Corn silage in dairy cow diets to reduce ruminal methanogenesis: Effects on the rumen metabolically active microbial communities

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ABSTRACT

Methane produced by the methanogenic *Archaea* that inhabit the rumen is a potent greenhouse gas and represents an energy loss for the animal. Although several strategies have been proposed to mitigate enteric CH₄ production, little is known about the effects of dietary changes on the microbial consortia involved in ruminal methanogenesis. Thus, the current study aimed to examine how the metabolically active microbes are affected when dairy cows were fed diets with increasing proportions of corn silage (CS). For this purpose, 9 ruminally cannulated lactating dairy cows were used in a replicated 3 × 3 Latin square design and fed a total mixed ration (60:40 forage:concentrate ratio on a dry matter basis) with the forage portion being either alfalfa silage (0% CS), corn silage (100% CS), or a 50:50 mixture (50% CS). Enteric CH₄ production was determined using respiration chambers and total rumen content was sampled for the determination of fermentation characteristics and molecular biology analyses (cDNA-based length heterogeneity PCR, quantitative PCR). The cDNA-based length heterogeneity PCR targeting active microbes revealed similar bacterial communities in cows fed 0% CS and 50% CS diets, whereas important differences were observed between 0% CS and 100% CS diets, including a reduction in the bacterial richness and diversity in cows fed 100% CS diet. As revealed by quantitative PCR, feeding the 100% CS diet increased the number of total bacteria, *Prevotella* spp., *Archaea*, and methanogenic activity, though it reduced protozoal number. Meanwhile, increasing the CS proportion in the diet increased propionate concentration but decreased ruminal pH, CH₄ production (L/kg of dry matter intake), and concentrations of acetate and butyrate. Based on these microbial and fermentation changes, and because CH₄ production was reduced by feeding 100% CS diet, this study shows that the use of cDNA-based quantitative PCR to estimate archaeal

growth and activity is not reliable enough to reflect changes in ruminal methanogenesis. A more robust technique to characterize changes in archaeal community structures will help to better understand the microbial process involved in ruminal methanogenesis and, hence, enabling the development of more effective dietary CH₄ mitigation strategies.

Key words: corn silage, alfalfa silage, ruminal methanogenesis, rumen microbe

INTRODUCTION

In ruminants, enteric CH₄ production is the result of CO₂ reduction by the methanogenic *Archaea* that use the hydrogen resulting from the anaerobic microbial fermentation of feeds. Methane is a potent greenhouse gas and represents about 25% of the annual anthropogenic CH₄ emitted into the atmosphere (Lassey, 2008). Moreover, methanogenesis is an energy waste for the ruminant, representing 2 to 12% of gross energy intake (Martin et al., 2010; Benchaar and Greathead, 2011). The level of CH₄ emission is mainly determined by feed intake and the composition of the diet (Boadi et al., 2004; Martin et al., 2010).

Because of its environmental and nutritional concern, several dietary strategies have been suggested to mitigate enteric CH₄ emissions from ruminants (Martin et al., 2010). It is well known that starch fermentation in the rumen favors propionate production at the expense of acetate and decreases ruminal pH, which reduces hydrogen availability and inhibits the activity of rumen methanogens (Martin et al., 2010; Hook et al., 2011a). Rumen protozoal numbers are also often decreased in ruminants fed high-starch diets, which also reduces the transfer of hydrogen from protozoa to methanogens (Morgavi et al., 2012). Compared with alfalfa silage (AS), corn silage (CS) contains higher amounts of starch, which make it an interesting means to reduce enteric CH₄ production.

Because of the symbiotic relationship between the ruminant and its microbiota, a greater understanding of how dietary changes affect the microbial consortia, especially the interactions between *Archaea* and other

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microorganisms, will enable the development of more robust mitigation strategies. So far, microbial quantification in the rumen was assessed by targeting DNA (Zhou et al., 2009; Lettat et al., 2012), which is not a reliable marker for metabolically active microbes because extracted DNA may arise from dead bacteria or bacteria in a dormant state, which are not implicated in the fermentation processes (Sheridan et al., 1998; de la Cruz-Leyva et al., 2011). On the contrary, RNA is more correlated with microbial growth rate and metabolic activity (Sheridan et al., 1998; de la Cruz-Leyva et al., 2011). Hence, its utilization is thought to be more reliable to quantify rumen microbial populations.

In a companion paper (Hassanat et al., 2013), we observed lower CH₄ energy losses in dairy cows when CS replaced AS in the diet. This was associated with lower ruminal pH, decreased protozoa numbers, and increased propionate proportion at the expense of acetate, suggesting possible shifts in microbial populations involved in substrate fermentation and CH₄ formation within the rumen. Therefore, the objective of this study was to examine the effects of replacing AS with CS in dairy cow diets on the metabolically active ruminal microbial communities (bacteria, *Archaea*, protozoa). This study also aimed to assess the reliability of length heterogeneity-PCR (**LH-PCR**) to fingerprint the bacteria and *Archaea* communities within the rumen.

