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Effect of Haylage and Monensin Supplementation on Ruminal Bacterial Communities of Feedlot Cattle

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Abstract The objective of this study was to investigate the ruminal bacterial communities as affected by monensin, haylage, and their interaction of feedlot cattle fed 60 % dried distillers grains with solubles in a replicated 4 × 4 Latin square design. Pyrosequencing analysis of the V1–V3 region (about 500 bp) of 16S rRNA gene from the four dietary treatments (3 treatment plus one control diets) collectively revealed 51 genera of bacteria within 11 phyla. *Firmicutes* and *Bacteroidetes* were the first and the second most predominant phyla, respectively, irrespective of the dietary treatments. Monensin supplementation decreased the proportion of Gram-positive *Firmicutes* while increasing that of Gram-negative *Bacteroidetes*. However, the monensin supplementation did not reduce the proportion of all genera of Gram-positive bacteria placed within *Firmicutes* and lowered that of some genera of Gram-negative bacteria placed within *Bacteroidetes*. Haylage supplementation appeared to attenuate inhibition of monensin on some genera of bacteria. Factors other than monensin and haylage could affect ruminal bacterial communities.

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Introduction

Monensin is an ionophore commonly fed to cattle because it decreases methane production [12, 24], increases propionate production [3], and improves feed utilization efficiency [3, 20]. Studies using pure cultures of bacteria suggested that these effects are attributed to modulation of ruminal bacterial communities through selective inhibition of Gram-positive bacteria [3]. However, some in vivo studies based on 16S rRNA genes [21, 25] did not show much effect of monensin on ruminal bacterial communities. Massively parallel deep sequencing provides opportunities to evaluate how monensin affects ruminal bacterial communities in a comprehensive manner.

Dried distillers grains with solubles (DDGS), the main byproducts of bioethanol production, are commonly used as a substitute for grain for feedlot cattle. However, the high levels of sulfate or sulfuric acid present in DDGS limit the proportion of DDGS in the diet because of increased risk of rumen acidosis and polioencephalomalacia (PEM) that results from H_2S production in the rumen [8, 9]. Strategies have been sought to reduce these risks. Felix and Loerch [8] recently showed that haylage supplementation can raise pH, while monensin can significantly reduce H_2S production in the rumen of feedlot cattle fed a diet contain 60 % DDGS. However, monensin was found to have mixed effect on H_2S production by rumen microbiome in vitro [16, 18]. The objective of this study was to investigate the ruminal bacterial communities as affected by monensin, haylage, and their interaction of feedlot cattle fed 60 % DDGS.

Materials and Methods

Experimental Design

The experimental design and the feeding have been reported previously [8]. Briefly, eight ruminally cannulated steers (BW = 347 ± 29 kg) were randomly allotted to one of the four dietary treatments in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: (1) C = control diet containing no monensin or haylage, (2) M = the control diet supplemented with 33 mg monensin/kg feed (dry matter, DM), (3) H = the control diet supplemented with 10 % haylage (DM), and (4) MH = the control diet containing 33 mg monensin/kg and 10 % haylage. The rest of the diet consisted of 10 % corn silage, 60 % DDGS, corn (5 for H and MH groups or 15 % for C and M groups), and 15 % supplement. The cattle were fed ad libitum. Rumen content samples were collected at 0, 3, and 6 post-feeding and then composited into one sample.

DNA Extraction and Pyrosequencing

Metagenomic DNA was extracted from each rumen content sample using the repeated bead-beating and column purification method as described previously [26]. The quality of the DNA was checked using agarose gel (0.8 %) electrophoresis, while the concentration was quantified using a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop products, Wilmington, DE). The DNA extracts were pooled based on diets, resulting in four composite metagenomic DNA samples (from eight cows on each diet) representing each of the four dietary treatments (C, H, M, and MH).

