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Rumen Microbiome Composition Determined Using Two Nutritional Models of Subacute Ruminal Acidosis[▽]

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Subacute ruminal acidosis (SARA) is a metabolic disease in dairy cattle that occurs during early and mid-lactation and has traditionally been characterized by low rumen pH, but lactic acid does not accumulate as in acute lactic acid acidosis. It is hypothesized that factors such as increased gut permeability, bacterial lipopolysaccharides, and inflammatory responses may have a role in the etiology of SARA. However, little is known about the nature of the rumen microbiome during SARA. In this study, we analyzed the microbiome of 64 rumen samples taken from eight lactating Holstein dairy cattle using terminal restriction fragment length polymorphisms (TRFLP) of 16S rRNA genes and real-time PCR. We used rumen samples from two published experiments in which SARA had been induced with either grain or alfalfa pellets. The results of TRFLP analysis indicated that the most predominant shift during SARA was a decline in gram-negative *Bacteroidetes* organisms. However, the proportion of *Bacteroidetes* organisms was greater in alfalfa pellet-induced SARA than in mild or severe grain-induced SARA (35.4% versus 26.0% and 16.6%, respectively). This shift was also evident from the real-time PCR data for *Prevotella albensis*, *Prevotella brevis*, and *Prevotella ruminicola*, which are members of the *Bacteroidetes*. The real-time PCR data also indicated that severe grain-induced SARA was dominated by *Streptococcus bovis* and *Escherichia coli*, whereas mild grain-induced SARA was dominated by *Megasphaera elsdenii* and alfalfa pellet-induced SARA was dominated by *P. albensis*. Using discriminant analysis, the severity of SARA and degree of inflammation were highly correlated with the abundance of *E. coli* and not with lipopolysaccharide in the rumen. We thus suspect that *E. coli* may be a contributing factor in disease onset.

The bovine rumen is a classical host-microbe symbiotic system, and disturbances in this exquisitely balanced ecosystem may lead to disease in the host. An example is subacute ruminal acidosis (SARA), or non-lactic acid acidosis, which has a disease etiology distinct from that of acute lactic acid acidosis because there is no accumulation of lactic acid (35). Field studies in the United States estimated that 19% of early lactating cows and 26% of mid-lactation cows suffered from SARA (11). In Germany and The Netherlands, approximately 11% of early lactation and 18% of mid-lactation cows suffered from this disease (22). In the acute form, lactic acid accumulates in the rumen, causing metabolic acidosis, and it usually occurs when animals are abruptly transitioned to a high-grain diet from a predominantly forage diet (38). If, however, the adaptation is gradual, slower-growing lactic acid-consuming bacteria, like *Megasphaera elsdenii*, convert the lactic acid to propionic acid (29). In SARA, lactic acid does not accumulate during low-pH conditions and other factors, like microbial population shifts and immune responses, appear to be associated with the disease etiology (35).

In both acute and subacute acidosis, there is an increase in lipopolysaccharide (LPS) concentrations in the rumen (8, 14, 16). LPS and/or the low-pH rumen conditions may increase the permeability of the gut to LPS, which could trigger systemic inflammation (4). We previously developed two animal models of SARA, one based on grain and one based on alfalfa pellets (20, 21). Even though both models resulted in substantial reductions in rumen pH and an accumulation of LPS, only the grain induction model resulted in inflammation and the appearance of LPS in the peripheral blood (20, 21).

In contrast to the rumen microbiome during lactic acid acidosis, the rumen microbiome during SARA has not been evaluated (13, 28). Even in acute acidosis, studies are largely culture based, and the uncultured members of the community have not been extensively assessed (31, 46, 49). In this article, we describe the rumen microbiome when two SARA induction models were used. The shifts in microbial community structure were assessed using terminal restriction fragment length polymorphism (TRFLP) analysis and real-time PCR of key microbial populations.

MATERIALS AND METHODS

Animal experiments were conducted at the Glenlea Dairy Research Unit at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of the Canadian Council on Animal Care (3). Data for rumen pH, feed intake, milk production and composition, and rumen and blood metabolites for these experiments have been reported previously (20, 21). In this article, we reinterpret the previous data (20, 21) in the context of the microbial changes evaluated in this research.

Animal models and sampling. As described previously (20, 21), SARA was induced in eight rumen-cannulated lactating dairy cows using two different induction models (total of eight animals from both experiments), one with grain, and the other with alfalfa pellets. In both experiments, animals were fed once daily at 9:00 a.m. and feed was always provided in an amount that met or

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exceeded the animal's nutritional requirements. In the first experiment (grain-induced SARA), cows received a basal diet with a forage-to-concentrate ratio of 50:50. The ration consisted of 25% as alfalfa silage, 25% as barley silage, 40% as an energy supplement, and 10% as a protein supplement. SARA was induced by replacing 21% of the dietary dry matter (DM) with wheat-barley pellets. In the second experiment (alfalfa pellet-induced SARA), cows received a basal diet that consisted of 50% of the DM as concentrate and 50% as chopped alfalfa hay. The concentrate fraction consisted of 39% as an energy supplement, 4.5% as a protein supplement, and 6.5% as roasted soybeans. SARA was induced by replacing 42% of the alfalfa hay DM with alfalfa pellets without changing the forage-to-concentrate ratio or the dietary starch content.

In both experiments, the severity of SARA in individual animals was determined based on objective criteria that included the duration of rumen pH below 5.6, free rumen LPS, and serum haptoglobin as an inflammatory marker (14). Rumen pH was monitored continuously using indwelling pH probes (21). Rumen fluids were collected from the ventral sac of the rumen 15 min before feeding (0 h) and at 6 h after feeding both during the control period and 4 days after induction of SARA during the SARA periods. Ruminal contents were strained through four layers of sterile cheesecloth, transferred to 50-ml sterile tubes, immediately frozen in liquid nitrogen, and stored at −20°C. The free rumen LPS content was determined with a chromogenic *Limulus* amebocyte lysate end-point assay (QCL-1000; Lonza group Ltd., Basel, Switzerland) (21). The serum concentration of haptoglobin was determined using an enzyme-linked immunosorbent assay kit (TP-801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ) (21).

