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ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle☆

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ABSTRACT

The bovine rumen undergoes substantial changes in environmental conditions during the animal's feeding cycle, but the effects of these changes on microbial populations have not been examined systematically. Two dairy cows fed a mixed forage/concentrate ration at 12 h intervals over 4 feeding cycles displayed substantial changes in ruminal pH and volatile fatty acid (VFA) concentrations. Automated ribosomal intergenic spacer analysis (ARISA) of solid- and liquid-associated bacterial populations in samples collected at 2, 4, 6, 9, and 12 h after feeding revealed a high degree of bacterial diversity. A total of 155 different amplicon lengths (ALs) were detected across all 83 samples, and 11–74 detected per sample. A substantial proportion (11%) of the ALs was detected in one cow but not in the other. The proportions of ALs that were detected only in the liquid phase or the solid phase were 13.5% and 1.9%, respectively. Correspondence analysis indicated that bacterial community composition differed between cows and between solid or liquid phases, but overall the solid-associated population displayed less change in composition within and across feeding cycles. The data support the notion that cows fed the same diets can have substantial differences in bacterial community composition, and that the solids-associated (biofilm) communities display greater stability than do associated planktonic communities.

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1. Introduction

Studies of microorganisms and their impact on, and interdependence with, various ecological systems have grown dramatically in recent years, owing in large part to advances in molecular characterization techniques. While environments such as freshwaters, sediments, and soils have been the focus of the majority of these studies [1–4], the same techniques can also be employed to investigate complex symbiotic relationships among microorganisms and their mammalian hosts [5–9]. One such environment is the rumen of ruminant animals. Ruminal bacteria perform functions vital to the health and productivity of the host, including the degradation and fermentation of cellulose and other polysaccharides, and the production of volatile fatty acids (VFA), microbial cell protein, and vitamins used by the host as energy sources, protein, and growth factors, respectively [10,11].

Bacterial populations in nature undergo dynamic turnover, although these dynamics are incompletely understood [12,13]. Thus, we would expect that the bacterial community composition (BCC) of the rumen should also display changes in response to changes in environmental conditions. However, the principal forces that influence these bacterial relationships in the ruminal environment and the variations in the diversity of ruminal bacterial populations over time have been largely unexplored. In the rumen, where environmental conditions such as temperature, ionic strength, and redox potential are relatively constant [10,11], the most important driver of bacterial population structure is likely to be the feeding cycle, during which the microbial community is supplied with large but discontinuous input of fermentable energy sources that support microbial catabolism and growth. Several enumeration studies, using culture-dependent techniques, have revealed only modest changes in the total culturable population [14,15] and in the populations of the principal physiological types (glucose-, starch-, pectin-, xylan-, and cellulose-degrading populations [16]) during the feeding cycle. However, changes in the composition of the entire bacterial community (including both cultured and uncultured members) during the feeding cycle have received relatively little attention.

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The purpose of this study was to characterize bacterial population dynamics in the rumen during the diurnal feeding cycle of the dairy cow, through the use of Automated Ribosomal Intergenic Spacer Analysis (ARISA) [1], a community fingerprinting technique that allows profiling of the culturable and currently unculturable residents of a microbial habitat. The ARISA technique was chosen for this study because of its ability to detect a greater extent of bacterial diversity than other techniques [17], and its usefulness in correlating changes in BCC with changes in various environmental factors [2,12]. In addition, its automated nature allows for rapid and efficient analysis of a substantial number of samples, to more accurately observe shifts in community composition over time. Individual peaks generated by ARISA analysis represent amplicons of a specific length, but not necessarily a single sequence (i.e., the peak may represent more than a single phylotype). Thus, ARISA does not provide a quantitative assessment of individual taxa. Nevertheless, comparison of ARISA profiles does permit broad-scale characterization of the extent of differences among different communities, or of changes in individual communities over time [1,12].

