Microbial community structure of the bovine rumen as affected by feeding cashew nut shell liquid, a methane-inhibiting and propionate-enhancing agent

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ABSTRACT

The effect of cashew nut shell liquid (CNSL) feeding on bacterial and archaeal community of the bovine rumen was investigated by analyzing clone libraries targeting 16S rRNA genes, methyl-coenzyme reductase A-encoding genes (*mcrA*), and their respective transcripts. Rumen samples were collected from three non-lactating cows fed on a hay and concentrate diet with or without CNSL supplementation. DNA and complementary DNA (cDNA) libraries were generated for investigating rumen microbial communities. MiSeq analysis also was performed to understand more comprehensively the changes in the microbial community structures. Following CNSL supplementation, the number of operational taxonomical unit (OTU) and diversity indices of bacterial and archaeal community were decreased. Bacterial OTUs belonging to *Proteobacteria*, including *Succinivibrio*, occurred at a higher frequency with CNSL feeding, especially in cDNA libraries. The methanogenic archaeal community became dominated by *Methanomicrobium*. A bacterial community shift also was observed in the MiSeq data, indicating that CNSL increased the proportion of *Succinivibrio* and other genera known to be involved in propionate production. Methanogenic archaeal community shifts to increase *Methanoplanus* and to decrease *Methanobrevibacter* also were observed. Together, these results imply the occurrence of significant changes in rumen communities, not only for bacteria but also for methanogens, following CNSL feeding.

Key words: *archaea, bacteria, cashew nut shell liquid, methane, rumen*

1. INTRODUCTION

Cattle emit methane gas, primarily via belching, accounting for approximately 20% of global anthropogenic methane emission (IPCC, 2007). There is an urgent need to reduce this source of methane strategically, for instance, by dietary manipulation to provide rumen fermentation changes without apparent decreases in animal productivity. The manipulation has to be implemented in an effective and safe manner, and functional feeds and feed additives for this purpose have been explored for the last two decades (Kobayashi et al. 2016; Broucek, 2018) after concerns were raised regarding antibiotic use (FAO 2011).

Cashew nut shell liquid (CNSL) is a potential feed additive for reducing methane synthesis and enhancing propionate production in the rumen of cattle (Watanabe et al., 2010; Shinkai et al. 2012) without adverse effects on the feed intake, feed digestibility, and milk yield of dairy cows (Coutinho et al. 2014). Such favorable fermentation changes also have been reported in sheep (Kang et al. 2018) and local ruminants, including Thai native cattle and swamp buffaloes (Konda et al. 2019). At the same time, some reports have failed to observe a strong impact of CNSL on rumen microbes and fermentation (Branco et al. 2015). The difference has been attributed to the use of heat-treated CNSL, which appears to have decreased functionality (Watanabe et al. 2010).

A key issue leading to significant fermentation changes with CNSL feeding is the selective antimicrobial action against rumen microbes by phenolics of CNSL, notably including anacardic acid (Oh et al. 2017a), a compound that possesses surfactant activity (Kubo et al. 1993; Oh et al. 2017b). Antimicrobial activities might induce in a shift of the rumen microbial community, resulting in decreased methane synthesis and a

higher propionate production, given that these pathways competitively consume metabolic hydrogen in the rumen (Ungerfeld 2015).

Responses of representative rumen bacterial species to CNSL and its compositional phenolics have been elucidated by pure culture studies (Watanabe et al. 2010; Oh et al. 2017a). That work indicated that growth of hydrogen- and formate-producing bacterial species were inhibited by those phenolics, while succinate- and propionate-producing species were not. The inhibited bacteria would normally contribute to methane synthesis by providing substrates for methanogenesis, while the uninhibited bacteria would enhance propionate production as an alternative metabolic hydrogen (electron) sink, thereby indirectly suppressing methane synthesis. Quantitative PCR (qPCR) analyses of the rumen contents in animals fed CNSL have supported these pure culture results, revealing that CNSL feeding results in increases in the proportions of *Prevotella* and *Succinivibrio* (Shinkai et al. 2012; Konda et al. 2019), both of which are involved in propionate production.

In contrast, deeper examinations of the influence of CNSL feeding on the rumen microbial community structure has (to our knowledge) been scarce. The sole exception appears to be the work of Konda et al. (2019), who described shifts in both the bacterial and archaeal communities upon feeding of CNSL to Thai local ruminants (native cattle and swamp buffaloes). Although in vitro analysis has elucidated microbial community shifts following CNSL supplementation (Danielsson et al. 2014; Schnurer et al. 2014), such short-term experiments may not fully explore the practical effects of CNSL on rumen microbes. When global application of CNSL is considered, comprehensive analysis using a broader range of ruminant animals fed CNSL is obviously necessary, because precise understanding of the actions of such a new additive can facilitate

97 approval of the additive by the animal industry and farmers in the targeted geographical
98 regions.

