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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 H₂ 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 H₂/mol CH₄. 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 CH₄ 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 H₂ emissions and microbial copy
19 number. It was concluded that the rate of H₂ production immediately after feeding
20 may lead to transient overloading of methanogenic archaea capacity to use H₂,
21 resulting in peaks in H₂ emissions from beef cattle.
22

23 Methane is a greenhouse gas with a global warming potential 25-fold that of CO₂⁽¹⁾.
24 Ruminant livestock production through the enteric fermentation of feed contributes
25 significantly to greenhouse gas production by agriculture; in the United Kingdom,
26 CH₄ accounted for 37% of all agricultural emissions in 2005⁽²⁾. Enteric production of
27 CH₄ also represents a loss of energy (from 2 to 12% of gross energy (GE) intake)⁽³⁾,
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 H₂ plays an important role in intermediary metabolism in the
33 rumen⁽⁴⁾. Hydrogen is formed by bacteria, protozoa and fungi from the fermentation
34 of carbohydrate. Hydrogen and CO₂ are the principal substrates for methane
35 formation by archaea^(5,6). Hydrogen is also a vital intermediate or substrate in other
36 reactions. Ruminal interspecies H₂ transfer is a process that affects the metabolism of
37 both the microbes that produce H₂ and those that utilise it⁽⁷⁾. Methanogenic archaea
38 require some accumulation of H₂ to grow rapidly enough to prevent them washing out
39 of the rumen⁽⁴⁾. On the other hand, the accumulation of H₂ exerts a thermodynamic
40 inhibitory effect on H₂-producing organisms and causes the fermentation products of
41 these and other microbial species to be changed⁽⁷⁾. As fibrolytic *Ruminococcus* spp.
42 are H₂ producers (via acetate formation), their growth and consequently fibre
43 degradation may be inhibited by H₂ accumulation^(4,7). These pure culture studies
44 indicate that decreasing H₂ concentrations in the rumen would be doubly beneficial in
45 terms of CH₄ emissions and fibre breakdown.

46 Several studies have measured H₂ concentrations in ruminal digesta, as
47 reviewed by Janssen⁽⁴⁾. Hydrogen concentrations increase *in vitro* after adding feed,
48 and the concentrations are diet-dependent. Fewer studies have reported H₂ emissions
49 *in vivo*. In one study⁽⁸⁾ involving two sheep, it was noted that the animals produced
50 two-fold different amounts of CH₄: the sheep with lower CH₄ emissions produced
51 more H₂. In another study using sheep, Takenaka et al.⁽⁹⁾, concluded that H₂
52 emissions were on average 2.1% (vol:vol) of CH₄ emissions based on exhaled gas
53 concentrations. There were periods of high H₂ emission when H₂ 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 H₂

57 and CH₄ 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 m³) 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/m²). Methane concentrations were measured by infrared absorption
101 spectroscopy and H₂ 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 CO₂ 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 CH₄ and H₂ 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⁽¹⁰⁾. 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⁽¹¹⁾. 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⁽¹²⁾. qPCR was carried out using a BioRad iQ5 as
136 described by Ramirez-Farias et al.⁽¹³⁾. 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.⁽¹³⁾. Template DNA from
139 *Roseburia hominis* A2-183 (DSM 16839^T) was used for bacterial calibration.
140 Archaeal amplification was using the primers described by Hook et al.⁽¹⁴⁾ 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⁽¹⁵⁾.
144 Coverage of qPCR primers was checked from original references and by use of the
145 Probe Match tool of the Ribosome Database Project⁽¹⁶⁾.

146

147 *Feed analysis*

148 Feed samples were analysed for DM, ash, crude protein, acid detergent fibre, neutral
149 detergent fibre, starch⁽¹⁷⁾ 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 2×2 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 2×2 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 CH₄
173 and H₂ 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 CH₄ than steers fed the Mixed diet
186 ($P < 0.001$). AAX steers produced more CH₄ (mol/d $P = 0.032$) than LIMx steers but
187 this difference disappeared when CH₄ production was expressed relative to DMI or
188 GEI.