DNA extraction. Rumen fluid samples ($n = 64$) were thawed at 32°C for 15 min and immediately centrifuged at 10,000 × *g* for 10 min. Supernatants were discarded, and pellets were resuspended in phosphate-buffered saline (1×) in new sterile tubes. Approximately 150 mg of microbial pellet was washed in 1 ml of phosphate-buffered saline (1×) and centrifuged at 10,000 × *g* for 2 min. The washing step was repeated twice. DNA was extracted from the pellets using a ZR fecal DNA kit (D6010; Zymo Research Corp., Orange, CA) which included a bead-beating step for the mechanical lysis of the microbial cells. DNA concentration and purity were determined spectrophotometrically by measuring the $A_{260/280}$ (Beckman DU/800; Beckman Coulter, Inc., Fullerton, CA). DNA quality was also evaluated by gel electrophoresis.

PCR amplification and TRFLP. The microbial composition in the rumen fluid was assessed using TRFLP as described by Sepehri et al. (41), with some modifications. In brief, the V1 and V2 regions of the 16S rRNA genes were PCR amplified using universal bacterial primers 27f (5′-GAAGAGTTTGATCATGG CTCAG-3′) and 342r (5′-CTGCTGCCTCCCGTAG-3′) (21). The forward primer was fluorescently labeled (WellRED D4dye; Sigma-Proligo, St. Louis, MO) to allow detection of the fragments by capillary electrophoresis. The PCRs were as follows: 37 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 5 min. The first cycle used a denaturation step of 5 min instead of 1 min. To produce terminal restriction fragments (T-RFs), the PCR products were digested with HhaI (15 μl of PCR product, 10 units of HhaI, 1× HhaI buffer, and 20 μg of bovine serum; New England Biolabs, Ipswich, MA) at 37°C for 3 h. The precise lengths of the T-RF amplicons were determined on a CEQ 8800 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). A 2-μl volume of fluorescently labeled fragments, 29.5 μl of sample loading solution, and 1 μl of 600-bp DNA size standard (Beckman Coulter, Inc., Fullerton, CA) was mixed and applied to the capillaries. An electropherogram with peaks of different sizes was obtained for each rumen fluid sample, and each peak of unique size represents an operational taxonomic unit (OTU). All rumen fluid samples were run in duplicate, and results were accepted when the variability in the sizes of the T-RFs in the duplicate electropherograms were in the range of ±2 bp.

Fragment analysis. CEQ software (version 9.0; Beckman Coulter, Inc., Fullerton, CA) with a binning parameter of 2 bp was used to analyze the fragment data. Signals were detected from electronic noise using a proportional threshold approach (40). In brief, the relative abundance of each T-RF within a profile was calculated as its peak height divided by the total peak height of all T-RFs in that profile. Only T-RFs with relative abundances higher than 1% of the total were included in the analysis. The incidence (presence/absence) data derived from the OTU profiles were grouped based on sampling times (0 h and 6 h) and dietary treatments and used for numerical analysis.

Richness, diversity, and similarity. Chao2 incidence-based index of richness and Shannon incidence-based diversity estimators were calculated using EstimateS (version 7.5; http://purl.oclc.org/estimates) to determine the richness and diversity of microbial communities in each time/treatment group. An upper abundance limit of 5 was used to determine rare or infrequent species. The order of the samples was randomized 500 times for each run to reduce the effect of sample order. Similarities among microbial communities were also determined using SPADE (version 2.1; http://chao.stat.nthu.edu.tw/softwareCE.html), based on the Jaccard multiple incidence-based index.

Bioinformatic analysis of TRFLP data. T-RFs alone do not allow for unequivocal identification of OTU to the genus or species level, and for high-resolution analysis, sequencing reactions need to be done. To partially circumvent this problem, we have previously described a bioinformatic approach in which we mined the Ribosomal Database Project (RDP-II) (6) for all sequences that were found in the digestive tract (1, 41). Essentially, a database was developed that only contained sequences that occur in the mammalian gut (42). This approach vastly improved data analysis because one does not have to account for taxa not found in the gut. We frequently update this database and it currently consists of over 200,000 curated near-full-length sequences from the RDP that only come from the mammalian and avian gut.

As described by Sepehri et al. (41), an in silico reference database based on 27f and 342r plus HhaI restriction digestion was created and submitted to the Phylogenetic Assignment Tool (PAT) (19) and compared to the experimentally produced data from capillary electrophoresis. The resultant libraries were then entered into the hierarchical browser of the RDP and converted to GenBank format. T-RFs with multiple accession numbers were assigned to taxonomic rank according to phylum, class, order, and family. Based on this analysis, reported values were expressed as the proportion of phylogenetic lineage for each library.

Primers and real-time PCR. The PCR primers used are listed in Table 1. Primers were assembled from the literature or newly designed (Primer Express, version 3.0; Applied Biosystems, Foster City, CA) and tested for specificity in silico. Those primers that did not meet our selection criteria for specificity and performance were redesigned from sequence alignments. The oligonucleotides were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada).

Real-time PCR was carried out using an AB 7300 system (Applied Biosystems, Foster City, CA) and sequence detection software (version 1.3; Applied Biosystems, Foster City, CA). Each reaction mixture was run in triplicate in a volume of 25 μl in optical reaction plates (Applied Biosystems, Foster City, CA) sealed with optical adhesive film (Applied Biosystems, Foster City, CA). Amplification reactions were carried out with Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) mixed with the selected primer set (Table 1) at a concentration of 0.5 μM for each primer and 2 μl (~12 ng) of genomic DNA. Different concentrations of forward and reverse primers were only used for *Prevotella brevis* (0.5 and 0.9 μM, respectively) and *Ruminobacter amylophilus* (0.3 μM each). Amplification consisted of one cycle of 95°C (10 min), 40 cycles of denaturation at 95°C (15 s), and annealing/extension at 60°C (1 min). The only exceptions were for methanogenic archaea and ciliate protozoa, where annealing/extension steps of 63°C (30 s)/72°C (30 s) and 54°C (30 s)/72°C (1 min) were applied, respectively. Final melting analysis was obtained by slow heating from 65°C to 95°C. To evaluate the efficiency (E) of the amplification of each primer set, DNA templates were pooled (50 ng/reaction mixture) and serially diluted eightfold. Amplification efficiency was calculated from the slope of the standard curve generated by plotting the threshold cycle (C_T) versus logarithmic values of different DNA concentrations using the following equation (7): $E = 10^{-1/\text{slope}}$. Relative quantitation was accomplished using the following mathematical model (34): $R_i = [(E_{\text{target}})\Delta^{CT}{}_{\text{target}}\ (\text{control}_i - \text{SARA}_i)]/[(E_{\text{ref}})\Delta^{CT}{}_{\text{ref}}\ (\text{control}_i - \text{SARA}_i)]$, where target is the specific microbial group of interest, ref is the 16S rRNA gene of the *Eubacteria*, Δ^{CT} is the C_T deviation of SARA versus control period, i is sampling time (0 h or 6 h after feeding), and R_i is the relative expression ratio of a target gene compared to a reference gene at a specific time point.