99 Here, we describe rumen bacterial and archaeal community shifts that were
100 observed in Holstein cows in response to CNSL feeding; as reported previously, feeding
101 with this supplement resulted in a significant decrease in methane emission and an
102 increase in propionate production (Shinkai et al. 2012). The microbial community
103 structure was evaluated by traditional clone library analysis based on DNA and
104 transcripts, together with more comprehensive MiSeq analysis.

105

106 **2. MATERIALS & METHODS**

107 **2-1. Animals, diets, and samplings**

108 Animal experiments, including feeding, management, and sampling, were
109 officially approved with the number of 09021201 by following the guidelines of the
110 Animal Care Committee of the National Institute of Livestock and Grassland Science,
111 Tsukuba, Japan. All procedures were the same as those described by Shinkai et al.
112 (2012).

113 In brief, 3 ruminally fistulated non-lactating Holstein cows were repeatedly
114 employed for two feeding trials. Cows were housed individually in stalls and fed twice
115 daily (0930 and 1630) with a basal diet consisting of a concentrate and chopped
116 Timothy hay in a 6:4 ratio. This diet was designed to provide 1.3-fold of the
117 maintenance energy requirement specified by the 2006 Japanese feeding standard for
118 dairy cattle. The concentrate contained 24.3% neutral detergent fiber and 18.5% crude
119 protein.

120 Raw CNSL, mechanically pressed from cashew nut shells, was provided by

121 Idemitsu Kosan Co., Ltd. (Tokyo, Japan). Two types of pellets (pellets A and B) were
122 formulated for the present feeding trials. For use in Trial 1, CNSL was mixed with silica
123 powder (6:4), and the mixture was pelleted (pellet A). For use in Trial 2, the palatability
124 of CNSL was improved by mixing with alfalfa meal (41.3%), defatted rice bran (20.0%),
125 silica powder (11.3%), crude sugarcane molasses (3.0%), and tapioca flour (2.4%), and
126 the mixture was pelleted (pellet B). The final concentrations of CNSL in pellets A and B
127 were 60% and 22%, respectively. Vehicle pellet B was prepared with the same feed
128 ingredients as in pellet B but omitting the CNSL; the resulting vehicle pellets were fed
129 as a control additive in Trial 2.

130 The cows were fed a basal diet without CNSL (Trial 1) or with the addition of
131 vehicle pellet B (Trial 2) for 4 weeks (control period), and then the same basal diet was
132 supplemented with CNSL-containing pellets (pellets A and B for Trials 1 and 2,
133 respectively) for 3 weeks (CNSL period). To erase or minimize a possible effect of
134 ambient conditions, the cows in the present experiment were maintained under strictly
135 fixed environmental conditions (20 °C, 60% humidity) throughout the experiment.
136 CNSL feeding level was set at 4 g CNSL per 100 kg body weight per day. The daily
137 allotment of each pellet was equally divided into two portions and given together with
138 the basal diet at the twice-daily feedings.

139 Rumen content was collected before the morning feeding via a ruminal cannula on
140 the first and third days of the last week in each period; the rumen content then was
141 filtered through four layers of cheese cloth, and stored at -80 °C. Upon thawing, the
142 filtrate was mixed prior to analyzing the rumen microbes.

143

144 **2.2 Analysis**

145 Total DNA was extracted from 1 mL of rumen fluid using the FastPrep FP100A bead
146 beating system with a Fast DNA Kit (Qbiogene, Carlsbad, CA, USA) and quantified by
147 absorbance using a SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA, USA).
148 Another portion of the rumen fluid (0.3 mL) was treated overnight with RNAlater
149 (Ambion, TX, USA) and centrifuged (14,000 x g for 5 min). The resulting pellet was
150 resuspended in 600 μL of RLT buffer (RNeasy mini kit, QIAGEN, Carlsbad, CA, USA)
151 supplemented with 6 μL of β-mercaptoethanol and packed in Lysing Matrix E (MP
152 Biomedicals, CA, USA) for RNA extraction. The FastPrep FP100A bead beating system
153 was employed at a speed setting of 6 for 2 min to extract total RNA, which then was
154 purified with a RNeasy mini kit (QIAGEN) and quantified by absorbance using a
155 SmartSpec 3000. cDNA was generated from 1 μg of purified RNA with the SuperScript
156 First-Strand Synthesis System (Invitrogen, Carlsbad, USA). DNA and cDNA were used
157 for clone library construction as described below.

