pH dynamics and bacterial community composition in the rumen of lactating dairy cows¹

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ABSTRACT

The influence of pH dynamics on ruminal bacterial community composition was studied in 8 ruminally cannulated Holstein cows fitted with indwelling electrodes that recorded pH at 10-min intervals over a 54-h period. Cows were fed a silage-based total mixed ration supplemented with monensin. Ruminal samples were collected each day just before feeding and at 3 and 6 h after feeding. Solid and liquid phases were separated at collection, and extracted DNA was subjected to PCR amplification followed by automated ribosomal intergenic spacer analysis (ARISA). Although cows displayed widely different pH profiles (mean pH = 6.11 to 6.51, diurnal pH range = 0.45 to 1.39), correspondence analysis of the ARISA profiles revealed that 6 of the 8 cows showed very similar bacterial community compositions. The 2 cows having substantially different community compositions had intermediate mean pH values (6.30 and 6.33) and intermediate diurnal pH ranges (averaging 0.89 and 0.81 pH units). Fortuitously, these 2 cows alone also displayed milk fat depression, along with markedly higher ruminal populations of 1 bacterial operational taxonomic unit (OTU) and reduced populations of another ARISA amplicon. Cloning and sequencing of the elevated OTU revealed phylogenetic similarity to *Megasphaera elsdenii*, a species reportedly associated with milk fat depression. The higher populations of both *M. elsdenii* and OTU246 in these 2 cows were confirmed using quantitative real-time PCR (qPCR) with species-specific primers, and the fraction of total bacterial rDNA copies contributed by these 2 taxa were very highly correlated within individual cows. By contrast, the fraction of total bacterial rDNA copies contributed by *Streptococcus bovis* and genus

Ruminococcus, 2 taxa expected to respond to ruminal pH, did not differ among cows (mean = <0.01 and 10.6%, respectively, of rRNA gene copies, determined by qPCR). The data indicate that cows with widely differing pH profiles can have similar ruminal bacterial community compositions, and that milk fat depression can occur at intermediate ruminal pH. The results support recent reports that milk fat depression is associated with shifts in bacterial community composition in rumine and is specifically related to the relative abundance of *Megasphaera elsdenii*.

Key words: bacterial community composition, *Megasphaera elsdenii*, milk fat depression, ruminal pH

INTRODUCTION

The effects of pH on ruminal fermentations and microbial growth both in vitro (Russell and Dombrowski, 1980; Hoover, 1986; Grant and Mertens, 1992; Mouriño et al., 2001) and in vivo (Mould and Ørskov, 1983–1984; Allen et al., 2006) are well known. Several studies have also demonstrated the effects of pH on appetite (Allen et al., 2006) and on fiber digestion (Terry et al., 1969; Hoover, 1986). Typically, in cows fed once daily, ruminal pH decreases after feeding for a period of a few hours, and then increases again because of VFA removal, rumination, and salivation. Other studies (Oba and Allen, 2000, 2003) have demonstrated that pH variations increase as the concentration of dietary starch increases. Thus, any consideration of ruminal pH should include not only the mean pH, but also the pH range during the feeding cycle. A pH profile having a narrow range suggests that salivary buffers and VFA removal can keep the pH from decreasing below the minimum threshold for particular microbial activities. This could mean more time for pH-sensitive processes such as fiber digestion. At the same mean pH value, a wider pH range could lead to both higher VFA accumulation and more prolonged period of higher pH known to be favorable for bacterial adherence, an important prerequisite for fiber digestion (Mouriño et al., 2001).

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pH has been shown to strongly affect both feed digestion in the rumen (Mould and Ørskov, 1983–1984) and the fermentation and growth of numerous species of ruminal bacteria in pure culture (Russell and Dombrowski, 1980). Some ruminal bacteria (e.g., *Streptococcus bovis*) are well known for their ability to rapidly outcompete other species at extremely low pH (Russell and Hino, 1985). Low pH is associated with several metabolic diseases thought to have a ruminal microbial origin, including ruminal acidosis (Russell, 2002) and laminitis (Russell et al., 2002), but these associations have largely been proposed based on in vitro studies, and there has been no systematic study on the effects of pH variations on the whole microbial community.

Even under conditions in which ruminal pH cycles within a normal range, pH may be expected to have a profound effect on populations of specific ruminal microbes; for example, the fibrolytic bacteria. Fiber digestion is a process that requires bacterial adhesion to the degradable surface to facilitate its hydrolysis before the fermentation of the carbohydrate oligomers (Weimer, 1996). Diets rich in fiber and physically effective NDF, as described by Mertens (1997), are known to yield different fermentation product profiles compared with diets rich in starch or concentrates, and these variations are considered to be strongly influenced by different pH profiles resulting from different rates of starch and fiber fermentation. The most intensively studied ruminal cellulolytic bacteria are known to cease growth at pH values below 6.0 (Russell and Dombrowski, 1980; Shi and Weimer, 1992; Weimer, 1993), although their enzymes can continue cellulose hydrolysis (Russell and Wilson, 1996). Thus, if the pH remains below certain levels, we can expect lower cellulolytic populations because of a reduced ability to bind cellulose or because of inhibition of catabolism of hydrolytic products. This in turn could lead to changes in the populations of sugar and cellodextrin-fermenting populations that could utilize products of continued enzymatic hydrolysis of fiber.