Statistical analysis. Discriminant multivariate analysis was conducted using JMP IN (version 5.1; SAS Institute, Inc., Cary, NC) to examine possible relationships among models of SARA induction and duration of rumen pH below 5.6, free rumen LPS, rumen microbial community dynamics, and serum haptoglobin as an inflammatory marker. Statistical significance ($P < 0.05$) was calculated using the least significant difference multiple comparison test to detect significant differences among times/treatments groups (39).

RESULTS

Significant variations in the animals' responses to SARA induction were observed with the grain-induced versus alfalfa-induced model (Tables 2 and 3) (20, 21). Multivariate discriminant analysis (Fig. 1) of time below pH 5.6, serum haptoglobin, and the concentration of free rumen LPS divided cows into three groups: severe grain-induced SARA, mild grain-induced SARA, and alfalfa pellet-induced SARA. The major differentiator between groups was the haptoglobin inflammatory marker.

TABLE 1. Primers used for real-time PCR quantification

Target organism(s)	Primer	Sequence (5′ → 3′)[a]	T_m (°C)	G+C (%)	Amplicon size (bp)	Source of primer
Eubacteria	341-357F	CCTACGGGAGGCAGCAG	55.2	70.6	189	25
	518-534R	ATTACCGCGGCTGCTGG	56.2	64.7		
Prevotella albensis	ProAlb4F	GCGCCACTGACGCTGAAG	58.3	66.7	110	This study
	ProAlb4R	CCCCAAATCCAAAAGGACTCAG	56.6	50.0		
Prevotella brevis	PreBre2F	GCGAACTGGTTTCCTTGAGTGTATT	58.8	50.0	153	This study
	PreBre2R	ACCTTCGAGCTTTAGCGTCAGTTAT	57.6	40.0		
Prevotella bryantii	ProBry4F	GAAGGCAGCTCGCTGTAGTGTT	60.6	54.5	145	This study
	ProBry4R	CTTAACGCTTTCGCTTAGCCACT	59.4	47.8		
Prevotella ruminicola	PreRum92862F	GCGAAAGTCGGATTAATGCTCTATG	58.5	44.0	78	This study
	PreRum92862R	CCCATCCTATAGCGGTAAACCTTTG	59.3	48.0		
Succinimonas amylolytica	SucAmy2F	CGTTGGGCGGTCATTTGAAAC	55.2	52.4	139	This study
	SucAmy2R	CCTGAGCGTCAGTTACTATCCAGA	56.2	50.0		
Succinivibrio dextrinisolvens	SucDex1F	TAGGAGCTTGTGCGATAGTATGG	57.4	47.8	174	This study
	SucDex1R	CTCACTATGTCAAGGTCAGGTAAGG	58.4	48.0		
Ruminobacter amylophilus	RumAmy2F	CTGGGGAGCTGCCTGAAT	55.3	61.1	100	43
	RumAmy2R	CATCTGAATGCGACTGGTTG	54.2	50.0		
Escherichia coli	EcoliFimH2F	GCCGGTGGCGCTTTATTTG	57.3	57.9	114	This study
	EcoliFimH2R	TCATCGCTGTTATAGTTGTTGGTCT	58.4	40.0		
Fibrobacter succinogenes	FibSuc4F	GGAGCGTAGGCGGAGATTCA	58.7	60.0	97	This study
	FibSuc4R	GCCTGCCCCTGAACTATCCA	58.5	60.0		
Anaerovibrio lipolytica	AnaLip2F	TGGGTGTTAGAAATGGATTCTAGTG	56.6	40.0	109	This study
	AnaLip2R	GCACGTCATTCGGTATTAGCAT	56.7	45.5		
Megasphaera elsdenii	MegEls1F	GACCGAAACTGCGATGCTAGA	57.7	52.4	129	32
	MegEls1R	CGCCTCAGCGTCAGTTGTC	58.2	63.2		
Selenomonas ruminantium	SelRum1F	GGCGGGAAGGCAAGTCAGTC	60.4	65.0	83	This study
	SelRum1R	CCTCTCCTGCACTCAAGAAAGACAG	61.1	52.0		
Ruminococcus albus	RumAlb1F	CCCTAAAAGCAGTCTTAGTTCG	54.3	45.5	176	48
	RumAlb1R	CCTCCTTGCGGTTAGAACA	53.8	52.6		
Ruminococcus flavefaciens	RumFla1F	CGAACGGAGATAATTTGAGTTTACTTAGG	57.5	34.5	132	7
	RumFla1R	CGGTCTCTGTATGTTATGAGGTATTACC	59.3	42.9		
Butyrivibrio fibrisolvens	ButFib2F	ACCGCATAAGCGCACGGA	58.8	61.1	65	43
	ButFib2R	CGGGTCCATCTTGTACCGATAAAT				
Lactobacillus spp.	Ulac16S1F	AGCAGTAGGGAATCTTCCA	51.5	47.4	345	24, 47
	Ulac16S1R	ATTCCACCGCTACACATG	51.1	50.0		
Streptococcus bovis	SBovis1F	TTCCTAGAGATAGGAAGTTTCTTCGG	57.9	42.3	127	43
	SBovis1R	ATGATGGCAACTAACAATAGGGGT	57.9	41.7		
Treponema bryantii	TrpBry1F	GAGAAACGCTTTGTGGTGACTGT	59.5	47.8	122	This study
	TrpBry1R	CCTACATGCCCTTTACGCTCAAT	58.7	47.8		
Methanogenic archaea	MB1174f	GAGGAAGGAGTGGACGACGGTA	60.6	59.1	232	30
	Arch1406-1389r	ACGGGCGGTGTGTGCAAG	60.0	66.7		
Ciliate protozoa	UPorCil1F	GCTTTCGWTGGTAGTGTATT	50.2	20.0	234	45
	UPorCil1R	CTTGCCCTCYAATCGTWCT	50.4	47.4		

[a] Primers were based on 18S rRNA genes for ciliate protozoa, 16S rRNA genes for bacterial groups except for *E. coli*, and the *fimH* gene for *E. coli*.

