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interval tended to be more efficient per unit time. In conclusion, rapidly growing, methane producing co-cultures of anaerobic fungi and methanogens from rumen digesta were easy to establish and maintain over considerable time periods and have potential in industrial-scale anaerobic digestion.

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3 **Composition and activity of mixed cultures of anaerobic fungi and**

4 **methanogens enriched from rumen contents and grown together in**

5 **consecutive batch culture**

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17 ## Abstract

18 Consecutive batch cultures (CBC), involving nine serial transfers at 3, 5 and 7 day
19 intervals and appropriate antibacterial antibiotics, were established to enrich for methane
20 generating co-cultures of anaerobic fungi and methanogens from rumen digesta.
21 Microbial diversity and fermentation end-products were measured at appropriate intervals
22 over each CBC time-course. While methanogenic populations remained diverse,
23 anaerobic fungal diversity was related to transfer interval and appeared to decrease with
24 transfer number. Fermentation profiling revealed minimal quantities of formate and (*D*+*L*)
25 lactate, and larger amounts of acetate. Methane and carbon dioxide were detected in all
26 co-cultures and the total amounts of gas generated per transfer were greater with transfer
27 intervals of 5 and 7 d compared with a 3 d interval, although the 3 d interval tended to be
28 more efficient per unit time. In conclusion, rapidly growing, methane producing co-
29 cultures of anaerobic fungi and methanogens from rumen digesta were easy to establish
30 and maintain over considerable time periods and have potential in industrial-scale
31 anaerobic digestion.

32

33 ## Keywords

34 Rumen; anaerobic fungi; methanogenic Archaea; consecutive batch culture; methane

35 ## 1. Introduction

36

37 The ability of a unique microbial consortium, normally resident in the digestive
38 tract of ruminants and other large mammalian herbivores, to generate methane from
39 lignocellulosic plant biomass was investigated within this study. Anaerobic fungi
40 (Neocallimastigales) and methanogenic Archaea are principle members of this
41 consortium, and it is well-known that they can form methane producing co-cultures when
42 grown together in batch culture on recalcitrant cellulosic substrates (Bauchop and
43 Mountfort, 1981). The nature of the anaerobic fungus-methanogen interaction has been
44 broadly described as cross-feeding, whereby particular fermentation end-products of the
45 cellulose and hemicellulose degrading anaerobic fungi are utilised as primary substrates
46 by the methane producing Archaea (Theodorou and France, 2005). However, as the
47 ability to produce methane is linked to inter-species hydrogen transfer and maintenance
48 of redox potential within cells, the anaerobic fungus-methanogen co-culture interaction is
49 intrinsically more complex than just cross-feeding. Methanogen growth influences
50 anaerobic fungal fermentation efficiency, catabolism pathways and specific enzyme
51 profiles, thus shifting fungal product formation away from more oxidised end-products,
52 such as lactate and ethanol, and towards production of the more reduced methanogenic
53 substrates, formate and acetate (Bauchop and Mountfort, 1981; Nakashimada et al., 2000;
54 Teunissen et al., 1992).

55 Studies on the nature of anaerobic fungus-methanogen co-culture interactions are
56 not extensive, and are generally concerned with activity in the ruminant digestive tract
57 and the contribution made by the consortium to the nutrition of the host animal. Research

58 to date has generally involved small-scale (10-100 ml) short-term (3-5 day) batch cultures
59 with co-cultures generated *de novo* from the combination of one or more axenic fungal
60 and methanogenic species (Bauchop and Mountfort, 1981; Mountfort et al., 1982;
61 Teunissen et al., 1992; Nakashimada et al., 2000). The population diversity of anaerobic
62 fungi and methanogens in the rumen however is considerably greater than this (Denman
63 et al., 2008; Janssen and Kirs, 2008), and their interactions are potentially more complex
64 than suggested by studies involving only a limited number of species *in vitro*. It is
65 therefore uncertain if interactions in defined co-cultures are truly representative of those
66 which occur in the rumen. The resilience of the anaerobic fungus-methanogen co-culture
67 and its ability to continue to produce methane over periods longer than just a few days is
68 also unknown. The potential exploitation of these fibre degrading, methane generating
69 co-cultures in industrial anaerobic digestion processes represents an intriguing possibility
70 that needs to be explored. The aim of this study was therefore to investigate the
71 enrichment, activity and diversity of methane generating co-cultures of anaerobic fungi
72 and methanogens from rumen digesta over longer periods of time using a consecutive
73 batch culture approach.
74
75 **2. Methods**
76
77 *2.1. Rumen inoculum and growth medium*
78 Rumen digesta was collected under license with the authority of the U.K. Animal
79 Scientific Procedures Act, 1986. Digesta contents were collected into pre-warmed
80 thermos flasks from two rumen cannulated, non-lactating Holstein-Friesian dairy cows

grazing a permanent ryegrass sward and immediately transported to the laboratory. Equal weights of the rumen contents from both cows were placed into a CO_2 gassed stomacher bag and 40 ml of anaerobic diluting solution (Bryant and Burkey, 1953) added prior to stomaching for 20 s at 230 rpm (Stomacher 400 Circulator, Seward, Worthing, UK). Three 10 ml aliquots were removed from the bag and inoculated into three pre-warmed bottles (39 ^{o}C) containing 90 ml of growth medium (one each for the 3, 5 and 7 d CBC series respectively). This process was repeated twice more in order to give triplicate cultures which served to initiate each of the 3, 5 and 7 d CBC series. The growth medium used was that of Davies et al., (1993) supplemented with 1% (w/v) barley straw (ground to pass through a 1 mm dry mesh screen (Cyclotec 1093 Sample Mill, Foss, Warrington, UK)) as a carbon source. The medium was dispensed in 160 ml glass serum bottles sealed with butyl rubber septa and aluminium crimp seals (Bellco Glass Inc., Vineland, New Jersey, USA).

2.2. Establishment and maintenance of anaerobic fungus-methanogen consecutive batch cultures

Consecutive batch culture (CBC) is an *in vitro* procedure which can be used to maintain actively fermenting mixed populations of rumen micro-organisms over long periods of time (Gascoyne and Theodorou, 1988). In the current study, each CBC consisted of a series of batch cultures inoculated in sequence (10% inoculum) at set transfer intervals with a microbial suspension originating from the previous culture, the first culture in each series being inoculated with a sample originating from the rumen (prepared as described above).

