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ORIGINAL ARTICLE

The effect of regular or reduced-fat distillers grains with solubles on rumen methanogenesis and the rumen bacterial community

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Abstract

Aims: The effect of feeding dried distillers grains with solubles (DDGS) or reduced-fat DDGS (RFDG) on ruminal methanogenesis and the rumen bacterial community of dairy cattle was evaluated.

Methods and Results: Treatments were CONT, a diet with no distillers grains; DG, inclusion of 20% DDGS; rFDG, inclusion of 20% RFDG; and MIX, inclusion of 10% DDGS and 10% RFDG. Methane emission was measured; rumen bacterial community was evaluated by sequencing the V4 region of the 16S rRNA gene. Total methane production remained unaffected. However, feeding distillers grains tended to reduce methanogenesis per unit of feed intake, decreased the abundance of the phylum *Bacteroidetes* and tended to increase *Firmicutes*. The abundance of *Prevotellaceae* positively correlated with feed intake; methane emission was positively correlated with the abundance of *Prevotellaceae* and was negatively correlated with the abundance of *Succinivibrionaceae*.

Conclusions: DDGS or RFDG may reduce methanogenesis per unit of feed intake; shifts in the abundance of predominant ruminal bacterial families may influence methane formation, likely because of their role on hydrogen liberation and utilization pathways.

Significance and Impact of the Study: Replacing corn and soybean meal with DDGS or RFDG in dairy rations may reduce the proportion of dietary energy wasted as methane, without detrimental effects on the overall bacterial population.

Introduction

The supply of corn milling by-products such as dried distillers grains with solubles (DDGS, a feedstuff that contains approx. 12% fat) from the ethanol industry has increased (Klopfenstein *et al.* 2008). More recently, the corn milling industry is partially removing the oil from the solubles stream by centrifugation and solvent extraction (Majoni *et al.* 2011) and the resulting by-product is commonly referred to as reduced-fat DDGS (RFDG) (Mjoun *et al.* 2010a) that contains approx. 5.5% fat. Both DDGS and RFDG have been shown to effectively support

lactation performance (Janicek *et al.* 2008; Mjoun *et al.* 2010b). Studies have also suggested that feeding DDGS may reduce methane production in ruminants possibly due to toxic effects of dietary fat on micro-organisms contributing to methane production (Hünerberg *et al.* 2013). Unfortunately, studies including the evaluation of these by-products on methane emission and the rumen microbiome using high-throughput DNA sequencing are limited. Thus, this research gap remains largely unknown; however, it can now be addressed using sequencing and bioinformatic analysis of the 16S rRNA gene to evaluate the rumen microbial community (Krause *et al.* 2013;

Chaucheyras-Durand and Ossa 2014), which provides new insights into variations occurring at each taxonomical level of the microbial population of the rumen due to dietary manipulations (Aldai *et al.* 2012; Thoetkiattikul *et al.* 2013; Castillo-Lopez *et al.* 2014a).

The ruminal microbial population plays a special role on production performance (Myer *et al.* 2015), fermentation end-products (Fernando *et al.* 2010; Anderson *et al.* 2016), ruminal pH and the extent of ruminal digestibility and fermentation efficiency (Mackie and Gilchrist 1979; Callaway *et al.* 2010), metabolizable protein supply (Castillo-Lopez *et al.* 2013) and milk composition in dairy cattle (Jami *et al.* 2014). Nonetheless, ruminal microorganisms contribute to methane formation. Methane emissions of enteric origin have nutritional and environmental impacts (Moss *et al.* 2000). For example, methane possesses strong greenhouse effects (Hünerberg *et al.* 2013), and may represent between 5 to 7% of dietary energy loss in the ruminant (Hristov *et al.* 2013).

Ruminal methane formation requires carbon dioxide and hydrogen (Danielsson *et al.* 2017). Thus, dietary approaches designed to mitigate ruminal methanogenesis can be evaluated not only in terms of their efficacy for inhibiting methanogens, belonging to the archaeal population, but also their effects on the broad bacterial population; especially major bacterial taxa that are associated with hydrogen liberation and utilization pathways. Therefore, improving our understanding not only on the efficacy of methods to decrease methane emissions, but also on potential detrimental effects on nutrient digestion and animal production performance, where bacteria play a crucial role. Many dietary intervention strategies have been explored to mitigate methane emissions, which include the use of inhibitors, electron acceptors, ionophores, inclusion of grain over fibrous feed, defaunation of protozoa (Hristov *et al.* 2013), inhibition of ruminal microorganisms with dietary tannins (Khiaosa-ard *et al.* 2015), or the use of 3-nitrooxypropanol, a compound designed to inhibit the activity of the enzyme responsible for formation of methane (Duval and Kindermann 2012). These strategies have resulted in various degrees of efficacy. However, further research is required to evaluate effects on the rumen bacterial community structure using high-throughput DNA sequencing (Danielsson *et al.* 2017), and to evaluate how shifts in the community composition may potentially be associated with methane emissions.

Therefore, the objective of this study was to evaluate the inclusion of DDGS and RFDG in diets fed to dairy cattle on enteric methane production and the ruminal bacterial community using Illumina DNA sequencing, and to evaluate potential associations between shifts in the bacterial community and methane emission. The

hypothesis of this experiment was that cows consuming diets with DDGS will produce less methane than those consuming diets with no DDGS or with RFDG because the inclusion of DDGS shifts the ruminal bacterial community structure, more specially affecting those microorganisms that directly or indirectly contribute to methane formation.

Materials and methods

This experiment was conducted at the Metabolism Research and Teaching facility of the Animal Science Department of University of Nebraska-Lincoln and was performed in accordance with Departmental guidelines. The protocols followed during this experiment were preapproved by the University of Nebraska-Lincoln Animal Care and Use Committee before the initiation of the study.

Experimental design and dietary treatments

Four multiparous, in mid-lactation Holstein cows (mean days in milk 93.5 ± 22.2 , mean body weight (BW) 657.3 ± 87.35 kg) were used in a 4×4 Latin square experimental design. These cows were fitted with permanent ruminal cannulae in order to facilitate sampling of representative whole ruminal digesta. At the initiation of the experiment, BW of cows was recorded. Each experimental period lasted 28 days to provide enough time for animal adaptation to treatments evaluated.

Treatments were formulated with the CPM-Dairy Model (ver. 3.0.8.1). Each total mixed ration was prepared using a small-batch mixing cart (Data Ranger, American Calan, Northwood, NH). Treatments (DM basis) were (i) CONT, a control diet that contained no distillers grains; (ii) DG, inclusion of 20% DDGS; (iii) rFDG, inclusion of 20% RFDG (Poet Nutrition, Sioux Falls, SD) and (iv) MIX, inclusion of 10% DDGS and 10% RFDG. In treatments DG, rFDG and MIX, distillers grains and solubles were included in replace of corn grain and soybean meal.

Animal care and housing

Cows were housed in individual tie-stalls with continuous access to water and feed. Wood shavings were used for bedding, and were replaced on a daily basis. Cows were individually fed once daily at 0930 to allow for approx. 5% refusals. A control concentrate mix, a DG-concentrate mix and an rFDG-concentrate mix were prepared. Individual feed intake was recorded daily during the experiment. Feed orts were collected and weighed daily to estimate intake for individual cows. In addition, cows

were milked at 0700 and 1800 using individual DEL-PRO™ 480 DeLaval portable milking units through a pipeline system (DeLaval International AB, Tumba, Sweden). Milk samples were taken on the AM and p.m. milkings of day 24 through 26 for analysis of chemical composition. Because this experiment was designed to evaluate methane emission and the ruminal bacterial community, data on milk production were recorded primarily to provide information on animal management and the physiological state of cows used.

Feed sampling and analysis

Samples of corn silage, alfalfa hay, brome hay, each concentrate mix, DDGS, RFDG and each TMR were collected on day 27 and 28 of each experimental period; these samples were then pooled within period and stored at -20°C until analysis. In addition, corn silage samples were collected twice a week for DM analysis. To do so, samples were dried in a forced-air oven for 48 h at 60°C . These results were then used for adjusting proper inclusion of ingredients in each treatment. Dried feed ingredients and orts were then ground to pass through a 1-mm screen (Willey mill, Arthur H. Thomas Co., Philadelphia, PA) and analysed for chemical composition by an external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD), which included dry matter (method no. 930-15; AOAC 2000), Nitrogen (method no. 990-03; Leco FP-528 Nitrogen Combustion Analyser, Leco Corp. St. Joseph, MI), neutral detergent fibre (Van Soest *et al.* 1991), starch (Hall 2009), crude fat using diethyl ether (method no. 2003-05; AOAC 2006) and ash (method no. 942-05; AOAC 2000). The nutrient composition of total mixed rations (Table 1) was calculated based on chemical analysis of individual ingredients (Table S1) and the rate of inclusion in the corresponding diet. This method of reporting chemical composition of dairy diets is highly recommended, because when sampling total mixed rations for analysis of chemical composition results may be affected by sampling variation (Weiss *et al.* 2016).

