Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant¹

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ABSTRACT

Some silage inoculants help to improve silage quality and promote an increase in milk production, possibly through altering the rumen microflora. We hypothesized that rumen bacterial community composition (BCC) would be different in cows fed alfalfa ensiled with the inoculant *Lactobacillus plantarum* MTD/1 (LP) compared with those fed alfalfa ensiled without the inoculant (Ctrl). Eight ruminally cannulated Holstein cows were allotted to 2 diets (Ctrl or LP) in a double crossover design with four 28-d periods. Diets were formulated to contain (% dry matter basis) 28.0% neutral detergent fiber and 16.2% crude protein, and contained alfalfa silage, 50.9; corn silage, 20.6; high-moisture shelled corn, 21.4; soy hulls, 4.7; plus minerals and vitamins, 2.4. Ruminal digesta were collected just before feeding on 3 consecutive days near the end of each period, and were separated into solid and liquid phases. Microbial DNA was extracted from each phase, amplified by PCR using domain-level bacterial primers, and subjected to automated ribosomal intergenic spacer analysis. The pH was 4.56 and 4.86 and the lactate-to-acetate ratio 9.8 and 4.4, respectively, for the treated and untreated alfalfa silages. Dry matter intakes and milk production data were not influenced by diets but showed a cow effect. Total volatile fatty acids (mM) tended to be greater for LP compared with Ctrl. Individual volatile fatty acids were not influenced by diets but showed a significant cow effect. Ruminal acetate (mol/100 mol) and acetate-to-propionate ratio were lower and propionate (mol/100 mol) greater for the 2 milk fat-depressed (MFD; <3.2% fat content) cows compared with the other 6 cows. Correspondence analysis of the 265 peaks in the automated ribosomal intergenic spacer analysis profile across the 188 samples revealed that the first 2 components contributed 7.1

and 3.8% to the total variation in the profile. The ordination points representing the liquid and solid phases clustered separately, indicating that these phases differed in BCC. The analysis of similarity data showed differences between Ctrl and LP. The lactic acid bacterial counts ($\log_{10}$ cfu/g of wet silage) were 3.94 and 4.53 for the untreated and treated silage, respectively, at ensiling. The relative population size (RPS) of *L. plantarum*, determined by real-time PCR of 16S rRNA gene copies, was greater in LP compared with Ctrl. The ordination points corresponding to certain individual cows clustered separately, and the most distinctive bacterial communities were those associated with MFD cows. The RPS of *Megasphaera elsdenii* was greater in 1 of the 2 MFD cows, although mean RPS of *M. elsdenii* did not differ between the treatments. In addition to the differences in rumen BCC between LP and Ctrl, MFD cows also displayed differences in BCC compared with cows with normal milk fat yield.

Key words: rumen, silage inoculant, microbial populations, milk fat depression

INTRODUCTION

Lactic acid bacterial (LAB) inoculants are used as additives in ensiling to enhance the preservation efficiency of silage and to improve animal performance. Desirable features of a silage inoculant include the ability to (1) rapidly lower the silage pH, (2) lower ethanol and ammonia nitrogen content, and (3) increase the lactate-to-acetate ratio and DM recovery (Weinberg and Muck, 1996). Silage additives containing LAB are commonly used in commercial inoculants to achieve the desired properties described above. However, it should be noted that LAB inoculants do not consistently yield the desirable silage properties if the crop is low in fermentable sugars due to excessive wilting, cloudy weather, or rain (Kung, 2009).

Several studies have reported the beneficial effects of LAB inoculants on silage fermentation (reviewed by Weinberg and Muck, 1996). In addition, some LAB inoculants have also been reported to improve animal performance by increasing milk yield and feed

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efficiency (Kung and Muck, 1997; Kung et al., 2003). An average increase in milk yield of 1.4 kg/cow per day was reported in 47% of the studies when inoculated silage was offered (Kung and Muck, 1997), with a more consistent increase in milk yield when silage was inoculated with the strain *Lactobacillus plantarum* MTD/1 (Kung et al., 2003). Some studies using silage inoculated with *L. plantarum* reported better animal performance even when the inoculant did not appear to improve the quality of the fermentation (Gordon, 1989; Steen et al., 1989), leading to the speculation that LAB inoculants may possibly act as probiotics. A probiotic is a viable culture of microorganisms that favors the growth of useful bacteria and inhibits the growth of pathogenic bacteria. Weinberg et al. (2003, 2004) demonstrated that silage inoculant LAB survived in rumen fluid in vitro. Recently, Contreras-Govea et al. (2011) reported that rumen microbial biomass yield determined in vitro was greater for the forage ensiled with the silage inoculants *L. plantarum*, *Lactobacillus pentosus*, and *Lactococcus lactis* than for those ensiled with *L. plantarum* + *Enterococcus faecium* and the uninoculated control. The ability of the LAB inoculants favoring increased microbial biomass led to the speculation that LAB inoculants could possibly affect rumen bacterial community composition (BCC) and population sizes of other bacteria in the rumen. However, the effect of specific strains of LAB on rumen BCC has not been explored.

We hypothesized that rumen BCC would be different in cows fed alfalfa ensiled with the inoculant *Lactobacillus plantarum* MTD/1 (LP) compared with those fed alfalfa ensiled without the inoculant (Ctrl). The objectives were to (1) examine rumen BCC of dairy cows as affected by feeding LP using the community fingerprinting technique, automated ribosomal intergenic spacer analysis (ARISA), and (2) determine the relative population size (RPS) of the inoculant (*L. plantarum* MTD/1).