TABLE 2. Time spent below pH 5.6, rumen LPS, plasma LPS, and serum haptoglobin level of dairy cows during control period and grain-induced SARA

Period	Mean value[a] for indicated variable and disease severity					
	Time below pH 5.6 (min/day)		Rumen LPS (EU/ml)		Serum haptoglobin (μg/ml)	
	Mild	Severe	Mild	Severe	Mild	Severe
Control	75 b	161 b	32,413 Bb	29,933 b	0.0 c	0.0 c
Grain-induced SARA	217 a	337 a	100,175 Ab	179,762 a	343.3 b	608.0 a

[a] EU, endotoxin unit. a, b, and c, within each variable, mean values in the same row or column with different symbols differ ($P < 0.05$). A and B, within each variable, mean values in the same row or column with different symbols differ ($P < 0.1$). Standard errors of the means are 72 for time below pH 5.6, 30,072 for rumen LPS, and 35.8 for serum haptoglobin.

The feeding method (grain or alfalfa pellet) and the amplitudes of inflammatory markers were used as criteria to group TRFLP data (Tables 4 and 5). In other words, we used the SARA induction method and the inflammatory markers to

TABLE 3. Time spent below pH 5.6, rumen LPS, plasma LPS, and serum haptoglobin of dairy cows during control period and alfalfa pellet-induced SARA

Period	Mean result[a] for indicated variable		
	Time below pH 5.6 (min/day)	Rumen LPS (EU/ml)	Serum haptoglobin (μg/ml)
Control	112 b	42,122 b	56 a
Alfalfa pellet-induced SARA	510 a	169,266 a	21 b

[a] EU, endotoxin unit. a and b, mean values within the same column with different symbols differ ($P < 0.05$). Standard errors of the means are 109.5 for time below pH 5.6, 16,780 for rumen LPS, and 6 for serum haptoglobin.

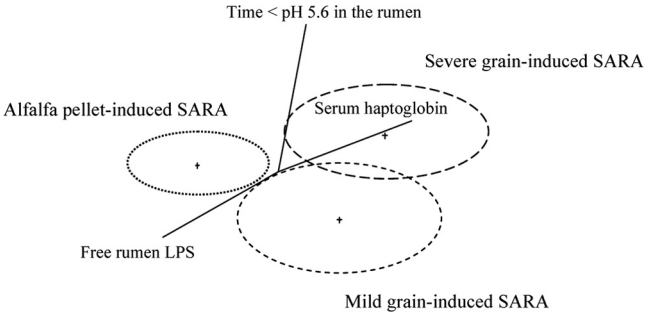

FIG. 1. Results of discriminant analysis of the duration of time below pH 5.6 in the rumen, free rumen LPS, and serum haptoglobin in response to SARA induction model. The circles are independent variables, and the distances between them reflect their dissimilarity. The straight lines are dependent variables, and their lengths and the angles between them are functions of relative effects of independent variables.

decide how to group animals. We conducted TRFLP analysis with primers 27f and 342r or 1100r, but the larger amplicon appeared to be less efficiently produced and resulted in fewer T-RFs. Nine predominant phyla were detected: *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Spirochaetes*, *Actinobacteria*, *Fusobacteria*, TM7, *Tenericutes*, and *Deinococcus-Thermus* (Tables 4 and 5). However, more than 98% of rumen bacteria were assigned to only three phyla (*Bacteroidetes*, *Proteobacteria*, and *Firmicutes*). *Fibrobacteres* was not detected with universal primers. We have determined that this is an artifact that has arisen because *Fibrobacter succinogenes* sequences in NCBI and RDP have not had the region upstream of the 27f site sequenced. Therefore, *F. succinogenes* is not included in the construction of our in silico database.

Mild and severe grain-induced SARA and alfalfa pellet-induced SARA produced different TRFLP profiles at the phylum level (Tables 4 and 5). The major differences between mild and severe grain-induced SARA were a significant increase in the phylum *Firmicutes* (79.7% versus 69.6%) and a significant decrease in the phylum *Bacteroidetes* (16.6% versus 26%) in severe versus mild disease (Tables 4 and 5). Within the *Firmicutes*, the family most affected by the severe form was the *Lachnospiraceae* (61.4% versus 44.7%), which is richly populated with known rumen bacteria. Alfalfa pellet-induced SARA also showed a signif-

TABLE 4. Comparison of putative microbial distribution generated from T-RF libraries of rumen fluid samples collected at 0 h

Taxonomic rank	% of T-RFs with taxonomic rank at 0 h during[a]:					
	Control period			SARA		
	Grain-induced SARA		Alfalfa pellet-induced SARA	Grain-induced SARA		Alfalfa pellet-induced SARA
	Mild	Severe		Mild	Severe	
Phylum *Bacteroidetes*	28.18 bc	28.56 bc	85.50 a	25.78 bc	17.75 c	36.19 b
Class *Bacteroidetes*	27.81 b	28.25 b	85.28 a	25.53 bc	17.66 c	36.06 b
Order *Bacteroidales*	27.81 b	28.25 b	85.28 a	25.53 bc	17.66 c	36.06 b
Family *Prevotellaceae*	2.71 c	2.82 c	25.49 a	2.47 c	2.24 c	10.70 b
Phylum *Proteobacteria*	4.99	3.83	1.14	4.02	1.97	0.78
Class *Epsilonproteobacteria*	0.39	0.28	0.35	0.33	0.03	0.15
Order *Campylobacterales*	0.39	0.28	0.35	0.33	0.03	0.15
Class *Gammaproteobacteria*	2.21	2.10	0.51	1.93	1.37	0.23
Order *Aeromonadales*	0.02	0.02	0.03	0.02	0.00	0.01
Family *Succinivibrionaceae*	0.02	0.02	0.03	0.02	0.00	0.01
Order *Enterobacteriales*	1.47	1.53	0.03	1.35	1.21	0.01
Family *Enterobacteriaceae*	1.47	1.53	0.03	1.35	1.21	0.01
Phylum *Firmicutes*	64.74 b	66.03 b	12.44 c	68.53 b	79.74 a	62.34 b
Class *Clostridia*	59.53 b	60.91 b	11.02 c	64.08 ab	75.89 a	57.24 b
Order *Clostridiales*	57.49 b	58.84 b	10.11 c	63.08 ab	75.17 a	56.31 b
Family *Clostridiaceae*	0.69	0.72	0.00	0.64	1.11	0.01
Family *Eubacteriaceae*	0.03	0.03	0.00	0.03	0.03	0.03
Family *Lachnospiraceae*	35.86 b	36.23 b	1.60 c	43.60 b	60.14 a	39.12 b
Family *Peptostreptococcaceae*	0.03	0.02	0.05	0.02	0.00	0.02
Family *Ruminococcaceae*	15.55 a	16.29 a	3.70 b	14.15 a	10.83 a	11.72 a
Family *Veillonellaceae*	3.83	3.98	3.80	3.46	2.31	7.70
Class *Bacilli*	2.54	2.33	1.12	2.01	1.61	2.65
Order *Lactobacillales*	2.39	2.17	0.84	1.87	1.49	2.53
Family *Streptococcaceae*	1.93	2.00	0.56	1.77	1.42	1.81
Phylum *Spirochaetes*	0.01	0.01	0.00	0.01	0.00	0.00
Phylum *Actinobacteria*	1.43	1.29	0.53	1.12	0.64	0.29
Phylum *Fusobacteria*	0.02	0.02	0.00	0.02	0.00	0.00
Phylum TM7	0.01	0.01	0.00	0.01	0.01	0.00
Phylum *Tenericutes*	0.10	0.02	0.00	0.07	0.04	0.03
Phylum *Deinococcus-Thermus*	0.01	0.01	0.00	0.01	0.00	0.00
Phylum-level unclassified bacteria	0.50	0.21	0.38	0.42	0.03	0.29

