Growth and morphologic response of rumen methanogenic archaea and bacteria to cashew nut shell liquid and its alkylphenol components

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ABSTRACT

The growth and morphology of rumen methanogenic archaea (15 strains of 10 species in 5 genera, including 7 strains newly isolated in the present study) and bacteria (14 species in 12 genera) were investigated using unsupplemented *in vitro* pure cultures and cultures supplemented with cashew nut shell liquid (CNSL) and its phenolic compound components, anti-methanogenic agents for ruminant animals. Growth of most of the methanogens tested was inhibited by CNSL and alkylphenols at different concentrations ranging from 1.56 to 12.5 μg/mL. Of the alkylphenols tested, anacardic acid exhibited the most potent growth inhibition. Three gram-negative bacterial species involved in propionate production were resistant to CNSL and alkylphenols (>50 μg/mL). All the methanogens and bacteria that were sensitive to CNSL and alkylphenols exhibited altered morphology; disruption of the cell surface was notable, possibly due to surfactant activity of the tested materials. Cells division was inhibited in some organisms, with cell elongation and unclear septum formation observed. These results indicate that CNSL and alkylphenols, particularly anacardic acid, inhibit both rumen bacteria and methanogens in a selective manner, which could help mitigate rumen methane generation.

Key words: anacardic acid, archaea, bacteria, growth, morphology

INTRODUCTION

Rumen fermentation yields short-chain fatty acids as an energy source for the host ruminant animal. However, this is accompanied by the production of undesirable methane gas, which diminishes feed energy (Johnson & Johnson, 1995) and acts as a greenhouse gas (FAO, 2021). To conserve dietary energy and ease the environmental burden of rumen fermentation, considerable research over the last few decades has focused on mitigation of methane production by ruminants. Through such studies, several different feed additives have been developed to modulate activity of the rumen microbiota and fermentation profiles with the goal of lowering methane generation (Kobayashi et al. 2016; Beauchemin et al. 2020). Recently developed functional additives that have been or are being commercialized include 3-nitrooxypropanol (Hristov et al. 2015), Oceanian red-algae *Asparagopsis taxiformis* (Roque et al. 2019), and cashew nut shell liquid (CNSL) (Shinkai et al. 2012; Konda et al. 2019). The former two materials inhibit methyl-coenzyme M reductase, which is involved in the final step of ruminal methanogenesis, whereas CNSL inhibits bacteria that produce hydrogen and formate, substrates for methane synthesis. All three additives can cause significant shifts in the rumen microbial community, based on sequencing analyses of bacterial and methanogenic archaeal genes (Hristov et al. 2015; Roque et al. 2019; Konda et al. 2019; Maeda et al. 2021; Su et al. 2021).

CNSL contains secondary plant compounds categorized as alkylphenols and represented by anacardic acid, cardanol, and caldol, which are distributed among quite a limited number of plant species belonging to the family Anacardiaceae. These alkylphenols exhibit wide-ranging activities, including anti-bacterial (Kubo et al. 1993a), anti-oxidative (Kubo et al. 2006; Trevisan et al. 2006), and anti-carcinogenic (Kubo et al.

72 1993b), such that multiple applications in the food, health promotion, and medical fields
73 can be expected. CNSL-formulated additives are already in practical use in cattle and
74 poultry in Japan (Rumi-Up™ and Clo-Stop™, Idemitsu Co., Ltd., Tokyo, Japan,
75 respectively), with more extensive and wider applications in the animal industry expected.
76 As such, more detailed information regarding animal and gut microbial responses to these
77 additives in different animal breeds and species is needed, including a better
78 understanding of the precise mechanisms involved in the beneficial effects in target
79 animals.

80 Although molecular analyses of the rumen microbiota have elucidated the
81 ecological responses to anti-methanogenic CNSL, biological data for microbial species
82 at the pure-culture level in relation to the efficacy of CNSL are extremely limited. Oh et
83 al. (2017a, 2017b) assessed the growth of representative rumen bacteria on alkylphenols
84 from ginkgo fruit, the components of which differ from those of CNSL, and found that
85 the bacteria exhibit different growth responses to each alkylphenol. As ginkgo fruit
86 contains alkylphenols that differ structurally from those of CNSL (carbon lengths 13, 15,
87 and 17 for ginkgo vs. only 15 for CNSL), the characteristics of CNSL alkylphenols cannot
88 be determined from evaluations of ginkgo compounds (Oh et al. 2017a). Moreover,
89 methanogens have never been employed for such analyses due to the greater difficulty of
90 maintaining and cultivating these organisms in comparison with bacteria (Buan 2018).

91 We describe here the growth and morphologic responses of representative rumen
92 microbes, including methanogens, to the methane-mitigating additive CNSL and its 9
93 alkylphenol components that differ in chemical structure. The methanogens tested
94 included type strains of representative species as well as wild strains newly isolated in the
95 present study from sheep rumen, cow feces, and cow slurry. We examined the relationship

between growth suppression of methanogen and bacterial species and specific alky-phenols and also related growth inhibition to changes in cell morphology following CNSL exposure. Such evaluations can deepen our understanding of the mechanisms involved in the methane-mitigating activity of CNSL.

MATERIALS & METHODS

CNSL and its alky-phenol components

CNSL and its alkylphenol components were provided by the Advanced Research Section, Idemitsu Co., Ltd. Nine molecular types of alkylphenols were isolated, including anacardic acid, cardanol, and caldol, each of which has an alkyl side chain with 1, 2, or 3 double bonds, as shown in Figure 1. Microbial sensitivity was evaluated against all 9 of these alkylphenols, along with CNSL.