The central hypothesis of this study was that cows whose ruminal pH dynamics differed (in terms of mean pH and diurnal pH variation) would also harbor different ruminal bacterial communities. To test this, we monitored pH profiles of ruminally cannulated cows and characterized bacterial populations in ruminal samples from these cows, using automated ribosomal intergenic spacer analysis (**ARISA**). The ARISA technique is a culture-independent technique that facilitates comparisons of entire bacterial communities, including both cultured and as-yet-uncultured taxa, and is further useful in identifying target taxa for more specific and detailed analysis by quantitative real-time PCR (**qPCR**). Moreover, these methods can identify specific animals whose bacterial communities differ dramatically from

Table 1. Total mixed ration fed during the pH dynamics experiment

Item	% (DM basis)
Ingredient	
Corn silage	26.7
Alfalfa haylage	24.6
Alfalfa hay	4.2
Whole cottonseed	5.0
High-moisture shell corn, finely ground	22.2
Corn grain, cracked	3.2
Distillers grains, dried	2.5
Roasted soybeans, rolled	6.0
Soybean meal, 48% CP	2.0
Blood meal, bovine	1.2
Vitamin and mineral mix ¹	2.5
Monensin ²	0.00118
Composition	
aNDF ³	28.3
ADF	19.3
Lignin	4.4
CP	17.3
Ether extract	5.2
Ash	7.5

¹Vita-Plus, Madison, WI.

²Rumensin 80 (Elanco Animal Health, Greenfield, IN) added with vitamin and mineral mix.

³Neutral detergent fiber determined after α -amylase treatment (Mertens, 2002).

those of other animals on the same diet, which encourages both retrospective examination of specific animal performance patterns and preselection of animals for nutritional studies.

MATERIALS AND METHODS

pH Dynamics

Animal experiments were conducted at the US Dairy Forage Research Center Farm near Prairie du Sac, Wisconsin, according to a protocol approved by the Animal Care and Use Committee, Research Animal Resource Center, University of Wisconsin-Madison. Eight lactating, ruminally cannulated Holstein cows were fed an alfalfa silage- and corn silage-based TMR (Table 1) formulated to meet NRC (2001) guidelines for lactating cows, and were supplemented with monensin. Cows were provided feed ad libitum every 24 h, along with a continuous supply of water.

To monitor pH dynamics, SenTix 41 pH electrodes (WTW, Weilheim, Germany) were inserted into custom-built, stainless steel housings having open slots on the sides that permitted contact with the ruminal contents. Each electrode was held in a medial position within the rumen by insertion of the metal housing into a length of thick Norprene tubing attached to a barbed fitting on the inside of the cannula plug. The wire leads from the electrode were passed through the Norprene tubing and the cannula plug, and connected

to a pH logger (model 340i, WTW) contained within an aluminum box fixed to the outer rim of the cannula by thick, plastic cable ties. The pH values were recorded at 10-min intervals for 54 consecutive hours, corresponding to 2 complete feeding cycles and the first 6 h of the third feeding cycle. Loggers and electrodes were calibrated before insertion using pH 7.00 and 4.00 buffer solutions (Fisher Scientific, Fair Lawn, NJ). At the end of the 54-h incubation *in rumine*, the electrodes were removed, rinsed, and immersed again in the buffer solutions to determine the drift in electrode response. Drift values from pH 7 and pH 4 were within 0.01 to 0.1 pH units. The pH readings ($n = 326$ per cow) were downloaded using MultiLab Pilot software (version 4.01, WTW) and then transferred to an Excel (Microsoft, Redmond, WA) file for calculations to correct for the measured drift. When a shift in the reading obtained for the pH 7 or pH 4 buffer occurred, this drift would have influenced the last recorded pH values more than the first. Based on this, we assumed a linear drift in pH and formulated the following equation to calculate the “weighted” drift at each individual pH reading:

$$\text{Drift} = \{[(N - 1) \times [(X - 7)/326] \times (pH_N - 4)] + [(N - 1) \times [(Y - 4)/326] \times (7 - pH_N)]\} / 3,$$

where N = ordered number of pH reading taken at 10-min intervals (from 1 to 326); X = pH reading in pH 7.00 buffer upon withdrawal from rumen at end of experiment; Y = pH reading in pH 4.00 buffer upon withdrawal from rumen at end of experiment; and pH_N = pH at the N th reading of the indwelling electrode during the experiment.

This drift value was then subtracted from the recorded pH value to obtain the corrected pH; the corrected pH values were used to calculate the mean pH within each day. Daily pH range was calculated from the maximum and minimum pH values during the first 6 h of each feeding cycle, based on observations that the maximum pH occurred just before feeding and the minimum generally occurred within 6 h after feeding. The pH data (mean pH, diurnal pH range, or both) on the 3 successive days were subjected to cluster analysis using Minitab software (Minitab, State College, PA) in a single linkage analysis at the indicated level of similarity.

