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2 **Response of the rumen archaeal and bacterial populations to anti-methanogenic**
3 **organosulphur compounds in continuous-culture fermenters**

4

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16 Running Head: Anti-methanogenic compounds and rumen ecosystem

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24

25

Abstract

The study of methanogenesis inhibitors effectiveness in the rumen have shown inconsistent results, mainly due to poorly understood effects on the key microbial groups involved in methane CH_4 synthesis pathways. This experiment was designed to assess the effect of propyl propane thiosulfinate (PTS), diallyl disulfide (DDS) and bromochloromethane (BCM) on rumen fermentation, methane production and microbial populations on continuous culture fermenters. No effects on total VFA were observed with PTS and DDS, while were decreased with BCM. Amylase activity increased with BCM compared to the other treatments. A decrease on methane production was observed with PTS (48%) and BCM (94%) compared to control. Concentration of methanogenic archaea decreased with BCM from day 4 onward and with PTS on days 4 and 8. The pyrosequencing analysis revealed that PTS and BCM decreased the relative abundance of Methanomicrobiales and increased that of *Methanobrevibacter* and *Methanosphaera*. The concentration of total bacteria was not modified by any treatment, although BCM treatment increased the relative abundance of *Prevotella* and decreased that of *Ruminococcus*. These results suggest that the inhibition of methane production in the rumen by PTS and BCM is associated with a shift in the archaeal biodiversity and changes in bacterial community with BCM.

Keywords: Garlic compounds; methanogens; microbial community; rumen.

44 ## Introduction

45 Enteric microbial fermentation in ruminants is an important source of anthropogenic methane
46 (CH_4), a potent greenhouse gas and its production represents a loss of around 2-12% energy
47 for the animal, and consequently, a decrease of productivity (Morgavi *et al*., 2010). The
48 microbial populations responsible for fermentation in the rumen are comprised of an
49 extremely diverse and complex mix of bacteria, protozoa, fungi and archaea. This enables the
50 animal to digest and metabolize plant structural carbohydrates that otherwise could not be
51 achieved with only its digestive enzymes. Methanogenic archaea fill the role in the rumen of
52 terminal reducers of carbon, producing CH_4 mainly from H_2 and CO_2.
53 In the last decade, a wide range of compounds has been tested for their ability to reduce
54 methane emissions (Benchaar & Greathead, 2011). Some plant secondary metabolites have
55 shown promise due to their antimicrobial activity, including garlic-derived compounds (Hart
56 *et al*., 2008). However, inconsistent results together with adverse effects on fiber digestion
57 and fermentation through inhibition of some bacterial groups have also been reported, with
58 the magnitude of these adverse effects varying depending upon the types and doses and diet
59 composition. Part of the inconsistency in the effects has been associated with the variety of
60 compounds included in the plant extracts, which highlights the importance of using pure
61 active compounds to understand the effects. In some cases, the effect is reversed after a few
62 days of treatment (Soliva *et al*., 2011). Thus, the research aiming to decrease CH_4 emissions
63 from ruminants has to be built upon a correct understanding on the mechanisms of action
64 involved in relation to the main microbial groups likely to be affected (bacteria and archaea).
65 We have recently observed that two organosulphur compounds, propyl propane thiosulfinate
66 (PTS) and dialyl disulphide (DDS), strongly inhibit methane production (up to 96 and 62 %,
67 respectively) in batch culture after 24 h incubation of goats' rumen fluid (Martínez-
68 Fernández *et al*., 2013). However, the persistency of such effect needs to be confirmed and

69 the microbial groups involved identified. This would enable an understanding into the
70 mechanisms of action and facilitate practical implementation as a feeding strategy. Therefore,
71 the aim of this work was to evaluate the effects of DDS and PTS on rumen fermentation,
72 microbial abundances and community structure and on methane production in continuous-
73 culture fermenters (CCF) inoculated with goats' rumen fluid.

74

75 Materials and methods

76 Fermenters, treatments, diet and animals

77 Eight CCF were used following the model of Muetzel *et al*. (2009) with an effective volume
78 of 1000 mL. The treatments were control (without additive), DDS (purity of 80%), PTS
79 (purity of 75%) and BCM that was included as antimethanogenic reference compound
80 (positive inhibition control). BCM was entrapped in an α-cyclodextrin matrix (May et al.,
81 1995) before being included in the diet to ensure its stability. The formulation was prepared
82 in our laboratory as dry white powder in 1 to 2 kg batches and contained 10% to 12% (wt/wt)
83 of BCM. The additives and doses were selected from previous results obtained in batch
84 cultures (Martínez-Fernández *et al*., 2013): 80 μL L^{-1} per day for DDS, 200 μL L^{-1} per day for
85 PTS and 160 mg L^{-1} per day for BCM. The DDS and BCM were provided by Sigma-Aldrich
86 Chemical (catalog numbers 317691 and 48067, respectively); PTS was provided by DMC
87 Research Center SL (Granada, Spain). The experimental diet (Table 1) was composed of
88 alfalfa hay and concentrate in a 50:50 ratio.

89 Eight Murciano-granadina goats fitted with permanent rumen cannula were used as donors of
90 rumen content for the experiment. Goats were adapted for 21 days to the experimental diet
91 and were fed once a day (9:00 h) with free access to water and mineral salt block (Pacsa
92 Sanders, Sevilla, Spain). Animals were cared by trained personnel in accordance with the
93 Spanish guidelines for animal protection (Royal decree, 2005) and the European

94 Convention for the Protection of Vertebrates used for Experimental and other Scientific
95 Purposes (European Directive, 2007). All the experimental procedures involved in this study
96 were approved (Proc. CB-INAN 2012001) by the Animal Welfare Committee at the Institute
97 of Animal Nutrition (CSIC, Spain).
98
99 **Experimental procedure and sampling**
100 Eight CCF (1L), as described by Muetzel *et al.* (2009), were used in two replicated
101 incubation runs of 12 days each. Eight adult Murciano-Granadina goats fitted with ruminal
102 canula were used as donors of ruminal contents. For each incubation run, two groups of four
103 fermenters were inoculated (700 mL per fermenter) with a different pool, each one obtained
104 from three different animals selected randomly. This resulted in four different pools as
105 follows: pool 1 (goats 1, 2 and 3) and pool 2 (goats 4, 5 and 6), used in the first run; pool 3
106 (goats 2, 5 and 7) and pool 4 (goats 3, 6 and 8), used in the second run. Treatments (control,
107 DDS, PTS and BCM) were randomly supplied to one of the 4 fermenters inoculated with
108 each rumen content pool, receiving each pool all the treatments. Each fermenter was fed 16 g
109 of fresh matter per day of the basal diet ground at 1 mm, in two equal portions at 09:00 and
110 14:00 h. Flow through fermenters was maintained by continuous infusion of artificial saliva
111 (Muetzel *et al.*, 2009) at a rate of 40 mL h^{-1} and CO_2 was continuously infused to keep
112 anaerobic conditions. Fermenters were maintained in a water bath at 39ºC. On days 0, 4, 8
113 and 12 of incubation 10 mL of the fermenters content were collected before the morning
114 feeding for VFA analysis and DNA extraction and kept at -20ºC and -80 ºC, respectively. On
115 days 4 and 12 of incubation 1 mL of the fermenters content was collected before feeding and
116 frozen at -80ºC for determination of amylase and xylanase activities.
117 On day 12 of each incubation run in CCF a batch culture trial (Theodorou *et al.,* 1994), was
118 carried out to incubate fermenters content for 24 h to measure CH_4 production. The content