Microbes

Methanogenic archaeal strains (type strains of 8 species belonging to 5 genera) were obtained from JCM and ATCC: *Methanobrevibacter smithii* PS (ATCC35061), *Methanobrevibacter wolinii* SH (BAA-170), *Methanomicrobium mobile* BP (JCM10551), *Methanobacterium bryantii* M.o.H (ATCC33272), *Methanosphaera stadtmanae* MCB-3 (JCM11832), *Methanobrevibacter ruminantium* M1 (JCM13430), *Methanobacterium formicicum* MF (JCM10132), and *Methanosarcina barkeri* MS (JCM10043). In addition to these type strains, 7 wild strains (*Methanobrevibacter olleyae* W1 and R1; *M. ruminantium* W2, W3, and W4; and *M. smithii* S1 and *Methanobrevibacter millerae* F2) were newly isolated and examined in the present study. The procedures for microbial isolation and identification are described in the next section. Detailed information

120 regarding all methanogens examined in the present study is summarized in Table 1, and
121 the phylogenetic relationship of these organisms is illustrated in Figure 2.
122 Rumen bacteria examined in this study included *Streptococcus bovis* ATCC33317,
123 *Lactobacillus ruminis* RF1 (ATCC27780), *Butyrivibrio proteoclasticus* ATCC51982,
124 *Eubacterium ruminantium* GA195 (ATCC17233), *Butyrivibrio fibrisolvens* D1
125 (ATCC19171), *Ruminococcus albus* 7 (ATCC27210), *Ruminococcus flavefaciens* C94
126 (ATCC19208), *Fibrobacter succinogenes* S85 (ATCC19169), *Prevotella ruminicola* 23
127 (ATCC19189), *Succinimonas amylolytica* $B_2$4 (ATCC19206), *Ruminobacter amylophilus*
128 ATCC29744, *Succinivibrio dextrinosolvens* 24 (ATCC19716), *Selenomonas*
129 *ruminantium* GA192 (ATCC12561), and *Megasphaera elsdenii* LC1 (ATCC25940). All
130 of the above organisms were type strains obtained from the ATCC.
131
132 **Methanogen isolation and identification**
133 Methanogens were isolated and identified according to the following procedures.
134 Rumen content donors included two ruminally cannulated sheep fed a hay and
135 concentrate diet (3:7 ratio, given at maintenance level) once daily (0830 hours). Rumen
136 contents were taken from each sheep before feeding, equally mixed, and strained using 4
137 layers of surgical gauze. The strained rumen fluid was diluted with an equal volume of
138 McDougal's buffer (pH 6.8) (McDougall 1948) and then incubated for 24 h at 38°C with
139 milled hay and concentrate (0.2 g at 3:7 ratio) in a tube (10 mL) equipped with a butyl
140 rubber stopper and plastic screw cap. The head space gas in the tube was replaced with
141 hydrogen for enrichment of methanogens. Hydrogen gas replacement was carried out
142 using a GR-8 gas replacement apparatus (Sanshin, Tokyo, Japan) to create an appropriate
143 pressure (0.1 MPa).

144 Slurry and feces samples were taken from a slurry tank and 2 dry cows on a hay
145 and concentrate diet (9:1 ratio, given at maintenance level), respectively, at the Hokkaido
146 University Field Science Center. These samples were mixed well, diluted, and incubated
147 as described above but without substrates. After 2 culture passages with confirmation of
148 the presence of methane in the headspace, each culture was serially diluted with anaerobic
149 dilution solution (Bryant and Burkey, 1953) for inoculation into the medium
150 recommended for *M. bryantii* (JCM) or PH medium (Paynter and Hungate, 1968) to
151 prepare roll tubes under hydrogen pressure. After 6 days of incubation at 38°C, colonies
152 were picked from tubes in which the presence of methane in the head space gas was
153 confirmed. The colonies picked were again diluted as above and subjected to the same
154 isolation procedure to confirm their purity and methane-generating potential. The purity
155 of the methanogen cultures was then further assessed by PCR to confirm that they were
156 negative for bacteria (primers 27F and 1525R, Rathsack et al. 2014) but positive for
157 archaea (primers Met86F and Met1340R, Saengkerdsub et al. 2007).
158 PCR products with archaeal primers were purified using a High-Pure PCR
159 Product Purification kit (Roche). The purified DNA was quantified (Nano Drop 2000,
160 Thermo Fisher Scientific, Waltham, MA, USA), diluted appropriately, and then
161 sequenced (Takara Bio Inc., Kusatsu, Japan). The sequences were searched using BLAST
162 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related species, and then
163 phylogenetic analyses were carried out using MEGA4 and Bio Align.
164 For taking above animal samples, we followed the Guidelines for Animal
165 Experiments, Hokkaido University (2007) and the Act on Welfare and Management of
166 Animal (2005).
167

168 **Growth response to CNSL and its alkylphenol components**

169 Each methanogen was cultivated on medium recommended by the ATCC or JCM
170 under hydrogen pressure (0.1 MPa) and supplementation with CNSL or each molecular
171 type of alkylphenol at 0, 1.56, 3.13, 6.25, 12.50 μg/mL (0 μg/mL for control). These
172 materials were dissolved in 99.5% ethanol and then added to the medium. For the control,
173 the same amount of ethanol (0.1 mL/tube) was added. The tubes were incubated at 38°C
174 for 7-11 days (up to stationary phase of the control culture, depending on the strain).
175 Growth of the methanogens tested was monitored by quantifying methane gas in the
176 head space of the culture (0.01ml in detection limit). In fact, methane production was
177 species/strain dependent, ranging from 0.48 to 5.60 ml/tube for un-supplemented control.
178 The minimum inhibitory concentration (MIC) was defined as the minimum concentration
179 required for growth inhibition (no detection of methane in the headspace). The
180 determination of MIC values for the rumen bacteria tested was essentially the same as
181 that described by Watanabe et al. (2010). Growth was monitored by measuring the optical
182 density at 660 nm using a MiniPhoto spectrophotometer (Taitec, Nagoya, Japan). For
183 bacteria, CNSL and alkylphenols were tested at concentrations ranging from 0-50 and 0-
184 12.5 μg/mL, respectively. These tested ranges were set by considering ruminal
185 distribution of oily CNSL when applied at the recommended feeding level (4g/100kg body
186 weight). Statistical analysis was not applied to the microbial growth data, because the
187 study aimed at defining minimum concentrations of tested materials for growth
188 inhibition.

