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Title	Feeding cashew nut shell liquid decreases methane production from feces by altering fecal bacterial and archaeal communities in Thai local ruminants
Author(s)	Tamori, Koya; Matsunaga, Bungo; Boonsaen, Phoompong et al.
Citation	Animal science journal, 92(1), e13569 <a href="https://doi.org/10.1111/asj.13569">https://doi.org/10.1111/asj.13569</a>
Issue Date	2021-01
Doc URL	<a href="https://hdl.handle.net/2115/83737">https://hdl.handle.net/2115/83737</a>
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Type	journal article
File Information	Anim Sci J_92_e13569.pdf



1 **Feeding cashew nut shell liquid decreases methane production from feces by altering fecal**  
2 **bacterial and archaeal communities in Thai local ruminants**

3

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26 **Abstract**

27 The effect of feeding cashew nut shell liquid (CNSL) on fecal fermentation products and  
28 microbiota were investigated in Thai native cattle and swamp buffaloes. Four of each animal  
29 were fed rice straw and concentrate diet with control pellets without CNSL for 4 weeks,  
30 followed by the same diet with pellets containing CNSL for another 4 weeks, so that CNSL  
31 was administered at a level of 4g/100 kg body weight. Feces were collected the last 2 days in  
32 each feeding period. CNSL alkyl phenols were recovered from feces (16-28%) in a similar  
33 proportion to those in the diet, indicating that most functional anacardic acid was not selectively  
34 removed throughout the digestive tract. In vitro production of gas from feces, particularly  
35 methane, decreased with CNSL feeding. The proportion of acetate in feces decreased with  
36 CNSL feeding, whereas that of propionate increased, without affecting total short-chain fatty  
37 acid concentration. CNSL feeding changed fecal microbial community, particularly in swamp  
38 buffaloes, which exhibited decreases in the frequencies of *Treponema*, unclassified  
39 Ruminococcaceae, and Methanomassiliicoccaceae. These results suggest that CNSL feeding  
40 alters not only rumen fermentation but also hindgut fermentation via modulation of the  
41 microbial community, thereby potentially attenuating methane emission from the feces of  
42 ruminant animals.

43

44 **Key words:** alkyl phenols, cashew nut shell liquid, feces, methane, microbiota

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## 46 **1 | INTRODUCTION**

47 Agriculture-associated methane accounts for 20% of global anthropogenic methane  
48 emissions, of which 40% is of ruminant origin (IPCC, 2007). Accordingly, mitigating methane  
49 production by ruminant animals has long been targeted. Methane is produced primarily in the  
50 rumen by symbiotic methanogenic archaea, typically hydrogenotrophic methanogens. These  
51 methanogens generate methane using hydrogen and CO<sub>2</sub> produced by other microbes, which  
52 is regarded as the major pathway for consuming metabolic hydrogen, which will hamper gut  
53 fermentation if it accumulates. Thus, a basic strategy for ruminal methane mitigation is to shift  
54 the hydrogen-utilizing process from methanogenesis to other pathways (Ungerfeld, 2015),  
55 represented by fumarate reduction leading to propionate production.

56 Over the past 2 decades, methane mitigation studies involving ruminants have proposed  
57 several different approaches based on various aspects of animal feed and management. More  
58 recent studies have suggested strategies for methane mitigation involving vaccination or  
59 genetic selection. The principles and efficacy of these approaches have been discussed in  
60 several reviews. For example, with regard to diet manipulation, a number of feed additives  
61 have been developed to reduce methane production through selection of rumen microbes  
62 (Kobayashi et al. 2016; Beauchemin et al. 2020). In addition, management practices such as  
63 feeding frequency and feeding level can influence methane production (Mathison et al. 1998;  
64 Benchaar & Hasaanat, 2020). Vaccination-based approaches to eliminate rumen methanogens  
65 have been improved due to more in-depth analyses that have identified methanogens that are  
66 ecologically significant and metabolically active (Baca-González et al. 2020). Identification of  
67 target methanogen candidates has enhanced the effectiveness and duration of vaccines.  
68 Selection of low-methane-emitting animals is a newer approach to provide more dramatic and  
69 sustainable mitigation, with research focusing on identifying rumen microbial indicators useful  
70 in screening for low-methane-emitting animals (Difford et al. 2018).

71           Manipulating the diet by feeding animals cashew nut shell liquid (CNSL) has been used  
72 in ruminants animals such as sheep (Kang et al. 2018), Holstein cows (Shinkai et al. 2012), and  
73 Thai native cattle and swamp buffaloes (Konda et al. 2019). A >50% decrease in methane  
74 production potential was achieved in Thai ruminant animals by altering the rumen microbial  
75 community structure through selective inhibition of specific microbes mediated by CNSL  
76 (Konda et al. 2019). A similar result was observed in Holstein cattle fed CNSL (Su et al. 2021).  
77 Konda et al. (2019) reported that the surfactant action of alkyl phenols, in particular anacardic  
78 acid, is stably maintained in the rumen, although anacardic acid is decarboxylated and becomes  
79 less active in the rumen of Japanese Holstein cows (Shinkai, unpublished results). If these  
80 phenolics pass through the entire digestive tract without degradation/transformation/absorption,  
81 the fermentation properties of the feces can be changed toward the production of less methane.  
82 This could provide an additional benefit of CNSL feeding in terms of decreasing methane  
83 emission from the feces of ruminants.

84           Although mitigation of methane emission from ruminants has focused on reducing  
85 ruminal methane production due to its large contribution, considerably less attention has  
86 focused on methane emission from excreta (feces and urine), which accounts for 10% of  
87 ruminal methane emissions (FAO, 2006). Although feces are treated in modern biogas and  
88 composting facilities, a large proportion of ruminant feces is not properly treated, and in some  
89 cases, feces are left for long periods without any special care. Such situations are often observed  
90 in developing countries, where local ruminants with low animal productivity are generally  
91 raised for a number of years without proper treatment of their excreta. This confers  
92 opportunities to study methods to reduce local methane production not only in the rumen but  
93 also reduce emission from the feces of ruminant animals.

94           The present study hypothesized that feeding CNSL to local Thai ruminant animals would  
95 decrease the fecal methane production potential by altering the microbial community through

96 alkyl phenols originating in the CNSL. The effect of CNSL feeding on methane production was  
97 evaluated by monitoring changes in alkyl phenols, fermentation products, and microbes in the  
98 feces of ruminants in comparison with control animals.