119 of each fermenter was filtered through two layers of cheesecloth while bubbling with CO_2.
120 The substrate incubated consisted of 500 mg of the diet fed to fermenters in 120 mL serum
121 bottles with 60 mL of the fermenter content. Three replicates and a blank of each fermenter
122 and treatment were used. Bottles were sealed with rubber stoppers and aluminum caps and
123 incubated at 39ºC in a water bath. At 24 h after inoculation, the total gas volume was
124 measured in each bottle and a sample of the gas was collected in a graduated syringe and
125 transferred to a 5 mL vacuum tube (Venoject, Terumo Europe N.V., Leuven, Belgium) and
126 then kept at room temperature before methane concentration was measured by gas
127 chromatography (GC).
128

129 **Chemical analysis and calculations**

130 Dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (method ID
131 7.045) and crude protein (CP, method ID 984.13) in samples were determined by the
132 procedures of the Association of Official Analytical Chemists (AOAC, 2005). Gross Energy
133 (GE) was measured with an adiabatic calorimeter (Model 1356, Parr Instrument Co., Moline,
134 IL). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin
135 (ADL) contents were analysed following the methodology described by Van Soest *et al.*
136 (1991) using an ANKOM Model 220 Fiber Analyzer (Macedon, NY). The α-amylase enzyme
137 was used for NDF analysis in the concentrate, and both NDF and ADF were expressed
138 exclusive of residual ash. The ADL content was determined by solubilization of cellulose in
139 the ADF residue with 72% sulphuric acid.

140 To measure the enzymatic activities in fermenters content, cells were lysed using a Mini-
141 Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) for one minute to release
142 intracellular enzymes, during all the process the samples were kept in ice. Cell material was
143 removed by centrifugation (10,000×g, 10 min, 4 ºC) and the supernatant was used for

144 analyses. Xylanase (EC 3.2.1.8.) and amylase (EC 3.2.1.1.) activities were determined
145 (Giraldo *et al*., 2008) using oat beachwood xylan and soluble starch, respectively, as
146 substrates. Enzymatic activities were expressed as micromoles of glucose or xylose released
147 in 1 min from the corresponding substrates per mL of sample at 39 ºC and pH 6.5.

148 The individual VFA concentrations were analysed using the gas chromatography technique
149 described by Isac *et al.* (1994).

150 The CH_4 concentration was determined by GC using a HP Hewlett 5890, Packard Series II
151 gas chromatograph (Waldbronn, Germany). A sample of 0.5 mL of gas was injected using a 1
152 mL Sample-Lock® syringe (Hamilton, Nevada, USA).

153 The amount of methane produced (micromoles) was calculated by multiplying the total gas
154 produced (micromoles) with the concentration of methane obtained.

155

156 **Real-Time PCR Analysis**

157 Samples from the fermenters content were collected on days 0, 4, 8 and 12 for DNA
158 isolation. Samples were freeze-dried and thoroughly mixed by physical disruption using a
159 bead beater (Mini-bead beater 8, BioSpec Products, Bartlesville, USA) for 1 min before using
160 QIAGEN QIAamp® DNA stool mini kit (Qiagen Ltd., UK) following the manufacturer's
161 instructions but with higher temperature (95ºC) for lysis incubation. The DNA samples were
162 used as templates for quantify the copy numbers of 16S rRNA (for bacteria), methyl
163 coenzyme M reductase A (mcrA) gene (for methanogenic archaea), and 18S rRNA (for
164 protozoa) by real-time quantitative PCR (qPCR).

165 The yield and purity of the extracted DNA were assessed using NanoDrop ND-1000
166 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

167 Primer sets used were as follows: forward: 5´-GTG-STGCAYGGYTGTCGTCA-3´and
168 reverse: 5´-ACGT- CRTCCMCACCTTCCTC-3´ for total bacteria (Maeda *et al*., 2003) and

169 forward: 5´-GCTTTCGWTGGTAGT-GTATT-3´ and reverse: 5´-CTTGCCCTCYAATCGT-
170 WCT-3´for protozoa (Sylvester *et al*., 2004). The primer sets for detection and enumeration
171 of methanogenic archaea (mcrA) were forward: 5´-TTCGGTGGATCD-CARAGRGC-3´ and
172 reverse: 5´-GBARGTCGWAWC- CGTAGAATCC-3´ (Denman *et al*., 2007). Three
173 replicates of each extract were used and a negative control was loaded on each plate run to
174 screen for possible contamination or dimer formation and to set the back- ground
175 fluorescence for plate normalization.

176 Real-time PCR analyses were performed on iQ5 multicolor Real-Time PCR Detection
177 System (BioRad Laboratories Inc., Hercules, CA). One microliter of DNA extract was added
178 to amplification reactions (25 μL) containing 0.2 μL of each primer (10 μM) and 12.5 μL of
179 iQ SYBR Green Supermix (BioRad Laborato- ries Inc.). Cycling conditions were 95°C for 5
180 min; 40 cycles of 95°C for 15 s, 60°C for 30s, and 72°C for 55 s; and 72°C for 1 min. The
181 threshold cycle (amplification cycle in which product formation exceeds background
182 fluorescence) of each sample was determined during the exponential phase of amplification.
183 The absolute amount for each microbial group, expressed as the number of DNA copies/g of
184 fresh matter, was determined using standards. The qPCR standards consisted of the plasmid
185 pCR 4-TOPO (Invitrogen, Carlsbad, CA) with an inserted 16S, mcrA, or 18S gene fragment
186 corresponding to a conserved sequence of total bacteria, methanogenic archaea, or protozoa,
187 respectively. The number of gene copies present in the plasmid extracts was calculated using
188 the plasmid DNA concentration and the molecular mass of the vector with the insert. The
189 concentrated plasmid was serially diluted (10-fold) to generate a standard curve.

