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Changes in the rumen microbial community composition of dairy cows subjected to an acidogenic diet

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ABSTRACT

In modern breeding systems, cows are subjected to many stress factors. Animals fed a high-grain diet may have a decreased rumen pH, which would lead to subacute ruminal acidosis syndrome. The aim of this study was to investigate the evolution of microbial community composition in cows undergoing a dietary stress challenge. Twelve cows were subjected to a challenge period that consisted of a rapid change of ration, from a normal (45.4:54.6 forage:concentrate) to a high-grain content diet (24.8:75.2 forage:concentrate) to induce subacute ruminal acidosis. Individual rumen fluid content samples were collected before (T0) and during the challenge (T3, T14, T28). The DNA from rumen contents was extracted, purified, and sequenced to evaluate bacterial populations, and sequencing was performed on Illumina MiSeq. The effect of animal conditions on rumen microbial community was quantified through a linear mixed model. The acidogenic diet created 2 main clusters: ruminal hypomotility (RH) and milk fat depression (MFD). The microbial composition did not differ in T0 between the 2 groups, whereas during the challenge *Ruminococcus* spp., *Treponema* spp., *Methanobrevibacter* spp., and *Methanosphaera* spp. concentrations increased in RH cows; and *Succinivibrio* spp. and *Butyrivibrio* spp. concentrations increased in MFD cows. *Prevotella* spp. and *Ruminococcus* spp. were negatively correlated, whereas the *Christenellaceae* family was positively correlated with both *Methanobrevibacter* spp. and *Methanosphaera* spp. Moreover, the same diet affected cows' microbiota composition differently, underlying the impact of the host effect. Other studies are necessary to deepen the relationship between microbiota composition and host.
Key words: dairy cows, rumen microbiota, milk fat depression, rumen hypomotility

INTRODUCTION

The ability of the ruminants to convert complex saccharides to nutritive food is due to the presence of rumen microbiota, which is characterized by bacteria, protozoa, fungi, and archaea. The rumen microbial community is involved in host physiology, health, feed efficiency, methane production, and gene regulation. Rumen microbiota degrade plant material, which results in its conversion into digestible compounds such as VFA (McCann et al., 2014; Weimer, 2015; Mizrahi and Jami, 2018). Bacteria are the most abundant microorganisms in the rumen (10^{10}–10^{11} cells/mL). The "bacteria core microbiome" is then characterized by particular phyla, including *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (McCann et al., 2014). All of the reactions in the rumen are influenced by the symbiotic host-microbiome relationship. The relationships between different microbial groups and genera of the same group could influence the whole microbial composition, but the role of each group in the rumen is still difficult to define (Kumar et al., 2013; Mizrahi and Jami, 2018).

Microbial populations in the rumen are influenced by several factors. Weimer (2015) explained that factors which are able to influence the microbiota composition are (1) the development of ruminal microbiome during the growth of cattle, (2) all environmental factors influencing the initial establishment of each community, and (3) the influence that all bacteria have on each other. Diet can affect microbial composition and its fermenting activities (Liu et al., 2021). All problems related to the hindgut tract can affect the animal's health, such as the increase of susceptibility to rumen disease (Polsky and von Keyserlingk, 2017; Cavallini et al., 2021b). For example, a nutritional stress condition can affect the motility of the rumen, decrease rumination time, and be manifested in rumen hypomotility. As shown in Cavallini et al. (2020), rumen hypomotility affected DMI and milk yield. Another example of nutritional stress is a high-grain content diet that results in a decrease of rumen pH and an acidosis condition in ruminants could occur (Buonaiuto et al., 2021b).

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Diet can also affect rumen microbiota composition: *Streptococcus bovis* grows faster in the presence of readily fermentable carbohydrates, and it slows when carbohydrates are fermented. Therefore, lactate influences *S. bovis* growth because it is a fermentation product of this bacterium (Ivany et al., 2002; Khafipour et al., 2009; Masih and Bhat, 2020). *Megasphaera elsdenii* and some strain of *Selenomonas ruminantium* are major lactate fermenters and can metabolize 60% to 80% of lactate under SARA condition (Mu et al., 2021). Also, only *M. elsdenii* can metabolize lactate to butyrate, which is important for rumen epithelial health and growth (Fan et al., 2022). Despite studies investigating the effects of SARA-inducing diet on rumen microbiota, there are no reports on how the same acidogenic diet could influence the development of different health issues and the influence of the microbiota.

Subacute ruminal acidosis is a multifactorial condition mainly caused by a decrease of rumen pH (around 5.2 to 5.8) for >3 h/d and ruminal D-lactate overproduction (Plaizier et al., 2008). A lower pH level affects rumen microbiota, increasing lysis of gram-negative bacteria and releasing cell-free bacterial LPS in rumen fluid, which can affect the permeability of rumen epithelium and increase inflammatory disease (Mao et al., 2013). Nutritional factors that can increase the risk of SARA development are inadequate ruminal buffering by saliva and a high-carbohydrate diet without rumen adaptation (Kleen et al., 2003). This digestive disorder causes feed intake depression, reduction of milk yield, laminitis, and poor fertility (Kleen et al., 2003). Another important consequence of SARA is milk fat depression (**MFD**) that can be induced by the inhibition of bacteria that biohydrogenate fatty acids in the rumen (Stone, 2004; Hua et al., 2017) or by a change in its pathway. Milk fat depression led to a 50% decrease of milk fat with no changes in the other milk components (Harvatine, 2016; Hackmann and Vahmani, 2023).

Therefore, the aim of the present study was to evaluate the impact of an acidogenic diet on rumen microbial community and the associations between rumen bacteria in 12 multiparous high-producing Italian Holstein-Friesian cows, during a 4-wk environmental-nutritional challenge design.

MATERIALS AND METHODS

This research was conducted at the dairy research farm of the Department of Veterinary Medical Sciences (DIMEVET; Alma Mater Studiorum, University of Bologna, Ozzano dell'Emilia, Italy). Unless stated differently, all of the laboratory procedures were conducted at the facilities of the DIMEVET Service of Animal Production and Food Safety. The animal study protocol was approved by the Ethics Committee of University of Bologna (protocol code 762, December 15, 2016).

Experimental Design

Twelve multiparous high-yielding Italian Holstein-Friesian cows (DIM = 51.90 ± 29.68 [SD]) were used in a 4-wk environmental-nutritional challenge design study. The cows had an average rumination time of 522.8 ± 79.6 min/d, and milk yield at the beginning of experiment was 40.27 ± 7.76 kg/d. The experiment was conducted over 28 d in which cows were subjected to a rapid change of ration, from a normal diet formulated to mimic the typical Parmigiano Reggiano rations (Mammi et al., 2018), to a high-grain diet (45.4:54.6 vs. 24.8:75.2 forage: concentrate) to induce SARA condition. Rations were balanced using a software based on the CNCPS model (DinaMilk5; Fabermatica) and offered ad libitum intake (approximately 1.10 × expected intake) distributed daily at 0900 h (Zago Mixer; Table 1). Samples of feedstuff and diets were collected twice a week and analyzed according to previous studies (Buonaiuto et al., 2021a; Mammi et al., 2022).