99

## 100 **2 | MATERIALS AND METHODS**

### 101 **2.1 | Animals and diets**

102 The present animal study protocol was reviewed by Institutional Animal Care and Use  
103 Committee of Kasetsart University, Thailand, in accordance with the Guidelines of Animal  
104 Care and Use under the Ethical Review Board of the Office of the National Research Council  
105 of Thailand. The study was approved under permission number ACKU60-AGK-002.

106 Four Thai native cattle ( $370 \pm 32$  kg body weight; 2 females and 2 males; 4 years old) and  
107 four swamp buffaloes ( $465 \pm 67$  kg body weight; all females; 4 years old) were fitted with  
108 permanent rumen cannula (Bar Diamond, Inc., Parma, ID, USA) for the 8-week feeding  
109 experiment. Animals were fed a locally formulated concentrate (1 kg/d) consisting of cassava,  
110 palm kernel meal, flaked corn, soybean meal, sugarcane molasses, urea, vitamin and mineral  
111 mixture, and rice straw (90% of ad libitum intake), as shown in Table 1. The feed formulation  
112 and chemical composition percentages in the table and the text were calculated on dry matter  
113 (DM) basis. The ad libitum intake of rice straw was determined by recording the daily intake  
114 for 7 days in advance of the experiments (i.e., the baselines for Thai native cattle and swamp  
115 buffaloes were 6 and 7 kg on average, respectively). The animals were fed the diet once daily  
116 at 8:00 AM. The experiment was conducted for a total of 8 weeks, consisting of a 4-week control  
117 period followed by a 4-week CNSL-feeding period. Because the residual effects of CNSL on  
118 the fecal microbiota and fermentation profile were uncertain, the CNSL treatment period  
119 occurred after the control period.

120 CNSL was fed using pellets containing 23.7% CNSL (CNSL pellets) (Shinkai et al. 2012),  
121 to which alfalfa meal (40.7%), defatted rice bran (18.9%), silica powder (11.6%), crude

122 sugarcane molasses (2.6%), and tapioca flour (2.4%) were added to improve palatability. The  
123 amount of CNSL fed was set at 4 g/100 kg body weight, as tested for Holstein cows (Shinkai  
124 et al. 2012). Control pellets were prepared using the same feed ingredients but without CNSL  
125 and fed to the animals during the control period. The pellets (11.9% crude protein [CP] and  
126 26.4% neutral detergent fiber [NDF] on DM basis) were 5 mm in diameter. Pellets were stored  
127 at 4°C in the dark until use. The total diet contained 7.8-8.2% CP and 56.8-57.7% NDF (Table  
128 1), which did not differ between the control and CNSL periods.

129

## 130 **2.2 | Sampling and measurement**

131 Feed intake was recorded daily by measuring the amount of feed offered and amount of  
132 feed remaining for each animal. Fresh feces were collected directly from the rectum of each  
133 animal just before feeding on the last two days (days 27 and 28) of each period. A portion of  
134 each fecal sample was mixed with distilled water (feces:water = 2:3) and subjected to pH  
135 measurement using a CyberScan pH510 electrode (Thermo Fisher Scientific, Waltham, MA).  
136 Another portion of each feces sample was used for the measurement of gas production potential  
137 (see below). The remainder of each feces sample was frozen (-80°C) for subsequent analyses  
138 of phenolics, fermentation products, and microbes. For calculating the recovery of CNSL  
139 components in feces, the amount of total feces of each animal was measured on the days 27 and  
140 28.

141 Gas production potential was evaluated essentially as described by Kang et al. (2018). In  
142 brief, feces (100g for each animal) was diluted with an equal volume of buffer (pH 6.8)  
143 (McDougall, 1948) and strained with a surgical gauze. The filtrate (10ml) was dispensed into  
144 a Hungate tube (18ml in volume), to which a butyl rubber stopper and plastic cap were fitted  
145 after flushing the head space with N<sub>2</sub> gas. Then, tubes were anaerobically incubated at 38°C  
146 for 24 h without substrate. Four replicates for each animal were monitored for gas production.

147 Total gas in the headspace of the tube was measured using a needle-attached pressure gauge,  
148 as described by Watanabe et al. (2010). Gas emitted from feces was expressed the gas  
149 production potential (mL/g of feces/day).

150

### 151 **2.3 | Chemical analysis**

152 Analysis of CP, crude fat, crude ash, and NDF of feces was done according to the AOAC  
153 method (AOAC, 2016). Non-fiber carbohydrate was determined based on the following  
154 equation:  $(100 - (\% \text{ NDF} + \% \text{ CP} + \% \text{ crude fat} + \% \text{ crude ash}))$ . CNSL-derived alkyl phenols  
155 (anacardic acids, cardanol, and cardol) were quantified by HPLC as described by Watanabe et  
156 al. (2010), using fecal samples collected before feeding in the control and CNSL-feeding  
157 periods. The original CNSL was also analyzed as a reference. Gas composition (CO<sub>2</sub>, CH<sub>4</sub>,  
158 and H<sub>2</sub>) was analyzed on a gas chromatograph equipped with a thermal conductivity detector  
159 (GC-8A; Shimadzu, Kyoto, Japan). Fecal short-chain fatty acids (SCFAs) and ammonia were  
160 extracted by mixing feces with distilled water (1:1) and then analyzed by gas chromatography  
161 on a GC-14B instrument (Shimadzu) equipped with a flame ionization detector and by  
162 spectrophotometry after indophenol reaction (Weatherburn, 1967). Gas chromatography  
163 analyses were carried out according to Oh et al. (2017).