189

190 **Morphologic response to CNSL and its alkylphenol components**

191 Cell morphology was observed using scanning electron microscopy (SEM) and

192 transmission electron microscopy (TEM), as follows. The methanogens and bacteria
193 tested were cultivated in the appropriate medium, as described above. When each microbe
194 reached the exponential phase, CNSL or ethanol (control) was added (200 μg/mL final
195 concentration), and incubation was continued for another 5 h. The cells were then
196 analyzed by electron microscopy as follows. For SEM observations, microbial samples
197 were washed with 20 mM potassium-phosphate buffer (pH 7.2), soaked in 2.5%
198 glutaraldehyde in the same buffer, and then fixed with 1% osmic acid in potassium-
199 phosphate buffer. The samples were then dehydrated using a graded ethanol series (50,
200 70, 90, and 99.5%), followed by isoamyl acetate and a critical point drier (HCP-2, Hitachi,
201 Tokyo, Japan). Samples were then coated with gold-palladium via ion spatter and
202 observed using a high-resolution scanning electron microscope (JSM-6301F, Japan
203 Electron, Tokyo, Japan). For TEM, samples were washed, soaked, and fixed in the same
204 manner described for SEM. Fixed samples were mixed with 2% agar, and after
205 solidification, an agar section was cut from each sample using a razor, and the section
206 was fixed and dehydrated as described for SEM (but using up to 100% ethanol). After
207 replacement of ethanol with QY-1 (n-butyl glycidyl ether), each sample was embedded in
208 resin (Epon 812, EMJapan Co., Ltd., Tokyo, Japan) and polymerized in an oven (60°C).
209 Each embedded sample was then sliced to 70-80 nm using an ultra-microtome (Reichert
210 Ultracuts, Leica, Wetzlar, Germany), stained with uranyl acetate followed by plumbum
211 citrate, and subjected to TEM (H-800, Hitachi, Tokyo, Japan) at an acceleration voltage
212 of 75 kV. All 15 methanogenic archaeal strains of 10 species, as well as 6 species of
213 bacteria (*R. albus*, *R. flavefaciens*, *F. succinogenes*, *B. fibrisolvens*, *S. ruminantium*, and
214 *M. elsdenii*), were observed by SEM, whereas only for *S. bovis* was analyzed by TEM.
215

RESULTS

Seven methanogen strains were isolated as a result of PCR, sequencing, and methane-producing potential analyses. These strains, identified as *M. olleyae*, *M. ruminantium*, *M. smithii*, and *M. millerae*, were then subjected to growth and morphologic characterizations. Sequence data for all of these strains were deposited in the DDBJ under accessions numbers AB928207-AB928209 and AB928211-AB928214.

Table 2 shows MIC values for CNSL and its alkylphenol components against different species of methanogenic archaea. Sensitivity was species dependent, with *M. formicicum*, *M. bryantii*, *M. ruminantium*, *M. olleyae*, and *M. millerae* exhibiting high sensitivity to CNSL (MIC, ≤3.13 μg/mL), whereas *M. smithii*, *M. wolinii*, *M. stadtmanae*, *M. barkeri*, and *M. mobile* were less sensitive or resistant to CNSL (MIC, ≥12.50 μg/mL). However, the sensitivity of *M. smithii* was strain dependent (MIC, >12.50 μg/mL for strain PS and 6.25 μg/mL for strain S1). Among the alkylphenols, anacardic acid and caldol exhibited greater inhibitory activity than cardanol, except that *M. ruminantium* was sensitive even to cardanol, irrespective of strain (MIC, ≤6.25 μg/mL). Although the number of double bonds in the side chains of all alkylphenols had minimal effect on growth inhibition, C15:1 anacardic acid more strongly inhibited *M. smithii* PS (MIC, ≤1.56 μg/mL) than the C15:2 and C15:3 anacardic acids (MIC, ≥12.50 μg/mL). Of the 15 methanogenic archaeal strains tested, those of rumen origin (*M. ruminantium* and *M. olleyae*), excluding *M. mobile*, were consistently sensitive to CNSL and all 9 molecular types of alkylphenol (MIC, ≤6.25 μg/mL).

The sensitivity of rumen bacterial species to CNSL and its alkylphenol components is summarized in Table 3. All gram-positive bacteria (*S. bovis*, *L. ruminis*, *B. proteoclasticus*, *E. ruminantium*, *B. fibrisolvens*, *R. albus*, and *R. flavefaciens*) were

240 sensitive, in some cases in a species-dependent manner (MIC, 50 μg/mL for *L. ruminis*,
241 1.56 μg/mL for *R. flavefaciens*). Gram-negative bacteria, including *R. amylophilus*, *S.*
242 *dextrinosolvens*, *S. ruminantium*, and *M. elsdenii*, were resistant to CNSL (MIC,
243 ≥50.00 μg/mL), whereas *F. succinogenes*, *P. ruminicola*, and *S. amylolytica* were
244 sensitive (MIC, 3.13-12.50 μg/mL). In regard to alkylphenol components, anacardic acid
245 was identified as the primary inhibitory component, whereas cardanol and caldol did not
246 inhibit the growth of any species examined, except for C15:2 and C15:3 caldols against
247 2 species of *Ruminococcus*. Aside from the growth-inhibition activity of these caldols
248 against ruminocci, the number of double bonds in the side chains of the alkylphenols was
249 found to have minimal impact on inhibitory activity.

250 Morphologic responses of methanogenic archaeal species to CNSL as determined
251 by SEM are shown in Figure 3. The surface of *M. ruminantium*, *M. bryantii*, and *M.*
252 *wolinii* cells was disrupted upon exposure to CNSL, with numerous bubble-like bumps
253 observed on the cell surface. Other CNSL-sensitive methanogenic archaeal species (*M.*
254 *formicicum*, *M. smithii*, *M. olleyae*, and *M. millerae*) exhibited similar changes on the cell
255 surface (data not shown). In contrast, CNSL-resistant *M. barkeri* exhibited no particular
256 changes in morphology, maintaining a consistently smooth cell surface. Other less
257 sensitive species exhibited differing responses to CNSL exposure; *M. stadtmanae*
258 retained a smooth surface, whereas bumps formed on the surface of *M. smithii* cells (data
259 not shown).

