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Effect of biochar produced from different biomass sources and at different process temperatures on methane production and ammonia concentrations *in vitro*

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I

1 ABSTRACT

2 The effects of different biochars on *in vitro* rumen gas production and fermentation
3 characteristics were investigated using a two (biochar inclusion level, 10 and 100 g biochar
4 /kg substrate) x two (process temperature, 550 or 700 °C) x five (biomass source, *Miscanthus*
5 straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw) factorial design. The
6 amount of biochar included in incubations had no effect on *in vitro* fermentation. Overall,
7 inclusion of biochar reduced total gas production to 0.96 (P<0.001) and methane (CH₄)
8 production to 0.95 (P<0.001) of that in control (no added biochar) incubations. There were no
9 differences in gas or CH₄ production between the biomass sources used to produce biochar
10 but total gas (P=0.058) and CH₄ (P=0.010) production were slightly greater when biochar
11 was produced at 700 rather 550 °C. Addition of biochar to incubations did not change total
12 amounts of volatile fatty acids (VFA) or acetic acid produced during *in vitro* fermentation;
13 however, the amounts of propionate (0.94; P<0.001) and butyrate (0.96; P=0.021) were
14 reduced when biochar was added to incubations. Process temperature had no effect on VFA
15 produced; however, total VFA and the amounts of acetic and butyric acids produced were
16 influenced by biochar biomass source. Ammonia concentrations at the end of incubations
17 were overall 0.84 of control concentrations (P<0.001) when biochar was added. Both process
18 temperature and biochar biomass source influenced ammonia concentrations which were
19 greater for biochar produced at 700 than 550 °C; concentrations were lowest for biochar
20 produced from *Miscanthus* straw and greatest for rice husk with oil seed rape straw, soft
21 wood pellets and wheat straw intermediate. Adding biochars with a range of compositions to
22 *in vitro* assays produced only small reductions in CH₄ production. However, the absence of
23 any negative effects of biochar coupled with the observed reduction in ammonia
24 concentrations makes it possible that including biochar in livestock feed could be a practical
25 means of applying biochar to pasture and soil.

26 Keywords: biochar; process temperature; biomass source; *in vitro* methane production;
27 ammonia concentration

28 **1. Introduction**

29 Methane (CH₄) emissions arising from the enteric fermentation of feed by ruminant
30 livestock is an important contributor to global greenhouse gas emissions. For example, in
31 2014 in the United Kingdom (Department of Energy and Climate Change, 2016), enteric CH₄
32 emissions were estimated to account for 23.8 Mt carbon dioxide equivalents or 48% of total
33 greenhouse gas emissions from the agriculture sector. There is therefore a need to develop
34 strategies to mitigate CH₄ emissions and Hristov et al. (2013) distinguished those that directly
35 address enteric fermentation; that focus on manure management and those that target animal
36 husbandry (where animal husbandry includes genetics, health and fertility).

37 Manufacture and use of biochar has the potential to mitigate the impact of agriculture
38 on greenhouse gas emissions in various ways. Biochar is the solid, carbon-rich product of
39 biomass pyrolysis; the controlled heating of biomass at high temperature with deliberate
40 exclusion of oxygen. Converting degradable biomass into recalcitrant biochar before adding
41 it to soil demonstrably sequesters carbon into the land. It has also been found to suppress soil-
42 based emissions of CH₄ and nitrous oxide (Sohi et al., 2010; Gurwick et al., 2013). The
43 reason for the latter effects remain uncertain, but probably involve the general porosity of
44 biochar and the large negative charge gradually developed across its large surface area
45 (Kammann et al. 2017).

46

47 The inclusion of biochar in ruminant diets has been investigated for two reasons.
48 First, biochar may reduce enteric CH₄ emissions (Leng et al., 2012; Hansen et al. 2012;
49 Calvelo Pereira et al. 2014) and secondly, faecal excretion of dietary biochar may provide an

50 effective means of transferring biochar into slurry or on to pasture (Calvelo Pereira et al.
51 2014; Joseph et al. 2015). Responses to the inclusion of biochar in rumen *in vitro* assays have
52 been variable ranging from no effect (Calvelo Pereira et al. 2014) to a 13% reduction (Leng
53 et al. 2012). As the properties of biochar are dependant on both the temperature of pyrolysis
54 and the biomass source from which it was prepared, such variation is not surprising. The
55 objective of the current work was therefore to determine whether biochar suppressed CH₄
56 production *in vitro* and by using a range of biochars with defined chemical and physical
57 compositions to investigate the attributes of biochar responsible for suppressing CH₄ *in vitro*.