164

### 165 **2.4 | Microbial analysis**

166 Fecal DNA was extracted using the RBB+C method (Yu & Morrison, 2004) and used for  
167 quantitative real-time PCR (qPCR) assays to quantify bacteria, as represented by total bacteria,  
168 *Ruminococcaceae*, *Bacteroides-Prevotella-Porphyrromonas*, genus *Prevotella*, *Clostridium*  
169 *coccoides-Eubacterium rectale* group, *Eubacterium hallii*, *Fecalibacterium prausnitzii*, genus  
170 *Lactobacillus*, genus *Bifidobacterium*, and total archaea. All qPCR details, including primers,  
171 standards, PCR conditions, and calculations, were as described by Koike et al. (2007) and

172 Ohene-Adajei et al. (2008). A LightCycler system and FastStart DNA master SYBR I reaction  
173 kit (Roche, Penzberg, Germany) were used with 10-fold serial dilutions of standard plasmid  
174 for the respective targets (16S rRNA gene sequence specific to each target microbe). Microbial  
175 quantity was calculated using amplification curves obtained from both standards and samples.  
176 PCR amplification specificity was confirmed by analysis of the melting curves of the PCR  
177 products (increasing the temperature from 70°C to 95°C at a rate of 0.1°C/s). Microbial  
178 abundance is reported as the copy number of the rRNA gene for total bacteria and as the relative  
179 proportion among the total bacterial copy number for total archaea and specific bacterial  
180 species and groups.

181 Clone library analysis for the bacterial and archaeal communities was carried out by  
182 sequencing the 16S rRNA and *McrA* (methyl-coenzyme reductase A) gene libraries,  
183 respectively, using feces obtained during the control and CNSL-feeding periods, as essentially  
184 described by Koike et al. (2003), except that PCR primers targeting *mcrA* for the archaeal  
185 library were as reported by Luton et al. (2002). Primers used were 27F (5'-  
186 AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3') for  
187 bacteria, whereas *mcrA* FW (5'-GGTGGTGTMGGATTCACCARTAYGCWACAGC-3') and  
188 *mcrA* RV (5'-TTCATTGCRTAGTTWGGRTAGTT-3') were used for methanogenic archaea.  
189 Sequencing was performed by a commercial service (TAKARA Bio Inc., Kusatsu, Japan).  
190 Almost the full-length of 16S rRNA genes (ca. 1300-1400 bases) were read, whereas only  
191 partial sequences (ca. 500 bases) of the *mcrA* genes were read. BLAST was employed to  
192 phylogenetically classify all cloned sequences. Diversity indices were obtained from  
193 FastGroup II (<http://biome.sdsu.edu/fastgroup/>).

194 To comprehensively analyze the microbial community, DNA samples were subjected to  
195 MiSeq analysis (Illumina, San Diego, CA, USA). Sequencing was performed by Hokkaido  
196 System Science Co., Ltd. (Sapporo, Japan). The V3 to V4 regions were amplified using two

197 primer sets, S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-  
198 a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') for bacterial rRNA genes (Herlemann et al.,  
199 2011), and arch349F (5'-GYGCASCAGKCGMGAAW-3') and arch806R (5'-  
200 GGACTACVSGGTATCTAAT-3') for archaeal rRNA genes (Takai and Horikoshi, 2000).  
201 PCR was carried out in a total volume of 50  $\mu$ L, consisting of 10  $\mu$ L of 5 $\times$  PrimeSTAR Buffer,  
202 4  $\mu$ L of dNTP mixture (2.5 mM each), 0.5  $\mu$ L of PrimeSTAR HS DNA polymerase (Takara Bio  
203 Inc., Kusatsu, Japan), 1  $\mu$ L of each primer (10 pmol/ $\mu$ L), 32.5  $\mu$ L of dH<sub>2</sub>O, and 1  $\mu$ L of  
204 template DNA (10 ng/ $\mu$ L). The following PCR conditions were used: 30 cycles for bacteria  
205 and 40 cycles for archaea, consisting of denaturation at 98°C (10 s), annealing at 55°C (15 s),  
206 and extension at 72°C (30 s). Amplicon sequencing was carried out using MiSeq, as described  
207 by Caporaso et al. (2012). Data quality control and analyses were performed using the QIIME  
208 (ver. 1.8.0) pipeline (Caporaso et al., 2010). Operational taxonomic units (OTUs) were  
209 generated from sequences clustered at a 97% similarity threshold using the UCLUST algorithm  
210 (Edgar, 2010). Chimeric sequences were removed from the analysis using the ChimeraSlayer  
211 algorithm. Taxonomy was assigned using the Greengenes database (ver. 13.8) at a 90%  
212 similarity threshold. Differences in biodiversity between samples in the control and CNSL-  
213 feeding periods were compared by alpha diversity metrics: Chao1, Shannon index, and  
214 observed number of OTUs. The sequences obtained were deposited in the DNA Data Bank of  
215 Japan (DDBJ) nucleotide sequence database under accession nos. LC028553-LC028891,  
216 LC029039-LC029391 and PRJNA682863.

217

## 218 **2.5 | Statistical analysis**

219 Data from the two sampling days in each period were averaged to obtain representative  
220 data for each animal, which were examined using the Student's *t*-test with SPSS software (ver.  
221 16.0 J; IBM, Tokyo, Japan). The qPCR and MiSeq microbial data were obtained by analyzing

222 samples after equally mixing two samples from each period.  $P < 0.05$  was considered to  
223 indicate statistical significance.

224

### 225 **3 | RESULTS**

226 Alkyl phenols present in the diet and feces are shown in Figure 1. Dietary proportions of  
227 anacardic acid, caldanol, and caldol were 67.2, 9.5, and 23.3%, respectively, whereas the fecal  
228 proportions of these alkyl phenols in native cattle were 62.5, 5.0, and 32.5%, respectively. The  
229 respective proportions in swamp buffalo were 70.0, 3.5, and 26.5%. Accordingly, the fecal  
230 proportion of most functional anacardic acid in the experimental animals was similar to its  
231 dietary proportion. Based on the recorded amount of total feces (12.2-21.1kg/d), total alkyl  
232 phenols in feces were 0.8-2.1 g/d in native cattle and 2.6-4.5 g/d in swamp buffaloes. These  
233 accounted for 16-20% and 16-28%, respectively, of total alkyl phenols ingested.

234 The fecal fermentation parameters influenced by CNSL feeding are shown in Table 2.  
235 Fecal pH was higher in the CNSL period than the control period both in native cattle and swamp  
236 buffalo. Although the concentration of total SCFAs did not differ between the control and  
237 CNSL-feeding periods, the molar proportions of SCFAs differed. A lower proportion of acetate  
238 and higher proportion of propionate was observed in both local ruminants, whereas a higher  
239 proportion of butyrate with CNSL feeding was observed only in swamp buffaloes. The  
240 ammonia level was not affected by CNSL feeding in either native cattle or swamp buffaloes.  
241 Production of total gas, CO<sub>2</sub>, methane, and hydrogen decreased with CNSL feeding in both  
242 native cattle and swamp buffaloes. In particular, the decrease in methane production was more  
243 marked than that of the other gases.

