Biohydrogenation of Fatty Acids and Digestibility of Fresh Alfalfa or Alfalfa Hay Plus Sucrose in Continuous Culture*

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ABSTRACT

The pattern of biohydrogenation of fatty acids from fresh alfalfa or alfalfa hay supplemented with 3 concentrations (0, 4, and 8%) of sucrose was studied at a constant pH of 6.2. Four continuous culture fermenters were used in a 4×4 Latin square design to test the hypothesis that fresh forage would increase flow of vaccenic acid (VA) from the fermenters compared with the same forage in hay form and that this difference would be diminished by adding sucrose to the hay diet by changing the bacterial community profile. Effluent was collected from each of the 4 fermenters during the last 3 d of each 10-d period. Nutrient digestibility, volatile fatty acids (VFA), and fatty acids in the effluent were measured. Flow of bacterial organic matter (OM) and neutral and acid detergent fiber and acid detergent fiber digestibilities were higher for fresh alfalfa than alfalfa hay. True OM digestibility of alfalfa hay tended to linearly decrease with sucrose supplementation. However, microbial efficiency and flow of bacterial OM (g/d) linearly increased with sucrose addition. There was no change in total VFA concentration; however, proportion of acetate linearly decreased and proportion of butyrate linearly increased with sucrose addition. Fresh alfalfa increased total biohydrogenation of fatty acids compared with hay. Vaccenic acid flow (mg/d) was much higher for fresh alfalfa compared with alfalfa hay (216 vs. 41) and VA was the predominant 18:1 isomer, followed by *trans*-13 18:1; however, sucrose had no effect on VA flow. The percentage of VA (of total *trans*-18:1) was not different between fresh alfalfa and hay, whereas percentage of *trans*-10 18:1 was much lower for fresh alfalfa. Therefore, the ratio of VA to *trans*-10 18:1 was higher for fresh alfalfa. Flow of *trans*-12 18:1 linearly increased, whereas flows of *cis*-12 and total *cis*-18:1 had quadratic responses to sucrose supplementa-

tion. Total biohydrogenation and biohydrogenation of linoleic and linolenic acids linearly decreased with sucrose; however, there was no effect of sucrose on total *trans* fatty acid flow. Sucrose may be more detrimental to the last step of biohydrogenation of VA. The effects of sucrose on biohydrogenation and concentration of VFA may have been caused by a shift in microbial population by mechanisms that are independent of pH.

(Key words: alfalfa, sucrose, fatty acids, biohydrogenation)

Abbreviation key: 18C = 18-carbon, BH = biohydrogenation, CLA = conjugated linoleic acid, FA = fatty acids, RIS = ribosomal intergenic spacer, RIS-LP = ribosomal intergenic spacer length polymorphism, UFA = unsaturated fatty acids, VA = vaccenic acid.

INTRODUCTION

Dairy products are one of the major sources of conjugated linoleic acid (CLA) in the human diet, and *cis*-9,*trans*-11 octadecadienoic acid (*cis*-9,*trans*-11 18:2) contributes the greatest proportion of all CLA isomers (Parodi, 1997). Much attention has been given to CLA because of its anticarcinogenic properties (Parodi, 1997). Dietary management of dairy cows to increase CLA concentration in milk may be beneficial for human health and for the dairy industry.

Conjugated linoleic acid in milk originates from CLA produced during ruminal biohydrogenation (BH) of linoleic acid and from desaturation of vaccenic acid (*trans*-11 18:1; VA) in the mammary gland. Most of the CLA in milk fat originates from VA (Kay et al., 2004); thus, many efforts have addressed ways to increase VA flow from the rumen. The factors affecting the flow of CLA isomers and *trans*-18:1 fatty acids (FA) to the duodenum as a result of ruminal BH need to be elucidated to increase the CLA concentration in milk and meat of ruminants used for human consumption.

Several researchers have reported that CLA is higher in milk from grazing animals than in that from animals fed TMR diets (Jahreis et al., 1997; Kelly et al., 1998; Dhiman et al., 1999; French et al., 2000). However, there are limited data addressing the basis for high concentration of CLA in milk from grazing cows. The

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higher concentration of CLA in milk fat from grazing animals could be a result of high concentration of octadecatrienoic acid (18:3) in fresh forage or specific plant chemicals (Lee et al., 2003), or higher concentration of soluble sugars in fresh plants (Kelly et al., 1998).

The concentrations of sugars are higher in fresh forages. Storing forages as silage or hay can decrease the concentration of FA (Boufaied et al., 2003a) and sugars in the forages (Van Soest, 1994). Traditional TMR diets have lower proportions of these nutrients compared with fresh pasture. For instance, the concentration of sugar in a TMR is about 1.5 to 3.0% of DM (Hoover and Miller-Webster, 1998), whereas the concentration of sugars in grasses averages 17% of DM, with fructosans being the major sugar, followed by sucrose, fructose, and glucose (Woolford, 1984). However, the effect of soluble sugars on BH has not been studied yet.

The dual-flow continuous culture system was used in the present study to assess the effect of diet on flow of BH intermediates without the confounding effects of variable or unpredictable passage rate, pH, fluid dilution, meal size, and feeding frequency. Because grazing cows have higher concentration of VA in milk and almost 50% of the sucrose is lost during hay making, we postulated that fresh forage would increase flow of VA from fermenters compared with the same forage in hay form and that sucrose addition would diminish this difference by changing the bacterial community profile.