58 **2. Material and methods**

59 This experiment was conducted at Scotland's Rural College (SRUC) Beef and Sheep
60 Research Centre in Edinburgh in 2013. The experimental protocol was approved by SRUC's
61 Animal Welfare and Ethical Review Body, the Animal Experiments Committee and was
62 conducted in accordance with the requirements of the UK Animals (Scientific Procedures)
63 Act, 1986.

64 *2.1. Biochar*

65 The experiment used 10 standard biochars provided by the UK Biochar Research
66 Centre, University of Edinburgh. These were manufactured in a scalable 20 minute process
67 optimised for research use, comprising a rotating kiln heated indirectly and electrically. The
68 biochars differ by their source biomass (five granular biomass sources: rice husks and
69 pelleted *Miscanthus* straw, oil seed rape straw, wheat straw or soft wood) and peak
70 processing temperature (550 or 700 °C). A summary of biochar composition is given in Table
71 1. Full details of biochar production and composition can be found at
72 (http://www.biochar.ac.uk/standard_materials.php; accessed 07/02/2017). To ensure that

73 particle size was small enough for inclusion in assays and to avoid gross differences between
74 biochars, the material used in the assay was the fraction that passed through a 2 mm screen.

75

76 2.2. *Experimental design*

77 A 2 (biochar inclusion) x 2 (process temperature) x 5 (biomass source) factorial
78 design was used where the factors were: biochar addition (10 or 100 g biochar/kg substrate
79 fresh weight); biochar process temperature (550 or 700 °C) and biomass source (*Miscanthus*
80 straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw). Each of the 20
81 individual treatments was incubated in triplicate in each replicate (week of experiment).
82 Within each replicate, control samples which contained substrate but no added biochar and
83 blank samples without substrate or biochar were also included in triplicate giving a total of 66
84 incubations on each replicate. There were a total of four replicates of the above carried out at
85 14 day intervals.

86 2.3. *Rumen fluid inocula*

87 To provide biological variation in rumen inocula, rumen samples were obtained from
88 a group of cross-bred beef cattle (approximately 16 months in age) fed *ad libitum* a diet
89 consisting of 500 g forage and 500 g concentrate /kg dry matter (DM). Steers were fed once
90 daily and rumen samples were obtained at approximately 08.00 h before fresh feed was
91 offered. Rumen samples were obtained using a stomach tube (16 × 2700 mm) introduced into
92 the oesophagus via a nostril and then passed down to the rumen. Samples were immediately
93 strained through two layers of muslin and transported in insulated flasks under anaerobic
94 conditions to the laboratory and used as inocula within one hour of collection. For each
95 replicate of the experiment, three different rumen inocula were prepared. Where possible

96 each inoculum (total volume 300 mL) was derived from an individual animal. On two
97 occasions, sample volume from an individual animal was inadequate and therefore, a
98 composite sample was produced by mixing samples from two animals. No animal contributed
99 rumen fluid on more than one occasion. Each of the triplicate incubations noted above
100 therefore contained three different rumen fluid inocula: that is of the 66 incubations per
101 replicate, 22 each contained rumen fluid from a different rumen fluid inoculum. Thus 12
102 different inocula were used in total for the four replicates.

103 *2.4. In vitro gas production*

104 Incubations took place in 160 mL serum bottles which contained 400 mg feed
105 substrate (343 mg DM) and biochar (4 or 40 mg) as appropriate. The feed substrate consisted
106 of a mixture (g/kg fresh weight) of hay (500), barley (400) and rapeseed meal (100) ground to
107 pass through a 2 mm screen. **The feed substrate was chosen to have a similar forage to
108 concentrate ratio and nutrient profile to the diet of the cattle used as rumen fluid donors and
109 thus was also typical of diets fed to finishing beef cattle.** Feed substrate was analysed for
110 DM, crude protein, acid hydrolysed ether extract and neutral cellulose and gamanase
111 digestibility according to Ministry of Agriculture Fisheries and Food (1992). Chemical
112 composition was: DM, 857 g/kg and (g/kg DM); crude protein, 105; acid hydrolysed ether
113 extract, 19; neutral cellulase plus gamanase digestibility, 760 and estimated metabolisable
114 energy, 11.2 MJ/kg DM.