244 The fecal abundance of representative bacterial groups as quantified by qPCR assays is  
245 indicated in Figure 2. Although the abundance of total bacteria was not affected by CNSL  
246 feeding (with the exception of a decreasing tendency in native cattle), the abundance of several  
247 bacterial groups changed with CNSL feeding. For example, there was a decrease in

248 Ruminococcaceae and increases in the abundance of the genus *Prevotella*, *E. halli*, and *F.*  
249 *prausizii* in native cattle and a decrease in Ruminococcaceae and increases in *Bacteroides-*  
250 *Prevotella-Porphyromonas*, the *C. coccoides–E. rectale* group, and *E. halli* in swamp buffaloes.

251 Table 3 and Figure 3 show the effect of CNSL feeding on fecal microbial diversity and  
252 composition as determined by clone library analysis in native cattle and swamp buffaloes. The  
253 number of OTUs and diversity indices did not differ markedly between treatments in either  
254 ruminant animal, based on a limited number of clones for the analysis (ranging from 153 to  
255 181 clones for each treatment). For bacteria, Ruminococcaceae, Lachnospiraceae, and  
256 Rikenellaceae were the 3 major families, whereas Mathanomicrobiales, Methanobacteriales,  
257 and Methanomassiliicoccales were the major orders for archaea. All of these classified families  
258 and orders did not exhibit changes with CNSL feeding, except that Methanosarcinales were  
259 detected only in CNSL-fed animals. LIBSHUFF analysis revealed a difference in community  
260 structure in swamp buffaloes but not native cattle.

261 The results of more-comprehensive MiSeq analyses of the community structures of fecal  
262 bacteria and archaea are shown in Tables 4 - 6. The number of OTUs declined with CNSL  
263 feeding, particularly in swamp buffaloes. CNSL feeding also altered the diversity indices in  
264 bacterial and archaeal communities, but the changes were not consistent between the two  
265 different ruminants. As seen in the LIBSHUFF analysis using clone library data, more  
266 differences between treatments were detected in swamp buffaloes than native cattle. In native  
267 cattle, CNSL feeding decreased *Treponema* and increased *Oscillospira*. In swamp buffaloes,  
268 CNSL feeding decreased the fecal abundance of unclassified Ruminococcaceae and *Treponema*  
269 but increased fecal abundance of 5-7N15 (belonging to the Bacteroidaceae), unclassified  
270 Lachnospiraceae, *Oscillospira*, and *Phascolarctobacterium*. No particular change in the  
271 archaeal community structure of native cattle was observed with CNSL feeding. In swamp  
272 buffaloes, however, CNSL feeding decreased the relative abundance of vadin CA11

273 (Methanomassiliicoccaceae).

274

## 275 **4 | DISCUSSION**

276 More than 70% of alkyl phenols appear to have disappeared during passage through the  
277 gastrointestinal tract, possibly due to degradation, transformation, and/or absorption. However,  
278 alkyl phenols reaching the lower digestive tract still exhibited anti-microbial activity that could  
279 lead to microbial community and fermentation changes. That is, fecal concentrations of these  
280 phenols (70-228 ppm) were higher than the minimum inhibitory concentration (< 25 ppm) for  
281 rumen bacteria sensitive to CNSL feeding (Watanabe et al. 2010; Oh et al. 2017), indicating  
282 that phenols can selectively inhibit specific bacteria (Kubo et al. 1993) even in the lower  
283 digestive tract and feces. In particular, the proportion of anacardic acid, the most-functional  
284 phenol, was similar as detected in dietary CNSL (Figure 1) and did not undergo selective  
285 decarboxylation throughout the digestive tract. These data support the hypothesis that feces  
286 from ruminants fed CNSL would emit less methane compared with feces from animals fed a  
287 control diet.

288 Fermentation parameters changed as well in animals fed CNSL, with less acetate and more  
289 propionate production among SCFAs and less gas production, particularly methane (83.2%  
290 decrease in native cattle and 74.5% decrease in buffaloes, Table 2). These results are in good  
291 agreement with reports of fermentation changes in the rumen of local Thai ruminants (Konda  
292 et al. 2019), Japanese Holsten cows (Shinkai et al. 2012), and sheep (Kang et al. 2018) fed  
293 CNSL. Thus, our findings demonstrate that CNSL feeding alters not only rumen fermentation  
294 but also hindgut fermentation, as observed in fresh feces of the present study.

295 Such fermentation shifts are presumably induced by shifts in the fecal microbiota  
296 composition (Figure 2) that can be explained in part by a decrease in Ruminococcaceae (both  
297 in native cattle and swamp buffaloes), to which hydrogen-producing bacteria could belong  
298 (Stewart & Flint, 1997). The increased abundance of *Prevotella* in native cattle could be

299 attributed to enhanced propionate production (Table 2) (Watanabe et al. 2010; Oh et al. 2017;  
300 Konda et al. 2019). Although LIBSHUFF analysis revealed that CNSL feeding altered the fecal  
301 bacterial and archaeal community structures in swamp buffaloes, groupings at the family and  
302 order levels exhibited no apparent change, except that Methnosarcinales became detectable  
303 with CNSL feeding. This order includes methanogens that use acetate in methanogenesis  
304 (Kendall & Boone, 2006), possibly leading to the lower acetate proportion in the feces of  
305 animals fed CNSL (Table 2). *Methanosarcina barkeri* in this order was confirmed to be quite  
306 tolerant to CNSL in our pure-culture study (Wakai et al., unpublished results). Rikeneraceae, a  
307 member of the gram-negative Bacteroidetes, is involved in succinate and propionate synthesis  
308 (Rautio et al. 2003), and its detection frequency increased numerically (but not significantly)  
309 with CNSL feeding (Fig. 3), which suggests the potential for increased propionate in the feces  
310 of animals fed CNSL (Table 2). These microbial changes could be the main factor to modify  
311 hindgut SCFA profile, though another possible factor might be the reflection of the rumen  
312 SCFA profile that had been altered.