104 Three CBC series, consisting of nine serial transfers at 3, 5 or 7 d transfer
105 intervals respectively (i.e. continuing for 27, 45 and 63 days respectively) were
106 established. Each series was performed in triplicate, with no mixing of the inoculum
107 between replicates. The growth medium was used with antibiotics (ampicillin or
108 streptomycin/penicillin, at final concentrations of 2 mg ml^{-1}, 1915U ml^{-1}, and 2031U ml^{-1}
109 respectively). The antibiotics were added to the CBC cultures alternately (i.e. ampicillin,
110 streptomycin/penicillin, ampicillin) to remove Eubacteria. Inoculated cultures were
111 incubated at 39 ºC without agitation.
112
113 *2.3. Establishment and maintenance of an anaerobic fungal consecutive batch culture*
114 An additional CBC series was prepared from spent bottles of the 3rd transfer of the
115 3 d series in order to allow assessment of the fermentation parameters typical of growth
116 by the anaerobic fungi alone. Triplicate bottles were treated with chloramphenicol (50μg
117 ml^{-1} final concentration) to remove methanogens and were transferred every 3 days. In all
118 other aspects the bottles were processed in exactly the same manner as for the other CBC
119 series. Gas production data is presented as the mean value of eight transfers
120 (corresponding to the 4th – 13th transfer of the original co-culture 3 d series), whereas
121 VFA, lactate and formate data is presented as the average of triplicate cultures from the
122 final transfer.
123
124 *2.4. Assessment of Eubacterial contamination of CBC cultures*
125 A cultivation method was used to determine Eubacterial contamination in CBC
126 cultures. Spent culture fluids from each replicate of the 3rd and 6th transfers were used to

127 assess whether the alternate use of antibacterial antibiotics had effectively removed
128 Eubacteria from the co-cultures. Spent culture fluids were inoculated into anaerobically
129 prepared peptone-yeast-glucose (PYG) medium (Holdeman and Moore, 1972; 10 ml
130 medium in 15 ml serum tubes) and the tubes chilled at 4 ^{o}C for 24 h. The chilling step
131 inhibited anaerobic fungal growth, and consequently that of methanogens, which are
132 unable to directly utilise PYG medium. The tubes were then incubated for 3 days at 39 ^{o}C
133 and culture (1 ml) was removed for measurement of turbidity at a wavelength of 650 nm
134 (Ultrospec 4000 UV/Visible Spectrophotometer, Pharmacia Biotech, Buckinghamshire,
135 UK). Antibiotics (at previously stated final concentrations) were included in some of the
136 PYG medium inoculated with spent CBC culture fluids from the 6th transfer series to
137 verify whether turbidity was due to eubacterial or methanogenic bacterial growth.
138
139 *2.5. Measurement and sampling of headspace gas*
140 The total volume of gas accumulated in the culture head-space over the previous
141 24 h period was measured on a daily basis using the pressure transducer technique (PTT)
142 described by Theodorou et al. (1994). Following readings, the head-space was vented,
143 returning the pressure back to ambient conditions, ready to accumulate gas for the next 24
144 h reading. At each reading, an aliquot (approx 7 ml) of the vented gas was transferred by
145 injection into an evacuated 11 mm diameter glass vial fitted with a crimp cap and PTFE
146 gas tight seals (Chromocol). Samples were stored at room temperature and analysed for
147 the relative abundance of CH_4 and CO_2 within 5 days of collection as described below.
148
149 *2.6. Determination of the relative abundance of carbon dioxide and methane*

150 The relative abundance of methane (CH_4) and carbon dioxide (CO_2) in head-space
151 gas was determined by electron impact ionisation (ionisation energy = 69.9 eV) mass
152 spectrometry scanning from 50 m z^{-1} to 2 m z^{-1} (Agilent 6890/ 5973N gas chromatograph/
153 mass spectrometer, Agilent Technologies UK Limited, Stockport, UK). All samples were
154 analysed in duplicate. A 4µl volume of each head-space gas sample (held within 11 mm
155 diameter glass vials) was injected by autosampler into a splitless mode split/splitless inlet
156 at 280 ºC using a 10 µl volume gas-tight syringe (Agilent). The sample was swept into
157 the mass detector in a stream of helium (1 ml min^{-1}) via a 30 m non-polar capillary
158 column (Varian CP9013 Factor 4) maintained at 35 ºC. The column, served only to act as
159 a gas tight link between the inlet and the mass detector. The temperatures of the source
160 and quadrupole were 230 ºC and 150 ºC respectively. The abundance of CH_4 and CO_2
161 were calculated by addition of the ion abundances at masses 15 m z^{-1}, 16m z^{-1} and 17 m z^{-}
162 ^{1} (CH_4) and 44 m z^{-1} and 45 m z^{-1} (CO_2) respectively and the amount (nmoles) of each
163 gas calculated using standard curves (eight point with triplicate estimations; R^2
164 coefficients of 0.999 and 0.993 for CH_4 and CO_2 respectively).
165

166 *2.7. Determination of culture pH and fermentation end-products*

167 Culture pH (recorded immediately upon removing crimp-seals and stoppers from
168 culture bottles) and the concentration of fermentation end-products was determined in
169 samples taken after each transfer and at the end of each consecutive batch culture series.
170 Volatile fatty acids were determined by gas chromatography as previously described
171 (Kim et al., 2001). Data collection and processing was performed using a Dionex
172 advanced computer interface and AI-450 data handling software (Dionex (UK) Ltd.,
173 Surrey, UK). Aliquots (1 ml) of culture supernatant were collected and stored at -20°C

174 prior to determination of formate and *L* and *D* lactate using dehydrogenase enzyme
175 methodology. Formate was determined using the method of Hopner and Knappe (1974)
176 and *L* and *D* lactate was analysed by using a Boehringer Mannheim *L*-lactate kit (R-
177 Biopharm Rhône Ltd., Glasgow, UK) and *D*-lactate dehydrogenase (Sigma). Each sample
178 for dehydrogenase assays was analysed in triplicate.

