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# Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype

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1 **Abstract**

2 This study determined hydrogen emissions by beef cattle under different dietary  
3 conditions and how cattle genotype and rumen microbial community affected  
4 emissions. Thirty-six Aberdeen Angus- (AAx) and thirty six Limousin-sired (LIMx)  
5 steers received two diets with forage:concentrate ratios (DM basis) of either 8:92  
6 (Concentrate) and 52:48 (Mixed). Eighteen animals of each genotype received each  
7 diet. Methane and H<sub>2</sub> emissions were measured individually in indirect respiration  
8 chambers. Hydrogen emissions (mmol/min) varied greatly throughout the day, being  
9 highest after feeding, and averaged about 0.10 mol H<sub>2</sub>/mol CH<sub>4</sub>. Hydrogen emissions  
10 were higher (mol/kg DM intake) with the Mixed diet. Methane emissions (mol/d and  
11 mol/kg DM intake) were higher from steers receiving the Mixed diet ( $P < 0.001$ );  
12 AAx steers produced more CH<sub>4</sub> on a daily (mol/d  $P < 0.05$ ) but not on a DM intake  
13 (mol/kg DM intake) basis. Archaea ( $P = 0.002$ ) and protozoa ( $P < 0.001$ ) were more  
14 and total bacteria ( $P < 0.001$ ) less abundant ( $P < 0.001$ ) in the Mixed diet. Relative  
15 abundance of *Clostridium* Cluster IV was greater ( $P < 0.001$ ) and Cluster XIVa ( $P$   
16 =0.025) less on the Mixed diet. Relative abundance of *Bacteroides* plus *Prevotella*  
17 was greater ( $P = 0.018$ ) and *Clostridium* Cluster IV less ( $P = 0.031$ ) in LIMx steers.  
18 There were no significant relationships between H<sub>2</sub> emissions and microbial copy  
19 number. It was concluded that the rate of H<sub>2</sub> production immediately after feeding  
20 may lead to transient overloading of methanogenic archaea capacity to use H<sub>2</sub>,  
21 resulting in peaks in H<sub>2</sub> emissions from beef cattle.  
22

23 Methane is a greenhouse gas with a global warming potential 25-fold that of CO<sub>2</sub><sup>(1)</sup>.  
24 Ruminant livestock production through the enteric fermentation of feed contributes  
25 significantly to greenhouse gas production by agriculture; in the United Kingdom,  
26 CH<sub>4</sub> accounted for 37% of all agricultural emissions in 2005<sup>(2)</sup>. Enteric production of  
27 CH<sub>4</sub> also represents a loss of energy (from 2 to 12% of gross energy (GE) intake)<sup>(3)</sup>,  
28 which might otherwise be available for growth or milk production. Understanding the  
29 mechanisms of methanogenesis and the microorganisms involved is important for  
30 devising sustainable mitigation strategies to lower the environmental impact of  
31 ruminant livestock production.

32 Molecular H<sub>2</sub> plays an important role in intermediary metabolism in the  
33 rumen<sup>(4)</sup>. Hydrogen is formed by bacteria, protozoa and fungi from the fermentation  
34 of carbohydrate. Hydrogen and CO<sub>2</sub> are the principal substrates for methane  
35 formation by archaea<sup>(5,6)</sup>. Hydrogen is also a vital intermediate or substrate in other  
36 reactions. Ruminal interspecies H<sub>2</sub> transfer is a process that affects the metabolism of  
37 both the microbes that produce H<sub>2</sub> and those that utilise it<sup>(7)</sup>. Methanogenic archaea  
38 require some accumulation of H<sub>2</sub> to grow rapidly enough to prevent them washing out  
39 of the rumen<sup>(4)</sup>. On the other hand, the accumulation of H<sub>2</sub> exerts a thermodynamic  
40 inhibitory effect on H<sub>2</sub>-producing organisms and causes the fermentation products of  
41 these and other microbial species to be changed<sup>(7)</sup>. As fibrolytic *Ruminococcus* spp.  
42 are H<sub>2</sub> producers (via acetate formation), their growth and consequently fibre  
43 degradation may be inhibited by H<sub>2</sub> accumulation<sup>(4,7)</sup>. These pure culture studies  
44 indicate that decreasing H<sub>2</sub> concentrations in the rumen would be doubly beneficial in  
45 terms of CH<sub>4</sub> emissions and fibre breakdown.

46 Several studies have measured H<sub>2</sub> concentrations in ruminal digesta, as  
47 reviewed by Janssen<sup>(4)</sup>. Hydrogen concentrations increase *in vitro* after adding feed,  
48 and the concentrations are diet-dependent. Fewer studies have reported H<sub>2</sub> emissions  
49 *in vivo*. In one study<sup>(8)</sup> involving two sheep, it was noted that the animals produced  
50 two-fold different amounts of CH<sub>4</sub>: the sheep with lower CH<sub>4</sub> emissions produced  
51 more H<sub>2</sub>. In another study using sheep, Takenaka et al.<sup>(9)</sup>, concluded that H<sub>2</sub>  
52 emissions were on average 2.1% (vol:vol) of CH<sub>4</sub> emissions based on exhaled gas  
53 concentrations. There were periods of high H<sub>2</sub> emission when H<sub>2</sub> formation occurred  
54 at a faster rate than methanogenesis, particularly when concentrate feeds were  
55 included in the diet. Similar investigations in cattle have to the best of our knowledge  
56 not been published. The aim of the present study was therefore to measure both H<sub>2</sub>

57 and CH<sub>4</sub> emissions from beef steers fed two contrasting finishing diets typical of  
58 production in the United Kingdom: a high concentrate diet based on barley and a  
59 mixed forage:concentrate diet including grass and whole crop barley silages, barley  
60 grain and maize distillers dark grains (similar to maize distillers grains with solubles).

61

## 62 **Materials and Methods**

63 This study was conducted at the Beef Research Centre of SRUC (6 miles south of  
64 Edinburgh, UK) in 2011. The experiment was approved by the Animal Experiment  
65 Committee of SRUC and was conducted in accordance with the requirements of the  
66 UK Animals (Scientific Procedures) Act 1986.