313 MiSeq analyses could give deeper insights into fecal microbial and fermentation changes  
314 with CNSL feeding. Feeding CNSL affected diversity indices in buffaloes to a greater extent  
315 than native cattle (Table 4). This is in good agreement with the results of clone library analyses  
316 (Table 3). Accordingly, swamp buffaloes may harbor a fecal microbiota in which community  
317 members are more sensitive to CNSL compared with the fecal microbiota of native cattle. This  
318 corresponds to the observation that rumen fermentation and microbiota composition exhibited  
319 greater changes with CNSL feeding in buffaloes than in native cattle (Konda et al. 2019). CNSL  
320 could thus be more functional in buffaloes than native cattle with regard to modulation of the  
321 gut microbiota and fermentation, both in the rumen and lower digestive tract and feces. Indeed,  
322 decreases in unclassified Ruminococcaceae and *Treponema* in buffaloes fed CNSL (Table 5)  
323 could explain the suppression of hydrogen and formate production for methane synthesis based

324 on their reported fermentation products (Stewart & Flint, 1997), even though the metabolic  
325 contribution of the former group is yet unclear. The functions of other shifting genera  
326 ([Bacteroides] 5-7N15, etc.) should also be evaluated.

327 The methanogenic archaeal community structure in feces also changed with CNSL  
328 feeding (Tables 4 & 6), together with a decrease of the detection frequency of vadin CA11  
329 (Methanomassiliicoccaceae) in swamp buffaloes. The ruminal abundance of  
330 Methanomassiliicoccaceae reportedly decreases with feeding of alkyl phenols, not only those  
331 derived from cashews (Konda et al. 2019) but also those derived from ginkgo fruit (Oh et al.  
332 2017). Therefore, this change should be consistent throughout the rumen and hindgut, possibly  
333 contributing to methane suppression in these gut fermentation sites. The contribution of  
334 Methanomassiliicoccaceae to methane production has been described in meta-transcriptomic  
335 analyses of the cattle rumen (Poulsen et al., 2013). Levels of methyl-coenzyme M reductase  
336 transcripts from this group decrease with feeding of rapeseed oil, together with a suppression  
337 of methane production. Ivy fruit saponin also decreases the abundance of this group of  
338 methanogens, with a concomitant decrease in methane production in experiments using the  
339 rumen simulation technique (Belanche et al., 2016). Therefore, the Methanomassiliicoccaceae  
340 may be an indicator of suppression of methane production in both the rumen and hindgut.

341 In conclusion, methane emission from ruminant animals can be suppressed by feeding  
342 CNSL. CNSL feeding suppresses methane production not only in the rumen but also the  
343 hindgut and even feces. This suppression is mediated via the selective anti-microbial surfactant  
344 activity of alkyl phenols in CNSL, which alter the gut microbial community structure, with  
345 resultant changes in the fermentation pattern. CNSL feeding might be useful for reducing  
346 methane emission from excreta in wider applications such as slurry storage and composting.  
347 Indeed, the addition of CNSL to flooded paddy soil decreases methane production without  
348 adversely affecting rice growth (Minamikawa et al. 2021). This could be another strategy for

349 CNSL feeding to reduce the environmental impact of agriculture.

350

## 351 **ACKNOWLEDGEMENTS**

352 This study was supported in part by Cabinet Office, Government of Japan, Cross-  
353 ministerial Moonshot Agriculture, Forestry and Fisheries Research and Development Program,  
354 “Technologies for Smart Bio-industry and Agriculture” (No. JPJ009237), and by a Grant-in-  
355 Aid for Scientific Research (B) to Y.K. (no. 18H02322) from the Ministry of Education, Culture,  
356 Sports, Science and Technology, Japan.

357

## 358 **CONFLICT OF INTEREST**

359 We certify that there is no conflict of interest related to the present study.

360

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Figure captions

FIGURE 1 Alkyl-phenols of cashew nut shell liquid (CNSL) in diet and in feces of Thai local ruminants fed CNSL

FIGURE 2 Effect of cashew nut shell liquid (CNSL) feeding on fecal bacterial abundance in Thai local ruminants (upper for native cattle and lower for swamp buffaloes) determined by quantitative PCR

FIGURE 3 Effect of cashew nut shell liquid (CNSL) feeding on fecal bacterial [A] and archaeal [B] community of Thai native cattle (left) and swamp buffaloes (right) determined by clone library analysis targeted for 16S rRNA and methyl coenzyme reductase A (mcrA) genes. Bacteria and archaea were classified at family and order levels, respectively, based on sequence similarity.

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TABLE 1 Ingredients and chemical composition of experimental diet

	Thai native cattle	Swamp buffaloes
Feed ingredient (% in dry matter)		
Concentrate		
Cassava chip	5.75	5.03
Corn	4.48	3.92
Soymeal	3.33	2.91
Urea	0.16	0.14
Salt	0.16	0.14
Vitamin and mineral mixture *	0.16	0.14
Rice straw	84.9	86.64
CNSL or control pellet	1.06	1.09
Chemical composition (% in dry matter)		
Crude protein	8.22	7.77
Ether extract	2.28	2.25
Crude ash	17.96	17.88
Neutral detergent fiber	56.83	57.70
Non-fiber carbohydrates	14.71	14.40

\* Agromix beef No.46 (A.I.O. Co. Ltd, Nakhon Pathom, Thailand): vitamin A = 2160000 IU, vitamin B3 = 100,000 IU, vitamin E = 5,000 IU, Mn = 8.5g, Zn = 6.4g, Cu = 1.6g, Co = 320mg., I = 800mg, Se = 32mg.

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TABLE 2 Effect of cashew nut shell liquid feeding on fecal fermentation parameters and gas production potential

Item	Thai native cattle		Swamp buffaloes	
	Control	CNSL	Control	CNSL
Fermentation parameters				
pH	6.89	7.45 *	6.84	7.12 **
Total SCFA (mmol/g feces)	0.068	0.073	0.057	0.046
Acetate (molar %)	76.5	60.9 **	78	68.4 **
Propionate (molar %)	12.5	22.5 **	12.9	19.6 **
Butyrate (molar %)	7.0	8.8	4.9	8.3 *
Ammonia (mg/g feces)	0.040	0.039	0.038	0.032
Gas production potential				
Total gas (ml/g feces)	0.058	0.018 **	0.027	0.017 **
CO <sub>2</sub> (ml/g feces)	0.046	0.016 **	0.022	0.015 **
CH <sub>4</sub> (ml/g feces)	0.011	0.002 **	0.0043	0.0011 **
H <sub>2</sub> (ml/g feces)	0.0012	0.0008 **	0.0012	0.0009 **

501 \*; \*\*, significantly different from control ( $P < 0.05$ ;  $P < 0.01$ ).