MATERIALS AND METHODS

Experimental Design

Experiments were conducted in accordance with a research protocol approved by the University of Wisconsin–Madison's Research Animal Resource Center. Eight ruminally cannulated Holstein cows (111 DIM, 46 kg/d average milk yield) were blocked by DIM and average milk yield forming 2 groups (4 cows each). Each group was randomly assigned to 2 experimental diets in a double crossover design with four 28-d periods. Diets were Ctrl and LP (*Lactobacillus plantarum* MTD/1 inoculant; Ecosyl Products Ltd., Yorkshire,

UK). The inoculant was applied to fourth-cut alfalfa at 1.0×10^5 cfu/g of the crop (in liquid form) at the forage harvester. Alfalfa was wilted in the field to approximately 50% DM. The inoculated treatment was harvested first from alternate windrows chopped to a theoretical chop length of 10 mm and ensiled in an oxygen-limiting tower silo. Later the same day the untreated alfalfa was harvested with the same equipment and ensiled in another oxygen-limiting tower silo. Total LAB counts were determined in alfalfa (treated and untreated) at ensiling, following the procedure described by Contreras-Govea et al. (2011). Duplicate samples of alfalfa silage (treated and untreated), corn silage and high-moisture corn were freeze-dried, ground through a 1-mm screen and analyzed for CP (Leco FP-2000A nitrogen analyzer; Leco Corp., St. Joseph, MI). Neutral detergent fiber (with heat-stable amylase) and ADF content were determined using the procedure of Robertson and Van Soest (1977) as modified by Hintz et al. (1996) to include sodium sulfite during refluxing. About 20 g of silage samples were diluted 10:1 with autoclaved distilled water, blended in a 0.5-L blender for 30 s and filtered through 4 layers of cheesecloth. The filtrate was immediately used for pH determination. An aliquot (20 mL) of the filtrate was centrifuged in a 50 mL polypropylene tube at $25,000 \times g$ for 25 min and the supernatant was stored at -20°C before analyzing the fermentation products using HPLC (Muck and Dickerson, 1988). The fermentation characteristics and chemical composition of alfalfa silage (treated and untreated), corn silage, and high-moisture corn is tabulated (Table 1). Diets were formulated to contain (% DM basis) 28.0% amylase-treated NDF, and 16.2% CP. Total mixed rations containing (% DM) were as follows: alfalfa silage, 50.9; corn silage, 20.6; high-moisture shelled corn, 21.4; soy hulls, 4.7; plus supplemental minerals and vitamins, 2.4 (Vita-Plus Corp., Madison, WI) were fed ad libitum once daily.

Milk samples were collected (a.m. and p.m.) on 4 d (d 20, 21, 27, and 28) in each period for the determination of fat, protein, lactose, and MUN contents using near infrared spectroscopy (AgSource Cooperative Services, Verona, WI). Rumen digesta were collected just before feeding on 3 consecutive days of each period (d 22–24) by manual mixing before sampling and spot sampled from midpoint along the length of the rumen (rumen mat) and from the midpoint along the height of the rumen contents (ventral rumen). It should be noted that rumen digesta confined to the rumen epithelium was not sampled. After collection, rumen digesta were squeezed through 4 layers of cheesecloth to separate the solid (SO) and liquid (L) phases. Volatile fatty acids were determined from the L phase of rumen digesta by HPLC (Weimer et al., 1991). Microbial DNA was

Table 1. Fermentation and nutritive characteristics of untreated alfalfa, treated alfalfa, corn silage, and high-moisture corn

Item	Untreated alfalfa silage (n = 16)	Treated ¹ alfalfa silage (n = 17)	Corn silage (n = 17)	High-moisture corn (n = 17)
DM (%)	49.3 ± 1.65	48.3 ± 1.11	31.4 ± 0.26	73.3 ± 0.17
pH	4.86 ± 0.06	4.56 ± 0.02	3.82 ± 0.01	4.60 ± 0.02
Lactate (g/kg of DM)	63.1 ± 5.79	75.8 ± 2.09	59.4 ± 2.55	11.7 ± 0.39
Acetate (g/kg of DM)	15.3 ± 1.88	8.3 ± 0.70	33.6 ± 2.28	2.6 ± 0.52
Lactate:acetate	4.4 ± 0.19	9.8 ± 0.54	2.0 ± 0.26	5.5 ± 0.38
Ethanol (g/kg of DM)	1.0 ± 0.12	0.7 ± 0.09	6.0 ± 0.54	2.0 ± 0.22
CP (% DM)	25.1 ± 0.23	25.1 ± 0.09	6.9 ± 0.07	7.3 ± 0.07
NDF (% DM)	31.7 ± 0.28	31.5 ± 0.24	37.2 ± 0.87	4.0 ± 0.16
ADF (% DM)	24.1 ± 0.25	23.8 ± 0.38	21.1 ± 0.59	1.1 ± 0.05
Ash (% DM)	10.4 ± 0.21	9.9 ± 0.17	3.8 ± 0.14	1.2 ± 0.03

¹Silage inoculant containing *Lactobacillus plantarum* (Ecosyl MTD/1) was applied to fourth-cut alfalfa silage at 1.0×10^9 cfu/g of crop.

extracted from the L phase directly, but the SO phase was first homogenized in a blender with chilled extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 0.15 M NaCl pH 8.0) to release the solids-associated bacteria as described by Stevenson and Weimer (2007). In brief, DNA was extracted from 25 mL of L phase and 25 g of SO phase of rumen digesta using a series of wash steps with the extraction buffer, followed by lysis of the microbial cells in a bead beater, extraction with combinations of phenol/chloroform, and precipitation with isopropanol (Stevenson and Weimer, 2007). The DNA obtained was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and its concentration measured by spectrophotometry.