Ruminal Sampling

During the same 3 consecutive feeding cycles in which pH data were obtained, ruminal samples were collected just prior (~5 min) to feeding and at 3 and 6 h post-feeding. Samples were collected from the medio-ventral

region, using new polyethylene gloves, and immediately squeezed through 4 layers of cheesecloth to separate solid and liquid phases. The 2 phases were placed in separate 50-mL centrifuge tubes (Corning, Corning, NY) and stored frozen at -80°C before DNA extraction. A total of 144 samples (72 each of liquids and solids) were collected.

Extraction of DNA was conducted as described by Welkie et al. (2009) using 25 mL of ruminal fluid or 25 g of ruminal solids for each sample. The DNA concentration for each sample was determined spectrophotometrically, and the DNA solution was diluted to a working concentration of 10 mg/mL. The ARISA technique was performed as described by Welkie et al. (2009), with the following modification. Amplified PCR product was processed in a Beckman Coulter CEQ8000 capillary electrophoresis Genetic Analysis System (Fullerton, CA) by mixing 0.5 μL of PCR product with 1 μL of WelRed #1 infrared fluorescent dye-labeled DNA standard ladder and 39 μL of Sample Loading Solution (Beckman Coulter). Relatedness among the bacterial community compositions of different samples was determined by correspondence analysis (Weimer et al., 2010) of the electrophoretically separated ARISA amplicons of different lengths (**AL**). Cluster analysis of points on the ordination biplot from correspondence analysis was performed using Minitab software at the indicated similarity levels. To characterize potential phylotypes associated with AL246 and AL383 peaks identified from the ARISA analysis, their PCR products were cloned into the pGem-T vector using the GoTAQ PCR product cloning system (Promega Corp., Madison, WI) to obtain the corresponding operational taxonomic unit (**OTU**), and the sequences determined at the University of Wisconsin-Madison Biotechnology Center.

Real-time PCR procedures, including methods to calculate the percentage of 16S rRNA gene copies represented by individual taxa, have been described previously (Stevenson and Weimer, 2007). Primers used included those for *M. elsdenii* and *S. bovis* (Stevenson and Weimer, 2007), and for genus *Ruminococcus* (Weimer et al., 2008); all were designed to amplify sequences within the 16S rRNA gene. Specific primers for individual OTU identified by ARISA were designed manually based on the cloned sequence and then analyzed using Primer Express Software (Applied Biosystems, Foster City, CA). Primer pairs were selected to have melting temperature values near 60°C , to have a G+C content of 35 to 70%, to amplify a region of approximately 190 bp in total, to avoid stable secondary structures, and to minimize the number of G+C residues within the last 5 nucleotides near the 3' end. For OTU246, the forward primer sequence, derived from the end of the 16S rRNA

Figure 1. Exemplary ruminal pH profile from cow 1819, showing that pH maximum occurred just before feeding and pH minimum approximately 6 to 7 h after feeding. Vertical lines indicate times of ruminal sampling for analysis of bacterial community composition. A zero-time prefeeding sample was also collected for analysis.

gene, was TCG TAC CTT GAC AAC TGC ATA AAA A. The reverse primer sequence, derived from the internally transcribed sequence (ITS) region, was GCG TCC TTC TTC GGC TGA TAT. For OTU383, the forward and reverse primer sequences, derived from the ITS, were GGA GCG TGT CCG AAG GCA and AGT ATT GTC AAT GAT CTG GCT CCT CA, respectively.

Statistics

Data for milk production and composition were averaged, and the least squares means were compared using PROC MIXED of SAS (version 7.0, SAS Institute, Cary, NC), with cow as class variable (SAS Institute, 1998). Results from qPCR quantification of individual taxa were analyzed using PROC MIXED with cow, day, hour, and phase as class variables and with all potential 2- and 3-way interactions. Interactions having $P > 0.4$ were removed from the model and the reduced model re-run. Least squares means were compared using the Bonferroni t -test at $P = 0.10$ to reduce the chance of type II error.

RESULTS

Ruminal pH Profiles

Milk yields from each cow over the 3-d period of the pH measurements were 86 to 104% (mean = 98.5%) of those of a 5-d period the week before or week after the pH measurements, indicating that insertion of the pH

monitoring devices did not affect milk yield. The pH profiles during the 54-h monitoring period generally resembled those shown in Figure 1. Ruminal pH was highest just before feeding and declined for approximately 5 to 7 h thereafter before gradually increasing. Although this general pattern was observed for all cows, both the daily mean pH and the daily range in pH varied within and across cows (Table 2). Daily mean pH over the 3 cycles varied from 0.04 units (cow 2097) to 0.32 units (cow 2430).