67

### 68 *Animals, experimental design and diets*

69 The seventy two cross-bred steers used were from a rotational cross between purebred  
70 Aberdeen Angus or Limousin sires and crossbred dams of those genotypes and  
71 referred to as AAX and LIMx, respectively. The steers were fed two complete diets  
72 using a forage wagon, consisting (g/kg DM) of either 480 forage: 520 concentrate  
73 (Mixed) or 75 forage: 925 concentrate (Concentrate). The composition of the diets  
74 and nutritional composition of the feeds are given in Tables 1 and 2 respectively.

75 Immediately before the experiment reported here, DM intake (DMI) and live-  
76 weight (LW) gain of the steers had been measured in a feeding trial for 8 weeks (to be  
77 reported elsewhere). The feeding trial was of a 2 × 2 factorial (genotype × diet) design  
78 with the steers being stratified by LW on entry. The experiment reported here was a  
79 continuation of the feeding trial and steers therefore continued on the diet they were  
80 fed during the feeding trial. Steers were allocated to the six respiration chambers over  
81 a 12-week period, using a randomised block design (6 chambers times 4 weeks)  
82 which was repeated three times. Within each block, each treatment of the 2 x 2  
83 factorial (genotype × diet) experimental design was replicated once in each respiration  
84 chamber. Steers were allocated to blocks to minimise variation in LW (mean LW (kg)  
85 674, SEM 4.2) on entry to the respiration chambers. Emissions from each of the 72  
86 steers were therefore measured once as described below.

87

### 88 *Respiration chamber design, operation and measurements*

89 Six indirect open-circuit respiration chambers were used (No Pollution Industrial  
90 Systems Ltd., Edinburgh, UK). The total chamber volume (76 m<sup>3</sup>) was ventilated by

91 recirculating fans set at 450 l/s. Air was removed from the chambers by exhaust fans  
92 set at 50 l/s giving approximately 2.5 air changes/h. Temperature and relative  
93 humidity were set at 15°C and 60% relative humidity respectively. Total air flow was  
94 measured by in-line hot wire anemometers which were validated by daily  
95 measurements made with an externally calibrated anemometer (Testo 417, Testo Ltd,  
96 Alton, Hampshire, UK). Temperature and humidity were measured using sensor  
97 probes in the exhaust air outlet (Johnson Controls, Milan, Italy) and atmospheric  
98 pressure, corrected for altitude, with a Vantage Pro2 weather station (Davis  
99 Instruments, Haywood, Ca, USA). Chambers were operated under negative pressure  
100 (50 N/m<sup>2</sup>). Methane concentrations were measured by infrared absorption  
101 spectroscopy and H<sub>2</sub> by a chemical sensor (MGA3000, Analytical Development Co.  
102 Ltd., Hoddesdon, UK). The analyser was calibrated with a gas mixture of known  
103 composition. Gas concentrations were recorded for each chamber and for inlet air  
104 every 6 min. Prior to the beginning of the experiment, gas recoveries were measured  
105 by releasing CO<sub>2</sub> at a constant rate into each chamber. The mean recovery was 98%  
106 (SEM 3.0) which was not different from 100%.

107 To accustom the steers to the chamber environment, 6 d prior to chamber  
108 measurements groups of steers were moved to the building in which chambers were  
109 located and loose-housed in single pens (4 × 3 m) of identical design to pens within  
110 the chambers. After 6 days, the steers were then moved to the chambers and remained  
111 there for 72 h, with CH<sub>4</sub> and H<sub>2</sub> measurements recorded in the final 48 h used in the  
112 analysis. Steers were fed once daily and weight of feed within the bins was recorded  
113 at 10 s intervals using load cells. Front doors of chambers were briefly opened at  
114 about 08.00 h daily to remove feed bins and again to replace bins with fresh feed at  
115 approximately 09.00 h. The pens were cleaned daily between 08.00 and 09.00 h.  
116 Exact times when doors were opened were recorded.

117

#### 118 *Rumen sampling and volatile fatty acid (VFA) analysis*

119 Immediately after the steers (within 2 h) left the respiration chambers, samples of  
120 rumen fluid were obtained (one per animal) by inserting a tube (16 × 2700 mm  
121 Equivet Stomach Tube, Jørgen Kruuse A/S, Langeskov, Denmark) nasally and  
122 aspirating manually. Approximately 50 ml fluid were strained through two layers of  
123 muslin and stored at -20 °C to await analysis. Samples for VFA analysis (1 ml) were  
124 deproteinised by adding 0.2 ml metaphosphoric acid (215 g/litre) and 0.1 ml internal

125 standard (10 ml 2-ethyl n-butyric acid /litre) and VFA concentrations determined by  
126 HPLC<sup>(10)</sup>. For DNA analysis, 5 ml strained rumen fluid were mixed with 10 ml  
127 phosphate buffered saline containing glycerol (30% v/v) and stored at -20 °C.

128

#### 129 *DNA analysis*

130 DNA extraction was carried out using a method based on repeated bead beating plus  
131 column filtration<sup>(11)</sup>. DNA concentrations were determined with a NanoDrop ND  
132 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA  
133 was diluted to 0.5 ng/μl in 5 μg/ml herring sperm DNA for amplification with  
134 universal bacterial primers UniF and UniR and 5 ng/μl in 5 μg/ml herring sperm DNA  
135 for amplification of other groups<sup>(12)</sup>. qPCR was carried out using a BioRad iQ5 as  
136 described by Ramirez-Farias et al.<sup>(13)</sup>. Calibration curves were prepared on three  
137 separate batches in different qPCR runs. Bacterial primer sets, methods development  
138 and target species may be found in Ramirez-Farias et al.<sup>(13)</sup>. Template DNA from  
139 *Roseburia hominis* A2-183 (DSM 16839<sup>T</sup>) was used for bacterial calibration.  
140 Archaeal amplification was using the primers described by Hook et al.<sup>(14)</sup> and  
141 calibrated using DNA extracted from *Methanobrevibacter smithii* PS, a gift from M.P.  
142 Bryant, University of Illinois. Protozoal 18S rRNA gene amplification was calibrated  
143 using DNA amplified from bovine rumen digesta with primers 54f and 1747r<sup>(15)</sup>.  
144 Coverage of qPCR primers was checked from original references and by use of the  
145 Probe Match tool of the Ribosome Database Project<sup>(16)</sup>.