Measurement of methane emissions

Methane emissions were measured through indirect calorimetry using headboxes (Foth *et al.* 2015). Prior to collections, three headboxes were used to test the rate of recovery of gas by burning 100% ethyl alcohol in the sealed headbox and comparing this measure to calculated gas concentrations. These calculations were based on weight of alcohol burned and a measured volume of gas sample. Cows were acclimated to the headboxes before the initiation of the collection. Methane collections consisted of two consecutive, 23-h periods on day 27 and 28

Table 1 Ingredient and chemical composition of diets including conventional distillers grains with solubles or reduced-fat distillers grains with solubles fed to lactating Holstein cows. Chemical composition is based on analysis of individual ingredients and the rate of inclusion

Item	Treatments			
	CONT	DG	rFDG	MIX
Ingredient, % DM				
Corn silage	31.7	31.7	31.7	31.7
Corn grain	21.8	10.2	10.2	10.2
Alfalfa hay	13.0	13.0	13.0	13.0
Soybean meal	10.0	3.0	3.0	3.0
Soybean hulls	8.8	8.8	8.8	8.8
Grass hay	6.0	6.0	6.0	6.0
SoyPass*	4.7	4.0	4.0	4.0
Limestone	1.1	1.1	1.1	1.1
Blood meal	0.7	0.0	0.0	0.0
Sodium bicarbonate	0.6	0.6	0.6	0.6
Megalac	0.6	0.6	0.6	0.6
Dicalcium phosphate	0.6	0.6	0.6	0.6
Magnesium oxide	0.2	0.2	0.2	0.2
Salt	0.2	0.2	0.2	0.2
Trace premix†	0.1	0.1	0.1	0.1
Vitamin premix‡	0.1	0.1	0.1	0.1
DDGS	0.0	20.0	0.0	10.0
RFDG	0.0	0.0	20.0	10.0
Chemical composition, % DM				
Crude protein	16.6	17.1	17.1	17.1
Neutral detergent fibre	34.1	38.1	37.9	38.0
Starch	27.2	21.4	21.3	21.3
Nonfibre carbohydrates	38.6	33.1	33.4	33.6
Crude fat	2.7	3.9	3.3	3.6
Ash	8.0	7.7	8.3	8.0
Sulphur	0.20	0.25	0.30	0.27
Metabolizable energy§, Mcal kg ⁻¹	2.64	2.55	2.51	2.53

CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rFDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG (Poet nutrition, Sioux Falls City South Dakota).

*LignoTech, Overland Park, KS.

†Contained 13.9% Ca, 0.03% P, 0.42% Mg, 0.20% K, 4.20% S, 0.08% Na, 0.03% Cl, 445 mg kg⁻¹ Fe, 60,021 mg kg⁻¹ Zn, 17,375 mg kg⁻¹ Cu, 43,470 mg kg⁻¹ Mn, 287 mg kg⁻¹ Se, 527 mg kg⁻¹ Co, and 870 mg kg⁻¹ I.

‡Formulated to supply approx. 1 20 000 IU per day vitamin A, 24 000 IU per day of vitamin D, and 800 IU per day of Vitamin E in total ration.

Analysed by Cumberland Valley Analytical Services, Hagerstown, MD.

§According to the CMP Dairy Ration Analyser v3.0.8.1.

of each experimental period. Gas concentrations were averaged for each interval. Feed offered continued to be recorded and adjusted throughout all collections, with the goal of *ad libitum* access. Cows and feed were placed in the headboxes at approx. 0900 h and the doors were closed and vacuum motor turned on for 15 min before

collections commenced to allow for air equilibration. Temperature and dew point within the box were recorded every minute using a probe (Model TRH-100, Pace Scientific Inc., Mooreville, NC) connected to a data logger (Model XR440, Pace Scientific Inc.). Line pressure was measured from a manometer (Item # 1221–8, United Instruments, Westbury, NY), and barometric pressure of the room was recorded using a barometer (Chaney Instruments Co., Lake Geneva, WI).

Total volume of gas was measured using a gas meter (Model AL425, American Meter, Horsham, PA), and continuous proportional samples of outgoing and incoming air were diverted to collection bags (61 × 61 cm, 44 L; PMC, Oak Park, IL) using glass tube rotameters (Model 1350E Sho-Rate “50,” Brooks Instruments, Hatfield, PA). Then, the collected gas samples were analysed to determine their composition (Emerson X-stream 3-channel analyser, Solon, OH) according to Nienaber and Maddy (1985) and corrected for pressure and temperature within the box.

Sampling of ruminal fluid for pH and ammonia measurements

Ruminal fluid samples were collected every 4 h on day 22 to 25 of each experimental period using a new palpation sleeve for each cow as an approach to minimize cross contamination among cows. To do so, grab samples were taken from the caudal ventral sac, cranial ventral sac, and two samples from the ruminal digesta mat in the dorsal rumen of each cow, and rumen contents were strained through four layers of cheesecloth. The pH was then measured and 40 ml of the strained fluid was placed in a 45-ml vial and immediately frozen at -20°C for later analysis of ammonia-N.

Collection time of ruminal fluid samples was advanced 1 h in subsequent collection day, so that every 60-min interval in a 24-h period was represented (six samples per cow per day and a total of 24 samples per cow per period; Castillo-Lopez *et al.* 2014b). Specifically, samples were collected on day 22 at 0100, 0500, 0900, 1300, 1700 and 2100; day 23 at 0200, 0600, 1000, 1400, 1800 and 2200; day 24 at 0300, 0700, 1100, 1500, 1900 and 2300; and day 25 at 0400, 0800, 1200, 1600, 2000 and 2400.

Sampling of whole ruminal contents for microbial community analysis

Samples of nonstrained ruminal contents, which included the solid and liquid fraction were collected for bacterial community analysis. For this procedure, grab samples were taken from the caudal ventral sac, cranial ventral sac, and two samples from the ruminal digesta mat in the dorsal rumen of each cow at 0900, 1300 and 2300 on day

26 of each experimental period. At each time point, the samples collected from the rumen of the same cow were composited, and a 40-g subsample was collected and placed in a sterile 50-ml conical tube and immediately snap frozen in liquid nitrogen. Thus, a total of 48 composited ruminal digesta content samples were collected during the experiment, which were used for microbial community analysis (four cows × four experimental periods × three sampling time points per period).

Microbial DNA extraction and library preparation for microbial community analysis

To evaluate the diversity of ruminal bacterial community composition, the composited ruminal content samples were homogenized using a flame-sterilized spatula, and DNA was extracted and purified utilizing the MoBio PowerMag Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to manufacturer's instructions. The quality of extracted DNA was verified by gel electrophoresis using a 1% agarose gel.

The concentration of DNA in each sample was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). Subsequently, the V4 hypervariable region of the 16S rRNA gene from the microbial communities was amplified as described previously (Kozich *et al.* 2013). The PCR reactions were performed in 20 μl volumes and contained 0.5 Units of Terra DNA polymerase (Clontech Laboratories, Mountain View, CA), 200 nmol l^{-1} of each primer, 50 ng of nucleic acid template or nontemplate control and 1X Terra PCR buffer. The cycling conditions included an initial denaturation at 98°C for 3 min, followed 25 cycles at 98°C for 30 s, 55°C for 30 s and 68°C for 45 s; and a final extension at 68°C for 4 min (Paz *et al.* 2016). The resulting amplicons from targeted region have been shown to be effective for describing the bacterial communities (Kozich *et al.* 2013; Danielsson *et al.* 2017). The quality of the amplified DNA was verified by resolving on a 1.5% agarose gel. Amplicons from each sample were normalized using the Sequel Prep normalization Plate (96) kit and the epMotion M5073 automated system (Eppendorf, Hauppauge, NY) and they were pooled. The resulting pooled library was sequenced on the Illumina MiSeq platform using 250 bp paired end sequencing using the MiSeq 500 cycle V2 kit as described by the manufacturer.

Bioinformatic analysis of the bacterial community

The sequenced reads were analysed using published bioinformatics pipelines *QIIME* (qiime.org; Caporaso *et al.* 2010) and *MOTHUR* (Schloss *et al.* 2009). Briefly, the forward and reverse reads were assembled to create a

contig read. The assembled sequences were then trimmed and analysed as described by Paz *et al.* (2016). The reads used for downstream analysis are deposited in the NCBI Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/sra> (accession # SRR5123529). Briefly, the size-trimmed high-quality sequences were assigned to operational taxonomic units (OTU) using UPARSE pipeline (drive5.com/uparse/; Edgar *et al.* 2011) after chimera removal using UCHIME (Edgar *et al.* 2011). Sequences from each OTU were then subjected to taxonomic classification using the latest ver. of the GreenGenes taxonomy database (gg_13_5_otus) (Wang *et al.* 2007). Then, based on the taxonomy information, any sequences associated with chloroplasts/Cyanobacteria (from plant origin, and thus, most likely from feed) were filtered and discarded. Then, sequences were aligned to the microbial 16S rRNA gene using the SILVA aligner tool (http://www.mothur.org/w/images/2/27/Silva.nr_v119.tgz); and those sequences that did not align with the target V4 hypervariable region were filtered, thus removing OTU sequences that did not align within the expected region. The resulting OTU table was then used for downstream analysis.

A Venn diagram was constructed to illustrate the distribution and OTU relationship among treatments. To do so, the venn function in the gplots package of R was used (Warnes *et al.* 2015). In addition, the OTU table was used to estimate sample richness (phylogenetic diversity whole tree, chao1 and observed species) and diversity index (Shannon), and rarefaction curves were calculated for the overall bacterial community using QIIME (qiime.org; Caporaso *et al.* 2010). Good's coverage test was performed to evaluate if adequate sampling depth was achieved. Subsequently, principal coordinate analysis plots for beta diversity based on sequence similarity were generated with QIIME.