MATERIALS AND METHODS

All animal procedures were conducted under the approval of the Animal Care Committee of the Dairy and Swine Research and Development Center (Sherbrooke, Canada) and were in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Experimental details (cows, diets, rumen content collection for the determination of pH and VFA, CH₄

measurements) of the present study have been described in a companion paper (Hassanat et al., 2013) and, therefore, will be briefly described in this section.

Dairy Cows and Diets

Nine multiparous Holstein lactating cows fitted with ruminal cannulas (10 cm, Bar Diamond Inc., Parma, ID) were used in a replicated 3 × 3 Latin square (31-d period). The cows averaged (mean ± SD) 688 ± 66 kg of BW, 82 ± 18 DIM, and 41 ± 4 kg/d of milk at the start of the experiment. They were fed a TMR (60:40 forage:concentrate ratio) twice daily (0900 and 1930 h) for ad libitum intake, with the forage portion being either alfalfa silage (**0% CS**), corn silage (**100% CS**) or a 50:50 mixture (**50% CS**). The cows were kept in individual stalls and had free access to water. Ingredient and chemical composition of the diets are shown in Table 1. More details on dairy cows and experimental diets are provided in a companion paper (Hassanat et al., 2013).

Rumen Fluid Collection for pH and VFA Determination

Sampling for the measurement of ruminal pH and VFA concentrations was performed as described in Benchaar et al. (2012). Briefly, ruminal fluid was collected from the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations within the rumen at 0 (i.e., before feeding), 1, 2, 4, 6, and 8 h after the morning feeding. Samples (250 mL/site) were withdrawn using a syringe screwed to a stainless tube ending with a probe covered by a fine metal mesh (RT Rumen Fluid Collection Tube, Bar Diamond Inc.). Ruminal fluid pH was measured immediately after sampling (Accumet pH meter; Fisher

Table 1. Ingredient and chemical composition of the experimental diets

Item	Treatment ¹		
	0% CS	50% CS	100% CS
Ingredient (% of DM)			
Corn silage	0.0	28.2	56.4
Alfalfa silage	56.4	28.2	0
Grass hay	3.2	3.2	3.2
Concentrate	37.7	37.5	37.4
Mineral and vitamin supplement	2.8	2.8	2.8
Chemical composition (% of DM)			
OM	91.7	92.8	94.2
ADF	23.4	20.2	17.0
CP	16.8	16.2	15.6
Starch	17.0	22.8	30.0

¹0% CS = diet with 0% corn silage; 50% CS = diet with 28.2% corn silage; 100% CS = diet with 56.4% corn silage.

Scientific, Montreal, Canada), and samples were acidified to pH 2 with 50% H₂SO₄ and frozen at -20°C for later determination of VFA concentrations as described in Hassanat et al. (2013).

Methane Production Measurement

Enteric CH₄ production was determined over 3 consecutive days using 2 air-flow-controlled chambers (1 animal/chamber). The measurements were based on the mass-balance approach, which consists of measuring flows of the air and the concentrations of CH₄ entering and leaving the chamber, and the difference between the incoming and the outgoing mass (i.e., air flow $\times$ concentration) of CH₄ was used to calculate the amount generated in the chamber by the animal. More details on enteric CH₄ measurements are given in the companion paper (Hassanat et al., 2013).

Rumen Sample Collection for RNA Extraction and cDNA Synthesis

Before (0 h) and 4 h after the morning feeding, ~ 2 kg of total ruminal content were collected from 5 different sites within the rumen. After manual homogenization, ~ 25 g of ruminal content were homogenized in ice using a PT 10/35 Polytron homogenizer (Kinematica GmbH, Bohemia, NY) at speed 6, for two 1-min cycles with 1 min of rest between cycles. Subsequently, representative aliquots of 1.5 g were stored at -80°C until RNA extraction. For each sampling time, samples of ruminal content were dried at 100°C for 72 h for DM determination.

Total RNA was recovered from frozen samples using the FastRNA Spin Kit (MP Biomedicals, Solon, OH) as previously described (Lettat et al., 2012) with minor modifications. Briefly, ~ 250 mg of frozen ruminal content were weighed into the provided tubes containing silica beads. For cell lysis, samples were subjected twice to a 1-min pulse, with 2 min of cooling at 4°C between the 2 pulses, using the Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK). The yield and the purity of the extracted RNA were assessed by optical density measurement with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Absorbance intensity at 260 nm was used to assess the concentration of RNA in 1 μL of sample, whereas sample purity was checked at 260:280 and 260:230 ratios.

For cDNA synthesis, extracted RNA was reverse transcribed using the AMV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions with minor modifications. Briefly, 1 μg of RNA was mixed with 0.5 μg of random primers in a 15- μL final volume and the mixture was brought to 70°C

for 5 min followed by 10 min on ice to allow annealing. Then, a 25- μL buffer mixture containing $1 \times$ AMV buffer, 1 μM of each dNTP, 1.6 U of RNasin and 3.6 U of AMV reverse transcriptase (Promega) was added. Reverse transcription was carried out at 37°C for 1 h using the C1000 thermal cycler (Bio-Rad Laboratories, Mississauga, Canada).