The internally transcribed region (ITS) between the bacterial 16S and 23S rRNA genes was amplified using domain-specific bacterial primers 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* (Cardinale et al., 2004). The PCR components and the cycling conditions have been described previously (Weimer et al., 2010b). The PCR product obtained was resolved in a Beckman Coulter CEQ8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA) using the run parameters as described by Weimer et al. (2010b). In brief, 0.5 µL of the PCR product was mixed with 1 µL of Beckman Coulter WelRed #1 infrared fluorescent dye-labeled DNA standard ladder (MapMarker 1000; Bio Ventures, Murfreesboro, TN) and 39 µL of sample loading solution (Beckman Coulter Inc.) and loaded into microtiter plates. Capillary electrophoresis was conducted following the manufacturer's instructions after covering the liquid surface of the wells with molecular biology-grade mineral oil.

The raw data obtained from capillary electrophoresis were imported into GeneMarker software (v. 1.75; Soft Genetics LLC, State College, PA) for further analysis. Peak detection and quantification were performed using the settings for amplicon fragment-length polymorphism analysis described in the GeneMarker manual. Peak sizes (bp) were determined using the DNA standard ladder described above. Baseline subtraction and peak smoothing was performed as described in the GeneMarker manual. The panel generated by the software was screened manually to remove any questionable peaks caused by pull-up from the dyed DNA standard. All peaks corresponding to amplicon length (AL) >112 bp were exported for correspondence analysis (described under the statistical analysis section).

Quantitative real-time PCR (qRT-PCR) assay was conducted with the L phase of rumen digesta. The primers used for targeting the *recA* gene of *L. plantarum* (LacPla1F 5'-AGG CGC GGC TGA TGT CA-3' and LacPla1R 5'-CGC GAT TGT CTT GGT TTT GTT-3') were previously validated for their specificity (Stevenson et al., 2006). These primers are complementary to the respective positions 503 to 570 of the *recA* gene of *Escherichia coli*. The size of the resulting amplicon was 68 bp. It was preferred to target the *recA* gene instead of the 16S rRNA gene because we have observed almost complete homology in the 16S rRNA genes of several *Lactobacillus* species found in plant material (Stevenson et al., 2006). The standards for the *L. plantarum* assay were prepared by making a serial dilution of the genomic DNA obtained from a pure culture of the target strain. The standards and the set of samples belonging to each period were run in the same plate in triplicate. The RPS of *L. plantarum* was determined as the ratio of the amplification of target species (*L. plantarum recA* copy numbers) to the amplification of the background obtained by amplifying the

16S rRNA gene with eubacterial primers (BAC338F and BAC805R, Yu et al., 2005). The qRT-PCR was conducted using POWER SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), forward and reverse primers (25 pmol of each primer/reaction) and approximately 20 ng of template DNA in a final volume of 25 μ L per reaction. Quantitative real-time PCR assays were conducted using the Applied Biosystems Prism 7300 sequence detection system. The cycling conditions for amplifying the *recA* gene of *L. plantarum* included 40 cycles of 95°C for 15 s and 59°C for 60 s and for the eubacterial 16S rRNA gene, 40 cycles of 95°C for 15 s, 50°C for 60 s, and 72°C for 90 s were used. The efficiency of the PCR was calculated as the negative reciprocal of the slope of the line obtained by plotting cycle threshold (Ct) versus log DNA concentrations of the standard dilution series.

The design and validation of primers for *Megasphaera elsdenii* (MegEls2F 5'-AGA TGG GGA CAA CAG CTG GA-3' and MegEls2R 5'-CGA AAG CTC CGA AGA GCC T-3') has been reported earlier (Stevenson and Weimer, 2007). Relative population size of *M. elsdenii* was determined as the ratio of 16S rRNA gene copies amplified with the specific primers to the 16S rRNA gene copies amplified with the eubacterial primers. The amplification conditions for *M. elsdenii* were 40 cycles of 95°C for 15 s and 59°C for 60 s. Readers should note that the determination of RPS of a rumen bacterium using the ratio of 16S rRNA gene copies is a relative quantification approach, and may sometimes over- or underestimate the absolute differences in the numbers of the bacterium under investigation.

Statistical Analysis

The data matrix resulting from the export of peak areas from GeneMarker were analyzed by correspondence analysis following the method of Ludwig and Reynolds (1988) using custom software written in the C programming language. Two ordination points (out of 192 total) corresponding to the L phase in 2 cows in the first period were identified as outliers. These 2 samples together with the corresponding samples in the SO phase were excluded from the analysis. The ordination points for the first 2 components were plotted as scatter plots. The ordination points for fitting vectors for the variables milk fat yield and VFA were calculated using the method of vector fitting described by McCune and Grace (2002).