A core ruminal microbiome, which was defined as those OTUs present in all animals, was calculated. From the core microbiome, taxa were summarized and plots were generated at the taxonomic levels of Phylum, Class, Order Family and Genus, with emphasis on representative OTUs that represented at least 0.1% of the microbial community at a given sample. To minimize animal to animal variation and to represent the shared OTUs within each diet, the core microbiome was analysed, which allows identification of the microbial community influenced by the treatment sorting through animal to animal variation. The hypothesis is that, if the treatment affects the microbial community, this effect should be present across multiple animals on the same treatment. Therefore, the core microbiome allows identification of the effects that might otherwise be hidden in the data (Benson *et al.* 2010; Castillo-Lopez *et al.* 2014a).

Statistical analysis

Data collected on ruminal fermentation and methane emissions were analysed according to a 4×4 Latin square using the MIXED procedure of SAS (ver. 9.1; SAS Institute Inc., Cary, NC, USA). Fixed effects included the treatment and period, with cow as the random effect. The statistical model for this experiment was as follows:

$$Y_{ijk} = \mu + \beta_i + \rho_j + \alpha_k + \varepsilon_{ijk},$$

where Y_{ijk} represents observation ijk ; μ represents the overall mean; β_i represents the random effect of cow i ; ρ_j represents the fixed effect of period j ; and α_k represents the fixed effect of treatment k . The residual term ε_{ijk} was assumed to be normally, independently, and identically distributed, with variance σ_ε^2 . In addition, data collected for Good's coverage, sample richness (phylogenetic diversity, chao1 and observed species), sample diversity (Shannon) and relative abundance of the microbial taxa Phylum, Family and Genus were compared with one-way ANOVA using the MIXED procedure of SAS. The comparison of treatment means was conducted using the PDIF option in the LSMEANS statement. The Pearson correlation coefficients between predominant bacterial families and DMI and methane production were calculated using SAS. Repeated measurements of rumen ammonia, ruminal pH and microbial abundance for samples collected at several time points were analysed by including a REPEATED model statement. Using the CONTRAST statement of SAS, CONT was compared to DG + rFDG + MIX, and DG was compared to rFDG.

In addition, the bacterial community composition differences were evaluated using the unweighted UniFrac distance matrix as an input for a permutational multivariate analysis of variance (PERMANOVA) in R using the vegan package (Adonis function) (Oksanen *et al.* 2015), where treatment was used as main effect. Treatment means are presented as least squares means. The largest standard error of the mean (SEM) is reported. Statistical significance was declared when $P \leq 0.05$ and tendency was discussed if $P > 0.05$ and ≤ 0.10 .

Results

Feed chemical composition, DMI and milk yield

The ingredient and chemical composition of experimental treatments fed in this study are listed in Table 1. The inclusion of conventional DDGS or RFDG in the diets increased the concentration of neutral detergent fibre, crude fat and sulphur, with only a slight increment in crude protein; while decreasing the concentration of starch compared to CONT. Total DMI (Table 2) was

Table 2 Effects of feeding conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles on dry matter intake (DMI), ruminal pH, ruminal ammonia, CO₂ and methane emissions for lactating Holstein cows

Item	Treatment				SEM*	P-Value†		
	CONT	DG	rfDG	MIX		TRT	DG	RF
DMI, kg per day	22.7	27.3	24.8	26.4	1.57	0.05	0.02	0.10
Rumen pH	5.77	5.81	5.84	5.72	0.078	0.06	0.62	0.42
Ammonia, mg dl ⁻¹	13.04	11.18	11.30	11.79	0.966	<0.01	<0.01	0.81
CO ₂ emission, l per day	4151.4	4487.8	4448.1	4462.1	191.74	0.52	0.16	0.87
Methane emission, l per day	426.6	456.6	438.0	454.3	20.62	0.69	0.35	0.53
Methane emission, l kg ⁻¹ DMI	19.2	16.7	17.9	17.3	1.22	0.19	0.06	0.28

CONT: a diet containing no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG (Poet nutrition, Sioux Falls City South Dakota).

*The largest standard error of the mean is shown.

†P-values for TRT: overall treatment effect; DG: CONT vs DG + rfDG + MIX; RF: DG vs rfDG.

affected ($P < 0.01$) by the inclusion of distillers grains with solubles compared to CONT. Specifically, on average, DMI was 4.1 kg greater for animals consuming treatments containing distillers grains with solubles compared to CONT. In addition, 3.5% fat-corrected milk yield ($P < 0.01$) was 30.0 and 36.1 ± 2.37 kg for CONT and diets containing distillers grains, respectively.

Ruminal pH, ruminal ammonia and ruminal methanogenesis

Treatment tended to have an effect on ruminal pH ($P = 0.06$); however, when CONT was compared with treatments containing distillers grains with solubles, ruminal pH was not different ($P = 0.62$) with an overall average of 5.78 ± 0.078 across treatments (Table 2). The concentration of ruminal ammonia was greater ($P < 0.01$) for CONT compared to treatments containing distillers grains with solubles with values of 13.04 and 11.42 ± 0.966 mg dl⁻¹, respectively. Ruminal enteric methane production was not affected ($P = 0.69$) when cows consumed diets containing distillers grains with solubles compared to CONT with an average of 443.8 ± 20.60 l per day across treatments. However, methane produced per unit of DMI tended ($P = 0.06$) to decrease for cows consuming distillers grains with solubles, with estimates of 19.2 and 17.3 ± 1.22 l kg⁻¹ DMI for CONT and treatments containing distillers grains, respectively (Table 2).

Microbial community composition, richness, diversity and distribution

A total of 5 263 141 DNA sequences were obtained from the 48 composited ruminal digesta samples, averaging 1 09 648 sequences per sample. Collectively, 4 083 327

high-quality DNA sequences were obtained after quality filtering and chimera removal, and were used for downstream analysis. The Good's coverage test showed that sequencing depth was able to characterize >98% of the bacterial community. Sample richness estimates were not affected by treatment, as revealed by phylogenetic diversity ($P = 0.19$), Chao1 ($P = 0.17$) and observed species ($P = 0.19$), respectively (Table S2). In addition, the Shannon diversity index tended to be lower ($P = 0.07$) for CONT compared to those treatments containing distillers grains with solubles.

Bacterial community analysis using the unweighted UniFrac distance matrix for treatment effects using PERMANOVA did not display a significant ($P = 0.56$) effect on bacterial community composition. In addition, the Beta diversity for sequence similarities (Fig. 1) based on principal coordinate analysis revealed no apparent clustering of microbial communities. This suggests that spatial sample heterogeneity was similar among treatments. More specifically, the Venn diagram revealed that each diet showed a number of unique OTUs; however, there were 2 357 OTUs shared by all four diets (Fig. 2), representing 74.4% of the total OTUs detected.

Effect of diet on rumen microbial community structure

Phylogenetic analysis of the core microbial community revealed the presence of 13 major bacterial phyla in the ruminal microbiome (Table 3). Although global effect of diet on the microbial community was not detected, significant shifts in the microbial community were detected at phylum level. Specifically, the abundance of the bacterial phylum *Bacteroidetes* was lower ($P = 0.04$) for treatments containing distillers grains with solubles ($54.07 \pm 1.028\%$) compared to CONT ($56.55 \pm 1.028\%$). However, the abundance of the phylum *Firmicutes* ($P = 0.06$) tended to

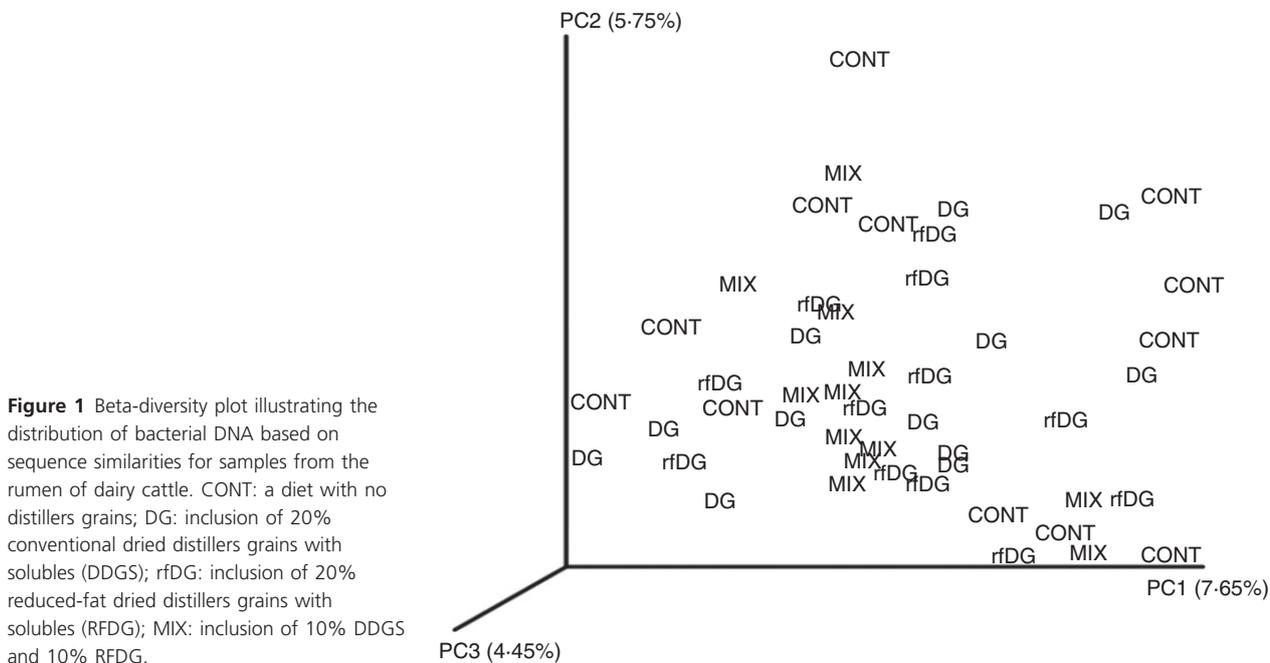


Figure 1 Beta-diversity plot illustrating the distribution of bacterial DNA based on sequence similarities for samples from the rumen of dairy cattle. CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