158 Bacterial and archaeal analysis was carried out by sequencing 16S rRNA and *mcrA*
159 (methyl-coenzyme reductase A) gene libraries, respectively, using 0-h individual rumen
160 samples that had been obtained from each cow in the control and CNSL periods. Library
161 construction was essentially as described in Koike et al. (2003), except that PCR
162 primers targeting *mcrA* (for the archaeal library) were as described by Luton et al.
163 (2002). The primers used included 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and
164 1492R (5'-GGYTACCTTGTTACGACTT-3') for bacteria, and mcrA FW
165 (5'-GGTGGTGTMGGATTCACCARTAYGCWACAGC-3') and mcrA RV
166 (5'-TTCATTGCRTAGTTWGGRTAGTT-3') for methanogenic archaea. Sequencing
167 was performed by a commercial service (TAKARA Bio, Inc., Kusatsu, Japan). Virtually
168 full-length 16S rRNA gene sequences (ca. 1300-1400 bases) were obtained for the 16S

169 rRNA genes, while partial sequences (ca. 500 bases) were obtained for *mcrA*. BLAST
170 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to phylogenetically classify all
171 cloned sequences. Diversity indices were obtained from FastGroup II
172 (http://biome.sdsu.edu/fastgroup/).

173 To more comprehensively analyze the microbial community structure, DNA
174 sample from each cow in Trial 2 was employed for MiSeq analysis (Illumina, San Diego,
175 CA, USA) as described by Konda et al. (2019). Sequencing was performed by
176 Hokkaido System Science Co., Ltd. (Sapporo, Japan). In brief, the procedures were as
177 follows. The V3 to V4 regions were amplified using two primer sets,
178 S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and
179 S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') for the bacterial
180 rRNA genes (Herlemann et al., 2011), and arch349F
181 (5'-GYGCASCAGKCGMGAAW-3') and arch806R
182 (5'-GGACTACVSGGGTATCTAAT-3') for the archaeal rRNA genes (Takai &
183 Horikoshi, 2000). PCR was carried out in a total volume of 50 µL, consisting of 10 µL
184 5x PrimeSTAR Buffer, 4 µL dNTP mixture (2.5 mM each), 0.5 µL PrimeSTAR HS
185 DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 1 µL each primer (10 pmol/µL),
186 32.5 µL dH_2O, and 1 µL template DNA (10 ng/µL). The following PCR conditions were
187 used: 30 cycles for bacteria and 40 cycles for archaea, with each cycle consisting of
188 denaturation at 98 °C (10 s), annealing at 55 °C (15 s) and extension at 72 °C (30 s).
189 Data quality control and analyses were performed using the QIIME ver. 1.8.0 pipeline
190 (Caporaso et al., 2012). OTUs were generated from sequences clustered at a 97%
191 similarity threshold using the UCLUST algorithm (Edgar, 2010). Chimeric sequences
192 were removed from the analysis using the ChimeraSlayer algorithm. Taxonomy was

193 assigned using the Greengenes database (ver. 13.8) at a 90% similarity threshold.
194 Differences in biodiversity between samples from the control and CNSL periods were
195 compared by alpha diversity metrics, including the observed number of OTUs, Shannon
196 index, Chao 1, and Good's coverage.

197 Comparison of clone library data between the control and CNSL periods was
198 performed using LIBSHUFF (http://libshuff.mid.uga.edu/). Diversity indices based on
199 MiSeq data, and proportional abundance of rumen bacterial and archaeal phyla/genera
200 classified by clone library and MiSeq analysis, were compared between control and
201 CNSL treatment by two-tailed non-paired Student's t-tests. Diversity indices in clone
202 library analysis were obtained from all sequence data of 3 cows and not allowed for
203 statistical analysis. Significance was defined at $P < 0.05$. The sequences obtained were
204 deposited in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under
205 Accession Nos. AB554982-555495, AB556949-AB557492 and PRJNA682280.
206

207 **3. RESULTS**

208 **3.1. Clone library analysis**

209 Comparisons of rumen bacterial community structure between control and CNSL
210 periods based on diversity indices and LIBSHUFF analysis are shown in Table 1. The
211 numbers of bacterial clones sequenced were 531 (Trial 1) and 517 (Trial 2) for DNA,
212 and 514 (Trial 1), and 548 (Trial 2) for cDNA. LIBSHUFF analysis revealed that the
213 community structure assessed by DNA and cDNA libraries differed between the control
214 and CNSL periods in both Trials 1 and 2. In fact, the values of Chao 1 and the Shannon
215 index, as well as the number of OTUs, in the DNA and cDNA libraries decreased with
216 CNSL feeding in both Trials 1 and 2.

Figure 1 illustrates rumen bacterial community structure classified at the phylum level in Trials 1 and 2. The detection frequency of *Proteobacteria* was increased by CNSL feeding, as judged by the cDNA library of Trial 1 and by both the DNA and cDNA libraries of Trial 2. This increase was more apparent in the cDNA libraries than in the DNA library of Trial 2. The increase of *Proteobacteria* was attributable to the increased detection of *Succinivibrio* and *Ruminobacter*, which was particularly pronounced in Trial 2. Notably, *Succinivibrio dextrinosolvens* was detected more frequently in the CNSL feeding period in both Trial 1 (0 vs. 1.1% (control vs. CNSL, respectively) for DNA and 0.4 vs. 3.6% for cDNA) and Trial 2 (0 vs. 3.1% for DNA and 0.7 vs. 6.5% for cDNA).