146

#### 147 *Feed analysis*

148 Feed samples were analysed for DM, ash, crude protein, acid detergent fibre, neutral  
149 detergent fibre, starch<sup>(17)</sup> and GE by adiabatic bomb calorimetry.

150

#### 151 *Calculations and statistical analysis*

152 To minimize bias caused by entry of air when doors were opened for feeding and, as  
153 during this period (54 min. SD 22.5) steers did not have access to feed, gas  
154 concentrations measured during this period were not used for further analysis. Instead,  
155 and to minimize bias, these values were replaced by the mean value of measurements  
156 (n=10) made in the last hour before doors were opened. If a steer had consumed food  
157 during that period, mean values for the hour preceding feed consumption were used.  
158 All data, including gas concentrations, air flow, temperature, humidity, atmospheric

159 pressure and records for feed consumption, were loaded into a database. Dry air flow  
160 was calculated and corrected to standard temperature and pressure for each individual  
161 record of gas concentration. Daily gas production was then calculated as the average  
162 of individual values.

163 Measurements were not made on one steer because of illness and data were  
164 rejected from three steers because of an air leak in one chamber; these consisted of  
165 two LIMx steers fed the Concentrate diet, one LIMx steer fed the Mixed diet and one  
166 AAX steer fed the Mixed diet. Data were analysed using Genstat (Version 11.1 for  
167 Windows, VSN Int. Ltd., Oxford, UK) using linear mixed models where the fixed  
168 factors were the  $2 \times 2$  arrangement of genotype and diet, and random factors, block  
169 and chamber. Since samples for VFA analysis were available for only seven weeks of  
170 the experiment, these data were analysed as a  $2 \times 2$  factorial arrangement of genotype  
171 and diet with week of experiment and chamber. Data are reported as means and SED  
172 unless otherwise stated. Multiple linear regression models were fitted to predict CH<sub>4</sub>  
173 and H<sub>2</sub> emissions from the whole dataset. Fitted terms included *Clostridium* Cluster  
174 IV, XIVa, *Bacteroides* + *Prevotella*, archaea and protozoa (expressed as copy  
175 number/ng DNA). To help with variable selection, all subsets of predictors were  
176 examined, with subsets compared using adjusted R-squared and Akaike's Information  
177 Criterion (AIC).

178

## 179 **Results**

180

181 Cattle offered the Mixed diet consumed less feed (Table 3) whether expressed as total  
182 daily DM intake (DMI,  $P < 0.001$ ) or as g/kg LW ( $P = 0.009$ ) than cattle offered the  
183 Concentrate diet. DMI was also greater ( $P = 0.002$ ) for AAX than for LIMx steers.

184 Whether expressed as mol/d, mol/kg DMI or kJ/MJ GE intake (GEI, Table 3),  
185 steers fed the Concentrate diet produced less CH<sub>4</sub> than steers fed the Mixed diet  
186 ( $P < 0.001$ ). AAX steers produced more CH<sub>4</sub> (mol/d  $P = 0.032$ ) than LIMx steers but  
187 this difference disappeared when CH<sub>4</sub> production was expressed relative to DMI or  
188 GEI.

189 Hydrogen production from the steers was on average 0.10 mol H<sub>2</sub>/mol CH<sub>4</sub>  
190 (Table 3). There was a significant diet  $\times$  genotype interaction such that Concentrate-  
191 fed AAX steers produced less total H<sub>2</sub> than LIMx steers but the opposite was found  
192 for the Mixed diet. When expressed as mol/kg DMI or kJ/MJ GEI, there was no



193 interaction, and Mixed diet-fed steers produced more H<sub>2</sub> than Concentrate-fed steers.  
194 However as a proportion of CH<sub>4</sub> production (mol H<sub>2</sub>/mol CH<sub>4</sub>), Concentrate-fed  
195 steers produced more H<sub>2</sub> than Mixed-fed steers ( $P < 0.001$ ).

196 Fig. 1 shows an example, comprising one steer fed the Concentrate and one  
197 fed the Mixed diet, of changes in the rate of CH<sub>4</sub> and H<sub>2</sub> production (mmol/min) over  
198 a 24-h period after fresh feed was offered. There were intermittent peaks, particularly  
199 in H<sub>2</sub> emission rates throughout the day. Aligning these peaks with records of feed  
200 consumption, it was apparent that the peaks in CH<sub>4</sub> and H<sub>2</sub> concentrations occurred a  
201 short time after feed consumption. Further analysis showed that whereas median H<sub>2</sub>  
202 production rates (0.63 vs 0.68 mmol/min, SED 0.060, Concentrate v Mixed), did not  
203 differ ( $P > 0.05$ ) between diets, the frequency of H<sub>2</sub> production more than 0.5  
204 mmol/min above median values (0.053 v 0.117, SED 0.210,  $P < 0.001$ ) was greater  
205 for Mixed- than Concentrate-fed steers. Thus, a substantial part of the greater H<sub>2</sub>  
206 output in Mixed-fed steers (mol/kg DMI) was related to peaks in H<sub>2</sub> concentration  
207 associated with feeding.

208 Molar proportions (mmol/mol, Table 4) of acetic ( $P < 0.001$ ), butyric ( $P =$   
209 0.013) and valeric acids ( $P = 0.01$ ) were greater and those of propionic acid ( $P <$   
210 0.001) less in rumen fluid samples from Mixed than Concentrate-fed animals.  
211 Genotype had no effect on VFA proportions.