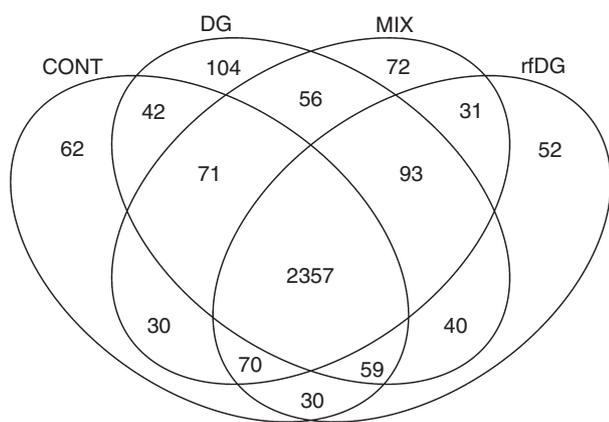


Figure 2 Venn diagram of OTU's relationship among treatments. Each circle represents the treatment; numbers in circles or overlapping areas indicating the number of OTU's in common to the corresponding treatments. CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

increase for treatments containing distillers grains with solubles ($32.97 \pm 0.783\%$) compared to CONT ($31.24 \pm 0.783\%$). Additionally, the abundance of *TM7* tended to increase ($P = 0.09$) and the abundance of *Tenericutes* increased ($P < 0.01$) for treatments containing distillers grains with solubles compared to CONT. No changes were detected in the abundance of the phyla *Spyrochaetes*, *Proteobacteria* and *Actinobacteria* across treatments.

Interestingly, the ratio of *Firmicutes*-to-*Bacteroidetes* increased when feeding distillers grains with solubles ($P = 0.05$) with estimates of 0.55 and 0.61 ± 0.024 for CONT and treatments containing distillers grains with solubles, respectively (Table 3). Only minor changes were observed in the abundance of bacterial phyla found in lower proportions in the rumen.

There were 177 bacterial families detected in all samples. The most abundant are presented in Table 4. No treatment effect was observed on the abundance of the major bacterial families *Prevotellaceae* ($P = 0.51$), *Lachnospiraceae* ($P = 0.67$), *Veillonellaceae* ($P = 0.69$), *Spirochaetaceae* ($P = 0.45$) and *Paraprevotellaceae* ($P = 0.87$). However, treatment effects were observed in the abundance of a few major bacterial families. Specifically, the abundance of *Ruminococcaceae* increased ($P = 0.03$) with inclusion of distillers grains with solubles. Contrasting this observation, the abundance of the family *BS11* decreased ($P < 0.01$) when treatments containing distillers grains with solubles were fed. Similarly, the abundance of an unclassified *RFP12* family ($P = 0.02$) and an unclassified *RF16* family ($P = 0.01$) were lower when treatments containing distillers grains with solubles were fed. Only minor variations were observed in the abundance of the rest of bacterial families.

Taxonomic classification of the core ruminal microbiome at the genus level revealed the presence of 317 major bacterial genera. Table 5 lists only the most abundant. No effect of diet was observed on the abundance of *Prevotella* ($P = 0.51$), an unclassified *Ruminococcaceae*

Table 3 Effects of feeding conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles on major ruminal bacterial phyla for lactating Holstein cows

Phylum, % of total	Treatment				SEM*	P-value†		
	CONT	DG	rFDG	MIX		TRT	DG	RF
<i>Bacteroidetes</i>	56.55	54.00	54.44	53.78	1.028	0.22	0.04	0.76
<i>Firmicutes</i>	31.24	32.96	32.75	33.20	0.783	0.29	0.06	0.84
<i>Spirochaetes</i>	3.21	3.02	2.95	3.05	0.218	0.85	0.42	0.83
<i>Tenericutes</i>	1.19	2.30	2.30	2.26	0.128	0.04	<0.01	0.99
<i>TM7</i>	2.19	2.55	2.47	2.50	0.164	0.38	0.09	0.73
<i>Proteobacteria</i>	0.34	0.30	0.45	0.40	0.096	0.45	0.58	0.31
<i>Actinobacteria</i>	0.13	0.14	0.11	0.15	0.016	0.33	0.49	0.21
<i>Plantomycetes</i>	0.61	0.80	0.71	0.54	0.092	0.01	0.23	0.30
<i>SR1</i>	0.53	0.62	0.65	0.51	0.145	0.63	0.52	0.84
<i>Fibrobacteres</i>	0.55	0.45	0.45	0.67	0.073	0.06	0.76	0.92
<i>Verrucomicrobia</i>	0.30	0.29	0.27	0.26	0.031	0.44	0.18	0.54
<i>Chloroflexi</i>	0.18	0.19	0.15	0.15	0.028	0.29	0.39	0.13
<i>WPS-2</i>	0.03	0.00	0.02	0.00	0.008	0.02	<0.01	0.16
Other‡	0.71	0.76	0.77	0.64	0.046	0.17	0.83	0.89
Bacteroidetes/Firmicutes	0.56	0.61	0.63	0.62	0.024	0.28	0.05	0.84

CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rFDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

*The largest standard error of the mean is reported.

†P-values for TRT: overall treatment effect; DG: CONT vs DG + rFDG + MIX; RF: DG vs rFDG.

‡Correspond to bacterial taxa not listed or unclassified bacterial operational taxonomic units.

genus ($P = 0.66$), *Succiniclaticum* ($P = 0.77$), and unclassified *Lachnospiraceae* genus ($P = 0.35$), *Treponema* ($P = 0.87$) and *Butyrivibrio* ($P = 0.74$). However, treatment effects were observed in the abundance of some major bacterial genera. For example, compared to CONT, the abundance of an unclassified bacterial genus belonging to *Bacteroidales* decreased ($P = 0.05$) when distillers grains with solubles were included in treatments. In addition, the abundance of an unclassified genus belonging to S24-7 decreased ($P = 0.01$) and the abundance of an unclassified genus corresponding to *BS11* decreased ($P = 0.01$) when treatments containing distillers grains with solubles were fed. However, when treatments included distillers grains with solubles, the abundance of *Ruminococcus* tended ($P = 0.06$) to increase, and the abundance of *Bulleidia* increased ($P < 0.01$). Similar to our observations at the taxonomical levels of phylum and family, only minor variations were observed on the abundance of bacterial genera found in lower proportions.

Correlation analysis of the abundance of bacterial families with DMI and methane production (Table 6) revealed that DMI was positively correlated ($P \leq 0.04$) with *Prevotellaceae*, *Veillonellaceae* and *Acetobacteraceae*; however, it was negatively correlated ($P < 0.05$) with *Coriobacteriaceae*, *BS11*, *Anaerolinaceae*, *Mogibacteriaceae* and *RFP12*. Methane emission was positively correlated with *Prevotellaceae* ($P = 0.02$), but was negatively

correlated ($P < 0.05$) with *BS11*, *Christensenellaceae* and *Succinivibrionaceae*.

Discussion

The effect of feeding DDGS (Klopfenstein *et al.* 2008) or RFDG (Mjoun *et al.* 2010b; Castillo-Lopez *et al.* 2014b) on milk production has been evaluated. However, there is limited research on their effects on rumen methanogenesis and the broad rumen microbial community. The gut microbiome influences physiology and metabolism, and disruption of this community has been linked to decreased performance and gastrointestinal problems (Guinane and Cotter 2013; Ridaura *et al.* 2013). In ruminants, gut microbes provide metabolizable protein (NRC 2000), digest feed (McAllister *et al.* 1996) and produce methane (Hristov *et al.* 2013). Thus, understanding the interaction of feeding distillers grains on methane production and rumen bacterial community is important to develop effective dietary intervention strategies for methane mitigation while preventing detrimental effects on nutrient utilization and animal productivity.