Bacterial Community Compositions

Relatedness in bacterial community composition (BCC) among ruminal samples was examined by ARISA profiling combined with correspondence analysis. The ARISA technique involves electrophoretic separation of PCR-amplified bacterial genes encoding the ITS between the 16S and 23S rRNA genes (Fisher and Triplett, 1999). Each individual peak in the capillary electropherogram corresponds to a discrete ITS length. Because a single peak can correspond to more than one bacterial species, individual peaks are assigned as amplicon lengths (Fisher and Triplett, 1999; Felske and Osborn, 2005). Thus, although the electropherograms do not unequivocally represent a complete bacterial profile, electropherograms from different samples do permit a general comparison of BCC (Fisher and Triplett, 1999). Correspondence analysis of the ARISA profiles involves multivariate regression of the AL-by-sample data matrix in the number of dimensions equal to the number of AL for each sample. The first cor-

Figure 2. Ordination biplot from correspondence analysis of the automated ribosomal intergenic spacer analysis (ARISA) profiles. Individual points represent the bacterial community composition from a single sample (combination of cow, phase, day, and time within feeding cycle). The horizontal and vertical axes represent the axes that account for the largest and second-largest amount of variation in the data matrix. See text for interpretation of plot.

response axis accounts for the largest fraction of the variation in the data, with subsequent axes accounting for successively smaller fractions. The ordination biplot (Figure 2) projects BCC of an individual sample as a single point along the first and second correspondence axes. In the biplot, samples having progressively divergent community compositions display progressively greater distances between the points. Figure 2 reveals that the BCC of 6 of the 8 cows were very similar. In contrast, the BCC of cow 1699 was distinctly different from those 6 cows, and cow 2097 displayed even further divergence in BCC from the other 7 cows. Using the biplot coordinates from the 143 total samples (one sample was lost during workup), cluster analysis at a 90% similarity level confirmed the separation of the samples into these 3 groups.

Included among the 6 cows that had similar BCC were cows that had the highest mean pH (cow 2653, mean pH = 6.51) and lowest mean pH (cows 1272 and 2088, mean pH = 6.11), along with cows that had the widest mean range of pH (cow 1819, mean daily range = 1.26 units) and the narrowest mean range of pH (the aforementioned cow 2653, mean range = 0.45). The 2 cows that had BCC that differed strikingly from one another and from the other 6 had intermediate pH profiles (cow 1699, mean pH = 6.30, mean daily range = 0.81 units; cow 2097, mean pH = 6.33, mean daily range = 0.89 units). These observations are consistent with results from attempting to match the 3 groups of cows based on their BCC with cows grouped by their ruminal pH dynamics (Table 2). In this case, cluster analysis was performed by relaxing the similarity level

Table 2. Mean pH and pH range by cow during each of the 3 d of the pH dynamics study, and results from cluster analysis grouping cows by pH dynamics

Cow	Mean pH			pH range			Cluster analysis results ¹		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Mean pH	pH range	Both ²
1272	6.22	6.22	5.89	0.77	0.93	0.56	A	A	A
1699	6.25	6.35	6.29	0.51	1.24	0.69	B	A	A
1819	6.26	6.41	6.27	1.39	1.13	1.25	B	B	B
2007	6.63	6.36	6.44	0.48	0.85	0.42	C	A	C
2088	6.19	6.26	5.90	1.19	1.28	0.95	A	B	B
2097	6.31	6.34	6.35	1.08	1.15	0.45	B	A	A
2430	6.06	6.11	6.38	1.24	1.25	0.95	B	B	B
2653	6.53	6.44	6.56	0.64	0.45	0.26	C	C	C
Cluster similarity level ³							58.6	70	57.5

¹Cows sharing same letter (A, B, or C) within column were contained in the same cluster group.

²Both mean pH and diurnal pH range were included in the cluster analysis.

³Highest similarity level that yielded 3 pH response groups for indicated analysis.

Table 3. Least squares means of milk yield and composition in individual cows used in the pH dynamics studies¹

Cow	Milk yield (kg/d)	Milk component (%)	
		Fat	CP
1272	40.6 ^{bc}	2.8 ^b	2.8 ^b
1699	37.0 ^c	2.4 ^b	2.8 ^b
1819	36.2 ^c	2.7 ^b	2.6 ^b
2007	23.3 ^c	3.5 ^{ab}	2.8 ^b
2088	41.2 ^b	3.4 ^{ab}	2.8 ^b
2097	46.1 ^a	2.2 ^b	2.4 ^b
2430	28.1 ^d	5.0 ^a	3.9 ^a
2653	22.4 ^e	3.8 ^{ab}	3.0 ^b
SE of difference	2.2	0.51	0.15

^{a-e}Mean values within column having different superscripts differ ($P < 0.10$).