190

191 **Pyrosequencing and sequence analysis**

192 The yield and purity of the extracted DNA from day 12 before feeding, were assessed using
193 NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). For

194 sequencing of the 16S rDNA gene using the Roche /454 system, previously published
195 primers were modified to include the 454 adaptor sequences and sample specific barcodes
196 allowing samples to be multiplexed. Amplification of the bacterial V1-V2 regions of 16S
197 rRNA was performed using the primer pair 27F and 357R (Liu *et al.*, 2007) . The archaeal
198 hypervariable V6 region of the 16S rRNA gene was amplified using the primer pair 958F and
199 1048 Rmajor (Galand *et al.*, 2009). The PCR was performed in triplicate, in a total volume of
200 25 µL containing 10x PCR buffer, 10 mM dNTP mix, 10 µM of forward and reverse primers,
201 1U FastStart Polymerase, and 1 µL of DNA template. The amplification conditions were: an
202 initial denaturation step at 95ºC for 2 min; 30 cycles of denaturation at 95ºC for 30s,
203 annealing at 55ºC for 30s, and elongation at 72ºC for 2 min; and a final extension step at
204 72ºC for 7 min. The size of the PCR products was then checked on a 1% agarose gel
205 electrophoresis. Then triplicates were pooled together and products were then purified using
206 the short fragment removal method described by Roche using their GS FLX amplicon DNA
207 preparation guide and AMPure beads. The purified PCR products were quantified using
208 Quant-iT PicoGreen dsDNA quantification kit (Invitrogen) and mixed in equimolar amounts
209 to 10^7 molecules μL^{-1} sample. The amplicon pooled libraries were pyrosequenced on a Roche
210 454 FLX Titanium. The flowgram (sff) files were converted to fasta DNA (fna) and quality
211 score (qual) file on the 454 cluster and transferred onto a Linux based workstation running
212 the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso *et al.*, 2010b).
213 Sequences were filtered to exclude those with mismatches in the primer sequence, exceeding
214 6 homopolymer base runs or sequences containing ambiguous bases. The libraries were split
215 according to the 10nt barcode incorporated into the forward primer. The error-corrections of
216 amplicon pyrosequences were made using Acacia (Bragg *et al.*, 2012). The OTUs were
217 generated by clustering at 97% sequence identity using UCLUST (Edgar, 2010) for bacteria
218 and CD-hit for archaea (Li & Godzik, 2006). Representative sequences were aligned to the

219 reads of the GreenGenes database (DeSantis Jr *et al.*, 2006) using PyNAST(Caporaso *et al.*,
220 2010a). Taxonomic classification was assigned using the Basic Local Alignment Search Tool
221 (BLAST). Beta diversity was used to create principal coordinate analysis (PCoA) plots using
222 weighted UniFrac distances. Unifrac (Lozupone & Knight, 2005).
223
224 **Statistical analysis**
225 Fermentation parameters and microbial population were analysed as a repeated measures
226 univariate analysis using GLM procedure of SPSS (IBM SPSS Statistics v.19, IBM Corp.,
227 Somers, NY). The linear model used for each dependent variable accounted for the effects of
228 treatment (T), day (d) and Txd interaction. Effects were considered significant at $P \leq 0.05$.
229 When significant differences were detected, differences among means were studied using the
230 LSD comparison test. An ANOVA analysis was used to establish differences in OTUS due to
231 the treatments using R software.
232

233 Results

234 **Ruminal fermentation**
235 Methane produced in batch cultures after 24 h of incubation (Table 2) was decreased ($P \leq$
236 0.005) by PTS (48 %) and BCM (94 %) compared with control. The pH values in fermenters
237 were not affected ($P = 0.308$) by the treatment, although tended to decrease as incubation
238 time progressed ($P = 0.072$). Within treatment total VFA and individual VFA molar
239 proportions differed ($P \leq 0.044$) for days 0 and 4 and then remained unchanged from the day
240 4 onward. Total VFA concentration decreased ($P < 0.001$) only with BCM addition from day
241 4 onward compared with control. Acetate molar proportion decreased ($P \leq 0.038$) from day 4
242 with all the treatments compared with control. Propionate molar proportion increased ($P \leq$
243 0.044) with all the treatments on day 4, although from day 4 remained higher ($P < 0.001$)

244 only for BCM treatment. *Iso*-butyrate molar proportion was not affected (P = 0.222) by any
245 of the treatments. Molar proportion of butyrate was higher ($P \leq 0.034$) with BCM compared
246 with control and the other two treatments from day 4 onward. Valerate and *Iso*-valerate molar
247 proportions were higher ($P < 0.001$) from day 4 and day 8 onward, respectively, with BCM
248 compared with control and all the treatments. Acetate to propionate ratio decreased ($P <$
249 0.001) from day 4 onward with BCM compared with other treatments and control. Only on
250 day 4 the ratio was higher ($P = 0.036$) for PTS and on days 4 and 12 it was lower ($P \leq 0.040$)
251 for DDS compared to the control.
252 The xylanase and amylase activities (Table 3) did not change from day 4 to 12 of incubation
253 ($P > 0.050$). Xylanase activity tended to be affected by PTS treatment ($P = 0.051$) as
254 compared to the control and BCM and DDS. Amylase activity increased ($P < 0.022$) on days
255 4 and 12 with BCM compared to the control and BCM and DDS.
256
257 **Microbial community**
258 The effects of the studied additives during 12 days of treatment on the numbers of bacteria,
259 archaea and protozoa are shown in table 4. The abundance of the total bacterial population
260 increased ($P \leq 0.040$) for all treatments on days 4 and 12, with a further increase by BCM
261 treatment as compared with the control. The number of archaeal mcrA gene copies was lower
262 ($P \leq 0.035$) with BCM and PTS from day 4 and from day 4 to 8, respectively, compared with
263 control. No effect ($P = 0.547$) on the numbers of protozoa was observed with any treatment.
264 However, a reduction ($P < 0.001$) of protozoa numbers was observed for all the treatments at
265 day 4 compared to 0. The treatment x day interaction was only significant ($P < 0.001$) for the
266 archaeal gene copy numbers.
267 The Roche/454 pyrosequencing analysis exhibited 21,530 and 36,183 input sequence reads of
268 bacteria and archaea, respectively. After the removal the low-quality reads, 18,182 bacterial

269 and 29,404 archaeal reads were used for the analysis.