2. Materials and methods

Two animals were chosen for this experiment to provide comparison in ruminal environments between individuals. Both were lactating, fistulated Holstein cows housed in indoor tie stalls adjacent to each other. The cows were maintained according to the protocol approved by the University of Wisconsin Animal Care and Use Committee. Cows were provided feed *ad libitum* at 12 h intervals (0500 and 1100) over a 48 h period (i.e., 4 feeding cycles), along with a continuous supply of water *ad libitum*, and were milked at 12 h intervals (0400 and 1600). The ration (32.5% neutral detergent fiber determined after α-amylase treatment, 39.3% non-fiber carbohydrates, and 17.4% crude protein, dry matter basis) was formulated to meet the National Research Council recommendations for lactating dairy cows, and consisted of the following ingredients (dry matter basis): 32.9% corn silage, 32.2% alfalfa haylage, 15.2% dry shell corn, 5.8% whole cottonseed, 4.4% soybean meal, 3.7% dried distiller grains, 2.8% roasted soybeans, 0.5% blood meal, plus supplemental vitamins and minerals. Within each cycle, ruminal samples were collected pre-feed and at 2, 4, 6, 9 and 12 h post-feeding, the last sample corresponding to the pre-feed sample of the next cycle.

Samples were collected medio-ventral in the rumen, using disposable, arm-length polyethylene gloves, and were transferred to pre-warmed thermos bottles that were immediately sealed and brought to the laboratory within 5 min of collection. The collected material was squeezed through 4 layers of cheesecloth into a CO_2-sparged flask. The separated solids and liquids were placed in separate Corning 50 ml centrifuge tubes and stored frozen at −80 °C prior to the analysis of VFA (liquid samples only) and DNA extraction. VFA were determined by HPLC [18]. DNA extraction was performed as described by Weimer et al. [19] using 25 ml of rumen fluid or 25 g of rumen solid for each sample. During the DNA purification procedure, 1 sample (cow 5003, cycle 1, 9 h post-feed liquid sample) was lost, leaving a total of 83 samples for ARISA analysis.

ARISA was performed generally as described by Fisher and Triplett [1], with the following modifications. The oligonucleotide primers used for ARISA were ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCAAC-3′). The primer ends were complementary to the respective positions 1423 and 1443 of the 23S rRNA and positions 38 and 23 of the 16S rRNA of *Escherichia coli* [20]. Primers were synthesized and labeled with Beckman Coulter (Fullerton, CA) WelRed #2 infrared fluorescent dye (Sigma-Proligo) and were reconstituted in water to a working concentration of 400 nM. Template DNA from the ruminal samples was prepared as described previously [19]. DNA concentrations were determined spectrophotometrically and diluted to a working concentration of 10 μg/ml. ARISA PCR reactions were carried out in 20 μl volumes using Promega (Madison, WI) GoTaq Flexi system reagents containing 4.0 μl Mg-free buffer (5 × concentrate), 2.0 μl of 2.5 mM $MgCl_2$, 0.5 μl of 200 μM dNTP mixture, 2.0 μl each of the 2 primers, 2.0 μl of template DNA, 7.0 μl nuclease-free water, and 0.5 μl of Taq polymerase. Molecular biology grade mineral oil (Sigma–Aldrich, St Louis, MO) was added to each tube in amounts sufficient to cover the reaction mixture. PCR reactions were carried out in an Applied Biosystems (Foster City, CA) thermocycler, under the following conditions: 94 °C for 2 min (1 cycle), followed by 30 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 120 s, and finally with 1 cycle of 72 °C for 120 s.

Amplified PCR product was resolved in a Beckman Coulter CEQ8000 capillary electrophoresis Genetic Analysis System by mixing 1.0 μl PCR product with 0.5 μl of Beckman Coulter (Fullerton, CA) WelRed #1 infrared fluorescent dye-labeled DNA standard ladder consisting of 23 sizes of DNA ranging from 50 to 1000 bp (MapMarker 1000, BioVentures, Murfreesboro, TN) and 39 μl sample loading solution (Beckman Coulter). This mixture was then loaded into microtiter plates, molecular biological grade mineral oil was added to cover the liquid surface, and the capillary electrophoresis conducted according to the manufacturer's directions. The run parameters consisted of a capillary temperature of 50 °C, a denaturation temperature of 90 °C for 120 s, an injection voltage of 2.0 kV for 30 s, and a separation voltage of 6.0 kV for 90 min.