115 The rumen fluid was mixed with buffer-mineral solution, prepared as described by
116 Menke et al. (1979) at a ratio 1:3 (v/v), rumen fluid: buffer. Rumen fluid: buffer mixture (40
117 mL / bottle) was dispensed under a stream of carbon dioxide, and the bottles were closed with
118 a butyl rubber stopper and placed in a water bath at 39 °C for 24 h. Contents were thoroughly
119 mixed periodically throughout the 24 h.

120 *2.5. Analytical methods*

121 Cumulative gas production during the 24 h incubation was measured by pressure
122 using a manual pressure transducer (Digitron 2023P, Digitron, Torquay, Devon, UK). The
123 pressure values were converted to the volumes of gas produced using the equation below
124 determined for local laboratory conditions.

125 $V = (P - 11.58) / 7.55$

126 where V = gas volume (mL) and P = pressure (mbar)

127 The gas produced due to fermentation of the feed substrate was corrected for gas
128 produced in appropriate blank incubations. After measurement of pressure, 20 mL gas
129 samples were transferred in duplicate to evacuated head-space vials and CH₄ was analysed by
130 gas chromatography (Agilent 7890, Agilent Technologies, Cheshire, UK) using a HayeSep Q
131 (80/100), 0.25m x 1mm internal diameter column with helium as carrier gas and detection by
132 flame ionisation using authenticated standards. At the end of the incubation, the bottles were
133 uncapped and pH measured immediately. Samples for volatile fatty acid (VFA) analysis (5
134 mL) were de-proteinized by adding 1 mL metaphosphoric acid (215 g/L) and 0.5 mL
135 methylvaleric acid (10 g/L) as an internal standard. These samples were stored at -20 °C
136 between collection and analysis. VFA concentrations were determined by HPLC as described
137 in Rooke et al. (1990). Samples for analysis for ammonia (NH₃-N) were diluted 1:1 (v/v)
138 with 1M-HCl and analysed using the phenol-hypochlorite method of Weatherburn (1967)
139 adapted for 96 well plates with absorbance measured at 625 nm.

140 *2.6. Calculations and statistical analyses*

141 Amounts of total gas, CH₄ and VFA produced were corrected for amounts produced
142 in blank incubations and expressed either as the total amount produced or per g substrate DM

143 incubated. To assess the overall effect of biochar inclusion, values were expressed as a
144 proportion of the control value for each of the 12 rumen fluid inocula and a single sample t-
145 test used to determine if the overall mean value differed from one (control value). Differences
146 between biochar treatments were analysed according to a factorial design using the Linear
147 Mixed Models procedure of GenStat (version 11.1 for Windows; VSN International Limited).
148 The model included the fixed effects of biochar inclusion, process temperature and biomass
149 source and their interactions. The different replicates and rumen fluid inocula (within
150 replicate) were included as random factors. Where significant differences ($P < 0.05$) were
151 detected between biomass sources, differences between means were identified using least
152 significant differences.

153

154 **3. Results**

155 *3.1. Rumen inocula*

156 Using rumen fluid inocula obtained from different animals (Table 2) to inoculate the
157 *in vitro* incubations achieved the objective of producing fermentations differing ($P < 0.001$)
158 not only in the extent (amounts of total gas, CH_4 and VFA produced) but also in the type of
159 fermentation (VFA molar proportions and $\text{NH}_3\text{-N}$ concentration).

160 *3.2. Gas and CH_4 production*

161 The amount of biochar included in incubations had no effect on *in vitro* fermentation
162 and there were also no interactions between the amount of biochar included in assays, process
163 temperature used to produce biochar or the source of biochar. Therefore Tables 3 and 4 report
164 only the main effects of biochar biomass source and process temperature. Overall, inclusion
165 of biochar in assays reduced total gas production to 0.96 (SEM 0.003, $P < 0.001$) and CH_4

166 production to 0.95 (SEM. 0.008, $P < 0.001$) of that in control (no added biochar) incubations.
167 There were no differences (Table 3) in gas or CH_4 production between the biomass sources
168 used to produce biochar but total gas ($P=0.058$) and CH_4 ($P=0.010$) production were slightly
169 greater when biochar was produced at 700 rather than 550 °C. When expressed as a ratio of
170 total gas production, CH_4 produced /mL total gas was 0.98 (SEM 0.040, $P=0.021$) of that in
171 control (no added biochar) incubations and lower for biochar produced at 550 than 700 °C
172 ($P=0.003$). However there were no differences between biomass sources.