189 Hydrogen production from the steers was on average 0.10 mol H₂/mol CH₄
190 (Table 3). There was a significant diet \times genotype interaction such that Concentrate-
191 fed AAX steers produced less total H₂ 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 H₂ than Concentrate-fed steers.
194 However as a proportion of CH₄ production (mol H₂/mol CH₄), Concentrate-fed
195 steers produced more H₂ 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 CH₄ and H₂ production (mmol/min) over
198 a 24-h period after fresh feed was offered. There were intermittent peaks, particularly
199 in H₂ emission rates throughout the day. Aligning these peaks with records of feed
200 consumption, it was apparent that the peaks in CH₄ and H₂ concentrations occurred a
201 short time after feed consumption. Further analysis showed that whereas median H₂
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 H₂ 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 H₂
206 output in Mixed-fed steers (mol/kg DMI) was related to peaks in H₂ 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 H₂ and CH₄ 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 H₂ emissions between individual animals. For
228 CH₄ (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: CH₄ (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 H₂. The
238 fate of H₂ depends on the animal species and its anatomical configuration. In man,
239 with a relatively rapid gut transit time, reductive acetogenesis (H₂ + CO₂ → acetate)
240 and H₂ gas tend to predominate as mechanisms for disposal of H₂. About 50% of
241 human subjects in Europe also produce CH₄; CH₄ production competes with other
242 metabolic processes but H₂ gas is still produced in these subjects⁽¹⁸⁾. Hydrogen
243 emissions from ruminants are known to be proportionally much smaller and CH₄
244 emissions much greater⁽¹⁹⁾. Van Zijderfeld et al.⁽²⁰⁾ measured H₂ 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 H₂ 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 H₂ concentrations for ruminants fed all-forage
252 diets than for diets containing various proportions of concentrate and forage whether
253 measured as concentrations of H₂ dissolved in rumen fluid⁽²¹⁾, in the rumen gas
254 phase⁽²²⁾ or in exhaled air⁽⁹⁾. There do not appear to be any reports of H₂ emissions for
255 high-concentrate diets in live animals. Here, daily H₂ emissions were similar with
256 both diets and genotypes, but when converted to units per DM intake, H₂ production
257 was greater on the Mixed than on the Concentrate diet. Total daily H₂ emissions were
258 about 1% and 10% of CH₄ 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 H₂ produced per unit carbohydrate fermented on the Concentrate diet was
262 less than on the Mixed diet (3.6 v 4.9 moles H₂/mole carbohydrate fermented),
263 estimates of total H₂ 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 H₂ emissions accounted for less than 2% of estimated total
267 H₂ production from fermentation. Further, after accounting for H consumed in
268 synthesis of microbial biomass, total recovery of hydrogen in microbial biomass, H₂
269 and CH₄ 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 H₂ emission rates (Fig. 1) were observed after feed was consumed
273 and these peak H₂ emission rates were greater on the Mixed diet. Increases in H₂
274 emission rates after feeding are consistent with measurements in sheep of H₂
275 concentrations in rumen fluid^(21,23), rumen head-space gas^(22,24) and respiration
276 chambers^(25,26). The larger size of the meal-related peaks in H₂ emissions on the
277 Mixed diet accounted for the differences in daily H₂ emissions (g/kg DMI) observed
278 for this diet. One might have expected that there would be correlations between the
279 ruminal microbiota and H₂ emissions particularly the balance between ciliate
280 protozoa and *Clostridium* Cluster IV as major H₂ producers and archaea as
281 consumers, but no relationships between H₂ 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 H₂ producing bacteria. Alternatively, the differences between diets in H₂
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 H₂ emissions may be
286 caused by physical displacement of gas from the rumen head space by the feed
287 consumed⁽²⁷⁾. 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 H₂ 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 H₂ 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 H₂. The peaks in H₂ emissions after consuming feed were
297 also more defined and discrete than the peaks in CH₄ emissions (Fig. 1). A possible
298 explanation for this is that while CH₄ is an end-product of metabolism of H₂ by
299 archaea, the H₂ present in the ruminal gas phase can either be emitted by eructation or
300 can redissolve in ruminal fluid and be utilised for CH₄ production by the archaea⁽²⁸⁾.
301 This may also explain the poor relationship between CH₄ and H₂ emissions (Fig. 2),
302 as H₂ emissions will depend not only on rates of production by H₂-generating
303 metabolism exceeding the capacity of archaea to consume H₂ but also the rate at
304 which dissolved/gaseous H₂ 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^(2,29), CH₄ 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 CH₄ 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⁽¹⁾ for estimating CH₄ emissions. Thus values predicted from IPCC⁽¹⁾ for CH₄
316 production for the Mixed diet differed little from those observed (predicted v
317 observed; 298 v 287 litre/d). However IPCC⁽¹⁾ predictions underestimated CH₄
318 production from the Concentrate diet (predicted v observed, 155 v 200 litre/d). The
319 reason for the higher CH₄ 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⁽²⁸⁾, CH₄
322 production of 0.028 and 0.040 of GEI were reported for maize and barley
323 respectively. Similarly, CH₄ 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⁽³⁰⁾. Finally, CH₄ values of 0.04 of GEI for a barley-based diet (900 g/kg diet
326 DM⁽³¹⁾), and recently 0.03 of per GEI for a maize-based concentrate⁽³²⁾ have been
327 reported. Thus, the value suggested by IPCC⁽¹⁾ 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^(29,32) 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⁽¹⁾ 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 CH₄ /MJ ME intake and 6.5 moles
338 CH₄/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 CH₄ in comparison with 1.58- fold expressed on a GE basis. Thus the
341 difference in CH₄ emissions between diets is amplified when expressed on a ME or
342 LW gain basis.