The resulting data were imported as an SCF 3.0 file for analysis using GeneMarker (v 1.70) software (SoftGenetics LLC, State College, PA). Settings to detect and quantify the peaks were based on the methods for AFLP analysis specified in the GeneMarker manual. The panel used for comparison was generated automatically using these parameters, and questionable peaks were screened manually. In addition, the software was used to correct "pull-up" between the 2 dyes used, and baseline subtraction and smoothing was performed. All peaks corresponding to amplicon lengths (ALs) of >238 bp were used for analysis. This length cutoff was selected based on our observation that accurate assignment of amplicon sizes using the size standards became difficult with amplicon peaks of <238 bp. Moreover, amplicons of <238 bp would be expected to have ITS sequences of <151 bp, and relatively few such ITS sequences have been identified [21]. The resulting peak area data were tabulated, with individual samples as columns and AL (in bp) as rows. Correspondence analysis was performed using the method of Ludwig and Reynolds [22]. Calculations were performed using custom software written in the C programming language. The first 3 eigenvalues and eigenvectors were found using the power method. Finally, vectors for pH, total mM VFA, and A:P ratio were added by tabulating these values as vectors and ordination points calculated by the method of vector fitting per McCune and Grace [23].

2.1. Statistical analysis

Milk production and composition data from the 2 cows were analyzed using PROC MIXED of the SAS statistical software program, v. 7.0 (SAS Institute, Cary, NC) [24], with cow, milking (AM or PM), and cow × milking interaction modeled as fixed effects, and with milking within cow as repeated measures. Ruminal chemistry (VFA and pH) data were analyzed with PROC MIXED, with feeding cycle, sampling time within cycle, and feeding cycle × sampling time interaction modeled as fixed effects; with cow × cycle and

cow × sampling time as random effects; and with sampling time within cow (cycle) as repeated measures.

The total numbers of ALs from ARISA were analyzed using a similar procedure, but the model also included sample phase (solid or liquid) as a fixed effect, and all significant 2-way interactions. Least-square means for parameter estimates were compared using the protected least significant differences (PDIFF) procedure, with significance at $P = 0.10$ to minimize type I errors with the small number of cows. The extent of BCC shifts across sampling times was determined by calculation of linear distances between coordinates from the 2-dimensional correspondence analysis ordination plots. These "internodal distances" were compared within sampling time across feeding cycles for different sample phases (solid or liquid) within individual cows, using PROC MIXED in SAS to obtain least-square means, and with means separation tests for significant parameter estimates using PDIFF.

3. Results

3.1. Animal production data

Feed intake, milk yield, and yield of major milk components were similar between the 2 cows, although there were significant differences in percentages of protein, lactose, and urea (Table 1).

3.2. Ruminal chemistry during the feeding cycle

The time courses of pH and VFA concentrations in the rumen over the 4 feeding cycles are shown in Figs. 1 and 2, and least-square mean data by cow are provided in Table 2. Ruminal pH varied during the feeding cycle ($P = 0.011$) and was highest just before feeding, and lowest approximately 6 h after feeding. Mean ruminal pH for all samples, or for samples collected across feeding cycles at a particular time point post-feeding, did not differ between the 2 cows. Individual or total VFA, the agents primarily responsible for acidification of the rumen, did not differ by cow or by feeding cycle, except for a few minor VFA (a cow effect for valerate and a cycle effect for isovalerate + 2 methylbutyrate). However, concentrations of all VFA varied with sampling time ($P < 0.05$). Within each cycle, VFA concentrations first increased due to microbial fermentation, reaching a maximum value at ~6 h post-feeding (i.e., near the pH minimum), then declined as VFA uptake by the host outpaced VFA production from microbial fermentation. Ruminal concentrations of acetate, butyrate, and total VFA were significantly higher, and ruminal pH was significantly lower in samples collected during the PM cycles relative to the AM cycles (Table 2). This suggests that individual cycles displayed significant variation despite the fixed (12 h) interval of feed presentation, likely due to different eating patterns between cows and among feeding cycles.

Table 1
Least-square means of production data from cows over a 5 day period terminating in the four 12 h feeding cycles used for collection of ruminal samples.

