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Author(s)	Tamori, Koya; Matsunaga, Bungo; Boonsaen, Phoompong; Khongpradit, Anchalee; Sawanon, Suriya; Nagashima, Kyo; Koike, Satoshi; Kobayashi, Yasuo
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Feeding cashew nut shell liquid decreases methane production from feces by altering fecal bacterial and archaeal communities in Thai local ruminants

Koya Tamori [1, 2], Bungo Matsunaga [1], Phoompong Boonsaen [3], Anchalee Khongpradit [3], Suriya Sawanon [3], Kyo Nagashima [2], Satoshi Koike [1] and Yasuo Kobayashi [1]

1 Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan

2 Agri-Bio Business Department, Idemitsu Kosan Co., Ltd., Tsukuba, Ibaraki 300-2646, Japan

3 Department of Animal Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand

Correspondence: Yasuo Kobayashi, Graduate School of Agriculture, Hokkaido University, Kita, Sapporo, Hokkaido 060−8589, Japan

E-mail: kyas@anim.agr.hokudai.ac.jp

Abstract

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

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

1 | INTRODUCTION

Agriculture-associated methane accounts for 20% of global anthropogenic methane emissions, of which 40% is of ruminant origin (IPCC, 2007). Accordingly, mitigating methane production by ruminant animals has long been targeted. Methane is produced primarily in the rumen by symbiotic methanogenic archaea, typically hydrogenotrophic methanogens. These methanogens generate methane using hydrogen and CO_2 produced by other microbes, which is regarded as the major pathway for consuming metabolic hydrogen, which will hamper gut fermentation if it accumulates. Thus, a basic strategy for ruminal methane mitigation is to shift the hydrogen-utilizing process from methanogenesis to other pathways (Ungerfeld, 2015), represented by fumarate reduction leading to propionate production.

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

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

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

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

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

2 | MATERIALS AND METHODS

2.1 | Animals and diets

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

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

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

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

2.2 | Sampling and measurement

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

Gas production potential was evaluated essentially as described by Kang et al. (2018). In brief, feces (100g for each animal) was diluted with an equal volume of buffer (pH 6.8) (McDougall, 1948) and strained with a surgical gauze. The filtrate (10ml) was dispensed into a Hungate tube (18ml in volume), to which a butyl rubber stopper and plastic cap were fitted after flushing the head space with N_2 gas. Then, tubes were anaerobically incubated at 38°C 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 − (% NDF + % CP + % crude fat + % 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_2, CH_4,
158 and H_2) 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-Porphyromonas*, 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

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

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

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

primer sets, S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') for bacterial rRNA genes (Herlemann et al., 2011), and arch349F (5'-GYGCASCAGKCGMGAAW-3') and arch806R (5'-GGACTACVSGGGTATCTAAT-3') for archaeal rRNA genes (Takai and Horikoshi, 2000). PCR was carried out in a total volume of 50 µL, consisting of 10 µL of 5× PrimeSTAR Buffer, 4 µL of dNTP mixture (2.5 mM each), 0.5 µL of PrimeSTAR HS DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 1 µL of each primer (10 pmol/µL), 32.5 µL of dH_2O, and 1 µL of template DNA (10 ng/µL). The following PCR conditions were used: 30 cycles for bacteria and 40 cycles for archaea, consisting of denaturation at 98°C (10 s), annealing at 55°C (15 s), and extension at 72°C (30 s). Amplicon sequencing was carried out using MiSeq, as described by Caporaso et al. (2012). Data quality control and analyses were performed using the QIIME (ver. 1.8.0) pipeline (Caporaso et al., 2010). Operational taxonomic units (OTUs) were generated from sequences clustered at a 97% similarity threshold using the UCLUST algorithm (Edgar, 2010). Chimeric sequences were removed from the analysis using the ChimeraSlayer algorithm. Taxonomy was assigned using the Greengenes database (ver. 13.8) at a 90% similarity threshold. Differences in biodiversity between samples in the control and CNSL-feeding periods were compared by alpha diversity metrics: Chao1, Shannon index, and observed number of OTUs. The sequences obtained were deposited in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under accession nos. LC028553-LC028891, LC029039-LC029391 and PRJNA682863.

2.5 | Statistical analysis

Data from the two sampling days in each period were averaged to obtain representative data for each animal, which were examined using the Student's *t*-test with SPSS software (ver. 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_2, 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 *Prevotell*a, *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

(Methanomassiliicoccaceae).