270 Alpha diversity based on Shannon diversity and richness of observed species measures
271 showed similar values for bacterial diversity within the treatments, ranging from 8.1, 8.4, 8.5
272 and 8.7 for BCM, Control, DDS and PTS respectively on Shannon diversity and from 261,
273 285, 286 and 295 for BCM, Control, DDS and PTS respectively on richness of observed
274 species. Similarly for Archaeal diversity, little variation in the observed sample diversity was
275 observed within treatments with 4.1, 4, 4.1 and 4.1 for BCM, Control, DDS and PTS
276 respectively on Shannon diversity and from 33, 37, 38 and 41 for BCM, Control, DDS and
277 PTS respectively on richness of observed species.

278 Between treatments diversity as measured with beta diversity analysis for the comparison of
279 the bacterial microbiome structures, found that the BCM treatment contributed the most
280 variance to the Principal Coordinate Analysis (PCoA) (Figure 1a). Although a smaller
281 percentage of variance was also explained in separating the PTS treated samples from the
282 control and DDS samples. Likewise for the archaeal beta diversity, the BCM sample
283 explained the most variance in the data (Figure 1b). However the PTS sample were also
284 clearly separated from the control and DDS samples along the first axis of variance.

285 Analysis of the Bacteria family level to its specific responses to the inhibitors (based on an
286 average of 4469 sequences per treatment) showed a substantial shift in the relative abundance
287 of some families in fermenters treated with BCM compared to the other three treatments (Fig.
288 2). For BCM treatment a reduction in *Anaeroplasmataceae* (2.6%) and *Ruminococcaceae*
289 (6.1%) compared with an average of 5.4% and 10.2%, respectively the other treatments. The
290 BCM treatment resulted in greater abundances of *Prevotellaceaea* (15.1%) and
291 *Streptococcaceae* (3.83%) families and of an unclassified family belonging to the order
292 *Bacteriodales* (17.1%) in comparison to the other treatments (9.1%, 1.3% and 12.3%,
293 respectively). Treatment with PTS resulted in higher relative abundance of the

294 *Spirochaetaceae* (5.9%) family compared to the other treatments (2.63%).

295 Specific OTU's that were significantly associated with an increase in abundance due to
296 treatment with BCM ($P \leq 0.05$) were classified to *Butyrivibrio* and *Prevotella* genus, while
297 those decreasing with BCM treatment were classified as *Ruminococcus* genus
298 (Supplementary figure 1). The PTS treatment increased ($P \leq 0.05$) the abundance of some
299 OTUs classified as *Ruminococcus* and *Prevotella* genus. The DDS only affected ($P < 0.001$)
300 one OTU classified as *Clostridium* genus.

301 Analysis of the methanogen genus level to its specific responses to the inhibitors (based on an
302 average of 7351 sequences per treatment) (Fig. 3) revealed higher relative abundance of
303 *Methanobrevibacter* genus in samples from fermenters treated with PTS (79.2%) and BCM
304 (73.7%) than for control (60.6%) and DDS (48.7%) treatments. The relative abundance of
305 archaea belonging to the *Methanosphaera* genus was higher for BCM (25.3%) than for the
306 other three treatments (6.5%). However, the relative abundance of *Methanomicrobium* genus,
307 from the *Methanomicrobiaceae* family was higher for control (32.9%) and DDS (41.4%) than
308 for PTS (11.7%) and BCM (0.3%).

309 In relation to the archaeal community, specific OTU's that were significantly associated with
310 an increase in abundance due to treatment with BCM and PTS ($P \leq 0.05$) were associated
311 (Supplementary figure 2) to *Methanosphaera* and *Methanobrevibacter* genus were increased,
312 while those decreasing with these treatments were classified as *Methanomicrobium* genus.

313

314 Discussion

315 In this study the compounds were provided together with the diet twice a day to mimic as
316 close as possible the conditions *in vivo*. Thus, fluctuations in compounds concentration are
317 expected thorough the day but no accumulation along the course of the trial. The selected
318 dosage was made based on previous in vitro trials conducted using batch culture systems

319 (Martinez-Fernandez *et al*., 2013). Forty ml saliva/h were infused which resulted in 960 ml/d
320 of dilution rate. In contrast to close systems (i.e. batch culture), continuous culture do not
321 have a steady concentration of active compound within the vessel, as it is the case of the
322 rumen.

323

324 **Ruminal fermentation**

325 Halogenated compounds such as BCM (McAllister & Newbold, 2008), and organosulphur
326 compounds (Patra & Yu, 2012; Mateos *et al*., 2013) such as PTS, have shown
327 antimethanogenic effect in ruminants. In the present work the addition of PTS and BCM
328 decreased methane production by 48% and 98%, respectively, values that are comparable to
329 those reported *in vitro* (Goel *et al*., 2009; Patra & Yu, 2012; Martínez-Fernández *et al*.,
330 2013). On the contrary, DDS did not decrease CH_4 production from fermenters content taken
331 on day 12. We recently reported a reduction in CH_4 production *in vitro* after 24 h treatment
332 with DDS, with equivalent doses as used here (Martínez-Fernández *et al*., 2013). The lack of
333 effect of DDS on methane production using fermenter content taken on day 12 of treatment
334 may suggest an adaptation of the microbial ecosystem over this period to the presence of this
335 compound, which agrees with the results obtained in sheep by Klevenhusen *et al.* (2011),
336 which suggested that some antimicrobial additives may be degraded by rumen
337 microorganisms. The analysis of the microbial community structure in the first days of
338 application of DDS would elucidate the adaptation mechanisms. The antimethanogenic effect
339 of PTS observed in the present work agrees with previous results obtained *in vitro* and *in vivo*
340 in our group (Martínez-Fernández *et al*., 2013). The significance of the treatment by time
341 interaction for some individual VFA molar proportions suggests that the rumen microbial
342 community is altering the fermentation profiles either through functional changes or
343 population shifts in the presence of the studied compounds. Total VFA concentration and

344 profile was only affected by BCM, in contrast to other observations (Goel *et al.*, 2009;
345 Mitsumori *et al.*, 2012; Abecia *et al.*, 2012), which could be due to the higher dose applied
346 here (136 μM) as compared to Goel *et al.* (2009) and Abecia *et al.* (2012) (10 and 23 μM,
347 respectively). Busquet *et al.* (2005) reported no effect of DDS and garlic oil after 9 days of
348 incubation in continuous-culture fermenters on total VFA and modifications of VFA profile
349 with decreased acetate and increased butyrate molar proportions. In the present work
350 acetate:propionate ratio decreased with BCM from day 4 to 12, in agreement with results
351 obtained in previous experiments using the same compound (Mitsumori *et al.*, 2012; Abecia
352 *et al.*, 2012). This reduction has been considered (McAllister & Newbold, 2008) a common
353 feature for antimethanogenic compounds, as a result of a redirection of hydrogen from
354 methane to propionic metabolic pathways, although the decrease in methane production not
355 necessarily always result in an increase of the propionate production. In this line, some
356 studies (Mitsumori *et al.*, 2012; Martínez-Fernández *et al.,* 2014) have reported that most of
357 the hydrogen produced in excess in the rumen when methanogenesis is inhibited is expelled
358 by the animal, which it is very likely to have occurred in our experiment. The potential
359 increase in propionate would result in more energy available for the animal (Abecia *et al.,*
360 2012), athough this effect might depend on the shifts caused in the bacterial populations as
361 discussed below.