Monitoring Production Parameters and Collection of Rumen Content

Individual live weight was recorded daily (Afiweight Scale, Afikim, Israel) as well as the individual DMI using an individual feed bunk (Dinamica generale), while water intake was recorded by individual water meter, as reported in previous research (Cavallini et al., 2021a, 2023). Rumination time was selected as an index of cows' health conditions, and the Hi-Tag rumination monitoring system (SCR Engineers) was used. Finally, individual daily milk yield was recorded using the Afimilk System. Cows were milked twice a day and milk samples from 2 consecutive milkings for each cow were collected on d 0 (baseline), 7, 14, 21, and 28 and analyzed within 12 h in the Artest S.P.A. (Modena, Italy) laboratory for fat, protein, and lactose percentage, and urea (mg/dL). Milk components were measured by mid-infrared analysis with MilkoScan 6000 FT (Foss Electric, Hillerød, Denmark). Precalibration procedures were performed according to International Dairy Federation Standards 141C:2000 (IDF, 2000), using total nitrogen for protein expression. Energy-corrected milk was calculated according to Cavallini et al. (2021a) using the following equation:

$$\text{ECM (kg/d)} = (\text{MY} \times 0.327) + (\text{MF} \times 12.86) + (\text{MP} \times 7.65),$$

where MY is milk yield, MF is milk fat, and MP is milk protein expressed in kilograms per day. Fat-corrected milk quantity calculated on a 4.0% butterfat energy basis was estimated according to Dairy Records Management Systems (2006) using the following equation:

$$\text{FCM 4\% (kg/d)} = (\text{MY} \times 0.4324) + (\text{MF} \times 16.2162).$$

Milk urea nitrogen was calculated according to Celis-Álvarez et al. (2016) starting from the milk urea.

Rumen contents were sampled 2 wk before the administration of the acidogenic diet (**T0**), on the first day of administration (**T3**), and 2 (**T14**) and 4 (**T28**) wk afterward. According to Palmonari et al. (2017), rumen fluid samples were collected through esophageal probe; after the first fractions of rumen content were discarded to avoid saliva contaminations, the samples were immediately frozen in Falcon tubes at −80°C.

Table 1. Characteristics and composition of diets

Item	T0 diet[1] (±SD)	Challenge diet[2] (±SD)
Ingredient, kg/head per day as fed		
Grass hay, finely chopped	9.5	6.0
Wheat straw, finely chopped	1.0	1.0
Corn flakes	6.0	13.0
Concentrate[3]	7.5	8.0
Cane molasses[4]	1.0	1.0
Forage:concentrate ratio[5]	45.4:54.6	24.8:75.2
Composition, % of DM		
DM	87.22 ± 3.00	88.11 ± 0.74
Ash	7.50 ± 1.28	8.06 ± 1.58
Ether extract	3.21 ± 0.47	3.27 ± 0.47
CP	14.79 ± 1.17	14.18 ± 0.84
aNDFom[6]	35.94 ± 4.16	28.38 ± 2.99
ADF	24.55 ± 2.56	16.29 ± 4.75
ADL	5.27 ± 1.09	3.37 ± 1.24
$uNDF_{240h}$[7]	9.93 ± 3.32	3.05 ± 0.18
Starch	22.95 ± 2.62	35.03 ± 2.18
peNDF[8]	17.56 ± 1.35	13.10 ± 0.85
Energy,[9] ME/Mcal/kg of DM	2.37	2.94

[1]Formulated following Parmigiano Reggiano regulation (Consorzio del Formaggio Parmigiano Reggiano, 2018).

[2]High-grain content diet.

[3]Lactation mix ingredient: 29.6% wheat bran, 29.4% sorghum grain, 21.6% canola meal, 14.7% flaked full-fat soybean, 2.2% calcium carbonate, 1% sodium chloride, 0.4% magnesium oxide, 0.9% sodium bentonite, and 0.3% vitamin and mineral premix (provided 40,000 IU of vitamin A, 4,000 IU of vitamin D_3, 30 mg of vitamin E 92% α-tocopherol, 5 mg of vitamin B_1, 3 mg of vitamin B_2, 1.5 mg of vitamin B_6, 0.06 mg of vitamin B_{12}, 5 mg of vitamin K, 5 mg of vitamin H_1 (para-aminobenzoic acid), 150 mg of niacin, 50 mg of choline chloride, 100 mg of Fe, 1 mg of Co, 5 mg of I, 120 mg of Mn, 10 mg of Cu, and 130 mg of Zn).

[4]Characterized as reported in Palmonari et al. (2021, 2023).

[5]Forage and concentrate ratio, % of forages and concentrates on a DM basis.

[6]aNDFom = amylase- and sodium sulfite-treated NDF with ash correction.

[7]Unavailable NDF estimated via 240-h in vitro fermentation.

[8]Physically effective NDF (aNDFom × physical effective factor), calculated using the Ro-Tap system (Cavallini et al., 2018).

[9]Estimated using DinaMilk5; Fabermatica, Ostiano, Italy.

DNA Extraction and Sequencing

The DNA was extracted and isolated from rumen samples using a specific protocol for rumen fluid as described in Stevenson and Weimer (2007). In brief, DNA was extracted using extraction buffer (100 m*M* Tris-HCl, 10 m*M* EDTA, 0.15 *M* NaCl, pH 8.0), 80 µL of 10% SDS, 700 µL of phenol (pH 8.0), transferred into an Eppendorf tube with 0.25 g of marbles and put into the TLyser (TissueLyser I, Qiagen) for 5 min. Subsequently, the samples were placed into a water bath (JULABO TN8) at 60°C for 10 min and put again into the TLyser (TissueLyser I, Qiagen) and spun in a centrifuge (MIKRO 200R, Hettich Zentrifugen) for 10 min at maximum speed to break the microbial cell wall. The samples were subjected to a combination of phenol/chloroform and then 50 µL of sodium acetate 3 *M* and 300 µL of isopropanol. The DNA was resuspended in 100 µL of TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and frozen at −80°C for the next analysis. The quality of DNA was evaluated with NanoDrop spectrophotometrically. For sequencing, each sample was PCR-amplified using 341F and 805R primers in the V3–V4 region of the 16S rRNA gene in 25-µL volumes containing 12.5 ng of microbial DNA, 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems), and 200 nmol/L of S-D-Bact 0341-b-S-17/S-D-Bact-0785-a-A-21 primers (33) carrying Illumina overhang adapter sequences (Bio-Fab Research). The thermal cycle consisted of an initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. Amplicons of about 460 bp were purified with a magnetic bead-based cleanup system (Agencourt AMPure XP; Beckman Coulter) and sequenced on Illumina MiSeq platform using a 2 × 300 bp paired end protocol, according to the manufacturer's instructions (Illumina). Briefly, indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with AMPure XP magnetic beads (Beckman Coulter). Libraries were pooled at equimolar concentrations, denatured, and diluted to 6 pmol/L before loading onto the MiSeq flow cell. Amplicon sequences were deposited in the MG-RAST database (http://metagenomics.anl.gov/linkin.cgi?project=17675).