260 CNSL-induced morphologic changes in rumen bacterial species as determined by
261 SEM and TEM are shown in Figure 4. CNSL-sensitive *R. albus*, *B. fibrisolvens*, and *S.*
262 *bovis* responded differently to CNSL exposure; numerous tiny bumps were observed on
263 the surface of *R. albus* and *S. bovis* cells, whereas *B. fibrisolvens* cells became elongated,

suggesting that cell division was disrupted. In contrast, CNSL-resistant *S. ruminantium* and *M. elsdenii* exhibited no particular changes in morphology upon CNSL exposure, based on SEM observations (data not shown). TEM observation of *S. bovis* indicated that the cytoplasm became heterogeneous, and septum formation was insufficient.

DISCUSSION

The present results provide a basic understanding as to what methanogen and bacterial species are inhibited by various components of CNSL, whether the inhibition is selective, and if selective, how the selection occurs. As certain characteristics of methanogens make their cultivation difficult (i.e., slow growth, substrate preference, etc.) (Buan 2018), evaluations as done in the present study have not been carried out to date. Therefore, this is the first study to demonstrate species- or strain-dependent inhibition of methanogen growth by CNSL and its alkylphenol components.

A few studies on alkylphenol–containing plant byproducts such as CNSL and ginkgo fruit have been performed to monitor rumen microbial community responses and revealed shifts in the methanogen community. These shifts included decreases in *Methanobrevibacter* (Oh et al. 2017a; Maeda et al. 2021; Su et al. 2021) and *Methanomassiliicoccaceae* (Oh et al. 2017a; Konda et al. 2019) and increases in *Methanomicrococcus* (Konda et al. 2019; Su et al. 2021) and *Methanoplanus* (Oh et al. 2017a; Su et al. 2021). Although the species tested in the present pure-culture study were limited, all species of *Methanobrevibacter*, except *M. wolinii* and a single strain of *M. smithii*, were sensitive to CNSL (Table 2). These results agree with the results of the above mentioned feeding (Konda et al. 2019; Su et al. 2021; Maeda et al.2021) and rumen simulation technique (Oh et al. 2017a) studies and strongly suggest that alkylphenols

288 selectively inhibit growth once delivered to the rumen environment.

289 SEM observations suggested that CNSL physically disrupts the surface of
290 methanogen cells, creating bubble-like bumps (Figure 3). The formation of these bumps
291 suggests that CNSL has surfactant activity, as described in an earlier study (Kubo et al.
292 1993a). The significance of such surfactant activity depends on the structure of the
293 microbial cell surface, as clearly shown in bacteria based on the presence of an outer
294 membrane (gram negatives) or naked cell wall (gram positives). Although the cell
295 structure of many methanogens has not been fully elucidated, various methanogens
296 possess a proteinaceous surface (S-) layer as the primary and outermost boundary of the
297 cell envelope (Klingl, 2014), as was reported for *M. barkeri* (Arbing et al. 2012). Indeed,
298 this species was quite resistant to CNSL (Table 2), and its cell surface was not affected
299 by CNSL exposure, judging from SEM observations (Figure 3). Another methanogen
300 tested in the present study that has an S-layer was *M. mobile* (Claus & Konig 2010), which
301 was resistant to CNSL and alkylphenols (Table 2). In contrast, methanogenic archaea
302 lacking an S-layer but possessing pseudomurein (Leathy et al. 2010), such as
303 *Methanobrevibacter,* were sensitive to CNSL (Table 2). This genus encompasses the
304 majority of the rumen methanogenic archaeal community in a broad range of ruminant
305 animals (Henderson et al. 2015). The S-layer thus might function as a barrier against
306 CNSL-derived alkylphenol components for which the side chain is thought to penetrate
307 and thus physically disrupt the microbial cell surface (Kubo et al. 1993a). However, the
308 presence of an S-layer does not ensure resistance of a methanogen to CNSL, as several
309 exceptions have been noted (S-layer–possessing *M. formicicum* and *M. bryantii* were
310 sensitive to CNSL, Table 2). Indeed, the complexity of the cell membrane structure has
311 been noted in studies of different archaea (Klingl, 2014). It remains to be determined as

312 to what factors are involved in maintaining the structural integrity of methanoarchaeal
313 cells in the presence of alkylphenols. Thus, the difficulty still exists to clearly explain the
314 mechanisms in the tolerance of methanogens to these surfactants.

315 The surfactant activity of alkylphenols is thought to be dependent on the molecular
316 type of the side chain (i.e., side chain length and number of double bonds) (Kubo et al.
317 1993a). According to the present results, the number of double bonds affected the
318 inhibitory potential only for strain PS of *M. smithii*, for which C15:1 anacardic acid was
319 more inhibitory than C15:2 and C15:3 anacardic acid (Table 2). However, this was strain-
320 dependent in this species (Table 2). Kubo et al. (1993a) found that structural differences
321 affect the surfactant activity of alkylphenols, with a chain length of 15 carbons associated
322 with the most potent bacterial cell penetration. In contrast, the presence of 3 double bonds
323 (C15:3) in the alkyl side chain exhibited the strongest antibacterial activity against gram-
324 positive bacteria. CNSL contains only 15-carbon alkyl side chains, all of which seemed
325 almost equally functional against the methanogens tested, except for *M smithii* PS, which
326 was inhibited by C15:1 anacardic acid to a greater degree (Table 2). Thus, methanogen
327 may exhibit a mode of response different from that of bacteria, based on the chemical
328 structure of the alkyl side chain.

329 According to Kubo et al. (2003), the balanced presence of hydrophobic and
330 hydrophilic groups in alkylphenols plays an important role in surfactant activity. The loss
331 of a hydrophilic carboxyl group may explain the lower surfactant activity of cardanol in
332 comparison with anacardic acid with 2 hydrophilic groups (carboxy and hydroxyl groups).
333 This was again confirmed in all rumen bacterial species sensitive to CNSL (Table 3),
334 whereas most of the CNSL-sensitive methanogens remained sensitive even to cardanol
335 (Table 2). Thus, methanogens seem to undergo greater physical damage by CNSL and its

components, possibly irrespective of the abovementioned mechanism theory regarding hydrophobicity/hydrophilicity. The details should thus be explored further, particularly for methanogens.