Measurement	Cow 5003	Cow 5184	Pooled S.E.
Feed intake (kg dry matter/d)	22.0	23.7	1.1
Yields (kg/d)			
Milk	37.5	33.8	2.6
Fat	1.45	1.28	0.18
Protein	1.06	0.92	0.10
Lactose	1.71	1.72	0.14
Milk composition			
Fat (%)	3.78	3.75	0.25
Protein (%)	2.79[a]	2.67[a]	0.05
Lactose (%)	4.50[a]	5.02[a]	0.06
Urea N (mg/dL)	9.3[a]	11.7[a]	1.1

[a] Mean values differ ($P < 0.10$).

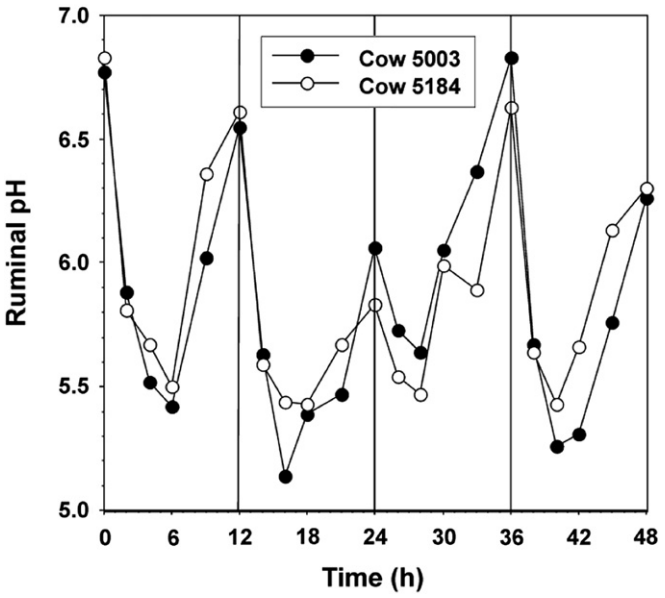

Fig. 1. Ruminal pH profiles of cows 5003 and 5184, determined over four 12 h feeding cycles.

3.3. Bacterial community dynamics

ARISA profiles of amplicons generated by PCR of bacterial ITS sequences across the full set of 83 ruminal samples yielded a total of 155 ALs. The number of ALs detected in the samples ranged from 11 to 74 (mean = 50, SEM = 10), and was not affected by cow ($P = 0.44$), sample phase ($P = 0.46$), feeding cycle ($P = 0.26$), or post-feed sampling time ($P = 0.33$). There was no significant cow-by-phase interaction ($P = 0.13$), but there was a phase-by-cycle interaction ($P = 0.005$) suggesting that the stability of total number of ALs detected across cycles was phase dependent. As shown in Fig. 3, 98 of the 155 ALs (63.2%) were detected in at least 1 sample in each phase (liquid or solid) from each cow. Twenty-one (13.5%) of the ALs were detected only in the liquid phase, and two-thirds of these were detected in both cows. By contrast, only 3 ALs (1.9%) were detected only in the solid phase, 2 of which were common to both cows. Across sample phases, 17 (11.0%) of the ALs were detected in one or the other cow but not in both, with cow 5003 harboring the greater number of unique ALs (12 versus 5).

To gauge shifts in bacterial community composition within and across feeding cycles, the matrix of data including all samples representing an individual cow and sample phase (solid or liquid) was subjected to correspondence analysis (CA). Fig. 4 shows a plot of the resulting ordination values. The ordination plot reduced the data from 155 dimensions (i.e., 155 ALs) to the 2 dimensions accounting for the largest variation in the data. Each point in the ordination plot represents the BCC of a single sample (cow–phase–cycle–sampling time combination), with a greater distance between points indicating a greater difference in BCC. For each cow–sample phase combination, plots contained all data points or nodes (representing the initial pre-feed community, and the communities at 2, 4, 6, 9, and 12 h post-feeding during each of the 4 successive cycles). For both liquid-associated and solids-associated communities in both cows, there was relatively little overlap of coordinates on the plot, suggesting substantial differences in BCC between cows, and between phases within cows. While shifts in