Bioinformatics

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012), QIIME 2 (Caporaso et al., 2010), and DADA2 (Callahan et al., 2016). High-quality reads were clustered into high-resolution amplicon sequence variants (**ASV**) and the taxonomy was assigned using SILVA as the reference database (Quast et al., 2013). The ASV tables were collapsed at all phyloge-

Table 2. Least squares means for cow condition effect on rumination time (total, prechallenge, and challenge), rumination drop, daily average reticular pH, pH (minutes below 5.8 and 5.5), days of SARA, daily DMI, and daily water intake[1]

Item	Production parameter		SEM	*P*-value
	MFD	RH		
Rumination time, min/d	513.37[A]	357.65[B]	13.73	<0.01
Prechallenge, min/d	545.85[A]	416.01[B]	15.89	<0.01
Challenge, min/d	479.10[A]	280.04[B]	12.43	<0.01
Rumination drop, min	65.13[B]	133.75[A]	17.43	<0.01
Daily average reticular pH	5.99	6.17	0.08	0.10
pH <5.8, min/d	394.73	120.65	113.12	0.12
pH <5.5, min/d	68.08	12.82	34.54	0.30
Days of SARA, %	45.09	12.44	0.13	0.12
Daily DMI, kg	26.23[a]	23.44[b]	0.93	<0.05
Daily water intake, L	152.85[A]	126.36[B]	6.73	0.01

[a,b]Within a row values with different lowercase superscripts differ ($P \leq 0.05$).
[A,B]Within a row values with different uppercase superscripts differ ($P \leq 0.01$).
[1]MFD = milk fat depression; RH = ruminal hypomotility.

Table 3. Least squares means for effect of cow condition on milk yield and quality[1]

Item	MFD	RH	SEM	*P*-value
Yield, kg/d	43.73[a]	36.78[b]	2.51	<0.05
Fat, %	2.48[B]	3.77[A]	0.11	<0.01
Protein, %	3.22	3.21	0.06	0.51
F:P ratio	0.77[B]	1.17[A]	0.03	<0.01
Lactose, %	4.99	5.09	0.03	0.15
ECM, kg/d	35.14	35.12	2.00	0.39
Urea, mg/dL	9.24[B]	14.96[A]	0.88	0.01
MUN, mg/dL	4.32[B]	6.99[A]	0.41	0.01

[a,b]Within a row values with different lowercase superscripts differ ($P \leq 0.05$).
[A,B]Within a row values with different uppercase superscripts differ ($P \leq 0.01$).
[1]MFD = milk fat depression; RH = ruminal hypomotility; F:P = fat:protein.

netic levels, from phylum to genus. Alpha diversity was computed using the number of observed ASV, Shannon index, and Faith's phylogenetic diversity metrics. Beta diversity was estimated by computing weighted and unweighted UniFrac distances, which were used as input for principal coordinates analysis.

Statistical Analysis

All the statistical analyses were carried out using the SAS software (SAS Institute Inc., Cary, NC). The distribution of each bacterial concentration was evaluated by visual inspection using PROC UNIVARIATE. Descriptive statistics, including mean, standard deviation, minimum, maximum, and 1st and 99th percentile were calculated using PROC MEANS. Data were analyzed using a linear mixed model (PROC MIXED) in which each bacterial concentration represented the dependent variable, and the independent variables were time of sampling (4 classes: T0, T3, T14, and T28), cow condition (2 classes: MFD and ruminal hypomotility [**RH**]), and the one-way interaction between time and condition. Time was also considered as a repeated effect over the subject cow. Based on both the Akaike information criterion and the Bayesian information criterion, the covariance structure which best fit the data was selected. Multiple comparison of least squares means was performed using Tukey adjustment, and significance was set at $P < 0.05$. Finally, Pearson correlation coefficients between each pair of bacterial concentrations were calculated using PROC CORR.

RESULTS

Animal Performance

The effects of cows' condition on animal performance and milk yield and quality are depicted in Tables 2 and 3. The abrupt change of diet produced 2 main clusters: 5 cows showed RH, whereas 7 cows manifested milk fat-to-protein ratio inversion or MFD. Cows in RH condition had a decrease in rumination time compared with MFD (357.65 vs. 513.37 min/d, $P < 0.01$); MFD animals had a pH below 5.8 for 394.73 min/d compared with RH animals (120.65 min/d). Subacute ruminal acidosis syndrome was declared as reticular pH below 5.8 for at least 330 min/d (Plaizier et al., 2008). During hypomotility conditions, cows reduced their metabolic activity and milk production compared with MFD animals (36.78 vs. 43.73 kg; $P < 0.01$). Fat-depressed animals had a reduction in milk fat content compared with those with hypomotility (2.48% vs. 3.77%, respectively; $P < 0.01$) and a fat-to-protein ratio of 0.77 compared with 1.17 in RH cows ($P < 0.01$). Energy-corrected milk was not significantly different between the 2 groups.

Rumen Microbial Composition

The sampling at T0 did not show differences between the 2 groups for the majority of bacterial families and genera (Tables 4 and 5), except for *Erysipelotrichaceae RFN20* genus, which was lower in MFD (0.38% vs. 2.18%, $P < 0.05$); *Succiniclasticum* spp., which was higher in MFD (1.95% vs. 0.56%, $P < 0.05$); and *Bacteroid_RF16_Un*, which was higher in MFD (1.59% vs. 0.45%, $P < 0.05$). A similar condition was observed during T3, T14, and T28, except for *Clostridium* spp.,

Table 4. Least squares means of concentrations of the main bacteria taxa and families at T0[1]

Bacteria	Bacteria concentration (%)		SEM	*P*-value
	MFD	RH		
Bacteroidaceae	0.06	0.1	0.22	0.86
Campylobacteraceae	0.03	0.02	0.03	0.81
Christensenellaceae	0.09	0.31	0.24	0.39
Clostridiaceae	0.22	0.31	0.24	0.73
Desulfobulbaceae	0.03	0.00	0.03	0.34
Desulfovibrionaceae	0.26	0.02	0.16	0.15
Elusimicrobiaceae	0.00	0.05	0.02	0.19
Enterobacteriaceae	0.00	0.02	0.04	0.58
Erysipelotrichaceae	1.95	3.61	1.14	0.16
Fibrobacteraceae	0.19	0.77	0.38	0.14
Lachnospiraceae	9.97	7.13	2.72	0.49
Lactobacillaceae	0.00	0.02	0.01	0.41
Methanobacteriaceae	0.06	0.26	0.12	0.12
Prevotellaceae	49.45	35.18	8.33	0.11
Pseudomonadaceae	0.03	0.02	0.05	0.89
Ruminococcaceae	8.33	10.82	2.57	0.34
Spirochaetaceae	2.08	4.20	1.69	0.22
Streptococcaceae	0.03	0.00	0.04	0.39
Succinivibrionaceae	0.54	0.92	0.64	0.56
Veillonellaceae	4.23	1.77	1.91	0.21

[1]MFD = milk fat depression; RH = ruminal hypomotility.

