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Source: Canadian Journal of Animal Science, 101(2) : 274-286

Published By: Canadian Science Publishing

URL: https://doi.org/10.1139/cjas-2020-0122

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NRC Research Press

ARTICLE

Effects of the dietary grain content on rumen and fecal microbiota of dairy cows

A. Kotz, P.A. Azevedo, E. Khafipour, and J.C. Plaizier

Abstract: Six non-lactating Holstein dairy cows received diets with forage to grain mixture ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50) that contained 0.5%, 10.0%, and 19.5% dry matter (DM) of starch, respectively. Rumen fluid and feces were sampled, and methane emissions were determined during the last week of 5 wk experimental periods. Taxonomic compositions of microbiota were determined using Illumina 16S rRNA sequencing. Increased grain feeding increased the acidity and volatile fatty acid concentrations of rumen fluid and feces, and decreased methane emissions expressed as L·kg^{-1} DM. Microbial diversities were highest for G25. The numbers of identified genera in rumen fluid were 185, 182, and 171 for G0, G25, and G50, respectively. In feces, these numbers were 197, 182, and 171 for these diets, respectively. In rumen digesta, seven genera were correlated positively to G0 and negatively to G50, and six genera were correlated negatively to G0 and positively to G50. In feces, 16 genera were correlated positively to G0 and negatively to G50, and 13 genera were correlated negatively to G0 and positively to G50. Increasing grain feeding affected a limited number of genera in rumen digesta and feces. This could not explain treatment effects on the functionalities of microbiota.

Key words: cattle, rumen, feces, grain, microbiota.

Résumé : Six vaches laitières holsteins non en lactation ont reçu des diètes avec rapport de mélange fourrage-grains de 100:0 (G0), 75:25 (G25), et 50:50 (G50) qui contenaient 0,5, 10,0, et 19,5 % d'amidon [selon les matières sèches (DM — « dry matter »)], respectivement. Le liquide du rumen et les fèces ont été échantillonnés et les émissions de méthane ont été déterminées pendant la dernière semaine de la période expérimentale de 5 sem. Les compositions taxonomiques du microbiote ont été déterminées au moyen du séquençage d'ARNr Illumina s16S. L'augmentation de grains a augmenté l'acidité et les concentrations d'acides gras volatils dans le liquide du rumen et les fèces, et a diminué les émissions de méthane exprimées en L·kg^{-1} DM. La diversité microbienne était la plus élevée pour G25. Le nombre de genres identifiés dans le liquide du rumen était de 185, 182, et 171 pour G0, G25, et G50, respectivement. Dans les fèces, c'était 197, 182, et 171 pour ces mêmes diètes, respectivement. Dans les digesta de rumen, sept genres ont corrélés de façon positive à G0 et de façon négative à G50, et six genres ont corrélées de façon négative à G0 et de façon positive à G50. Dans les fèces, 16 genres ont corrélé de façon positive à G0 et de façon négative à G50, et 13 genres ont corrélés de façon négative à G0 et de façon positive à G50. Augmenter la proportion de grains dans la diète a eu un effet sur un nombre limité de genres dans le liquide du rumen et les fèces. Ceci ne pouvait pas expliquer les effets des traitements sur les fonctions du microbiote. [Traduit par la Rédaction]

Mots-clés : bovins, rumen, fèces, grain, microbiote.

Introduction

Increasing the starch content of moderate-grain diets alters the taxonomic composition and reduces the richness and diversity of rumen microbiota in cattle (Fernando et al. 2010; Khafipour et al. 2016; Tun et al. 2020). These dietary changes increase the relative abundance of Firmicutes and reduce that of Bacteroidetes (Mao et al. 2013; Plaizier et al. 2017*a*; Tun et al. 2020).

Received 24 July 2020. Accepted 11 November 2020.

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However, the magnitudes of these changes vary greatly among studies and among animals within studies (Derakhshani et al. 2016*a*; Khafipour et al. 2016). These changes of the rumen microbiota may reflect changes in the availabilities of substrates for rumen microorganisms and a change in the rumen environment, such as a decrease in the pH (Russell and Rychlik 2001; Plaizier et al. 2018). In addition, when excessive amounts of starch are fed, the decrease of the rumen pH can be excessive, and rumen dysbiosis can occur (Khafipour et al. 2009, 2016; Tun et al. 2020). Increases in the dietary starch content also alter the functionality of rumen microbiota, e.g., by increasing the concentrations of volatile fatty acids (VFA) and free lipopolysaccharide endotoxins from Gram-negative bacteria in rumen digesta, by reducing the acetate to propionate ratio, as well as by reducing the methane production per amount of ingested dry matter (DM) (Hatew et al. 2015; Owens and Basalan 2016; Plaizier et al. 2018).

The effects of increased grain feeding on the microbiota in the large intestine have not been studied as much as those in the rumen (Li et al. 2012*b*). Studying these effects is, however, important, as the composition and functionality of hindgut microbiota greatly affect the health of the cow and the excretion of pathogens in feces (Callaway et al. 2010; Plaizier et al. 2012). The diversity of the rumen microbiome appears to be comparable to that of the large intestine when high-forage diets are fed, but it is lower when high-grain diets are fed (Khafipour et al. 2009; Krause et al. 2013; Plaizier et al. 2017*a*). Reasons for these responses are that feeding high-grain diets to cows increases the amount of dietary starch that escapes ruminal microbial degradation and digestion in the small intestine and that passes to the large intestine (Callaway et al. 2010). This increases the fermentation of starch in the large intestine, which can result in acidosis and dysbiosis in the hindgut (Gressley et al. 2011; Plaizier et al. 2012). In agreement, Plaizier et al. (2017*a*) showed that experimental induction of subacute ruminal acidosis (SARA) by adding grain to a moderate grain diet altered the taxonomic composition of the bacterial community in the cecum.

Due to the difficulty of sampling digesta from the large intestine, feces have been used as a proxy (Gressley et al. 2011; Krause and Khafipour 2010; Mao et al. 2012). In agreement of this approach, Plaizier et al. (2017*a*) showed that the changes in the populations of common bacterial species as determined by quantitative polymerase chain reaction (qPCR) resulting from this SARA were similar in cecal digesta and feces. Mao et al. (2012) also showed that induction of SARA by excessive grain feeding shifted the structure of the fecal microbial community, as the relative abundances of 88 of the 2116 detected operational taxonomic units (OTU) were affected by the SARA induction. In the studies from Mao et al. (2012) and Plaizier et al. (2017*a*), changes in the composition of the feces microbiota were determined when grain was added to a moderate grain diet to induce grain-based SARA. These changes are likely very different when grain is added to a diet where forage- and grain-based SARA is not induced. Such an addition of grain increases the complexity of the diet and thereby, provides substrate that can be utilized by more microbial taxa (Tapio et al. 2017; Matthews et al. 2019; Noel et al. 2019). Therefore, the main goals of this experiment were to determine the changes in the conditions and the taxonomic composition of microbiota in the rumen and feces resulting from increasing the grain content of forage-based diet to achieve a higher grain diet without inducing SARA.