¹Cows and values in italics indicate bacterial community compositions substantially different from each other and from those of the other cows.

until cows were grouped (on the basis of mean pH, diurnal pH range, or both) into 3 clusters for each analysis (i.e., the number of clusters obtained from the BCC grouping). Clustering based on mean pH alone, or on pH range alone, assigned cows 1699 and 2007 to the same group (despite large differences in their BCC), and in each case (mean pH or pH range), the group also contained 2 of the 6 cows that had BCC very similar to one another. On the other hand, grouping of cows based on a combination of mean pH and pH range placed cows 1699 and 2007 into a single group that contained one other cow, but split the 5 remaining cows having similar BCC into 2 other groups. Overall, the data indicate that ruminal BCC in cows fed the same diet can be quite different in cows displaying similar pH profiles, and can be quite similar in cows having substantially different ruminal pH dynamics.

Retrospective examination of the production data from cows 1699 and 2097 indicated that they had numerically lower values of milk fat (<2.5%, even lower than their protein content) than did the other 6 cows (Table 3). In an attempt to identify specific bacterial taxa that contributed to the unique BCC of these 2 cows, the areas of individual peaks in the capillary electrophoregrams obtained during ARISA analysis were compared across cows. The 2 cows that had the lowest fat content displayed unusually large peaks for AL246 and substantially reduced peak areas for AL383 (similar to a pattern observed among milk fat-depressed cows in a companion study; Weimer et al., 2010). The distribution of these AL across all cows examined in this study is shown in Figure 3. These 2 AL were detected together in only 21% of the 143 ruminal samples collected across all 8 cows. Samples in which AL246 accounted for <3% of total ARISA peak area were well populated with AL383 (0.4 to 1.5% of total ARISA peak area), but

samples in which AL246 accounted for >4% of the total ARISA peak area appeared to have much less AL383 (0 to 0.5% of total ARISA peak area).

Sequencing of a 443-bp cloned PCR amplicon (sequence deposited in GenBank with accession no. FJ687347) of a DNA fragment from a gel band corresponding to AL246 yielded a 150-bp segment of the adjacent 16S rRNA gene. Comparison of the sequence of this OTU246 with the GenBank database revealed 100% identity to partial 16S rRNA gene sequences for 2 uncultured ruminal bacterial entries (AB034086.1 and AB034085.1) and 96% identity to the partial 16S rRNA gene sequence of an uncultured *Megasphaera* sp. clone SNH07 (entry AY672079.2; Tajima et al., 2000). Based on this putative taxonomic identification, real-time PCR was performed on solid-phase samples from the 2 fat-depressed cows (1699 and 2097) and 2 of the 6 other cows (1272 and 2088), using primers specific for OTU246 and primers specific for *M. elsdenii*. These latter 2 cows were selected for analysis because in the companion study (Weimer et al., 2010) they had displayed monensin-induced milk fat depression (MFD), which they had since overcome upon prolonged (8-mo) exposure to monensin. As indicated in Table 4, real-time PCR analysis indicated that the 2 MFD cows had elevated levels of both OTU246 and *M. elsdenii*, with cow 2097 displaying the higher populations consistent with its more displaced position in the correspondence analysis (Figure 2). For each sample, the percentage of the bacterial gene copies attributable to these 2 taxa (OTU246 and *M. elsdenii*) were remarkably similar, and linear regression of all qPCR analyses within samples

Figure 3. Distribution of amplicon lengths (AL)246 and AL383 in 143 ruminal samples from 8 cows, as detected by automated ribosomal intergenic spacer analysis (ARISA) profiling.

Table 4. Least squares means from real-time PCR analysis of 16S rRNA gene copies of *Megasphaera elsdenii* and operational taxonomic unit (OTU)246 in solid-phase ruminal samples from 4 cows

Milk fat status	Cow	Percentage of total bacterial 16S rRNA gene copies	
		<i>M. elsdenii</i>	OTU246
Fat-depressed	1699	0.320 ^b	0.289 ^b
	2097	1.946 ^a	1.582 ^a
Not fat-depressed	1272	0.018 ^b	0.014 ^b
	2088	0.032 ^b	0.028 ^b
SE of difference		0.27	0.08

^{a,b}Means having different letters within column differ ($P < 0.10$).

from the 4 cows revealed a very strong correlation between the 2 taxa ($r = +0.99$, $P < 0.0001$).

Sequencing of a 574-bp cloned PCR amplicon from a gel band corresponding to AL383 yielded a 143-bp segment of the adjacent 16S rRNA gene. Comparison of this sequence with the GenBank database revealed up to 99% identity to partial 16S rRNA gene sequences of uncultured ruminal bacteria and 93% identity to the 3 closest identified relatives (*Prevotella ruminicola*, *Prevotella denticola*, and *Prevotella* sp.). Subsequent qPCR assays of the corresponding OTU383 indicated that the relative number of gene copies of this apparent *Prevotella* did not vary among samples even though the AL383 ARISA peak areas varied substantially, suggesting that AL383 contained an additional, yet-undefined phylotype whose gene copy amounts varied among samples.