The MICs of the alkylphenols against rumen bacteria were almost the same as those reported for ginkgo fruit (Oh et al. 2017a). Thus, selective inhibition of formate- and hydrogen-producing species occurs, leading to a deficiency of substrates for methanogenesis and indirect mitigation of methane production. Methane substrate providers include the Ruminococcaceae, *Treponema*, and *Butyrivibrio*, the abundances of which decline consistently with CNSL feeding (Watanabe et al. 2010; Shinkai et al. 2012; Kang et al. 2018; Konda et al. 2019; Maeda et al. 2021). The lack of reports of a clear reduction in methane production with CNSL feeding (Branco et al. 2016) could be due to the use of heated CNSL, in which most functional anacardic acid is decarboxylated (Philip et al. 2008) and transformed into less functional cardanol (Himejima & Kubo et al. 1991) that cannot fully select rumen bacteria as shown for anacardic acid.

Another beneficial aspect of CNSL is the inhibition of *S. bovis*, a candidate bacterium linked to feedlot bloat and lactic acidosis (Nagaraja & Titgemeyer, 2007). CNSL-mediated suppression of *S. bovis* growth decreases the viscosity (Watanabe et al. 2010), foam formation, and foam stability (Kang et al. 2018) of rumen fluid. These are useful indices with regard to the prevention and cure of the abovementioned metabolic disorders of cattle fed a high-grain diet.

The present study provides the first indication that CNSL selectively inhibits not only bacteria but also methanogens. In addition, the effects of alkylphenol components of CNSL on cell morphology, in particular anacardic acid, were clearly visualized using microscopic observations. Taken together, these data suggest that the rumen microbial

community can be altered to reduce methane emissions and enhance propionate production via selective suppression of hydrogen- and formate-producing bacteria, as reported previously (Watanabe et al. 2010, Shinkai et al. 2012, Kang et al. 2018; Konda et al. 2019; Maeda et al. 2021; Su et al. 2021). Specific genera/species/strains of methanogenic archaea affected by CNSL were also identified in the present study.

Recent deep sequencing analyses of the rumen microbiota have facilitated efforts to elucidate the functional contribution of each microbial group in the complex rumen ecosystem, although cultivation studies such as the present work provide clear indications of the response of each microbe to a test material. Thus, combinations of both approaches (culture-dependent and -independent methods) can provide insights into the biological function of the rumen and its possible regulation.

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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. Chemical structure of alkylphenols in cashew nut shell liquid
From left to right: anacardic acid, caldanol, caldol, each possessing 3 different alkyl side chains (9 different molecules in total)

FIGURE 2. Phylogenetic relationship of methanogenic archaeal strains used in the present study, based on full sequence of 16S rRNA gene

566 Red, type strains for each species obtained from international culture collections
567 Blue, newly isolated strains in the present study
568
569 FIGURE 3. Morphology of methanogenic archaea as affected by cashew nut shell liquid
570 (CNSL) exposure (Scanning electron microscope × 30,000). From top to bottom:
571 *Methanobrevibacter ruminantium Methanobacterium bryantii, Methanobrevibacter*
572 *wolinii* and *Methanosarcina barkeri,* (left for control; right for CNSL (200μg/ml)). Note
573 that cell surface was disrupted for all methanogens except *M. barkeri*.
574
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576 FIGURE 4. Morphology of rumen bacteria as affected by cashew nut shell liquid (CNSL)
577 exposure. From top to bottom: *Ruminococcus albus* (SEM, ×30,000)*, Butyrivibrio*
578 *fibrisolvens* (SEM, ×10,000)*,* and *Streptococcus bovis* (SEM, ×30,000 and TEM,
579 ×60,000)) (left for control; right for CNSL (200μg/ml)). Note that cell surface was
580 disrupted for *R. albus* and *S. bovis*, while cell division was inhibited for *B. fibrisolvens*.
581 Cytoplasm of *S. bovis* became heterogeneous with no clear septum formation by CNSL.

TABLE 1 Methanogenic archaea strains used in the present study. (upper table for representative strains from international culture collections, lower table for newly isolated strains in the present study)

Species	Strain	Gram staining	Source	Morphology	Methanogeneisis from					Reference
					H_2/CO_2	Formate	Methanol	Acetate	Methylamine	
Methanobacterium formicicum	MF (JCM10132)	+	Swege sludge digester	rods	+	+	-	-	-	Chua & Robinson (1983)
Methanobacterium bryantii	M.o.H (ATCC33272)	+	Syntrophic culture of *Methanobacterium omelianskii*	rods	+	-	-	-	-	Godsy (1980)
Methanobrevibacter ruminantium	M1 (JCM13430)	+	Cattle rumen	rods	+	+	-	-	-	Leathy et al. (2010)
Methanobrevibacter smithii	PS (ATCC35061)	+	Swege digester	cocci	+	+	-	-	-	Balch et al. (1979)
Methanobrevibacter wolinii	SH (BAA-1170)	+	Sheep feces	cocci	+	-	-	-	-	Miller & Lin (2002)
Methanosphaera stadtmanae	MCB-3 (JCM11832)	+	Human stool	cocci	-	-	+	-	-	Miller & Wolin (1985)
Methanosarcina barkeri	MS (JCM10043)	-	Swege sludge digester	cocci	+	+	+	+	+	Hook et al. (2010)
Methanomicrobium mobile	BP (JCM10551)	-	Cattle rumen	rods	+	+	-	-	-	Claus & Konig (2010)

Species	Strain	Gram staining	Source	Morphology	Closest relative (Similarity %)	Reference
Methanobrevibacter ruminantium	W2	+	Sheep rumen	rods	*Methanobrevibacter* sp. Z8 (99.9)	This study
	W3	+	Sheep rumen	rods	*Methanobrevibacter* sp. Z8 (99.7)	This study
	W4	+	Sheep rumen	cocci	*Methanobrevibacter* sp. Z8 (99.9)	This study
Methanobrevibacter smithii	S1	+	Cattle slurry	rods	*Methanobrevibacter smithii* 4F_4_E02 (99.5)	This study
Methanobrevibacter olleyae	W1	+	Sheep rumen	cocci	*Methanobrevibacter olleyae* KM1H5-1P (99.8)	This study
	R1	+	Sheep rumen	cocci	*Methanobrevibacter olleyae* KM1H5-1P (99.6)	This study
Methanobrevibacter millerae	F2	-	Cattle feces	rods	*Methanobrevibacter millerae* ZA-10 (98.3)	This study