4 | DISCUSSION

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

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

Such fermentation shifts are presumably induced by shifts in the fecal microbiota composition (Figure 2) that can be explained in part by a decrease in Ruminococcaceae (both in native cattle and swamp buffaloes), to which hydrogen-producing bacteria could belong (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

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

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

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

CNSL feeding to reduce the environmental impact of agriculture.

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CONFLICT OF INTEREST

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

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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 **
CO2 (ml/g feces)	0.046	0.016 **	0.022	0.015 **
CH4 (ml/g feces)	0.011	0.002 **	0.0043	0.0011 **
H2 (ml/g feces)	0.0012	0.0008 **	0.0012	0.0009 **

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

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	*	**	**	*

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

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 *

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

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)

	Thai native cattle		Swamp buffaloes	
Taxon	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
Akkermansia	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
Oscillospira	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
Dorea	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
Phascolarctobacterium	0.8	0.9	0.7	1.1 *
Paludibacter	1.1	1.2	0.6	0.3 †
Treponema	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

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

	Thai native cattle		Swamp buffaloes	
Taxon	Control	CNSL	Control	CNSL
Methanocorpusculum	52.4	48.7	36.1	22.2
Methanobrevibacter	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
Methanosphaera	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

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

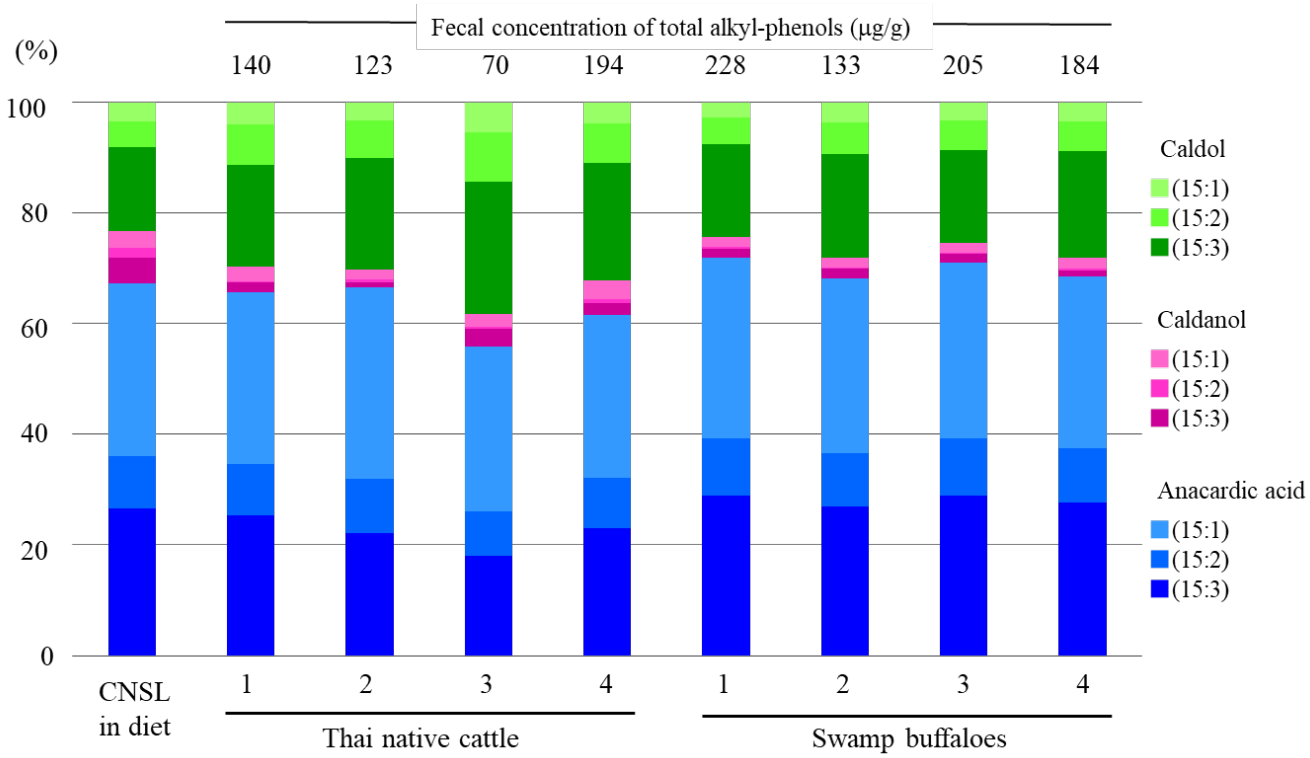

Fecal concentration of total alkyl-phenols (μg/g)
(%)
140
123
70
194
228
133
205
184
100
80
60
40
20
0
Caldol
(15:1)
(15:2)
(15:3)
Caldanol
(15:1)
(15:2)
(15:3)
Anacardic acid
(15:1)
(15:2)
(15:3)
CNSL in diet
1
2
3
4
1
2
3
4
Thai native cattle
Swamp buffaloes