The relative peak areas obtained from ARISA were analyzed for the shifts in BCC using analysis of similarity (ANOSIM; Clarke, 1993). Four data matrices were constructed from the ARISA data (L and SO phase for LP and Ctrl treatments) corresponding to each cow.

Each data matrix contained 265 rows (corresponding to the different AL) and 12 columns (3 daily samples for each of the 4 periods). The ANOSIM statistic, conventionally termed the R-value, was determined using Bray-Curtis dissimilarity rankings (Clarke, 1993). A higher R-value indicates greater differences in the BCC and the probability values represent the fraction of these R-values that exceeded the R-value obtained from the randomized comparison.

Dry matter intakes, production data, rumen VFA data and the RPS of *L. plantarum* and *M. elsdenii* averaged per cow per period ($n = 32$) were analyzed using the MIXED procedure of SAS. Cow within diet was the experimental unit with group, cow, treatment, and period designated as fixed effects in the model. Because group and period were not significant ($P > 0.05$) for all the variables (except for RPS of *L. plantarum*), they were removed subsequently from the model.

RESULTS

Fermentation and Nutritive Characteristics of Silages

Prior to ensiling, alfalfa contained 50% DM, and (DM basis) 24% CP, 30% NDF, 22% ADF, and 5.6% acid detergent lignin. The nutritive value of alfalfa silage remained closer to that of the original crop with a negligible loss in DM (Table 1). The fermentation characteristics of treated alfalfa silage were better than those of untreated alfalfa silage based on the lower pH and greater lactate content and lactate-to-acetate ratio of the former relative to the latter. Butyrate was not detected and propionate was negligible in alfalfa silage (data not shown).

Intakes and Production

Dry matter intakes, milk yield, and milk components (content and yield), and MUN were not influenced by diet but a cow effect was observed without any interaction between cow and treatment (Table 2). Milk fat concentration was lower for cows 3016 and 3436 compared with the other cows, whereas milk fat yield (kg/d) was lower for cows 3436 and 3295 than for the other cows.

Rumen VFA

Total VFA (mM) tended to be greater ($P = 0.08$) for LP than for Ctrl (Table 3). However, individual VFA proportions were not influenced by diets. A significant cow effect was observed for all of the individual VFA. Ruminal acetate (molar proportion) was lower and propionate greater for the milk fat-depressed (MFD;

Table 2. Dry matter intake and production data in dairy cows offered alfalfa silage inoculated (LP), or not (Ctrl), with a lactic acid bacterial inoculant¹

Variable	Treatment			<i>P</i> -value	Cow								SEM	<i>P</i> -value	Cow × treatment
	Ctrl	LP	SEM		2623	2674	3016	3035	3039	3085	3295	3436			
DMI (kg/d)	24.8	25.5	0.39	0.27	29.0 ^a	27.2 ^{ab}	23.2 ^c	28.5 ^a	24.2 ^{bc}	26.2 ^{ab}	22.9 ^c	20.2 ^d	0.79	<0.01	0.21
Milk yield (kg/d)	36.9	37.7	1.17	0.68	39.8 ^a	42.6 ^a	41.9 ^a	45.5 ^a	33.6 ^b	31.7 ^b	27.8 ^b	35.5 ^{ab}	2.34	<0.01	0.58
Milk yield/kg of DMI	1.49	1.48	0.03	0.88	1.37 ^{bc}	1.56 ^{bc}	1.76 ^a	1.60 ^b	1.38 ^{bc}	1.21 ^b	1.21 ^c	1.76 ^a	0.06	<0.01	0.87
Milk fat (%)	3.71	3.84	0.07	0.19	4.09 ^{ab}	3.72 ^{bc}	3.08 ^d	3.61 ^{bc}	4.55 ^a	4.51 ^a	3.81 ^b	2.84 ^d	0.14	<0.01	0.76
Fat yield (kg/d)	1.37	1.43	0.04	0.31	1.67 ^a	1.60 ^a	1.29 ^b	1.61 ^a	1.52 ^{ab}	1.44 ^{ab}	1.07 ^c	1.01 ^c	0.08	<0.01	0.39
Protein (%)	2.82	2.81	0.02	0.63	2.98 ^b	2.79 ^c	2.51 ^d	2.33 ^e	3.07 ^b	3.13 ^{ab}	3.25 ^a	2.47 ^d	0.04	<0.01	0.26
Protein (kg/d)	1.04	1.04	0.03	0.88	1.22 ^a	1.21 ^a	1.05 ^{ab}	1.05 ^{ab}	1.03 ^b	0.98 ^b	0.90 ^b	0.88 ^b	0.06	<0.01	0.78
Lactose (%)	4.85	4.90	0.04	0.32	4.68 ^c	4.77 ^c	4.99 ^{ab}	4.70 ^c	5.17 ^a	4.85 ^c	4.95 ^b	4.89 ^{bc}	0.08	<0.01	0.29
Lactose (kg/d)	1.82	1.83	0.07	0.84	1.91 ^{ab}	2.07 ^a	2.10 ^a	2.12 ^a	1.74 ^b	1.53 ^{bc}	1.39 ^{bc}	1.75 ^{ab}	0.14	0.01	0.56
MUN (mg/dL)	12.5	12.1	0.43	0.54	12.6 ^{ab}	11.4 ^{bc}	14.3 ^a	13.6 ^{ab}	11.5 ^b	13.6 ^{ab}	9.4 ^c	12.2 ^{ab}	0.86	0.02	0.55

^{a-e}Least squares means within cows in a row with different superscripts differ ($P < 0.05$).