212 Both diet and genotype influenced microbial numbers (Table 5). The  
213 Concentrate diet supported lower copy numbers of archaea ( $P = 0.002$ ) and protozoa  
214 ( $P < 0.001$ ) but larger copy numbers of total bacteria ( $P < 0.001$ ) than the Mixed diet.  
215 *Clostridium* Clusters IV and XIVa and *Bacteroides* + *Prevotella* accounted for  
216 between 0.7 and 0.8 of copy numbers represented by total bacteria and there were no  
217 differences in this proportion due to diet or genotype. The relative abundance of  
218 *Clostridium* Cluster IV (proportion of total bacteria, Table 5) was greater ( $P < 0.001$ )  
219 and that of *Clostridium* Cluster XIVa ( $P = 0.025$ ) was less on the Mixed diet than the  
220 Concentrate diet ( $P < 0.001$ ). Proportionally, AAX steers supported larger copy  
221 numbers of *Clostridium* Cluster IVa ( $P = 0.031$ ) and lower numbers of *Bacteroides* +  
222 *Prevotella* ( $P = 0.018$ ).

223 There was a significant correlation between H<sub>2</sub> and CH<sub>4</sub> production (mol/kg  
224 DMI) for the Mixed but not the Concentrate diet (Fig. 2). For the Mixed diet, linear  
225 regression analysis found a significant slope (0.088, SE 0.0041,  $P < 0.001$ ) with  
226 intercept not different from 0. No microbial predictors were able to explain a

227 significant amount of variability in H<sub>2</sub> emissions between individual animals. For  
228 CH<sub>4</sub> (mol/kg DMI), there was a relationship ( $r^2 = 0.30$ ) with copy numbers ( $\times 10^3$ /ng  
229 DNA) of archaea and Clostridium cluster XIVa: CH<sub>4</sub> (mol/g DMI) = 1.07 - 0.00298  
230 Cluster XIVa (s.e. 0.00083,  $P = 0.001$ ) + 0.0094 Archaea (s.e. 0.0024,  $P < 0.001$ )

231

## 232 **Discussion**

233 Enteric fermentation in animals occurs predominantly in the absence of oxygen.  
234 Under such conditions, microbial communities adapt differently to the disposal of the  
235 reducing equivalents that are generated by glycolysis. Some microorganisms use an  
236 internal redox mechanism, such as in the formation of propionate and succinate.  
237 However, most microbial fermentation results in the formation of molecular H<sub>2</sub>. The  
238 fate of H<sub>2</sub> depends on the animal species and its anatomical configuration. In man,  
239 with a relatively rapid gut transit time, reductive acetogenesis (H<sub>2</sub> + CO<sub>2</sub> → acetate)  
240 and H<sub>2</sub> gas tend to predominate as mechanisms for disposal of H<sub>2</sub>. About 50% of  
241 human subjects in Europe also produce CH<sub>4</sub>; CH<sub>4</sub> production competes with other  
242 metabolic processes but H<sub>2</sub> gas is still produced in these subjects<sup>(18)</sup>. Hydrogen  
243 emissions from ruminants are known to be proportionally much smaller and CH<sub>4</sub>  
244 emissions much greater<sup>(19)</sup>. Van Zijderfeld et al.<sup>(20)</sup> measured H<sub>2</sub> production from  
245 dairy cows hourly for 9 h and reported greater concentrations when nitrate was  
246 included in the diet but, to the authors' knowledge, this is the first report in which  
247 total daily H<sub>2</sub> emissions by cattle have been quantified on a large scale using indirect  
248 respiration chambers.

249

### 250 *Hydrogen emissions*

251 Previous studies have reported lower H<sub>2</sub> concentrations for ruminants fed all-forage  
252 diets than for diets containing various proportions of concentrate and forage whether  
253 measured as concentrations of H<sub>2</sub> dissolved in rumen fluid<sup>(21)</sup>, in the rumen gas  
254 phase<sup>(22)</sup> or in exhaled air<sup>(9)</sup>. There do not appear to be any reports of H<sub>2</sub> emissions for  
255 high-concentrate diets in live animals. Here, daily H<sub>2</sub> emissions were similar with  
256 both diets and genotypes, but when converted to units per DM intake, H<sub>2</sub> production  
257 was greater on the Mixed than on the Concentrate diet. Total daily H<sub>2</sub> emissions were  
258 about 1% and 10% of CH<sub>4</sub> emissions on a mass and molar basis respectively. A total  
259 H balance was constructed from estimates of the amounts of carbohydrate fermented  
260 in the rumen and observed mean VFA molar proportions for each diet. Whilst the

261 amount of H<sub>2</sub> produced per unit carbohydrate fermented on the Concentrate diet was  
262 less than on the Mixed diet (3.6 v 4.9 moles H<sub>2</sub>/mole carbohydrate fermented),  
263 estimates of total H<sub>2</sub> produced were not dissimilar between diets (169 v 177  
264 moles/day, Concentrate v Mixed) because of both the lower fermentability (due to the  
265 presence of fermentation end-products in the silages) and the lower daily feed intakes  
266 of the Mixed diet. Thus H<sub>2</sub> emissions accounted for less than 2% of estimated total  
267 H<sub>2</sub> production from fermentation. Further, after accounting for H consumed in  
268 synthesis of microbial biomass, total recovery of hydrogen in microbial biomass, H<sub>2</sub>  
269 and CH<sub>4</sub> was similar between diets (108 and 114% of H produced for Concentrate  
270 and Mixed diets) indicating that there were no major H-consuming processes  
271 unaccounted for or that differed between diets.