One amplicon library was prepared from each composite DNA using 454BactF (5′-AKRGTTYGATYNTGGCTCAG-3′) and 454BactR (5′-GTNTBACCGCDGCTGCTG-3′) primers that were modified to increase their inclusiveness compared to the original 27F and 519R primers and to include unique barcodes and the adapters needed for pyrosequencing on the 454 GS FLX Titanium system [17]. After the V1–V3 hypervariable region of the bacterial 16S rRNA gene was amplified from each composite DNA sample using the above primers, each amplicon library was diluted with the Elution buffer of the QIAquick Gel Extraction Kit to a concentration of 20 ng/μl and pooled by combining an equal volume from each amplicon library. This pool was sequenced at the Plant–Microbe Genomics Facility at The Ohio State University using a 454 GS FLX Titanium system.

The four composite metagenomic DNA samples were also sent to the Research and Testing Laboratories (Lubbock, TX), where amplicon libraries (spanning the V1–V3 region of bacterial rRNA gene) were prepared using the Gray28F (5′-GAGTTTGATCNTGGCTCAG-3′) and Gray519R (5′-GTNTTACNGCGGCKGCTG-3′) primers and then sequenced similarly using a 454 GS FLX Titanium system [15].

Bioinformatic Analysis

The programs in the QIIME software package 1.5.0 [4] were used to process and analyze the sequences. First, sequences obtained from each of the two sequencing facilities were subjected to quality screening; and sequences with a length <200 bases, a mean quality score <Q25, or a homopolymer stretches >8 bases were removed. Second, the sequences that met the above quality criteria were subjected to the default denoising process [19] implemented in QIIME to identify and remove erroneous sequences. Third, possible chimeric sequences were identified and removed using the UCHIME program [7]. Fourth, the quality-checked sequences obtained were normalized based on the smallest number of sequences among the samples. Finally, the sequences were assigned to phylum, class, order, family, and genus using the RDP naïve Bayesian rRNA Classifier [23]. Species-level OTUs were calculated at 0.03 % dissimilarity using the uclust method [6].

Diversity indices, including the number of observed OTUs, Chao1, PD_whole_tree, and Shannon-Wiener diversity index, were calculated using QIIME [4]. Principal Coordinates Analysis (PCoA) based on unweighted UniFrac was conducted using QIIME. Canonical Correspondence Analysis (CCA) was used to examine possible associations between OTUs and environmental variables of the rumen including H_2S and pH using the vegan package (http://r-forge.r-project.org/projects/vegan/). The average values of H_2S and pH at 0, 3, and 6 h post-feeding [8] were each used in CCA. Since no significant difference in ruminal VFA concentrations was noted among the diet groups, CCA analysis was not done on ruminal VFA.

Results

Data Summary

A total of 30,464 quality-checked sequences were obtained, with a normalized 7,616 sequences representing each of the four dietary treatments. Collectively, these sequences were classified to 11 phyla, 15 classes, 17 orders, 26 families, and 51 genera of bacteria, and more than 1 % of these sequences could not be assigned to any known phylum. *Firmicutes* was the most predominant phylum accounting for about 50–58 % of total sequences,

Table 1 Major taxa detected in the four dietary groups

Rank	Taxon	Relative abundance (%)			
		C	H	M	MH
Phylum	*Firmicutes*	55.4	57.8	49.8	49.7
	Bacteroidetes	37.7	34.0	41.6	41.3
	Proteobacteria	3.1	4.4	4.3	5.0
	Actinobacteria	2.1	1.5	2.8	2.3
Genus	*Prevotella*	22.9	20.8	28.8	22.6
	Dialister	7.2	4.5	9.9	4.3
	Anaerobiospirillum	2.4	2.1	3.5	4.0
	Syntrophococcus	3.0	2.2	2.8	1.3
	Olsenella	1.7	1.2	2.2	1.6
	Mitsuokella	0.7	1.6	1.2	1.4
	Succiniclasticum	1.6	1.6	0.5	1.2
	Butyrivibrio	0.8	2.1	0.6	1.1
	Selenomonas	1.4	0.6	1.8	0.5
	Megasphaera	0.5	1.0	0.9	1.4
	Sharpea	1.1	0.9	0.4	1.0
	Oscillibacter	0.8	0.5	0.4	0.7
	Succinivibrio	0.1	1.5	0.1	0.2
	Ruminococcus	0.4	0.8	0.1	0.5
	Acidaminococcus	0.4	0.5	0.3	0.3
Unclassified group[a]	*Lachnospiraceae*	23.1	27.1	17.8	20.0
	Prevotellaceae	12.9	9.7	11.1	16.6
	Ruminococcaceae	7.1	5.7	3.5	6.3
	Veillonellaceae	2.5	3.8	6.0	5.8
	Clostridiales	2.2	2.7	2.0	1.6
	Bacteria	1.4	2.2	1.3	1.5
	Erysipelotrichaceae	1.6	1.0	1.1	0.9
	Bacteroidetes	1.1	1.6	0.8	0.9
	Bacteroidales	0.7	1.7	0.9	1.1
	Coriobacteriaceae	0.4	0.2	0.5	0.6