¹Silage inoculant containing *Lactobacillus plantarum* (Ecosyl MTD/1) was applied to fourth-cut alfalfa at 1.0×10^5 cfu/g of crop.

Table 3. Rumen fermentation and milk fat variables in dairy cows offered alfalfa silage inoculated (LP), or not (Ctrl), with a lactic acid bacterial inoculant¹

Variable	Treatment			<i>P</i> -value	Cow								SEM	<i>P</i> -value	Cow × treatment
	Ctrl	LP	SEM		2623	2674	3016	3035	3039	3085	3295	3436			
Total VFA (mM)	120.4	129.6	3.45	0.08	109.2	139.3	123.1	118.3	120.9	139.2	123.2	126.8	6.91	0.09	0.31
mol/100 mol															
Acetate (Ac)	68.3	68.5	0.36	0.72	70.7 ^a	69.9 ^{ab}	63.6 ^c	69.9 ^{ab}	71.2 ^a	68.2 ^b	70.8 ^a	63.1 ^c	0.72	<0.01	0.55
Propionate (Pr)	18.6	18.7	0.33	0.82	17.5 ^{bc}	16.9 ^{bc}	23.2 ^a	18.0 ^b	16.4 ^{bc}	18.2 ^b	15.9 ^c	23.1 ^a	0.66	<0.01	0.63
Butyrate (Bu)	9.10	9.03	0.09	0.62	8.17 ^c	9.06 ^b	9.43 ^{ab}	8.62 ^{bc}	8.90 ^b	9.70 ^a	9.15 ^{ab}	9.46 ^{ab}	0.19	<0.01	0.46
Isobutyrate	0.84	0.79	0.02	0.29	0.92 ^a	0.89 ^a	0.64 ^c	0.73 ^{bc}	0.85 ^{ab}	0.81 ^{ab}	0.93 ^a	0.73 ^{bc}	0.06	0.02	0.39
Valerate	1.66	1.52	0.06	0.15	1.16 ^b	1.56 ^b	1.84 ^{ab}	1.44 ^b	1.40 ^{bc}	1.49 ^b	1.67 ^b	2.17 ^a	0.13	<0.01	0.41
Isovalerate	1.47	1.43	0.05	0.49	1.56 ^a	1.64 ^a	1.22 ^b	1.29 ^b	1.62 ^a	1.57 ^a	1.57 ^a	1.43 ^{ab}	0.09	0.02	0.84
Ac-to-Pr ratio	3.78	3.76	0.07	0.88	4.07 ^{ab}	4.11 ^{ab}	2.77 ^c	3.88 ^b	4.36 ^a	3.76 ^b	4.47 ^a	2.74 ^c	0.14	<0.01	0.35
Ac + Bu-to-Pr ratio	4.28	4.25	0.08	0.84	4.54 ^b	4.65 ^{ab}	3.18 ^c	4.36 ^b	4.91 ^a	4.29 ^b	5.04 ^a	3.15 ^c	0.16	<0.01	0.41

^{a-c}Least squares means within cows in a row with different superscripts differ ($P < 0.05$).

¹Silage inoculant containing *Lactobacillus plantarum* (Ecosyl MTD/1) was applied to fourth-cut alfalfa at 1.0×10^5 cfu/g of crop.

<3.2% fat content) cows (3436 and 3016) compared with the other cows. Consistent with this, the ratios acetate to propionate and acetate + butyrate to propionate were also lower for the MFD cows than for the other cows. A tendency ($P = 0.09$) existed for a cow effect for total VFA. Cow by treatment interactions for total and individual VFA were not significant.

BCC

Total AL detected by the analysis of raw data generated from capillary electrophoresis of PCR products was 265 (range = 61–798 bp). The mean number of AL detected in individual samples was 113, ranging from a minimum of 36 to a maximum of 143. The maximum number of AL detected in both digesta types were similar (138 in L and 143 in SO). The minimum number of AL detected in the SO digesta were 62 compared with 36 in L digesta, indicating greater fluctuations in the latter. Correspondence analysis of the ARISA profile across the entire data set revealed that the first 2 components contributed 7.1 and 3.8%, respectively, to the total variation in the profile; these percentages, while small, are not unexpected due to the high dimensionality of the data set (36 to 143 amplicons per sample).

The ordination points representing the L and SO phases clustered separately (Figure 1A). In Figure 1B, ordination points corresponding to individual cows were spread along the first component (x-axis) with the majority of the ordination points clustering on the left, whereas a small but distinct set of ordination points clustered toward the right. This distinctly separated group of ordination points represented the MFD cows (3016 and 3436). It should be noted that the vector for milk fat yield showed in the direction of the first component (x-axis) but away from the ordination points corresponding to the MFD cows, indicating that ARISA profiles in this study were helpful in identifying the MFD cows from the normal cows. Vectors for milk fat content and acetate-to-propionate ratio were also in the same direction as milk fat yield (not shown in the figure). The vector for VFA was also in the same direction as milk fat yield, but not very strong, indicating a lower contribution of VFA to the variation in BCC.