[a] Rumen fluid samples were collected 4 days after SARA induction during SARA periods. a, b, and c, mean values within a row with different symbols differ ($P < 0.05$).

TABLE 5. Comparison of putative microbial distribution generated from T-RF libraries of rumen fluid samples collected at 6 h

Taxonomic rank	% of T-RFs with taxonomic rank at 6 h during[a]:					
	Control period			SARA		
	Grain-induced SARA		Alfalfa pellet-induced SARA	Grain-induced SARA		Alfalfa pellet-induced SARA
	Mild	Severe		Mild	Severe	
Phylum *Bacteroidetes*	24.58 bc	20.04 bc	47.41 a	26.27 bc	15.34 c	34.65 ab
Class *Bacteroidetes*	24.07 bc	19.81 bc	47.14 a	26.09 bc	15.29 c	34.46 ab
Order *Bacteroidales*	24.07 bc	19.81 bc	47.14 a	26.09 bc	15.29 c	34.46 ab
Family *Prevotellaceae*	2.41 b	2.03 b	13.53 a	2.60 b	7.65 ab	9.99 a
Phylum *Proteobacteria*	4.49	3.04	1.98	1.94	3.66	2.99
Class *Epsilonproteobacteria*	0.26	0.23	0.19	0.35	0.00	0.14
Order *Campylobacterales*	0.26	0.23	0.19	0.35	0.00	0.14
Class *Gammaproteobacteria*	2.03	1.45	0.62	0.40	2.96	1.92
Order *Aeromonadales*	0.02	0.02	0.03	0.00	0.00	0.02
Family *Succinivibrionaceae*	0.02	0.02	0.03	0.00	0.00	0.02
Order *Enterobacteriales*	1.31	1.12	0.03	0.37	1.22	1.28
Family *Enterobacteriaceae*	1.31	1.12	0.03	0.37	1.22	1.28
Phylum *Firmicutes*	69.33 b	75.65 ab	49.52 c	70.72 ab	79.58 a	60.68 bc
Class *Clostridia*	64.03 bc	71.83 ab	43.76 cd	66.11 abc	78.03 a	56.44 c
Order *Clostridiales*	62.23 bc	70.34 ab	42.78 cd	65.01 abc	78.19 a	55.46 c
Family *Clostridiaceae*	0.61	0.53	1.10	0.66	1.13	0.60
Family *Eubacteriaceae*	0.03	0.03	0.00	0.03	0.03	0.03
Family *Lachnospiraceae*	42.16 bc	54.85 ab	32.76 c	45.73 bc	62.60 a	37.00 c
Family *Peptostreptococcaceae*	0.08	0.00	0.03	0.02	0.00	0.07
Family *Ruminococcaceae*	13.90 a	11.69 ab	5.62 b	13.70 a	11.26 ab	13.30 ab
Family *Veillonellaceae*	4.09	2.14	2.66	3.69	2.34	3.37
Class *Bacilli*	2.90	1.67	2.46	2.11	0.41	1.93
Order *Lactobacillales*	2.77	1.56	2.29	1.97	0.29	1.80
Family *Streptococcaceae*	1.71	1.46	2.13	1.84	0.17	1.68
Phylum *Spirochaetes*	0.01	0.01	0.11	0.00	0.00	0.01
Phylum *Actinobacteria*	1.22	0.87	0.63	0.84	1.01	1.14
Phylum *Fusobacteria*	0.02	0.02	0.00	0.02	0.02	0.00
Phylum TM7	0.01	0.01	0.01	0.01	0.01	0.00
Phylum *Tenericutes*	0.10	0.02	0.12	0.00	0.08	0.11
Phylum *Deinococcus-Thermus*	0.01	0.01	0.01	0.01	0.00	0.01
Phylum-level unclassified bacteria	0.23	0.34	0.30	0.17	0.21	0.40

[a] Rumen fluid samples were collected 4 days after SARA induction during SARA periods. a, b, and c, mean values within a row with different symbols differ ($P < 0.05$).

icant increase in the phylum *Firmicutes* and a decrease in the phylum *Bacteroidetes* (Tables 4 and 5). However, in the alfalfa pellet-induced model, *Bacteroidetes* were 35.4% and *Firmicutes* were 61.5% of the population (Tables 4 and 5). Microbial composition did not change over time, with the exception of the control period during alfalfa pellet-induced SARA, where a significant shift from *Bacteroidetes* to *Firmicutes* was observed at 6 h postfeeding (Table 5).

Species richness was not affected by the SARA induction model but was numerically lower (in both the grain and the alfalfa pellet model) than in the control period (Table 6). Species diversity was significantly lower during mild grain-induced SARA and tended to be lower in severe grain-induced SARA but was not different for alfalfa pellet-induced SARA (Table 6). The similarity between microbial communities was greater for the control period and alfalfa pellet-induced SARA and mild grain-induced SARA than for severe grain-induced SARA (Table 7).