MATERIALS AND METHODS

Treatments and Experimental Design

Fresh alfalfa samples (prebloom/early bloom) were obtained from a greenhouse at the Ohio Agricultural Research and Development Center (Wooster, OH). Samples were harvested randomly and immersed immediately in liquid nitrogen using a metal grid. Samples were freeze-dried, ground at 1 mm, and stored at -20°C . Purchased alfalfa hay was also ground at 1 mm, and stored at -20°C .

Four continuous culture fermenters were used (Hoover et al., 1976; Hannah et al., 1986) in a 4×4 Latin square design. Each period consisted of 7 d for adaptation and 3 d for sample collection. A priori analysis of sucrose from both forages showed that 4% of supplemental sucrose added to the hay treatment would match the percentage of sucrose in the fresh alfalfa; therefore, the treatments were: 1) fresh alfalfa, 2) alfalfa hay, 3) alfalfa hay plus 4% sucrose (as a percentage of DM), and 4) alfalfa hay plus 8% sucrose. The percentage of nutrients from each forage source is shown in Table 1. The actual concentration of sucrose in the hay used for the project was higher than expected, resulting

Table 1. Nutrient composition of fresh alfalfa and alfalfa hay.

Item	Fresh alfalfa	Alfalfa hay
DM	21.2	91.5
	(% of DM)	
OM	90.5	92.2
CP	23.3	20.2
NDF	31.1	37.9
ADF	23.5	26.8
Sucrose	8.64	6.78
Total fatty acids (FA)	2.28	1.07
	(% of FA)	
Individual FA		
16:0	20.8	30.9
18:0	3.40	5.41
<i>Cis</i> -9 18:1	2.65	3.78
18:2	17.0	19.7
18:3	48.6	24.1

in more sucrose in the alfalfa hay plus 4% sucrose treatment than in the fresh alfalfa.

Continuous Culture Operation

Ruminal inoculum was obtained from 2 ruminally cannulated Holstein cows fed a TMR diet. Within 20 min of collection, the inocula from both cows were pooled, squeezed through 2 layers of cheesecloth, and divided among the 4 fermenters. The dual-flow continuous culture system used in this study was similar to that described by Hoover et al. (1976) and modified by Hannah et al. (1986). Diets were fed (100 g of DM) continuously throughout the day using an automated augur system to provide steady state conditions. Volumes of the 4 fermenters were between 1700 and 1800 mL. Sucrose was mixed once daily with hay before the initiation of the 24-h feeding cycle. The liquid and solid dilution rates were maintained at 10 and 5.5%/h, respectively, by regulation of filtrate removal rates and buffer input. Filtrate removal rate was regulated using automatic pumps. The pH was maintained at 6.2 ± 0.1 by automatic administration of 5 N NaOH or 3 N HCl. The fermenters were continuously purged with N_2 to maintain anaerobiosis, and the temperature was held at 39°C . Temperature, acid and alkali use, filtrate flow, and pH were recorded and recalibrated, if needed, every 6 h. Solid and liquid effluents were weighed once daily to determine flow rates.

Sample Collection and Analysis

One daily sample of effluent (1 L) was taken on d 8, 9, and 10 and composited by fermenter for analysis. Effluents were collected in bottles kept at 8°C until the end of the 24-h period. The contents were blended using a Waring blender for 30 s, and the effluent was strained through 2 layers of cheesecloth. Differential centrifuga-

tion was used to first separate the feed from the bacteria (15 min at 500 × g) and then to separate the bacteria from the liquid supernatant (15 min at 23,300 × g). An aliquot of the effluent was acidified using 3 mL of 6 N HCl per 50 mL of sample to stop fermentation before analysis of VFA (Firkins et al., 1990). Effluent and bacteria samples were frozen, freeze-dried, and stored at -20°C. Both samples were analyzed for N by microKjeldahl (AOAC, 1990), and effluent samples were analyzed for NDF and ADF (Van Soest et al., 1991). Forage sources were also analyzed for sucrose (Hall, 2000).

The FA in the dried forages and effluent samples, stored at -20°C, were methylated with 0.5 M sodium methoxide (10 min at 50°C), followed by 5% methanolic HCl (10 min at 80°C). Nonadecanoic acid (19:0) was used as an internal standard. Retention times and response factors were determined with methyl ester standards purchased from Nu-Check Prep (Elysian, MN; cat. no. GLC-60) and Matreya, Inc. (Pleasant Gap, PA; FIM-FAME-7). The 18:1 FA that were not available commercially (*trans*-6/8, *trans*-9, *trans*-12, *trans*-13, *trans*-15, *cis*-12, and *cis*-15) were identified by order of elution (Molkentin and Precht, 1995). The FA methyl esters were separated by GLC. The column was a fused silica capillary (SP-2380, 100 m × 0.25 mm i.d. × 0.2-μm film thickness; Supelco Inc., Bellefonte, PA). Nitrogen was used as the carrier gas. Detector and injector temperatures were set at 250 and 220°C, respectively, and the split ratio was set at 100:1. Oven temperature was set for 160°C for 10 min, increased by 3.0°C/min to 180°C, held for 60 min, increased by 5.0°C/min to 220°C, held for 50 min, and decreased by 20°C/min to 160°C for 1 min. The BH of FA was calculated as described by Wu et al. (1991).

