. Anim. Sci.* 70:3562–3577.
- Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. *J. Dairy Sci.* 88:2083–2095.
- Udén, P., P. E. Colucci, and P. J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta: Rate of passage studies. *J. Sci. Food Agric.* 31:625–632.
- Vagnoni, D. B., G. A. Broderick, M. K. Clayton, and R. D. Hatfield. 1997. Excretion of purine derivatives by Holstein cows abomasally infused with incremental amounts of purines. *J. Dairy Sci.* 80:1695–1702.
- Zinn, R. A., and F. N. Owens. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Can. J. Anim. Sci.* 66:157–166.