In contrast to *M. elsdenii*, the genus *Ruminococcus* and the species *Streptococcus bovis*—2 taxa whose populations would be expected to be strongly dependent on pH (Russell, 2002)—on average accounted for 10.6 and 0.01% of bacterial 16S rRNA gene copies, respectively (Table 5), generally did not differ by cow, despite substantial differences in their ruminal pH profiles. *Streptococcus bovis* was slightly elevated in cow 2088, which had moderately low pH but normal fat levels.

DISCUSSION

For each feeding cycle within each cow, pH measured in the medial ventral region of the rumen reached its maximum just before feeding, then decreased to its minimum over an approximately 6-h period, similar to the time reported by Duffield et al. (2004) and somewhat longer than the 3 h reported by Marden et al. (2005). Over the next ~18 h, a gradual decrease in acid production and increased VFA absorption (Whitelaw et al., 1970), combined with salivary buffer production during rumination (Maekawa et al., 2002), returned pH to near its previous value before feeding. All of the pH traces exhibited some variation in pH around the trend

line, likely because of local microenvironmental differences resulting from fluid movement and from alternating contact between rumen liquor and solid digesta.

Although these general patterns were observed in all cows, there were slight to substantial variations in mean pH and in diurnal pH range for each of the cows during the 3-d study period. Looking more broadly across 3 feeding cycles, there were substantial differences among cows in overall mean pH (6.11 to 6.51) and in average diurnal pH range (0.45 to 1.26). The pH ranges observed here thus encompassed values that varied from the rather low diurnal ranges (~0.3 units) reported by Marden et al. (2005) to the rather high diurnal ranges (~1.2 units) reported by Maekawa et al. (2002). Although the cows in this study were all fed the same diet, it is likely that these differences in diurnal range were due to a variety of factors, including the timing of individual feeding and rumination bouts after feed presentation; the rate of passage of digesta and VFA from the rumen, the effectiveness of VFA absorption from the rumen; and the effectiveness of buffering from salivation into the rumen (Russell, 2002).

Despite these differences in pH dynamics, there were marked similarities in BCC in 6 of the 8 cows studied, even though these 6 accounted for the complete spectrum of variation in mean pH and mean diurnal pH range. These similarities were observed in ARISA profiles, which largely represent the uncultured members of the ruminal bacterial community. However, the populations of even some culturable taxa whose growth is known to be affected by pH did not differ among cows. Although both *Ruminococcus albus* and *R. flavefaciens* are unable to grow at pH <6.0 in continuous culture (Russell and Dombrowski, 1980; Shi and Weimer, 1992), real-time PCR analysis of the ruminal samples revealed similar levels of genus *Ruminococcus* in all 8 cows (approximately 10.6% of 16S rRNA gene copies). The abundance of *Ruminococcus* is consistent

Table 5. Least squares means from real-time PCR (qPCR) analysis of 16S rRNA gene copies of genus *Ruminococcus* and of *Streptococcus bovis* in cows from the pH profile study

Cow	Percentage of total 16S rRNA gene copies	
	<i>Ruminococcus</i>	<i>S. bovis</i>
1272	9.96	0.0045 ^b
1699	13.01	0.0043 ^b
1819	8.35	NT ¹
2007	12.17	NT
2088	10.68	0.0222 ^a
2097	10.80	0.0089 ^b
2430	9.19	NT
2653	11.03	NT
SE of difference	1.11	0.0051

^{a,b}Means having different letters within column differ ($P < 0.10$).

¹NT = not tested.

with previous real-time PCR data (Weimer et al., 2008) and the frequent appearance of sequences related to *R. flavefaciens* (cellulolytic), and *Ruminococcus bromii* and *Ruminococcus callidus* (amylolytic) in clone libraries (Tajima et al. 2000). *Streptococcus bovis*, a bacterium considered to be more abundant under conditions of ruminal acidosis (Russell, 2002), was maintained in very low levels (<0.01% of 16S rRNA gene copy number) in all 8 cows studied. This low abundance is consistent with previous qPCR studies (Stevenson and Weimer, 2007; Weimer et al., 2008) and the absence of *S. bovis* sequences in clone libraries (Tajima et al., 2000). A partial explanation of the lack of an *in rumine* pH effect on these taxa may involve the short-term disparity between population sizes and their metabolic activity over the course of the feeding cycle. For example, although cellulolytic ruminococci may cease growth at pH <6 (Russell and Wilson, 1996), they remain adherent and viable on digesta particles at somewhat lower pH, and they can probably rapidly re-initiate growth upon return to a permissive pH later in the feeding cycle (Mouriño et al., 2001). The effects of pH on amylolytic ruminococci have not been reported.