272 Peaks in H<sub>2</sub> emission rates (Fig. 1) were observed after feed was consumed  
273 and these peak H<sub>2</sub> emission rates were greater on the Mixed diet. Increases in H<sub>2</sub>  
274 emission rates after feeding are consistent with measurements in sheep of H<sub>2</sub>  
275 concentrations in rumen fluid<sup>(21,23)</sup>, rumen head-space gas<sup>(22,24)</sup> and respiration  
276 chambers<sup>(25,26)</sup>. The larger size of the meal-related peaks in H<sub>2</sub> emissions on the  
277 Mixed diet accounted for the differences in daily H<sub>2</sub> emissions (g/kg DMI) observed  
278 for this diet. One might have expected that there would be correlations between the  
279 ruminal microbiota and H<sub>2</sub> emissions particularly the balance between ciliate  
280 protozoa and *Clostridium* Cluster IV as major H<sub>2</sub> producers and archaea as  
281 consumers, but no relationships between H<sub>2</sub> emissions and any of the different groups  
282 of micro-organisms were found. It is possible that the primers used may not have  
283 detected all H<sub>2</sub> producing bacteria. Alternatively, the differences between diets in H<sub>2</sub>  
284 emissions are more likely to be related to the nature of the diets fed and the  
285 consumption patterns of individual cows. First the peaks in H<sub>2</sub> emissions may be  
286 caused by physical displacement of gas from the rumen head space by the feed  
287 consumed<sup>(27)</sup>. Because the Mixed diet contained larger proportions of long forage and  
288 had a higher moisture content (443 v 853 g DM/kg fresh weight), the bulkier Mixed  
289 diet may have caused greater displacement of rumen head space gas and hence greater  
290 H<sub>2</sub> emissions. Secondly, compared to the Concentrate diet, the Mixed diet contained  
291 higher concentrations of more slowly fermented cell wall carbohydrates and less  
292 starch and also higher concentrations of soluble feed constituents derived from the  
293 silages fed, particularly amino acids and fermentation products. Therefore there may  
294 be increased production of H<sub>2</sub> from rapid fermentation of soluble feed components

295 immediately after consumption of the Mixed diet which exceeded the capacity of  
296 methanogens to utilise the H<sub>2</sub>. The peaks in H<sub>2</sub> emissions after consuming feed were  
297 also more defined and discrete than the peaks in CH<sub>4</sub> emissions (Fig. 1). A possible  
298 explanation for this is that while CH<sub>4</sub> is an end-product of metabolism of H<sub>2</sub> by  
299 archaea, the H<sub>2</sub> present in the ruminal gas phase can either be emitted by eructation or  
300 can redissolve in ruminal fluid and be utilised for CH<sub>4</sub> production by the archaea<sup>(28)</sup>.  
301 This may also explain the poor relationship between CH<sub>4</sub> and H<sub>2</sub> emissions (Fig. 2),  
302 as H<sub>2</sub> emissions will depend not only on rates of production by H<sub>2</sub>-generating  
303 metabolism exceeding the capacity of archaea to consume H<sub>2</sub> but also the rate at  
304 which dissolved/gaseous H<sub>2</sub> is utilised. Both of these will depend on the meal size  
305 and rate of feed consumption of individual animals.

306

### 307 *Methane production*

308 As found in other studies<sup>(2,29)</sup>, CH<sub>4</sub> production (mol/d) was substantially lower when  
309 the diet containing more than 900 g concentrate/kg DM was fed compared to the  
310 mixed forage:concentrate diet, thus confirming the well-established strategy of  
311 reducing CH<sub>4</sub> emissions by increasing the concentrate proportion of the diet. Mean  
312 methane yields (MJ/MJ GEI) were 0.039 and 0.062 for the Concentrate and Mixed  
313 diets respectively. These compare with values of 0.030 (“for diet containing more  
314 than 900 kg concentrates / kg DM”) and 0.065 (“for all other diets”) adopted by  
315 IPCC<sup>(1)</sup> for estimating CH<sub>4</sub> emissions. Thus values predicted from IPCC<sup>(1)</sup> for CH<sub>4</sub>  
316 production for the Mixed diet differed little from those observed (predicted v  
317 observed; 298 v 287 litre/d). However IPCC<sup>(1)</sup> predictions underestimated CH<sub>4</sub>  
318 production from the Concentrate diet (predicted v observed, 155 v 200 litre/d). The  
319 reason for the higher CH<sub>4</sub> production for the Concentrate diet in the current  
320 experiment was probably that the cereal fed was barley rather than maize. When high-  
321 concentrate diets based on maize and barley were fed to feedlot cattle<sup>(28)</sup>, CH<sub>4</sub>  
322 production of 0.028 and 0.040 of GEI were reported for maize and barley  
323 respectively. Similarly, CH<sub>4</sub> production of 0.033 and 0.046 of GEI were reported for  
324 maize and barley-based concentrates (800 g concentrate /kg DM) albeit in different  
325 years<sup>(30)</sup>. Finally, CH<sub>4</sub> values of 0.04 of GEI for a barley-based diet (900 g/kg diet  
326 DM<sup>(31)</sup>, and recently 0.03 of per GEI for a maize-based concentrate<sup>(32)</sup> have been  
327 reported. Thus, the value suggested by IPCC<sup>(1)</sup> of 0.030 for high concentrate diets is  
328 probably inappropriate for diets based on barley and 0.04 per GEI might be more

329 appropriate. The reasons for the difference between barley and maize have been  
330 discussed<sup>(29,32)</sup> and are most likely due to the more rapid and complete fermentation of  
331 barley grain in the rumen and the higher fibre concentration in barley. The simple  
332 approach used by IPCC<sup>(1)</sup> does not account for variations in diet digestibility or  
333 differences in the efficiency of utilisation of absorbed nutrients for productive  
334 purposes. Methane emissions from the present study were estimated relative first to  
335 ME (estimated from feed analysis) intake as a proxy for digestibility and secondly  
336 with respect to steer LW gain during the feeding trial which preceded this experiment.  
337 For the Concentrate diet, estimates were 0.058 MJ CH<sub>4</sub> /MJ ME intake and 6.5 moles  
338 CH<sub>4</sub>/kg LW gain compared to 0.101 and 11.7 for the Mixed diet. Relative to the  
339 Concentrate diet, the Mixed diet produced 1.74- (ME basis) and 1.80-fold (LW gain  
340 basis) more CH<sub>4</sub> in comparison with 1.58- fold expressed on a GE basis. Thus the  
341 difference in CH<sub>4</sub> emissions between diets is amplified when expressed on a ME or  
342 LW gain basis.

343 Although total daily CH<sub>4</sub> emissions were greater for AAx steers, this  
344 difference was accounted for by differences in DM intake. Thus CH<sub>4</sub> emissions  
345 (mol/kg DMI) did not differ between the similar genotypes, although there were  
346 effects of individual sires<sup>(33)</sup>.