DMI, milk yield, ruminal ammonia and ruminal pH

Data collected are in agreement with previous reports indicating that DDGS (Anderson *et al.* 2006; Janicek *et al.* 2008) or RFDG (Mjoun *et al.* 2010a, 2010b;

Table 4 Effects of feeding conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles on major ruminal bacterial families for lactating Holstein cows

Family, % of total	Treatment				SEM*	P-value†		
	CONT	DG	rfDG	MIX		TRT	DG	RF
<i>Prevotellaceae</i>	28.65	30.20	28.81	29.85	1.430	0.51	0.33	0.77
<i>S24-7</i>	10.58	8.85	8.91	11.35	0.585	0.01	0.20	0.93
<i>Lachnospiraceae</i>	7.12	7.15	7.20	7.46	0.367	0.86	0.67	0.92
<i>Ruminococcaceae</i>	7.60	8.68	8.36	7.79	0.454	0.02	0.03	0.20
<i>Veillonellaceae</i>	6.13	5.49	6.39	5.57	0.963	0.74	0.69	0.35
<i>Spirochaetaceae</i>	3.19	3.00	2.95	3.05	0.218	0.87	0.45	0.85
<i>BS11</i>	3.22	2.20	2.43	1.98	0.386	0.01	<0.01	0.45
<i>Paraprevotellaceae</i>	2.20	2.30	2.25	2.10	0.151	0.64	0.87	0.77
<i>F16</i>	2.36	2.65	2.60	2.62	0.186	0.61	0.19	0.83
<i>Erysipelotrichaceae</i>	1.26	1.38	1.33	1.39	0.084	0.43	0.14	0.55
<i>Mogibacteriaceae</i>	0.58	0.60	0.59	0.61	0.054	0.91	0.60	0.75
<i>Pirellulaceae</i>	0.60	0.80	0.71	0.54	0.092	0.01	0.23	0.30
<i>Fibrobacteraceae</i>	0.55	0.45	0.45	0.67	0.073	0.06	0.76	0.92
<i>Clostridiaceae</i>	0.53	0.50	0.55	0.59	0.044	0.11	0.55	0.23
<i>Coriobacteriaceae</i>	0.10	0.10	0.10	0.12	0.046	0.89	0.45	0.68
<i>RFP12</i>	0.30	0.27	0.25	0.25	0.028	0.09	0.02	0.23
<i>Christensenellaceae</i>	0.25	0.22	0.24	0.21	0.039	0.66	0.48	0.46
<i>Bifidobacteriaceae</i>	0.22	0.06	0.04	0.08	0.102	0.52	0.14	0.85
<i>Anaerolinaceae</i>	0.18	0.19	0.15	0.15	0.028	0.29	0.39	0.13
<i>RF16</i>	0.16	0.10	0.10	0.12	0.017	0.03	0.01	0.51
<i>Succinivibrionaceae</i>	0.10	0.06	0.10	0.12	0.068	0.73	0.84	0.44
<i>Bacteroidaceae</i>	0.15	0.15	0.17	0.16	0.028	0.90	0.70	0.53
<i>Anaeroplasmataceae</i>	0.18	0.15	0.15	0.13	0.057	0.61	0.28	0.97
<i>Desulfovibrionaceae</i>	0.03	0.02	0.10	0.07	0.032	0.05	0.21	0.01
Other‡	22.01	22.34	22.80	21.34	0.918	0.61	0.87	0.68

CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

*The largest standard error of the mean is reported.

†P-values for TRT: overall treatment effect; DG: CONT vs DG + rfDG + MIX; RF: DG vs rfDG.

‡Correspond to bacterial taxa not listed or unclassified bacterial operational taxonomic units.

Castillo-Lopez *et al.* 2014b) can effectively be included in dairy rations and maintain or increase feed intake and milk yield. However, increments observed were greater than previous reports, which have shown an increase in DMI (Janicek *et al.* 2008; Ramirez Ramirez *et al.* 2015) or milk yield (Anderson *et al.* 2006; Janicek *et al.* 2008; Paz *et al.* 2013) when cows are fed distillers grains. The increment in DMI could reflect greater palatability of diets; the exact reason for the increment in milk yield remains still unknown, but could be partially explained by an enhancement in total tract digestibility of some nutrients when distillers grains are fed (Castillo-Lopez *et al.* 2014b).

When dietary crude protein enters the rumen, microbes attack the ruminally degradable protein (RDP) generating ammonia. Part of the ammonia, is used for microbial protein synthesis (Hristov and Ropp 2003). In this study, despite the increase in dietary protein with distillers grains inclusion, ruminal ammonia decreased.

Although, RDP and microbial protein were not measured, there may have been an improved efficiency in the use of ammonia for microbial growth with distillers grains inclusion, lowering the concentration of ammonia in ruminal fluid. Ruminal pH measured in this study was lower compared to previous reports in lactating cattle being fed distillers grains using indwelling pH meters (Castillo-Lopez *et al.* 2014b), this may be explained by the fact that pH of particle associated ruminal fluid is lower compared to pH of free ruminal fluid (Neubauer *et al.* 2017).

The rumen bacterial community structure when feeding DDGS and RFDG

Consistent with previous reports (Petri *et al.* 2012), predominant phyla observed in the rumen were *Bacteroidetes* and *Firmicutes*, representing more than 85% of the bacterial community. The influence of diet on community

Table 5 Effects of feeding conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles on major ruminal bacterial genera for lactating Holstein cows

Genera, % of total	Treatment				SEM*	P-value†		
	CONT	DG	rfDG	MIX		TRT	DG	RF
<i>Prevotella</i>	28.65	30.20	28.81	29.85	1.430	0.51	0.33	0.77
Unclassified <i>bacteroidales</i>	11.57	10.18	10.73	9.26	0.602	0.05	0.03	0.50
Unclassified <i>S24_7</i>	10.58	8.85	8.91	11.35	0.585	0.01	0.20	0.93
Unclassified <i>Ruminococcaceae</i>	5.77	6.00	5.70	6.02	0.389	0.66	0.60	0.34
<i>Succinivasticum</i>	5.46	4.90	5.75	5.00	0.948	0.77	0.75	0.36
Unclassified <i>Lachnospiraceae</i>	3.36	3.19	3.56	3.40	0.249	0.35	0.89	0.07
<i>Treponema</i>	3.19	3.00	3.05	2.95	0.218	0.87	0.45	0.85
Unclassified <i>BS11</i>	3.22	2.20	2.43	1.98	0.386	0.01	<0.01	0.45
<i>Butyrivibrio</i>	2.36	2.62	2.45	2.61	0.197	0.74	0.38	0.55
<i>Ruminococcus</i>	1.72	2.55	1.95	2.17	0.230	0.08	0.06	0.06
<i>CF231</i>	1.06	1.20	1.05	1.04	0.078	0.37	0.70	0.14
<i>YRC22</i>	0.50	0.55	0.56	0.54	0.058	0.62	0.22	0.75
<i>Coprococcus</i>	0.49	0.45	0.47	0.55	0.065	0.39	0.95	0.76
<i>p-75-a5</i>	0.56	0.55	0.58	0.56	0.055	0.97	0.95	0.65
<i>Fibrobacter</i>	0.55	0.45	0.45	0.67	0.073	0.06	0.76	0.92
<i>Moryella</i>	0.40	0.45	0.35	0.45	0.041	0.07	0.68	0.03
<i>Bulleidia</i>	0.35	0.48	0.43	0.49	0.027	0.01	<0.01	0.13
<i>Clostridium</i>	0.36	0.35	0.39	0.40	0.051	0.30	0.44	0.28
<i>SHD-231</i>	0.18	0.19	0.15	0.15	0.028	0.29	0.39	0.13
<i>Mogibacterium</i>	0.22	0.23	0.25	0.27	0.037	0.61	0.37	0.55
Other‡	16.14	18.00	18.88	17.71	0.577	0.05	<0.01	0.86

CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

*The largest standard error of the mean.

†P-values for TRT: overall treatment effect; DG: CONT vs DG + rfDG + MIX; RF: DG vs rfDG.

‡Correspond to bacterial taxa not listed or unclassified bacterial operational taxonomic units.

composition of ruminal contents has been recognized (Fernando *et al.* 2010). In agreement with our observations, Callaway *et al.* (2010) and Ramirez Ramirez *et al.* (2012) used DNA pyro-sequencing and found that feeding DDGS shifts the rumen community structure. However, contrasting our observations those authors reported a decrease in the ratio *Firmicutes*-to-*Bacteroidetes*. The discrepancies between these observations may be related to the concentration of DDGS and chemical composition of the diets and the available substrates for microbial growth, and finally the region of the 16S rRNA gene sequenced (V1–V3 vs V4). Callaway *et al.* (2010) and Ramirez Ramirez *et al.* (2012) included greater concentrations of DDGS, 30 and 50% of DM respectively, and there was greater variation in diet composition, namely crude fat and starch. In this study, however, there were minor variations in dietary substrates available for bacterial fermentation, which may have prevented drastic changes in the abundance of most predominant bacterial phyla.

Our findings from bacterial taxonomic analyses at the family and genus levels are consistent with previous findings indicating that the ruminal microbiome is largely

composed of the families *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* and the genera *Prevotella*, *Succinivasticum* and *Ruminococcus* (Zened *et al.* 2013). Although a significant correlation does not necessarily indicate a direct cause–effect relationship, the positive association observed between the abundance of the family *Prevotellaceae* and DMI may reflect the relevant role of this family on nutrient digestion. *Prevotellaceae*, the largest bacterial family in the rumen, is composed of versatile species that can utilize a variety of nutrients, including protein, starch, pectins and hemicellulose (Russell 2002). Thus, the abundance of *Prevotellaceae* may have a direct relationship with feed degradation and DMI. Other studies have also shown the predominance of this bacterial family in the rumen of cattle fed forage-based diets supplemented with corn distillers grains (Ramirez Ramirez *et al.* 2012; Castillo-Lopez *et al.* 2014a).