Taxa accounted for ≥0.5 % of total sequences each in at least one dietary group

[a] Unclassified groups that could not be assigned to a known genus and that were represented by >0.5 % of total sequences each in at least one dietary treatment

while *Bacteroidetes* was the second most predominant phylum accounting for about 34–42 % of total sequences (Table 1). *Proteobacteria* and *Actinobacteria* were the third and the fourth largest phyla and accounted for about 3–5 and 1.5–2.8 % of total sequences, respectively.

Prevotella was the most predominant genus and accounted for >20 % of total sequences in all the four diet groups, followed by *Dialister*, *Anaerobiospirillum*, *Syntrophococcus,* and *Olsenella* (Table 1). Another 10 genera were each represented by ≥0.5 % of total sequences in at least one diet group, and they were regarded as "major genera." In addition, a large portion of the sequences could not be classified to a known genus. Unclassified *Lachnospiraceae* and unclassified *Prevotellaceae* were the first and the second most abundant among unclassified groups, respectively. In total, 2,549 species-level (0.03 sequence dissimilarity) OTUs were identified across the four dietary groups, but about 65 % of these OTUs were singletons. *Firmicutes* and *Bacteroidetes* were represented by about 58 and 32 % of all the OTUs, respectively. Seventy OTUs were each represented by ≥0.5 % of total sequences in at least one dietary group and regarded as predominant OTUs (Suppl Table 1).

Effect of Monensin on Bacterial Communities

The proportion of Gram-positive phylum *Firmicutes* was decreased in the M diet group compared to the C diet group, whereas that of Gram-negative phylum *Bacteroidetes* was increased (Table 1). This trend is consistent with that shown in a previous in vitro study [3]; however, the magnitude of decrease in *Firmicutes* and increase in *Bacteroidetes* was small. The proportion of Gram-positive phylum *Actinobacteria* was slightly increased by monensin. Of the known major genera, *Succiniclasticum* (a Gram-negative genus), *Ruminococcus* (a Gram-positive genus), *Sharpea* (a Gram-positive genus), *Oscillibacter* (a Gram-negative genus), and some sequences unclassifiable within the family *Ruminococcaceae* were at least twofold less abundant in the M diet group than in the C diet group. On the contrary, some sequences unclassifiable within the family *Veillonellaceae* were more than twofold abundant in the M diet group than in the C diet group.

Five species-level OTUs had a relative abundance >3-fold greater in the M than in the C diet groups (Suppl Table 1). These included OTUs classified to Gram-negative taxa (e.g., *Prevotella*) and Gram-positive taxa (e.g., *Lachnospiraceae* and *Veillonellaceae*). On the other hand, 10 OTUs had lower relative abundance in the M than in the C diet groups. Again, these included OTUs classified to both Gram-positive taxa (e.g., *Ruminococcaceae*, *Succiniclasticum*, *Syntrophococcus*, *Sharpea*, and *Lachnospiraceae*) and Gram-negative taxa (e.g., *Prevotella*). These 10 OTUs might have been inhibited by monensin. The stimulatory and inhibitory effects varied among these OTUs.

