

# Pure

## Scotland's Rural College

### **The effect of dietary addition of nitrate or increase in lipid concentrations, alone or in combination, on performance and methane emissions of beef cattle**

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1 **The effect of dietary addition of nitrate or increase in lipid concentrations,**  
2 **alone or in combination, on performance and methane emissions of beef cattle**

3

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12

13 Short title: Methane mitigation strategies for finishing steers

14

15

16 **Abstract**

17 Adding nitrate to or increasing the concentration of lipid in the diet are established  
18 strategies for reducing enteric methane (CH<sub>4</sub>) emissions, but their effectiveness  
19 when used in combination has been largely unexplored. This study investigated the  
20 effect of dietary nitrate and increased lipid included alone or together on CH<sub>4</sub>  
21 emissions and performance traits of finishing beef cattle. The experiment was a 2 × 4  
22 factorial design comprising two breeds (AAx, cross-bred Aberdeen Angus; LIMx,  
23 cross-bred Limousin steers) and four dietary treatments (each based on 550 g  
24 forage: 450 g concentrate /kg DM). The four dietary treatments were assigned  
25 according to a 2 x 2 factorial design where the control treatment contained rapeseed  
26 meal as the main protein source which was replaced either with nitrate (21.5 g  
27 nitrate/kg DM); maize distillers dark grains (MDDG, which increased diet ether  
28 extract from 24 to 37 g/kg DM) or both nitrate and MDDG. Steers (n = 20 /dietary  
29 treatment) were allocated to each of the four treatments in equal numbers of each  
30 breed with feed offered *ad libitum*. After 28 days adaptation to dietary treatments,  
31 individual animal intake, performance and feed efficiency were recorded for 56 days.  
32 Thereafter, CH<sub>4</sub> emissions were measured over 13 weeks (six steers / week).  
33 Increasing dietary lipid did not adversely affect animal performance and showed no  
34 interactions with dietary nitrate. In contrast, addition of nitrate to diets resulted in  
35 poorer live-weight gain (P<0.01) and increased feed conversion ratio (P<0.05)  
36 compared with diets not containing nitrate. Daily CH<sub>4</sub> output was lower (P<0.001) on  
37 nitrate-containing diets but increasing dietary lipid resulted in only a non-significant  
38 reduction in CH<sub>4</sub>. There were no interactions associated with CH<sub>4</sub> emissions between  
39 dietary nitrate and lipid. AAx steers achieved greater live-weight gains (P<0.01), but  
40 had greater DM intakes (P<0.001), greater fat depth (P<0.01) and poorer residual

41 feed intakes ( $P<0.01$ ) than LIMx steers. AAx steers had higher daily CH<sub>4</sub> outputs  
42 ( $P<0.001$ ) but emitted less CH<sub>4</sub> per kg DM intake than LIMx steers ( $P<0.05$ ). In  
43 conclusion, inclusion of nitrate reduced CH<sub>4</sub> emissions in growing beef cattle  
44 although the efficacy of nitrate was less than in previous work. When increased  
45 dietary lipid and nitrate inclusion were combined there was no evidence of an  
46 interaction between treatments and therefore combining different nutritional  
47 treatments to mitigate CH<sub>4</sub> emissions could be a useful means of achieving  
48 reductions in CH<sub>4</sub> while minimizing any adverse effects.

49 **Keywords:** beef cattle, greenhouse gas, methane, nitrate, dark grains.

50

## 51 **Implications**

52 The ability of individual nutritional strategies to reduce methane (CH<sub>4</sub>) emissions  
53 from cattle is limited by potential adverse consequences such as reduction in fibre  
54 digestion for increased lipid or toxicity for added nitrate. The reduction in CH<sub>4</sub>  
55 emissions when dietary nitrate was fed was not influenced by the presence of lipid.  
56 Combining different nutritional strategies to mitigate CH<sub>4</sub> emissions could be a useful  
57 means of achieving reductions in CH<sub>4</sub> while minimizing any adverse effects on cattle  
58 health and performance.