347

#### 348 *Diet and microbial numbers*

349 Analysis of the rumen microbial community provided information about how diet  
350 affected the main groups of bacteria, total ciliate protozoa and archaea. The three  
351 groups of bacteria were chosen to represent the main groups of bacteria (*Firmicutes*  
352 and *Bacteroidetes*) that are known to colonise the rumen<sup>(34-36)</sup>, but it should be noted  
353 that the primers used would not account for all species of *Firmicutes* or *Bacteroidetes*.  
354 The three groups of bacteria accounted for more than 0.70 of total bacteria copy  
355 numbers and this proportion was not influenced by diet or genotype. The *Clostridium*  
356 groups form part of the *Firmicutes* phylum, which are usually more abundant than  
357 *Bacteroidetes* in rumen samples<sup>(34-36)</sup> and this was true for the AAx but not LIMx  
358 steers in this experiment. Part of the variation in relative abundance (proportion of  
359 total bacteria) of the two *Clostridium* Clusters was due to diet. Cluster IV,  
360 encompassing the highly cellulolytic *Ruminococcus* and several *Eubacterium* spp.<sup>(37)</sup>  
361 were more abundant with the Mixed diet. The Cluster XIVa grouping, whose  
362 abundance was lower in the Mixed diet, would contain *Butyrivibrio* and related

363 spp<sup>(37)</sup>, none of which are known to possess the ability to break down crystalline  
364 cellulose<sup>(38)</sup>. Ciliate protozoa were more numerous with the Mixed diet, a result  
365 which seems to be at odds with the general observation that adding concentrate to a  
366 forage diet usually increases protozoal numbers<sup>(19,39)</sup>. There is a limited number of  
367 reports on the rumen microbial community when diets containing high proportions of  
368 concentrate were fed. The abundance of archaea increased when concentrate was  
369 increased from 100 to 500 g/kg diet<sup>(40)</sup> and decreased when dietary concentrate was  
370 increased from 500 to 900 g/kg<sup>(41)</sup> (similar to the present experiment). However when  
371 Popova et al.<sup>(42)</sup> compared starch and fibre-rich concentrates in a diet containing 870 g  
372 concentrate /kg there were no difference in numbers of methanogens between diets.  
373 When dietary concentrates were increased<sup>(43)</sup> from 0 to 700 g/kg, increasing  
374 concentrate reduced the numbers of *Fibrobacter succinogenes* and increased the  
375 numbers of genus *Prevotella* but there were no differences between diets in the  
376 populations of *Ruminococcus albus* or *R. flavefaciens*. This is in contrast to the  
377 decrease in *Clostridium* Cluster IV and no change in *Bacteroides* plus *Prevotella*  
378 numbers when concentrate was increased in the present study. Similarly, increases in  
379 protozoal numbers were reported<sup>(42,43)</sup> when concentrate or dietary starch was  
380 increased, again in contrast to the decrease in numbers reported here and  
381 elsewhere<sup>(31)</sup>. These differences are probably explained by the different dietary  
382 protocols and approaches to community analysis used in the experiments. For  
383 example Carberry et al<sup>(43)</sup> compared 0 and 700 g concentrate /kg whilst the  
384 comparison was between 500 and 920 g concentrate /kg in the present study.

385 In terms of our focus on H<sub>2</sub> emissions, it was perhaps surprising that the H<sub>2</sub>-  
386 producing *Ruminococcus* spp. of Cluster IV and total protozoa which produce  
387 abundant H<sub>2</sub><sup>(44)</sup> were not more correlated with CH<sub>4</sub>, as H<sub>2</sub> is the main substrate for  
388 methanogenesis in the rumen<sup>(28,45)</sup>. There is no obvious explanation, except perhaps  
389 that any effect of the abundance of H<sub>2</sub> producers was swamped by effects of long-  
390 term adaptation to the diets fed. Alternatively, a more detailed taxonomic description  
391 within the groups, best derived from metagenomic information, might identify key  
392 genera and species that dictate H<sub>2</sub> production and thereby influence methanogenesis.

393 Many researchers believe, and some studies are beginning to show, that the  
394 host animal exerts a controlling effect on its own gut microbiota<sup>(46-48)</sup>. The findings  
395 here that the relative abundance of *Bacteroides* plus *Prevotella* was less and cluster  
396 IV greater in AAx than LIMx steers on the corresponding diets would support such a

397 hypothesis and may provide a mechanism for the greater feed intakes observed with  
398 the AAx steers.

399

#### 400 *Implications*

401 Recently, when interactions between H<sub>2</sub> and other gases in the atmosphere were  
402 considered<sup>(49)</sup>, it was proposed that H<sub>2</sub> is an indirect greenhouse gas with a global  
403 warming potential of 5.8 compared to 25 for CH<sub>4</sub> on a carbon dioxide mass  
404 equivalent basis. On a daily basis, total (CH<sub>4</sub> plus H<sub>2</sub>) mean emissions from enteric  
405 fermentation were 3.6 and 5.1 kg CO<sub>2</sub> for the Concentrate and Mixed diets of which  
406 H<sub>2</sub> contributed 12 and 13 g CO<sub>2</sub> daily. Thus, although inefficiency of capture of H<sub>2</sub>  
407 during inter-species H<sub>2</sub> transfer is a loss of energy from the system, in terms of  
408 overall greenhouse gas production by ruminants, its contribution will be negligible  
409 with the exception of circumstances where methanogenesis is severely disrupted, e.g.  
410 when halogenated compounds are used to inhibit methanogenesis<sup>(25)</sup>.