DNA Extraction and PCR

One sample per fermenter during each period was taken for DNA extraction and frozen at -80°C. Total community DNA was extracted from the effluent samples using the bead beating method (Yu and Mohn, 1999) followed by purification of the DNA using a QIAamp column (Qiagen, Valencia, CA). The DNA samples were used in PCR amplification with primers S926f (5'-CTYAAAKGAATTGACGG-3') and L189r (5'-TACTGAGATGYTTMARTTC-3'). The resultant ribosomal DNA-RIS PCR products contain the complete ribosomal intergenic spacers (**RIS**) and parts of the flanking ribosomal DNA genes (~600 bp of 16S rDNA and 190 bp of 23S rDNA). The PCR conditions were as follows: initial denaturation at 94°C for 3 min, annealing at 45°C for 1.5 min, and extension at 72°C for 2.5 min. Subsequent cycles consisted of a 1.5-min denaturation step at 94°C, a 1.5-min annealing step at 45°C, and a 2-min extension

step at 72°C. After 30 cycles, there was a final 7-min extension step at 72°C.

Phylogenetic Analysis Based on RIS-Length Polymorphism

The PCR amplified rDNA-RIS fragments were separated on a 4% polyacrylamide (37:1) gel, and the gel was stained with GelStar nucleic acid stain (BioWhittaker, Inc., Walkersville, MD). The RIS-length polymorphism (**RS-LIP**) banding patterns were documented using a FluorChem Imaging System (Alpha Innotech Corporation, San Leandro, CA). The gel image was then exported into the GelCompar II analysis software in the BioNumerics package (BioSystematica, Devon, UK) for cluster analysis and dendrogram construction. The unweighted pair group method with arithmetic averages (UPGMA) method was used by the software for dendrogram construction.

Statistical Analyses

Data were analyzed by the PROC MIXED procedure of SAS (SAS Institute, 1999), according to the following model:

$$Y_{ijk} = \mu + T_i + c_j + p_k + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable for treatment i on fermenter j and period k ; μ = overall mean; T_i = fixed effect of treatment i ; c_j = random effect of fermenter j ; p_k = random effect of period k ; and ε_{ijk} = residual error associated with the ijk observation.

Preplanned contrasts were used to compare the treatment effects: 1) fresh alfalfa vs. alfalfa hay without sucrose, 2) linear effect for concentration of sucrose, and 3) quadratic effect for concentration of sucrose. Significance was declared at $P < 0.05$.

RESULTS

Digestibility Measures

True OM digestibility was 14% higher ($P = 0.09$) in fresh alfalfa than alfalfa hay (Table 2). The digestibility of N tended ($P = 0.11$) to be higher and digestibilities of the NDF and ADF fractions were higher for fresh alfalfa than hay.

Addition of sucrose tended ($P = 0.09$) to decrease true OM digestibility. There was no effect of sucrose on NDF and ADF digestibilities. Adding 8% sucrose decreased NDF digestibility of alfalfa hay by 14%. Sucrose addition did not affect true N digestibility. However, there was a positive linear effect of sucrose concentration on efficiency of microbial protein synthesis and flow of

Table 2. Nutrient digestibility in continuous culture fermenters receiving fresh alfalfa or alfalfa hay supplemented with 3 concentrations of sucrose.

Item	Treatment					Probability of contrasts		
	Fresh alfalfa	Hay + sucrose (%)			SE	Fresh vs. hay	Linear	Quadratic
		0	4	8				
Digestibility, %								
OM ¹	46.1	39.5	40.0	33.0	2.6	0.09	0.09	0.25
NDF	39.1	31.2	31.9	26.8	2.6	0.03	0.18	0.29
ADF	40.2	29.7	30.3	25.0	2.4	0.01	0.28	0.15
Nonbacterial N	48.1	29.5	23.8	24.5	5.6	0.01	0.36	0.50
Bacterial OM, g/d	14.8	12.3	12.5	14.4	0.8	0.02	0.05	0.25
Efficiency ²	37.6	36.7	36.3	56.4	6.2	0.92	0.04	0.17

¹True OM digestibility.²Grams of microbial N produced per kilogram of OM truly digested.

bacterial OM in the effluent. Flow of bacterial OM was higher for fresh alfalfa; however, no difference was found on efficiency of microbial protein synthesis between alfalfa sources.

VFA

Only isobutyrate and isovalerate concentrations were affected by alfalfa source, being higher for fresh alfalfa (Table 3). There was a quadratic trend ($P = 0.12$) for the concentration of total VFA to change with increasing sucrose concentration. Addition of sucrose linearly decreased molar percentage of acetate; however, no effects were observed on propionate concentration or the acetate to propionate ratio. Butyrate and valerate concentrations linearly increased with increasing sucrose addition, whereas isobutyrate and isovalerate concentrations linearly decreased as sucrose level increased.

Fatty Acids and Biohydrogenation

Flow (mg/d) of 18:0, 18:2, and 18:3 in the effluent was higher for fresh alfalfa than alfalfa hay (Table 4), but

no difference was observed for *cis*-9 18:1. However, addition of sucrose had a quadratic effect on the flow of *cis*-9 18:1 without affecting the flow of the other 18-carbon (18C) FA.