Bacteria and Archaea Quantification by Quantitative PCR

In this study, we quantified methanogenic *Archaea*, total bacteria, and specific bacteria that were selected based on their role in ruminal fermentation processes (i.e., starch and fiber degradation) and the utilization or production of hydrogen. The examined bacteria included *Prevotella* genus, which is the dominant amylolytic bacterial group and an efficient hydrogen utilizer for propionate production; the lactate-utilizing and propionate-producing bacterium *Megasphaera elsdenii*; the cellulolytic bacteria *Fibrobacter succinogenes*, a nonhydrogen-producing organism; and *Ruminococcus albus*, a hydrogen producing bacterium (Chaucheyras-Durand et al., 2010).

The SYBR green chemistry-based quantitative PCR was carried out using the 7500 Fast System (Applied Biosystems, Mississauga, Canada). Total and selected bacteria were quantified by targeting the small ribosomal subunit (*rrs*) RNA (Lettat et al., 2012), whereas the *rrs* and methyl coenzyme-M reductase (*mcrA*) RNA were used for methanogenic *Archaea* detection (Mosoni et al., 2011). In a 20- μL total volume, the quantitative PCR mixture was composed of $0.75 \times$ SYBR Premix Ex TaqII (Clontech Laboratories Inc., Madison, WI), 40 ng of cDNA template, and 0.25 μM of each forward and reverse primer, except for *Archaea* and *Prevotella* for which 0.3 and 0.5 μM were used, respectively. Each reaction was run in triplicate in 96-well plates (Applied Biosystems). The primer sequences and amplification programs used are summarized in Tables 2 and 3. An absolute quantification of the bacteria was performed using specific *rrs* DNA standards from *R. albus* 7 (ATCC 27210), *F. succinogenes* S85 (ATCC 19169), *Prevotella bryantii* B14 (DSM 11371), and *M. elsdenii* (DSMZ 20460). For methanogenic *Archaea*, *rrs* and *mcrA* DNA standards from *Methanobrevibacter smithii* (DSM 861) were used. Results of quantification are expressed as log₁₀ gene copies/g of DM of rumen content.

Protozoa Quantification by Quantitative PCR

The ruminal protozoa were quantified by targeting the 18S rRNA in the same conditions as those for

Table 2. Primers sequence used to target the *rrs* and *mcrA* ruminal RNA

Target	Primer set	Primer sequence 5' - 3'	Used for ¹	Source
Bacteria	520-F	AGC AGC CGC GGT AAT	qPCR	(Edwards et al., 2007)
	799-R2	CAG GGT ATC TAA TCC TGT T	qPCR	
<i>Prevotella</i> spp.	gPrevo-F	CAC RGT AAA CGA TGG ATG CC	qPCR	(Bekele et al., 2010)
	gPrevo-R	GGT CGG GTT GCA GAC C	qPCR	
<i>Fibrobacter succinogenes</i>	FibSuc3-F	GCG GGT AGC AAA CAG GAT TAG A	qPCR	(Stevenson and Weimer, 2007)
	FibSuc3-R	CCC CCG GAC ACC CAG TAT	qPCR	
<i>Ruminococcus albus</i>	RumAlb3-F	TGT TAA CAG AGG GAA GCA AAG CA	qPCR	(Stevenson and Weimer, 2007)
	RumAlb3-R	TGC AGC CTA CAA TCC GAA CTA A	qPCR	
<i>Megasphaera elsdenii</i>	MegEls1-F	GAC CGA AAC TGC GAT GCT AGA	qPCR	(Ozutsumi et al., 2006)
	MegEls1-R	CGC CTC AGC GTC AGT TGT	qPCR	
Archaea (<i>rrs</i>) ²	1174-F	GAG GAA GGA GTG GAC GAC GGT A	qPCR	(Ohene-Adjei et al., 2008)
	1406–1389-R	ACG GGC GGT GTG TGC AAG	qPCR	
Archaea (<i>mcrA</i>) ³	qmcrA-F	TTC GGT GGA TCD CAR AGR GC	qPCR	(Denman et al., 2007)
	qmcrA-R	GBA RGT CGW AWC CGT AGA ATC C	qPCR	
Protozoa	316-F	GCT TTC GWT GGT AGT GTA TT	qPCR	(Sylvester et al., 2004)
	539-R	CTT GCC CTC YAA TCG TWC T	qPCR	
Bacteria	27-F	6FAM-AGA GTT TGA TCM TGG CTC AG	LH-PCR	(Suzuki et al., 1998)
	355-R	GCT GCC TCC CGT AGG AGT	LH-PCR	
Archaea	mcrA-F	6FAM-GGT GTM GGD TTC ACH CAR TAY GC	LH-PCR	(Gagnon et al., 2011)
	mcrA-R	TTC ATN GCR TAG TTH GGR TAG TT	LH-PCR	

¹qPCR = quantitative PCR; LH-PCR = length heterogeneity-PCR.²*rrs* = small ribosomal subunit.³*mcrA* = methyl coenzyme-M reductase.**Table 3.** Amplification programs used for quantitative PCR (qPCR), length heterogeneity-small ribosomal subunit (LH-*rrs*), and length heterogeneity-methyl coenzyme-M reductase (LH-*mcrA*)