Although many families and genera remained unaffected when corn by-products were included in the diets, it is interesting to note shifts in some of these taxa. For example, the increase in the abundance of *Ruminococcus*, a fibre digesting bacterial genus, may be due to the

Table 6 Correlation coefficients between the abundance of bacterial families in the rumen and DMI and methane emission for Holstein dairy cattle fed treatments* including distillers grains

DMI or methane emission	Bacterial family	Correlation coefficient (r)	P-value
DMI, kg	<i>Coriobacteriaceae</i>	-0.299	0.03
	<i>BS11</i>	-0.541	<0.01
	<i>Prevotellaceae</i>	0.288	0.04
	<i>Anaerolinaceae</i>	-0.298	0.03
	<i>Veillonellaceae</i>	0.283	0.05
	<i>Mogibacteriaceae</i>	-0.349	0.01
	<i>Acetobacteraceae</i>	0.294	0.04
	<i>RFP12</i>	-0.352	0.01
Methane emission, l per day	<i>BS11</i>	-0.489	<0.01
	<i>Prevotellaceae</i>	0.319	0.02
	<i>Christensenellaceae</i>	-0.373	<0.01
	<i>Succinivibrionaceae</i>	-0.284	0.04

*CONT: a diet with no distillers grains; DG: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rFDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG.

increase in dietary neutral detergent fibre with distillers grains inclusion, providing additional substrate for microbial proliferation. Bacteria belonging to BS11 are usually favoured by low-starch diets (Zened *et al.* 2013; De Nardi *et al.* 2016), despite the decrease in dietary starch with distillers grains inclusion, the abundance of BS11 decreased. This is possibly due to sensitivity of this bacterial family to dietary fat (van Lingen *et al.* 2017).

Distillers grains, ruminal methanogenesis and association with bacterial taxa

A reduction in ruminal methanogenesis has been reported when distillers grains with solubles are fed to lactating dairy cattle (Benchaar *et al.* 2013; Foth *et al.* 2015) or beef heifers (Hünerberg *et al.* 2013). In addition, Birkelo *et al.* (2004) reported a 14% decrease in methane energy loss when cows were fed 31% wet corn distillers grains with solubles. In this study, no change in total methane emission was found; however, when methane production was expressed on the basis of DMI, animals fed distillers grains with solubles produced less methane. Therefore, these data suggest that when feeding distillers grains with solubles to dairy cows, dietary energy lost through methane emissions as a proportion of feed cost may be reduced, improving farm profitability. Ruminal methanogens generate methane by utilizing carbon dioxide and hydrogen (Danielsson *et al.* 2017). Thus, methods to decrease enteric methane formation may include direct mitigation of methanogens or decreasing the availability of hydrogen in the rumen. For example,

Khiaosa-ard *et al.* (2015) reported that fortification of DDGS with grape seed meal decreased methane emission, and they concluded that methane mitigation was due to the decreased numbers of *Ruminococcus flavefaciens*, total fungi and total protozoa. They also indicated that the inhibition of those microbes was likely caused by dietary tannins contained in the grape seed meal, which limited hydrogen supply for methane formation.

Dietary fat may affect methane emissions by affecting the growth of microbes contributing to methanogenesis (Martin *et al.* 2008; Yang *et al.* 2009). Benchaar *et al.* (2013) reported that, on average, a 1% increase in dietary fat decreased methane production by 0.5 g kg⁻¹ DMI. In addition, Hales *et al.* (2017) reported a linear decrease in methane production with an increase of dietary corn oil in high-concentrate finishing diets containing from 3.0 to 8.7% dietary fat, and they suggested that this reduction was likely due to ruminal fatty acid biohydrogenation, which sequestered part of the hydrogen in the rumen. In this study, the increase in dietary fat between 0.5 and 1.2% tended to lower methane emission when expressed per unit of feed intake. The lack of an effect of dietary fat on total methane emission may have been due to the smaller increase in dietary fat compared to Hales *et al.* (2017). Hales *et al.* (2013) fed 0, 15, 30 and 45% wet distillers grains and solubles and found that methane production increased linearly as a percent of gross energy intake in Jersey steers fed diets containing between 5.9 and 8.3% dietary fat. This increment may be explained by the decrease in dietary starch and the increase in NDF, which likely favoured acetate production with subsequent hydrogen liberation (Philippeau *et al.* 2017).

Methane emissions usually increase with increasing dietary fibre content (Johnson and Johnson 1995). Fibre degradation in the rumen promotes acetate production, which liberates hydrogen as a by-product; therefore, enhancing methane generation (Van Soest 1994). This indicates that a reduction in ruminal fibre degradability may contribute to reduce methane emissions (Martin *et al.* 2008; Beauchemin *et al.* 2009; Benchaar *et al.* 2013). In this study, the inclusion of distillers grains with solubles in diets increased dietary fibre concentration, with no impact on ruminal methanogenesis. Although volatile fatty acids were not measured, results indicate a positive correlation between total methane produced and the abundance of the family *Prevotellaceae*. *Prevotellaceae* is largely composed of organisms that can digest a variety of substrates including hemicellulose and produce acetate (Russell 2002). Therefore, a shift in the abundance of *Prevotella* may shift, in the same direction, hydrogen liberation in the rumen; thus, influencing methane production, which could explain the observed positive correlation.

In agreement with our observations, a negative correlation between methane emissions and the abundance of *Succinivibrionaceae* has been reported (Wallace *et al.* 2015). Members of this family produce succinate, an intermediate product in propionate production. Propionate formation is not associated with any hydrogen production (Wallace *et al.* 2015; Danielsson *et al.* 2017); rather, propionate production utilizes hydrogen, and is considered a competitive pathway for methane formation (Moss *et al.* 2000; Ungerfeld 2015), which may explain the negative correlation between *Succinivibrionaceae* abundance and the amount of methane emitted. This observation is consistent with other reports indicating that an increase in propionate production in cattle fed high-concentrate diets is associated with lower methane emission compared to cattle fed high-fibre diets (Hristov *et al.* 2013). We still do not know the potential underlying mechanisms for the negative association of some acetate- and butyrate-producing bacterial taxa with methane formation, namely the families *BS11* and *Christensenellaceae* (Ribeiro *et al.* 2017). *BS11* is composed of yet uncultured organisms that can degrade hemicellulose and produce acetate and butyrate (Ribeiro *et al.* 2017). *Christensenellaceae* has also been associated with low body mass index and a reduction in adiposity gain in nonruminants (Goodrich *et al.* 2014), suggesting that this bacterial family may influence animal nutrient metabolism. Further investigation on the activity and role of members of these bacterial families in the rumen of dairy cattle, and why their abundance was negatively correlated with methane production is warranted.

Methane production may also be affected by dietary sulphur, sulphate can act as an alternative electron acceptor in the rumen given that the reduction of sulphate to sulphide is thermodynamically more favourable than the reduction of CO₂ to methane (McAllister *et al.* 1996). In this study, the inclusion of DDGS in rations increased the concentration of sulphur. However, this increment was not accompanied by a significant effect on total methane emitted.

Overall, contrasting our hypothesis, no reduction in total methane emissions was observed when distillers grains with solubles were included in rations formulated for lactating dairy cows. However, our results demonstrate that DDGS or RFDG may be used to replace corn and soybean meal in dairy rations resulting in a reduction of ruminal methane emitted per unit of DMI. In addition, the inclusion of DDGS or RFDG shifted the abundance of some ruminal bacterial taxa, including an increase in the genus *Ruminococcus*; however, no detrimental effects were detected on the overall bacterial community structure. Furthermore, the abundance of

Prevotellaceae, the most predominant bacterial family in the rumen was positively correlated with DMI and methane emissions. However, a negative correlation was found between methane emission and the abundance of *Succinivibrionaceae*, a family that includes organisms that produce succinate, an intermediate product in propionate. Predominant bacterial families associated with methane emissions may influence the amount of hydrogen liberated in the rumen and the proportion of hydrogen utilized in metabolic pathways other than methanogenesis, and thus, affecting methane formation. Future research should consider the evaluation of the archaeal community within the rumen in order to determine whether distillers grains shift the abundance of methanogenic archaeal taxa, and if those changes are associated with enteric methane production.

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Conflict of Interest

Samodha C. Fernando and Paul J. Kononoff, author(s) of this publication have disclosed a significant financial interest in NuGUT LLC. In accordance with its Conflict of Interest policy, the University of Nebraska-Lincoln's Conflict of Interest in Research Committee has determined that this must be disclosed.

References

- Aldai, N., Klieve, A.V., Dugan, M.E.R., Kramer, J.K.G., Ouwerkerk, D., Aalhus, J.L., McKinnon, J.J., McAllister, T.A., *et al.* (2012) Evaluation of rumen fatty acid hydrogenation intermediates and differences in bacterial communities after feeding wheat- or corn-based dried distillers grains to feedlot cattle. *J Anim Sci* **90**, 2699–2709.
- Anderson, J.L., Schingoethe, D.J., Kalscheur, K.F. and Hippen, A.R. (2006) Evaluation of dried and wet distillers grains included at two concentrations in the diets of lactating dairy cows. *J Dairy Sci* **89**, 3133–3142.
- Anderson, C.L., Schneider, C.J., Erickson, G.E., MacDonald, J.C. and Fernando, S.C. (2016) Rumen bacterial communities can be acclimated faster to high concentrate diets than currently implemented feedlot programs. *J Appl Microbiol* **120**, 588–599.