362 The treatment with PTS tended to decrease xylanase activity, while BCM and DDS did not
363 show such effect. Xylanase degrades the linear polysaccharide beta-1,4-xylan into xylose,
364 thus breaking down hemicellulose, one of the major components of plant cell walls in
365 forages. The impact that this could have on plant fibre degradation in the rumen and on
366 intakes by animals subjected to high feeding levels deserves further attention. On the other
367 hand, only BCM treatment increased amylolytic activity on days 4 and 12. Other authors
368 (Hristov *et al.*, 2003) reported decreased amylolytic activity by several bioactive agents using

369 bovine rumen fluid, in contrast to our results. These differences could be due to the different
370 activity, chemical structure or mechanism of action of the compounds used in both studies.
371 Janssen (2010) described that cellulolytic microbes produce more acetate and H_2, while
372 amylolytic microbes produce less H_2 and more propionate, which explains why more CH_4 is
373 formed, per unit of fermented matter, from forage based diets as compared to those including
374 concentrate. That could explain the different mechanisms of action of PTS and BCM, so
375 BCM could affect competitors of amylolytic microorganisms, thus increases amylolytic
376 activity and produces less H_2, while PTS could affect other microbial groups as discussed in
377 following sections.
378
379 **Microbial community analyses**
380 In agreement with the methane reduction observed with the addition of PTS and BCM, the
381 abundance of methanogenic archaea was decreased on days 4 and 8 (and on day 12 only with
382 BCM). Goel *et al*. (2009) reported a sharp decrease in biomass of methanogenic archaea as a
383 result of adding BCM in batch and CCF, in agreement with our results. On the contrary
384 Abecia *et al*. (2012) reported no changes in the concentration of methanogenic archaea in
385 goats treated with BCM for 60 days. This disagreement could be due to the different duration
386 of the treatments (Williams *et al*., 2009) and the inherent differences between *in vivo* and *in*
387 *vitro* conditions (Soto *et al*., 2012; 2013). Some works reported that adding garlic compounds
388 does not induce changes in the abundance of methanogenic archaea (Ohene-Adjei *et al*.,
389 2008; Kongmun *et al*., 2011) while others reported decreased archaeal population size (Patra
390 & Yu, 2012). The variability in concentration of active compounds in plant extracts generates
391 confusion because the effects can be contradictory, according to the content of the active
392 component in the extract and the dose used (Patra & Saxena, 2009). Therefore, it seems
393 necessary either to report concentrations and active compounds in plant extracts or to use

394 pure products to unequivocally define activities, doses, and mechanisms of action.

395 The taxonomic assignment of bacteria revealed that the most abundant families were
396 *Lachnospiraceae, Ruminococcaceae*, *Prevotellaceae* and unclassified *Bacteroidales* in
397 accordance with other studies (Kong *et al*., 2010; Zened *et al*., 2012). The treatment with
398 BCM increased the relative abundance of *Prevotella* and decreased those belonging to
399 *Ruminococcus,* which is in the line of what Mitsumori *et al*. (2012) observed using the same
400 compound. Increased abundance of *Prevotella* promoted by treatment with BCM is likely
401 associated to the increase in branched chain fatty acids, propionate and amylolytic activity
402 observed in that study. The abundance of *Prevotella* has been linked to hydrogen
403 accumulation due to decreased methane production. Likewise, the effect of BCM on
404 *Ruminococcus* is in agreement with Mitsumori *et al*. (2012) that reported a decrease in *R.*
405 *albus* as a result of treating goats with BCM, due to the high sensitivity to high partial
406 pressure of hydrogen. This decrease might be compensated by greater abundances of other
407 fibrolytic bacteria such as *F. succinogenes* that does not produce H_2 and is not susceptible to
408 H_2 accumulation. This agrees with Kittelmann *et al.* (2014), who reported that two different
409 bacterial community types are linked with the low-methane emission trait in sheep,
410 hypothesizing that lower CH_4 yields are the result of bacterial communities that ferment
411 ingested feed to relatively less hydrogen, which results in less CH_4 being formed.
412 Furthermore, BCM interferes with the cobalamin-dependent pathway to reduce CO_2 to CH_4
413 and thus decreasing methanogenesis. Some cellulolityc bacteria have been shown to be
414 dependent on vitamin B12 (Scott & Dehority, 1965), which could explain the shift in the
415 distribution of some bacterial groups observed here and the reduction in the abundance of
416 *Ruminococcus* when treating with BCM. With regards to PTS, although a decrease in
417 methane production was observed, it did not induce detectable changes in the bacterial
418 taxonomic distribution, which could be explained by the fact that the reduction in methane

419 was not as dramatic as for BCM (48 and 94 %, respectively for PTS and BCM) and
420 theoretically no major shift in metabolic H_2 transfer occurred. Indeed, this is confirmed by
421 the PCoA plot in which a distinct group including BCM samples was recognized and
422 separated from the rest.