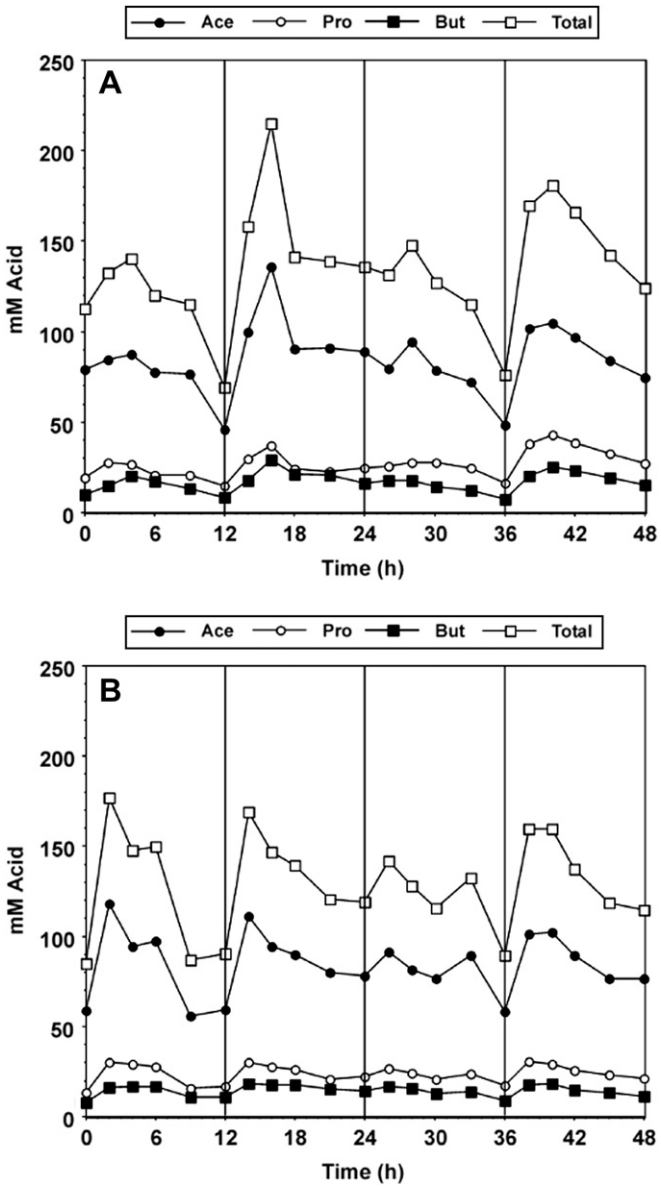

Fig. 2. Ruminal VFA concentrations in the 2 cows from samples collected over four 12 h feeding cycles.(A) Cow 5003,(B) cow 5184. Ace = acetic, Pro = propionic, and But = butyric acid.

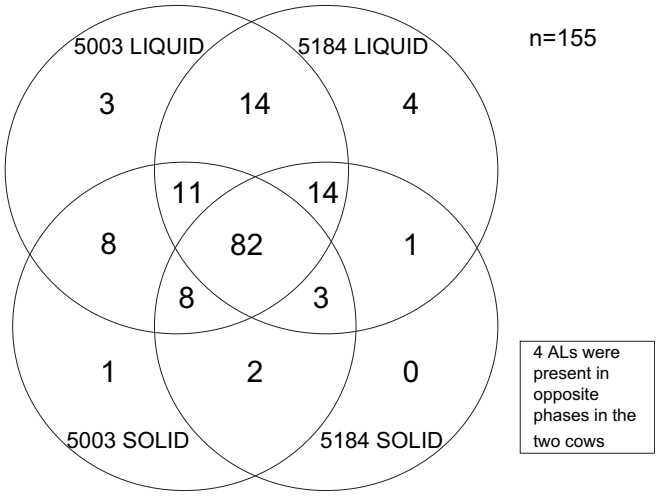

Fig. 3. Venn diagram showing distribution of amplicon lengths (ALs) detected from ARISA analysis of solid and liquid samples from cows 5003 and 5184.