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TABLE 3. Effect of cashew nut shell liquid (CNSL) feeding on microbial biodiversity by clone library analysis for bacterial 16S rRNA and methanogenic archeal methyl coenzyme reductase A genes in feces of Thai local ruminant animals

Diversity indices	Thai native cattle				Swamp buffaloes			
	Bacterial community		Archaeal community		Bacterial community		Archaeal community	
	Control	CNSL	Control	CNSL	Control	CNSL	Control	CNSL
No of clones	153	168	169	163	165	171	171	181
OTU	125	127	16	16	120	120	14	13
Shannon index	4.74	4.73	1.6	1.8	4.59	4.67	1.8	1.7
Chao 1	473	321	34	16	329	368	15	22
LIBSHUFF	ns	ns	ns	ns	*	**	**	*

504 \*, \*\*, significantly different from another treatment ( $P < 0.05$ ;  $P < 0.01$ ).

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TABLE 4. Effect of cashew nut shell liquid (CNSL) feeding on microbial biodiversity by MiSeq analysis for bacterial 16S rRNA and methanogenic archaeal methyl coenzyme reductase A genes in feces of Thai local ruminant animals

Diversity indices	Thai native cattle				Swamp buffaloes			
	Bacterial community		Archaeal community		Bacterial community		Archaeal community	
	Control	CNSL	Control	CNSL	Control	CNSL	Control	CNSL
No of sequence	45,611	40,332	40,123	47,814	52,001	49,889	45,550	43,721
OTU	2,501	2,311	161	121	2,100	1,899 *	153	105 *
Shannon index	7.74	6.38	1.60	1.80 *	6.12	5.82 *	1.53	1.28 *
Chao 1	1356	1233 *	232	145 *	1363	1211 *	202	180 *

507 \*, significantly different from control ( $P < 0.05$ ).

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Table 5 Effect of cashew nut shell liquid (CNSL) feeding on fecal bacterial community in Thai local ruminants determined by MiSeq analysis (shown at genus level)

Taxon	Thai native cattle		Swamp buffaloes	
	Control	CNSL	Control	CNSL
Unclassified Ruminococcaceae	34.2	35.8	42.4	38.0 #
Unclassified Bacteroidales	8.8	8.7	8.6	7.1
Unclassified Clostridiales	6.8	6.9	7.1	8.3
<i>Akkermansia</i>	9.4	6.9	3.9	4.3
Unclassified Bacteroidaceae	3.8	4.4	4.8	3.9
5-7N15	3.6	3.9	3.7	5.7 *
Unclassified Lachnospiraceae	3.0	3.5	2.9	4.8 †
Unclassified Rikenellaceae	3.0	2.8	2.6	2.7
<i>Oscillospira</i>	1.5	1.9 *	1.7	2.0 *
Unclassified RF16	2.0	2.3	1.4	1.5
CF231	1.5	2.2	1.9	0.7
Unclassified YS2	1.4	1.2	1.3	1.4
<i>Dorea</i>	1.0	1.0	1.5	1.4
Unclassified p-2534-18B5	1.3	1.5	1.1	0.6
Unclassified Clostridiaceae	0.9	0.9	1.2	1.3
Unclassified Victivallaceae	1.3	1.1	1.1	0.7
Unclassified RFP12	1.4	1.0	0.5	0.9
<i>Phascolarctobacterium</i>	0.8	0.9	0.7	1.1 *
<i>Paludibacter</i>	1.1	1.2	0.6	0.3 †
<i>Treponema</i>	1.2	0.6 *	0.8	0.6
[Prevotella]	0.3	0.5	0.4	1.5
Unclassified Pirellulaceae	1.0	0.8	0.5	0.4
Others	10.7	9.8	9.4	10.9

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Table 6 Effect of cashew nut shell liquid (CNSL) feeding on fecal archaeal community in Thai local ruminants determined by MiSeq analysis (shown at genus level)

Taxon	Thai native cattle		Swamp buffaloes	
	Control	CNSL	Control	CNSL
<i>Methanocorpusculum</i>	52.4	48.7	36.1	22.2
<i>Methanobrevibacter</i>	38.4	41.7	55.5	70.0
vadin CA11	5.9	6.1	5.0	2.3 †
Methanimicrococcus	2.3	2.4	0.5	3.2
<i>Methanosphaera</i>	0.3	0.6	1.3	1.9
Unclassified Methanobacteriaceae	0.4	0.1	1.3	0.3
Unclassified Methanocorpusculaceae	0.4	0.3	0.2	0.2

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† , tended to be different from control ( $P < 0.1$ ).