423 The dominant archaea belonged to the orders *Methanobacteriales* and *Methanomicrobiales,*
424 in accordance with previous works (Janssen & Kirs, 2008; Zhou *et al*., 2009; GU *et al*.,
425 2011). Both BCM and PTS increased the relative abundance of *Methanobrevibacter* and
426 decreased that of *Mathenomicrobium* compared with control and DDS. The BCM treatment
427 also increased archaea from *Methanosphaera*. These results are in agreement with previous
428 results obtained in goats treated with BCM for two months (Abecia *et al*., 2014). At least 60
429 genes are involved in methanogenesis and hydrogen transfer. The first five steps of the
430 pathway result in the sequential reduction of CO2 by electrons sourced from H_2 to form N5-
431 methyl-H4MPT (Thauer *et al*., 1993). The methyl group is then transferred to coenzyme M
432 via the action of methyl-H4MPT:CoM-methyltransferase which is encoded by the mtr gene
433 cluster and this is the step inhibited by BCM. This is a multi-subunit enzyme encoded by
434 more than 10 different genes (Attwood & McSweeney, 2008) that include the methyl CoM
435 reductase cluster (Mcr B, D, C, G, A) and the CoM methyltransferase cluster (Mtr E, D, C, B,
436 A, G, H, X) and a set of genes whose function is currently not known. Within the
437 hydrogenotrophic methanogens, a further 10 genes are conserved (Gao & Gupta, 2007) and
438 include a cluster of genes that encode subunits of [Ni-Fe] hydrogenases (Eha B, C, D, E, F,
439 G) that catalyse the reversible reduction of protons to molecular hydrogen. Although some of
440 the genes are known to be conserved across methanogens, some differ between families
441 (Attwood & McSweeney, 2008). Since our results are consistent with other work using BCM
442 (Abecia *et al*., 2014), we hypothesize that the different sensitivity of Methanobacteriales and
443 Methanomicrobiales is a result of genes differently expressed that make some species more

444 suitable to cope with the new environment.

445 Our results support previous observations by Ohene-Adjei *et al*. (2008) who reported changes
446 in the archaeal banding profile by DGGE as a result of treating sheep with garlic oil. Based
447 on the taxonomy derived from pyrosequencing we hypothesize that the extent in which
448 groups are shifted is directly associated to the extent of methane inhibition. However, the
449 different mechanisms of action of BCM and PTS could also explain the different impact
450 observed. Bromochloromethane directly reacts with reduced vitamin B12 and results in the
451 inhibition of cobamide-dependent methyl group transfer in methanogenesis (Wood *et al*.,
452 1968), while the antimicrobial effect of thiosulfinates (Focke *et al*., 1990; Ruiz *et al*., 2010) is
453 associated with chemical reaction with thiol groups of various enzymes such as the acetyl-
454 CoA-forming system. Some authors (Busquet *et al*., 2005; Benchaar & Greathead, 2011)
455 reported the relationship of antimethanogenic effect of organosulphur and the inhibition of
456 HMG-CoA reductase, which play an important role in the synthesis of isoprenoid ethers, the
457 main component of archaeal cell membranes. Therefore, the sensitivity of key archaeal
458 groups to these inhibitory compounds may be explained by the different mechanisms of
459 action exhibited, as discussed above, which would need to be further tested using deep
460 metagenomic and metatranscriptomic sequencing transcription studies combined with pure
461 culture *in vitro* incubations. Kang *et al*., (2013) used a combined RNA and DNA-derived
462 analysis and concluded that less abundant but highly active methanogens may make a greater
463 contribution to total methane formation than their abundance may suggest. Shi et *al*. (2014)
464 have recently observed that in despite of minor changes in the abundance of different
465 methanogens groups in the rumen of sheep that are consistently high or low CH_4 yield
466 phenotypes, the transcription of methanogenesis pathway genes was substantially different.
467 This differential transcription pattern needs to be assessed in the future when effective anti-
468 methanogenesis compounds are applied.

469 In conclusion, the inhibition of methane production in the rumen by BCM (94%) and PTS
470 (48%) is associated with a shift in the archaeal biodiversity that involves an increase in
471 *Methanosphaera* and a decrease in *Methanomicrobium*. In the case of BCM the effect causes
472 changes in the bacterial population that are clearly reflected in the fermentation profile. The
473 potential negative impact of PTS on fibre degradation deserves further studies. The treatment
474 with DDS over 12 days does not confirm the antimethanogenic effect previously reported.

475

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481

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651

651 Table 1. Chemical composition of alfalfa hay and concentrate (g kg^{-1} dry matter) and
652 ingredients (g kg^{-1}) of concentrate.

Item	Alfalfa hay	Concentrate
DM (g kg^{-1} fresh matter)	907	915
OM	875	884
NDF	513	245
ADF	330	118
ADL	99.2	36.3
CP	203	168
Ether Extract	8.1	15.3
GE (MJ Kg^{-1} DM)	18.4	19.5
Ingredients		
Barley		174
Faba beans		233
Maize		174
Sunflower meal		233
Maize gluten meal		116
Rumen-inert fat		70

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654
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Table 2. Effects of additives on VFA concentration (mM), profiles (mol 100^{-1} mol) and pH on days 0, 4, 8 and 12 of incubation in continuous-culture fermenters and on CH_4 production (µmol) after 24 hours of incubation in batch culture inoculated with fermenters content after 12 days of incubation.