The BH of total 18C FA and the BH of oleic, linoleic, and linolenic acids were higher for fresh alfalfa than alfalfa hay with no supplemental sucrose. Increasing sucrose concentration linearly decreased BH of total 18C FA and BH of linoleic and linolenic acids, but there was no difference in the BH of oleic acid (Table 4).

Fresh alfalfa had a higher flow of all the intermediates of BH than alfalfa hay (Table 5) except for CLA and *trans*-10 18:1. Among the *trans*-18:1 FA, addition of sucrose linearly increased the flow of *trans*-12. Additionally, total *cis*-18:1 and *cis*-11 18:1 had a quadratic change in flow by sucrose supplementation, whereas *trans*-6/8 and *cis*-12 18:1 tended ($P < 0.10$) to decrease linearly.

The intermediates of BH were also reported as a percentage of the total *trans*- or *cis*-18:1 FA to compare treatment-induced changes in the pathways within each isomer group (Table 6). The percentage of *trans*-6/8, *trans*-9, *trans*-10, *trans*-12, and *cis*-11 were lower

Table 3. Proportions of volatile fatty acids in continuous culture fermenters receiving fresh alfalfa or alfalfa hay supplemented with 3 concentrations of sucrose.

Item	Treatment				SE	Probability of contrasts		
	Fresh alfalfa	Hay + sucrose (%)				Fresh vs. hay	Linear	Quadratic
		0	4	8				
Total VFA, mM	100.9	97.2	82.8	88.5	6.2	0.58	0.22	0.12
		(mol/100 mol)						
Acetate	73.6	74.8	72.8	71.3	0.9	0.38	0.02	0.81
Propionate	14.8	13.5	14.1	14.9	0.8	0.28	0.26	0.96
Butyrate	8.3	8.7	10.1	11.2	0.4	0.22	<0.01	0.43
Isobutyrate	0.58	0.38	0.25	0.14	0.04	<0.01	<0.01	0.73
Valerate	1.37	1.61	1.98	2.20	0.1	0.20	<0.01	0.65
Isovalerate	1.31	1.02	0.75	0.37	0.10	0.05	<0.01	0.60
Acetate:propionate	4.99	5.58	5.20	4.92	0.33	0.23	0.18	0.90

Table 4. Flow and biohydrogenation (BH) of 18:1, 18:2, 18:3 and BH of total 18-Carbon (18C) fatty acids (FA) in continuous culture fermenters receiving fresh alfalfa or alfalfa hay supplemented with 3 concentrations of sucrose.

	Treatment				SE	Probability of contrasts		
	Fresh alfalfa	Hay + sucrose (%)				Fresh vs. hay	Linear	Quadratic
		0	4	8				
Effluent	(mg/d)							
18:0	920	315	247	256	106	<0.01	0.68	0.75
<i>cis</i> -9 18:1	44.4	44.1	38.4	47.8	5.7	0.93	0.33	0.04
18:2	192	141	129	163	26	0.02	0.22	0.16
18:3	261	151	138	177	27	<0.01	0.30	0.23
18C FA	1854	765	660	763	203	<0.01	0.99	0.59
BH ¹								
Total BH ²	66.0	45.0	41.2	35.9	2.5	<0.01	0.01	0.77
<i>cis</i> -9 18:1	28.4	12.8	13.4	6.3	5.2	0.05	0.36	0.52
18:2	55.3	47.2	43.8	38.8	3.3	<0.01	<0.01	0.66
18:3	78.4	52.6	50.1	44.5	2.3	<0.01	0.02	0.50

¹Percentage of fatty acids that disappeared corrected for the proportion of 18C FA fed and in the effluent (Wu et al., 1991).

²Total 18C FA.

for fresh alfalfa than alfalfa hay. Conversely, percentages of *trans*-13 and *cis*-15 were higher for fresh alfalfa.

RIS-LP Analysis

Distinct RIS-LP banding patterns were obtained from gel electrophoresis of the PCR amplicons (Figure 1). Cluster analysis showed that the treatments tended to group together, but no distinct pattern was detected because samples taken in the same period tended to cluster together irrespective of the treatment group from which the samples were collected. However, all the sucrose treatments (hay plus 4 and 8% sucrose) grouped together, suggesting that sucrose had an effect on the bacterial population in the fermenters.

The RIS-LP followed by cluster analysis based on pairwise comparison of the banding patterns obtained from gel electrophoresis of rDNA-RIS amplicons provided a reliable and fast method for comparison of microbial community profiles in environmental samples. The banding patterns suggest that sucrose supplementation influenced bacterial community structure. Because each period was initiated by a new inoculation and the fermentations were controlled for pH and continuous feeding of forages, periods also had an effect on the banding patterns. However, minor alterations in bacterial species might not be easily detected because of the presence of other bacterial species in larger numbers. The use of genera- and species-specific primers

Table 5. Flow (mg/d) of the intermediates of biohydrogenation in the effluent from continuous culture fermenters receiving fresh alfalfa or alfalfa hay supplemented with 3 concentrations of sucrose.