Assay	Target	Initial heating	Denaturation	Annealing or elongation	Final extension	PCR efficiency (%)
qPCR	<i>Bacteria</i>	95°C, 10 s	95°C, 15 s	58°C, 1 min	—	92
	<i>Prevotella</i>	95°C, 10 s	95°C, 15 s	55°C, 5 s then 72°C, 30 s	—	95
	<i>Fibrobacter succinogenes</i>	95°C, 10 s	95°C, 15 s	60°C, 30 s	—	102
	<i>Ruminococcus albus</i>	95°C, 10 s	95°C, 15 s	60°C, 30 s	—	92
	<i>Megasphaera elsdenii</i>	95°C, 10 s	95°C, 15 s	60°C, 30 s	—	92
	Archaea (<i>rrs</i>)	95°C, 10 s	95°C, 15 s	63°C, 15 s then 72°C, 30 s	—	100
	Archaea (<i>mcrA</i>)	50°C, 2 min then 95°C, 1 min	95°C, 15 s	60°C, 1 min	—	99.5
	Protozoa	95°C, 10 s	95°C, 15 s	60°C, 30 s	—	92
LH- <i>rrs</i>	<i>Bacteria</i>	94°C, 2 min	94°C, 1 min	55°C, 1 min then 72°C, 1 min	72°C, 20 min	—
LH- <i>mcrA</i>	Archaea	94°C, 2 min	94°C, 1 min	55°C, 1.30 min then 72°C, 1 min	72°C, 20 min	—

bacteria and *Archaea*, using 0.25 μM of each forward and reverse primer (Tables 2 and 3). An absolute quantification was made using 18S rRNA standards prepared as described in Sylvester et al. (2004) and total protozoal DNA extracted from fresh protozoal preparations. Briefly, protozoa were counted microscopically (Neubauer Improved Bright-Line counting cell, 0.1 mm depth; Hausser Scientific, Horsham, PA) and then were diluted to a range of 1.2×10^6 to 1.2×10^2 cells/mL. Genomic DNA was extracted using the FastDNA Spin Kit (MP Biomedicals) according to the manufacturer instructions and was used as a standard for protozoa quantification. The results were expressed as $\log_{10}$ 18S rRNA copies or $\log_{10}$ cells/g of DM. Only quantitative PCR assays that fell within the range of 90 to 110% of efficiency and that showed $R^2 \geq 0.99$ were considered for further analysis.

LH-PCR Fingerprints of Bacteria (*rrs* Gene) and Archaea (*mcrA* Gene)

Length heterogeneity-PCR based on natural length variations in the *rrs* (LH-*rrs*) and *mcrA* (LH-*mcrA*) genes was used to fingerprint the bacterial (Suzuki et al., 1998) and archaeal communities (Gagnon et al., 2011). Briefly, in a final volume of 25 μL , the LH-*rrs* and LH-*mcrA* mixtures were composed of 40 ng of cDNA template, 0.5 μM of each forward and reverse primer (Applied Biosystems), 0.1 mM of dNTPs (Promega), 1 $\times$ Taq buffer, 1.5 mM of MgCl_2 , and 0.625 U of Taq polymerase (BioShop Inc., Burlington, Canada) in a C1000 thermal cycler (Bio-Rad Laboratories). The primer sequences and amplification programs used are summarized in Tables 2 and 3. After PCR amplification, 0- and 4-h samples were pooled and capillary electrophoresis was performed as described in Barret et al. (2012). Briefly, 1 μL of template was mixed with 12.34 μL of Hi-Di Formamide (Applied Biosystems) and 0.06 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems). The mixture was then heated at 95°C for 5 min and chilled on ice. Capillary electrophoresis was performed for 40 min in the GeneScan mode on an ABI Prism 310 47-cm capillary DNA sequencer using POP-4 polymer (Applied Biosystems). The GeneMapper software (Applied Biosystems) was used for fingerprints analysis.

Statistical Analyses

Data were analyzed using the MIXED procedure of SAS with repeated measurements statement (SAS Institute Inc., Cary, NC). The statistical model included treatment and period as fixed effects and square and cows within square as random effects. For CH_4 data,

the fixed effect of the chamber was initially included in the model but was removed because it was not significant. Significant effect of treatment on least squares means was declared when $P \leq 0.05$, whereas tendencies were declared when $0.05 < P \leq 0.10$. The principal component analysis (PCA) was performed on the LH-PCR fingerprints using the PRINCOMP procedure of SAS using the COV option. Only the first 2 principal components were plotted. Indicator species analysis and multi-response permutation procedure were performed using the PC-ORD software (McCune and Mefford, 1999).

RESULTS AND DISCUSSION

Effects of Increasing CS Proportion of the Diet on Ruminal Fermentation Pattern and CH_4 Production

As expected, feeding the 100% CS diet decreased ruminal pH, acetate, and butyrate concentrations but increased propionate concentration (Table 4). This ruminal fermentation pattern is commonly observed in ruminants fed starch-rich diets (Benchaar et al., 2007; Khafipour et al., 2009). Methane production, expressed in liter per day, was lower for cows fed 0% CS and 100% CS diets compared with cows fed 50% CS diet. However, when expressed per kilogram of DMI to account for variation in feed intake between treatments, CH_4 production was 14% lower for cows fed 100% CS compared with cows fed 0% CS and 50% CS diets. This decrease is consistent with the shift in VFA pattern toward more propionate and less acetate observed in the current study.