TABLE 2 Sensitivity of rumen methanogenic arcaea to cashew nut shell liquid and its componential alkyl-phenols as indicated by minimal inhibitory concentration (μg/ml of culture) (upper table for representative strains from international culture collections, lower table for newly isolated strains in the present study)

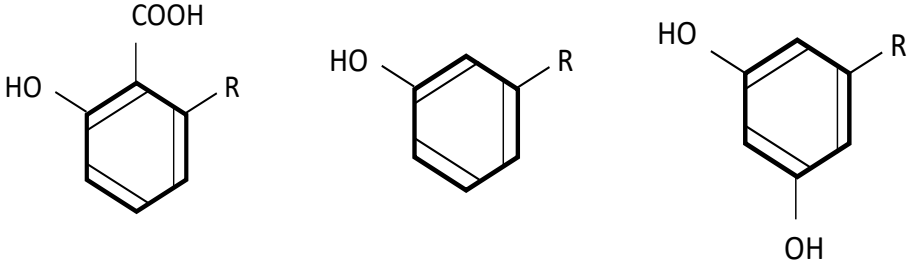
Species	Strain	CNSL	Anacardic acid			Caldanol			Caldol		
			C15:1	C15:2	C15:3	C15:1	C15:2	C15:3	C15:1	C15:2	C15:3
Methanobacterium formicicum	MF	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	3.13	3.13	3.13	≤ 1.56	≤ 1.56	≤ 1.56
Methanobacterium bryantii	M.o.H	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56
Methanobrevibacter ruminantium	M1	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	3.13	6.25	≤ 1.56	≤ 1.56	≤ 1.56
Methanobrevibacter smithii	PS	> 12.50	≤ 1.56	> 12.50	> 12.50	6.25	6.25	> 12.50	12.50	12.50	6.25
Methanobrevibacter wolinii	SH	12.50	3.13	12.5	3.13	12.50	> 12.50	> 12.50	3.13	3.13	3.13
Methanosphaera stadtmanae	MCB-3	> 12.50	3.13	6.25	3.13	> 12.50	> 12.50	> 12.50	6.25	6.25	6.25
Methanosarcia barkeri	MS	> 12.50	3.13	6.25	3.13	> 12.50	> 12.50	> 12.50	6.25	6.25	3.13
Methanomicrobium mobile	BP	12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50
Methanobrevibacter ruminantium	W2	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56
	W3	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56
	W4	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56
Methanobrevibacter smithii	S1	6.25	12.5	6.25	12.50	> 12.50	> 12.50	> 12.50	> 12.50	> 12.50	12.50
Methanobrevibacter olleyae	W1	3.13	3.13	3.13	≤ 1.56	3.13	6.25	≤ 1.56	≤ 1.56	≤ 1.56	6.25
	R1	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	3.13	3.13	3.13	3.13
Methanobrevibacter millerae	F2	≤ 1.56	≤ 1.56	≤ 1.56	≤ 1.56	> 12.50	> 12.50	> 12.50	> 12.50	12.50	12.50

≤ 1.56, minimum inhibitory concentration could be 1.56 μg/ml or lower.
>12.50, growth inhibition did not occur at 12.50 μg/ml.

Table 3 Sensitivity of rumen bacteria against cashew nut shell liquid and its componential alkyl^phenols as indicated by minimal inhibitorybconcentration (µg/ml)

Species	CNSL	Anacardic acid			Caldanol			Caldol		
		C15:1	C15:2	C15:3	C15:1	C15:2	C15:3	C15:1	C15:2	C15:3
Streptococcus bovis	25.00	>12.50	12.50	12.50	>12.50	>12.50	>12.50	>12.50	12.50	>12.50
Lactobacillus ruminis	50.00	>12.50	>12.50	6.25	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
Butyrivibrio proteoclasticus	6.25	6.25	6.25	6.25	>12.50	>12.50	>12.50	12.50	12.50	>12.50
Eubacterium ruminantium	6.25	3.13	6.25	6.25	>12.50	>12.50	>12.50	>12.50	12.50	12.50
Butyrivibrio fibrisolvens	3.13	3.13	6.25	6.25	12.50	>12.50	>12.50	12.50	12.50	12.50
Ruminococcus albus	6.25	3.13	3.13	3.13	12.50	>12.50	>12.50	12.50	6.25	6.25
Ruminococcus flavefaciens	1.56	3.13	3.25	6.25	>12.50	>12.50	>12.50	12.50	6.25	6.25
Fibrobacter succinogenes	12.50	12.50	12.50	6.25	>12.50	>12.50	>12.50	>12.50	12.50	12.50
Prevotella ruminicola	12.50	12.50	>12.50	6.25	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
Succinimonas amylolytica	3.13	3.13	6.25	3.13	12.50	>12.50	>12.50	12.50	12.50	12.50
Ruminobacter amylophilus	50.00	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
Succinivibrio dextrinosolvens	>50.00	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
Selenomonas ruminantium	>50.00	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
Megasphaera elsdenii	>50.00	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50