Effects of Haylage on Bacterial Communities

The proportion of *Firmicutes* was slightly increased in the H diet group compared to the C diet group, whereas the opposite held true for that of *Bacteroidetes* (Table 1). Among the major genera, several genera had a relative abundance at least twofold greater in the H diet group than in the C diet group: *Succinivibrio*, *Butyrivibrio*, *Ruminococcus*, *Megasphaera*, and *Mitsuokella*. On the other hand, *Selenomonas* had a lower relative abundance in the H than

in the C diet groups. Among groups that could not be classified to a known genus, only unclassified *Bacteroidales* and unclassified *Coriobacteriaceae* differed in relative abundance between the H and the C diet groups, with the former having a greater relative abundance in the H diet group, while the latter having a greater relative abundance in the C diet group.

Several OTUs (e.g., OTU-1949, OTU-2145, and OTU-2300), which were assigned to *Bacteroidales*, *Succinivibrio,* and *Prevotellaceae*, respectively, were represented by ≥0.5 % of total sequences in the H diet group but not detected in the C diet group (Suppl Table 1). Besides, OTU-2281 and OTU-2443 (both assigned to *Succinivibrio*), and OTU-2424 (*Mitsuokella*) had a relative abundance ≥3-fold greater in the H than in the C diet groups. The two *Succinivibrio* OTUs were particularly abundant in the H diet group. In addition, the relative abundance of OTU-1949, OTU-2145, OTU-2281, and OTU-2443 was greater in the H diet group, but not in the MH diet group, reflecting probable stimulation of these OTUs by haylage but inhibition by monensin. On the contrary, OTU-2300 had a greater relative abundance in both the H and the MH diet groups than in the other two diet groups, indicating stimulation by haylage irrespective of monensin. Some OTUs had a greater relative abundance in the C than in the H diet group. These included OTUs assigned to *Prevotella* (OTU-2451 and OTU-2515), *Selenomonas* (OTU-2541), *Prevotellaceae* (OTU-2441 and OTU-2460), and *Erysipelotrichaceae* (OTU-2529). These differential OTUs might be involved in degradation of grain rather than plant cell wall materials. In addition, the relative abundance of OTU-2460 was greater in the C than in the other three diet groups, suggesting inhibition by either haylage or monensin. However, OTU-2451, OTU-2515, OTU-2541, OTU-2441, and OTU-2529 had a greater relative abundance in both the C and the M diet groups than in the other diet groups receiving haylage, suggesting that these OTUs might be inhibited by haylage supplementation.

Effects of Both Haylage and Monensin on Bacterial Communities

The proportions of individual phyla in the MH diet group were similar to those in the M diet group but were slightly different from those in the C and H groups, suggesting a main effect of monensin on bacterial communities (Table 1). Of the major genera, only *Megasphaera* and *Succinivibrio* were relatively more abundant (<3-fold) in the MH diet group than in the C diet group, whereas *Selenomonas* and *Syntrophococcus* exhibited opposite trend. The proportions of Gram-negative *Succiniclasticum* and *Oscillibacter*, and Gram-positive *Sharpea* and *Ruminococcus*, which were all inhibited in the M diet group, were similar between the MH and the C diet groups. In addition, none of the major genera differed in relative abundance by ≥2-fold between the MH and the H diet groups. To some extent, the haylage supplementation alleviated the effect of monensin on bacterial communities. Between the M and the MH diet groups, *Dialister* and *Selenomonas* were >2-fold more abundant in the M diet group than in the MH diet group, whereas the opposite held true for *Succiniclasticum*, *Sharpea*, and *Ruminococcus*. Among the unclassified taxa, only unclassified *Veillonellaceae* had a greater (2.4-fold) relative abundance in the MH group than in the C group.