Table 1. Ingredient and chemical composition of experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50) (%, dry matter basis).

	Diets		
Item	G0	G25	G50
Ingredients			
Grass hay	80	60	40
Alfalfa hay	20	15	10
Grain mixture[a]	0	25	50
Chemical composition			
Dry matter	95.2	95.4	95.6
Crude protein	13.6	13.5	13.4
ADF	36.6	30.0	23.3
NDF	56.0	47.3	38.7
Starch	0.5	10.0	19.5

Note: ADF, acid detergent fiber; NDF, neutral detergent fiber.

[a]The grain mixture included 34.6% wheat middlings, 30% barley, 10% corn, 10% wheat, calcium (1.63%), phosphate (0.770%), sodium (0.407%), chloride (0.665%), potassium (0.648%), magnesium (0.562%), and sulfur (0.208%) at a percent inclusion rate and iron (248.5 $mg \cdot kg^{-1}$), manganese (65.5 $mg \cdot kg^{-1}$), zinc (45.7 $mg \cdot kg^{-1}$), copper (7.9 $mg \cdot kg^{-1}$), selenium (0.34 $mg \cdot kg^{-1}$), cobalt (0.17 $mg \cdot kg^{-1}$), and iodine (0.035 $mg \cdot kg^{-1}$).

Materials and Methods

Experimental design

The experiment was conducted in accordance with Canadian Council on Animal Care guidelines (CCAC 2009), and it was pre-approved by the University of Manitoba Animal Care Committee. The study used six mature, non-pregnant, non-lactating, and non-cannulated Holstein dairy cows, including three primiparous and three multiparous cows. The cows were housed in a tie-stall facility at the University of Manitoba's Glenlea Research Station and received total mixed rations that contained grass hay and alfalfa hay, as well as either 0%, 25%, or 50% of a grain mixture (G0, G25, and G50) ad libitum (Tables 1 and 2).

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Table 2. Chemical composition of the grass hay (GH), alfalfa hay (AH), and grain mixture (G).

	GH	AH	G
DM (%)	95.3	95.1	96.0
Crude protein (% DM)	8.8	16.2	13.6
ADF (% DM)	34.1	30.5	13.3
NDF (% DM)	58.4	46.7	19.4
Starch (% DM)	0.5	0.46	38.5

Note: DM, dry matter; ADF, acid detergent fiber; NDF, neutral detergent fiber.

The experiment was set up as a replicated 3 × 3 Latin square design and consisted of three 5 wk experimental periods. In the first four weeks of each experimental period, cows were adapted to the experimental diets. During the days 2 and 4 of the fifth week of experimental periods, fecal and rumen fluid samples were taken at 0830 prior to AM feeding, and at 1500, i.e., at 6 h after the initial feeding of the day. Rumen fluid samples were collected using an oral stomach tube (Bassert and Thomas 2014). Fecal grab samples were collected using a sterile glove (Bassert and Thomas 2014). The pH of samples was determined with a pH probe (Fisher Scientific, Toronto, ON, Canada). Prior to samples being collected, the pH meter was calibrated using standard solutions of pH 4 and 7 (Fisher Scientific). The day following rumen and feces sampling, i.e., days 3 and 5, the cows were placed for 24 h in an open-hood calorimetric system to measure enteric methane as described by Odongo et al. (2008). Cows were familiarized with the hoods and chambers prior to the beginning of the experiment.

Processing and analysis of rumen and fecal samples

A representative subset of the fecal sample (5 g) was put in a 60 g whirltop bag (Fisher Scientific) and placed in liquid nitrogen for 2 min. The sample was then removed and stored at −80 °C until further analysis. A subset of the rumen fluid sample (10 mL) was collected and thoroughly mixed before being put in a 15 mL conical centrifuge tube (Corning Life Sciences, Fisher Scientific) and placed in liquid nitrogen for 2 min. The sample was then removed and stored at −80 °C until further analysis.

After the samples were thawed at room temperature, 3 mL of subsamples was taken into test tubes and mixed thoroughly with 0.6 mL 25% metaphosphoric acid. Subsequently, 0.24 mL of 25% sodium hydroxide and 0.384 mL of 0.3 mol·L^{-1} oxalic acid were added to the test tubes and vortexed. The mixture in the test tubes were then centrifuged at 3000*g* for 20 min, and 2 mL of supernatant was transferred into vials for VFA analysis. Concentrations of VFA were determined by gas chromatography (model 3900 Star, Varian, Walnut Creek, CA, USA) using a 1.83 m packed glass column (model 2-1721, Supelco, Oakville, ON, Canada). The injector and detector temperatures were set at 170 and 195 °C, respectively, with initial and final column temperatures set at 120 and 165 °C, respectively. The run time was 4 min followed by a 2 min thermal stabilization period.

Monitoring of methane emissions

Enteric methane emissions were measured using a modified open-hood calorimetric system similar to that from Odongo et al. (2008). Hence, only methane emissions from the forestomachs were recorded. The system allowed for real-time data analysis to be displayed and stored on a windows-based laptop computer using LabVIEW software version 11.0 (Professional, National Instruments, Vaudreuil-Dorion, QC, Canada) (Odongo et al. 2008). Modifications were made to the procedure described by Odongo et al. (2008). These included amendments to the calibration frequency, which now occurred daily and to the procedure that involved adding known concentrations of methane, carbon dioxide (CO_2), and dioxide (O_2) to calibrate the analyzers, the addition of valves to ensure consistent flow rate for both the chambers and the background air, and the installation of gauges to ensure that there was an optimum amount of each gas for the sensor to function. In brief, analyzer calibration of methane, CO_2, and O_2 occurred daily preceding the animal's entry into the chamber. The methane recovery rate of both chambers used in this study was measured at the end of each sample period by adding a known amount and concentration of methane into the chamber hood and comparing that to the amount of methane determined by the analyzer. These recovery rates were 95.1% ± 0.7% (mean ± standard deviation).