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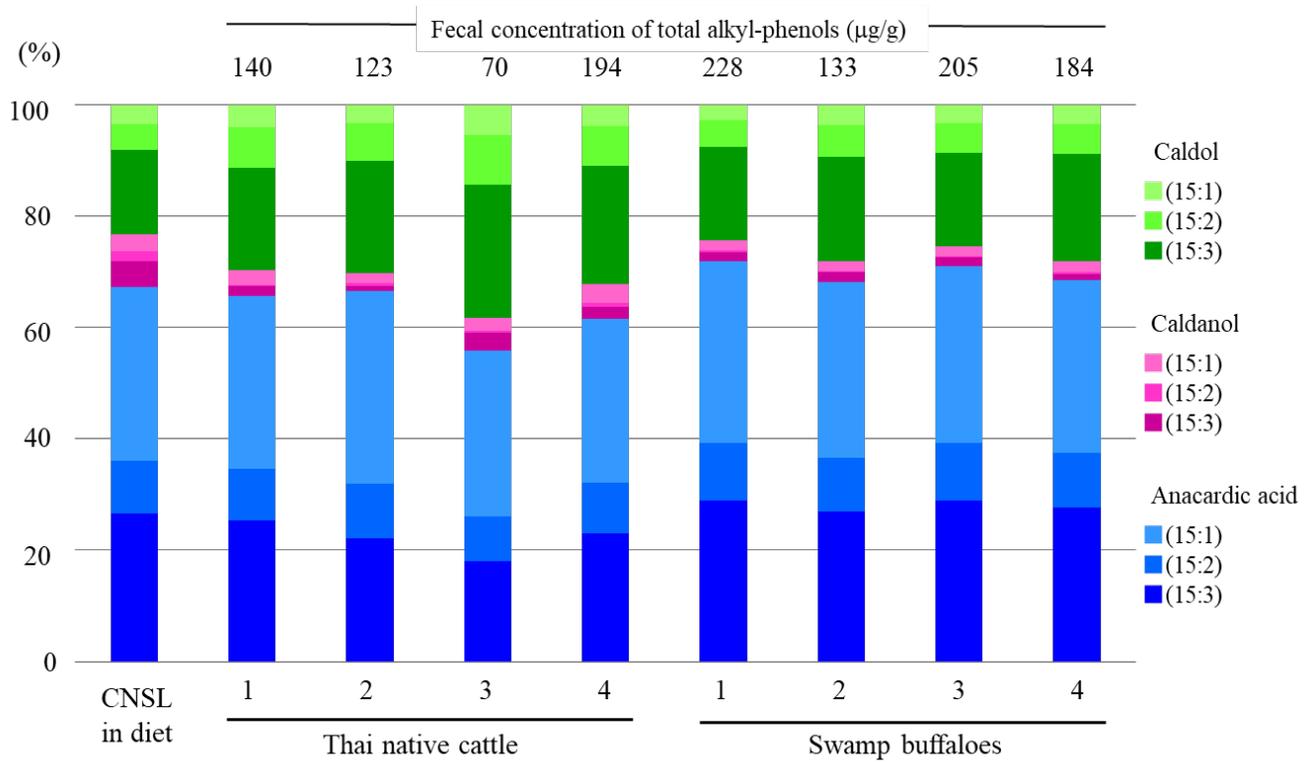


Fig.1

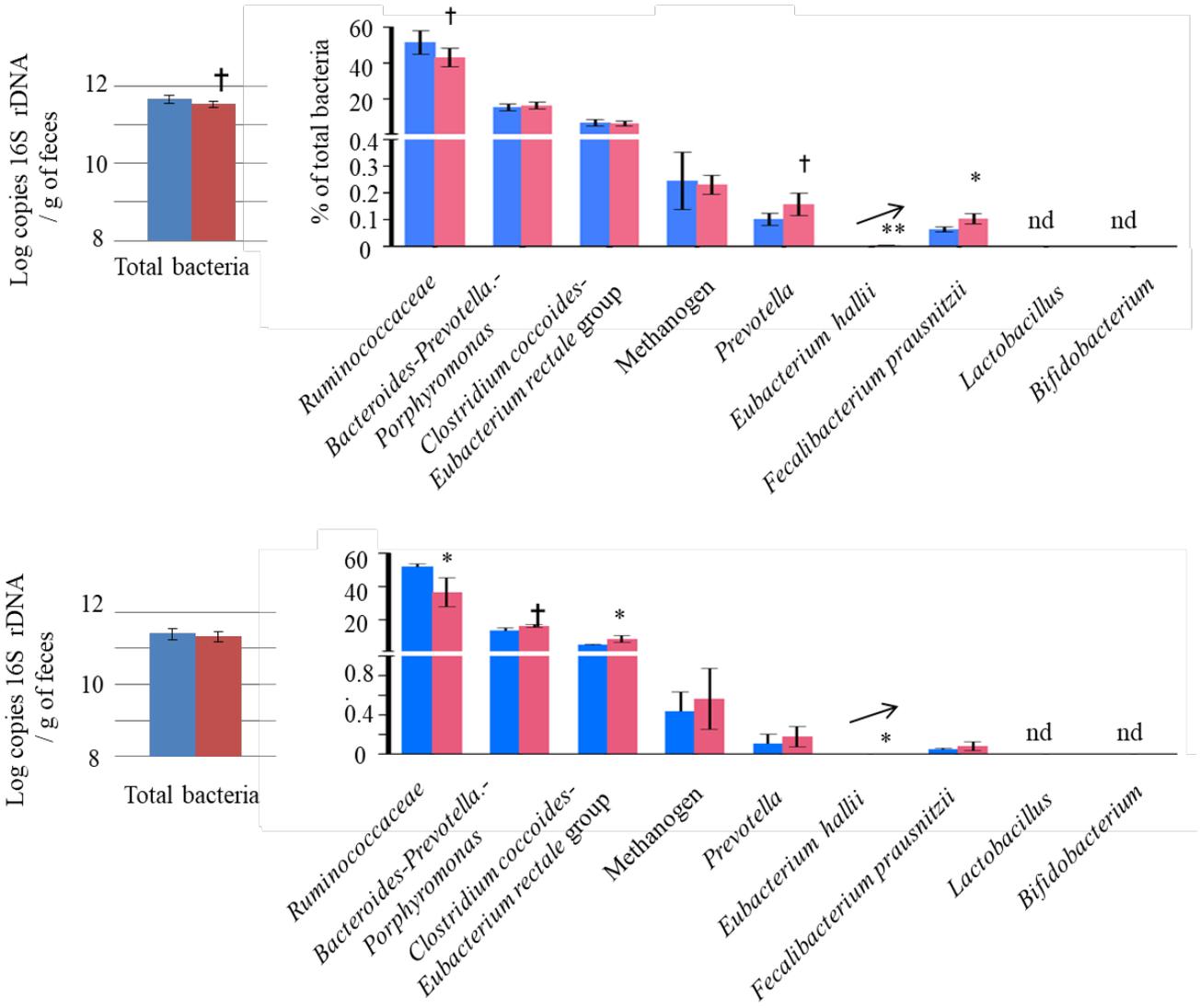
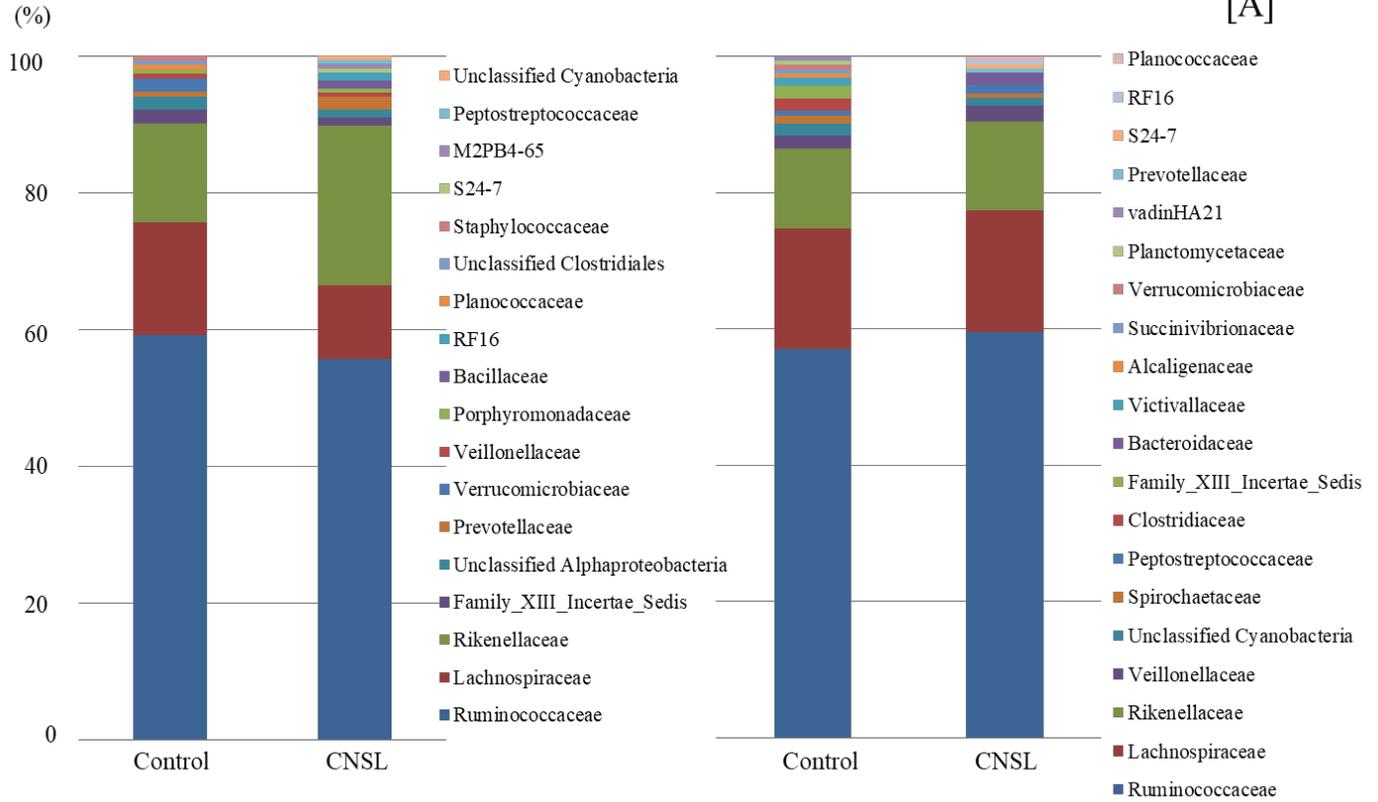