Item	Treatment					Probability of contrasts		
	Fresh alfalfa	Hay + sucrose (%)			SE	Fresh vs. hay	Linear	Quadratic
		0	4	8				
CLA ¹	15.5	9.7	5.8	11.0	2.9	0.12	0.71	0.16
Total <i>trans</i> -18:1	352.8	67.6	73.3	74.9	48.4	< 0.01	0.91	0.97
<i>Trans</i> -6/8	11.7	5.25	2.40	0.74	2.20	0.04	0.06	0.75
<i>Trans</i> -9	7.57	3.27	2.70	2.20	1.10	<0.01	0.09	0.92
<i>Trans</i> -10	11.2	5.7	2.9	3.9	3.90	0.17	0.59	0.64
<i>Trans</i> -11	216.0	41.0	40.9	36.5	30.5	<0.01	0.91	0.95
<i>Trans</i> -12	15.7	7.30	9.17	15.5	2.34	<0.01	<0.01	0.14
<i>Trans</i> -13	68.9	9.4	9.6	12.2	10.4	<0.01	0.83	0.92
<i>Trans</i> -15	27.1	4.43	9.19	5.83	4.70	<0.01	0.78	0.36
Total <i>cis</i> -18:1	104.8	70.9	60.9	72.9	10.23	<0.01	0.50	<0.01
<i>Cis</i> -11	19.7	15.6	12.9	16.3	1.97	0.01	0.59	0.03
<i>Cis</i> -12	10.7	6.85	5.88	5.49	1.52	<0.01	0.07	0.61
<i>Cis</i> -15	29.9	4.38	3.72	3.40	3.07	<0.01	0.81	0.96

¹Geometrical and positional isomers of conjugated linoleic acid (CLA).

Table 6. Percentage of the intermediates of biohydrogenation in the effluent from continuous culture fermenters receiving fresh alfalfa or alfalfa hay supplemented with 3 concentrations of sucrose.

Item	Treatment				SE	Probability of contrasts		
	Fresh alfalfa	Hay + sucrose (%)				Fresh vs. hay	Linear	Quadratic
		0	4	8				
<i>Trans</i> -18:1 ¹								
<i>Trans</i> -6/8	3.29	5.21	3.37	1.53	0.42	<0.01	<0.01	0.99
<i>Trans</i> -9	2.34	4.10	2.98	2.05	0.37	0.02	0.01	0.85
<i>Trans</i> -10	1.12	8.48	6.63	5.42	2.11	0.05	0.12	0.94
<i>Trans</i> -11	60.6	60.7	57.9	48.5	3.96	0.96	<0.01	0.27
<i>Trans</i> -12	4.73	11.0	13.1	21.0	1.34	<0.01	<0.01	0.07
<i>Trans</i> -13	19.6	14.3	12.6	16.4	2.02	0.05	0.38	0.20
<i>Trans</i> -15	7.97	6.69	9.22	7.72	2.81	0.73	0.78	0.53
<i>Cis</i> -18:1 ²								
<i>Cis</i> -11	19.1	22.2	21.5	22.1	1.13	<0.01	0.94	0.46
<i>Cis</i> -12	10.2	9.55	9.69	7.73	1.50	0.56	0.13	0.29
<i>Cis</i> -15	27.8	6.01	6.09	4.89	1.46	<0.01	0.60	0.73

¹Percentage of the total *trans*-18:1 FA.
²Percentage of the total *cis*-18:1 FA.

Figure 1. Comparison of bacterial community structure in fermenter samples; dendrogram shows cluster analysis performed based on sample similarities of the communities. P = Period 1 to 4; 0, 4, and 8% = percentage of sucrose supplementation on the hay treatments; and Alf = fresh alfalfa. The length of scale depicts the percentage similarity between different lanes.

would allow more specific monitoring of the minor population changes.

DISCUSSION

We reported the isolated effect of sucrose on BH and FA concentrations in the effluent of continuous fermenters because pH, passage rates, and nutrient composition did not vary among the hay treatments. Fresh alfalfa was used as a positive control to show how VA and other intermediates of BH accumulate in the effluent and to compare the differences in BH attributed to forage conservation.

Biohydrogenation of Fatty Acids

The BH values observed in this experiment are similar to those reported by Qiu et al. (2004). However, other researchers have reported BH values for 18:2 and 18:3 higher than 80% for in vitro (Loor et al., 2003) and in vivo studies (Sackmann et al., 2003). Source of FA, level of DM intake, concentration of FA in the diet, and ruminal pH are some of the factors that may contribute to the differences in estimates of BH among experiments.

The decrease in BH of FA in alfalfa hay by sucrose addition may be associated with a decrease in cellulolytic microbes. Any shift in bacterial population in favor of noncellulolytic species could inhibit the BH of FA, because cellulolytic bacteria play a major role in ruminal BH (Harfoot and Hazlewood, 1997). Support for this hypothesis was the lower BH of total 18C FA and the numerically lower NDF and ADF digestibilities when alfalfa hay was supplemented with sucrose.

The BH of linoleic and linolenic acids was 14.6 and 32.9% higher, respectively, for fresh alfalfa when compared with alfalfa hay with no supplemental sucrose. Higher BH of 18:2 and 18:3 also has been reported for fresh grass compared with hay (Boufaied et al., 2003b). The composition of individual FA between grass and legumes is variable, depending on season, species, and N fertility (Boufaied et al., 2003a). Nutrient digestibility was higher, especially the fiber fraction, for fresh alfalfa. The lower degradability observed for hay may have physically trapped the FA within the organic matrix, decreasing release, and therefore BH of 18:2 and 18:3 (Boufaied et al., 2003b). Conversely, the flow of bacterial OM was 14% lower for hay, indicating that for a similar bacterial mass, the biohydrogenating microflora were much more active in fresh alfalfa.