>12.50 and >50.00, growth inhibition did not occur at 12.50 and 50.00 µg/ml, respectively.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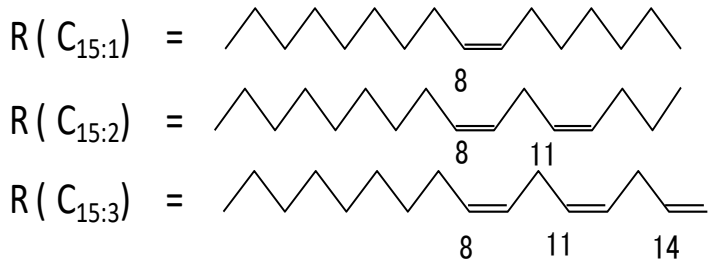
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R (C15:2) =
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R (C15:3) =
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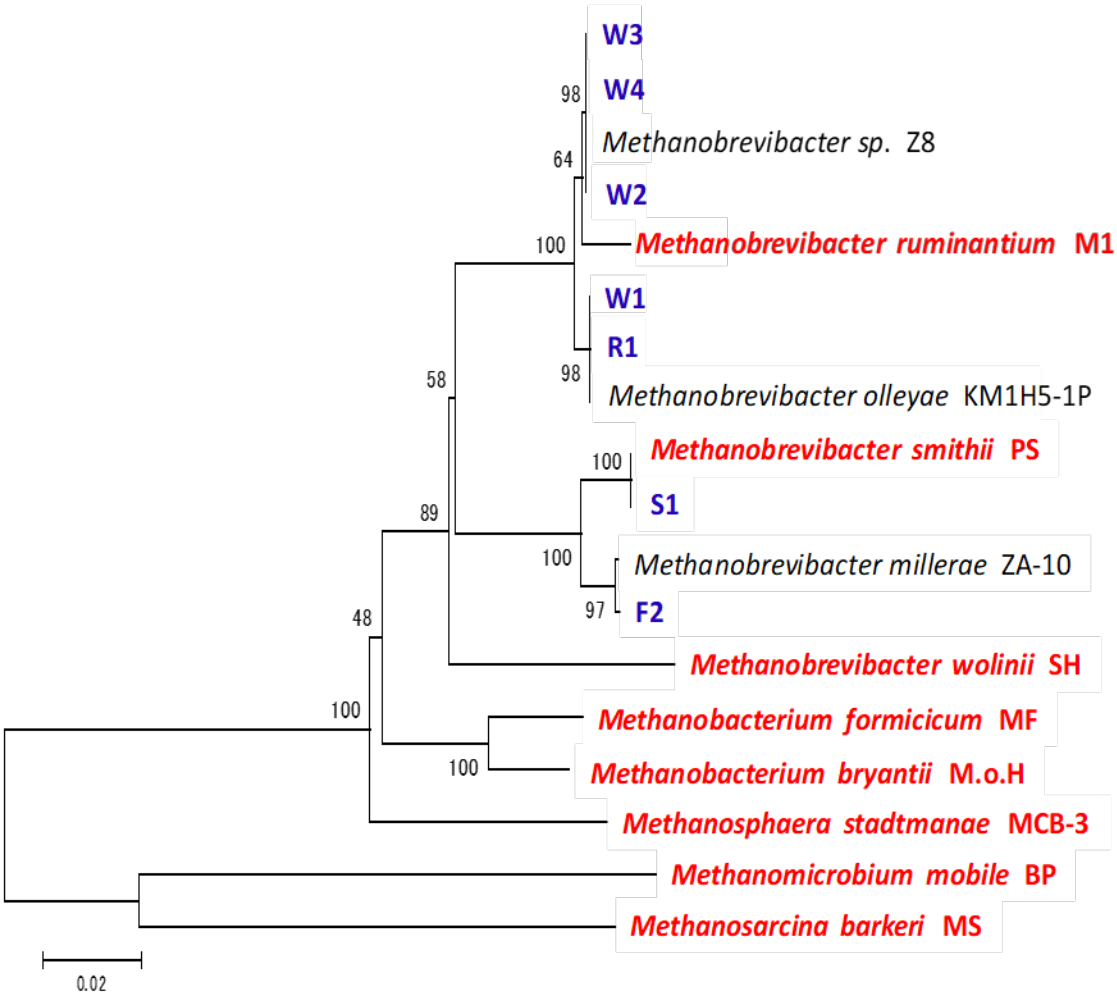
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W4
Methanobrevibacter sp. Z8
W2
Methanobrevibacter ruminantium M1
W1
R1
Methanobrevibacter olleyae KM1H5-1P
Methanobrevibacter smithii PS
S1
Methanobrevibacter millerae ZA-10
F2
Methanobrevibacter wolinii SH
Methanobacterium formicicum MF
Methanobacterium bryantii M.o.H
Methanosphaera stadtmanae MCB-3
Methanomicrobium mobile BP
Methanosarcina barkeri MS
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89
100
97
48
100
100
0.02


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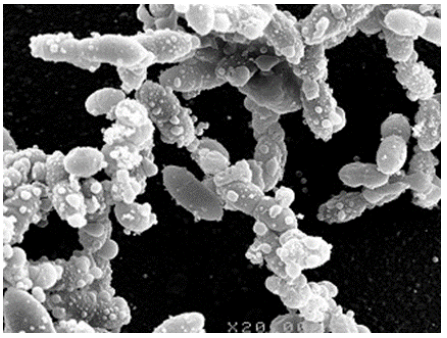

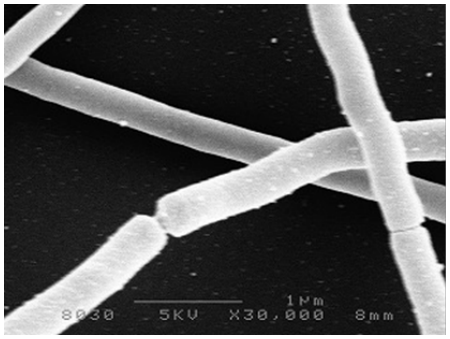
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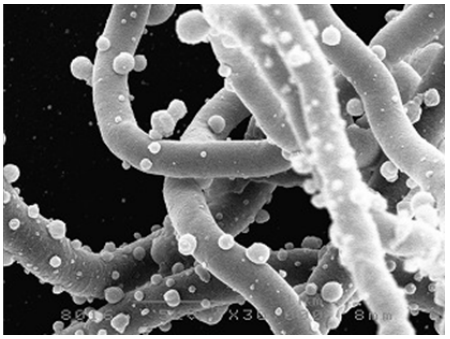