Fig.2

[A]



[B]

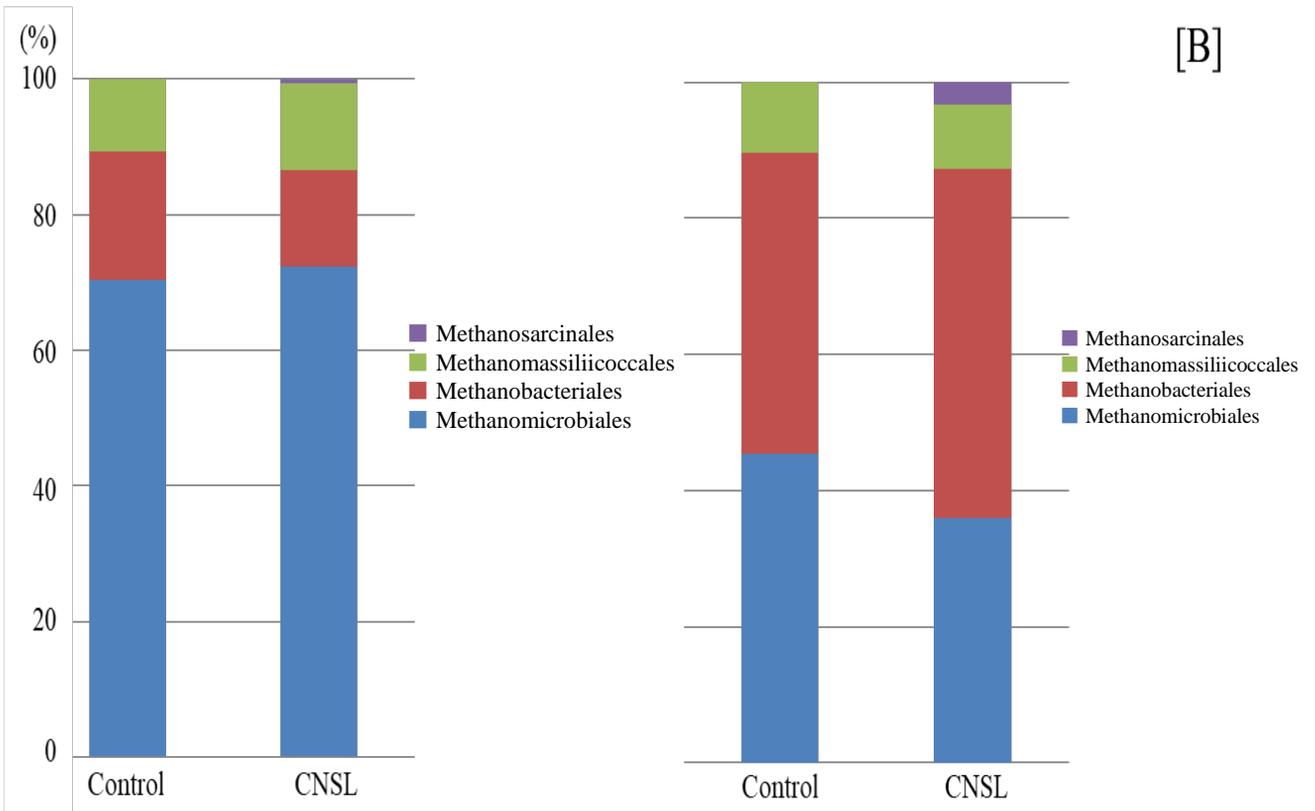


Fig.3