Relative quantification of major rumen bacteria, methanogenic archaea, and ciliate protozoa during SARA compared to the control period is shown in Fig. 2 and 3. At 0 h,

TABLE 6. Richness and diversity indices calculated from TRFLP incidence profiles of rumen fluid[a]

Time	Period	Mean value[b] under indicated conditions for:					
		Richness[c]			Diversity[d]		
		Grain-induced SARA		Alfalfa pellet-induced SARA	Grain-induced SARA		Alfalfa pellet-induced SARA
		Mild	Severe		Mild	Severe	
0 h	Control	69.9	46.8	41.3	3.1 a	2.8	2.8
	SARA	48.5	29.6	31.7	2.4 b	2	2.2
6 h	Control	93.6	48.1	25.2	3.2 a	2.8 ab	2.4 b
	SARA	45	49.2	42.5	2.3 b	2.4	2.8

[a] Rumen fluid samples were collected 4 days after SARA induction during SARA period.

[b] a and b, mean values within the same row or column with different symbols differ ($P < 0.05$). Standard errors of the means were 35.3 for richness and 0.2 for diversity.

[c] Based on Chao-2 richness estimator.

[d] Based on Shannon diversity estimator.

TABLE 7. Multiple-incidence-based similarities of rumen bacterial communities between control period and grain-induced and alfalfa pellet-induced SARA calculated from TRFLP incidence profiles of rumen samples amplified using 27f and 342r primers

Induction model	Period, disease severity	Time (h)	% Similarity[a] (mean ± SE) of bacterial community under indicated conditions											
			Grain induction								Alfalfa pellet induction			
			Control				SARA				Control		SARA	
			Mild		Severe		Mild		Severe					
			0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h
Grain induced	Control, mild	0	100											
		6	77 ± 8	100										
	Control, severe	0	72 ± 8	66 ± 9	100									
		6	73 ± 8	61 ± 9	74 ± 10	100								
	SARA, mild	0	69 ± 9	58 ± 9	77 ± 8	71 ± 10	100							
		6	59 ± 9	55 ± 8	73 ± 9	60 ± 9	86 ± 8	100						
	SARA, severe	0	31 ± 8	30 ± 8	38 ± 9	46 ± 10	42 ± 9	50 ± 10	100					
		6	29 ± 7	28 ± 7	29 ± 8	40 ± 10	41 ± 9	43 ± 10	67 ± 10	100				
Alfalfa pellet induced	Control	0	28 ± 8	28 ± 8	41 ± 9	31 ± 9	32 ± 8	39 ± 10	42 ± 11	42 ± 9	100			
		6	41 ± 9	44 ± 9	57 ± 10	46 ± 10	54 ± 10	57 ± 6	35 ± 10	36 ± 9	50 ± 11	100		
	SARA	0	40 ± 10	52 ± 9	54 ± 10	44 ± 9	46 ± 10	54 ± 10	33 ± 10	34 ± 9	48 ± 10	52 ± 10	100	
		6	54 ± 8	64 ± 8	44 ± 8	46 ± 9	47 ± 8	48 ± 9	38 ± 9	46 ± 8	30 ± 8	42 ± 9	45 ± 9	100

[a] Based on Jaccard index of similarity.

severe grain-induced SARA was dominated by *Escherichia coli*, *Streptococcus bovis*, *Megasphaera elsdenii*, and *Lactobacillus* spp.; mild grain-induced SARA was dominated by *M. elsdenii*, *Selenomonas ruminantium*, *Prevotella bryantii*, and *Anaerovibrio lipolytica*; and alfalfa pellet-induced SARA was dominated only by *Prevotella albensis*. At 6 h after feeding, the severe grain-induced SARA group was dominated by *M. elsdenii* and *S. bovis*, while the mild group was dominated by *M. elsdenii*, *Succinivibrio dextrinisolvens*, *P. bryantii*, and *Ruminococcus flavefaciens*. In contrast, the alfalfa pellet-in-

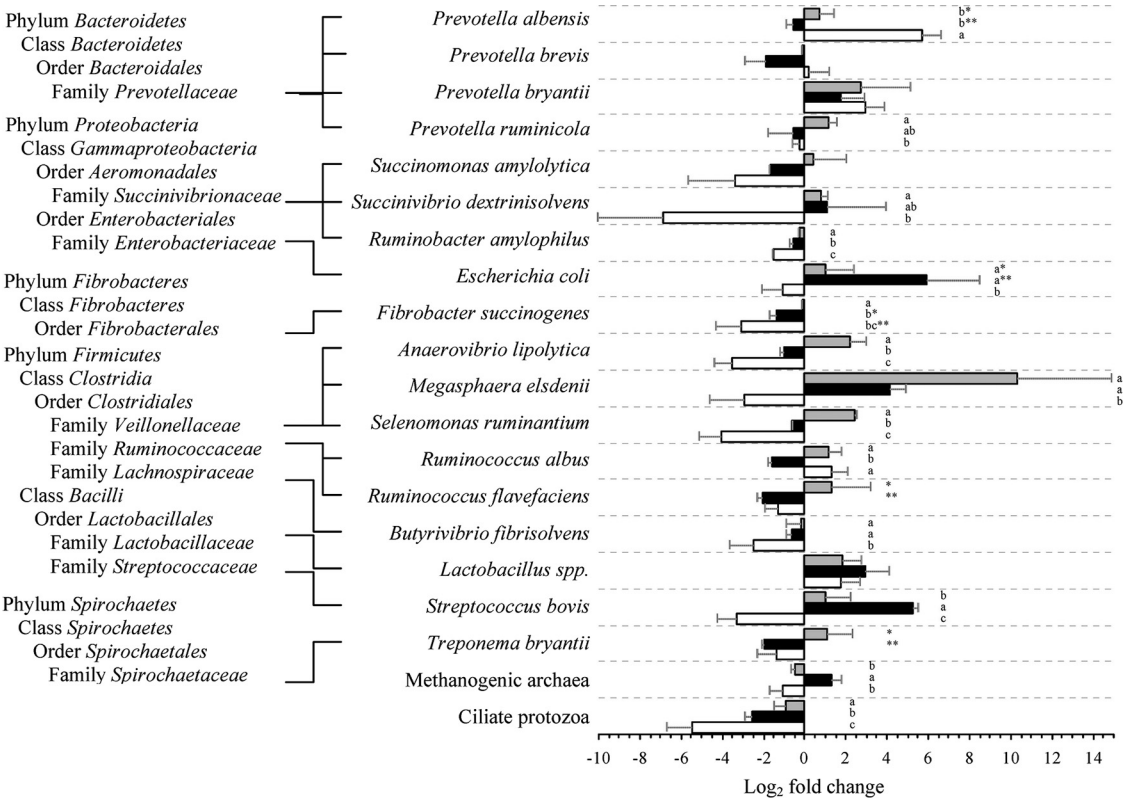

FIG. 2. Changes ($\log_2$) in predominant rumen microorganisms at 0 h during mild (gray bars) and severe (black bars) grain-induced and alfalfa pellet-induced (white bars) SARA compared to the control period, determined with real-time PCR. The letters a, b, and c indicate statistical differences within species at a P value of <0.05; * and **, $P < 0.1$. Error bars show standard errors.