Measurements were gathered for a duration of 100 s sequentially from both chambers and background air then cycling back to the chambers again. For the background measurements, the tubing was changed to 8.5 cm to ensure a flow rate consistent with both chambers. Because the measurements occur in a negative pressure system, the values in the total production numbers were subtracted from the value of methane present in the background air.

DNA extraction and quality check

Approximately 200 mg of rumen fluid and feces sample was used for DNA extraction. The day preceding DNA extraction, samples were removed from the −80 °C freezer and placed in a 4 °C refrigerator overnight to thaw. The samples were then cryogenically homogenized with a Geno/Grinder® 2010 (SPEX SamplePrep, Metuchen, NJ, USA), subsequently, the DNA was extracted using a ZR-96 Fecal DNA Kit (Zymo Research, Irvine, CA, USA) that included a bead-beating step for mechanical lysis of bacterial cells. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA was then normalized to a concentration of 20 ng·μL^{-1}, and the quality of the amplified DNA was verified by polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal primers 27F (5′-GAAGAGTTTGATCATGGCTCAG-3′) and 342R

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(5′-CTGCTGCCTCCCGTAG-3′) as described by Khafipour et al. (2009). The amplicons were quality checked via agarose gel electrophoresis.

Library construction and Illumina sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. (2016*b*). In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (Caporaso et al. 2012). The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. Duplicates were run for all PCRs, with the contents of each reaction being 1.0 μL of prenormalized DNA, 1.0 μL of both forward and reverse primers (10 μmol·L^{-1}), 12 μL of high-performance liquid chromatography grade water (Fisher Scientific, Ottawa, ON, Canada), and 10 μL of 5 Prime Hot MasterMix (5 Prime, Inc., Gaithersburg, MD, USA). The PCR reactions occurred in an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany) and were composed of an initial denaturing step at 94 °C for 3 min followed by 35 amplification cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s. Upon completion of the amplification cycles, the final step was an extension step that occurred at 72 °C for a duration of 10 min. Upon completion, the PCR products were purified using the ZR-96 DNA Clean-up Kit (Zymo Research) to remove primers, deoxyribose nucleotide triphosphates, and reaction components. A 200 ng of product from each sample was pooled into the allocated V4 library.

The pooled samples were then quantified fluorometrically using Picogreen dsDNA (Invitrogen, Burlington, ON, Canada). This was followed by multiple dilution steps using a prechilled hybridization buffer (HT1; Illumina, San Diego, CA, USA) in an effort to bring the pooled amplicons to a final concentration of 5 pmol·L^{-1}. Following this, the final concentration of the pooled amplicons was measured using a Qubit version 2.0 Fluorimeter (Life Technologies, Burlington, ON, Canada). Finally, 15% of PhiX control library was administered into the amplicon pool to enhance the composition of unbalanced and biased bases, a known characteristic of low-diversity 16S rRNA libraries. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3′) were synthesized and subsequently purified via polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq reagent kit V2 (300-cycle; Illumina). The 150 paired-end sequencing reaction was performed using the MiSeq platform (Illumina) at the Gut Microbiome and Large Animal Biosecurity Laboratories (Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada). The sequencing data were deposited into the sequence read archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed via accession number SRR3202872.

Bioinformatic analyses

The FLASH assembler (Magoč and Salzberg 2011) was employed to merge the paired-end Illumina fastq files which overlapped. All sequences possessing mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was further analyzed by downstream computational pipelines of the open-source software package QIIME (Caporaso et al. 2010*b*). Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality filters to discard reads with ambiguous calls and those with Phred quality scores (*Q*-scores) below 20. Chimeric reads were filtered using UCHIME (Edgar et al. 2011), and sequences were assigned to OTU using the QIIME implementation of UCLUST (Edgar et al. 2010) at 97% pairwise identity threshold using an open-reference OTU picking process (Rideout et al. 2014).

Taxonomies were assigned to the representative sequence of each OTU using RDP classifier (Wang et al. 2007), and they were aligned with the Greengenes Core reference database (version 13.5) (DeSantis et al. 2006) using PyNAST algorithms (Caporaso et al. 2010*a*). To allow for further microbial community comparisons, a phylogenetic tree was built using FastTree version 2.1.3. (Price et al. 2010). Within community, diversity (α-diversity) was calculated using QIIME (Caporaso et al. 2010*b*). An alpha rarefaction curve was generated using Chao1 estimator of species richness (Chao 1984) with 10 sampling repetitions at each sampling depth. An even depth of approximately 15 700 sequences per sample was used for calculation of richness and diversity indices.

To compare the microbial composition between communities in the rumen and fecal samples, β-diversity was measured by calculating the unweighted UniFrac distances (Lozupone et al. 2012) using QIIME default scripts (Caporaso et al. 2010*b*). Principal coordinate analysis was applied on the resulting distance matrices to generate two-dimensional plots using PRIMER version 6 software (Warwick and Clarke 2006). Permutational multivariate analysis of variance (Anderson 2005) was employed to determine *P* values and test for significant differences of β-diversity among treatment groups.

Statistical analysis

Statistical analyses were performed as described by Li et al. (2012*b*). In brief, for DM intake (DMI), methane, as well as microbiota richness and diversity indices, statistical analyses were conducted using a completely randomized design using MIXED procedure of SAS version 9.3 (2011). The model included the fixed effects of period, cow, diet, and the period × diet interaction, and random effect of cow. Differences between means were determined using Tukey's test, and they were considered significant at $P < 0.05$. Trends were discussed at $P < 0.10$. The UNIVARIATE procedure of SAS was used for testing the normality of residuals.

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Table 3. Dry matter intake (DMI) and methane (CH_4) emissions of cows on experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50).

	Treatment[a]				
Item	G0	G25	G50	SEM	*P* value
DMI (kg)	11.4c	13.7b	15.6a	0.7	<0.01
CH_4 ($L \cdot d^{-1}$)	354.5c	423.3b	445.0a	10.2	<0.01
CH_4 ($L \cdot kg^{-1}$ DMI)[b]	31.1b	30.9b	28.5a	1.1	0.05

Note: SEM, standard error of means of samples. Statistical analysis was conducted using MIXED procedure of SAS.

[a]Barley grain ratio inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%).

[b]Adjusted DMI was an average of DMI on sample collection and CH_4 collection days.

Table 4. pH and volatile fatty acids (VFA) in rumen fluid and feces of cows on experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50).