173 3.3 VFA production

174 Overall addition of biochar to incubations did not change total amounts of VFA or
175 acetic acid produced during *in vitro* fermentation; however, the amounts of propionate (0.94;
176 SEM 0.011, $P < 0.001$) and butyrate (0.96; SEM 0.015, $P=0.021$) were reduced when biochar
177 was added to incubations. Process temperature had no effect on VFA produced during *in*
178 *vitro* incubations (Table 4). However, total VFA and the amounts of acetic and butyric acids
179 produced were influenced by biochar biomass source with extent of production ranked;
180 lowest for *Miscanthus* straw and highest for rice husks with oilseed rape straw; wheat straw;
181 soft wood pellets intermediate.

182 3.4. $\text{NH}_3\text{-N}$ and pH

183 $\text{NH}_3\text{-N}$ concentrations at the end of incubation were overall reduced to 0.84 of control
184 concentrations (SEM 0.022, $P < 0.001$) by addition of biochar. Both process temperature and
185 biochar biomass source influenced $\text{NH}_3\text{-N}$ concentrations (Table 4). Concentrations were
186 greater for biochar produced at 700 than 550 °C. $\text{NH}_3\text{-N}$ concentrations for biomass sources
187 were lowest for *Miscanthus* straw and greatest for rice husk with oil seed rape straw, soft
188 wood pellets and wheat straw intermediate (in order of ascending concentration). Although

189 (Table 4) there were significant differences in final incubation pH between treatments, these
190 differences were small with mean values ranging from 6.51 to 6.55.

191

192 **4. Discussion**

193 Including biochar in *in vitro* rumen fluid incubations reduced total gas, VFA and CH₄
194 production to a limited extent and NH₃-N concentrations to a greater extent.

195 *4.1 Source of rumen fluid*

196 When using the *in vitro* gas production technique, it is usually recommended that the
197 most consistent results are obtained by taking rumen samples after an overnight fast and by
198 combining rumen fluid from a minimum of three animals (Williams 1990). However, such an
199 approach precludes assessment of variation between animals in response and in the current
200 experiment, animal to animal variation was specifically addressed by using 12 different
201 sources of rumen fluid (in most cases from individual animals) in the incubations. Despite the
202 fact that there was substantial animal to animal variation, estimated to be four (gas produced /
203 g substrate DM) to ten (CH₄ produced / g substrate DM) times greater than the variation
204 associated with the biochar treatments imposed, the overall effects of and differences
205 between biochar types were successfully captured. It should be noted that at least some of the
206 animal to animal variation will be related to when feed was last consumed and nutritional
207 quality of feed, as although fresh feed was last offered 24h before rumen samples were
208 obtained, patterns of feed intake would undoubtedly have differed from animal to animal and
209 the nutritional quality of feed from week to week and thus could have led to differences in the
210 chemical and microbiome composition of different inocula.

211 *4.2 Effects of biochar on fermentation*

212 Biochar reduced the overall extent of fermentation (gas production) to 0.96 and CH₄
213 emissions to 0.95 of control values. More importantly the ratio of CH₄ to total gas in samples
214 to which biochar had been added was 0.98 of control values. Therefore biochar caused only a
215 small reduction in CH₄ production. In previous *in vitro* studies, variable results have been
216 obtained. At one extreme, Leng et al. (2012) reported a significant reduction in CH₄
217 production to 0.86 of control with no depression in total gas production while Calvelo Pereira
218 et al. (2014) found no overall effect of biochar addition on either gas or CH₄ production.
219 Hansen et al. (2012) reported non-significant reductions by three different biochars of 0.89 to
220 0.92 (of control) for total gas and 0.84 to 0.86 for CH₄ production. Overall CH₄ production
221 when expressed as proportion of total gas production ranged from 0.86 of control values
222 (Leng et al. 2012) to 1.00 (Calvelo Pereira et al. 2014) with Hansen et al. (2012), 0.91 and the
223 present study (0.98) intermediate. Since the biochars used in the above studies were produced
224 from different biomass sources and at different temperatures and little (pH, surface area, C, N
225 and ash; Calvelo Pereira et al., 2014) or no detail (Hansen et al., 2012, Leng et al., 2012)
226 reported on biochar composition, then the range of results is not surprising and difficult to
227 analyse. For example, Leng et al. (2012) prepared biochar at 900 °C whereas Calvelo Pereira
228 et al. (2014) reported no differences between biochars prepared at 350 or 550 °C; in the
229 present study, the reduction in CH₄ was greater with biochar prepared at 550 than 700 °C.
230 Kammann et al. (2017) have suggested that the high pyrolysis temperature used by Leng et
231 al. (2012) could explain why a reduction in CH₄ production was observed. However, the
232 effects of biochar *in vitro* may not be realised *in vivo* because although batch *in vitro* systems,
233 as used in this experiment, are valuable screening tools, they do not reproduce the complex
234 situation *in vivo*; in particular the ability of the rumen microbiome to adapt to novel materials
235 cannot be reproduced and many novel compounds effective *in vitro* are not effective *in vivo*
236 (Hristov et al., 2013).