Figure 2 displays the ordination points corresponding to treatments (Ctrl and LP) in individual cows. The ordination points above and below the dotted line represent SO and L phases, respectively. A closer examination of this figure revealed that diets tended to cluster together in each phase for most of the cows (3295, 2623, 3436, 3016, and 3085), indicating treatment differences. Consistent with this, the ANOSIM data (Table 4) also showed differences in the pairs of dietary treatments compared within cows (greater R-values indicate

greater differences). However, the ANOSIM data also showed differences when cows were offered the same diet (Ctrl vs. Ctrl or LP vs. LP) but in a different period, indicating an effect of the period or possibly adaptation of the bacterial community over time.

Epiphytic LAB Counts and Relative Population Size of *L. plantarum* and *M. elsdenii*

The epiphytic LAB counts for the untreated and treated alfalfa ($\log_{10}$ cfu/g) at ensiling were 3.94 and 4.53, respectively. The RPS of *L. plantarum* (expressed as percent of 16S rRNA gene copy number) was greater for LP compared with Ctrl without any significant cow effect (Table 5). The RPS of *M. elsdenii* showed a significant cow effect with greater values observed for 1 of the MFD cows (3436) compared with the normal cows. However, the RPS of *M. elsdenii* was not different between treatments.

DISCUSSION

Automated ribosomal intergenic spacer analysis has been used as a microbial fingerprinting technique for studying BCC (Weimer et al., 2010a,b; Welkie et al., 2010). It should be noted that ARISA is well suited to obtain a broader picture of the shifts in the BCC, although it does not provide specific taxonomic information about the individual AL (Fisher and Triplett, 1999). Automated ribosomal intergenic spacer analysis is more sensitive compared with the alternative fingerprinting techniques [e.g., Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)] considering the greater mean number of AL detected (113 in this study) compared with the number of detectable bands in other fingerprinting techniques.

BCC

The BCC reported here reflects the bacterial communities in the rumen contents only and does not include bacteria confined to the rumen epithelium. The separation between the L and SO phases, evident from the lack of overlap in the ordination points between the 2 phases (Figure 1A), was consistent with the findings of Welkie et al. (2010) and Weimer et al. (2010b). This indicates that the L- and SO-associated rumen bacterial communities are substantially different, consistent with their preference for nutrient utilization in the SO and L phases. Ordination points for individual cow/treatment combinations, from samples collected on the last 3 d of each period were very similar in position, indicating that the BCC had reached a substantial degree of

Figure 1. Scatter plot of the ordination points obtained from correspondence analysis of the automated ribosomal intergenic spacer analysis (ARISA) data matrix from 8 cows across 4 periods with 3 replicate samples per cow in a period obtained on 3 consecutive days ($n = 188$; 94 from each phase). Both Figures 1A and 1B were prepared with the same scale but are separated for clarity. In Figure 1A, the filled and open circles represent the ordination points corresponding to the solid and liquid phases of rumen digesta, respectively. In Figure 1B, the 8 different markers represent the ordination points corresponding to the 8 individual cows. The ordination points corresponding to the liquid phase are shown in the oval and the other points above the oval correspond to the solid phase. Note that the vector for milk fat yield is in a direction opposite to that of the ordination points, corresponding to the milk fat-depressed cows (3016 and 3436).

stability by the end of each 28-d period. These results were in accord with previous studies in which a 28-d period was sufficient to achieve stability following either substantial dietary changes (Weimer et al., 2010b) or severe perturbation resulting from almost complete exchange of ruminal contents (Weimer et al., 2010a). The ordination points corresponding to individual cows (Figure 1B) within each phase showed separation along the first component (which contributed greater variation), indicating that individual cows displayed shifts in the BCC. The clustering of the ordination points corresponding to each cow within a phase (evident in

Figure 2) is consistent with the findings that ruminal BCC displays substantial host specificity (Weimer et al., 2010a).

Several possible reasons exist for individual cows to be different in ruminal BCC even when offered the same diet. Some of the differences could be due to receipt of unique inocula from the dam (and possibly from other cows/calves) following birth, the nutritional history of the cow before the experiment, and the contribution of yet-undefined genetic factors that regulate the host-microbe symbiosis. Differences in BCC among individual cows on the same diet are also likely to occur due to

Figure 2. Scatter plot of the ordination points obtained from correspondence analysis of the automated ribosomal intergenic spacer analysis (ARISA) data matrix from 8 cows across 4 periods with 3 replicate samples per cow in a period obtained on 3 consecutive days. In this figure, individual cows are plotted separately for the purpose of clarity ($n = 22$ for the cows 3016 and 3436 and $n = 24$ for the other cows). The ordination points above and below the dotted lines represent solid and liquid phase of rumen digesta, respectively. All of the figures here have the same scale. The open and filled circles represent the control (Ctrl) and *Lactobacillus plantarum* MTD/1 inoculated silage (LP), respectively. Note the distinct scatter of the ordination points for the milk fat-depressed cows (3016 and 3436) compared with the other cows.

differences in rumen pH among cows (not measured). Low rumen pH decreases the efficiency of conversion of complex feed components into simpler end products that can affect the growth of rumen bacteria. However, differences in rumen pH alone do not explain differences in BCC (Palmonari et al., 2010). Thus, the differences among cows in their feeding behavior, salivary secretion, and nutrient absorption capacity of the epithelium are all likely to contribute to the variation in individual BCC among cows.