	Treatment[a]				
	G0	G25	G50	SEM	*P* value
Rumen fluid					
pH	6.80a	6.68b	6.46c	0.05	<0.01
Acetate (%)	70.7a	67.4ab	66.6b	1.2	<0.05
Propionate (%)	16.4	16.2	18.4	1.8	0.15
Butyrate (%)	9.2b	10.8ab	11.8a	0.8	<0.01
Total VFA ($mmol \cdot L^{-1}$)	67.1c	76.8b	83.0a	2.5	<0.01
Ac:Pr	4.3a	4.2a	3.6b	0.08	<0.01
Feces					
pH	7.38a	7.02b	6.87c	0.06	<0.01
Acetate (%)	68.2	70.2	69.6	2.9	0.21
Propionate (%)	13.8	13.6	14.3	0.6	0.28
Butyrate (%)	8.0b	7.9b	9.1a	0.4	<0.05
Total VFA ($mmol \cdot L^{-1}$)	26.1b	26.5b	38.5a	4.2	0.02
Ac:Pr	5.1	5.1	4.9	0.2	0.5

Note: Means within a row with different lowercase letters differ ($P < 0.05$). SEM, standard error of means; Ac:Pr, ratio between acetate and propionate.

[a]Barley grain ratio inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%).

Partial least square discriminant analysis (SIMCA P+ 13.0, Umetrics, Umea, Sweden) was conducted on the genus data to ascertain the effects of treatments according to Li et al. (2012*a*). For this analysis, the *X* variables were bacterial genera, and the *Y* variables were the diets that varied most in grain concentration (G0 and G50).

Results

Dry matter intake, methane production, and conditions in the rumen and feces

Increasing the dietary grain content from 0% to 50% of DM increased the DMI from 11.4 to 15.6 $kg \cdot d^{-1}$ ($P \leq 0.01$) (Table 3). This change in grain feeding also increased the oral methane emissions from 354.5 to 44.0 $L \cdot d^{-1}$ ($P < 0.01$) (Table 3). However, methane emissions expressed per kilogram of DMI were 30.3, 30.1, and 27.6 $L \cdot kg^{-1}$ for the G0, G25, and G50 treatments, respectively, with that of the G50 treatment being lower ($P = 0.01$) than those of the G0 and G25 treatments. The increase in grain feeding from 0% to 50% of DM also reduced ($P < 0.01$) the rumen pH from 6.80 to 6.46 and the feces pH from 7.38 to 6.87 (Table 4). This increase also reduced the acetate to propionate ratio and the molar proportion of acetate, and increased the concentration of total VFA and the molar proportion of butyrate in rumen digesta. The concentrations of total VFA and the molar proportion of butyrate in the feces were increased by the increase in grain feeding, but the molar proportion of acetate and propionate and the acetate to propionate ratio in feces were not affected.

Rumen microbiota

The reads were quality filtered on the basis of the quality and length of the reads. The OTUs were aligned at a 97% similarity threshold against the Greengenes database.

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Table 5. Statistical summary for richness and diversity indices in rumen communities of cows on experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50).

	Treatment[a]				
	G0	G25	G50	SED	*P* value
Number of sequences per sample	10 000	10 000	10 000	—	—
Observed number of species	1043b	1122a	1051b	19.22	<0.001
Goods-coverage (%)	96.9b	96.6b	96.8ab	0.05	0.007
Chao1[b]	1307b	1418a	1325c	28.50	0.001
Shannon[c]	8.14b	8.43a	8.15c	0.09	0.003
Simpson[c]	0.986b	0.991a	0.986b	0.001	0.03

Note: Means within a row with different lowercase letters differ ($P < 0.05$). SED, standard error of difference between least square means of treatments.

[a]Barley grain ratio inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%)

[b]Based on Chao1 estimator of species richness.

[c]Based on Shannon and Simpson diversity estimators.

Although the majority of sequences are classified at the genus level, some could only be assigned to the phylum, class, order, and family levels. The average sequencing lengths for the V4 region were 253 nucleotides, and there was an average of 35 600 sequences per sample.

Table 5 shows the numerical values pertaining to the within-community diversity found in the rumen fluid samples. At a cut-off of 10 000 sequences per sample, the G25 treatment had the highest number of observed species ($P \leq 0.001$) compared with G0 and G50. The Chao1 estimator of richness values were 1307, 1418, and 1325 ($P = 0.001$) for the diets G0, G25, and G50, respectively. The values for the Shannon's diversity index for G0, G25, and G50 were 8.14, 8.43, and 8.14 ($P = 0.003$). The Simpson's diversity index showed values of 0.96, 0.99, and 0.99 ($P = 0.03$) for G0, G25, and G50, respectively. Based on the unweighted UniFrac distances, the bacterial communities of the three treatments were distinct ($P = 0.001$) (Fig. 1).

At the phylum level, there were 21 phyla present in the rumen fluid of animals on the G0 diet. Among those, Bacteroidetes, Firmicutes, Proteobacteria, Fibrobacteres, and Tenericutes were the most abundant in the rumen fluid. Investigation at the genus level showed that there were 185 genera present. Among those, 106 were classified genera, and 79 were unclassified. There were 22 phyla present in the rumen fluid of animals on the G25 diet. Among those, Firmicutes, Bacteroidetes, Proteobacteria, Fibrobacteres, and Tenericutes were the most abundant. With regards to the genera present in the rumen fluid at the G25 level, there were a total of 182 genera present. Of those 182 genera, 109 were classified, whereas 73 were unclassified. Finally, in the rumen fluid collected from animals offered diet G50, there were 22 phyla present. Among those, Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, and Tenericutes were the five most abundant phyla. For the rumen fluid collected from animals offered diet G50, there were also a total of 171 genera present. Of those 171, there were 100 classified genera as well as 71 unclassified genera present.

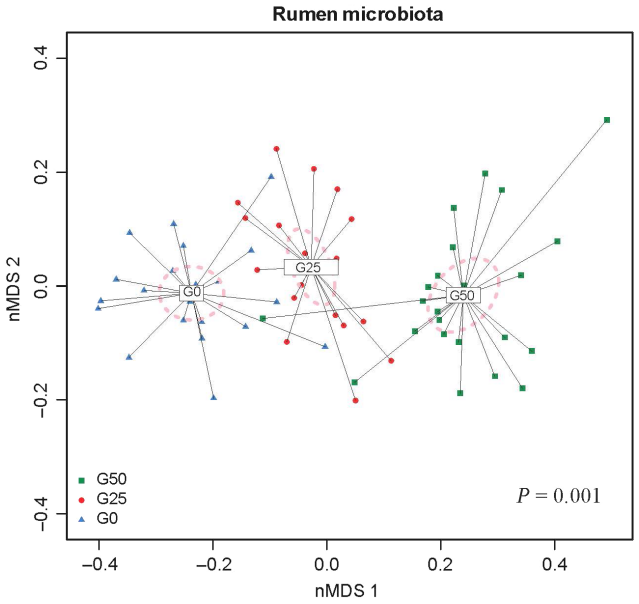


Fig. 1. Two-dimensional principal coordinate analysis plot based on the unweighted UniFrac distance matrix illustrating variation in rumen bacterial communities of experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50). [Colour online.]