The BCC of 2 of the 8 cows were markedly different from each other and from those of the other 6. These cows were distinguished by their very low milk fat content. Examination of the ARISA data revealed that both cows had much higher levels of one AL (AL246). Cloning and sequencing of a portion of the 16S rRNA gene associated from this AL yielded an OTU246 that had a strong sequence similarity to *M. elsdenii*, a bacterium whose population size has long been known to increase under conditions of MFD (Latham et al., 1972). Real-time PCR experiments with samples from all 8 of the cows, using primer sets separately designed for OTU246 and *M. elsdenii*, confirmed that both taxa were elevated only the 2 fat-depressed cows (Table 4), and that these 2 taxa were very strongly correlated ($r^2 = 0.99$) with one another across different cows. The data support a companion study (Weimer et al., 2010) in which OTU246 was strongly elevated in 6 cows subjected to diet-induced MFD, but was not elevated in 2 cows that did not display MFD under the same dietary treatments. It is noteworthy that 2 of the cows (cows 1272 and 2088) that did not display MFD or high populations of OTU246 in the present study displayed both MFD and high levels of AL246 in the study conducted 8 mo previously (Weimer et al., 2010), indicating that cows that display MFD on diets containing both rapidly fermented starch and monensin can overcome this condition upon prolonged monensin exposure, and that overcoming this condition is associated with changes in the relative abundance of some bacterial OTU. Moreover, when considered together,

these studies provide the first molecular evidence of an association between MFD and *M. elsdenii* populations *in rumine*, and are in agreement with culture-based evidence that this species displayed elevated cell densities in cows that were fed high-concentrate diets and that displayed MFD (Latham et al., 1972). As noted in our companion study, however, it appears that the association between OTU246 (*M. elsdenii*) and MFD is not absolute, as we have observed a few situations in which this taxon is barely detectable during MFD, or appears at substantial levels in advance of MFD (Weimer et al., 2010).

A second AL (AL383) was found to have reduced ARISA peak areas in ruminal samples that had elevated peak areas for OTU246; AL383 was first noted to have reduced ARISA peak areas in cows that displayed MFD when fed diets containing rapidly fermented starch and monensin (Weimer et al., 2010). In the present study, AL383 was found to contain at least 2 phylotypes: A *Prevotella* sp. whose contribution to ITS gene copies did not vary among samples and an unidentified taxon that likely contributed to the variation in ARISA peak area. These observations support reports that ARISA AL can contain more than one phylogenetically distinct taxon (i.e., more than one phylotype; Fisher and Triplett, 1999). Isolation and characterization of the unknown phylotype in AL383 and elucidation of its interactions with *M. elsdenii* may provide important information on the mechanism of MFD.

The potential of *M. elsdenii* as a causative agent of MFD is interesting in light of its possible production of *trans*-10, *cis*-12 conjugated linoleic acid, a known substrate for ruminal biohydrogenation reactions to produce C18:1 acids that can suppress mammary lipogenesis (Bauman and Griinari, 2003). Kim et al. (2002) reported that several strains of this species produce *trans*-10, *cis*-12 conjugated linoleic acid, although Maia et al. (2007) reported that none of the strains they tested produced this compound. Resolution of this issue will require testing of *M. elsdenii* isolates from MFD and non-MFD cows for *trans*-10, *cis*-12 conjugated linoleic acid production. However, MFD and its microbial underpinnings are complex issues that may also demand consideration of the possible ruminal interconversion of long-chain fatty acid isomers (Wallace et al., 2007) and of the activities of other taxa, particularly the unknown strain represented by AL383.

CONCLUSIONS

Ruminal pH dynamics, including both mean ruminal pH and diurnal pH range, varies considerably between cows and even within cows during successive feeding cycles. Despite these variations, cows having quite

different ruminal pH dynamics can have considerable similarities in their BCC. In this study, cows that displayed unusual BCC exhibited intermediate pH profiles but produced milk having a low fat content, and the differences in BCC from those of other cows was largely because of differences in the relative amounts of only a few ruminal bacterial taxa, particularly *M. elsdenii*. Controlling populations of these few taxa warrants examination as a potential means of controlling MFD.