Although some overlap exists in the ordination points corresponding to individual cows, 2 cows (3016 and 3436 in Figure 1B and Figure 2) displayed profiles that differed from those of the other 6. These 2 cows were identified as MFD cows (see Table 2). Previously,

Palmonari et al. (2010) and Weimer et al. (2010b) also reported that MFD cows displayed a distinct BCC demonstrating a strong association between MFD and shifts in BCC.

Treatment and Cow Effects

A 6-fold increase in total LAB counts at ensiling in the treated alfalfa compared with the untreated alfalfa could be attributed to the inoculant treatment. The increase in RPS of *L. plantarum* in LP compared with Ctrl was proportionately small but significant (Table 5). This discrepancy could be due to the use of species-specific (rather than group-specific) primers for quantifying *L. plantarum* in rumen fluid, in contrast

Table 4. Analysis of similarity (ANOSIM)¹ of bacterial community composition in liquid and solid phases of rumen digesta from dairy cows offered alfalfa silage inoculated with (LP) and without (Ctrl) a commercial silage inoculant²

Treatment/period ³	Group 1 cow ⁴				Treatment/period ³	Group 2 cow ⁴			
	3295	2623	2674	3016		3039	3035	3436	3085
Liquid phase					Liquid				
Ctrl-LP (period 1–2)	0.704 (0.009)	0.259 (0.009)	0.111 (0.029)	1.000 (0.009)	LP-Ctrl (period 1–2)	0.444 (0.009)	0.333 (0.009)	1.000 (0.009)	0.704 (0.009)
Ctrl-LP (period 3–4)	0.296 (0.021)	−0.037 (0.958)	0.074 (0.030)	0.000 (0.311)	LP-Ctrl (period 3–4)	0.220 (0.021)	0.481 (0.009)	0.037 (0.039)	0.333 (0.009)
Ctrl-Ctrl (period 1–3)	0.778 (0.009)	0.296 (0.009)	0.148 (0.009)	0.500 (0.009)	LP-LP (period 1–3)	0.926 (0.009)	0.111 (0.028)	0.000 (0.289)	0.370 (0.009)
LP-LP (period 2–4)	0.148 (0.020)	0.000 (0.325)	0.148 (0.018)	0.667 (0.009)	Ctrl-Ctrl (period 2–4)	0.000 (0.326)	1.000 (0.009)	1.000 (0.009)	0.185 (0.031)
Solid phase					Solid				
Ctrl-LP (period 1–2)	0.185 (0.021)	0.778 (0.009)	0.333 (0.02)	−0.250 (0.931)	LP-Ctrl (period 1–2)	0.778 (0.009)	0.667 (0.009)	1.000 (0.009)	0.444 (0.021)
Ctrl-LP (period 3–4)	0.259 (0.028)	0.407 (0.021)	0.296 (0.018)	0.926 (0.009)	LP-Ctrl (period 3–4)	0.667 (0.009)	0.222 (0.009)	0.667 (0.009)	0.518 (0.020)
Ctrl-Ctrl (period 1–3)	0.259 (0.019)	0.704 (0.009)	0.741 (0.009)	0.500 (0.009)	LP-LP (period 1–3)	0.185 (0.019)	0.667 (0.009)	1.000 (0.009)	0.333 (0.020)
LP-LP (period 2–4)	0.037 (0.041)	0.185 (0.039)	0.000 (0.328)	0.370 (0.009)	Ctrl-Ctrl (period 2–4)	0.407 (0.009)	0.296 (0.021)	0.037 (0.048)	0.555 (0.021)

¹The ANOSIM R-values were determined from Bray-Curtis dissimilarity measurements of the relative peak areas obtained from capillary electrophoresis. Higher ANOSIM R-values indicate greater differences in bacterial community composition between the 2 diets compared (+1 = maximally distinct; −1 = maximally similar). Values in parentheses indicate probability of obtaining a larger ANOSIM value from randomized rankings of between-group similarities.

²Silage inoculant containing *Lactobacillus plantarum* (Ecosyl MTD/1) was applied to fourth-cut alfalfa at 1.0×10^5 cfu/g of crop.

³LP = *L. plantarum* MTD/1-treated silage.

⁴The 8 cows in this study were blocked by DIM and average milk yield into 2 groups of 4 cows each. Cows in group 1 were allotted to Ctrl in periods 1 and 3, respectively, and to LP in periods 2 and 4, respectively. Cows in group 2 were allotted to LP in periods 1 and 3, respectively, and to Ctrl in periods 2 and 4, respectively.

Table 5. Percentages of target species in rumen liquid samples relative to total eubacterial content determined by quantitative real-time PCR (qRT-PCR) analysis¹

Species ²	Treatment				Cow										Cow × treatment
	Ctrl	LP	SEM	P-value	2623	2674	3016	3035	3039	3085	3295	3436	SEM	P-value	
<i>Lactobacillus plantarum</i>	0.016	0.032	0.002	<0.01	0.024	0.020	0.032	0.016	0.023	0.017	0.033	0.026	0.013	0.97	0.44
<i>Megasphaera elsdenii</i>	0.040	0.060	0.019	0.47	0.004 ^b	0.003 ^b	0.075 ^b	0.004 ^b	0.004 ^b	0.003 ^b	0.004 ^b	0.298 ^a	0.038	<0.01	0.93

^{a,b}Least squares means within cows in a row with different superscripts differ ($P < 0.05$).