The BH of 18:3 from fresh plants has been reported to be in the range of 80 to 90% (Loor et al., 2003; Sackmann et al., 2003). The lower BH of 18:3 for fresh alfalfa observed in the present study could be attributed to the

lower digestibility of the OM fraction observed in our trial. If the plant matrix has a lower digestibility, release of FA as substrate for BH would be diminished.

Flow of VA

The higher CLA percentage in milk of grazing cows is largely a result of endogenous synthesis by Δ^9 -desaturase, with VA as a substrate (Kay et al., 2004). The BH of linolenic acid results in VA as an intermediate but not CLA (Harfoot and Hazlewood, 1997). High concentrations of VA in the duodenal digesta have been reported for ruminants consuming diets with either 18:2 or 18:3 FA as the major unsaturated FA (UFA) (Sackmann et al., 2003). Therefore, factors regulating VA synthesis and flow out of the rumen must be defined to improve our understanding of ruminal BH.

The fresh alfalfa diet had a much higher flow of VA in the effluent than alfalfa hay with no sucrose (216 vs. 41 mg/d, respectively), consistent with our hypothesis. This was most likely due to the higher percentage of 18:3 in fresh forage (Table 1). Although there was a linear decrease in BH of total 18C FA in the effluent as sucrose concentration increased, no differences were detected in the flow of VA and total *trans* FA. This observation does not support our hypothesis that the difference in VA flow between fresh alfalfa and the same forage in hay form would be diminished by adding sucrose to the hay diet. Therefore, soluble carbohydrates do not appear to be solely responsible for the higher flow of VA from the rumen of grazing cows or may not play any role.

Limitations of our study to determine the exact role of sucrose on VA accumulation in the rumen may be a result of the compartmentalization of sucrose in the fresh alfalfa samples and lack of viable protozoa in the fermenters. The location of sucrose and soluble protein in fresh alfalfa is entirely inside the plant tissue, whereas the supplemented sucrose to the hay treatments can be fermented faster; thus, the synchronism between availability of sucrose and soluble protein was not similar among treatments. Additionally, soluble sugars may be responsible for a higher number of protozoa in the rumen of cows fed fresh pasture compared with hay (Holden et al., 1994), which could not be reproduced in our fermenters. Although we are not aware of any data showing the importance of protozoa on VA flow in cows on pasture, protozoa might delay complete BH of UFA by engulfing bacteria and solid particles rich in *trans*-18:1 FA.

Supplemental sucrose decreased BH of total 18C FA and disappearance of 18:2 and 18:3, with similar flows of *trans* FA in the effluent of all the hay treatments. Although BH of total 18C FA was decreased by 20%

when 8% sucrose was added, flow of VA and 18:0 decreased 13 and 19%, respectively. Therefore, sucrose may be more detrimental to the last BH step (synthesis of 18:0 from *trans*-18:1 FA) than to the isomerization/reduction step of 18:2 and 18:3 to *trans*-18:1 FA.

The concentration of VA in the effluent of continuous culture fermenters fed the fresh alfalfa was lower than reported by Loor et al. (2003). These authors reported ~16% of VA (proportion of total FA) in the effluent from red clover and orchardgrass; whereas, we observed 3 to 9% VA (data not shown). Our lower values were likely a result of 1) the lower OM digestibility observed in this trial, 2) type of forage and conservation method, 3) passage rate, and 4) constant pH.

An early vegetative state of alfalfa could have increased the amount of VA formed because of its higher concentration of UFA. Because grasses have a higher proportion of 18:3 than legumes (Boufaied et al., 2003a), researchers using grasses in continuous culture reported higher concentrations of VA in the effluent (Loor et al., 2003). Moreover, alfalfa has a lower proportion of total FA and 18:3 compared with red clover (Boufaied et al., 2003a). The flow of VA in the effluent of the hay treatment was 19% of that for the fresh alfalfa. Conserved forages (hay) have a lower concentration of FA (Boufaied et al., 2003a), which decreases the amount of substrate for BH, thus diminishing the concentration of VA in the rumen.

Higher solid and liquid passage rates may increase the flow of intermediates of BH (Qiu et al., 2004) (i.e., a higher flow rate decreases the opportunity for complete BH). In the experiment of Loor et al. (2003), the liquid and solid passage rates were 0.18 and 0.07/h, respectively. Those values were higher than used in this trial (0.10 and 0.05/h). In addition, diurnal variation in ruminal pH of grazing animals with values falling below 6.2 (Kolver and de Veth, 2002) may inhibit BH of *trans*-18:1 to 18:0 (Qiu et al., 2004). The result would be higher VA flow in the ruminal fluid.

Intermediates of Biohydrogenation

The flow of the intermediates of BH did not follow a specific pattern with sucrose addition. We cannot explain the quadratic effect of sucrose observed on the flows of total *cis*-18:1, *cis*-9, and *cis*-11 18:1 FA. Additionally, the cause for a linear decrease in *cis*-12 and increase in *trans*-12 18:1 FA was not apparent. Changes in the duodenal flows of *trans*-9, *trans*-12, and *trans*-10 varied with the proportion of forage in the diet (Sackmann et al., 2003). Moreover, the initial concentration of UFA affects the rates of BH for 18:2 and 18:3 (Czerkawski, 1967). The initial concentration of UFA in the fermenters was less than 1 mg/mL. Therefore, we could

isolate the effect of sucrose on the parameters studied from pH and high initial amounts of 18:2 and 18:3.