411 In conclusion, this large-scale study of the effect of diet, feeding pattern and  
412 cattle genotype on H<sub>2</sub> emissions by cattle has revealed that H<sub>2</sub> emissions can be up to  
413 10% on a molar basis of CH<sub>4</sub> emissions from beef cattle on commonly used diets.  
414 Most H<sub>2</sub> was produced shortly after feeding, and the concentration followed that of  
415 CH<sub>4</sub>. However the feeding-related increases in H<sub>2</sub> were not related to the microbial  
416 populations and therefore are more likely due to between-diet differences in feeding  
417 patterns and the nutrients rapidly fermented upon feed ingestion. Cattle genotype  
418 affected H<sub>2</sub> emissions *via* differences in feed intake and this may be related to  
419 differences in microbial community structure. The observations are consistent with  
420 the review by Janssen<sup>(4)</sup> that the capacity for archaeal methanogenesis is in balance  
421 with rates of H<sub>2</sub> production, such that some accumulation of H<sub>2</sub> is required for  
422 methanogenesis to occur. The quantities of H<sub>2</sub> emitted and the lower radiative forcing  
423 potential of H<sub>2</sub> suggest that H<sub>2</sub> emissions present a minor environmental problem in  
424 comparison with those of methane.

425

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436

437 **Conflicts of interest**

438 The authors declare no conflict of interest.

439

440 **Authorship**

441 T.W., R.J.W. and R.R. initiated the research. CAD, JJH, DWR. participated in  
442 planning and facilitating the animal work. N.McK. and S. M. de S. carried out DNA  
443 extraction and qPCR. J.A.R supervised the respiration chamber studies and wrote the  
444 manuscript with input from R.J.W. All authors provided feedback on the manuscript.  
445 The authors declare no conflict of interest.

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**Table 1.** Ingredient composition (fresh weight basis; g/kg) of high- concentrate and mixed forage: concentrate diets

Ingredient	High concentrate	Mixed forage:concentrate
Barley straw	81	0
Grass silage	0	413
Whole crop barley silage	0	340
Barley grain	688	156
Maize distillers dark grains	200	86
Molasses	20	0
Minerals-vitamin supplement*	10	5

\*Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; ( $\mu\text{g/kg}$ ): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500

**Table 2.** Chemical composition of feeds incorporated into high-concentrate and mixed forage: concentrate diets\*

	Barley	MDDG	Silage	WCBS	Straw
DM(g/kg)	850	865	211	329	825
(g/kg DM)					
Ash	22	47	67	60	37
Crude protein	104	273	147	111	21
Acid detergent fibre	69	216	345	312	519
Neutral detergent fibre	163	377	567	540	826
Starch	592	22	6	141	3
pH			3.9	4.7	
Gross energy (MJ/kg DM)	18.8	21.8	19.0	19.1	17.1

Barley, barley grain; MDDG, maize distillers dark gains; silage, grass silage; WCBS, whole crop barley silage, Straw, barley straw.

\*Molasses contained 688 g DM /kg and Gross Energy 15.3 MJ/kg DM

**Table 3.** Intakes, methane and hydrogen production from steers fed either a high concentrate or mixed forage:concentrate diets

(Means with SED for 17 observations per mean)

Diet Genotype	Concentrate		Mixed		SED	Probability		
	AAx	LIMx	AAx	LIMx		Genotype	Diet	GxD
DMI								
kg/d	11.4	10.0	10.2	8.7	0.52	0.002	<0.001	NS
g/kg LW	16.1	15.1	15.2	13.4	0.76	0.016	0.009	NS
Hydrogen								
mol/d	0.92	1.08	1.18	1.05	0.106	NS	NS	0.027
mol/kg DMI	0.084	0.112	0.116	0.122	0.0111	NS	0.006	NS
kJ/MJ GEI	1.27	1.66	1.74	1.84	0.168	NS	0.004	NS
Methane								
mol/d	9.4	8.5	13.6	12.0	0.72	0.032	<0.001	NS
mol/kg DMI	0.83	0.87	1.34	1.38	0.077	NS	<0.001	NS
kJ/MJ GEI	39.0	39.9	61.7	64.2	3.31	NS	<0.001	NS
H <sub>2</sub> :CH <sub>4</sub> mol/mol	0.101	0.126	0.086	0.088	0.0135	NS	<0.001	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.

AA, Aberdeen Angus; LIM, Limousin; G x D, genotype x diet; DMI, dry matter intake; GEI, Gross Energy intake.



**Table 4.** Volatile fatty acid (VFA) molar proportions (mmol/mol) in rumen fluid samples obtained from steers fed either a high concentrate or mixed forage:concentrate diets

(Means with SED for 8 observations per mean)

Diet Genotype	Concentrate		Mixed		SED	Probability		
	AAx	LIMx	AAx	LIMx		Genotype	Diet	GxD
Acetic	557	562	670	670	27.9	NS	<0.001	NS
Propionic	290	306	172	173	34.9	NS	<0.001	NS
Butyric	105	92	114	125	13.4	NS	0.013	NS
Valeric	16	16	12	13	1.8	NS	0.010	NS
Branched chain	32	24	30	20	6.2	Ns	NS	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.

AA, Aberdeen Angus; LIM, Limousin; G x D, genotype x diet; Branched chain: iso-butyric plus isovaleric acids

**Table 5.** Microbial numbers in samples of ruminal digesta

(Means with SED for 13 observations per mean)

Diet Genotype	Concentrate		Mixed		SED	Probability		
	AAx	LIMx	AAx	LIMx		Genotype	Diet	GxD
Archaea <sup>1</sup>	30.4	25.7	46.4	36.7	5.84	NS	0.002	NS
Protozoa <sup>2</sup>	37.2	40.0	102.1	71.4	16.1	NS	<0.001	NS
Total bacteria	669	761	492	513	57.7	NS	<0.001	NS
<i>Clostridium</i>								
Cluster IV <sup>1</sup>	138	122	179	135	32.7	NS	NS	NS
Cluster XIVa <sup>1</sup>	127	122	75	69	18.9	NS	<0.001	NS
<i>Bacteroides</i> plus <i>Prevotella</i> <sup>1</sup>	218	302	157	202	29.1	0.002	<0.001	NS
Relative abundance <sup>3</sup>								
<i>Clostridium</i>								
Cluster IV <sup>1</sup>	0.21	0.17	0.35	0.26	0.046	0.031	<0.001	NS
Cluster XIVa <sup>1</sup>	0.19	0.16	0.15	0.13	0.023	NS	0.025	NS
<i>Bacteroides</i> plus <i>Prevotella</i> <sup>1</sup>	0.33	0.40	0.32	0.40	0.041	0.018	NS	NS
Sum <sup>3</sup>	0.74	0.73	0.82	0.79	0.057	NS	NS	NS

Concentrate, high concentrate diet; Mixed, mixed forage: concentrate diet.