Item	Day	Treatment				SEM[1]	*P*-value[2]		
		Control	BCM	DDS	PTS		T	d	Txd
Total VFA	**0**	30.6^{B}	31.6^{B}	28.8^{B}	28.7^{B}				
	4	76.2^{aA}	62.9^{bA}	74.9^{aA}	73.1^{aA}	11.4	0.015	<0.001	0.013
	8	73.9^{aA}	60.2^{bA}	74.82^{aA}	72.4^{aA}				
	12	77.4^{aA}	65.7^{bA}	78.1^{aA}	78.9^{aA}				
Acetate	**0**	69.0^{A}	68.4^{A}	68.2^{A}	68.4^{A}				
	4	63.7^{aB}	50.0^{cB}	61.4^{bB}	62.8^{abB}	1.8	<0.001	<0.001	<0.001
	8	64.9^{aB}	49.4^{cB}	62.6^{bB}	62.6^{bB}				
	12	64.6^{aB}	49.6^{bB}	61.9^{aB}	62.1^{aB}				
Propionate	**0**	13.8^{B}	14.1^{C}	14.1^{B}	13.9^{B}				
	4	16.0^{bA}	25.8^{aA}	17.6^{bA}	17.1^{bA}	1.5	<0.001	<0.001	<0.001
	8	17.0^{cA}	24.1^{aA}	17.5^{bA}	17.8^{bA}				
	12	17.8^{bA}	23.4^{aB}	18.6^{bA}	18.0^{bA}				
Isobutyrate	**0**	1.88^{A}	1.85^{A}	1.80^{A}	1.83^{A}				
	4	1.48^{B}	1.23^{B}	1.38^{B}	1.43^{B}	0.16	0.163	0.013	0.118
	8	1.33^{B}	1.25^{B}	1.35^{B}	1.23^{B}				
	12	1.30^{B}	1.22^{B}	1.35^{B}	1.35^{B}				
Butyrate	**0**	11.2	11.4^{B}	12.1^{B}	11.9				
	4	13.8^{b}	16.3^{aA}	14.5^{bA}	13.9^{b}	0.7	0.002	<0.001	<0.001
	8	12.2^{b}	15.4^{aA}	13.3^{bAB}	13.8^{ab}				
	12	11.7^{c}	15.6^{aA}	12.9^{bcAB}	13.7^{b}				
Isovalerate	**0**	2.93	3.03	2.70	2.83				
	4	2.78	2.73	2.75	2.50	0.39	<0.001	0.405	<0.001
	8	2.38^{b}	5.38^{a}	2.68^{b}	2.20^{b}				
	12	2.38^{b}	5.90^{a}	2.58^{b}	2.33^{b}				
Valerate	**0**	1.28^{B}	1.28^{B}	1.25^{C}	1.20^{B}				
	4	2.20^{bA}	4.00^{aA}	2.38^{bB}	2.35^{bA}	0.32	<0.001	<0.001	<0.001
	8	2.20^{cA}	4.43^{aA}	2.50^{bAB}	2.38^{bcA}				
	12	2.33^{bA}	4.38^{aA}	2.68^{bA}	2.55^{bA}				
Acetate:Propionate	**0**	5.02^{A}	4.88^{A}	4.90^{A}	4.97^{A}				
	4	3.98^{aB}	1.94^{dB}	3.48^{cB}	3.67^{bB}	0.46	<0.001	<0.001	<0.001
	8	3.85^{aB}	2.06^{bB}	3.57^{aB}	3.52^{aB}				
	12	3.67^{aB}	2.13^{cB}	3.34^{bB}	3.46^{abB}				
pH	**0**	6.55^{A}	6.58	6.55^{A}	6.58				
	4	6.39^{AB}	6.45	6.43^{AB}	6.39	0.22	0.138	0.033	0.668
	8	6.31^{B}	6.44	6.41^{AB}	6.40				
	12	6.36^{AB}	6.44	6.38^{B}	6.40				
Methane	**13**	249^{a}	14^{c}	248^{a}	129^{b}	13	<0.001	n.d.	n.d.

660 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane
661 thiosulfinate) and BCM (Bromochloromethane).
662 [1]SEM: Standard error of the mean.
663 [2] T: treatment effect; d: day effect; Txd: Treatment x day interaction.
664 [a-c] within a row treatment means without a common superscript differ, $P < 0.05$.
665 [A-B] within a column treatment without a common superscript differ, $P < 0.05$.
666
667

667 Table 3. Effect of the additives on xylanase and amylase activities in CCF content sampled

668 on day 4 and 12 after inoculation.

Item	Day	Treatment				SEM[1]	P-value[2]		
		Control	BCM	DDS	PTS		T	d	Txd
Xylanase	**4**	5.72	6.52	5.65	5.14	1.58	0.051	0.458	0.186
	12	6.91	7.10	6.08	5.22				
Amylase	**4**	1.03[b]	1.33[a]	1.02[b]	1.04[b]	0.23	0.022	0.571	0.867
	12	1.10[b]	1.23[a]	1.06[b]	1.08[b]				

669

670 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane

671 thiosulfinate) and BCM (Bromochloromethane).

672 [1]SEM: Standard error of the mean.

673 [2] T: treatment effect; d: day effect; Txd: Treatment x day interaction. [a-c] within a row

674 treatment means without a common superscript differ, $P < 0.05$.

675 Amylase activity is expressed as micromoles of glucose released from soluble starch by 1 mL

676 of ruminal fluid in 1 min at 39ºC and pH=6.5. Xylanase activity is expressed as micromoles

677 of xylose liberated from oat beachwood xylan by 1 mL of ruminal fluid in 1 min at 39ºC and

678 pH=6.5.

679

679 Table 4. Effects of the additives on the concentration (log copy gene numbers g^{-1} fresh
680 matter) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea
681 (mcrA gene) in fermenters after 0, 4, 8 and 12 days of incubation.

		Treatment				SEM[1]	*P*-value[2]		
Item	Day	Control	BCM	DDS	PTS		T	d	Txd
Methanogens	**0**	8.72	8.63^{A}	8.69^{AB}	8.68^{A}	0.10	<0.001	0.027	<0.001
	4	8.62^{a}	7.91^{bB}	8.17^{abB}	7.96^{abAB}				
	8	8.69^{a}	7.19^{cC}	8.37^{bAB}	8.26^{bB}				
	12	8.81^{a}	6.90^{bC}	8.71^{aA}	8.26^{aAB}				
Protozoa	**0**	8.11^{A}	7.96^{A}	8.14^{A}	8.05^{A}	0.51	0.551	<0.001	0.033
	4	7.23^{B}	7.13^{B}	7.28^{B}	6.96^{B}				
	8	6.93^{B}	7.02^{B}	6.48^{C}	6.74^{B}				
	12	6.79^{B}	6.99^{B}	6.63^{BC}	6.92^{B}				
Bacteria	**0**	9.86^{A}	9.82^{A}	9.88^{A}	9.79^{A}	0.09	0.010	0.002	0.099
	4	9.49^{bB}	9.76^{aA}	9.58^{abB}	9.54^{bABC}				
	8	9.50^{B}	9.56^{B}	9.45^{B}	9.44^{C}				
	12	9.51^{B}	9.74^{A}	9.53^{B}	9.68^{AB}				

682 Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane
683 thiosulfinate) and BCM (Bromochloromethane).
684 [1]SEM: Standard error of the mean.
685 [2] T: treatment effect; d: day effect; Txt: Treatment x day interaction.
686 $^{a-c}$ within a row treatment means without a common superscript differ, $P < 0.05$.
687 $^{A-B}$ within a column treatment without a common superscript differ, $P < 0.05$.