Multivariate regression analysis comparing the differences in microbial communities in the rumen fluid between G0 and G50 is shown in Fig. 2. The R^2 and Q^2 of the regression estimates were 0.876 and 0.810, respectively. There were seven bacterial members that were positively correlated to G0 and negatively correlated to G50. These bacterial members belonged to the phylum Cyanobacteria (order YS-2), Proteobacteria (class Alphaproteobacteria), Bacteroidetes (genera BF311), Tenericutes (order ML615J-28 and RF-39),

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Fig. 2. Partial least squares discriminant analysis between an experimental diet with a forage to grain ratio of 100:0 (G0) and that with a forage to grain rate of 50:50 (G50) of rumen fluid samples. [Colour online.]

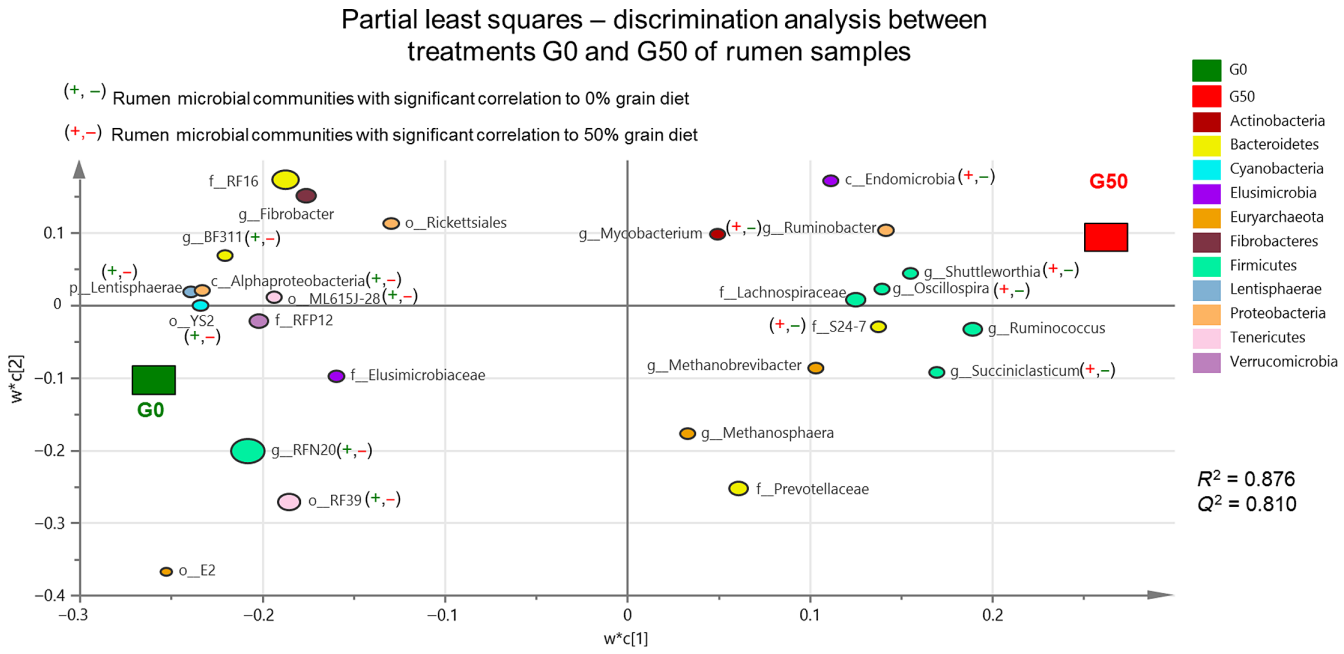


Table 6. Statistical summary for richness diversity indices in fecal bacterial communities of cows on experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50).

	Treatment[a]				
	G0	G25	G50	SED	*P* value
Number of sequences per sample	10 000	10 000	10 000	—	—
Observed number of species	1004b	1035a	961c	14.55	<0.001
Goods-coverage (%)	97.2b	97.1b	97.4a	0.001	<0.001
Chao1[b]	1244b	1289a	1182c	15.0	<0.001
Shannon[b]	8.39b	8.49a	8.31c	0.03	<0.001
Simpson[c]	0.99	0.99	0.99	—	—

Note: Means within a row with different lowercase letters differ ($P < 0.05$). SED, standard error of difference between least square means of treatments.

[a]Barley grain ratio inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%).

[b]Based on Chao1 estimator of species richness.

[c]Based on Shannon and Simpson's diversity estimators.

Firmicutes (genera RFN20), and the phyla Lentisphaerae itself. There were six bacterial members showing positive correlation to G50, while being negatively correlated to G0. They included members belonging to the phylum Elusimicrobia (class Endomicrobia), Proteobacteria (genus *Ruminobacter*), Bacteroidetes (family S24-7), and Firmicutes (genera *Shuttleworthia*, *Oscillospira*, and *Succiniclasticum*).

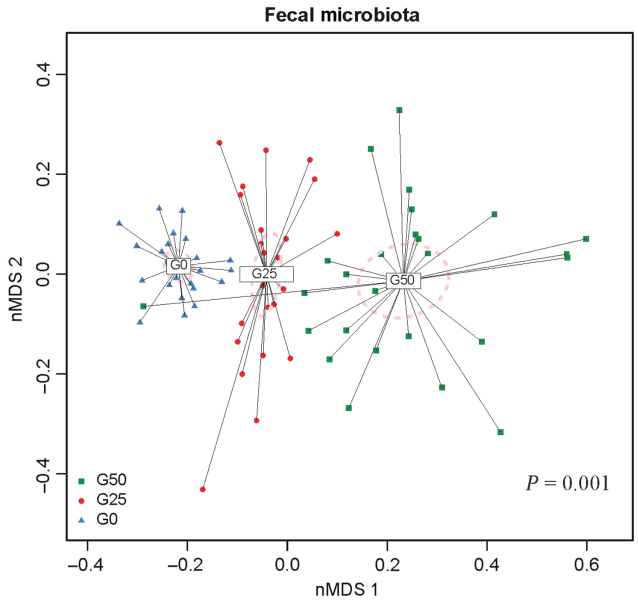
Feces microbiota

The reads were quality filtered based on the quality and length of the reads. The OTU were aligned at a 97% similarity threshold against the Greengenes database. Some sequences could only be determined to the phyla, class, order, and family levels rather than the genus level. The average sequencing lengths for the V4 region were 253 nucleotides, and there was an average of 28 500 sequences per sample.