Ten OTUs had a relative abundance >3-fold greater in the MH diet group than in the C diet group (Suppl Table 1). These included OTUs assigned to *Prevotella* (OTU-2498 and OTU-2444), *Sharpea* (OTU-2539), *Megasphaera* (OTU-2359), *Prevotellaceae* (OTU-1951, OTU-2386, OTU-2402 and OTU-2466), and *Lachnospiraceae* (OTU-2472 and OTU-2457). The relative abundance of OTU-2444, OTU-2359, OTU-1951, OTU-2386, OTU-2402, OTU-2466, and OTU-2472 was greater in the MH diet group than in the other three diet groups, indicating possible additive effect of both haylage and monensin. OTU-2457 had a greater relative abundance in the MH and the M diet groups, while OTU-2498 was more abundant in the MH and H diet groups than in the other two diet groups. The relative abundance of OTU-2359 was smaller in the C diet group than in the other three diet groups. On the contrary, four OTUs (OTU-2340 and OTU-2458 assigned to *Prevotella*, OTU-2540 of *Prevotellaceae*, and OTU-2264 of *Lachnospiraceae*) were less abundant in the MH diet group than in the C diet group. These OTUs, however, had different relative abundance in the other diet groups. Specifically, the relative abundance of OTU-2340, OTU-2540, and OTU-2264 was greater in the C diet group than in the other three diet groups, while OTU-2458 was more abundant in both the C and the M diet groups than in the other two diet groups supplemented with haylage.

Diversity Statistics

The H diet group had the greatest OTU richness, Chao1estimate, PD_whole_tree distance, and Shannon diversity index among the four diet groups (Table 2). These results indicate that the bacterial communities in the H diet group are more diverse than in the other three diet groups. The opposite held true for the M diet group, probably reflecting inhibition of some bacteria by monensin and attenuation of inhibition by the haylage. On the unweighted PCoA plot, PC1 separated the diet groups supplemented with haylage (H and MH) from those without haylage (C and M), while PC2 separated the diet groups

Table 2 Diversity indices

Dietary group	# of observed OTUs	Chao1	PD_whole_tree	Shannon
C	856	2,033	54.60	7.21
M	733	2,185	44.24	6.92
H	1,218	3,399	73.26	8.02
MH	993	2,862	60.58	7.47

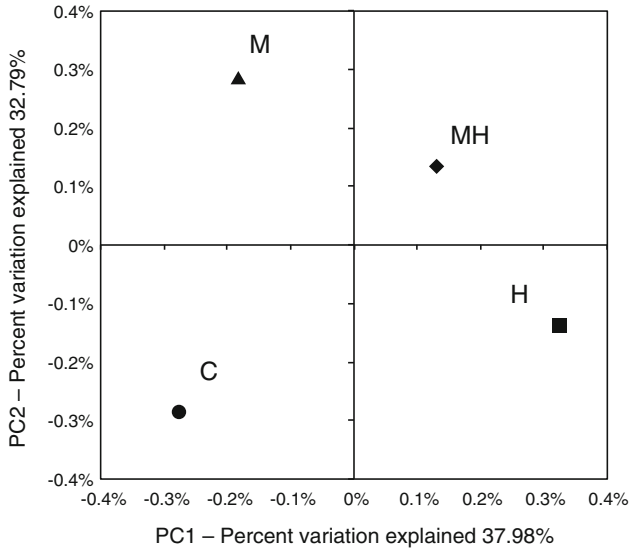

Fig. 1 Unweighted UniFrac Principal Coordinates Analysis (PCoA) showing correlations among the four dietary groups. PC1 separated bacterial communities based on haylage supplementation, while PC2 separated bacterial communities based on monensin supplementation. *C* control diet, *H* control diet supplemented with haylage, *M* control diet supplemented with monensin, and *MH* control diet supplemented with both haylage and monensin

that received monensin (M and MH) from those that did not receive monensin (C and H; Fig. 1).