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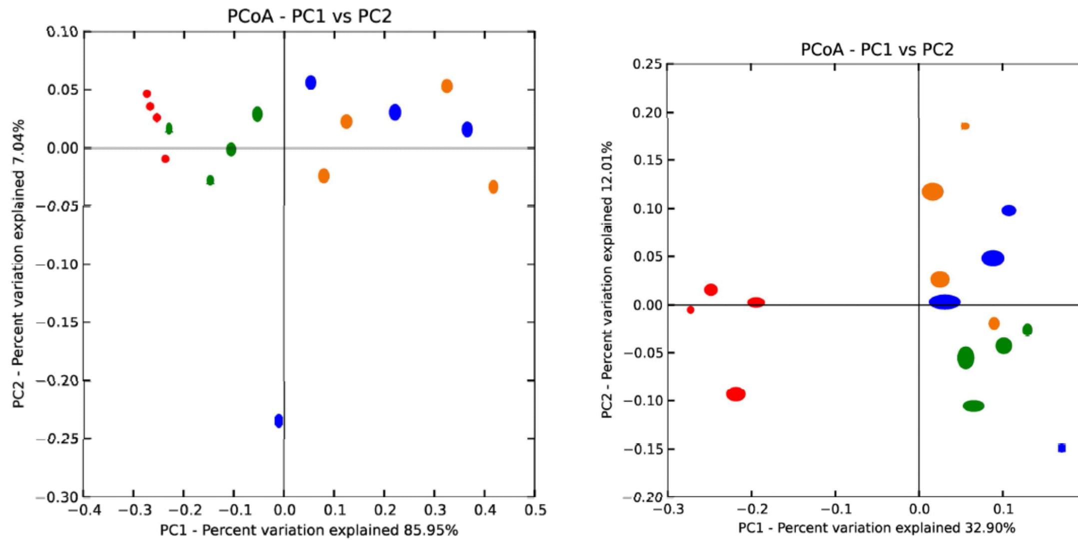

690
691 (A) (B)

692 Figure 1. Principal coordinate analysis showing the relationships of weighted bacterial (a) and
693 archaeal (b) communities with jackknife support of the fermenters content treated with BCM (Red),
694 PTS (Green), DDS (Orange) and without treatment (Blue) after 12 days of incubation. Size of spots
695 represent robustness of principal coordinate analysis based on jackknife for 1000 subset
696 resamplings.
697

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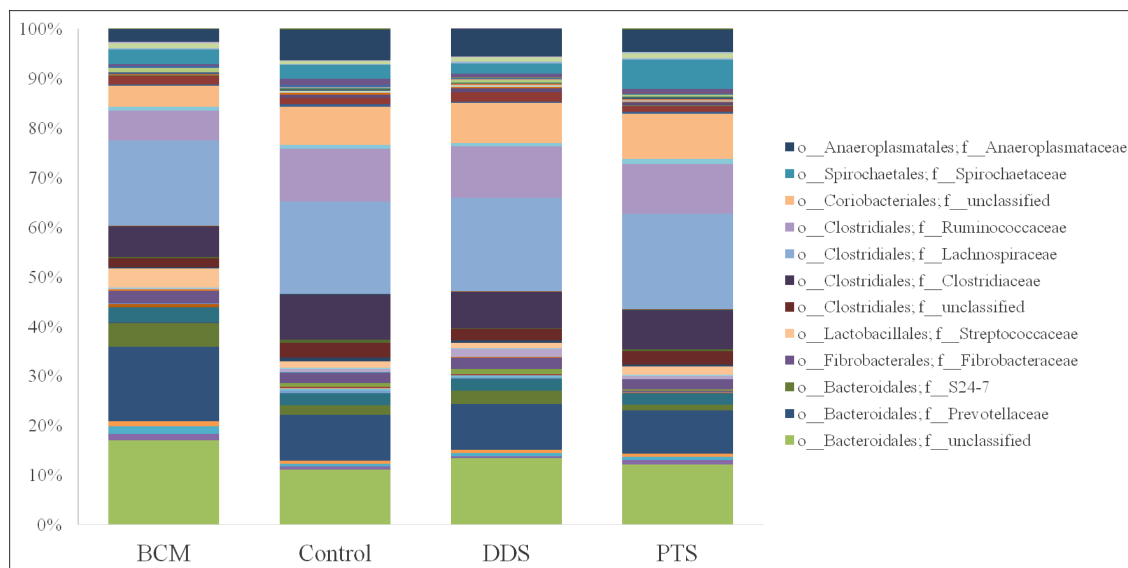
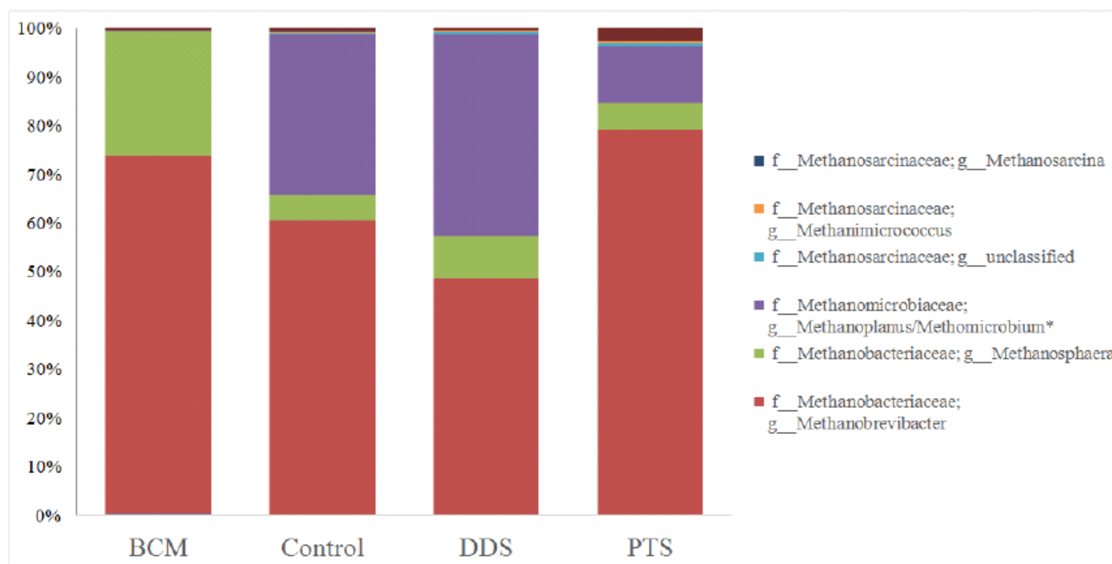


698
699 Figure 2. Bacterial taxonomic composition of the fermenters content without treatment
700 (Control), or treated with BCM, DDS and PTS after 12 days of incubation at family level.
701 Sequences were classified using BLAST with a 97% similarity level. Further information
702 regarding the complete family level taxonomic classification is available in supplementary
703 table 1.
704



704
705
706 Figure 3. Archaeal taxonomic composition of the fermenters content without treatment (Control), or
707 treated with BCM, DDS and PTS after 12 days of incubation. Sequences were classified using BLAST
708 with a 97% similarity level. Further information regarding the complete family level taxonomic
709 classification is available in supplementary table 2. * Conflict of taxonomy for GreenGenes database.
710 They were most associated to *Methanomicrobium mobile*.
711
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