Fig.1

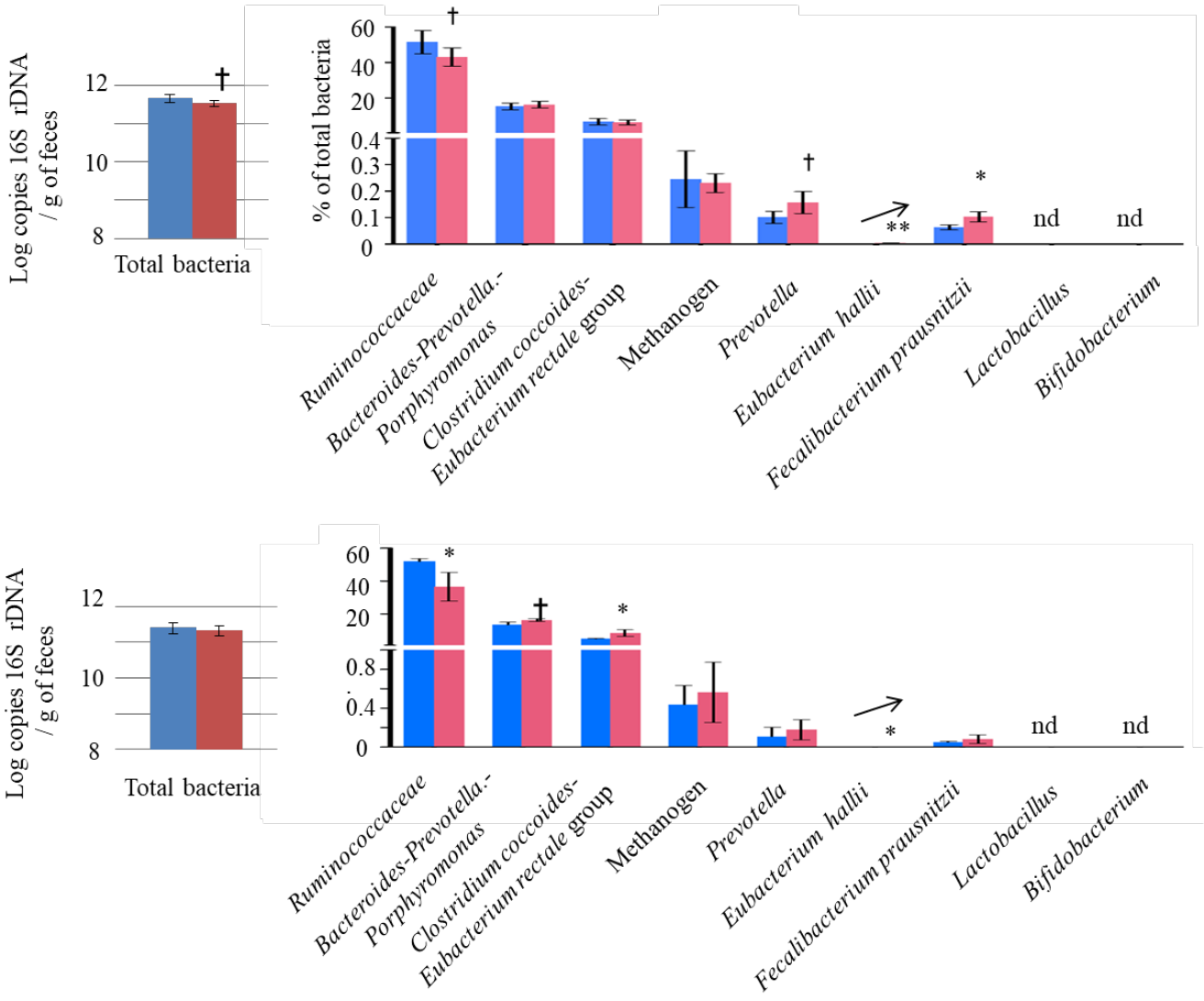