Canonical Correspondence Analysis

Thirteen OTUs were positively associated with the concentration of H_2S (Fig. 2), and they were assigned to *Prevotella* (OTU-2340, OTU-2362 and OTU-2493), *Syntrophococcus* (OTU-2403), *Mitsuokella* (OTU-1901), unclassified *Prevotellaceae* (OTU-2460), unclassified *Erysipelotrichaceae* (OTU-2529), unclassified *Clostridiales* (OTU 2348 and OTU-2449), and unclassified *Lachnospiraceae* (OTUs-2264, OTU-2425, OTU-2440, and OTU-2500; Suppl Table 1). Of these OTUs, OTU-1901, OTU-2348, OTU-2362, OTU-2425, OTU-2449, and OTU-2493 were minor OTUs accounting for <0.5 % of total sequences (data not shown). Another 14 OTUs were inversely associated with the concentration of H_2S, and they were assigned to *Prevotella* (OTU-2115, OTU-2169, OTU-2385, OTU-2398, OTU-2473, OTU-2476, and OTU-2498), *Mitsuokella* (OTU-2395 and OTU-2424), *Megasphaera* (OTU-2359), *Succiniclasticum* (OTU-2448), unclassified *Veillonellaceae* (OTU-2365), and unclassified *Prevotellaceae* (OTU-2300 and OTU-2360; Suppl Table 1). Some of these OTUs (OTU-2115, OTU-2169, OTU-2360, OTU-2365, OTU-2395, OTU-2398, OTU-2424, and OTU-2448) were minor OTUs accounting for <0.5 % of total sequences (data not shown). The OTUs that were positively associated with H_2S concentration were inversely associated with the values of rumen pH.

Discussion

The species richness and Shannon diversity index were reduced in the M diet group (Table 2), suggesting that monensin supplementation inhibits some bacteria. However, although the abundance of *Firmicutes*—the major Gram-positive phylum in the rumen—was reduced, that of *Actinobacteria*—another Gram-positive phylum—was not. Because small variations in proportion of major taxa can cause large variations in that minor taxa [15], it is likely that the abundance of *Actinobacteria* was reduced by the monensin supplementation but variations in relative abundance of the major phyla (e.g., *Firmicutes* and *Bacteroidetes*) rendered the changes in *Actinobacteria* proportion not significant. Pure cultures of a few *Firmicutes* species have been shown to be sensitive to monensin [3], but no study has been reported that examined the effect of monensin on species of *Actinobacteria*. Future studies using pure cultures and direct quantification of *Actinobacteria* are needed to further assess the effect of monensin on *Actinobacteria*. Of particular interest were some genera of *Firmicutes* (e.g., *Ruminococcus* and *Sharpea*) that were reduced, whereas others (e.g., *Dialister* and *Butyrivibrio*) were not. A group of *Veillonellaceae* (a Gram-positive family) was even increased in the M diet group. On the other hand, the relative abundance of several Gram-negative genera (e.g., *Succiniclasticum* and *Oscillibacter*) was reduced by >2-fold in the M diet group. The above mixed effects of monensin on Gram-positive and Gram-negative bacteria were also observed for many OTUs. Thus, the conclusion drawn from studies, using pure cultures that monensin inhibits Gram-positive bacteria but not Gram-negative bacteria, does not apply to all bacteria. The results also suggest that monensin decreases bacterial diversity and species richness by inhibiting numerous minor species rather than Gram-positive bacteria in general. Interestingly, the species richness and Shannon diversity index were greater in the MH diet group than in the M diet group but smaller than in the H diet group, suggesting interaction