Estimation of the Growth of the Metabolically Active Microbes

Bacteria. In our study, we aimed to quantify the metabolically active microbes by targeting RNA instead of DNA, which, up to now, has been widely used to monitor microbial communities within the rumen (Hook et al., 2011a; Mohammed et al., 2011). Regardless the type of the diet fed to cows, the bacterial number observed in the current study was less than 4×10^9 *rrs* copies/g of DM (Table 5), which is in agreement with values reported when traditional culture methods were applied (Mackie et al., 1978; Olumeyan et al., 1986). On the contrary, the total bacteria number observed in this study is much lower than the 10^{10} to 10^{12} *rrs* copies/g of DM classically reported when DNA was used as a marker (Mosoni et al., 2007; Lettat et al., 2012), which is surprising knowing that active cells harbor several rRNA copies. Similar to total bacteria number, *Prevotella* spp., *F. succinogenes*, *R. albus*, and

Table 4. Ruminal pH, VFA concentrations, and enteric CH₄ production in lactating cows fed diets with 0, 50, or 100% corn silage (CS)

Item	Treatment ¹			SE
	0% CS	50% CS	100% CS	
pH				
Mean	6.26 ^a	6.18 ^a	6.03 ^b	0.07
Minimum	6.05 ^a	5.95 ^a	5.66 ^b	0.09
VFA (mM)	99.6	97.8	95.0	4.27
Acetate	66.6 ^a	63.4 ^{ab}	57.3 ^b	2.57
Propionate	20.9 ^a	22.1 ^a	27.4 ^b	1.89
Butyrate	12.1 ^a	12.3 ^a	10.2 ^b	0.56
CH ₄				
L/d	612 ^b	671 ^a	604 ^b	31.9
L/kg of DMI	28.3 ^a	28.8 ^a	24.6 ^b	1.14

^{a,b}Means in a row that do not share a common superscript significantly differ ($P \leq 0.05$).

¹0% CS = diet with 0% corn silage; 50% CS = diet with 28.2% corn silage; 100% CS = diet with 56.4% corn silage.

M. elsdenii numbers were also 1 to 3 log units lower than reported in previous studies based on DNA quantification (Wanapat and Cherdthong, 2009; Lettat et al., 2012).

Archaea. For *Archaea*, the *rrs* mRNA was used to estimate their number, whereas the *mcrA* transcripts

were used as a biomarker for their methanogenic activity because this gene encodes the α -subunit of the *mcrA*, which catalyzes the last step of CH₄ production by methanogens (Thauer, 1998). In the present study, *mcrA* transcripts were lower than those of *rrs* (5.32 vs. 7.94 copies/g of DM, on average; Table 5), which can be

Table 5. Ruminal microbial numbers and archaeal methanogenic activity in ruminal fluid collected before (0 h) and 4 h after feeding from lactating cows fed diets with 0, 50, or 100% corn silage (CS)

Item ¹		Treatment ²			SE
		0% CS	50% CS	100% CS	
Bacteria (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	9.12 ^a	9.14 ^a	9.43 ^b	0.08
	4 h	9.20 ^a	9.31 ^a	9.55 ^b	0.08
	Mean	9.16 ^a	9.22 ^a	9.49 ^b	0.06
<i>Prevotella</i> spp. (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	8.18 ^a	8.17 ^a	8.51 ^b	0.08
	4 h	8.19	8.27	8.37	0.08
	Mean	8.19 ^a	8.22 ^a	8.44 ^b	0.05
<i>Fibrobacter succinogenes</i> (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	6.65	6.71	6.82	0.11
	4 h	6.74	6.77	6.94	0.11
	Mean	6.70	6.74	6.88	0.10
<i>Ruminococcus albus</i> (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	5.51	5.54	5.40	0.09
	4 h	5.70	5.75	5.74	0.09
	Mean	5.61	5.64	5.57	0.06
<i>Megasphaera elsdenii</i> (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	4.93	4.86	5.00	0.19
	4 h	4.75	5.02	5.14	0.19
	Mean	4.84	4.94	5.07	0.16
<i>Archaea</i> (log ₁₀ <i>rrs</i> copies/g of DM)	0 h	7.81 ^a	7.81 ^a	8.08 ^b	0.08
	4 h	7.96	7.94	8.05	0.08
	Mean	7.88 ^a	7.87 ^a	8.07 ^b	0.05
<i>Archaea</i> (log ₁₀ <i>mcrA</i> copies/g of DM)	0 h	5.25 ^a	5.35 ^{ab,A}	5.62 ^{b,B}	0.11
	4 h	5.27	5.12	5.31	0.11
	Mean	5.26 ^{AB}	5.23 ^A	5.46 ^B	0.09
Protozoa (log ₁₀ 18S copies/g of DM)	0 h	8.69 ^{a,A}	8.41 ^{a,B}	7.95 ^b	0.12
	4 h	8.60 ^a	8.43 ^a	8.01 ^b	0.12
	Mean	8.64 ^{a,A}	8.42 ^{a,B}	7.98 ^b	0.09
Protozoa (log ₁₀ protozoa/g of DM)	0 h	6.65 ^a	6.49 ^a	6.03 ^b	0.15
	4 h	6.89 ^a	6.74 ^a	6.22 ^b	0.15
	Mean	6.77 ^a	6.62 ^a	6.12 ^b	0.11

^{a,b}Means in a row that do not share a common superscript differ significantly ($P \leq 0.05$).