179

180 *2.8. Sample DNA extraction*

181 DNA was extracted from samples of the rumen inoculum (20 mg dry weight) and
182 the liquid phase of the cultures (5 ml) from the 2nd, 4th and 9th transfers of all of the CBC
183 co-cultures. Culture samples were centrifuged at 13,000 *g* for 5 min after collection, and
184 the pellet washed with 1 ml of TE buffer (20 mM Tris-HCl, 2 mM EDTA) prior to DNA
185 extraction. DNA was extracted from the rumen inoculum and washed pelleted cultures
186 using the BIO101 FastDNA SPIN kit for soil (QBiogene). Manufacturer's guidelines
187 were followed with the exception that the samples were processed for 3 x 30 s at speed
188 6.0 in the FastPrep instrument (QBiogene), with incubation for 30 s on ice between bead-
189 beating. Integrity of the DNA was verified by agarose gel electrophoresis and DNA
190 quantified using a NanoDrop® ND-1000 UV-Visible spectrophotometer (Labtech
191 International, East Sussex, UK).

192

193 *2.9. PCR-based method for the detection of ciliate protozoa*

194 PCR amplification was used to check for the presence of ciliate protozoa in the
195 cultures from the 2nd, 4th and 9th transfers. Amplification of the 18S rRNA gene of ciliate
196 protozoa was carried out using the primers of Huws et al. (2008) without the GC clamp

197 on the reverse primer, i.e. 316F (5'- GCTTTCGWTGGTAGTGTATT -3') and 539R (5'-
198 ACTTGCCCTCAAATCGT -3'). After PCR, amplification of products was verified by
199 agarose gel electrophoresis.
200

201 *2.10. Neocallimastigales specific automated ribosomal intergenic spacer (ARISA)*
202 *analysis*

203 Size polymorphism analysis of the anaerobic fungal (Neocallimastigales) internal
204 transcribed spacer 1 (ITS1) region was carried out with the primer pair Neo 18S For (5'-
205 AAT CCT TCG GAT TGG CT-3' labelled with 6-FAM on the 5' end) and Neo 5.8S Rev
206 (5'- CGA GAA CCA AGA GAT CCA -3') as previously described (Edwards et al.,
207 2008). For each sample, triplicate PCR reactions with approx. 20 ng of template DNA
208 were performed. After PCR amplification was verified by agarose gel electrophoresis,
209 PCR products from the same sample were then combined and run on an ABI 3130xl
210 Genetic Analyser (Applied Biosystems) with GeneScan 500 LIZ size standard. The data
211 was exported from the software package Genemapper (Applied Biosystems) as a table of
212 peaks. Peak heights that were less than 1% of the largest peak were included in
213 subsequent cluster analysis as uncertain bands so that their presence/absence would not
214 penalise the analysis.
215

216 *2.11. Methanogenic Archaea specific terminal restriction fragment length polymorphism*
217 *(T-RFLP) analysis*

218 Primers specific for the methyl-coenzyme M reductase α-subunit (*mcrA*) gene
219 were used to generate ~ 0.5 kb PCR amplicons from methanogenic Archaea (Luton et al.,

220 2002). The forward primer MLf (5'-GGTGGTGTMGGATTCACACARTAYGCWAC-

221 AGC-3') was used along with the reverse primer MLr (5'-TTCATTGCRTAGTTWGG-

222 RTAGTT-3') labelled with the D4 fluorophore at the 5' end. PCR amplification was

223 carried out in 25 µl reaction mixtures, with three replicates prepared for each sample. The

224 reaction mixture contained 1 × PCR amplification buffer (50 mM KCl, 10 mM Tris-HCl

225 pH 8.5, 0.1% Triton X-100,), 1.75 mM $MgCl_2$, 800 µM deoxynucleoside triphosphates,

226 500 nM of each primer, 25 ng of template DNA and 1.25 U of *Taq* polymerase (Promega,

227 Southampton, UK). Amplification conditions were as follows: 95 ºC for 5 min, 30 cycles

228 of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min, and a final extension of 72 ºC for 7

229 min.

230 After PCR, amplification of products was verified by agarose gel electrophoresis.

231 Triplicate tubes of each sample were then combined and purified using a MultiScreen

232 $PCR_{\mu 96}$ Filter Plate (Millipore, Watford, UK) with an exclusion size of 60 bp. Purified

233 PCR products were then quantified using a NanoDrop® ND-1000 UV-Visible

234 spectrophotometer. After checking the digestion sites of *Taq*I, *Msp*I, *Hha*I, *Hae*III and

235 *Sau96* I which were reported previously (Luton et al., 2002; Merila et al., 2006; Lueders

236 et al., 2001) in all of the available *mcrA* gene sequences from the rumen environment

237 (NCBI), *Taq* I and *Msp* I were selected as restriction enzymes. Restriction digestion with

238 *Taq* I was carried out in a reaction volume of 20 µl containing 50 ng of purified PCR

239 product, 1 × buffer and 10 U of *Taq*I, with digestion for 5 h at 65 ºC. For *Msp*I a similar

240 reaction mix was used except 2 µg of BSA was added and the digestion was incubated for

241 5 h at 37 ºC. Digestion products were then purified using a Montage SEQ_{96} Cleanup Kit

242 (Millipore). The purified digested products were then run on a CEQ 8000 Genetic

243 Analysis system (Beckman Coulter, High Wycombe, UK) using a DNA Size Standard
244 Kit - 600 (Beckman Coulter) as an internal size standard.
245 All the raw data was exported from CEQ analysis software (Beckman Coulter) as
246 a table of peaks. Peaks that were less than 4.75% of the maximum peak height were
247 removed from the analysis along with any peaks corresponding to the position of the
248 undigested PCR product. The 4.75% value was determined by assessing the maximum
249 background peak height value present in an undigested control T-RFLP sample derived
250 from the rumen inoculum, and its percentage relative to the maximum peak heights
251 present in the sample set.
252
253 *2.12. Cluster analysis*
254 Tables of peaks generated from the ARISA and T-RFLP analysis were imported
255 into the Fingerprint Type and Cluster Analysis modules of the Fingerprinting software
256 package (Bio-Rad UK Ltd, Hemel Hempstead, UK). Cluster analysis was performed
257 using the different bands similarity coefficient, with a position tolerance of 0.5 bases and
258 an optimisation parameter of 0.5 %.
259
260 *2.13. Statistics*
261 Total gas production during the first transfer was noticeably higher than from
262 subsequent transfers as a result of nutrients originating from the rumen digesta from
263 which the inoculum was derived. In the assessment of whether there was any systematic
264 effect of transfer number on fermentation products, data from the first transfer were
265 excluded. Mean values were calculated for each transfer at each transfer interval and, in
266 the absence of systematic patterns in residuals to suggest a nonlinear response, a linear