Yu and Mohn (2001) developed a composite method for investigating bacterial community structure in an aerated lagoon. The method is based on analyses of PCR amplicons containing the RIS and its flanking partial 16S rRNA gene. Community structure similarity was determined based on RIS-LP. The 16S-23S rDNA-RIS region has a highly variable length and can thus be used as a marker to distinguish different bacterial species.

Using this methodology, we observed that sucrose caused a change in the bacterial community, although there also was a period effect influencing the analysis. Similar period effects have been reported previously (Noftsker et al., 2003) when bacterial community structure was analyzed in continuous culture fermenters using RIS-LP. The shift in bacterial population may have been associated with the observed effects of sucrose on some of the intermediates of BH. Different *trans*-18:1 isomers (positions 6 to 16) can be formed from *cis*-9 18:1 (Mosley et al., 2002). However, data are not in the literature regarding how all *cis* and *trans* isomers are formed during BH or how the major ruminal microbial species are involved. Future research should focus on determining quantitative shifts in bacterial species by real time-PCR and correlate the results with the linear decrease in BH. Recently, Karnati et al. (2005) excised bands from RIS-LP gels and sequenced the PCR products to determine specific changes in the ruminal bacterial community from dairy cows. Such an approach can facilitate quantification of individual bacterial genera or species as affected by different dietary treatments. We are not aware of any research showing the pH-independent effect of sucrose on the quantitative and qualitative changes in bacterial profile using molecular techniques.

The percentage of *cis*-15 18:1 (of total *cis*-18:1 isomers; Table 6) was much higher for fresh alfalfa than for hay. Body (1976) reported that as the percentage of 18:3 increased, the proportion of *cis*-15 18:1 also increased in *in vitro* incubations. Therefore, the higher percentage of this *cis*-18:1 isomer reported in our trial resulted from the higher 18:3 in fresh alfalfa.

Sucrose tended ($P = 0.12$; Table 6) to linearly decrease the proportion of *trans*-10 18:1 in the effluent. Based on flow of FA (Table 5), there was a 100% increase in the ratio of VA to *trans*-10 18:1 with 4% supplemental sucrose vs. hay alone. Milk fat depression has been associated with an increase in *trans*-10 18:1 in milk (Grinari et al., 1998). This fatty acid is probably an intermediate of BH of *trans*-10, *cis*-12 18:2, the CLA isomer responsible for inhibiting Δ^9 -desaturase (Park et al., 2000). The exact pathway and major player in-

volved in the BH of 18:2 to *trans*-10 18:1 in the rumen is not known. However, manipulation of ruminal BH toward an increase in the ratio of VA to *trans*-10 18:1 would benefit herds experiencing milk fat depression.

We observed a higher ratio of VA to *trans*-10 in the effluent of fresh alfalfa compared with hay (19.3 vs. 7.2; Table 5). Sackmann et al. (2003) observed a linear decrease in this ratio as the proportion of forage increased in the diet of steers. These observations agree with the proposed BH pathway where VA is a major intermediate of BH of 18:3. Those authors also observed synthesis of *cis*- and *trans*-15 18:1 from BH of 18:3. Besides the much higher flows of those 2 FA in the effluent of the fresh alfalfa treatment (Table 5), *trans*-13 18:1 was the second most prevalent intermediate of BH. We are not aware of any proposed BH pathway that accounts for the formation of this FA. Loor et al. (2003) have also reported *trans*-13 18:1 as the second major 18:1 FA in the effluent of continuous culture fermenters. There is still a need to understand how the major factors (diet composition, ruminal parameters, and population of microorganisms) of ruminal BH are correlated.

Digestibility

Although there is a large variation in NDF digestibilities reported in the literature, the NDF digestibilities observed in this trial for hay and fresh alfalfa are in agreement with a previous study (Bach et al., 1999). The variation observed in NDF digestibility among experiments using continuous culture fermenters may be caused by differences in forage sources and maturity, pH, inoculum, and liquid and solid passage rates (Qiu et al., 2004).

True OM digestibilities for hay and hay plus 4% sucrose were very similar, whereas the value for hay plus 8% sucrose was 16% lower than hay alone. The true OM digestibilities are in agreement with previous published data (Griswold et al., 1996, 2003; Bach et al., 1999; Qiu et al., 2004). Sucrose addition has been reported to reduce the rates of fiber fermentation in situ (Huhtanen and Khalili, 1992). Addition of 8% sucrose negatively affected true OM digestibility of hay, most likely reflecting the numerical decrease in nonbacterial N, NDF, and ADF digestibilities by a pH-independent mechanism. A decrease in NDF digestibility by addition of soluble sugars with pH higher than 6.2 has been reported previously (Piwonka and Firkins, 1996).