Comparison of methanogenic archaeal community structure between control and CNSL periods was based on diversity indices and LIBSHUFF analysis; results are presented in Table 2. The numbers of clones sequenced were 545 (Trial 1) and 551 (Trial 2) for DNA, and 544 (Trial 1) and 549 (Trial 2) for cDNA. Methanogenic community structure differed between control and CNSL periods, as judged by LIBSHUFF analysis in both DNA and cDNA libraries and also in both Trials 1 and 2. All of the diversity indices and OTU numbers in the DNA and cDNA libraries decreased with CNSL feeding in both Trials 1 and 2.

A shift of the methanogen community was observed with CNSL feeding in Trial 2, although the changes fell short of significance (apparently because of animal-to-animal variation). Notably, *Methanomicrobium* (primarily consisting of *Methanomicrobium mobile*) became predominant following CNSL feeding, as judged by both DNA and cDNA libraries in Trial 2 (Figure 2). However, such changes with CNSL feeding were not observed in Trial 1.

241

242 **3.2. MiSeq analysis**

243 Table 3 indicates diversity indices of rumen bacterial and methanogenic archaeal
244 communities as influenced by CNSL feeding in Trial 2. The numbers of sequences
245 employed for the analysis were 58,765 (control) and 60,175 (CNSL) for bacteria, and
246 101,048 (control) and 65,036 (CNSL) for methanogenic archaea. Following CNSL
247 feeding, the values of the Shannon index and Chao 1, as well as the OTU numbers, were
248 decreased for bacteria. As for the methanogenic archaeal community, only the OTU
249 numbers were decreased following CNSL feeding. Notably, coverages of bacterial and
250 methanogenic archaeal community analysis were satisfactorily high (99-100%).

251 CNSL feeding altered the bacterial community as classified at the family level,
252 with increased detection of *Prevotellaceae*, *Veillonellaceae*, and *Succinivibrionaceae*,
253 and decreased detection of unclassified *Bacteroidales*, *Lachnospiraceae*, and some other
254 unclassified families (data not shown). CNSL feeding also altered the methanogenic
255 archaeal community as classified at the family level, with increased detection of
256 *Methanomicrobiaceae* and decreased detection of *Methanobacteriaceae* (data not
257 shown).

258 Community structure classified at the genus level is shown in Figure 3. For
259 bacteria, CNSL feeding increased the detection frequency of *Prevotella*,
260 *Succiniclasticum*, *Selenomonas*, unclassified *Succinivibrio*, unclassified *Veillonellaceae*,
261 and some other unclassified genera, while decreasing the detection frequency of
262 *Butyrivibrio*, unclassified *Ruminococcaceae*, unclassified *Bacteroidales*, and
263 unclassified *Clostridiales*. For methanogenic archaea, CNSL increased the detection
264 frequency of *Methanoplanus*, while decreasing that of *Methanobrevibacter*.

4. DISCUSSION

In the present study, the methane mitigation rates (% per unit of dry matter intake) by CNSL feeding were reported to be 38.3 and 19.3% in Trials 1 and 2, respectively (Shinkai et al. 2012). Those authors observed shifts of rumen bacterial abundance with CNSL (increases of *Prevotella ruminicola*, *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, and *Succinivibrio dextrinosolvens*, all involved in propionate production), as assessed by quantitative PCR. Such changes are consistent with those reported in other feeding studies using sheep (Kang et al.2018) and Thai local ruminants (Konda et al. 2019). The present study attempted to evaluate rumen microbial responses to CNSL in a more detailed manner by using clone libraries and MiSeq analysis targeting not only bacteria but also methanogenic archaea. Libraries covering the expressed 16S rRNA and *mcrA* genes also were constructed and analyzed to understand metabolically significant changes in specific microbial groups. Therefore, the present analysis provides further insights into rumen microbial community shifts caused by CNSL. These results are expected to facilitate understanding from scientific, feed industrial, and even practical perspectives, permitting further application of this additive in animal production.

As seen in many other reports, typical bacterial community shifts induced by CNSL feeding, which causes propionate enhancement, were confirmed in 16S rDNA clone library analysis, and by MiSeq analysis. These observations are in good agreement with the susceptibility of bacterial species to CNSL and its compositional phenolic compounds in pure culture studies (Watanabe et al. 2010; Oh et al. 2017a). One such shift in the present study was the increased abundance of *Proteobacteria*, a bacterial group that includes *Succinivibrio*, which is a known producer of succinate, a