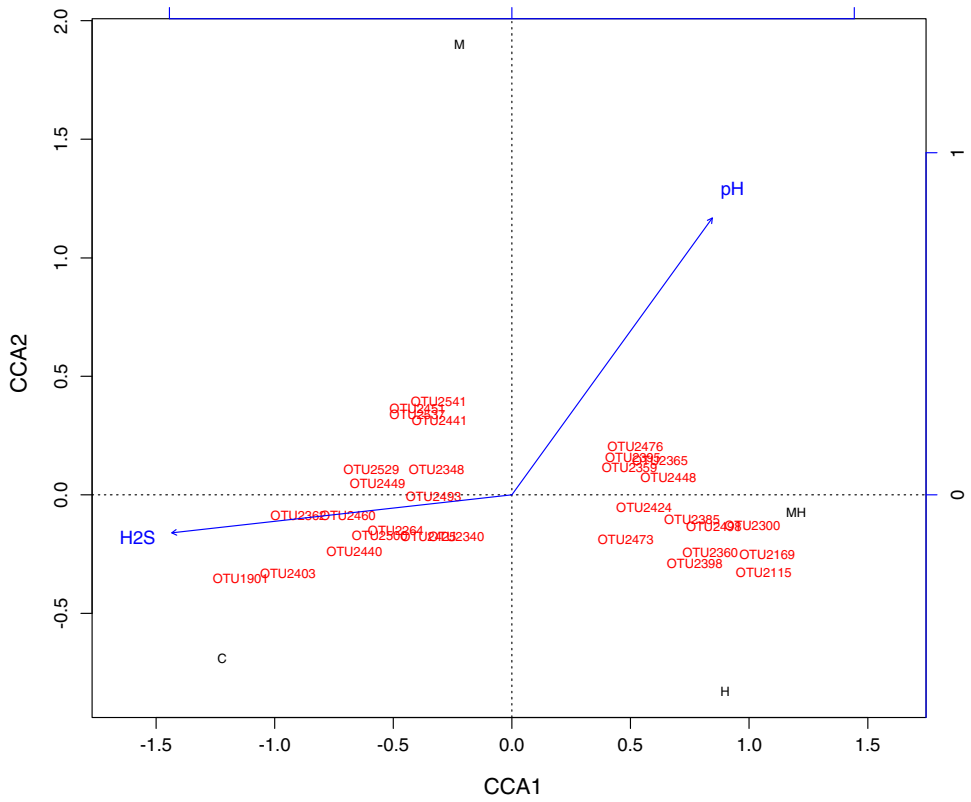

Fig. 2 Canonical Correspondence Analysis showing the relationship between OTU abundance and the concentration of H_2S, and pH values in the rumen. *C* control diet, *H* control diet supplemented with haylage, *M* control diet supplemented with monensin; and *MH* control diet supplemented with both haylage and monensin

between monensin and haylage in affecting rumen microbial community. The interaction was supported by recovery of some taxa and OTUs by the haylage supplementation, which were inhibited by monensin supplementation (Tables 1 and 2).

Unclassified *Lachnospiraceae*, unclassified *Ruminococcaceae,* and unclassified *Clostridiales* of rumen origin were the largest groups of novel bacteria in the global diversity dataset [14] and in several other studies [11, 13, 22, 27]. In the present study, unclassified *Lachnospiraceae*, unclassified *Prevotellaceae*, and unclassified *Ruminococcaceae* contained the first, second, and third largest numbers of sequences that could not be assigned to any known genus, respectively. The inclusion of high levels of DDGS, which is primarily composed of protein, fat, crude fiber, and starch, might have increased novel species of the family *Prevotellaceae* containing xylanolytic, amylolytic, and proteolytic bacteria, while reducing species of *Clostridiales* that are cellulolytic.

Two issues can hinder inclusion of high level of DDGS in diets fed to ruminants: acidosis and PEM. Acidosis was attributed to the sulfuric acid contained in the DDGS, while PEM is caused by inhaling of H_2S. Most DDGS contains high levels of proteins and sulfuric acid, both of which can be source of H_2S production via fermentation of sulfur-containing amino acids (i.e., methionine and cysteine) and sulfate reduction, respectively. As of 2009, 220 species within 60 genera were reported as sulfate-reducing bacteria (SRB) [1], with 23 genera being in the class *Deltaproteobacteria* and three genera (*Desulfotomaculum*, *Desulfosporomusa* and *Desulfosporosinus*) within the phylum *Firmicutes*. Although *Desulfovibrio desulfuricans* and *Desulfotomaculum ruminis* were isolated in the rumen [5, 10], few studies on ruminal SRB have been performed. In the present study, 13 OTUs were positively associated with H_2S concentration in the rumen (Fig. 2), but none of them could be assigned to any of the known genera of SRB. Two of the 13 OTUs were assigned to *Prevotella*. *Prevotella* is the most predominant among known genera of rumen origin, but numerous *Prevotella* strains have not been isolated [2]. The present study indicates that some uncultured *Prevotella* strains might be associated with sulfate reduction. Further studies will need to be performed to verify this assumption. In the present study, eight minor OTUs were assigned to the family *Desulfovibrionaceae*. However, it was difficult to assess if and to what extent these minor OTUs have contributed to sulfate reduction in the rumen. Targeted analysis for SRB may help further determine the effect of monensin and DDGS on SRB.

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