BCC over the course of 4 feeding cycles were observed for each sample set, the linear distances on the CA trace between the first and last sampling point for the solids-associated samples (0.38 for cow 5003, 0.39 for cow 5184) were 5- to 7-fold smaller than for the liquid samples (2.05 for cow 5003, 2.63 for cow 5184), indicating a smaller overall shift in BCC during the course of the experiment. Similarly, to compare population shifts at a particular post-feed time point across individual cycles, the linear distances between nodes on the ordination plot for adjacent cycles (e.g., 2 h post-feed time points from cycle 1 to cycle 2, cycle 2 to cycle 3, and cycle 3 to cycle 4) were compared across time points (Fig. 5). Overall, LS means of this internodal distance were lower for the solid-associated community than for the liquid-associated community of both cow 5003 (solids, 0.68; liquid, 1.05; SED = 0.12; $P = 0.003$) and cow 5184 (solids, 0.34; liquid, 0.92; SED = 0.10; $P < 0.001$). Taken together, these data suggest that BCC was significantly more stable within the solids-adherent bacterial populations than within the planktonic populations.

4. Discussion

Ribosomal intergenic spacer analysis has been used in a gel format to characterize the diversity in the ruminal bacterial community of sheep fed diets differing in forage:concentrate ratio [25]. More recently, ARISA has been used with other

Table 2
Least-square means of ruminal pH and VFA concentrations from cows over four 12 h feeding cycles.

Measurement	LS means			$P > F$[a]				Contrast
	Cow	Cow	Pooled	Cow	Cycle	Time	Cycle ×	AM versus PM
	5003	5184	S.E.			Post-feed	Time post-feed	
pH	5.79	5.83	0.09	0.753	0.103	*0.011*	*0.009*	*0.035*
Acetate (mM)	86.2	85.9	4.2	0.939	0.150	*0.035*	0.483	*0.047*
Propionate (mM)	27.5	24.1	1.8	0.306	0.269	*0.010*	0.345	0.114
Butyrate (mM)	17.9	14.7	1.4	0.119	0.204	*<0.001*	*0.078*	*0.065*
Isobutyrate (mM)	0.6	0.7	0.1	0.330	0.764	*0.037*	*0.008*	0.721
Isoval + 2 MB (mM)[b]	3.0	3.2	0.1	0.118	0.070	*0.006*	0.227	0.125
Valerate (mM)	2.4	2.1	0.2	*0.001*	0.296	*<0.001*	0.045	0.189
Total (mM)	137.3	132.1	9.8	0.619	0.175	*0.025*	0.448	*0.051*
A:P ratio	3.18	3.52	0.20	0.183	0.339	0.604	0.720	0.831

[a] Probability of a greater *F*-value in *F*-test. Italicized values are significant ($P < 0.10$).
[b] Isovaleric plus 2-methylbutyric acid (isomers co-elute in HPLC assay).