289 propionate precursor. This shift was more pronounced in the cDNA library than in the
290 DNA library (Figure 1). Therefore, the accumulation of *Proteobacteria* ribosomal RNA
291 suggests that this group might be metabolically activated by CNSL feeding, with the
292 effects appearing to be greater than those reported in previous evaluations based on
293 ribosomal DNA abundance (Watanabe et al. 2010; Shinkai et al. 2012). This increase of
294 *Proteobacteria* may be attributable to the increased detection of *S. dextrinosolvens*.
295 Thus, the hydrogen sink may be converted (at least in part) to propionate production, a
296 change that would competitively decrease methanogenesis (Russell & Strobel, 1989;
297 Ungerfeld, 2015). This shift could be one of the explanations for the decreased
298 production of methane in CNSL-fed ruminants (Shinkai et al. 2012; Mitsumori et al.
299 2014). Recent studies on rumen microbial communities have indicated that the rumen of
300 low-methane-emitting cattle has a higher molar proportion of propionate and higher
301 abundance of *Succinivibrionaceae* (Danielsson et al. 2017); these observations coincide
302 with the decreased methane production seen in CNSL-fed cows in the study related to
303 the present work (Shinkai et al 2012). Accordingly, CNSL might shift the rumen
304 bacterial community toward the community observed in potentially
305 low-methane-yielding cattle.

306 The analyses in the previous study described that (in both Trials 1 and 2), CNSL
307 feeding led to decreased abundances of both the *mcrA* gene and its transcript (Shinkai et
308 al, 2012), indicating decreases in the abundance of methanogenic archaea and their
309 metabolism, which contribute to methane synthesis. The present sequencing results also
310 suggest that CNSL feeding decreases the diversity of the methanogen community (Table
311 2). This decrease in diversity is evidenced by the increased detection frequency of
312 *Methanomicrobium* in Trial 2 (retrieved from the *mcrA* library, Figure 2), which is

supported by the increased detection of *Methanoplanus* (retrieved from 16S rDNA library, Figure 3). These two genera are phylogenetically close members of the family *Methanomicrobiaceae*. The results are suggestive of the low susceptibility of these two genera to CNSL. It is known that methanogens belonging to *Methanomicrobiaceae* possess an S-layer in their cell surface (Sowers, 2009). We postulate that this layer might be a barrier against alkyl-phenols, make these archaea tolerant to the surfactant activity of CNSL. In contrast, another methanogen (*Methanobrevibater*) that dominated in samples from the control period belongs to the *Methanobacteriales*, archaea known to have thin cell walls that are stained as Gram positives and are composed of pseudomurein but lack the S-layer (Sowers, 2009). This difference in cell wall structure may correspond to a difference in susceptibility to the surfactant activity of CNSL; this difference would lead to a shift in the archaeal community structure, possibly decreasing *mcrA* expression followed by the attenuation of methane synthesis, as reported by Shinkai et al. (2012).

Changes of representative methanogens with CNSL feeding have not been consistent among feeding studies; Kang et al. (2018) described the replacement of *Methanobrevibacter ruminantium* by *Methanobrevibacter wolnii* in the rumen of sheep fed CNSL, while Konda et al. (2019) reported the decrease of *Methanomassilicoccaceae* and the increase of *Methanomicrococcus* in Thai local ruminants fed CNSL. These inconsistent responses to CNSL feeding presumably reflect differences between animal species and/or breeds in which the respective methanogen communities originally developes. Thus, it is difficult currently to specify which methanogens play pivotal role in the decrease of methane production caused by CNSL. However, it is clear that CNSL feeding changes not only the bacterial community but

337 also the methanogenic community, shifting metabolic hydrogen sinks away from
338 methane toward propionate production.

339 Methanogen diversity has been reported to reflect the host animal species and
340 geography (de la Fuente et al. 2019). Notably, *Methanomicrobium* is known to
341 constitute one of the representative methanogens in Asia but not in other regions
342 (Henderson et al. 2015). In fact, this group of methanogens does not contribute to the
343 methanogen community in high- and low-methane-producing dairy cows in Europe
344 (Danielsson et al. 2017). Therefore, the increase of *Methanomicrobium* with CNSL
345 feeding, as observed in the present study, may be a key aspect of the community shift
346 only in specific regions of Asia. Metabolic differences among methanogenic species
347 continue to be the subject of scientific exploration, and the rate and extent of metabolic
348 hydrogen flow may vary among hydrogenotrophic methanogens such as
349 *Methanobrevibacter* and *Methanomicorbiaceae*. In fact, the phylogeny of *mcrA* clearly
350 differs between these two families (Evans et al. 2019).

351 For assessing the diversity of methanogen communities, the use of *mcrA* (rather
352 than 16S rRNA genes) has been recommended, together with correlation of *mcrA*
353 transcripts and methane production in anaerobic digesters (Wilkins et al. 2015). In
354 previous work, we reported the relative gene expression of *mcrA* in relation to decreased
355 methane production in the present samples (Shinkai et al. 2012). Therefore, it may be
356 relevant to discuss those results in the context of the results from the *mcrA* transcript
357 library (Table 2, Figure 2), even though the number of library clones sequenced was
358 limited. Notably, of the sequences that were read, the majority represented uncultured
359 methanogens, followed by *Methanomicrobium* (Figure 2). Obviously, further study will
360 be necessary for more detailed taxonomical and functional characterization of rumen

methanogens, for instance, via transcriptomic analysis.