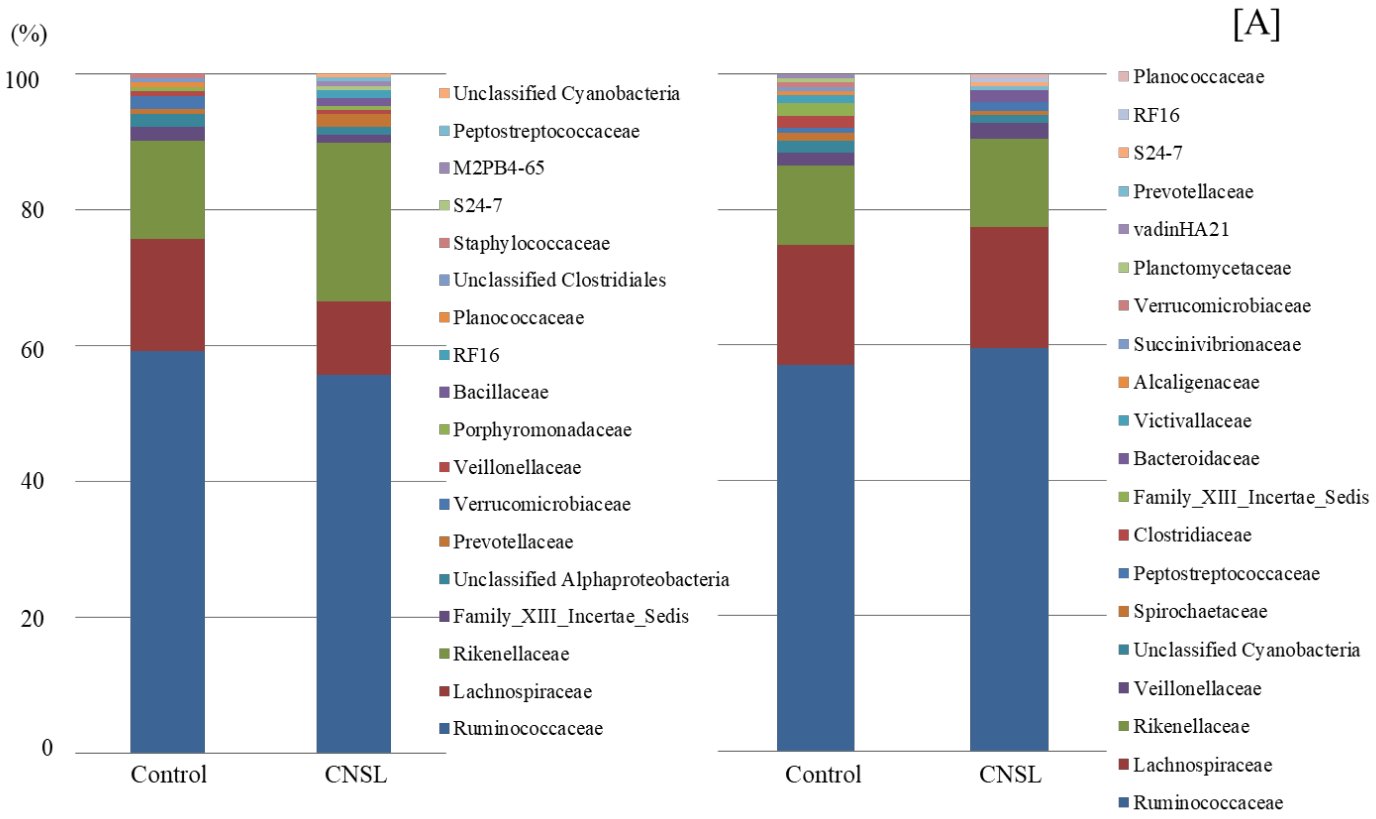