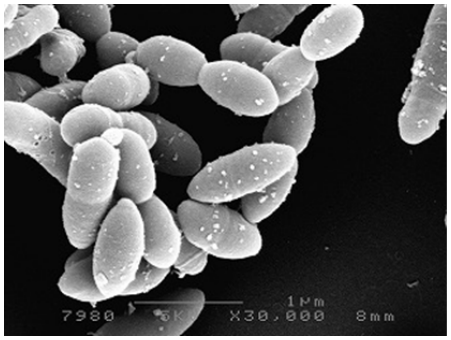
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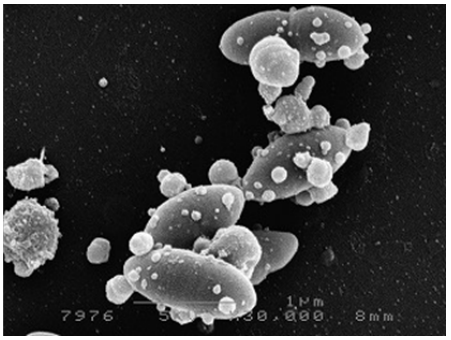
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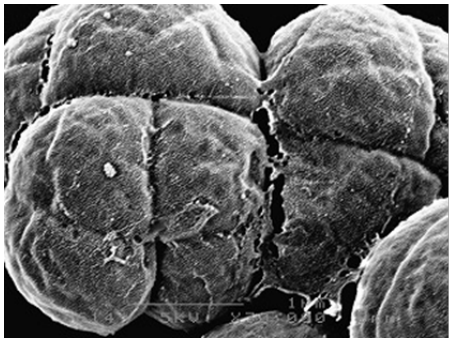

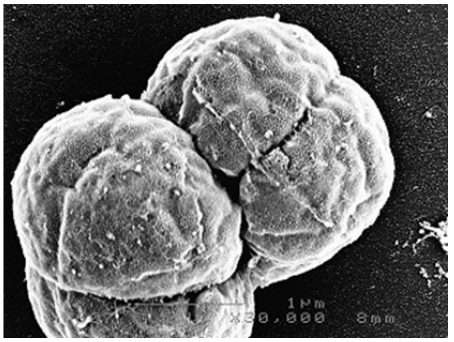
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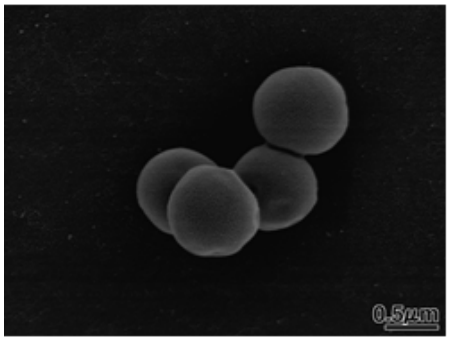

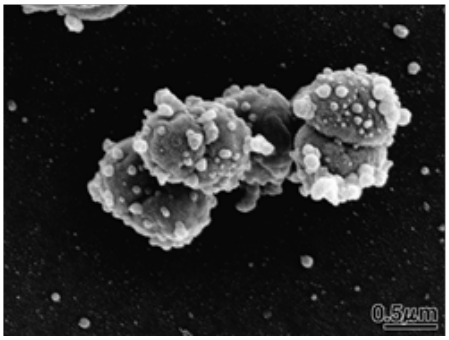

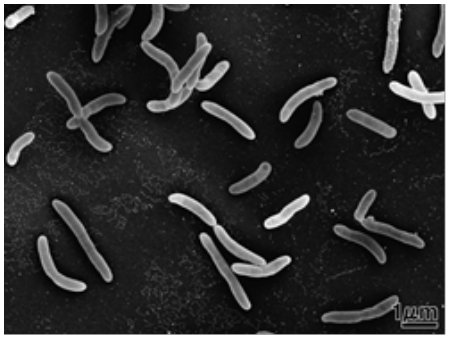

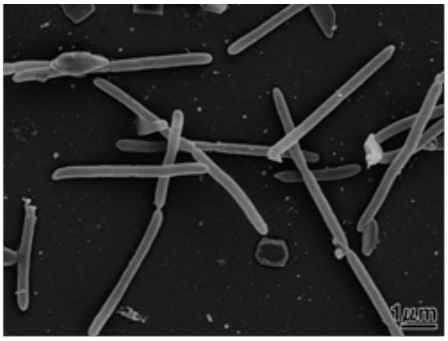

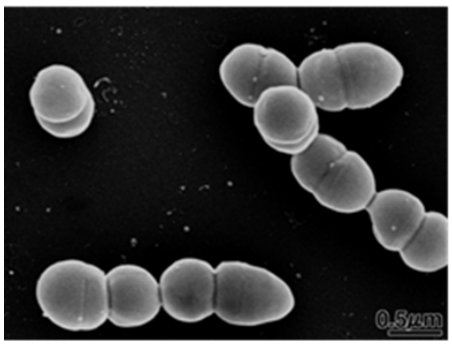

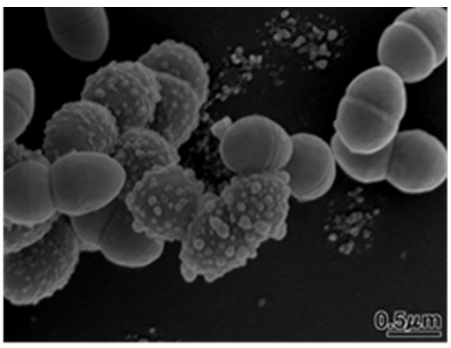

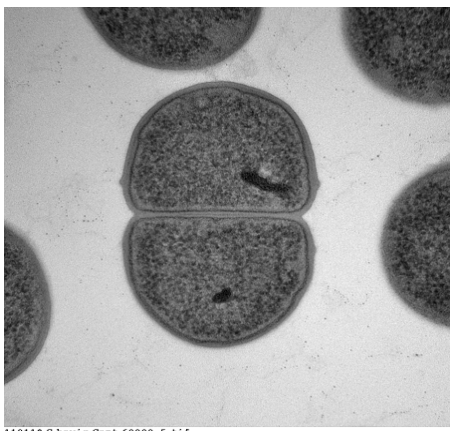

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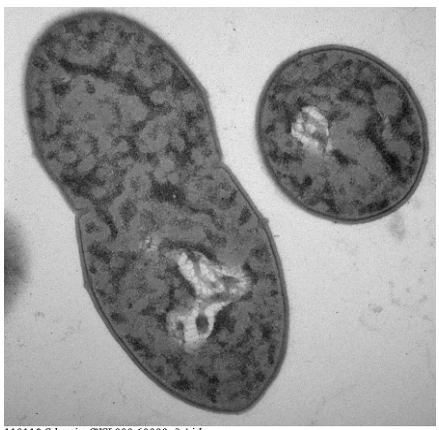

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