In conclusion, the effects of CNSL on rumen fermentation, whereby methane production is decreased and propionate production is increased, can be explained by CNSL-imposed microbial selection not only for bacteria but also for methanogenic archaea, leading to a less diverse community structure. The mode of such community shifts seems to differ among animal species/breeds, as indicated by comparison of the results of the current and previous studies. Nonetheless, it appears that the methane mitigation rate may be influenced by the extent of functional changes in methanogens, along with the profile of propionate-producing bacteria, both of which are altered by CNSL.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the present publication.

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Figure captions

Figure 1. Effect of cashew nut shell liquid (CNSL) feeding on rumen bacterial community structure revealed by clone library analysis targeting 16S rRNA gene in trials 1 [A] and 2 [B], and their transcripts [C, D].
Detection frequency (%) at phylum level was shown (n=3). *, Significantly different from control.

Figure 2. Effect of cashew nut shell liquid (CNSL) feeding on rumen methanogenic archaeal community structure revealed by clone library analysis targeting methyl-coenzyme reductase A gene in trials 1 [A] and 2 [B], and their transcripts [C, D].
Detection frequency (%) at genus level was shown (n=3).

Figure 3. Effect of cashew nut shell liquid (CNSL) feeding on rumen bacterial [A] and methanogenic archaeal [B] community structure revealed by Miseq analysis using samples in trial 2. Detection frequency (%) at genus level was shown (n=3). Arrows with direction represent significant increase/decrease by CNSL feeding.

505

Table 1. Effect of cashew nut shell liquid (CNSL) feeding on diversity indices of rumen bacterial community as shown in clone library analysis targeting 16S rRNA gene and its transcript

Trial / Library / Treatment	Chao 1	Shannon index	No. of OTU	No. of clone	LIBSHUFF [a]
Trial 1					
DNA library					
Control	848.1	4.9	176	261	*
CNSL	264.7	4.3	129	270	*
cDNA library					
Control	641.7	4.8	165	262	*
CNSL	208.9	4.2	103	252	*
Trial 2					
DNA library					
Control	1558	5.3	213	260	*
CNSL	532.7	4.8	160	257	*
cDNA library					
Control	868	5.2	204	272	*
CNSL	522.9	4.7	154	276	*

[a] Significantly different (vs. another treatment).

506 Diversity indices were obtained from all sequence data from 3 cows in each treatment.

507

508

Table 2. Effect of cashew nut shell liquid (CNSL) feeding on diversity indices of rumen methanogenic archaeal community as shown in clone library analysis targeting methyl coenzyme reductase A gene (*mcrA*) and its transcript

Trial / Library / Treatment	Chao 1	Shanon index	No. of OTU	No. of clone	LIBSHUFF [a]
Trial 1					
DNA library					
Control	49.4	2.5	35	275	*
CNSL	36	2	20	270	*
cDNA library					
Control	75.1	3	45	272	*
CNSL	58.7	1.6	26	272	*
Trial 2					
DNA library					
Control	58.1	3	37	275	*
CNSL	23.1	1.5	20	276	*
cDNA library					
Control	92.1	3	48	270	*
CNSL	39	1.7	23	279	*

[a] Significantly different (vs. another treatment).

509 Diversity indices were obtained from all sequence data from 3 cows in each treatment.

510

511

Table 3. Effect of cashew nut shell liquid (CNSL) feeding on diversity indices of rumen bacterial and methanogenic archaeal community as shown by Miseq analysis using samples in trial 2