Table 5. Least squares means of concentrations of the bacteria genera and species at T0[1]

Bacteria	Bacteria concentration (%)		SEM	*P*-value
	MFD	RH		
Paraprev_CF231	0.48	0.92	0.25	0.32
Paraprev_YRC22	0.44	0.82	0.27	0.38
Alphaproteo_Un	0.00	0.02	0.01	0.41
Anaerostipes spp.	0.00	0.02	0.01	0.41
Bacteroid_RF16_Un	0.45[b]	1.59[a]	0.46	<0.05
Blautia spp.	0.22	0.15	0.12	0.68
Butyrivibrio spp.	2.98	1.56	0.64	0.16
Clostridium spp.	0.19	0.26	0.24	0.8
Desulfobulbus spp.	0.03	0.00	0.02	0.29
Desulfovibrio spp.	0.26	0.02	0.08	0.11
Erysipelot_RFN20	0.38[b]	2.18[a]	0.33	<0.05
Fibrobacter spp.	0.19	0.77	0.25	0.27
Lachnospira spp.	0.06	0.20	0.12	0.57
Lactobacil_Un	0.03	0.00	0.02	0.29
Methanobrevibacter spp.	0.06	0.23	0.07	0.18
Methanosphaera spp.	0.00	0.03	0.01	0.41
Molli_RF39_Un	0.13	0.20	0.04	0.41
Prevotella spp.	49.45	35.18	5.35	0.11
Rickett_Un	0.06	0.18	0.11	0.54
Ruminococcus spp.	3.27	5.10	0.74	0.18
Shuttleworthia spp.	0.26	0.74	0.42	0.55
Succiniclasticum spp.	1.95[a]	0.56[b]	0.37	<0.05
Succinivibrio spp.	0.26	0.33	0.17	0.8
Treponema spp.	2.05	4.20	0.96	0.21

[a,b]Within a row values with different superscripts differ ($P \leq 0.05$).
[1]MFD = milk fat depression; RH = ruminal hypomotility.

Lachnospira spp., *Lactobacil_Un*, and *Succiniclasticum* spp. in MFD cows, as shown in Tables 6, 7, 8, and 9. As expected, *Prevotellaceae* was the dominant family in both MFD and RH cows, with a significant difference among the 2 groups showing a higher abundance in milk fat–depressed cows (45.86% and 31.49% in MFD and RH, respectively; $P < 0.01$, Table 10), whereas *Paraprevotellaceae* family had a lower abundance in MFD cows (1.53% and 2.69% in MFD and RH, respectively; $P < 0.05$, Table 10). Another important family in the rumen, *Ruminococcaceae*, was significantly different among the 2 groups, being higher in relative abundance in RH compared with MFD animals (10.89% vs. 4.76% in RH and MFD, respectively; $P < 0.01$, Table 10). *Methanobacteriaceae* family was more abundant in RH animals (0.24% and 0.004% in RH and MFD, respectively; $P < 0.01$, Table 10). *Succinivibrionaceae* family increased in MFD cows compared with RH (1.59% vs. 0.64%, $P < 0.05$, Table 10), whereas *Veillonellaceae* was higher in MFD ($P < 0.01$). The RH cows showed a greater abundance of *Paraprevotella YRC22* than MFD cows (1.09% vs. 0.58%, respectively; $P < 0.05$). Another important genus, *Ruminococcus* spp., had a higher abundance in RH cows compared with those with MFD (3.35% and 1.99%, respectively; $P < 0.05$). *Lactobac_Un* was higher in MFD compared with RH animals (2.86% vs. 0; $P < 0.01$). Least squares means for *Treponema* spp. differed significantly ($P < 0.01$) in RH (4.97%) compared with MFD cows (0.94%). *Butirivibrio* spp. displayed a numerically higher concentration in MFD cows than RH. *Methanobrevibacter* spp. and *Methanosphaera* spp. concentrations were significantly greater in RH animals ($P < 0.05$, Table 11) as well *Erysipelot_RFN20* ($P < 0.01$, Table 11) Finally, *Prevotella* spp. was higher in the MFD condition compared with RH (45.86% vs. 31.49%, respectively; $P < 0.01$, Table 11).

Correlation Coefficients Between Bacteria and Bacteria-Methanogens

Results of the pairwise Pearson correlation coefficients between rumen microbial species are in Figure 1. Methanogen species such as *Methanobrevibacter* spp. and *Methanosphaera* spp. were negatively correlated, as expected, to *Succinivibrio* spp. (−0.27 and −0.22, respectively). The aforementioned methanogen species were also positively correlated with the *Christenellaceae* family. This family had also a strong and positive correlation (0.68) with the *Ruminococcus* genus. *Prevotella* and *Ruminococcus* genera were negatively correlated (−0.65), whereas *Sphingomonas* spp. had a positive correlation with *Butyrivibrio* spp. (0.66); *Fibrobacter* spp. and *Treponema* spp. were positively correlated (0.59; Figure 1).

Table 6. Least squares means of concentrations of the main bacteria taxa and families at T0, T3, T14, and T28 in MFD cows[1]

	Bacteria concentration (%) MFD					
Bacteria	T0	T3	T14	T28	SEM	*P*-value
Bacteroidaceae	0.06	0.06	0.54	0.03	0.16	0.40
Campylobacteraceae	0.03	0.00	0.00	0.00	0.02	0.51
Christensenellaceae	0.09	0.03	0.13	0.10	0.18	0.59
Clostridiaceae	0.22	0.58	0.29	0.00	0.18	0.12
Desulfobulbaceae	0.03	0.00	0.00	0.06	0.02	0.30
Desulfovibrionaceae	0.26	0.13	0.22	0.06	0.12	0.43
Enterobacteriaceae	0.00	0.00	0.10	0.00	0.03	0.17
Erysipelotrichaceae	1.95	1.15	1.54	3.43	0.85	0.22
Fibrobacteraceae	0.19	0.55	0.16	0.00	0.28	0.28
Lachnospiraceae	9.97	7.72	9.52	7.69	2.49	0.87
Lactobacillaceae	0.00	0.00	0.00	0.06	0.03	0.12
Methanobacteriaceae	0.06	0.03	0.03	0.06	0.09	0.90
Prevotellaceae	49.45	55.41	48.78	33.40	6.21	0.10
Pseudomonadaceae	0.03	0.03	0.06	0.03	0.04	0.90
Ruminococcaceae	8.33	5.03	5.22	4.03	1.91	0.18
Spirochaetaceae	2.08	1.47	1.02	0.35	1.26	0.07
Streptococcaceae	0.03	0.03	0.00	0.06	0.03	0.66
Succinivibrionaceae	0.54	1.89	2.02	0.87	0.48	0.44
Veillonellaceae	4.23	6.31	6.99	1.41	1.42	0.25

[1]MFD = milk fat depression.