267 model was fitted to examine effects of transfer number, transfer interval and any
268 interaction. Bootstrapped (500 repetitions) estimates of intercepts, slopes and in the
269 absence of a non-zero slope overall mean values for each transfer interval and their
270 associated standard errors were obtained.
271

272 3. Results

273

274 *3.1. Assessment of the removal of Eubacteria from the CBC series*

275 Anaerobic fungal growth was evident in all the PYG cultures inoculated with
276 spent culture fluids from the 3rd transfer of each CBC series, along with a limited
277 turbidity (OD_{650} of <0.3). The growth of anaerobic fungi was unexpected as actively
278 growing cultures are generally considered to be intolerant of low temperatures (Lowe et
279 al., 1987). Consequently, as anaerobic fungal fermentation end-products can provide the
280 primary growth substrates for methanogens, it was not possible to directly confirm
281 whether the low amounts of turbidity observed was due to the growth of Eubacteria or
282 methanogens. Therefore spent culture fluids from the 6th transfer of each CBC series were
283 tested again in a similar manner, but with and without the addition of antibiotics. PYG
284 cultures treated with ampicillin and/or streptomycin/penicillin generally resulted in
285 turbidity comparable to that previously observed, but with chloramphenicol (50 μg/ml)
286 the turbidity was consistently lower (OD_{650} of <0.15). As only chloramphenicol can
287 inhibit methanogens as well as Eubacteria, it was concluded that the limited turbidity
288 observed in the 3rd and 6th transfers was likely to be due mainly to growth of
289 methanogens.

290

291 *3.2. Assessment of the removal of ciliate protozoa from the CBC series*

292 PCR amplification using primers targeting the ciliate protozoal 18S rRNA gene

293 and DNA extracted from the original rumen fluid inoculum gave a strong signal. By way

294 of contrast, PCR amplification of DNA extracted from the 2nd, 4th and 9th transfers from

295 all the CBC series gave no PCR amplification for protozoal 18S rDNA, with the

296 exception of the cultures from the 2nd transfer period of the 3-day CBC series. These

297 cultures however only gave a weak amplification signal when compared to the original

298 inoculum. Thus in all the transfer series, protozoal signals were negligible prior to and

299 were not detected beyond the 3rd CBC transfer.

300

301 *3.3. Detection and characterisation of the anaerobic fungi in the CBC series*

302 The presence and population composition of anaerobic fungi was assessed by

303 ARISA analysis of DNA extracted from the original rumen inoculum and the culture

304 fluids of each of the 2nd 4th and 9th transfer replicates from all three of the CBC series.

305 The DNA from the original inoculum and all of the CBC culture fluids gave a positive

306 amplification signal for anaerobic fungi, with the size of the amplicons obtained ranging

307 from 330 to 438 bases. Amplicons with sizes of 426, 437 and 438 bases (see Fig 1. box 5)

308 were absent from the ARISA profile of the original inoculum, but showed substantial

309 intensity in the 2nd transfers of the 3 d CBC series. The reason for this is not clear, but it

310 would seem probable that the corresponding amplicon sizes in the inoculum and all other

311 CBC cultures were below the detection level of the method.

312 Diversity within the anaerobic fungal populations, as assessed by the number and

313 intensities of different amplicon sizes, appeared to differ between and within CBC series

314 (Fig. 1). Generally it was observed that for all of the CBC series there was an associated
315 decrease in the number of amplicon sizes as the number of transfers increased (Fig. 1).
316 Within the 3 d CBC series the rumen inoculum clustered with two of the 2nd transfer
317 samples, in contrast to both the 5 d and 7 d series where the rumen inoculum formed an
318 outlier. The basis of this observation appeared to be due to a group of amplicon sizes
319 (367- 375 bases; Fig 1. box 2) that were consistently lost by the time of the 4th transfer in
320 the 3 d CBC series and by the 2nd transfer in the 5 d and 7 d CBC series. In the 5 d series
321 the 2nd transfers formed a tight cluster, distinct from the 4th and 9th transfer due to a group
322 of amplicons (413-419 bases; Fig. 1 box 4) that were absent from these samples,
323 compared to the original inoculum and 2nd transfer.

324 In all three CBC series there was no clear difference between the 4th and 9th
325 transfers, although there was a loss of one group of amplicon sizes (383-392 bases; Fig 1.
326 box 3) by the 9th transfer in the some of the 5 d and 7 d CBC series bottle replicates.
327 Another group of amplicons (354-358 bases; Fig 1. box 1), while generally present in all
328 of the 5 d and 7 d CBC cultures, were only weakly represented in the 3 d CBC series
329 where they did not persist beyond the 2nd transfer. The relative intensity of this group of
330 fragments in the 5 d and 7 d CBC series was much higher than in the original inoculum,
331 indicating that they were able to persist more favourably in cultures where the transfer
332 interval was greater than three days. In contrast the amplicon sizes in the region of 413-
333 419 bases (Fig. 1 box 4) generally showed a relative and consistent increase in intensity
334 and occurrence in the 3 d CBC series, compared to the 5 d and 7 d CBC series. Despite
335 all the differences observed over the duration of the transfers, it was noted that cluster
336 analysis of all the 9th transfer samples did not result in any clusters that could be directly
337 correlated with the different CBC series (data not shown).

338

339 *3.3. Detection and characterisation of the methanogenic archaea in the CBC series*

340 The presence and population composition of methanogenic Archaea within each

341 of the d CBC series was assessed by PCR amplification and T-RFLP analysis of the *mcrA*

342 gene. Amplification of the *mcrA* gene was carried out using DNA extracted from the

343 original inoculum and the culture fluid of each of the 2nd 4th and 9th transfer replicates

344 from all three of the CBC series. The DNA from the original inoculum and from all CBC

345 culture fluids gave a positive amplification signal. T-RFLP analysis of the gene was

346 performed using two different enzymes that were both found *in silico* to restrict *mcrA*

347 sequences obtained from the rumen environment. Only the results from the enzyme *Taq* I

348 are presented however as the *Msp* I digests generated just a limited number of restriction

349 fragments with some of the samples, and the undigested amplicon peak was large in all of

350 the samples. For example, with the rumen inoculum raw data 91 % of the total peak

351 height was represented by the undigested amplicon.