^{A,B}Means in a row that do not share a common superscript tended ($0.05 \leq P \leq 0.10$) to differ.

¹*rrs* = small ribosomal subunit; *mcrA* = methyl coenzyme-M reductase.

²0% CS = diet with 0% corn silage; 50% CS = diet with 28.2% corn silage; 100% CS = diet with 56.4% corn silage.

explained by the fact that *Archaea* have a single copy of *mcrA* but multiple *rrs* copies (Nunoura et al., 2006; Freitag and Prosser, 2009). Based on the *rrs* gene, the methanogenic *Archaea* number observed in this study is in accordance with previous reports with dairy cows (Zhou et al., 2011), heifers (Mohammed et al., 2011), steers (Zhou et al., 2009), and sheep (Mosoni et al., 2011) where DNA was used for methanogens quantification. This suggests that the methanogen community is mostly composed of living cells. On the contrary, the *mcrA* transcripts presented here are lower than previously reported by Popova et al. (2011) in bulls (1×10^9 copies/g of DM), Mosoni et al. (2011) in sheep (7.9×10^9 copies/g of DM), Hook et al. (2011a) in dairy cows (5.4×10^6 copies/g of wet weight), and Denman et al. (2007) in steers (10^8 to 10^9 copies/mL). The low *mcrA* transcript numbers observed in the current study could be related to amplification biases. Indeed, because *mcrA* transcripts were less abundant in the total RNA extracts, it may be possible that they were less accessible to the DNA-polymerase due to a dilution effect.

Protozoa. For a more accurate quantification, protozoa were quantified using standards made with 18S rRNA fragments and genomic DNA extracted from preparations with known cell number. Independent of the type of diet fed to cows, the protozoa numbers were greatest when expressed as 18S rRNA copies compared to when they were expressed as cell numbers (8.35 vs. 6.50 log copies/g of DM; Table 5), which is a normal pattern because a single protozoan cell carries several copies of the 18S rRNA gene (Sylvester et al., 2005). Moreover, the protozoa can adjust their 18S rRNA gene copy number to adapt to ruminal environmental conditions, such as substrate availability (Sylvester et al., 2009).

Effects of Increasing CS Proportion of the Diet on Active Microbial Populations

Bacteria. As expected, total bacteria were more abundant ($P < 0.05$) in cows fed the 100% CS diet compared with cows fed 0% CS and 50% CS diets (Table 5). This was likely due to a greater bacterial proliferation caused by the supply of more readily fermentable carbohydrates (i.e., starch) in the 100% CS diet (Table 1). This higher bacterial population was partly due to an increment ($P < 0.05$) in the number of the amylolytic bacterial group *Prevotella* spp. Previous studies also reported an increase in amylolytic bacteria that grow rapidly when more fermentable substrate is available (Tajima et al., 2001; Callaway et al., 2010). Moreover, as already evidenced by previous studies that targeted DNA, the use of RNA confirms that *Prevotella*

spp. is the dominant bacterial group within the rumen (Nagaraja and Titgemeyer, 2007; Bekele et al., 2010). *Prevotella* spp. is a well-known propionate-producing bacterial group via the succinate pathway (Purushe et al., 2010), and its proliferation in cows fed the 100% CS diet resulted in a greater ruminal propionate concentration at the expense of acetate and butyrate (Table 4). The lactate-utilizing bacterium *M. elsdenii* was numerically more abundant ($P = 0.15$) at 4 h after feeding in cows fed the 100% CS diet compared with cows fed the 0% CS diet. Higher numbers of *M. elsdenii* may be an indication of a greater lactate production at 4 h after feeding in cows fed the 100% CS diet, as this bacterium has been reported to metabolize up to 60 to 80% of ruminal lactate (Counotte et al., 1981) and is also known as the only bacterium that metabolizes lactate to propionate through the acrylate sink (Hino and Kuroda, 1993).

Replacing AS with CS in the diet reduced ($P < 0.05$) mean and minimum daily ruminal pH (Table 4) but did not affect the number of the cellulolytic bacteria *R. albus* and *F. succinogenes* (Table 5). However, although mean ruminal pH decreased ($P < 0.05$) from 6.47 before feeding to 5.69 after feeding (data not shown), *R. albus* numbers increased ($P < 0.05$) by 0.34 log units in cows fed the 100% CS diet (Table 5). It is generally assumed that low ruminal pH has a negative effect on the growth of cellulolytic bacteria (Bekele et al., 2010; Fernando et al., 2010), but this has not been always the case, as others reported no change (Martin et al., 2001; Stevenson and Weimer, 2007) or even an increase (Olumeyan et al., 1986; Khafipour et al., 2009) in the number of cellulolytic bacteria. According to our findings and findings in the literature, it seems that the effect of diets on the growth of cellulolytic bacteria is variable and multifactorial because ruminal pH, the capacity of the different species to adapt to dietary changes, and the interactions among different species are all factors that may account for the discrepancies between studies (Lettat, 2011).