DISCUSSION

Animal Performance

A high-grain diet can cause SARA condition in cows, which is characterized by an average pH lower than 5.8 for at least 330 min (Plaizier et al., 2008) and related to the occurrence of MFD. Cows in our challenge were fed with the same acidogenic diet, but they manifested 2 different situations: rumen hypomotility and MFD. The productivity of cows was influenced by the diet: compared with fat-depressed cows, milk production decreased in those with hypomotility, and this condition is related to the reduction in rumination time and rumen motility of cows. On the other hand, fat-depressed cows were characterized by SARA condition. According to Kleen et al. (2003), ruminal acidosis is one of the causes of MFD and is influenced by the inhibition of the bacteria activity that

Table 7. Least squares means of concentrations of the main bacteria taxa and families at T0, T3, T14, and T28 in RH cows[1]

	Bacteria concentration (%) RH					
Bacteria	T0	T3	T14	T28	SEM	*P*-value
Bacteroidaceae	0.10	0.20	0.15	0.15	0.14	0.87
Campylobacteraceae	0.02	0.00	0.00	0.08	0.18	0.07
Christensenellaceae	0.31	0.15	0.33	0.41	0.16	0.83
Clostridiaceae	0.31	0.87	0.43	0.26	0.16	0.20
Desulfobulbaceae	0.00	0.02	0.02	0.02	0.02	0.85
Desulfovibrionaceae	0.02	0.26	0.02	0.10	0.10	0.54
Enterobacteriaceae	0.02	0.05	0.05	0.00	0.03	0.69
Erysipelotrichaceae	3.61	2.08	1.56	2.49	0.76	0.30
Fibrobacteraceae	0.77	0.87	0.38	0.25	0.25	0.48
Lachnospiraceae	7.13	9.26	7.59	6.46	2.22	0.85
Lactobacillaceae	0.02	0.05	0.00	0.00	0.02	0.60
Methanobacteriaceae	0.26	0.31	0.23	0.18	0.08	0.81
Prevotellaceae	35.18	29.89	26.64	37.95	5.55	0.37
Pseudomonadaceae	0.02	0.05	0.05	0.02	0.03	0.95
Ruminococcaceae	10.82	10.05	13.49	9.15	1.71	0.44
Spirochaetaceae	4.2	6.64	4.82	3.51	1.13	0.45
Streptococcaceae	0.00	0.02	0.00	0.00	0.03	0.51
Succinivibrionaceae	0.92	0.43	0.67	0.82	0.43	0.47
Veillonellaceae	1.77	2.46	1.05	1.02	1.27	0.44

[1]RH = ruminal hypomotility.

Table 8. Least squares means of concentrations of the main bacteria genera and species at T0, T3, T14, and T28 in MFD cows[1]

	Bacteria concentration (%) MFD					
Bacteria	T0	T3	T14	T28	SEM	*P*-value
Paraprev_CF231	0.48	0.70	0.64	0.13	0.25	0.10
Paraprev_YRC22	0.45	0.67	0.86	0.19	0.30	0.12
Alphaproteo_Un	0.00	0.00	0.00	0.00	0.02	1
Anaerostipes spp.	0.00	0.06	0.00	0.00	0.08	0.51
Bacteroid_RF16_Un	0.45	0.16	0.45	0.22	0.34	0.55
Blautia spp.	0.22	0.03	0.00	0.00	0.08	0.18
Butyrivibrio spp.	2.98	1.60	2.59	2.56	0.85	0.49
Clostridium spp.	0.19^{B}	0.57^{A}	0.29^{B}	0.00^{B}	0.18	≤0.01
Desulfobulbus spp.	0.03	0.00	0.00	0.06	0.02	0.30
Desulfovibrio spp.	0.26	0.13	0.23	0.06	0.11	0.43
Erysipelot_RFN20	0.38	0.51	0.25	0.13	0.33	0.06
Fibrobacter spp.	0.19	0.54	0.16	0.00	0.29	0.28
Lachnospira spp.	0.06^{B}	0.13^{B}	0.73^{A}	0.22^{B}	0.18	≤0.05
Lactobacil_Un	0.03^{B}	0.00^{B}	0.00^{B}	8.59^{A}	0.88	≤0.01
Methanobrevibacter spp.	0.06	0.03	0.03	0.06	0.08	0.91
Methanosphaera spp.	0.00	0.00	0.00	0.00	0.03	1
Molli_RF39_Un	0.13	0.19	0.19	0.03	0.09	0.36
Prevotella spp.	49.45	55.42	48.78	33.40	6.20	0.19
Rickett_Un	0.06	0.06	0.16	0.10	0.09	0.64
Ruminococcus spp.	3.27	1.83	2.08	2.08	0.80	0.27
Shuttleworthia spp.	0.26	0.99	1.05	0.25	0.43	0.11
Succiniclasticum spp.	1.95^{a}	2.98^{a}	1.54^{a}	0.06^{b}	0.52	≤0.05
Succinivibrio spp.	0.26	0.32	0.29	0.29	0.16	0.97
Treponema spp.	2.05	1.44	1.02	0.35	1.26	0.06

^{a,b}Within a row values with different lowercase superscripts differ ($P \leq 0.05$).

^{A,B}Within a row values with different uppercase superscripts differ ($P \leq 0.01$).

[1]MFD = milk fat depression.

Table 9. Least squares means of concentrations of the main bacteria genera and species at T0, T3, T14, and T28 in RH cows[1]

	Bacteria concentration (%) RH					
Bacteria	T0	T3	T14	T28	SEM	*P*-value
Paraprev_CF231	0.92	1.10	1.00	0.69	0.23	0.73
Paraprev_YRC22	0.82	0.95	1.26	1.08	0.27	0.77
Alphaproteo_Un	0.03	0.03	0.03	0.00	0.02	0.85
Anaerostipes spp.	0.02	0.23	0.03	0.00	0.07	0.27
Bacteroid_RF16_Un	1.59	1.08	1.41	1.20	0.31	0.79
Blautia spp.	0.15	0.10	0.08	0.15	0.07	0.87
Butyrivibrio spp.	1.56	2.49	2.07	1.69	0.76	0.87
Clostridium spp.	0.26	0.80	0.36	0.20	0.16	0.23
Desulfobulbus spp.	0.00	0.03	0.03	0.03	0.02	0.84
Desulfovibrio spp.	0.02	0.26	0.00	0.10	0.10	0.45
Erysipelot_RFN20	2.18	1.36	0.77	1.41	0.30	0.09
Fibrobacter spp.	0.77	0.87	0.38	0.26	0.25	0.48
Lachnospira spp.	0.20	0.15	0.28	0.05	0.16	0.80
Lactobacil_Un	0.00	0.00	0.00	0.00	0.79	1
Methanobrevibacter spp.	0.23	0.23	0.15	0.18	0.07	0.92
Methanosphaera spp.	0.03	0.08	0.08	0.00	0.03	0.51
Molli_RF39_Un	0.20	0.36	0.16	0.10	0.08	0.22
Prevotella spp.	35.18	29.90	26.64	37.95	5.55	0.36
Rickett_Un	0.18	0.18	0.23	0.08	0.08	0.70
Ruminococcus spp.	5.10	2.46	3.51	4.08	0.71	0.17
Shuttleworthia spp.	0.74	0.31	0.67	0.21	0.38	0.85
Succiniclasticum spp.	0.56	0.69	0.31	0.28	0.47	0.80
Succinivibrio spp.	0.33	0.10	0.20	0.39	0.14	0.67
Treponema spp.	4.20	6.64	4.80	3.49	1.13	0.44

[1]RH = ruminal hypomotility.