237 The current study investigated the effects of biochar biomass source and process
238 temperature on *in vitro* fermentation. Overall, the differences between biochars were small
239 but there were consistent effects of biomass source. Biochar prepared from *Miscanthus*
240 reduced gas, CH₄ and VFA production to the greatest extent and biochar prepared from rice
241 husk and soft wood pellets were least effective. In other studies, Hansen et al. (2012) found
242 that straw-derived biochar numerically reduced CH₄ to a greater extent than wood-derived
243 biochar but Calvelo Pereira et al. (2014) reported no difference between wood and crop
244 residue-derived biochars. In addition, no significant correlations were found between mean
245 values for the 10 different biochars for gas and CH₄ production and the compositional
246 information from Table 1. Thus there was no clear evidence for relationships between biochar
247 composition and *in vitro* activity from this or other studies investigating biochar as a means
248 of reducing CH₄; this is possibly not surprising as the same conclusion has been drawn from
249 meta-analysis of the more widely studied area of biochar as a greenhouse gas mitigation
250 strategy when applied to soils (Gurwick et al. 2013).

251 In the light of the above it is difficult to comment on mechanisms by which biochar
252 could reduce CH₄. In the soil and composting environments, the balance between
253 methanogenic archaea and methanotrophic organisms was altered favourably towards
254 methanotrophism rather than methanogenesis with biochar application (Feng et al., 2012;
255 Sonoki et al 2013) but this is unlikely in the anaerobic rumen environment which precludes
256 growth of the aerobic methanotrophs. Other possibilities suggested have included creation of
257 micro-environments by the large surface area of biochar. The absence of any relationship
258 between biochar surface area and CH₄ production in the current study is probably related to two
259 factors. First, the small differences in CH₄ production between biochars made it difficult to
260 discriminate between surface areas of different biochars. Secondly, gross surface area (m²/g,

261 Table 1) may not adequately describe the optimum micro-environment for colonisation of
262 biochar by rumen microbes as this may require description of structure not only at the μm
263 scale but also to account for distribution of charged particles (Leng 2014).

264 4.3 Effects of biochar on $\text{NH}_3\text{-N}$ concentrations

265 Unexpectedly $\text{NH}_3\text{-N}$ concentrations after 24 h incubation were reduced when biochar was
266 added to incubations. The reduction was most marked in *Miscanthus*-derived biochar (0.58 of
267 control) and biochar prepared at 550 °C had a greater effect than preparation at 700 °C. Since
268 the *in vitro* incubation is a sealed system, there are two possible reasons for this difference.
269 First, the differences in $\text{NH}_3\text{-N}$ concentrations could be due to a reduction in proteolysis and
270 deamination of nitrogenous constituents of the feed substrate, increased incorporation of
271 $\text{NH}_3\text{-N}$ into microbial protein or a combination of these two processes. As the differences
272 between treatments in energy supply for microbial growth (gas or VFA production) were
273 small then a reduction in proteolysis / deamination seems more likely but as no direct
274 measurements of these processes were made, then this is speculative. Only Calvelo Pereira et
275 al (2014) also reported $\text{NH}_3\text{-N}$ concentrations *in vitro* with biochar addition but these authors
276 found no differences between treatments. Secondly, in the soil environment, biochar has in
277 some studies reduced leaching of $\text{NH}_3\text{-N}$ (Ding et al. 2010). This has been attributed to the
278 cation exchange capacity of the negatively charged biochar and indeed in laboratory studies,
279 $\text{NH}_3\text{-N}$ is adsorbed (Gai et al. 2014; Winning 2014). In these laboratory studies, the efficacy
280 of biochars in adsorbing $\text{NH}_3\text{-N}$ was inversely related to the temperature at which biochar
281 was produced (increased pyrolysis temperature reduces cation exchange capacity) and to the
282 influence of biomass source on cation adsorption. In the present experiment, $\text{NH}_3\text{-N}$
283 concentrations were lower when biochar was produced at 550 rather than 700 °C was
284 included in the assay, which is consistent with the laboratory studies. Thus, an alternative