59

## 60 **Introduction**

61 Methane emissions arising from the enteric fermentation of feed by ruminant  
62 livestock contribute significantly to greenhouse gas emissions. In the United  
63 Kingdom (Department of Energy and Climate Change, 2016), enteric CH<sub>4</sub> emissions  
64 were estimated to account for 23.8 Mt carbon dioxide equivalents or 48% of total  
65 greenhouse gas emissions from the agriculture sector in 2014. Strategies to mitigate

66 CH<sub>4</sub> emissions have been classified (Hristov *et al.*, 2013) as addressing enteric  
67 fermentation, manure management or animal husbandry (where animal husbandry  
68 included genetics, health and fertility).

69 Many nutritional strategies which target CH<sub>4</sub> emissions have been tested but  
70 convincing evidence for long-term efficacy *in vivo* for many is lacking. Increasing  
71 dietary lipid and inclusion of nitrate in the diet are effective mitigation strategies  
72 (Hristov *et al.*, 2013) and their use has been recently reviewed (Martin *et al.*, 2010;  
73 Patra, 2014; Lee and Beauchemin, 2014; Yang *et al.*, 2016). However, the extent to  
74 which either strategy can be included in the diet is limited by potential adverse  
75 effects: a reduction in fibre digestion and consequently feed intake from increased  
76 lipid in the diet and nitrate / nitrite toxicity from adding nitrate. As the mechanisms by  
77 which lipid (reduction in fermentable carbohydrate intake, inhibition of micro-  
78 organisms; Martin *et al.*, 2010; Patra, 2013) and nitrate (alternative hydrogen  
79 acceptor; Yang *et al.* 2016) reduce CH<sub>4</sub> emissions are different, it may be practically  
80 useful to combine these mitigation strategies.

81 Klop *et al.* (2016) fed lipid and nitrate alone or in combination to dairy cows  
82 and found no evidence for any negative interactions between strategies for either  
83 CH<sub>4</sub> emissions or animal performance. Similarly in non-lactating dairy cows  
84 (Guyader *et al.*, 2015), there were no interactions between nitrate and tea saponins.  
85 Interactions between mitigation strategies have not been explored to date in beef  
86 cattle. The main hypotheses addressed in this study were that the effects on CH<sub>4</sub>  
87 emissions of increasing lipid or including nitrate in diets of finishing beef cattle would  
88 be additive and that there would be no adverse effects upon animal performance.  
89 The nutritional strategies used were based on those reported previously (Troy *et al.*,  
90 2015; Duthie *et al.*, 2016).

91

## 92 **Materials and Methods**

93 This experiment was conducted at Scotland's Rural College (SRUC) Beef and  
94 Sheep Research Centre in Edinburgh in 2014. The experimental protocol was  
95 approved by SRUC's Animal Welfare and Ethical Review Body, the Animal  
96 Experiments Committee and was conducted in accordance with the requirements of  
97 the UK Animals (Scientific Procedures) Act, 1986.

98

### 99 *Experimental design, animals and diets*

100 The experiment was a 2 × 4 factorial (breed × dietary treatment) design. The basal  
101 diet contained 550 forage (grass and whole crop barley silages): 450 concentrate  
102 (g/kg DM). The four dietary treatments were assigned according to a 2 x 2 factorial  
103 arrangement where the control diet (CTL) contained rapeseed meal as the main  
104 protein source which was replaced either with nitrate (NIT, 21.5 g nitrate/kg DM) or  
105 maize distillers dark grains (MDDG) to increase diet lipid concentration or both  
106 nitrate and MDDG (COMB). Forage to concentrate ratio was maintained constant by  
107 varying the amounts of barley included in the diets. Nitrate was added in the form of  
108 calcium ammonium nitrate (Calcinit; Yara, Oslo, Norway). The ingredient and  
109 nutritional composition of each dietary treatment are given in Table 1. The steers  
110 were offered diets *ad libitum*. Feed samples were analysed for DM, ash, CP, ADF,  
111 NDF, starch and ether extract (Ministry of Agriculture Fisheries and Food, 1992), and  
112 gross energy (GE) by adiabatic bomb calorimetry.