Log copies 16S rDNA / g of feces
12
10
8
†
Total bacteria
% of total bacteria
60
40
20
0.4
0.3
0.2
0.1
0
†
†
**
*
nd
nd
Ruminococcaceae
Bacteroides-Prevotella-Porphyromonas
Clostridium coccoides-Eubacterium rectale group
Methanogen
Prevotella
Eubacterium hallii
Fecalibacterium prausnitzii
Lactobacillus
Bifidobacterium
Log copies 16S rDNA / g of feces
12
10
8
Total bacteria
60
40
20
0.8
0.4
0
*
‡
*
*
nd
nd
Ruminococcaceae
Bacteroides-Prevotella-Porphyromonas
Clostridium coccoides-Eubacterium rectale group
Methanogen
Prevotella
Eubacterium hallii
Fecalibacterium prausnitzii
Lactobacillus
Bifidobacterium

Fig.2


[A]
(%)
100
80
60
40
20
0
Control
CNSL
Unclassified Cyanobacteria
Peptostreptococcaceae
M2PB4-65
S24-7
Staphylococcaceae
Unclassified Clostridiales
Planococcaceae
RF16
Bacillaceae
Porphyromonadaceae
Veillonellaceae
Verrucomicrobiaceae
Prevotellaceae
Unclassified Alphaproteobacteria
Family_XIII_Incertae_Sedis
Rikenellaceae
Lachnospiraceae
Ruminococcaceae
Control
CNSL
Planococcaceae
RF16
S24-7
Prevotellaceae
vadinHA21
Planctomycetaceae
Verrucomicrobiaceae
Succinivibrionaceae
Alcaligenaceae
Victivallaceae
Bacteroidaceae
Family_XIII_Incertae_Sedis
Clostridiaceae
Peptostreptococcaceae
Spirochaetaceae
Unclassified Cyanobacteria
Veillonellaceae
Rikenellaceae
Lachnospiraceae
Ruminococcaceae

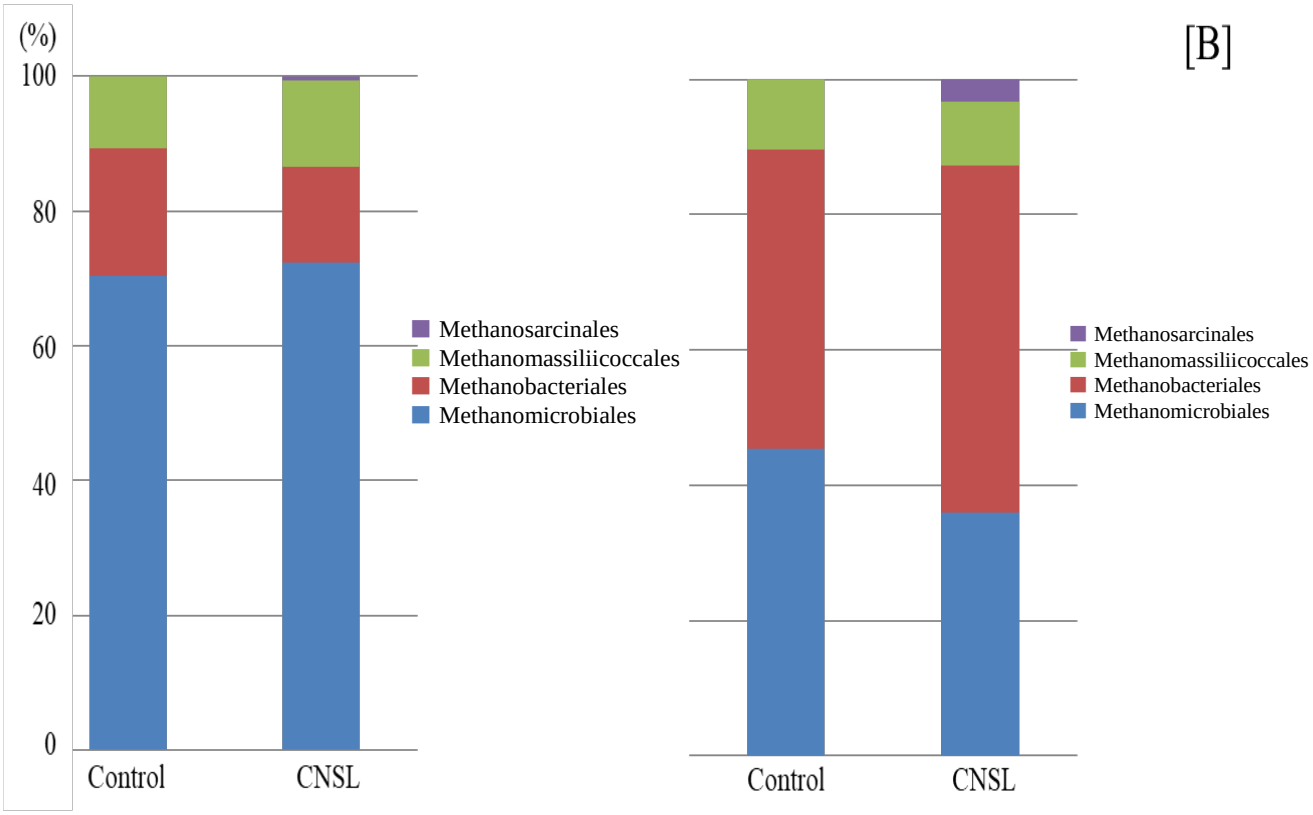

[B]
(%)
100
80
60
40
20
0
Control
CNSL
Methanosarcinales
Methanomassiliicoccales
Methanobacteriales
Methanomicrobiales
Control
CNSL
Methanosarcinales
Methanomassiliicoccales
Methanobacteriales
Methanomicrobiales

Fig.3