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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 (CH<sub>4</sub>)  
8 production to 0.95 (P<0.001) of that in control (no added biochar) incubations. There were no  
9 differences in gas or CH<sub>4</sub> production between the biomass sources used to produce biochar  
10 but total gas (P=0.058) and CH<sub>4</sub> (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 CH<sub>4</sub> 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 (CH<sub>4</sub>) 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 CH<sub>4</sub>  
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 CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub>  
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 CH<sub>4</sub> *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](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 CH<sub>4</sub> 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 (NH<sub>3</sub>-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, CH<sub>4</sub> 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,  $\text{CH}_4$  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 $\text{CH}_4$ 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  $\text{CH}_4$

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  $\text{CH}_4$  production between the biomass sources  
168 used to produce biochar but total gas ( $P = 0.058$ ) and  $\text{CH}_4$  ( $P = 0.010$ ) 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,  $\text{CH}_4$  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 CH<sub>4</sub>  
194 production to a limited extent and NH<sub>3</sub>-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 (CH<sub>4</sub> 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 CH<sub>4</sub>  
213 emissions to 0.95 of control values. More importantly the ratio of CH<sub>4</sub> 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 CH<sub>4</sub> production. In previous *in vitro* studies, variable results have been  
216 obtained. At one extreme, Leng et al. (2012) reported a significant reduction in CH<sub>4</sub>  
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 CH<sub>4</sub> 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 CH<sub>4</sub> production. Overall CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> 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, CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub>; 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 CH<sub>4</sub>. 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 CH<sub>4</sub> production in the current study is probably related to two  
259 factors. First, the small differences in CH<sub>4</sub> production between biochars made it difficult to  
260 discriminate between surface areas of different biochars. Secondly, gross surface area (m<sup>2</sup>/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  $\mu\text{m}$   
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 NH<sub>3</sub>-N concentrations is that NH<sub>3</sub>-N was adsorbed  
286 by biochar. In the *in vivo* situation, binding of NH<sub>3</sub>-N by biochar may be beneficial as any  
287 NH<sub>3</sub>-N bound when NH<sub>3</sub>-N concentrations are high immediately after feeding would be  
288 released when NH<sub>3</sub>-N concentrations declined and therefore would improve synchrony  
289 between the supply of NH<sub>3</sub>-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* CH<sub>4</sub>  
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* NH<sub>3</sub>-N concentration observed with  
305 biochar may change the balance of nitrogenous constituents in animal excreta as less NH<sub>3</sub>-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 NH<sub>3</sub>-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 CH<sub>4</sub> production which may not be reproduced *in vivo*. However, the  
313 absence of any negative effects of biochar coupled with the observed reduction in NH<sub>3</sub>-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 <sup>2</sup> /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](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 <sub>4</sub> 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 CH<sub>4</sub> 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 <sup>0</sup>	267	<b>P&lt;0.001</b>	252	255	255	256	255	2.6	<b>P=0.11</b>	<b>P=0.058</b>
700 <sup>0</sup>			254	255	262	258	257			
CH <sub>4</sub> (ml /g DM)										
550 <sup>0</sup>	35.2	<b>P&lt;0.001</b>	32.1	33.3	33.5	33.4	33.1	0.75	<b>P=0.055</b>	<b>P=0.010</b>
700 <sup>0</sup>			33.3	33.5	35.0	34.3	33.7			
CH <sub>4</sub> / gas ratio										
550 <sup>0</sup>	0.159	<b>P=0.021</b>	0.151	0.155	0.152	0.154	0.152	0.0034	<b>P=0.55</b>	<b>P=0.003</b>
700 <sup>0</sup>			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 (NH<sub>3</sub>-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 <sup>0</sup>	4.86	<b>P=0.15</b>	4.38 <sup>a</sup>	4.58 <sup>a</sup>	4.52 <sup>bc</sup>	4.61 <sup>bc</sup>	4.68 <sup>ab</sup>	0.166	<b>P=0.018</b>	<b>P=0.28</b>
700 <sup>0</sup>			4.51	4.31	4.96	4.83	4.57			
Acetate (mmol /g DM)										
550 <sup>0</sup>	2.72	<b>P=0.70</b>	2.46	2.58	2.54	2.58	2.64	0.103	<b>P=0.051</b>	<b>P=0.23</b>
700 <sup>0</sup>			2.54	2.43	2.81	2.73	2.57			
Propionate (mmol /g DM)										
550 <sup>0</sup>	1.45	<b>P&lt;0.001</b>	1.31 <sup>a</sup>	1.34 <sup>ab</sup>	1.31 <sup>b</sup>	1.33 <sup>ab</sup>	1.36 <sup>ab</sup>	0.043	<b>P=0.025</b>	<b>P=0.53</b>
700 <sup>0</sup>			1.32	1.27	1.42	1.38	1.34			
Butyrate (mmol /g DM)										
550 <sup>0</sup>	0.58	<b>P=0.021</b>	0.49 <sup>a</sup>	0.53 <sup>ab</sup>	0.54 <sup>c</sup>	0.56 <sup>c</sup>	0.55 <sup>bc</sup>	0.023	<b>P&lt;0.001</b>	<b>P=0.071</b>
700 <sup>0</sup>			0.54	0.51	0.59	0.59	0.54			
NH <sub>3</sub> -N (mmol/g DM)										
550 <sup>0</sup>	1.89	<b>P&lt;0.001</b>	1.03 <sup>a</sup>	1.32 <sup>b</sup>	1.58 <sup>b</sup>	1.51 <sup>b</sup>	1.45 <sup>b</sup>	0.072	<b>P&lt;0.001</b>	<b>P=0.007</b>
700 <sup>0</sup>			1.16	1.55	1.56	1.48	1.58			
pH										
550 <sup>0</sup>	6.54	<b>P&lt;0.001</b>	6.51	6.52	6.54	6.53	6.54	0.011	<b>P&lt;0.001</b>	<b>P=0.52</b>
700 <sup>0</sup>			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).