¹The 8 cows in this study were blocked by DIM and average milk yield into 2 groups of 4 cows each. Cows in group 1 were allotted to control (Ctrl) in periods 1 and 3, respectively, and to *L. plantarum* MTD/1-treated silage (LP) in periods 2 and 4, respectively. Cows in group 2 were allotted to LP in periods 1 and 3, respectively, and to Ctrl in periods 2 and 4, respectively. Therefore, group and period within group were also included in the statistical model. A significant group ($P = 0.04$) and period within group ($P < 0.01$) effect was observed for *L. plantarum*.

²The relative population size (RPS) of *L. plantarum* was determined as the ratio of copies of the *recA* gene to copies of the 16S rRNA gene amplified with eubacterial primers and expressed as percentage. The RPS of *M. elsdenii* was determined as the ratio of copies of the 16S rRNA gene of the target species to copies of the 16S rRNA gene amplified with eubacterial primers and expressed as percentage.

Figure 3. Individual cow differences in milk fat content (%; left y-axis) and relative population size of *Megasphaera elsdenii* (%; right y-axis). Note the inverse trend between the 2 variables. ^{a-d}Least squares means for milk fat content with different letters differ ($P < 0.05$); ^{y,z}least squares means for relative population size of *M. elsdenii* with different letters differ ($P < 0.05$)

to the LAB counts determined from the silage, which are based on plating onto selective media designed to cultivate a range of LAB genera.

An improvement in silage quality for the treated alfalfa compared with untreated alfalfa was evident from the lower pH and increase in lactate and lactate-to-acetate ratio for the former than the latter. However, a lack of difference in DMI and milk yield between LP and Ctrl was not anticipated. Muck (1993) reported that the incidence of positive responses for intake and milk production was 25 and 40%, respectively, in his review of inoculant studies from 1985 to 1992. Reviewing studies in which *L. plantarum* MTD/1 was used as an inoculant (Weinberg and Muck, 1996), it could be noticed that 6 of the 10 studies reported an improvement in both silage fermentation and animal performance; 2 studies reported only increased animal performance;

whereas 1 study reported improvement in silage quality without any effect on animal performance. In the current study, cows were allotted to the diets in a double crossover design, resulting in all cows going through both diets twice. A lack of positive response in intakes and production could, therefore, be attributed to an increased variation in animal performance resulting from dietary changes across the 4 periods and possibly to the small number of animals used.

No treatment effects on milk fat content or yield were observed, but individual cow differences in milk fat yield resulted in a cow effect. Although we did not anticipate MFD in the current study due to the high forage content of diets, ARISA profiles showing distinct BCC for the 2 cows described above prompted us to explore this further. An increase in the RPS of *M. elsdenii* has been reported earlier in MFD cows (Palmonari et

al., 2010; Weimer et al., 2010b). Consistent with their findings, the RPS of *M. elsdenii* in this study was also greater for 1 of the cows experiencing MFD (Table 5; Figure 3). It should be noted that cows experiencing MFD were also low in milk protein content and had greater efficiency of milk yield.

Although individual VFA were not influenced by treatments, a tendency for the increase in total VFA for LP compared with Ctrl demonstrates a positive effect of the inoculant. In a previous study, Contreras-Govea et al. (2011) reported a greater microbial biomass yield in the inoculated silage (third-cut alfalfa) compared with the control, despite the lack of difference in in vitro gas production and total VFA between treatments. It is worth exploring if a difference in microbial biomass yield could exist with or without a difference in total VFA.

The treatment effect is also evident in some of the cows showing clustering of the ordination points by diet (Figure 2). Furthermore, the greater ANOSIM R-values when cows moved from Ctrl to LP or vice versa also demonstrates a treatment effect. However, ANOSIM R-values were also greater when cows offered the same diets in different periods were compared (Ctrl vs. Ctrl or LP vs. LP), indicating a period effect as well. A period effect can occur if a specific diet offered during a period is different in nutrient composition from the same diet offered in other periods. However, no differences existed in nutrient composition measured across periods (results not shown). We also used the same set of reagents for molecular analyses of samples to rule out possible effects on ARISA profiles. The feeding trial began in early April, and was completed in late July. It is possible that some of the period effect may be due to environmental effects on the cows (e.g., heat stress) that were not recorded.

CONCLUSIONS

This study evaluated the effect of alfalfa ensiled with or without the inoculant *L. plantarum* MTD/1 on silage quality, intakes, production, and rumen BCC in dairy cows. Inoculated alfalfa silage was better in quality compared with the untreated control based on the lower pH and greater lactate-to-acetate ratio for the former than the latter. No effect of the inoculant on animal performance occurred. Feeding silage prepared with the inoculant resulted in higher ruminal levels of *L. plantarum* and, in some cows, a detectable shift in bacterial community composition (BCC). However, differences in BCC between treatments were relatively minor compared with major differences in BCC between the SO and L phases of rumen digesta, and among cows that did, versus did not, display MFD. Milk fat de-

pression was associated with an increase in the relative population size of the bacterium *Megasphaera elsdenii*. In addition to the differences in rumen BCC between LP and Ctrl, MFD cows also displayed differences in BCC compared with the cows with normal milk fat yield.

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