Table 6 shows the numerical values pertaining to the within-community diversity found in the feces. At a cut-off of 10 000 sequences per sample, the number of observed species for diets G0, G25, and G50 were 1004, 1035, and 961 ($P \leq 0.01$), respectively. With regards to the Chao1 estimator of richness, the values were 1244, 1289, and 1182 ($P \leq 0.01$) for the diets G0, G25, and G50, respectively. The Good's coverage estimate values were 97.2%, 97.1%, and 97.4% ($P \leq 0.01$) for the diets G0, G25,

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Fig. 3. Two-dimensional principal coordinate analysis plot based on the unweighted UniFrac distance matrix illustrating variation in fecal bacterial communities as affected by experimental diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50). [Colour online.]



and G50, respectively. The Shannon's diversity index values for G0, G25, and G50 were 8.39, 8.49, and 8.31 ($P \leq 0.01$), respectively, showing the highest diversity in diet G25. Based on the unweighted UniFrac distances, the bacterial communities of the three treatments were distinct ($P = 0.001$) (Fig. 3).

At the phylum level, there were 16 phyla present in the diet G0. Among those, Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, and Verrucomicrobia were the most prevalent in the fecal samples. Investigation at the genus level showed that there were 197 genera present. Among those, there were 114 classified genera and 83 unclassified genera. There were 16 phyla present in the diet G25. Among those, Firmicutes, Bacteroidetes, Verrucomicrobia, Tenericutes, and Cyanobacteria were the most abundant. With regards to the genera present at the G25 level, there were a total of 182 genera present. Of those 182 genera, 109 were classified, and 73 were unclassified. Finally, in the diet G50, there were 17 phyla present. Among those, Firmicutes, Bacteroidetes, Tenericutes, Spirochaetes, and Cyanobacteria were the five most abundant phyla. For the diet G50, there were also a total of 171 genera present. Of those 171, there were 100 classified genera as well as 71 unclassified genera present.

Multivariate regression analysis comparing the G0 and G50 treatments is shown in Fig. 4. The R^2 and Q^2 estimates of this analysis were 0.957 and 0.914, respectively. There were 16 taxa that were positively correlated to G0 and negatively correlated to G50. They included members belonging to the phyla Actinobacteria (family Bifidobacteriaceae and Coriobacteriaceae), Proteobacteria (family Desulfovibrionaceae, genera *Campylobacter* and *Sutterella*), Verrucomicrobia (genus *Akkermansia*), Bacteroidetes (family BS11 and Bacteroidaceae), and Firmicutes (families Ruminococcaceae, Christensenellaceae, and Peptococcaeace, and the genera *Bulledia*, *Butyrivibrio*, L7AE11, *Oscillospira*, and *Dehalobacterium*). There were 13 taxa that were positively related to G50 and negatively correlated to G0. They included members of phyla Proteobacteria (family Enterobacteriaceae), Bacteroidetes (family S24-7, genera *Bacteroides*, *Prevotella*, and *Parabacteroides*), and Firmicutes (family Lachnospiraceae, genera *Blautia*, *Ruminococcus*, *Turicibacter*, *Sarcina*, SMB53, RC4-4, and *Anaerovibrio*).

Discussion

Conditions in rumen digesta and feces

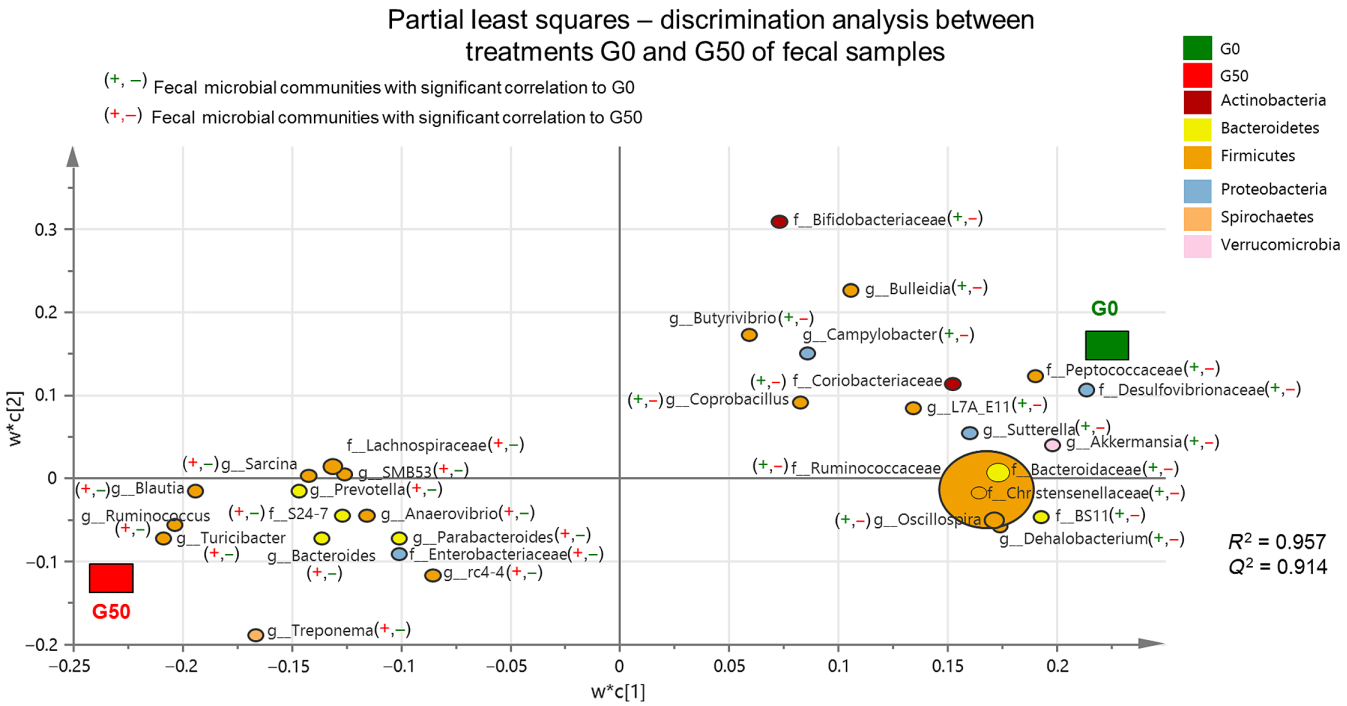
The reductions of the rumen pH and the acetate to propionate, and increases in the concentrations of VFA in the rumen due to the increases in grain feeding in our study confirm previous research (Petri et al. 2012; Mao et al. 2013; Plaizier et al. 2018). The rumen pH of the G50 diet was well above the threshold for SARA, indicating that this disorder did not occur (Plaizier et al. 2018). The rumen pH was assessed in rumen fluid collected with an oral stomach tube (Duffield et al. 2004). As methane emissions were determined in hoods, methane leakage from a rumen cannula would have compromised the accuracy of the measurement of the oral methane emissions (Beauchemin et al. 2012). In addition, rumen cannula would have allowed oxygen to enter the rumen, which could have affected its anaerobic microorganisms (Plaizier et al. 2018). The use of a stomach tube resulted in the inability to collect both solid and liquid digesta, likely resulting in a conservative reporting of fibrolytic bacteria (Kong et al. 2010; Mao et al. 2012; Petri et al. 2013).