Archaea and Protozoa. *Archaea* (at 0 h and mean) were more abundant ($P < 0.05$) in cows fed the 100% CS diet compared with cows fed the 0% CS and 50% CS diets (Table 5). Similarly, their methanogenic activity at the 0-h sampling time estimated with the number of *mcrA* transcripts was greatest ($P < 0.05$) for cows fed 100% CS diet compared with cows fed 0% CS and 50% CS ($P = 0.10$) diets. At 4 h after feeding, archaeal methanogenic activity was similar among diets, but the mean activity tended ($P = 0.10$) to be greater in cows fed 100% CS diet than in cows fed 50% CS diet. For cows fed the 100% CS diet, archaeal methanogenic activity decreased ($P < 0.05$) after feeding, but their

Figure 1. Electropherograms of DNA fragments from *Archaea* [length heterogeneity-methyl coenzyme-M reductase (LH-*mcrA*)] and bacteria [length heterogeneity-small ribosomal subunit (LH-*rrs*)] within the rumen of a single lactating cow fed the 100% corn silage (CS) diet.

number remained unaffected. This was probably due to the decrease in ruminal pH (-0.78 pH units; $P < 0.05$) and is supported by previous reports that showed that low ruminal pH impairs methanogens activity but not their growth (Van Kessel and Russell, 1996; Hook et al., 2011a).

Contrary to our observations on bacteria and *Archaea*, increasing the proportion of CS in the diet was accompanied by a decrease in the protozoal population, which is in line with the decrease in butyrate concentration (Table 4), as protozoa are known to be an important contributor to butyrate production within the rumen (Eugène et al., 2004). When expressed as 18S rRNA copies, the protozoal number was reduced ($P < 0.05$) in cows fed 100% CS compared with cows fed the 0% CS and 50% CS diets. Moreover, the protozoal population (at 0 h and mean) tended ($P = 0.10$) to be lower in cows fed the 50% CS diet compared with cows fed the 0% CS diet. When expressed as cell number, feeding cows the 100% CS diet decreased ($P < 0.05$) the number of protozoa compared with feeding 0% CS and 50% CS diets. To the best of our knowledge, studies dealing with the effects of forage type on the protozoal population are very scarce, and those reporting diet change effects have been inconsistent and revealed either an increase (Hook et al., 2011a), a decrease (Khafipour et al., 2009), or no change (Benchaa et al., 2007) in the protozoal population. One possible explanation for the decrease in protozoa number in the present study could be a toxic effect of propionate associated with a low ruminal pH (minimum pH = 5.66; Table 4), as previously suggested (Fonty et al., 1995; Lettat, 2011).

Effects of Increasing CS Proportion of the Diet on Microbial Diversity

The LH-PCR fingerprint method used to characterize the bacterial community detected a total of 56 raw peaks. An iterative process of standardization (Dunbar et al., 2001) was applied so that the total peak height reflects an equivalent amount for each profile. This process ensures eliminating peaks with low original percentages (less than 2.5%). The standardization reduced the number to 26 true comparable peaks of specific amplicons with a length ranging from 327 to 370 base pairs.

Compared with *Bacteria*, only 1 single peak (464 bp; Figure 1) was observed for *Archaea* using LH-*mcrA*, which means that archaeal community is less diverse than the bacterial one (Aller and Kemp, 2008). Moreover, as recently pointed out in a study on swine manure (Barret et al., 2012), our results showed that the LH-PCR technique is not sensitive enough to study the archaeal species structure and diversity within the rumen compared with DGGE and clone library construction (Zhou et al., 2009; Popova et al., 2011). According to recent reports on swine manure (Gagnon et al., 2011; Barret et al., 2012), the 464-bp amplicon could be identified as *Methanobrevibacter* spp., which is consistent with numerous reports that showed the dominance of *Methanobrevibacter* within the rumen (Hook et al., 2011a; Zhou et al., 2011).

Principal component analysis allows identification of the most important directions to explain the variation in a multivariate data matrix in a graphical plot. Thus,

Figure 2. (A) Principal component (PC) analysis ordination of bacterial length (L) heterogeneity-small ribosomal subunit amplicons for samples collected from lactating cows fed diets with 0, 50, or 100% corn silage (CS). The percentage of total variance accounted for by each of the 2 principal components (PC1 and PC2) is shown in parentheses. (B) Diversity indices of the bacterial community from lactating cows fed diets with 0, 50, or 100% CS. Error bars represent SEM. Lowercase letters (a,b) indicate significant differences ($P \leq 0.05$) among diets.