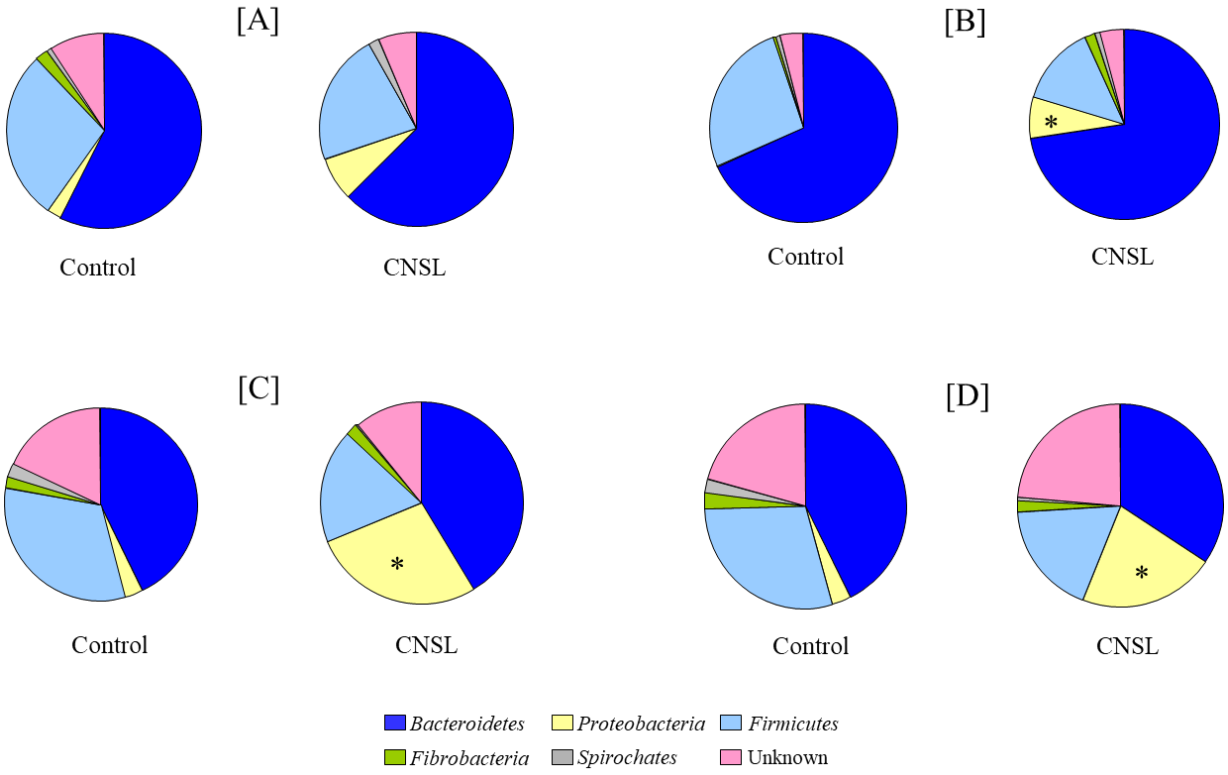
	Bacterial community			Archaeal community		
	Control	CNSL		Control	CNSL	
OTU	1283±30	743±61	*	76±9	48±2	*
Shannon index	7.9±0.1	6.6±0.2	*	3.0±0.1	2.7±0.5	ns
Chao 1	1592±27	994±38	*	91±19	62±13	ns
Cgood's coverage	99.1±0.2	99.4±0.1	ns	100	100	ns

* Significantly different.

512 Samples taken before feeding during control and CNSL feeding periods are analyzed (n=3).

513

514



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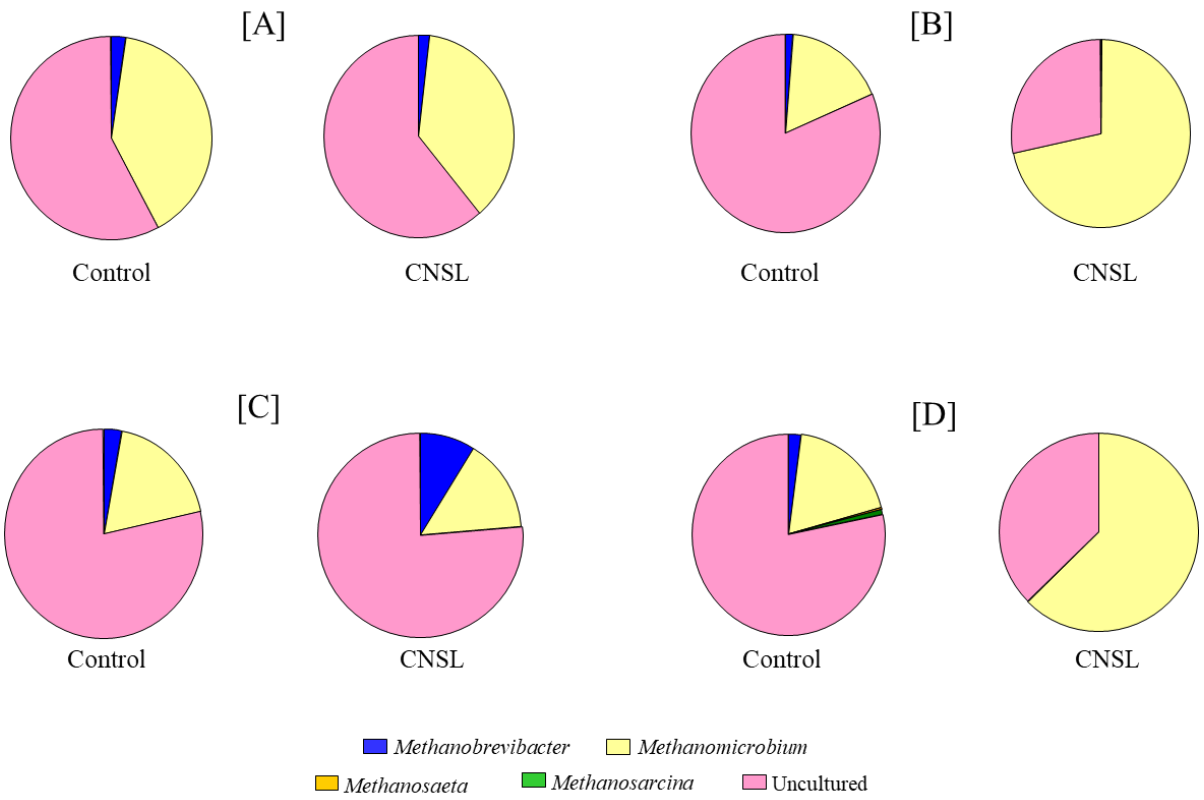