The increase in the dietary starch content also reduced the pH and increased the concentrations of VFA in the feces. This suggests that this increase in dietary grain increased fermentation in the large intestine (Gressley et al. 2011; Mao et al. 2012; Plaizier et al. 2017*a*). The feces pH of the highest grain diet (G50) showed that hindgut acidosis was not induced by any of the experimental treatments (Gressley et al. 2011; Plaizier et al. 2018).

The reduction of the oral methane emissions per kilogram of DMI also agrees with earlier studies (Knapp et al. 2014; Hatew et al. 2015; Owens and Basalan 2016) and confirms that fermentation by amylolotic bacteria produces more propionate than that by fibrolytic bacteria (Russell and Rychlik 2001). The increase in absolute methane emissions expressed as litres per day due to the higher grain feeding was the result of an increase in DMI. This increase in DMI may have been the result of the reduction of the physical fill of the diet when the dietary content of grain increased (Allen 2000; Plaizier et al. 2008).

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Fig. 4. Partial least squares discriminant analysis between an experimental diet with a forage to grain ratio of 100:0 (G0) and that with a forage to grain rate of 50:50 (G50) of feces samples. [Colour online.]



The above shows that the increase in grain feeding changed the conditions in the rumen and large intestine as expected and reported previously. These changes suggest shifts in the functionality and taxonomic composition of rumen and hindgut microbiota (Russell and Rychlik 2001; Mao et al. 2012; Petri et al. 2012).

Rumen and fecal microbiota

In agreement with our study, several studies have shown that adding grain to moderate grain diets reduces the richness and diversity of rumen, hindgut, and fecal microbiota (Fernando et al. 2010; Mao et al. 2012; Petri et al. 2012; Plaizier et al. 2017*a*). The sizes of these reductions vary greatly among studies, which may be the result of differences in the dietary type and contents of grain, as well as experimental designs and methodologies (Khafipour et al. 2016). Reductions in the richness and diversity of microbiota may be detrimental, as this reduces their functionality and resilience, and make them more susceptible to invasion by other microorganisms, including pathogens (Khafipour et al. 2011; Levine and D'antonio 1999; Lozupone et al. 2012). However, benefits of less diverse rumen microbiota have also been suggested, as this may increase the efficiencies of nutrient utilization (Belanche et al. 2019).

The G25 treatment had the highest richness, diversity, and number of species of rumen and fecal bacteria. This was likely due to the fact that the G25 diet contained substantial amounts of cellulose and hemi-cellulose, as well as starch, which provided substrates to more groups of bacteria in the rumen and large intestine than the diet without concentrate (G0) (Russell and Rychlik 2001; Petri et al. 2012; Henderson et al. 2015). These results confirm that increasing diet complexity increases the richness and diversity of microbiota in the digestive tract of cattle (Tapio et al. 2017; Matthews et al. 2019; Noel et al. 2019).

Similar to our study, earlier studies have also used sequencing methodology to assess changes in the taxonomic composition of rumen microbiota due to increased grain feeding at the genus level (Asma et al. 2013). These studies agree that increased grain feeding increases the relative abundance of Firmicutes and reduces that of Bacteroidetes (Mao et al. 2013; Plaizier et al. 2017*a*, 2017*b*). However, at the genus level, results vary greatly among studies. Asma et al. (2013) reported that higher grain feeding increased the relative abundances of *Barnesiella*, *Oribacterium*, and *Olsenella* and decreased these abundances of 19 taxa, including Rikenellaceae, *RC9*, *Butyrivibrio*, and *Pseudobutyrivibrio*. Mao et al. (2013) identified 155 bacterial genera in rumen digesta and showed that feeding more grain reduced the relative abundances of *Prevotella*, *Treponema*, *Anaeroplasma*, *Papillibacter*, *Acinetobacter*, and unclassified Lentisphaerae, whereas it increased these abundances of *Ruminococcus*, *Atopobium*, unclassified Clostridiales, and *Bifidobacterium*. Plaizier et al. (2017*a*) found that increased grain feeding altered the relative rumen abundances of 9 out of the 90 identified genera. This included decreases in the abundances of *Rickettsiales*, Acholeplasmatales, Victivallaceae, *Sutterella*, S24-7,

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WCHB1-41, RFP12, and *Shuttleworthia*, and increases in the abundance of *Succinivibrio*. Increasing the starch content of dairy cow diets also increased the abundance of *Sharpea*, tended to increase those of *Ruminococcus*, *Megasphaera*, and *Shuttleworthia*, and decreased those of CF231 and BF31 in the study by Plaizier et al. (2017*b*). Tun et al. (2020) observed that higher grain feeding affected the relative abundances of 29 out of the 49 identified genera. This included reductions in the relative abundances of *Paludibacter*, *Prevotella*, BF311, CF231, YRC22, L7A_E1, *Succiniclasticum*, *Treponema*, *Anaeroplasma*, *Pyramidobacter*, and *Sutterella*, and increases in these abundances of *Butyrivibrio*, *Shuttleworthia*, *Staphylococcus*, and *Lactobacillus.*

Our study identified 185 genera in the rumen digesta, which is more than in the earlier studies. In agreement with these earlier studies, the relative abundances of most genera were not affected by the level of grain feeding. Of the 13 genera in our study whose relative abundances were positively related to higher grain feeding, only an increase in the abundance of *Shuttleworthia* was reported in an earlier study (Tun et al. 2020). In addition, the relative rumen abundances of major amylolytic genera such as *Prevotella*, *Streptococcus*, *Ruminobacter*, *Streptococcus*, and *Selenomas* (Russell and Rychlik 2001) were not increased by higher grain feeding in our study and in the earlier studies. This suggests that the changes in the relative abundances on microbiota genera in the rumen due to increased grain feeding cannot be explained by increases in substrates for the predominant amylolytic bacteria. This contrasts with studies on these populations of predominant bacterial species in the rumen with qPCR, as these populations are greatly affected by the availability of their substrates (Petri et al. 2012; Plaizier et al. 2017*a*; Tun et al. 2020).