285 explanation for the effect of biochar on NH₃-N concentrations is that NH₃-N was adsorbed
286 by biochar. In the *in vivo* situation, binding of NH₃-N by biochar may be beneficial as any
287 NH₃-N bound when NH₃-N concentrations are high immediately after feeding would be
288 released when NH₃-N concentrations declined and therefore would improve synchrony
289 between the supply of NH₃-N and energy (from degraded carbohydrates) for rumen
290 microbial protein synthesis.

291 *4.4 Feeding biochar as a means of reducing greenhouse gas emissions from soil and pasture*

292 Including biochar in animal feed as a means of applying biochar to soil and pasture
293 has been suggested (Calvelo Pereira et al. 2014; Joseph et al. 2015) either directly through
294 grazing and defaecation or more likely through the application of animal waste to land, as is
295 standard practice in livestock farming systems. Incorporating biochar into ensiled grass had
296 no adverse effects on the resulting silage (Calvelo Pereira et al. 2014) and Joseph et al. (2015)
297 reported little change in the recalcitrant carbon structure of biochar as it passed through the
298 gut of cattle. Adding biochar up to 100g /kg feed substrate in the current experiment did not
299 adversely effect rumen fermentation. Although direct mitigating effects on *in vitro* CH₄
300 production were small this suggests that biochar is inert in terms of digestion and using the
301 animal to apply biochar to pasture is possible. However, one factor limiting the use of biochar
302 is that it is inert and therefore including biochar in feed will dilute the energy content of feed
303 and therefore may reduce energy supply to the animal if it cannot increase feed intake to
304 compensate. On the other hand, the reductions in *in vitro* NH₃-N concentration observed with
305 biochar may change the balance of nitrogenous constituents in animal excreta as less NH₃-N
306 may be absorbed from the digestive tract and excreted as urea in urine, thus decreasing the
307 soluble nitrogenous constituents of manure or slurry. If NH₃-N is excreted bound to biochar

308 it may then contribute to improved N retention in soils as well as and lower emission of
309 ammonia to air.

310 **5. Conclusions**

311 Adding biochars with a range of compositions to *in vitro* rumen assays produced
312 small reductions in CH₄ production which may not be reproduced *in vivo*. However, the
313 absence of any negative effects of biochar coupled with the observed reduction in NH₃-N
314 concentrations makes it possible that feeding biochar to livestock could be a means of
315 applying biochar to pasture and soil.

316 **Conflict of interest**

317 There is no conflict of interest.

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384

385 **Table 1**

386 Composition of biochars manufactured at different peak temperatures and prepared from different biomass sources (g/kg DM unless stated otherwise)

Source	<i>Miscanthus</i> straw pellets		Oil seed rape straw pellets		Rice husks		Soft wood pellets		Wheat straw pellets	
	550	700	550	700	550	700	550	700	550	700
Dry matter (g/kg)	982	988	974	964	985	985	985	990	986	978
Ash	122	116	195	219	479	473	125	189	213	238
pH	9.77	9.72	9.78	10.40	9.71	9.81	7.91	8.44	9.94	10.00
C	754	792	689	677	487	473	855	902	683	690
H	24	13	18	11	12	6	28	18	21	12
O	92	70	89	78	25	21	104	60	69	53
N	8	10	16	13	10	9	<1	<1	14	13
P	2	8	3	3	1	5	<1	<1	1	3
K	10	26	29	30	4	6	2	3	16	15
Surface area (m ² /g)	34	37	16	22	20	42	26	162	26	23
Volatile matter	116	77	164	252	75	50	142	67	106	74

387 Data reproduced from UK Biochar Research Centre (http://www.biochar.ac.uk/standard_materials.php; accessed 07/02/2017).