PCA was used to visualize how the bacterial communities are affected when cows were fed different proportions of CS. The first 2 principal components explain 77.3% of the total variance (Figure 2A). According to the PCA analysis, the 343-, 350-, and 351-bp amplicons seem to be the most important. Moreover, the 343- and 350-bp amplicons were related to the 100 and 0% CS diets, respectively. Using the indicator species analysis, which allows identification of amplicons responsible for differences among groups (Dufrêne and Legendre, 1997),

we observed a higher abundance of the 334-, 340-, and 370-bp amplicons in cows fed the 0% CS diet, whereas the 327-, 343-, and 362-bp amplicons were more represented in those fed the 100% CS diet (Table 6). The diversity indices calculated from the LH-*rrs* fingerprints revealed that feeding the 100% CS diet decreased ($P < 0.05$) the bacterial community richness (6.44 vs. 7.89) and diversity (1.47 vs. 1.75; Figure 2B). The evenness remained similar among diets, suggesting that the relative abundance of the different species making up the richness of the bacterial community was similar. In line with previous reports, this study confirms that providing more readily fermentable carbohydrates decreases the bacterial richness and diversity (Khafipour et al., 2009; Hook et al., 2011b).

Microbial Population Shifts and Ruminal Methanogenesis

Results of the current study revealed that increasing starch supply via increasing the proportion of CS in the diet reduced CH_4 production (L/kg of DMI). Our results are in line with previous reports, confirming that ruminal methanogenesis is not strongly linked to archaeal number (Popova et al., 2011; Morgavi et al., 2012). Popova et al. (2011) reported that methanogenesis reduction in feedlot bulls fed a starch-rich diet supplemented with lipids was associated with a decrease in the archaeal methanogenic activity. This is in opposition to the findings of the present study, where a greater archaeal methanogenic activity was associated with a reduction in CH_4 production (L/kg of DMI). This discrepancy may be explained by the experimental conditions and the methodology used by Popova et al. (2011) for RNA extraction and *mcrA* quantification. Compared with our work, in the study by Popova et al. (2011), ruminal content was collected from slaughtered animals (after 24 h of fasting) and archaeal methanogenic activity was estimated using a relative quantification procedure.

In the present study, feeding the 100% CS diet decreased the number of protozoa, which is consistent with the lower CH_4 production (L/kg of DMI) observed. Several studies also reported lower CH_4 production when the number of protozoa decreased (Morgavi et al., 2010; Patra and Yu, 2012). Because protozoa are important hydrogen producers, a decrease in the transfer of hydrogen between the protozoa and the methanogens is probably at the origin of the observed reduction in ruminal methanogenesis (Martin et al., 2010; Morgavi et al., 2012). Moreover, feeding a 100% CS diet increased the number of total bacteria and the propionate-producing group *Prevotella* spp. as well as propionate concentration. These microbial and fer-

Table 6. Indicator value (%) of the bacterial length heterogeneity-small ribosomal subunit fingerprints from lactating cows fed diets with 0, 50, or 100% corn silage (CS)¹

Amplicons (bp)	Treatment ²			SD	P-value ³
	0% CS	50% CS	100% CS		
327	0	0	33	7.4	0.08
334	40	10	0	8.5	0.06
340	33	0	0	7.3	0.08
343	4	12	46	7.4	0.02
362	1	7	46	8.7	0.03
370	41	31	0	7.4	0.07

¹Indicator value (IV) = relative abundance × relative frequency. Maximum IV for each amplicon is indicated in bold.

²0% CS = diet with 0% corn silage; 50% CS = diet with 28.2% corn silage; 100% CS = diet with 56.4% corn silage.

³Indicator values were tested for significance using a Monte Carlo technique.

mentation shifts represent competitive pathways that divert away hydrogen from methanogenesis, and could therefore explain the observed reductions in CH₄ (L/kg of DMI) production (Martin et al., 2010; Morgavi et al., 2010).

Regarding the microbial structure, previous studies reported a decrease in CH₄ production when the archaeal richness and diversity were reduced (Zhou et al., 2009; Popova et al., 2011). Although our attempt to estimate archaeal diversity was unsuccessful using the LH-PCR technique, the present study suggests that a less diverse bacterial community may result in lower CH₄ production. As evidenced by indicator species analysis and multi-response permutation procedure analyses, feeding the 100% CS diet affected the bacterial community composition and, therefore, the fermentation pathways compared with feeding the 0% CS diet. The identification of the amplicons revealed in the current study, and especially those highly represented in cows fed the 0% CS (334-, 340-, and 370-bp) and the 100% CS (327-, 343-, and 362-bp) diets, may contribute to enhancing our understanding of how the bacterial community is affected by dietary changes and enabling prediction of CH₄ response and, hence, the development of more robust mitigation strategies.

CONCLUSIONS

This work aimed to investigate how the active microbial communities are affected by replacing AS with CS in dairy cow diets. Increasing the CS proportion in the diet (i.e., increasing starch supply) reduced the richness and the diversity of the bacterial community but increased the number of total bacteria and *Prevotella* spp., and favored propionate production. In this study, CH₄ reduction can be linked to the decrease in protozoa but not to *Archaea* number or methanogenic activity that were increased by the 100% CS diet. Based on mi-

crobial and fermentation changes, this study shows that the use of cDNA-based quantitative PCR to estimate archaeal growth and activity is not reliable enough to reflect changes in ruminal methanogenesis. In addition to the shifts observed within the bacterial community, a modification in the archaeal community structure toward less efficient CH₄-producing species is probable and deserves further investigation.

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