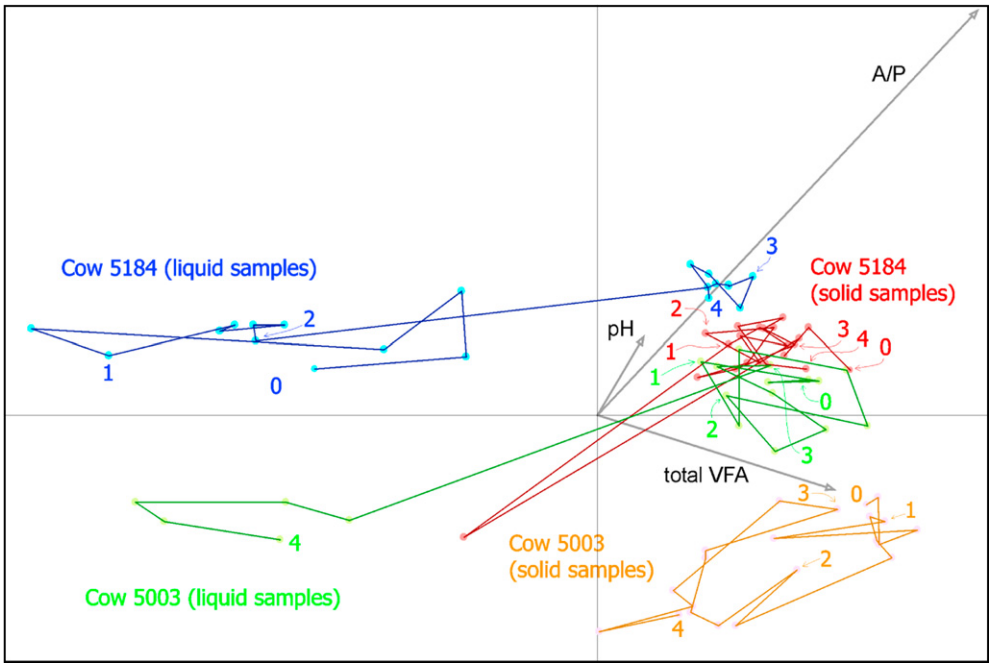

Fig. 4. Ordination biplot from correspondence analysis of ARISA data on bacterial communities, with vectors added showing selected environmental variables. Different ruminal samples are displayed as a series of lines representing shifts of planktonic (liquid-associated) and adherent (solid-associated) bacterial community composition during the course of 4 feeding cycles. For each feeding cycle, 0 represents the bacterial community just prior to the start of the first 12 h cycle, and numbers 1 through 4 represent the communities at the end of the indicated feeding cycle, with intermediate points representing, successively, 2, 4, 6, and 9 h post-feeding within the cycle.

molecular methods to characterize fungal populations in the rumens of pastured reindeer [26]. In this study, we used ARISA to systematically examine changes in ruminal bacterial communities within and across the feeding cycle of lactating dairy cows. Taken together, these studies indicate that intergenic spacer analysis is particularly well suited to detecting subtle changes in ruminal BCC, although it cannot provide specific taxonomic identification of individual ALs [1]. Examination of the ARISA data obtained in our study reveals that a total of 155 ALs were detected in the 83 samples analyzed from 2 cows over the 4 feeding cycles. Although

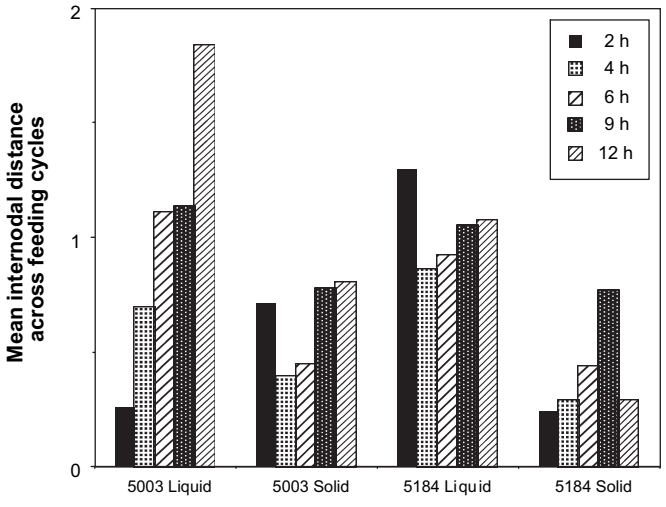

Fig. 5. Magnitude of shifts in ruminal BCC at specific times after feeding, across feeding cycles. Data for each combination of cow and sample phase (liquid-associated and solids-associated) are mean distances between coordinates on the biplot of the first and second axes from correspondence analysis, for individual post-feed time points across 3 successive feeding cycles (cycles 1–2, 2–3, and 3–4).

the 2 cows were fed the same diet and were housed in close physical proximity, and displayed similar yields of milk and milk components and similar ruminal pH and VFA profiles, a substantial fraction of the ruminal bacterial ALs detected in one cow was not detected in the other cow. Moreover, ordination plots from the correspondence analysis revealed that, despite substantial shifts in BCC within each cow over the course of the 4 feeding cycles, the plots of the 2 cows displayed little or no overlap, suggesting that there were also differences in relative population sizes of many of the ALs between the cows. The mechanisms underlying differences in BCC are yet to be elucidated, but could potentially include, among others, different eating patterns (e.g., small meals taken frequently versus large meals consumed more intermittently) or ill-understood differences in feedback between the cow and its microflora. The observation that cows having substantially different ruminal BCCs can have similar ruminal microbial outputs (concentrations and proportions of VFA) suggests that there is considerable niche overlap and capacity for niche replacement within the ruminal bacterial community. Further research is warranted to determine the extent to which individual cows differ in BCC even under apparently similar feeding and management practices, and to determine how different bacterial communities must be to yield significant differences in fermentation end products.