352 Cluster analysis of the *Taq* I T-RFLP profiles was performed and, as with the

353 anaerobic fungi, there was no obvious tendency for the cultures to cluster by bottle

354 replicate (Fig. 2). In general, there was no obvious grouping either by the number of

355 transfers, although in the 3 d CBC series the 2nd transfers sub-clustered with the original

356 inoculum. Unlike the anaerobic fungi, there was no simplification of the profiles with

357 number of transfers with instead a slight tendency for an increase in the number of *Taq* I

358 restriction fragments observed, relative to the original rumen inoculum.

359

360 *3.4. Gas, carbon dioxide and methane production*

361 The total volume of gas and amounts of CO_2 and CH_4 produced during each
362 transfer interval in the 3, 5 and 7 d co-culture CBC series are presented in Fig. 3. Gas,
363 CO_2 and CH_4 were produced throughout the entire time course of each CBC series and
364 the amounts produced, while differing between CBC series, appeared stable and
365 reasonably consistent from the second transfer, with minimal deviation between replicate
366 cultures. There was no statistical evidence of any change in total gas production or CO_2
367 production per transfer with increasing transfer number (P>0.05). Gas and CO_2
368 production per transfer was higher (P<0.05) with 5 and 7 d transfer intervals than with the
369 3 d transfer interval (Fig 3a and b).

370 Methane production increased with each increment in transfer interval (1.23 (s.e.
371 0.176), 1.67 (s.e. 0.123) and 1.99 (s.e. 0.132) mmoles/transfer with 3, 5 and 7 d transfers
372 respectively) but only the difference between the two extreme intervals was statistically
373 significant (P<0.05). Taking the number of days of culturing time into account, gas (ml/d)
374 and carbon dioxide and methane (mmoles/d) production decreased with increasing
375 transfer interval. Significantly less (P<0.05) gas and carbon dioxide was produced from
376 the 7d series compared with either the 3 or 5 d series which did not differ significantly
377 (P>0.05). Differences in methane production per day between transfer intervals did not
378 reach statistical significance but there was a tendency (P>0.05) for the 3d series to be
379 more efficient in terms of methane production per unit time than the longer intervals
380 (0.41 (s.e. 0.058), 0.33 (s.e. 0.025), 0.28 (s.e. 0.019) mmoles/d for 3, 5 and 7 d intervals
381 respectively)).

382 Mean gas production from the anaerobic fungal 3d CBC bottles was lower
383 (P<0.001) than from the 3d methanogenic co-culture bottles (83.4 (s.e. 3.88) *v* 149.2 (s.e.

384 8.32) ml/transfer respectively). Following chloramphenicol treatment, production of
385 methane was not evident in any of the anaerobic fungal 3d CBC series.

386

387 *3.5. pH, formate, lactate and VFA*

388 The pH recorded at the end of each CBC transfer was within the range of 6.21 –
389 6.80. Therefore, the pH of all the cultures was generally maintained at a level appropriate
390 for fibre degradation and was supportive of the growth of rumen micro-organisms.

391 Formate and lactate concentrations were determined in the culture fluid of each
392 replicate at the end of each transfer for each co-culture CBC series. Formate was
393 consistently ≤ 0.1 mM in the culture supernatant of all the CBC series which contained
394 methanogens, and after the first transfer, was not detected at all in the 3 d series. This
395 contrasted with the concentration of formate in the anaerobic fungal 3-day CBC series
396 where values were higher (13.8 mM, s.e.m. 0.99) and similar to those recorded previously
397 for axenic fungal cultures (Lowe et al., 1987).

398 Lactate concentrations were generally low for the 3 and 5-d co-culture series with
399 ranges of 0.16-0.85 mM and 0.18-1.17 mM respectively. With the 7d CBC series values
400 were slightly higher, 0.28-2.82 mM. Again, co-culture values were lower than the amount
401 produced during the final transfer of the anaerobic fungal 3-day CBC series (12.3 mM,
402 s.e.m. 0.44) which was typical of previously reported values for axenic fungal cultures
403 (Lowe et al., 1987).

404 Concentrations of the volatile fatty acids, iso-butyrate, n-valerate and iso-valerate,
405 were low in all the CBC series co-culture supernatants (and the anaerobic fungal 3-d CBC
406 series) with maximum levels of 0.9, 1.4 and 1.3 mM respectively. Concentrations of
407 propionate and n-butyrate were slightly higher with means of 2.89 mM and 2.77 mM

408 respectively. These values were consistent with the amounts of VFA present in
409 uninoculated culture medium, which contained 15% (v/v) clarified rumen fluids as an
410 ingredient. By contrast, concentrations of acetate in all co-cultures of the CBC series (Fig.
411 4.) were substantially higher than in the uninoculated culture medium. Mean acetate
412 concentrations for the 3 d, 5 d and 7 d CBC series over transfers 2 to 9 were 36.6 mM (s.e.
413 1.38), 45.4 mM (s.e. 1.61) and 44.9 mM (s.e. 1.46) respectively. The 3 d CBC series
414 produced significantly less ($P<0.05$) acetate than either the 5 d or 7 d CBC series. All
415 three of the methanogenic CBC series produced more acetate compared to the final
416 transfer of the anaerobic fungal 3 d CBC series (23.3 mM, s.e.m. 1.21), as reported
417 previously (Bauchop and Mountfort, 1981; Mountfort et al., 1982; Wood et al, 1986;
418 Teunissen et al., 1992) suggesting increased efficiency of fungal metabolism and
419 enhanced fibre degradation in co-cultures.

420 ## 4. Discussion

421

422 Syntrophic co-cultures of individual anaerobic fungal species with up to three
423 strains of methanogen have been described in the literature (Bauchop and Mountfort,
424 1981; Mountfort et al., 1982; Teunissen et al., 1992). In one publication, the influence of
425 the fibrolytic bacterium *Fibrobacter succinogenes* on the methanogenic co-culture was
426 also investigated (Joblin et al., 2002). Generally, the methodology used to generate
427 methanogenic co-cultures in these publications are similar, relying upon the growing
428 together of previously isolated axenic cultures of an anaerobic fungus and a methanogen
429 for relatively short periods (up to 7 days) in anaerobic batch culture. The methodology
430 used to generate methanogenic co-cultures in this study, and in the related studies by
431 Cheng et al. (2006; 2007), however was distinctly different. It was reliant upon an
432 enrichment procedure to isolate a co-culture consortium of syntrophic fungi and
433 methanogens directly from rumen digesta.