Table 10. Least squares means of concentrations of the main bacteria taxa and families during the challenge[1]

Bacteria	Bacteria concentration (%)		SEM	*P*-value
	MFD	RH		
Methanobacteriaceae	0.004^{B}	0.24^{A}	0.07	<0.01
Bifidobacteriaceae	0.05	0.08	0.08	0.68
Coriobacteriaceae	3.21^{A}	0.79^{B}	0.83	<0.01
Bacteroidales	6.05^{B}	11.51^{A}	1.31	<.01
Bacteroidaceae	0.21	0.17	0.12	0.74
Prevotellaceae	45.86^{A}	31.49^{B}	4.81	<0.01
Paraprevotellaceae	1.53^{b}	2.69^{a}	0.46	<0.05
Cyanobacteria	0.36^{B}	1.12^{A}	0.26	<0.01
Elusimicrobiaceae	0.11	0.22	0.10	0.28
Fibrobacteraceae	0.23	0.50	0.22	0.23
Lactobacillaceae	0.02^{A}	0.50^{B}	0.22	0.23
Streptococcaceae	0.03	0.01	0.02	0.28
Clostridiales	7.27	7.00	7.07	0.81
Christensenellaceae	0.08	0.29	0.14	0.14
Clostridiaceae	0.29	0.52	0.14	0.11
Lachnospiraceae	8.31	7.77	1.93	0.78
Ruminococcaceae	4.76^{B}	10.89^{A}	1.48	<0.01
Veillonellaceae	4.90^{A}	1.51^{B}	1.11	<0.01
Erysipelotrichaceae	2.04	2.04	0.65	0.99
Alphaproteobacteria	0.00	0.02	0.01	0.25
Desulfobulbaceae	0.02	0.02	0.02	0.87
Desulfovibrionaceae	0.14	0.13	0.09	0.9
Campylobacteraceae	0.00	0.02	0.01	0.11
Succinivibrionaceae	1.59^{a}	0.64^{b}	0.37	<0.05
Enterobacteriaceae	0.03	0.03	0.03	0.94
Pseudomonadaceae	0.04	0.04	0.03	1
Sphaerochaetaceae	0.16	0.09	0.09	0.47
Spirochaetaceae	0.95^{B}	4.99^{A}	0.98	<0.01
Mollicutes	0.14	0.21	0.07	0.34
Tenericutes	0.00^{b}	0.08^{a}	0.04	<0.05

^{a,b}Within a row values with different lowercase superscripts differ ($P \leq 0.05$).

^{A,B}Within a row values with different uppercase superscripts differ ($P \leq 0.01$).

[1]MFD = milk fat depression; RH = ruminal hypomotility.

Table 11. Least squares means of bacteria genera and species during the challenge[1]

Bacteria	Bacteria concentration (%)		SEM	*P*-value
	MFD	RH		
Methanobrevibacter spp.	0.04^{b}	0.19^{a}	0.06	<0.05
Methanosphaera spp.	0.00^{b}	0.05^{a}	0.02	0.05
*Bacteroid_BS11_*	0.04^{b}	0.84^{a}	0.31	<0.05
Prevotella spp.	45.86^{A}	31.49^{B}	4.81	<0.01
Paraprev_CF231	0.49^{b}	0.93^{a}	0.19	<0.05
Paraprev_YRC22	0.58^{b}	1.09^{a}	0.23	<0.05
Fibrobacter spp.	0.23	0.50	0.21	0.23
Lactobac_Un	2.86^{A}	0.00^{B}	0.68	<0.01
Clostridium spp.	0.29	0.45	0.14	0.25
Lachnospira spp.	0.36	0.16	0.13	0.16
Anaerostipes spp.	0.02	0.09	0.06	0.28
Butyrivibrio spp.	2.25	2.08	0.66	0.8
Ruminococcus spp.	1.99^{b}	3.35^{a}	0.62	<0.05
Succiniclasticum spp.	1.53^{A}	0.43^{B}	0.41	0.01
Desulfobulbus spp.	0.02	0.03	0.02	0.82
Succinivibrio spp.	0.30	0.23	0.13	0.59
Treponema spp.	0.94^{B}	4.97^{A}	0.98	<0.01
Molli_RF39_Un	0.14	0.21	0.07	0.33
Blautia spp.	0.01	0.11	0.06	0.1
Shuttleworthia spp.	0.77	0.39	0.33	0.27
Erysipelot_RFN20	0.29^{B}	1.18^{A}	0.26	<0.01
Alphaproteo_Un	0.00	0.02	0.01	0.25
Ricket_Un	0.11	0.16	0.07	0.44

^{a,b}Within a row values with different lowercase superscripts differ ($P \leq 0.05$).

^{A,B}Within a row values with different uppercase superscripts differ ($P \leq 0.01$).

[1]MFD = milk fat depression; RH = ruminal hypomotility.

bio-hydrogenates fatty acids in the rumen. Also, DMI influenced SARA development, but in our trial, the cows showing lower DMI were those with rumen hypomotility.

Rumen Microbial Composition Reflected RH and MFD Conditions

Despite the possible individual differences, the microbial composition of each cow changed and created one of 2 clusters with the same microbial difference among each group. The differences in the microbial community of the cows were also observed in the varieties of species: MFD cows had a lower number of identified microbial species than RH cows (data not shown). Plaizier et al. (2017) asserted that a great diversity of microbiota in the digestive tract is associated with adaptability, functionality, and host health.

Bacteroidetes decreased their concentration in milk fat–depressed cows as shown by Mao et al. (2013). During the high-grain diet administration, gram-negative bacteria such as *Bacteroidetes* and *Proteobacteria* could have been lysed by the low pH and caused the release of free LPS in the rumen. The negative correlation between *Bacteroidetes* and LPS was demonstrated also in Mao et al. (2013), but in this study we did not collect data about LPS concentration in the rumen.

Hua et al. (2017) observed that cows fed with a high-grain diet decreased the overall VFA (m*M*) and acetate production, which is related to fiber degradation, and it represents key product for milk fatty acid synthesis in the mammary gland. Moreover, Plaizier et al. (2017) reported that SARA increased the concentration of lactic acid-utilizing bacteria: in the present study the concentration of *Lactobacillaceae* and *Veillonellaceae* was greater in milk fat–depressed cows. *Megasphaera elsdenii* belongs to the *Veillonellaceae* family and is related to the production of branched-chain VFA in the rumen. It metabolizes lactate into formic and acetic acid. According to Palmonari et al. (2010), *M. elsdenii* had a higher abundance in cows fed a high-grain diet, and some strains are involved in MFD syndrome development.

Moreover, amylolytic bacteria such as *Streptococcaceae* and *Clostridiales* increased during rumen acidosis.