- AOAC. (2000) *Official Methods of Analysis*. Vol. 1 and 2. 17th edn. Gaithersburg, MD: AOAC International.
- AOAC. (2006) *Official Methods of Analysis*. 18th edn. Gaithersburg, MD: AOAC International.
- Beauchemin, K.A., McGinn, S.M., Benchaar, C. and Holtshausen, L. (2009) Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: effects on methane production, rumen fermentation, and milk production. *J Dairy Sci* **92**, 2118–2127.
- Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., Julien, C., Petit, H.V. and Massé, D.I. (2013) Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. *J Dairy Sci* **96**, 2413–2427.
- Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M. and Oh, P.L. (2010) Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA* **44**, 18933–18938.
- Birkelo, C.P., Brouk, M.J. and Schingoethe, D.J. (2004) The energy content of wet corn distillers grains for lactating dairy cows. *J Dairy Sci* **87**, 1815–1819.
- Callaway, T.R., Dowd, S.E., Edrington, T.S., Anderson, R.C., Krueger, N., Bauer, N., Kononoff, P.J. and Nisbet, D.J. (2010) Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *J Anim Sci* **88**, 3977–3983.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez, Pena A., *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336.
- Castillo-Lopez, E., Klopfenstein, T.J., Fernando, S.C. and Kononoff, P.J. (2013) In vivo determination of rumen undegradable protein of dried distillers grains with solubles and evaluation of duodenal microbial crude protein flow. *J Anim Sci* **91**, 924–934.
- Castillo-Lopez, E., Klopfenstein, T.J., Anderson, C., Alugthge, N.D., Fernando, S.C. and Kononoff, P.J. (2014a) Effect of feeding dried distillers grains with solubles on rumen biohydrogenation, intestinal fatty acid profile and gut microbial diversity evaluated through DNA pyrosequencing. *J Anim Sci* **92**, 733–743.
- Castillo-Lopez, E., Ramirez Ramirez, H.A., Klopfenstein, T.J., Hostetler, D., Fernando, S.C. and Kononoff, P.J. (2014b) Ration formulations containing reduced-fat dried distillers grains with solubles and their effect on lactation performance, rumen fermentation, and intestinal flow of microbial nitrogen in Holstein cows. *J Dairy Sci* **97**, 1578–1593.
- Chaucheyras-Durand, F. and Ossa, F. (2014) Review: the rumen microbiome: composition, abundance, diversity, and new investigative tools. *Prof Anim Sci* **30**, 1–12.
- Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Müller, B., Schnürer, A. and Bertilsson, J. (2017) Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front Microbiol* **8**, 226.
- De Nardi, R., Marchesini, G., Li, S., Khafipour, E., Plaizier, K.J.C., Ganesella, M., Ricci, R., Andrighetto, I., *et al.* (2016) Metagenomic analysis of rumen microbial population in dairy heifers fed a high grain diet supplemented with dicarboxylic acids or polyphenols. *BMC Vet Res* **12**, 29.
- Duval, S. and Kindermann, M. (2012) Use of Nitrooxy Organic Molecules in Feed for Reducing Enteric Methane Emissions in Ruminants, and/or to Improve Ruminant Performance. World Intellectual Property Organization. International Patent Application WO 2012/084629 A1.
- Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–2200.
- Fernando, S.C., Purvis, H.T., Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G., Roe, B.A. and Desilva, U. (2010) Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environ Microbiol* **76**, 7482–7490.
- Foth, A.J., Brown-Brandl, T., Hanford, K.J., Miller, P.S., Garcia Gomez, G. and Kononoff, P.J. (2015) Energy content of reduced-fat dried distillers grains with solubles for lactating dairy cows. *J Dairy Sci* **98**, 7142–7152.
- Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Bleckhman, R., Beaumont, M., Treuren, V., *et al.* (2014) Human genetics shape the gut microbiome. *Cell* **159**, 789–799.
- Guinane, A.M. and Cotter, P.D. (2013) Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Ther Adv Gastroenterol* **6**, 295–308.
- Hales, K.E., Cole, A.E. and MacDonald, J.C. (2013) Effects of increasing concentrations of wet distillers grains with solubles in steam-flaked, corn-based diets on energy metabolism, carbon-nitrogen balance, and methane emissions of cattle. *J Anim Sci* **91**, 819–828.
- Hales, K.E., Foote, A.P., Brown-Brandl, T.M. and Freetly, H.C. (2017) The effects of feeding increasing concentrations of corn oil on energy metabolism and nutrient balance in finishing beef steers. *J Anim Sci* **95**, 939–948.
- Hall, M.B. (2009) Analysis of starch, including maltooligosaccharides, in animal feeds: a comparison of methods and a recommended method for AOAC collaborative study. *JAOACI* **92**, 42–49.
- Hristov, A.N. and Ropp, J.K. (2003) Effect of dietary carbohydrate composition and availability on utilization of ruminal ammonia nitrogen for milk protein synthesis in dairy cows. *J Dairy Sci* **86**, 2416–2427.
- Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P., Adesogan, A.T., *et al.* (2013)

- SPECIAL TOPICS-Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *J Anim Sci* **91**, 5045–5069.
- Hünerberg, M., McGinn, S.M., Beauchemin, K.A., Okine, E.K., Harstad, O.M. and McAllister, T.A. (2013) Effect of dried distillers grains plus solubles on enteric methane emissions and nitrogen excretion from growing beef cattle. *J Anim Sci* **91**, 2846–2857.
- Jami, E., White, B.A. and Mizrahi, I. (2014) Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS ONE* **9**, e85423.
- Janicek, B.N., Kononoff, P.J., Gehman, A.M. and Doane, P.H. (2008) The effect of feeding dried distillers grains plus solubles on milk production and excretion of urinary purine derivatives. *J Dairy Sci* **91**, 3544–3553.
- Johnson, K.A. and Johnson, D.E. (1995) Methane emissions from cattle. *J Anim Sci* **73**, 2483–2492.
- Khiaosa-ard, R., Metzler-Zebeli, B.U., Ahmed, S., Muro-Reyes, A., Deckardt, K., Chizzola, R., Böhm, J. and Zebeli, Q. (2015) Fortification of dried distillers grains plus solubles with grape seed meal in the diet modulates methane mitigation and rumen microbiota in Rusitec. *J Dairy Sci* **98**, 2611–2626.
- Klopfenstein, T.J., Erickson, G.E. and Bremer, V.R. (2008) Board-invited review: use of distillers by-products in the beef cattle feeding industry. *J Anim Sci* **86**, 1223–1231.
- Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl Environ Microbiol* **79**, 5112–5120.
- Krause, D.O., Nagaraja, T.G., Wright, A.D.G. and Callaway, T.R. (2013) Board-invited review: rumen microbiology: leading the way in microbial ecology. *J Anim Sci* **91**, 331–341.
- van Lingen, H.J., Edwards, J.E., Vaidya, J.D., van Gastelen, S., Saccanti, E., van den Bogert, B., Bannink, A., Smidt, H., et al. (2017) Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Front Microbiol* **8**, 425.
- Mackie, R.I. and Gilchrist, F.M.C. (1979) Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high concentrate diet. *Appl Environ Microbiol* **38**, 422–430.
- Majoni, S., Wang, T. and Johnson, L.A. (2011) Physical and chemical processes to enhance oil recovery from condensed corn distillers solubles. *J Am Oil Chem Soc* **88**, 425–434.
- Martin, C., Rouel, J., Jouany, J.P., Doreau, M. and Chilliard, Y. (2008) Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J Anim Sci* **86**, 2642–2650.
- McAllister, T.A., Okine, E.K., Mathison, G.W. and Cheng, K.J. (1996) Dietary, environmental and microbiological aspects of methane production in ruminants. *Can J Anim Sci* **76**, 231–243.
- Mjoun, K., Kalscheur, K.F., Hippen, A.R. and Schingoethe, D.J. (2010a) Performance and amino acid utilization of early lactation dairy cows fed regular or reduced-fat dried distillers grains with solubles. *J Dairy Sci* **93**, 3176–3191.
- Mjoun, K., Kalscheur, K.F., Hippen, A.R., Schingoethe, D.J. and Little, D.E. (2010b) Lactation performance and amino acid utilization of cows fed increasing amounts of reduced-fat dried distillers grains with solubles. *J Dairy Sci* **93**, 288–303.
- Moss, A.R., Jouany, A.R. and Newbold, J. (2000) Methane production by ruminants its contribution to global warming. INRA, EDP Sciences. *Annal Zootech* **49**, 231–253.
- Myer, P.R., Smith, P.L., Wells, J.E., Kuehn, L.A. and Freetly, H.C. (2015) Rumen microbiome from steers differing in feed efficiency. *PLoS ONE* **10**, e0129174.
- Neubauer, V., Humer, E., Kröger, I., Braid, T., Wagner, M. and Zebeli, Q. (2017) Differences between pH of indwelling sensors and the pH of fluid and solid phase in the rumen of dairy cows fed varying concentrate levels. *J Anim Physiol Anim Nutr.* <https://doi.org/10.1111/jpn.12675>.
- Nienaber, J. and Maddy, A. (1985) Temperature controlled multiple chamber indirect calorimeter-design and operation. *Trans ASAE* **28**, 555–560.
- NRC. (2000) *Nutrient Requirements of Beef Cattle*. 7th revised edn.: Update 2000. Washington, DC: National Academic Press.
- Oksanen, J., Guillaume Blanchet, F., Kindt, R., Legendre, P., Minchin, P. R. and O'Hara, R. B. (2015) *Vegan: Community Ecology Package*. R Package Version 2.3-0. Available at: <http://CRAN.R-project.org/package=vegan>.
- Paz, H., Castillo-Lopez, E., Ramirez-Ramirez, H.A., Christensen, D.A., Klopfenstein, T.J. and Kononoff, P.J. (2013) Invited review: ethanol co-products for dairy cows: there goes our starch... now what? *Can J Anim Sci* **93**, 407–425.
- Paz, H.A., Anderson, C.A., Muller, M.J., Kononoff, P.J. and Fernando, S.C. (2016) Rumen bacterial community composition in Holstein and Jersey cows is different under same dietary condition and is not affected by sampling method. *Front Microbiol* **7**, 1206.
- Petri, R.M., Forster, R.J., Yang, W., McKinnon, J.J. and McAllister, T.A. (2012) Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J Appl Microbiol* **112**, 1152–1162.
- Philippeau, C., Lettat, A., Martin, C., Silberberg, M., Morgavi, D.P., Ferlay, A., Berger, C. and Nozière, P. (2017) Effects of bacterial direct-fed microbials on ruminal characteristics, methane emission, and milk fatty acid composition in cows fed high- or low-starch diets. *J Dairy Sci* **100**, 2637–2650.