434 The enrichment procedure uses antibacterial antibiotics to selectively remove
435 Eubacteria. Although in this study the culture-based check for bacteria was inconclusive
436 by itself, due to the unexpected growth of anaerobic fungi after chilling, the lack of net
437 production of propionate and butyrate, which are typical end-products of bacterial mixed
438 acid fermentation, confirmed that this selective removal had been effective. The
439 procedure also made use of the fact that protozoa do not survive indefinitely in batch
440 culture to exclude these Eukaryotes, as was demonstrated by their absence after the 3rd
441 transfer of all the CBC series.

442 The use of a recalcitrant cellulosic substrate enriched the fibrolytic anaerobic
443 fungal populations within the CBC series. In turn, this selected for a consortium of
444 methanogenic Archaea due to their sole reliance upon fungal end-products in the absence
445 of bacteria and protozoa. Analysis of the population composition of anaerobic fungi and
446 methanogens in the initial rumen inoculum showed both populations to be diverse, as has
447 been previously reported (Edwards et al., 2008; Janssen and Kirs, 2008). In the
448 subsequent CBC cultures however the complexity of anaerobic fungal populations
449 simplified with transfer, in contrast to the methanogen populations which generally
450 remained diverse throughout the CBC series regardless of transfer interval. These
451 findings are in line with previous observations by Cheng et al. (2007) who also found that
452 while methanogen populations remained more diverse, anaerobic fungal populations
453 simplified in a 3 d transfer CBC series.
454 Anaerobic fungi use a wide range of polysaccharides and disaccharides for growth,
455 although growth on monosaccharides is generally restricted to glucose, fructose and
456 xylose (Lowe et al., 1987; Phillips & Gordon, 1995). These substrates are fermented via a
457 mixed acid fermentation to give end-products such as: formate, acetate, lactate, succinate,
458 ethanol, CO_2, and H_2 (Lowe et al.1987; Marvin-Sikkema et al., 1990). Some of these
459 products (formate, ethanol, lactate and succinate) are not typical end-products of rumen
460 fermentation. They are however electron sink products formed during glycolysis from the
461 re-oxidation of reduced pyridine nucleotides (NADH and NADPH). Their accumulation
462 in axenic anaerobic fungal culture is thought to be due to the absence of hydrogen
463 consuming microorganisms.
464 Cultivation of anaerobic fungi in the presence of methanogens enables inter-
465 species hydrogen transfer, resulting in the disposal of electrons via CH_4. According to

466 Hungate (1966), this process is energetically more favourable than disposal via other
467 electron sink products such as those mentioned above. Thus, in the presence of up to
468 three different methanogens, the fermentation profiles of *Neocallimastix* and *Piromyces*
469 altered to include increased production of acetate and CO_2 and decreased production of
470 ethanol, lactate and succinate (Bauchop and Mountfort, 1981; Mountfort et al, 1982;
471 Teunissen et al., 1992).

472 Methanogens can maintain low hydrogen levels in the co-cultures due to the
473 ability of their hydrogenase enzymes to catalyse the production of hydrogen from reduced
474 pyridine nucleotides at very low partial pressures of H_2 (Marvin-Sikkema et al., 1990).
475 Given that ATP formation is coupled to acetate synthesis in many anaerobes (Gottschalk
476 and Andreesen, 1979), metabolism alters to provide more energy for the cellulose
477 degrading primary organism and enhances cellulose fermentation kinetics. Higher
478 cellulolytic activities have been recorded in several anaerobic fungus-methanogen co-
479 culture studies (Bauchop and Mountfort, 1981; Mountfort et al., 1982; Wood et al., 1986;
480 Teunissen et al., 1992). Although the kinetics of fibre degradation was not assessed in
481 this study, the results indicated that the anaerobic fungi were able to degrade more fibrous
482 substrate in the presence of methanogens than in their absence. The co-cultures produced
483 more gaseous end-products relative to the fungal cultures alone, and this has been
484 correlated to enhanced fibre degradation (Theodorou et al., 1998).

485 Significant differences in fungal metabolism, towards the production of more
486 reduced fermentation end-products with increased production of acetate and minimal
487 production of lactate were observed in the presence of methanogens. The decrease in
488 formate in CBC co-cultures to negligible amounts reflected its utilisation as a primary
489 substrate for methane production by the formate-utilizing methanogens (Ellis et al., 1990).

490 These changes in metabolism within the co-culture persisted throughout the entire CBC
491 series. The ability of these stable co-cultures to continue to degrade fibre and produce
492 methane for up to 63 days is novel and requires further investigation, particularly in terms
493 of potential industrial anaerobic digestion processes.
494

495 5. Conclusions

496 In this study, a simple enrichment procedure was performed to obtain methane
497 producing co-cultures of anaerobic fungi and methanogens from rumen digesta. By
498 transferring a proportion of the co-culture to fresh culture media at regular intervals, a
499 stable syntrophic consortium of anaerobic fungi and methanogens was maintained.
500 Diverse populations of methanogens persisted in the consortium, which consistently
501 produced methane as a gaseous end-product from the degradation of a plant derived
502 recalcitrant lignocellulosic substrate. Our results demonstrate that these diverse fibre
503 degrading methanogenic co-cultures from the rumen ecosystem require additional
504 attention, as they may be exploitable in industrial anaerobic digestion processes.

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506

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513

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606 **Fig. 1.** Cluster analysis of ARISA profiles of anaerobic fungi present in 3 d (a), 5 d (b)
607 and 7 d (c) CBC series. Dendrogram labels (i.e. 9b2) represent the number of transfers
608 (i.e. 9 is the 9th transfer) and the replicate bottle (i.e. b2 is the second replicate bottle) that
609 was serially transferred. The rumen inoculum used to start the CBC series is designated
610 INO. Band intensity and position represents the ARISA peak intensity and corresponding
611 amplicon size (increasing from left to right). Boxed regions (1-5) of the ARISA profiles
612 are used to indicate bands discussed within the results. The scale bar indicates percentage
613 similarity.