343 Although total daily CH₄ emissions were greater for AAx steers, this
344 difference was accounted for by differences in DM intake. Thus CH₄ emissions
345 (mol/kg DMI) did not differ between the similar genotypes, although there were
346 effects of individual sires⁽³³⁾.

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⁽³⁴⁻³⁶⁾, 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⁽³⁴⁻³⁶⁾ 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.⁽³⁷⁾
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⁽³⁷⁾, none of which are known to possess the ability to break down crystalline
364 cellulose⁽³⁸⁾. 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^(19,39). 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⁽⁴⁰⁾ and decreased when dietary concentrate was
370 increased from 500 to 900 g/kg⁽⁴¹⁾ (similar to the present experiment). However when
371 Popova et al.⁽⁴²⁾ 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⁽⁴³⁾ 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^(42,43) when concentrate or dietary starch was
380 increased, again in contrast to the decrease in numbers reported here and
381 elsewhere⁽³¹⁾. 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⁽⁴³⁾ 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 H₂ emissions, it was perhaps surprising that the H₂-
386 producing *Ruminococcus* spp. of Cluster IV and total protozoa which produce
387 abundant H₂⁽⁴⁴⁾ were not more correlated with CH₄, as H₂ is the main substrate for
388 methanogenesis in the rumen^(28,45). There is no obvious explanation, except perhaps
389 that any effect of the abundance of H₂ 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 H₂ 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⁽⁴⁶⁻⁴⁸⁾. 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 H₂ and other gases in the atmosphere were
402 considered⁽⁴⁹⁾, it was proposed that H₂ is an indirect greenhouse gas with a global
403 warming potential of 5.8 compared to 25 for CH₄ on a carbon dioxide mass
404 equivalent basis. On a daily basis, total (CH₄ plus H₂) mean emissions from enteric
405 fermentation were 3.6 and 5.1 kg CO₂ for the Concentrate and Mixed diets of which
406 H₂ contributed 12 and 13 g CO₂ daily. Thus, although inefficiency of capture of H₂
407 during inter-species H₂ 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⁽²⁵⁾.

411 In conclusion, this large-scale study of the effect of diet, feeding pattern and
412 cattle genotype on H₂ emissions by cattle has revealed that H₂ emissions can be up to
413 10% on a molar basis of CH₄ emissions from beef cattle on commonly used diets.
414 Most H₂ was produced shortly after feeding, and the concentration followed that of
415 CH₄. However the feeding-related increases in H₂ 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 H₂ 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⁽⁴⁾ that the capacity for archaeal methanogenesis is in balance
421 with rates of H₂ production, such that some accumulation of H₂ is required for
422 methanogenesis to occur. The quantities of H₂ emitted and the lower radiative forcing
423 potential of H₂ suggest that H₂ 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 ₂ :CH ₄ 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 ¹	30.4	25.7	46.4	36.7	5.84	NS	0.002	NS
Protozoa ²	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 ¹	138	122	179	135	32.7	NS	NS	NS
Cluster XIVa ¹	127	122	75	69	18.9	NS	<0.001	NS
<i>Bacteroides</i> plus <i>Prevotella</i> ¹	218	302	157	202	29.1	0.002	<0.001	NS
Relative abundance ³								
<i>Clostridium</i>								
Cluster IV ¹	0.21	0.17	0.35	0.26	0.046	0.031	<0.001	NS
Cluster XIVa ¹	0.19	0.16	0.15	0.13	0.023	NS	0.025	NS
<i>Bacteroides</i> plus <i>Prevotella</i> ¹	0.33	0.40	0.32	0.40	0.041	0.018	NS	NS
Sum ³	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 10³)/ng DNA as determined by qPCR of 16S rRNA¹ and 18S rRNA².³ 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