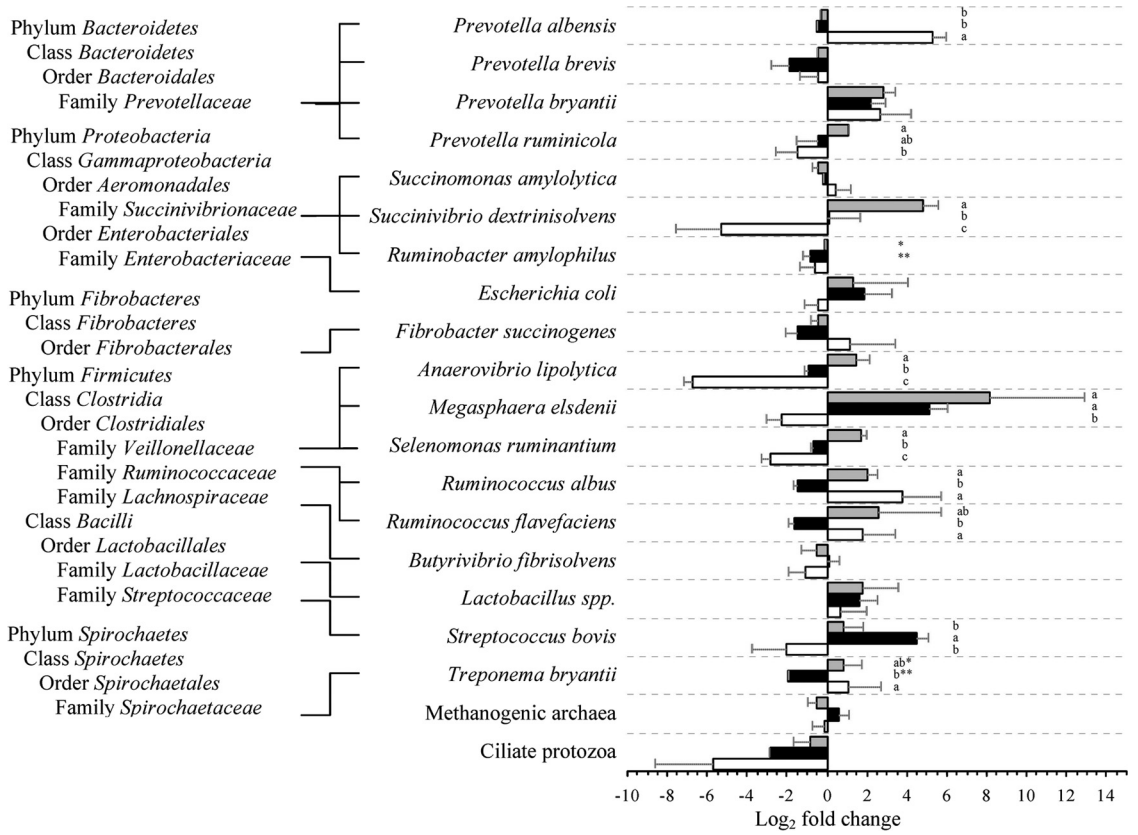

FIG. 3. Changes ($\log_2$) in predominant rumen microorganisms at 6 h during mild (gray bars) and severe (black bars) grain-induced and alfalfa pellet-induced (white bars) SARA compared to the control period, determined with real-time PCR. The letters a, b, and c indicate statistical differences within species at a P value of <0.05; * and **, $P < 0.1$. Error bars show standard errors.

duced SARA group was dominated by *P. albensis*, *P. bryantii*, and *Ruminococcus albus*. Multivariate discriminant analysis of real-time PCR data indicated that the best predictor of severe grain-induced SARA was *E. coli*.

DISCUSSION

Current definitions of SARA are based on the low rumen pH typically generated on high-starch diets (35). However, there is no general agreement on the pH threshold that defines SARA, and moreover, rumen pH may not even be highly correlated with disease symptoms (2, 9). To refine the definition of SARA, Gozho et al. (14) suggested that the free rumen LPS concentration should be considered. In our previous work (20, 21), we demonstrated that the feed type and physical form of the diet had an effect on the induction of SARA. When we induced low rumen pH with highly fermentable alfalfa-pellets, inflammatory markers were absent even though the free rumen LPS concentrations were high (20). Thus, rumen fermentation conditions by themselves are not sufficient to explain SARA. In fact, we speculate that SARA is a historical artifact derived from the pH-related definition of acute ruminal acidosis.

To understand the underlying causes of SARA, we examined the microbial changes that occur with grain- and alfalfa pellet-induced SARA using rumen samples from our previous experiments (20, 21). A critical part of our analysis was to separate animals into mild and severe SARA groups based on objective criteria using multivariate statistical techniques (Table 2; Fig. 1). Clustering of cows into mild and severe groups was conducted using the animal as the independent variable and the free rumen LPS concentration, time below pH 5.6, and serum haptoglobin as dependent variables. Variation in animal response is one of the diagnostic criteria used to identify SARA on a herd basis. The coefficient of variation among animals is often used as an objective criterion to determine whether SARA is present (12). At this time, it is not clear in the literature whether the various SARA symptoms are the result of feed intake variation among individuals, natural variations in saliva buffering capacity, or rumen epithelial potential for uptake of short-chain fatty acids (10, 33). Differences in endotoxin tolerance and immune responses that predispose certain animals to the disease may also be a factor contributing to the variation in disease etiology seen in the field (17). Although the number of animals used in this study was small, which is typical of this type of experiment in dairy cattle (22), we were still able to detect a significant difference among individual responses to SARA, leading us to believe that our SARA induction models were characteristic of a field situation. We are confident that our experiment replicated the variation in disease etiology seen in the field.