	Methanobr	Methanosp	Prevot	Paraprev	Cyanoba	Fibrobact	Lactobac	Christen	Butyrivibrio	Ruminococ	Erysipelot	Sharpea	Sphingom	Succinivi	Anaeropl	Bulleidia
Methanobr	1.00	0.22	-0.32	0.03	0.24	0.20	-0.14	0.23	0.00	0.36	-0.20	-0.10	0.16	-0.27	0.37	-0.10
Methanosp	0.22	1.00	-0.20	-0.12	0.61	0.25	-0.10	0.12	-0.06	0.26	0.04	-0.06	0.21	-0.22	-0.02	-0.17
Bifidobac	0.22	-0.19	0.18	0.11	-0.15	-0.14	-0.12	-0.21	0.03	-0.19	0.05	0.12	-0.12	-0.06	0.00	-0.03
Bacteroid_Un	0.17	0.23	-0.45	0.22	0.28	0.24	-0.07	0.43	-0.26	0.52	-0.34	-0.24	-0.05	-0.23	0.18	-0.36
Bacteroid_BS11_Un	0.11	0.10	-0.42	0.06	0.14	0.01	-0.12	0.82	-0.18	0.66	-0.16	-0.15	0.24	-0.28	-0.03	-0.20
Bacteroid_BF311	0.27	-0.05	-0.18	0.19	0.10	0.39	-0.14	-0.11	-0.15	0.15	-0.05	-0.11	0.10	0.16	0.29	-0.13
Prevot	-0.32	-0.20	1.00	-0.12	-0.30	-0.10	-0.03	-0.45	-0.35	-0.65	0.14	0.07	-0.52	0.13	0.01	-0.15
Bacteroid_RF16_Un	0.24	0.05	-0.25	0.39	0.31	0.46	-0.25	-0.11	-0.14	0.14	-0.23	-0.27	-0.06	-0.10	0.50	-0.30
Bacteroid_S24_7_Un	0.08	-0.07	-0.03	0.16	-0.14	-0.15	-0.17	0.12	-0.22	0.18	0.18	0.09	-0.12	-0.17	-0.27	-0.04
Paraprev_Un	0.03	-0.12	-0.12	1.00	0.07	0.12	-0.28	-0.01	-0.08	0.09	-0.18	-0.16	-0.04	0.14	0.33	-0.24
Paraprev_CF231	0.28	0.05	-0.18	0.22	0.13	0.57	-0.31	-0.11	-0.34	0.31	-0.30	-0.17	-0.03	-0.11	0.38	-0.40
Paraprev_YRC22	0.19	-0.01	0.01	0.35	0.01	0.40	-0.27	-0.04	-0.56	0.20	-0.05	-0.25	-0.22	-0.07	0.33	-0.31
Prevotella2	0.11	-0.15	0.17	0.14	0.00	-0.20	0.05	-0.19	0.03	-0.18	0.17	-0.03	-0.16	-0.05	-0.13	-0.03
Cyanobac_4C0d_2_YS2_Un	0.24	0.61	-0.30	0.07	1.00	0.23	-0.01	0.05	0.04	0.11	-0.06	-0.22	0.34	-0.08	0.14	-0.17
Fibrobacter	0.20	0.25	-0.10	0.12	0.23	1.00	-0.19	-0.24	-0.42	0.02	-0.15	-0.09	-0.16	-0.05	0.62	-0.25
Lactobac_Un	-0.14	-0.10	-0.03	-0.28	-0.01	-0.19	1.00	-0.05	0.07	-0.29	-0.01	-0.02	0.00	-0.07	-0.20	0.26
Streptococcus	0.15	-0.04	-0.03	0.18	0.10	-0.15	-0.09	-0.04	0.14	0.04	-0.01	-0.06	0.01	0.12	-0.07	-0.12
Clostrid_Un	0.12	-0.11	-0.26	0.20	0.03	-0.04	-0.15	0.21	0.03	0.32	-0.10	0.09	0.17	-0.17	0.08	-0.16
Christen_Un	0.23	0.12	-0.45	-0.01	0.05	-0.24	-0.05	1.00	0.09	0.68	-0.27	-0.17	0.29	-0.23	-0.16	-0.06
Clostridium	0.20	0.11	-0.30	-0.03	0.09	0.08	-0.23	0.06	0.44	0.36	-0.06	0.09	0.62	0.10	0.05	-0.23
Lachno_Un	0.01	0.04	-0.33	-0.18	0.06	-0.19	-0.09	0.02	0.53	0.04	0.39	0.46	0.36	0.00	-0.31	0.48
Anaerostipes	0.22	0.53	-0.01	-0.16	0.47	0.20	-0.10	0.03	-0.07	0.00	0.01	-0.12	0.01	-0.03	0.06	-0.18
Blautia	-0.06	-0.15	-0.07	0.12	-0.08	0.02	-0.17	0.11	-0.05	0.19	0.04	0.01	-0.08	-0.24	-0.08	-0.02
Butyrivibrio	0.00	-0.06	-0.35	-0.08	0.04	-0.42	0.07	0.09	1.00	0.09	0.03	0.14	0.66	0.10	-0.28	0.20
Coprococcus	-0.09	-0.10	0.15	0.13	-0.25	0.24	-0.32	-0.26	0.02	-0.11	0.03	0.11	-0.09	0.25	0.20	-0.14
Lachnospira	-0.25	0.05	0.10	-0.27	-0.03	-0.13	-0.02	-0.17	0.10	-0.30	0.42	0.40	0.03	0.24	-0.31	0.21
Pseudobut	0.32	0.25	-0.48	-0.08	0.23	-0.04	-0.14	0.23	0.57	0.42	-0.13	-0.07	0.58	-0.01	-0.05	-0.05
Shuttle	-0.15	0.11	0.20	-0.24	-0.01	0.06	-0.12	-0.17	-0.09	-0.25	0.48	0.62	-0.09	0.09	-0.24	0.08
Ruminococ_Un	0.36	0.26	-0.65	0.09	0.11	0.02	-0.29	0.68	0.09	1.00	-0.35	-0.15	0.44	-0.34	-0.03	-0.28
Selenomonas	-0.17	-0.10	0.22	-0.02	-0.20	0.08	-0.12	-0.08	-0.02	-0.10	-0.08	-0.01	-0.10	0.43	0.00	-0.12
Succinicl	-0.25	-0.15	0.34	0.05	-0.04	-0.05	-0.17	-0.18	0.10	-0.24	-0.06	-0.08	-0.16	0.48	0.05	-0.12
Erysipelot_Un	-0.20	0.04	0.14	-0.18	-0.06	-0.15	-0.01	-0.27	0.03	-0.35	1.00	0.60	-0.05	0.02	-0.25	0.34
Bulleidia	-0.10	-0.17	-0.15	-0.24	-0.17	-0.25	0.26	-0.06	0.20	-0.28	0.34	0.09	0.01	0.03	-0.26	1.00
Sharpea	-0.10	-0.06	0.07	-0.16	-0.22	-0.09	-0.02	-0.17	0.14	-0.15	0.60	1.00	0.02	-0.05	-0.17	0.09
Sphingomonas	0.16	0.21	-0.52	-0.04	0.34	-0.16	0.00	0.29	0.66	0.44	-0.05	0.02	1.00	-0.06	-0.12	0.01
Desulfobulbus	0.08	-0.12	-0.34	0.06	0.06	-0.30	0.05	0.05	0.60	0.07	-0.05	-0.08	0.37	0.01	-0.15	0.44
Desulfovibrio	0.29	-0.15	-0.19	-0.12	-0.29	-0.12	-0.08	0.03	0.39	0.20	-0.07	-0.03	0.12	0.00	-0.20	0.09
Succinivi_Un	-0.27	-0.22	0.13	0.14	-0.08	-0.05	-0.07	-0.23	0.10	-0.34	0.02	-0.05	-0.06	1.00	0.08	0.03
Pseudomonas	0.00	-0.04	-0.07	0.11	-0.21	0.05	0.00	-0.13	0.07	0.09	0.24	0.32	-0.01	0.05	-0.21	0.03
Treponema	0.07	0.17	-0.38	0.21	0.24	0.59	-0.25	-0.03	-0.26	0.36	-0.16	-0.22	0.05	-0.05	0.36	-0.39
Anaeropl_Un	-0.11	-0.12	0.22	-0.13	-0.13	0.12	0.35	-0.22	0.02	-0.30	0.21	0.21	-0.09	0.23	0.05	0.09