388

389 **Table 2**390 Fermentation characteristics of different sources of rumen fluid inocula (n=12) used in *in vitro* incubations.

	Mean	SD	Minimum	Maximum
Gas produced (ml/g substrate DM)	267	35.9	212	317
CH ₄ produced (ml/g substrate DM)	35	6.1	27	44
Volatile fatty acids produced (mmol/g substrate DM)	1.62	0.373	0.95	2.38
Molar proportions (mmol/mol)				
Acetate	567	25.3	527	599
Propionate	250	36.0	213	312
Butyrate	136	21.2	107	175
Ammonia (mmol/g substrate DM)	1.89	0.437	1.11	2.58

391

392 Table 3

393 Gas and CH₄ production in the absence (control) or presence of biochar prepared from different biomass sources and at different temperatures.

Treatment	Control	Significance‡	Biomass source					SED	Significance‡	
			<i>Miscanthus</i> straw pellets	Oil seed rape straw pellets	Rice husks	Soft wood pellets	Wheat straw pellets		Substrate	Temperature
Gas (ml/g DM)										
550 ⁰	267	P<0.001	252	255	255	256	255	2.6	P=0.11	P=0.058
700 ⁰			254	255	262	258	257			
CH ₄ (ml /g DM)										
550 ⁰	35.2	P<0.001	32.1	33.3	33.5	33.4	33.1	0.75	P=0.055	P=0.010
700 ⁰			33.3	33.5	35.0	34.3	33.7			
CH ₄ / gas ratio										
550 ⁰	0.159	P=0.021	0.151	0.155	0.152	0.154	0.152	0.0034	P=0.55	P=0.003
700 ⁰			0.155	0.156	0.162	0.158	0.157			

394 ‡Significance for control is for mean effect of biochar calculated by expressing individual values as a proportion of control for each rumen fluid
 395 source. For differences between substrate and temperature, there were no interactions between substrate and temperature, nor were there any effects of
 396 amount of biochar included in assay.

397 **Table 4**

398 Volatile fatty acid (VFA) production, ammonia (NH₃-N) concentrations and pH after incubation in the absence (control) or presence of biochar
 399 prepared from different substrates and at different temperatures.

Treatment	Control	Significance‡	Biomass source					SED	Significance‡	
			<i>Miscanthus</i> straw pellets	Oil seed rape straw pellets	Rice husk	Soft wood pellets	Wheat straw pellets		Substrate	Temperature
Total VFA (mmol/g DM)										
550 ⁰	4.86	P=0.15	4.38 ^a	4.58 ^a	4.52 ^{bc}	4.61 ^{bc}	4.68 ^{ab}	0.166	P=0.018	P=0.28
700 ⁰			4.51	4.31	4.96	4.83	4.57			
Acetate (mmol /g DM)										
550 ⁰	2.72	P=0.70	2.46	2.58	2.54	2.58	2.64	0.103	P=0.051	P=0.23
700 ⁰			2.54	2.43	2.81	2.73	2.57			
Propionate (mmol /g DM)										
550 ⁰	1.45	P<0.001	1.31 ^a	1.34 ^{ab}	1.31 ^b	1.33 ^{ab}	1.36 ^{ab}	0.043	P=0.025	P=0.53
700 ⁰			1.32	1.27	1.42	1.38	1.34			
Butyrate (mmol /g DM)										
550 ⁰	0.58	P=0.021	0.49 ^a	0.53 ^{ab}	0.54 ^c	0.56 ^c	0.55 ^{bc}	0.023	P<0.001	P=0.071
700 ⁰			0.54	0.51	0.59	0.59	0.54			
NH ₃ -N (mmol/g DM)										
550 ⁰	1.89	P<0.001	1.03 ^a	1.32 ^b	1.58 ^b	1.51 ^b	1.45 ^b	0.072	P<0.001	P=0.007
700 ⁰			1.16	1.55	1.56	1.48	1.58			
pH										
550 ⁰	6.54	P<0.001	6.51	6.52	6.54	6.53	6.54	0.011	P<0.001	P=0.52
700 ⁰			6.51	6.52	6.52	6.54	6.55			

400 ‡Significance for control is for mean effect of biochar calculated by expressing individual values as a proportion of control for each rumen fluid
 401 source. For differences between substrates and temperature; there were no interactions between substrate and temperature, nor were there any effects
 402 of amount of biochar included in assay. . Means with different superscripts are different (P<0.05).