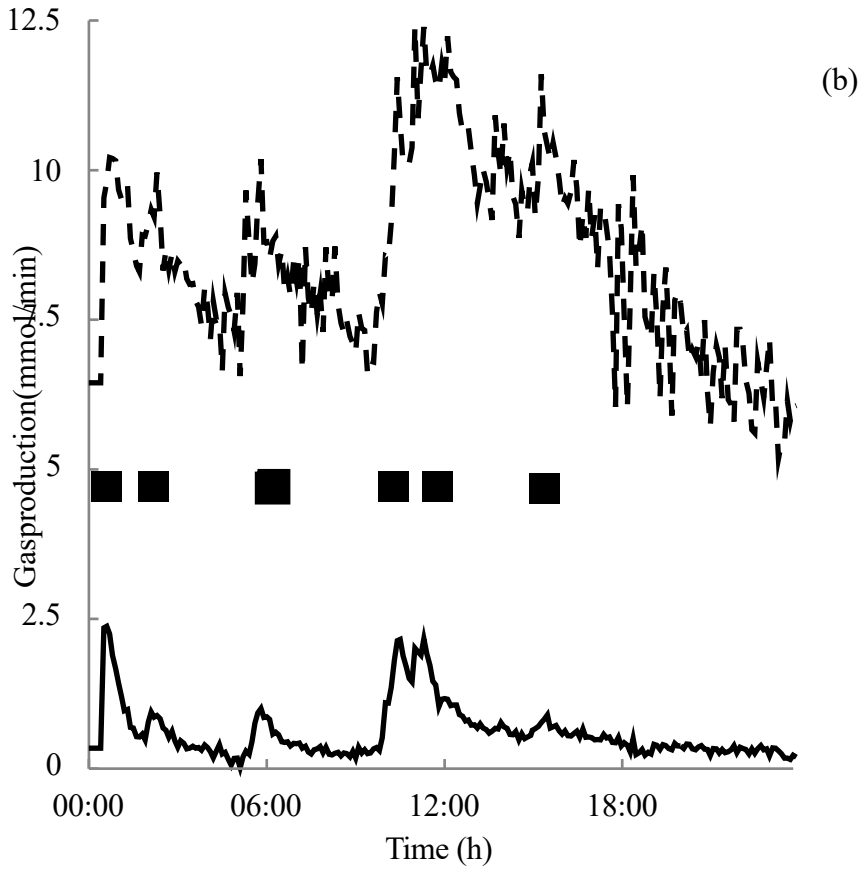
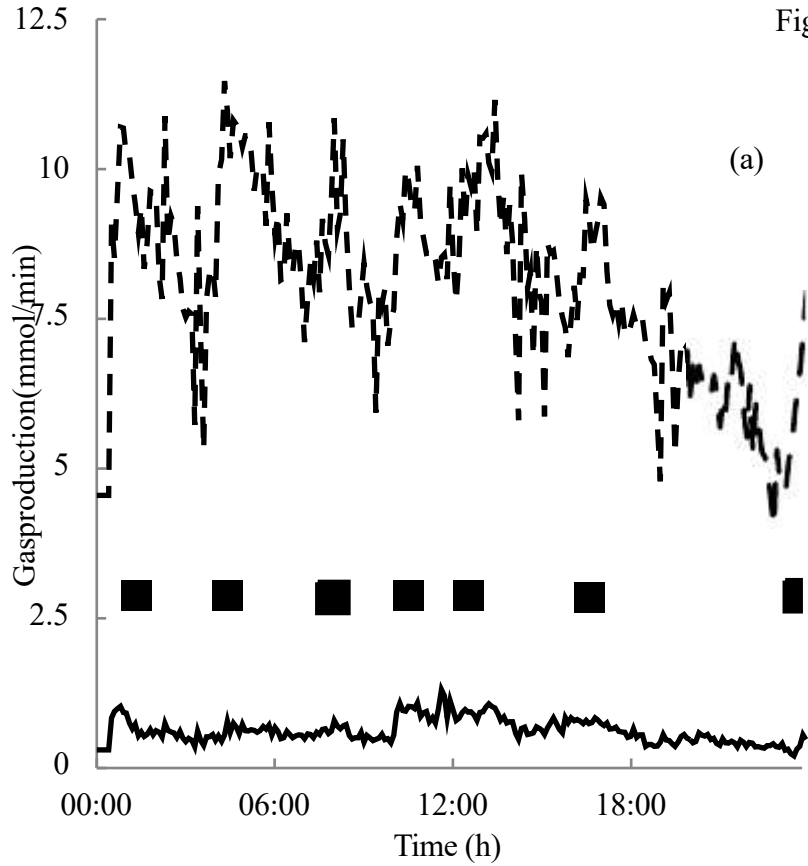


Figure 2