AAx, Aberdeen Angus cross; LIMx, Limousin cross; G x D, genotype× diet.

Results are expressed as copy numbers (x 10<sup>3</sup>)/ng DNA as determined by qPCR of 16S rRNA<sup>1</sup> and 18S rRNA<sup>2</sup>.<sup>3</sup> Relative abundance as a proportion of total bacteria; sum is that of *Clostridium* Cluster IV plus Cluster XIVa plus *Bacteroides* plus *Prevotella*.

Legends for figures

Figure 1. Changes in methane (dashed line) and hydrogen (solid line) concentrations during a 24 h period (beginning after fresh feed offered at 09.00h). Examples are given for (a) one steer fed a high concentrate and (b) one steer fed a mixed forage:concentrate diet. Diets were fed *ad libitum* and solid bars denote when feed was consumed.

Figure 2 Relationships between daily hydrogen and methane (mol/kg DM intake) production for cattle fed either (a) a high concentrate (●) or (b) a mixed forage:concentrate (○) diet. Significant regression line is shown for the mixed forage:concentrate diet: ( $y = 0.088x$ ; SE 0.0041;  $P < 0.001$ )

Figure 1

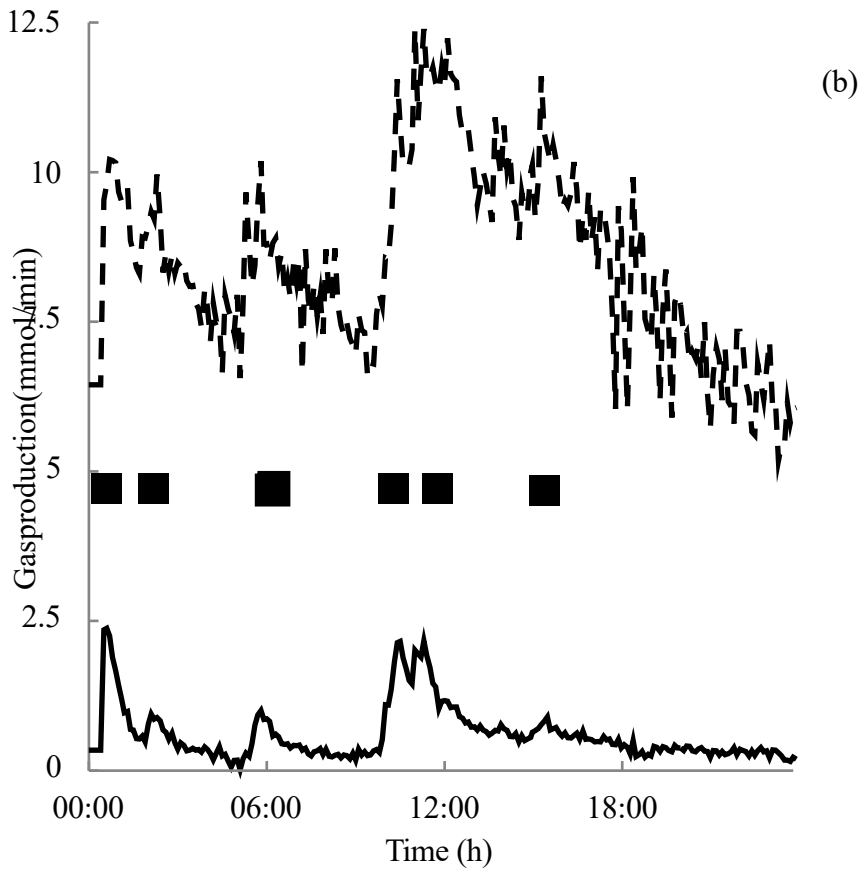
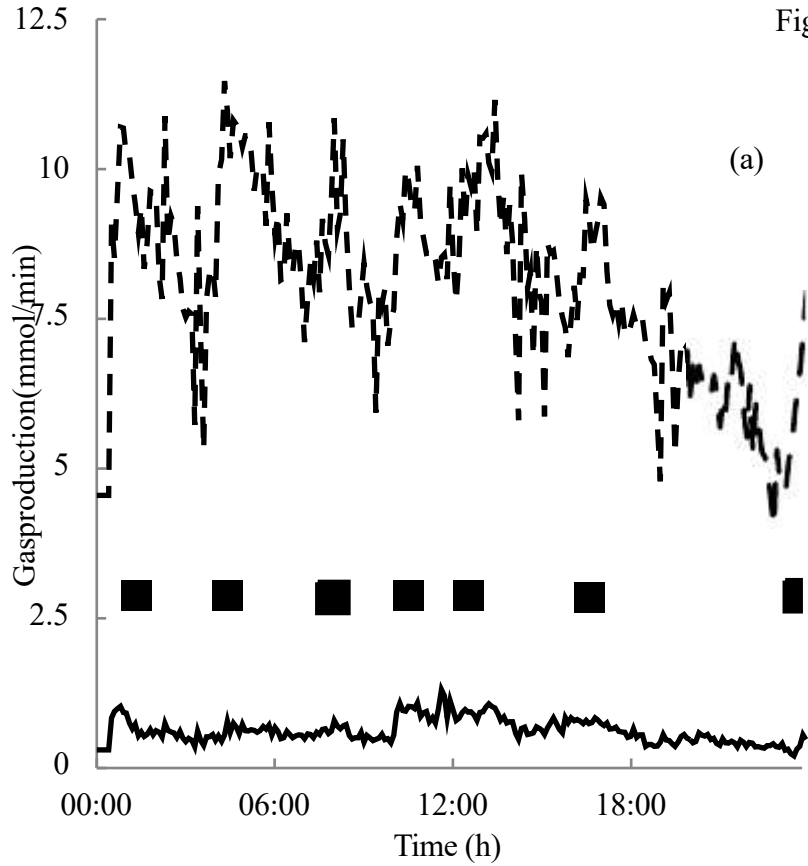


Figure 2