We used TRFLP to evaluate the structure, dynamics, and diversity of the rumen microbiome in mild and severe grain-induced SARA and alfalfa pellet-induced SARA. We previ-

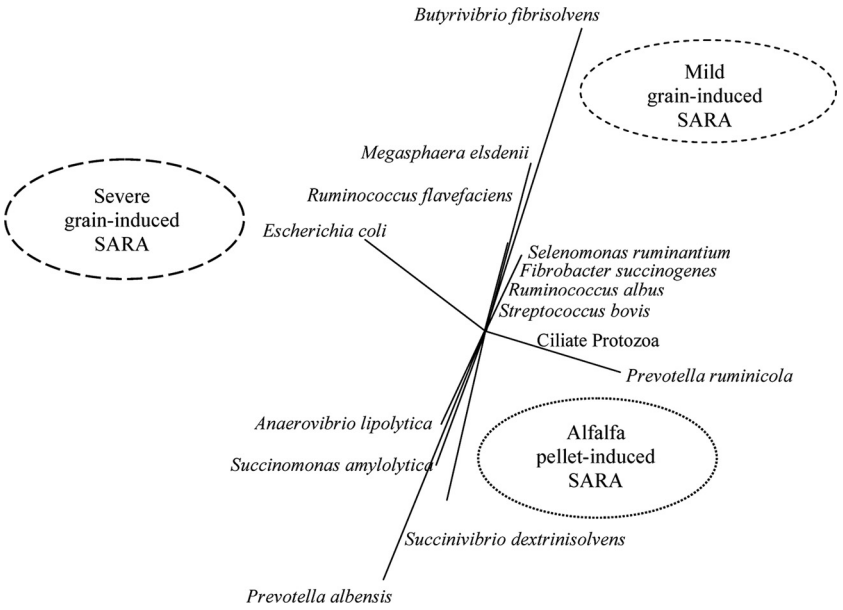

FIG. 4. Results of discriminant analysis of major rumen bacteria and ciliate protozoa in response to SARA induction model. The circles are independent variables, and the distances between them reflect their dissimilarity. The straight lines are dependent variables, and their lengths and the angles between them are functions of the relative effects of independent variables.

ously demonstrated (1, 41) that this analysis was not able to unambiguously identify peaks to the genus or family levels, but over 95% of T-RFs were unambiguous at the order level (41). The general population shifts we noted in our analysis were similar to those obtained with the 16S rRNA gene library studies of Tajima et al. (46) and Whitford et al. (49). However, these two studies (46, 49) evaluated typical high-grain diets but did not evaluate the possibility of reducing ruminal pH with highly fermentable nonstarch substrates like alfalfa pellets. By changing the particle size of the alfalfa (hay versus pellets), we could change the rate of fermentation without changing the chemical composition of the diet. Thus, fermentation substrate was not a confounding factor in the analysis.

The real-time PCR data (Fig. 2 and 3) evaluated with multivariate statistics (Fig. 4) indicated that the major predictor of severe grain-induced SARA was *E. coli*. The TRFLP prevalence analysis also indicated changes in the *Proteobacteria* (contains *E. coli*), although the differences between treatments were not significant. Given that the rumen LPS concentration was high in both grain- and alfalfa pellet-induced SARA and that only grain-fed animals exhibited inflammatory responses, this excludes the possibility that LPS alone is the cause of inflammation.

The most predominant shifts in microbial populations were among the gram-negative *Bacteroidetes*. These shifts were evident from the TRFLP prevalence data, as well as the real-time PCR data, for *P. albensis*, *P. brevis*, and *Prevotella ruminicola*, which are members of the *Bacteroidetes*. We suspect that a majority of the LPS produced came from these bacteria. *Bacteroides* spp. LPS is much less toxic than LPS from *E. coli* or *Salmonella* spp. (18, 44). A human study indicated that LPS from *Salmonella typhi*, *E. coli*, and *Pseudomonas aeruginosa* reached toxicity thresholds at ~0.1, 1.0, and 60 ng/kg of body weight, respectively (15). In an earlier rumen study, LPS was extracted from mixed rumen bacteria from either grain-fed or hay-fed animals and compared to *E. coli* LPS in a rodent endotoxin model. The most-toxic LPS came from *E. coli*, followed by LPS from grain-fed and then hay-fed animals (26). *M. elsdenii* is a gram-negative bacterium and almost always in high abundance in grain-fed animals. To test the possibility that the LPS from this bacterium was toxic, its LPS was compared to that of *E. coli* in a rodent model and found to be much less potent (27).

The classical view of acidosis in the rumen is that as grain increases, so does the prevalence of starch-fermenting bacteria like *S. bovis* (38). Thus, there should be a high population ratio of lactate-consuming bacteria like *M. elsdenii* to prevent lactic acid acidosis (37). Our real-time PCR data indicated that the amylolytic bacterium whose abundance most closely mirrored the severity of SARA was *S. bovis*, and other major amylolytic bacteria were less affected. *M. elsdenii* populations were synchronized with the *S. bovis* numbers, indicating that this bacterium was effectively eliminating rumen lactate. In contrast, even though the rumen pH conditions created by the alfalfa pellet diet were typical of SARA, the levels of *S. bovis* and, consequently, *M. elsdenii* were low.

The results of multivariate analysis (Fig. 1 and 4) indicated that there was a significant association between alfalfa-fed animals, microbial populations, and the absence of inflammation. The microbial species most closely associated with this diet were *P. ruminicola*, *A. lipolytica*, *Succinimonas amylolytica*, *P. albensis*, and *S. dextrinosolvens*. In particular, *P. albensis* was in high abundance in this diet. Thus, *Prevotella* spp. may potentially be used as probiotics in SARA. Rodríguez (36) selected strains of *Prevotella* spp. that grew rapidly on starch and produced succinate and propionate as major end products. When this strain was inoculated into three lactic acid-challenged goats, it was found that lactic acid levels declined.

Chiquette et al. (5) dosed *Prevotella* spp. into dairy cattle and observed small changes in lactic acid levels, but appropriate acidotic rumen conditions were not produced, so the effect of the *Prevotella* was equivocal. Our study is the first to our knowledge to demonstrate a clear association between SARA and particular microbial populations that might be protective, and it provides objective evidence for the use of *Prevotella* spp. as a probiotic in SARA.

In conclusion, we have demonstrated that grain- and alfalfa pellet-induced SARA result in different rumen microbial population structures even though rumen fermentation conditions are similar. We hypothesized that LPS concentrations in the rumen were not a predictor of SARA because high LPS levels could be created with an alfalfa pellet diet but no inflammation occurred. As the inflammatory response may have been due in part to translocation of LPS from the gut into the bloodstream, we conclude that LPS is indicative of a barrier function defect and that the key to understanding SARA is to identify potential microbial and physiological factors that result in increased epithelial permeability. Because *E. coli* is a major contributor to the rumen LPS pool and its LPS is highly toxic (17), we hypothesize that *E. coli* may be a contributing factor to disease onset.

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