Some cellulolytic bacteria are able to use soluble sugars as well as fiber (Huhtanen and Khalili, 1992), changing their metabolism according to substrate availability (Russell and Baldwin, 1978). We postulate that sucrose decreased nutrient digestibility by shifting ei-

ther bacterial ecology or metabolism, or both. Russell and Baldwin (1978) reported alterations in bacterial growth rates with a combination of different carbohydrates. Therefore, sucrose addition may have favored growth of bacteria species that have faster growth rates when fermenting this sugar. Furthermore, sucrose may decrease fiber digestibility by decreasing fibrolytic enzyme activity (Huhtanen and Khalili, 1992; Piwonka and Firkins, 1993), possibly explaining the numerical decrease in fiber digestibility in this trial.

Bacterial OM flow increased 14% and microbial efficiency increased 35% from 0 to 8% sucrose. A similar increase in efficiency has been reported in Holstein heifers supplemented with dextrose (Piwonka et al., 1994). We reported higher values for microbial efficiency (Table 2), likely caused by the lower values observed for true OM digestibilities. Meng et al. (1999) observed a higher microbial efficiency for nonfibrous carbohydrate sources compared with fibrous sources in continuous culture fermenters at higher dilution rates (0.15 and 0.20/h). However, these observations were different at lower dilution rates. Supplemental sucrose provided a more readily available energy source, contributing to higher efficiency and bacterial OM flow.

Nutrients in fresh alfalfa were more digestible than those in hay. Harvest of alfalfa to represent the selection of the immature plants during grazing conditions resulted in a lower percentage of NDF compared with hay. Higher nutrient digestibilities for fresh alfalfa resulted in higher bacterial OM flow. Sucrose content of fresh alfalfa was 2 percentage units higher than for alfalfa hay (Table 1). A possible synchronism between N and sucrose release from the plant matrix and fermentation may have contributed to the almost 2-fold increase in true N digestibility for fresh alfalfa. Moreover, fresh forages have higher concentrations of other rapidly fermented sugars that are lost during wilting and higher concentration of more digestible protein (Van Soest, 1994).

VFA

Total VFA concentrations observed in this experiment were similar to previous reports that used continuous culture fermenters (Bach et al., 1999) and grazing animals (Reis and Combs, 2000). The reason for the quadratic effect of sucrose addition on VFA concentration is not certain; such a quadratic response was not observed elsewhere in this trial, except for a quadratic trend observed for the percentage of CLA in the effluent.

Degradation of fiber in the rumen is associated with a higher molar proportion of acetate to propionate (Van Soest, 1994). Changes in fermentation patterns likely reflect shifts in bacterial population that respond to

changes in fermentable substrates. The decrease in acetate concentration as sucrose concentration increased is consistent with the numerically lower NDF digestibility observed for the highest concentration of sucrose. The change in molar proportion of butyrate was the most pronounced of all, with a 22% increase in concentration at the highest concentration of sucrose. Crawford et al. (1980) also reported reciprocal changes in acetate and butyrate concentrations by varying liquid dilution rate. Addition of sucrose also linearly increased the concentration of valerate, whereas isobutyrate and isovalerate concentrations were decreased. When taken together, these observations support our hypothesis that addition of sucrose caused a shift in the bacterial population.

The molar proportion of butyrate has been reported to increase with addition of soluble sugars (Piwonka and Firkins, 1996) and concentrate feeds (Reis and Combs, 2000). Soluble sugars are the main carbohydrate substrate for holotrichs (Van Soest, 1994). However, continuous culture fermenters are associated with a decrease in protozoal population and even greater reductions in protozoa numbers were observed with solid dilution rates higher than 0.04/h (Hoover et al., 1976). Therefore, the higher butyrate concentration observed in this experiment was not likely caused by an increase in protozoal cells. The higher butyrate concentration could be attributed to a change in fermentation pathways to accommodate the higher flux of hydrogen from the rapidly fermented sugar source (Piwonka and Firkins, 1996). Moreover, the higher molar proportion of butyric acid could be attributed to its synthesis from lactate by *Megasphaera elsdenii* (Klieve et al., 2003).

No difference was observed for the major VFA between fresh alfalfa and hay; however, isobutyrate and isovalerate were higher for fresh alfalfa compared with hay, whereas these branched-chain VFA linearly decreased with sucrose addition. Valerate was not different between fresh and hay but linearly increased with sucrose. The branched-chain VFA resulted from fermentation of branched-chain amino acids. The change in branched-chain VFA likely is related to the difference in N digestibility observed in this trial.

CONCLUSIONS

Our data reject the hypothesis that increasing sucrose supplementation to alfalfa hay would increase flow of VA from the fermenters. However, addition of sucrose changed microbial populations; as concentration of sucrose increased, BH of total 18C FA decreased by a pH-independent process. Synthesis of VA and *trans* FA were not changed by sucrose addition. The final step of BH, reduction of *trans*-18:1 to 18:0, may be more

sensitive to the suppressing effect of sucrose supplementation.

The VA was the major octadecenoic acid in the effluent of all treatments, followed by *trans*-13 18:1. Adding sucrose to hay shifted the intermediates of BH independently of pH and concentrations of UFA.

Fresh alfalfa had a higher digestibility of NDF and ADF and tended to have a higher true OM digestibility than alfalfa hay. Addition of sucrose tended to decrease true OM digestibility of hay and improve efficiency of microbial protein synthesis. Sucrose addition decreased acetate and increased butyrate concentrations. The BH of total 18C FA for fresh alfalfa was higher than for alfalfa hay. Future research needs to link the sucrose-induced changes in BH with changes in metabolites and a shift in microbial species in the rumen.

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