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REFERENCES

- Allen, M. S., J. A. Voelker, and M. Oba. 2006. Physically effective fiber and regulation of ruminal pH: More than just chewing. Pages 270–278 in *Production Diseases in Farm Animals*. N. P. Joshi and T. H. Herdt, ed. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. *Annu. Rev. Nutr.* 23:203–227.
- Duffield, T., J. C. Plazier, A. Fairfield, R. Bagg, G. Vessie, P. Dick, J. Wilson, J. Aranini, and B. McBride. 2004. Comparison of techniques for measurement of rumen pH in lactating dairy cows. *J. Dairy Sci.* 87:59–66.
- Felske, A., and A. M. Osborn. 2005. DNA fingerprinting of microbial communities. Pages 65–96 in *Molecular Microbial Ecology*. A. M. Osborn and C. J. Smith, ed. Taylor and Francis, New York, NY.
- Fisher, M. M., and E. W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 63:4630–4636.
- Grant, R. J., and D. R. Mertens. 1992. Influence of buffer pH and raw corn starch addition on *in vitro* fiber digestion kinetics. *J. Dairy Sci.* 75:2762–2768.
- Hoover, W. H. 1986. Chemical factors involved in ruminal fiber digestion. *J. Dairy Sci.* 69:2755–2766.
- Kim, Y. J., R. H. Liu, J. L. Rychlik, and J. B. Russell. 2002. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12 isomer of conjugated linoleic acid. *J. Appl. Microbiol.* 92:976–982.
- Latham, M. J., J. E. Storrey, and M. E. Sharpe. 1972. Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. *Appl. Microbiol.* 24:871–877.
- Mackawa, M., K. A. Beauchemin, and D. A. Christensen. 2002. Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. *J. Dairy Sci.* 85:1165–1175.
- Maia, M. R. G., L. C. Chaudhary, L. Figueres, and R. J. Wallace. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie van Leeuwenhoek* 91:303–314.
- Marden, J. P., C. Bayourthe, F. Enjalbert, and R. Moncoulon. 2005. A new device for measuring kinetics of ruminal pH and redox potential of dairy cattle. *J. Dairy Sci.* 88:277–281.
- Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cows. *J. Dairy Sci.* 80:1463–1481.
- Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. *J. AOAC Int.* 85:1217–1240.
- Mould, F. L., and E. R. Ørskov. 1983–1984. Manipulation of rumen fluid pH and its influence on cellulolysis in sacco, dry matter degradation and the rumen microflora of sheep offered either hay or concentrate. *Anim. Feed Sci. Technol.* 10:1–14.
- Mourinho, F., R. Akkarawongsa, and P. J. Weimer. 2001. Initial pH as a determinant of cellulose digestion rate by mixed ruminal microorganisms *in vitro*. *J. Dairy Sci.* 84:848–859.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Oba, M., and M. S. Allen. 2000. Effects of brown midrib 3 mutation in corn silage on productivity of dairy cows fed two concentrations of dietary neutral detergent fiber: 1. Feeding behavior and nutrient utilization. *J. Dairy Sci.* 83:1333–1341.
- Oba, M., and M. S. Allen. 2003. Effects of corn grain conservation method on feeding behavior and productivity of lactating dairy cows at two dietary starch concentrations. *J. Dairy Sci.* 86:174–183.
- Russell, J. B. 2002. *Rumen Microbiology and its Role in Ruminant Nutrition*. J. B. Russell, Ithaca, NY.
- Russell, J. B., and D. B. Dombrowski. 1980. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Appl. Environ. Microbiol.* 39:604–610.
- Russell, J. B., M. R. Garner, and J. P. Flint. 2002. *Allisonella histiformans*, gen. nov., sp. nov., a novel bacterium that produces histamine, utilizes histidine as its sole energy source, and could play a role in bovine and equine laminitis. *Syst. Appl. Microbiol.* 25:498–506.
- Russell, J. B., and T. Hino. 1985. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis. *J. Dairy Sci.* 68:1712–1722.
- Russell, J. B., and D. B. Wilson. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *J. Dairy Sci.* 79:1503–1509.
- SAS Institute. 1998. *SAS User's Guide*, Version 7. SAS Inst., Inc., Cary, NC.
- Shi, Y., and P. J. Weimer. 1992. Response surface analysis of the effects of pH and dilution rate on *Ruminococcus flavefaciens* FD-1 in cellulose fed continuous culture. *Appl. Environ. Microbiol.* 58:2583–2591.
- Stevenson, D. M., and P. J. Weimer. 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75:165–174.
- Tajima, K., A. Shozo, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R. Aminov, and Y. Benno. 2000. Rumen bacterial community transition during adaptation to a high-grain diet. *Anaerobe* 6:273–284.
- Terry, R. A., J. M. A. Tilley, and G. E. Outen. 1969. Effect of pH on the cellulose digestion under in vitro conditions. *J. Sci. Food Agric.* 20:317–320.
- Wallace, R. J., N. McKain, K. J. Shingfield, and E. Devillard. 2007. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J. Lipid Res.* 48:2247–2254.
- Weimer, P. J. 1993. Effect of dilution rate and pH on the ruminal cellulolytic bacterium *Fibrobacter succinogenes* S85 in cellulose-fed continuous culture. *Arch. Microbiol.* 160:288–294.
- Weimer, P. J. 1996. Why don't ruminal bacteria digest cellulose faster? *J. Dairy Sci.* 79:1496–1500.
- Weimer, P. J., D. M. Stevenson, and D. R. Mertens. 2010. Shifts in bacterial community composition in the rumen of lactating dairy cows under milk fat depressing conditions. *J. Dairy Sci.* 93:265–278.
- Weimer, P. J., D. M. Stevenson, D. R. Mertens, and E. E. Thomas. 2008. Effect of monensin feeding and withdrawal on populations of individual bacterial species in the rumen of lactating dairy cows fed high-starch rations. *Appl. Microbiol. Biotechnol.* 80:135–145.
- Welkie, D. G., D. M. Stevenson, and P. J. Weimer. 2009. ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. *Anaerobe* doi:10.1016/j.anaerobe.2009.07.002
- Whitlaw, F. G., J. Hyldgaard-Jensen, R. S. Reid, and M. G. Kay. 1970. Volatile fatty acid production in the rumen of cattle given an all-concentrate diet. *Br. J. Nutr.* 24:179–195.