Figure 1. Correlation coefficients among families and genera of bacteria. Colors indicate positive (blue) or negative (red) correlation. Methanobr = *Methanobrevibacter* spp., Methanosp = *Methanosphaera* spp., Bifidobac = *Bifidobacterium*, Bacteroid = *Bacteroidales*; Prevot = *Prevotella* spp., Paraprev = *Paraprevotellaceae* spp., Cyanobac = *Cyanobacteria*, Fibrobact = *Fibrobacter* spp., Lactobac = *Lactobacillus*, Clostrid = *Clostridium* spp., Christen = *Christensenella*, Lachno = *Lachnospira*, Pseudobut = *Pseudobutyrivibrio* spp., Shuttle = *Shuttleworthia* spp., Ruminococ = *Ruminicoccus*, Succinicl = *Succiniclasticum* spp., Erysipelot = *Erysipelothrix*, Sphingom = *Sphingomonas* spp., Succinivi = *Succinivibrio*, Anaeropl = *Anaeroplasma*. Where a genus or family is listed singly multiple species were considered; where they are followed by an underscore, the designation after the underscore is the subspecies.

Streptococcaceae are more acid tolerant than other bacteria. *Streptococcus bovis* represents the main lactic acid producer in the rumen and grows faster when there are readily fermentable carbohydrates, and it is associated with rumen acidosis development during a high-grain diet. Cellulolytic bacteria are negatively affected by low pH: *Ruminococcaceae* and *Fibrobacteraceae* families decreased their concentration in milk fat–depressed cows, and this condition triggers the decrease of acetate synthesis (Hua et al., 2017).

Prevotellaceae family represents the most abundant family in the rumen and, in our trial, decreased in hypomotility cows. The Pearson correlation coefficient was negative between *Prevotella* spp. (*Prevotellaceae* family) and *Ruminococcus* spp. (*Ruminococcaceae* family). Indeed, these bacteria belong to different phyla (*Bacteroidetes* and *Firmicutes*) that characterize the core microbiome, and they seem to compete for the same substrates. In fact, in the present study during the challenge *Ruminococcus* spp. concentration increased more in hypomotility animals than in milk fat–depressed ones. During the challenge, the different abundance of *Prevotella* spp. observed in hypomotility cows could be related to lower DMI, resulting in less available fermentable substrates and reduction of milk produced. *Prevotellaceae* family has a versatile metabolic capability, and uses a broad range of substrates including peptides, proteins, monosaccharides, and plant polysaccharides. Schären et al. (2018) estimated a negative correlation of some *Prevotella* spp. to milk production, fat yield, and feed efficiency. Another important genus is *Butyrivibrio* spp. that belongs to *Lachnospiraceae* family. The high abundance of this genus, and family as well, in MFD cows was probably due to the increase of lactate production: butyrate that is produced by *Butyrivibrio* spp. downregulated lactate accumulation, which had negative effects on the health of the rumen. Butyrate is important for the growth and health of rumen epithelium, and a certain degree of its production is useful.

Fibrobacter spp. is one of the most important cellulolytic bacteria that produce substrates for metabolic activity of other bacteria such as *Treponema* spp. Consistent with the literature (Xie et al., 2018), the aforementioned bacteria species abundances were positively correlated. On the other hand, the correlation between *Succinivibrio* spp. and *Selenomonas* spp., as observed in the present study, was negative because they competed for the same substrates. Indeed, milk fat–depressed cows had a higher abundance of *Selenomonas* spp. and a low concentration of *Succinivibrio* genus. The metabolic activity of *Selenomonas* spp. occurred before *Succinivibrio* spp. and the availability of soluble sugar in MFD animals' rumen increased the activity of its bacterium and decreased substrates for the *Succinivibrio* genus.

Methanogens

An important observation was the increase of the *Methanobacteriaceae* family in hypomotility cows. The greater concentration of archaea could be associated with the decreased productivity of these animals. Moreover, an increased amount of methane emission is a main concern for farmers, not only because it stands as a loss of energy, but also because of the negative impact of greenhouse gases on the environment. Also, methanogens had a negative correlation with *Succinivibrio* spp., as demonstrated in Liu et al. (2021). Methanogens were positively correlated with the *Christenellaceae* family and *Fibrobacter* spp. because they produced substrates for methane production (formate). The positive correlations observed in Figure 1 would suggest cross feeding mechanisms occurring among different bacteria (Williams et al., 1991, 1994). Some of these patterns have been described, in in vitro studies, and considered as mutualistic relationships. However, these processes are not well characterized, and such relationships remain unknown for the majority of rumen bacteria.

CONCLUSIONS

As expected, the type of diet administered affected rumen microbial composition. The challenge diet grouped cows in 2 clusters: one characterized by RH, the other characterized by MFD. The increase of soluble sugars in MFD cows favored a greater concentration of *Megasphaera elsdenii*, a lactate fermenter, and an increase of *Butyrivibrio* spp., which decreased lactate concentration due to its butyrate production. A low ruminal pH brought a decrease in cellulolytic bacteria concentrations and an increase in lactic acid–utilizing microorganisms, such as *Lactobacillaceae*, and acidosis could occur. In contrast, the decrease of productivity of RH cows could be related to the increase of methanogens, which produce methane that represents a loss of energy for the cows' production. Other studies are necessary to improve the knowledge about the association between methanogens and hypomotility cows and the role of the *Prevotella* genus when this condition occurs to better understand the ability of different microorganisms in preventing paraphysiological situations (such as MFD and RH) for the animal.

NOTES

This study received no external funding. The animal study protocol was approved by the Ethics Committee of University of Bologna (protocol code 762, December 15, 2016). The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: ASV = amplicon sequence variant; MFD = milk fat depression; RH = ruminal hypomotility; T0 = rumen contents sampled 2 wk before administration of the acidogenic diet; T3 = sampling the first day of administration; T14 = sampling 2 wk after administration; T28; sampling 4 wk after administration.

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