In addition to differences in BCC between cows, there appear to be substantial differences in the BCC of the liquid and solid phases of each cow as well. Approximately 14% of the detected ALs was only observed in the liquid phase, and 2% was detected only in the solid phase. This observation, combined with the lack of overlap in the ordination plot between the solid and liquid phase for each cow, suggests that substantial differences existed between the liquid- and solid-phase populations, even within individual cows. For both cows, changes in BCC over the course of the feeding cycles were smaller for the solid-adherent fraction of the population than for the population in the liquid phase. This difference was probably not

due to differences in numbers of taxa, as both solid and liquid phases had similar total numbers of ALs. The smaller shifts in overall BCC of the particle-associated ruminal bacterial fraction are in accordance with modern concepts of microbial biofilms as ecosystems having substantial stability in BCC [27] and a more stable BCC than its associated planktonic community [13]. On the other hand, in some aquatic habitats (viz., San Francisco Bay, CA) there appear to be substantial similarity in both the structure and function of the solids-associated and liquid-associated bacterial communities [28]. The 2 communities in the rumen appear to be less similar, probably because feed particles are the dominant source of nutrients for the bacterial population. The major species of plant cell wall degraders require adherence to insoluble substrates to effect their degradation [11], and are known to be concentrated on the surfaces of plant particles in the rumen [29].

Several other studies have used other community fingerprinting techniques to characterize ruminal bacterial communities. Most of these studies have involved comparing bacterial populations in animals fed different diets. Miyagawa et al. [30] used terminal restriction fragment length polymorphism to demonstrate differences in the bacterial communities of sheep fed different diets. Denaturating gradient gel electrophoresis (DGGE) has been used to show the effect of methionine source on bacterial community profiles of steers [31]; the different bacterial community profiles of steers fed hay or corn diets [32]; and the different bacterial community profiles in sheep fed diets that varied in fiber content (the aforementioned study of Larue et al. [25] that incorporated the gel configuration of RISA).

DGGE has also been used to compare microbial diversity in the rumen in animals fed similar diets. Sadet et al. [33] demonstrated fundamental differences between the epimural (rumen wall associated) bacterial community and that of the rumen liquor. McEwan et al. [34] examined Soay sheep fed the same diet over different photoperiods (8 h "short day" versus 16 h "long day"). In this last study, the DGGE profiles of the bacterial populations (but not the ciliate protozoal populations) displayed clear clustering with photoperiod, and the differences were ascribed to the large differences in total feed intake during the 2 photoperiods, although the ruminal samples for each sheep were collected only during a single sampling event immediately after slaughter. All of these studies, along with our own, reinforce the conclusion that the diversity of the ruminal bacterial flora exceeds that which has been gained by decades of isolation of pure cultures, and that the number of ruminal bacterial species has long been underestimated [35].

Although our data clearly show changes in BCC over the feeding cycle, deciphering these changes at the level of individual taxa, and relating these changes to their ecological function, remains a formidable challenge. The 2 cows examined here displayed similarities in ruminal pH and VFA profiles, and in performance (milk production and composition). Further research with larger numbers of animals is required to establish the relationship between BCC, ruminal chemistry, and, ultimately, animal production. Community fingerprinting techniques such as ARISA are useful as a means of rapidly obtaining broad-scale profiles of BCC in different ruminal samples and for estimating the diversity of the bacterial population that neither culture-dependent methods nor molecular methods geared toward specific taxa would fully capture. ARISA may be particularly useful as a means of identifying groups of animals that differ substantially in BCC or in grouping animals having similar BCC for more refined studies that employ methods that can yield more specific phylogenetic information on community members (e.g., clone library construction and sequencing for phylogenetic assignment [36–38]) or qPCR to quantify specific taxa [39].

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