- Ramirez Ramirez, H.A., Nestor, K., Tedeschi, L.O., Callaway, T.R., Dowd, S.E., Fernando, S.C. and Kononoff, P.J. (2012) The effect of brown midrib corn silage and dried distillers' grains with solubles on milk production, nitrogen utilization and microbial community structure in dairy cows. *Can J Anim Sci* **92**, 380.
- Ramirez Ramirez, H.A., Castillo-Lopez, E., Jenkins, C.J.R., Aluthge, N.D., Anderson, C., Fernando, S.C., Harvatine, K.J. and Kononoff, P.J. (2015) Reduced-fat dried distillers grains with solubles reduces the risk for milk fat depression and supports milk production and ruminal fermentation in dairy cows. *J Dairy Sci* **99**, 1912–1928.
- Ribeiro, D.O., Oss, D.B., He, Z., Gruninger, J.R., Elekwachi, C., Forster, R.J., Yang, W., Beauchemin, K.A., *et al.* (2017) Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. *Sci Rep* **7**, 1276.
- Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffith, N.W., Lombard, V., *et al.* (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **341**, 1241–1244.
- Russell, J.B. (2002) *Rumen Microbiology and its Role in Ruminant Nutrition*. Ithaca, NY: Cornell University.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**, 7537–7541.
- Thoetkiattikul, H., Mhuantong, W., Laothanachareon, T., Tangphatsornruang, S., Pattarajinda, V., Eurwilaichitr, L. and Champreda, V. (2013) Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S Rna gene pyrosequencing. *Curr Microbiol* **67**, 1–8.
- Ungerfeld, E.M. (2015) Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Front Microbiol* **6**, 37.
- Van Soest, P.J. (1994) *Nutritional Ecology of the Ruminant*. Ithaca, NY: Comstock Pub.
- Van Soest, P.J., Robertson, J.B. and Lewis, B.A. (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**, 3583–3597.
- Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.A., Hyslop, J.J., Ross, A., Waterhouse, A., Watson, M., *et al.* (2015) The rumen microbial metagenome associated with high methane production in cattle. *BMC Genom* **16**, 839.
- Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261–5267.
- Warnes, G. R., Bolker, B., Bonebakker, L., Gentleman, R., Andy Liaw, W. H. and Lumley, T. (2015) Gplots: Various R Programming Tools for Plotting Data. R Package Version 2.17.0. Available at: <http://CRAN.Rproject.org/package=gplots>.
- Weiss, B., Zhang, P., Goeser, P. and St-Pierre, N. (2016) TMR sampling: valuable exercise or a random number generator?. Tri-State Dairy Nutrition Conference. Available at: <http://tristatedairy.org/Proceedings%202016/Bill%20Weiss.pdf>.
- Yang, S.L., Bu, D.P., Wang, J.Q., Hu, Z.Y., Li, D., Wei, H.Y., Zhou, L.Y. and Looor, J.J. (2009) Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows. *Animal* **3**, 1562–1569.
- Zened, A., Combes, S., Cauquil, L., Mariette, J., Klopp, C. and Bouchez, O. (2013) Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol Ecol* **83**, 504–514.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Analysed chemical composition* (mean and standard deviation) of feedstuffs used in diets containing conventional dried distillers grains with solubles (DDGS) or reduced-fat dried distillers grains with solubles (RFDG) and fed to lactating dairy cows.

Table S2. Average of treatments for microbial richness estimates and diversity index of ruminal contents of Holstein cows consuming conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles.

Table S1. Analyzed chemical composition* (mean and standard deviation) of feedstuffs used in diets containing conventional dried distillers grains with solubles (DDGS) or reduced-fat dried distillers grains with solubles (RFDG) and fed to lactating dairy cows.

Component	FEED INGREDIENT							
	Alfalfa hay	Brome hay	Corn silage	Control concentrate	High fat concentrate	Low fat concentrate	DDGS	RFDG†
Dry matter	88.1 ± 1.2	89.2 ± 0.8	44.4 ± 2.4	88.5 ± 0.3	88.7 ± 0.2	89.2 ± 0.2	89.2 ± 0.7	89.1 ± 0.5
Crude protein	16.6 ± 0.3	8.1 ± 0.1	8.1 ± 0.4	23.1 ± 0.7	24.2 ± 0.8	24.2 ± 0.8	28.0 ± 4.2	28.3 ± 3.7
Neutral detergent fiber	45.5 ± 1.3	65.5 ± 0.8	41.8 ± 3.1	22.3 ± 3.5	30.4 ± 1.6	30.1 ± 0.7	31.2 ± 1.0	30.6 ± 2.3
Starch	2.8 ± 0.5	1.3 ± 0.7	33.1 ± 2.6	33.4 ± 2.0	21.1 ± 2.7	21.0 ± 1.7	10.7 ± 5.4	12.0 ± 4.9
Crude fat	1.5 ± 0.2	2.1 ± 0.4	3.2 ± 0.1	2.7 ± 0.4	5.3 ± 0.1	3.9 ± 0.2	7.2 ± 2.2	4.8 ± 1.2
Ash	10.3 ± 0.1	10.0 ± 0.6	5.0 ± 0.2	9.1 ± 0.4	8.5 ± 0.9	9.6 ± 0.6	7.6 ± 2.8	7.2 ± 2.2
Calcium	1.19 ± 0.07	0.37 ± 0.04	0.25 ± 0.01	1.75 ± 0.16	1.50 ± 0.23	1.75 ± 0.32	0.89 ± 0.80	0.87 ± 0.82
Phosphorous	0.33 ± 0.02	0.25 ± 0.03	0.23 ± 0.02	0.66 ± 0.01	0.84 ± 0.07	0.83 ± 0.06	0.85 ± 0.01	0.79 ± 0.03
Magnesium	0.23 ± 0.02	0.11 ± 0.01	0.18 ± 0.01	0.48 ± 0.05	0.52 ± 0.07	0.57 ± 0.03	0.48 ± 0.12	0.45 ± 0.10
Potassium	3.44 ± 0.36	2.29 ± 0.27	1.27 ± 0.08	1.32 ± 0.05	1.22 ± 0.06	1.34 ± 0.05	1.40 ± 0.23	1.26 ± 0.03
Sulphur	0.23 ± 0.01	0.12 ± 0.00	0.12 ± 0.00	0.25 ± 0.00	0.35 ± 0.00	0.47 ± 0.01	0.47 ± 0.10	0.66 ± 0.19

*Analyzed by Cumberland Valley Analytical Services, Hagerstown, MD.

†RFDG, reduced-fat dried distillers grains with solubles (Poet nutrition, Sioux Falls City South Dakota).

Table S2. Average of treatments for microbial richness estimates and diversity index of ruminal contents of Holstein cows consuming conventional dried distillers grains with solubles or reduced-fat dried distillers grains with solubles.

Item	Treatment*				SEM†	P-Value‡		
	CONT	DG	rfDG	MIX		TRT	DG	RF
Good's coverage, %	98.3	98.2	98.3	98.3	0.03	0.29	0.21	0.58
Richness estimates								
Phylogenetic diversity	79.4	81.6	78.3	80.3	2.40	0.19	0.34	0.47
Chao1	2338.9	2394.8	2346.7	2386.2	687.78	0.17	0.11	0.77
Observed species	2032.1	2169.7	2000.6	2115.0	80.43	0.19	0.30	0.57
Diversity index								
Shannon	8.70	8.92	8.82	8.73	0.103	0.09	0.07	0.33

*CONT: a diet with no distillers grains; DGS: inclusion of 20% conventional dried distillers grains with solubles (DDGS); rfDG: inclusion of 20% reduced-fat dried distillers grains with solubles (RFDG); MIX: inclusion of 10% DDGS and 10% RFDG (Poet nutrition, Sioux Falls City South Dakota).

†The largest standard error of the mean is shown.

‡P-values for TRT: overall treatment effect; DG: CONT vs. DG+rfDG+MIX; RF: DG vs. rfDG.