614

615 **Fig. 2.** Cluster analysis of T-RFLP fragments produced by *Taq* I digestion of the *mcrA*
616 gene amplified from methanogens present in the 3 d (a), 5 d (b) and 7 d (c) co-cultures.
617 Dendrogram labels are as previously described (see Fig. 1 legend). Band intensity and
618 position represents the peak intensity and corresponding size of the T-RFLP fragment
619 (increasing from left to right). The scale bar indicates percentage similarity.

620

621 **Fig. 3.** Gaseous fermentation products generated during each transfer of the 3 d (■), 5 d
622 (▲) and 7 d (○) CBC series. Cumulative total gas (a), carbon dioxide (b) and methane (c)
623 are presented as the average of the replicate CBC series cultures (n=3). Error bars
624 (present on all graphs) represent the standard error of the mean.

625

626 **Fig. 4.** Concentration of acetate in culture supernatants during each transfer of the 3 d (■),
627 5 d (▲) and 7 d (○) CBC series. Values presented are the average of the replicate CBC
628 series cultures (n=3). Error bars represent the standard error of the mean.

Figure 1

Fig. 1.

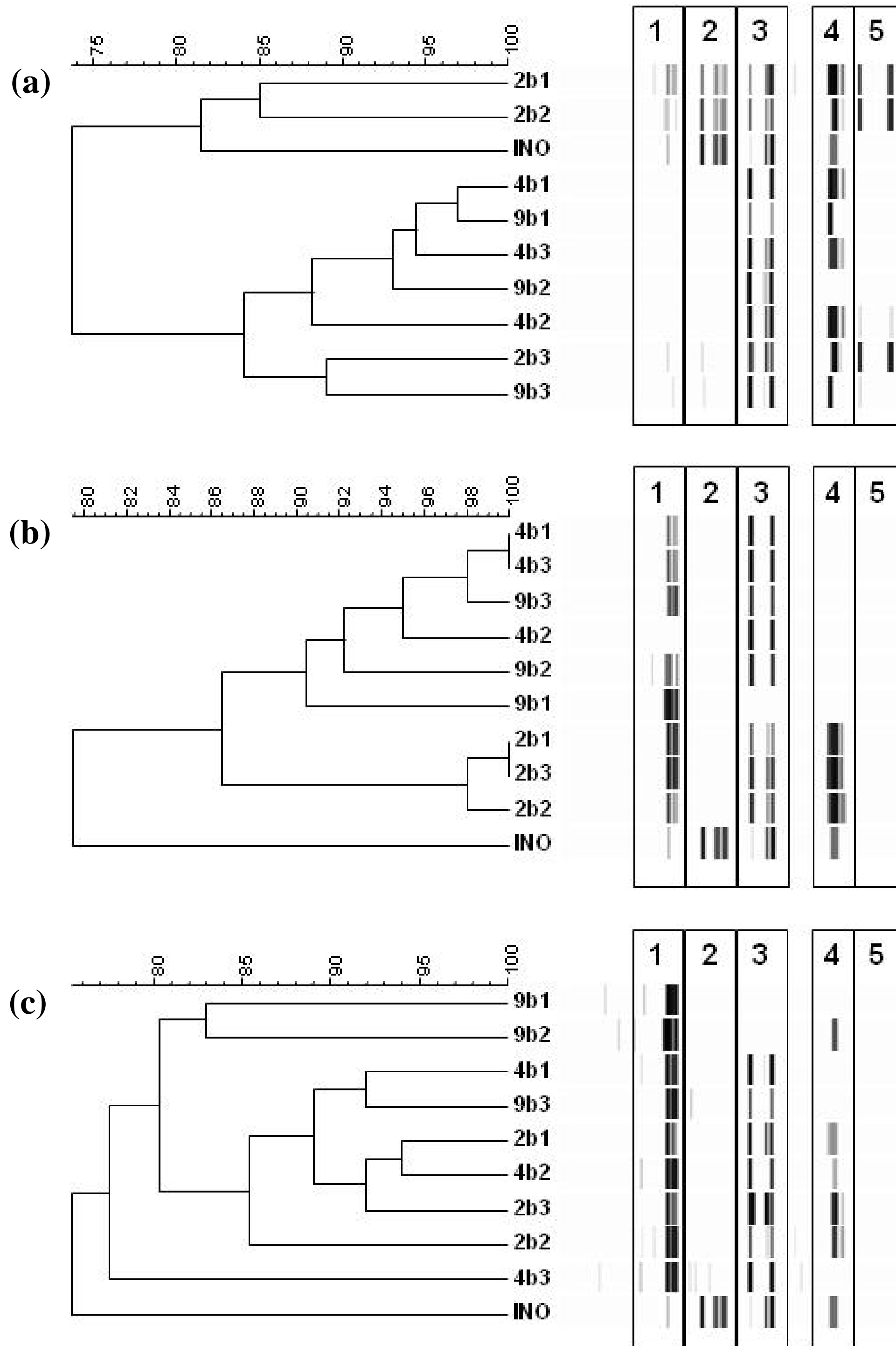

Figure 2

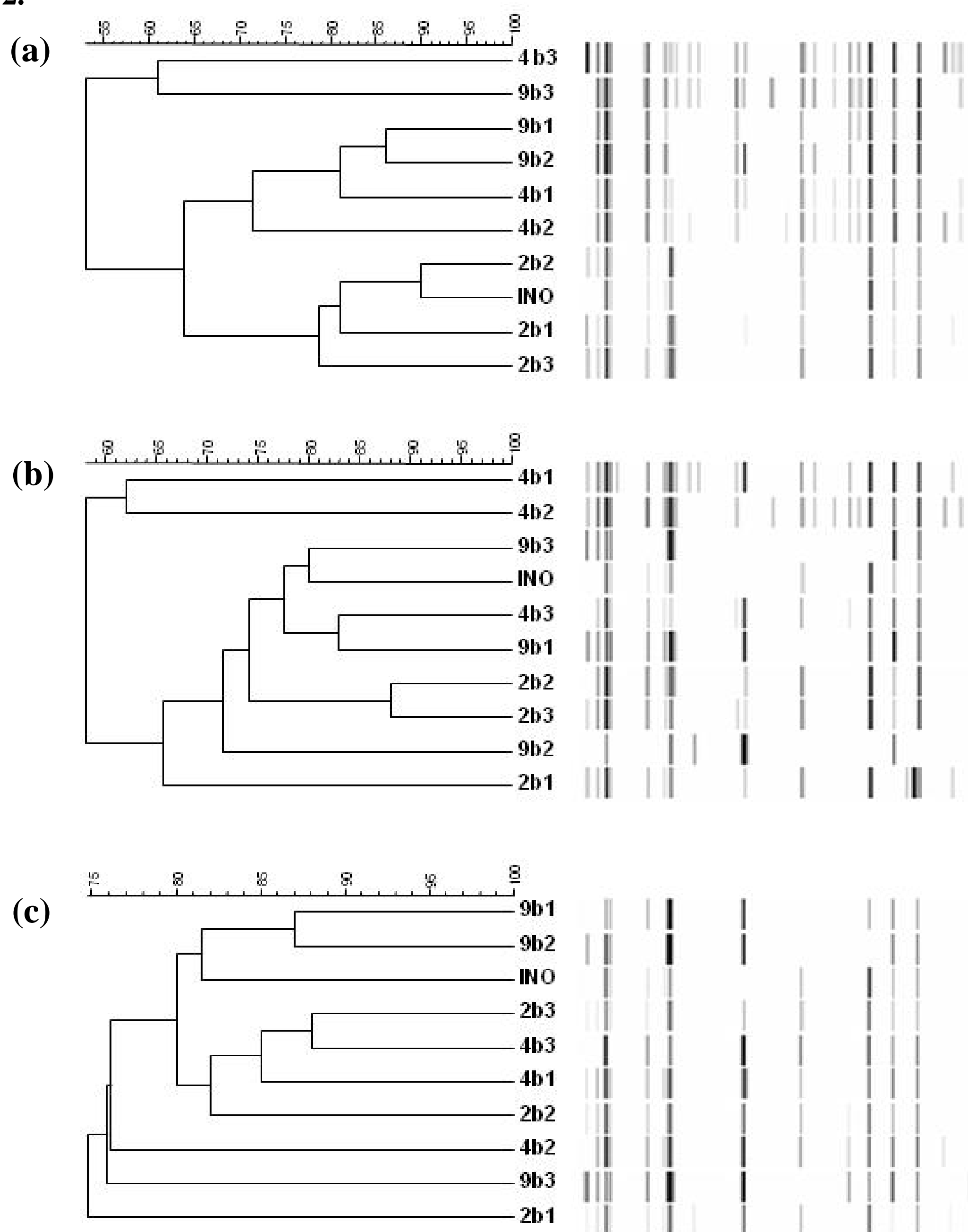

Figure 3

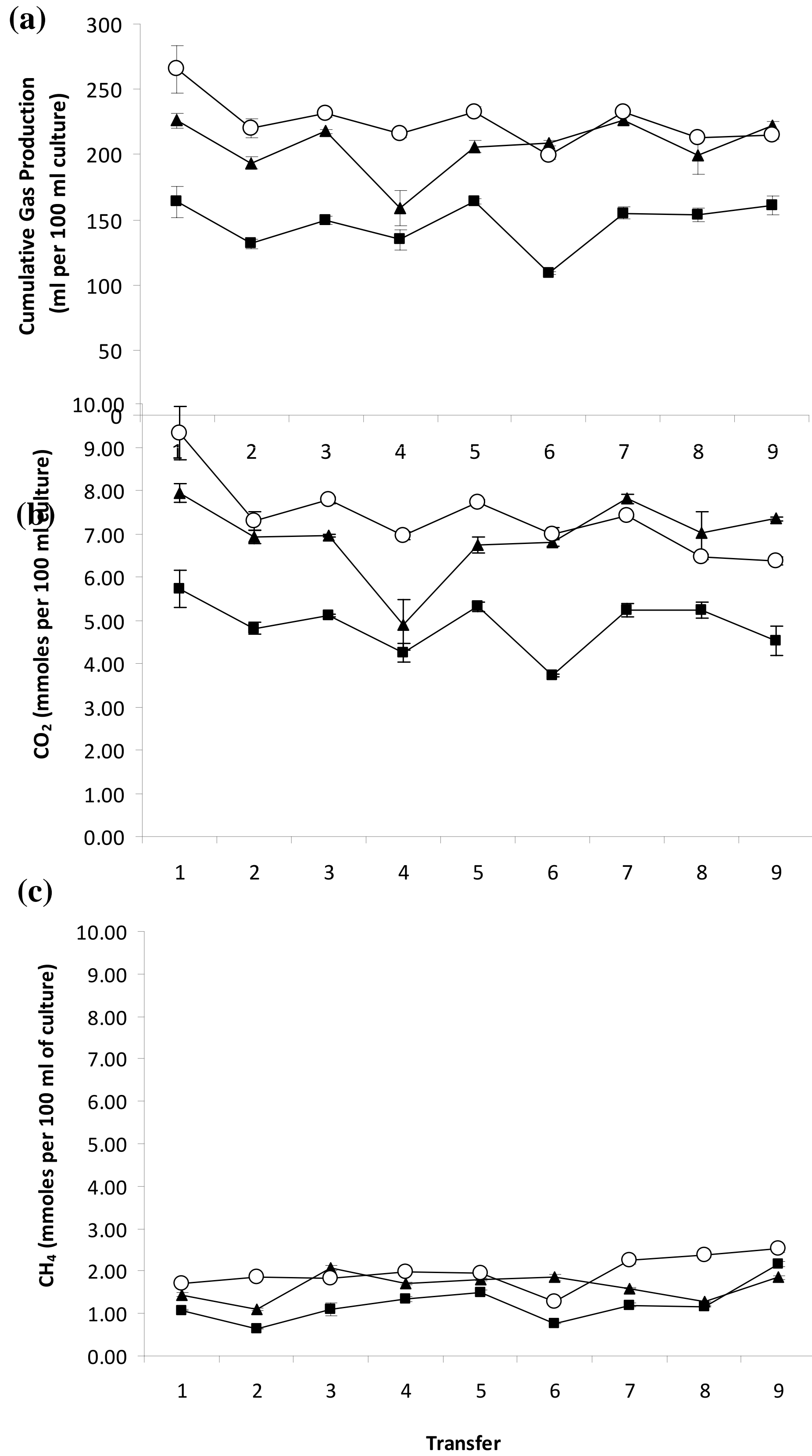

Figure 4

Fig. 4.