Khafipour et al. (2016) suggested that differences among experiments on the effects of grain feeding on rumen microbiota at the genus level may be caused by differences in the methodologies of the collection and processing of rumen digesta, the methodologies for sequencing and bioinformatics, the types of grain and forages used, and the cows used in the experiments. Differences in methodologies are illustrated by the differences in the number of genera that are identified in each study. These studies also include small numbers of cows, varying from 4 to 12. As cows differ in the composition of their rumen microbiome, and this difference may be partially due to genetics, the use of a such small number of cows may have contributed to the differences among studies (Weimer 2015). In contrast to the results on the microbial sequencing, the effects of the increased grain feeding on the pH and VFA concentrations in the rumen were similar among studies. That may suggest that difference in taxonomic composition of rumen microbiota do not necessarily translate into differences in functionality of these microbiota. This was also suggested by Weimer (2015) and Tun et al. (2020), who showed that functionality of rumen bacteria is shared by different bacterial taxa. This implies that the prediction of the changes in functionality of gut microbiota based on changes in the abundances of individual taxa is not very accurate. Assessing the changes in this functionality may require metatranscriptomics (Tun et al. 2020).

As higher grain feeding increases the starch content of digesta and fermentation in the large intestine, higher grain feeding can be expected to alter the composition of microbiota in the hindgut and in the feces (Mao et al. 2013; Plaizier et al. 2017*a*). The decrease of the pH and the increase of the VFA concentrations in the feces due to the higher grain feeding in our study confirms that this also occurred in this study. The differences in β diversity of the dietary treatments confirmed that the level of grain feeding affected the taxonomic composition at the genus level of fecal microbiota. In agreement, the increase in the dietary grain content in our study altered the relative abundances of 88 out of the 2166 identified OTU. Out of these, the relative abundances of *Turicibacter* and *Stenotrophomonas* and the family Lachnospiraceae were increased, whereas these abundances of *Solibacillus* and *Lysinibacillus* were decreased. In agreement, Plaizier et al. (2017*a*) also demonstrated that increases in the starch content of dairy cow diets, where 73 bacterial genera were identified in the feces, and reported that the relative abundances of CF231 and YRC22 tended to be increased and those of *Paludibacter* and *Epulopiscium* tended to be decreased by the higher grain feeding. Plaizier et al. (2017*b*) also increased the starch content of dairy cow diets and reported that this affected the relative abundances of 25 out of the 89 identified bacterial genera. Of the affected bacteria, the relative abundances of S24-7, *Parabacteroides*, Bifidobacteriaceae, *Streptococcus*, Lachnospiraceae, *Clostridium*, and *Blautia* were increased. In contrast, the relative abundances of Pirellulaceae, *Akkermansia*, YS2, RF32, M2PT2-76, Desulfovibrionaceae, Victivallaceae, unclassified Fibrobacteraceae, Bacteroidaceae, RFP12, ML615J-28, Erysipelotrichacea, Gracilibacteraceae, Clostridiaceae, Peptococcaceae, Christensenellaceae, and *Anaerofustis* were decreased.

The increase in grain feeding in our study reduced the fecal pH, which indicates that the pH of hindgut digesta was also reduced (Plaizier et al. 2017*a*). However, this decrease in pH was smaller than that in the rumen, and the fecal pH of the G50 diet was well above the threshold for hindgut acidosis and a reduced functionality of most hindgut bacteria (Gressley et al. 2011; Li et al. 2016). Hence, it is unlikely that the increase in grain feeding in our study caused dysbiosis in the large intestine.

Similar to what was observed for rumen microbiota, the effects of increased starch feeding on the taxonomic composition of fecal microbiota at the genus level varied between our study and earlier studies, and among these earlier studies. Reasons for these differences may be similar as for these differences among rumen microbiota. However, in contrast to the rumen studies, feces were

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sampled, and the collection and processing of feces was similar among studies. Hence, the variation in the composition of fecal microbiota among studies could have been less than that of rumen microbiota. Still, similar to what is observed in the rumen, high grain feeding reduces the richness and diversity of feces microbiota. The effects of grain feeding on the taxonomic composition at the genus level are limited and variable, and it is difficult to be explained by the changes in available substrates for microorganisms. It can be assumed that the increase in starch availability in the large intestine increased populations of amylolytic bacteria, but as many different microorganisms are amylolytic, it is difficult to predict which amylolytic taxa will be most affected (Russell and Rychlik 2001; Khafipour et al. 2016; Plaizier et al. 2018). In our study, there were no genera in the feces that stood out as being affected by high grain feeding.

Conclusions

Increasing the grain content of non-lactating Holstein dairy cows from 0% to 50% of DM increased the concentrations of VFA and reduced the pH in rumen fluid and feces, reduced the acetate to propionate ratio in rumen fluid, and reduced the methane emissions per kilogram of DMI. This suggests that this increase in grain feeding altered the functionality of microbiota in the rumen and the large intestine. The highest microbial richness and diversity were found when the diet containing 25% DM of grain and 75% DM of forage was fed. The increase in grain feeding changed the taxonomic composition at the genus level of the microbiota in the rumen fluid and feces, and affected the relative abundances of a limited number of genera in rumen digesta and feces. The changes in the pH and the concentrations of VFA in rumen fluid and feces, and in the methane emissions, could not be explained by the changes in the relative abundances of these genera.

Acknowledgements

This study was supported by grants from the Agriculture and Agri-Food Canada's Agricultural Greenhouse Gas Program (to E. Khafipour) and Natural Sciences and Engineering Research Council of Canada (to J.C. Plazier). We thank Shucong Li, Hein Min Tun, and Hooman Derakhshani for their advice on the planning and execution of the experiments and the interpretation of the data. We also thank Terri Garner and Deanne Fulawka for their technical assistance, and the staff of the Dairy Research Unit at the Glenlea Research Station, University of Manitoba, for the maintenance and care of the animals.

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