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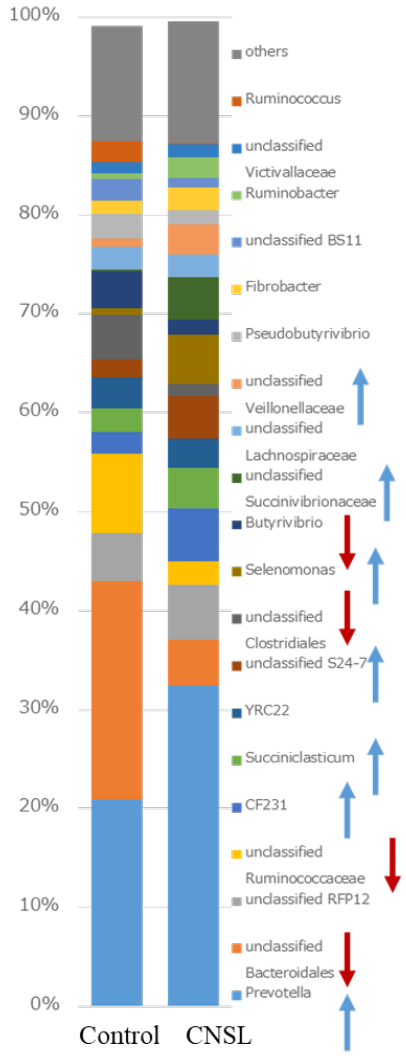
[A]


100%
90%
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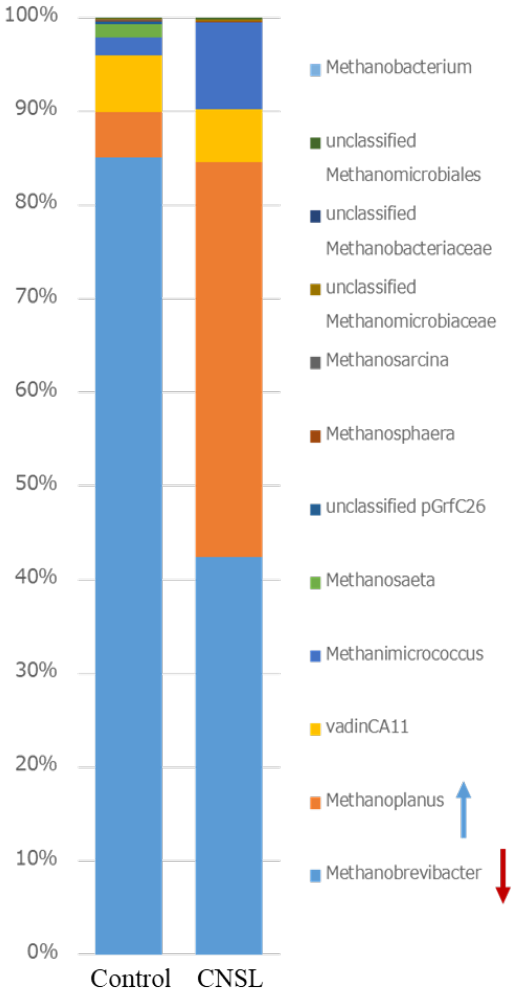
Control
CNSL
others
Ruminococcus
unclassified Victivallaceae
Ruminobacter
unclassified BS11
Fibrobacter
Pseudobutyrivibrio
unclassified Veillonellaceae
unclassified Lachnospiraceae
unclassified Succinivibrionaceae
Butyrivibrio
Selenomonas
unclassified Clostridiales
unclassified S24-7
YRC22
Succiniclasticum
CF231
unclassified Ruminococcaceae
unclassified RFP12
unclassified Bacteroidales
Prevotella

[B]


100%
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Control
CNSL
Methanobacterium
unclassified Methanomicrobiales
unclassified Methanobacteriaceae
unclassified Methanomicrobiaceae
Methanosarcina
Methanosphaera
unclassified pGrfC26
Methanosaeta
Methanimicrococcus
vadinCA11
Methanoplanus
Methanobrevibacter

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