113 The 80 cross-bred steers (13 to 15 months of age at start of performance trial)  
114 used were from a rotational cross between pure-bred Aberdeen Angus or Limousin  
115 sires and cross-bred dams of those genotypes and are referred to as AAx and LIMx,

116 respectively. Thus, 20 steers (10 of each breed) were allocated to each dietary  
117 treatment. To avoid animals not adapted to nitrate gaining access to dietary nitrate,  
118 each treatment was allocated to one pen (four pens in total). Treatments were  
119 balanced for sire within each breed and BW at the start of the experiment. Fresh  
120 water was provided *ad libitum* using a water trough, and diets were offered using 32  
121 electronic feeders (eight per pen, HOKO, Insentec, Marknesse, The Netherlands) to  
122 record individual animal feed intakes. All steers were bedded on wood fibre and  
123 sawdust to ensure that consumption of bedding did not contribute to nutrient intake.

124 Steers were adapted to the experimental diets in two stages. In stage one  
125 (days -64 to -36 in relation to the start of the performance test on day 0), the animals  
126 were all adapted to the control diet and trained to use the electronic feeders. In stage  
127 two (days -35 to 0), steers were adapted to the treatments over a 5 week period.  
128 Dietary treatments (nitrate and MDDG) were progressively incorporated into the NIT,  
129 MDDG and COMB treatments at 25% (day -35), 50% (day -28), 75% (day -21) and  
130 100% (day -14) of the final dietary inclusion.

131

### 132 *Blood met-haemoglobin (MetHb) measurements*

133 Blood MetHb is formed when nitrite, arising from reduction of nitrate in the rumen,  
134 reacts with haemoglobin to form MetHb which is incapable of oxygen transport and  
135 responsible for acute toxicity (Bruning-Fann and Kaneene, 1993). Blood MetHb  
136 concentrations were monitored in all steers receiving dietary nitrate. Blood samples  
137 were taken 3 h after fresh feed was offered, when MetHb concentrations were  
138 expected to be greatest (van Zijderveld *et al.*, 2010). Samples were taken on days -  
139 34 (25%), -27 (50%), -13 (100%), -6 (100%) and 1 (100%) where 100% is the final  
140 dietary inclusion of nitrate. Blood samples were taken from the caudal vein into two

141 evacuated tubes (Vacuette 9 ml LH Lithium Heparin, Vacuette, Griener Bio One Ltd.,  
142 Gloucestershire, UK). The samples were immediately combined to give one tube  
143 from which air was excluded, sealed and kept on ice until blood MethHb  
144 concentrations were measured within 2 h of sampling by co-oximetry (Stat Profile  
145 Critical Care Xpress, Nova Biomedical U.K., Cheshire, UK).

146

147 *56 day performance test*

148 After full adaptation to the experimental diets, performance and feed efficiency were  
149 characterised for all steers over a 56 day test period (days 0 to 56). Steers were  
150 maintained under controlled conditions, where group sizes within the pen remained  
151 constant. Individual DM intake (DMI, kg/day) was recorded for each animal using the  
152 electronic feeding equipment and BW was measured weekly, before fresh feed was  
153 offered, using a calibrated weigh scale. For all steers, ultrasonic fat depth was  
154 obtained at the 12th/13th rib at the start (FD0) and end (FD1) of the 56 day test  
155 using industry-standard equipment (Aloka 500, BCF Technology LTD, UK). Images  
156 were analysed using Matrox Inspector 8 software (Matrox Video and Imaging  
157 Technology Europe Ltd., Middlesex, UK).

158

159 *Respiration chamber measurements*

160 Seventy-two of the steers, balanced for breed and treatment were chosen for  
161 respiration chamber measurements. The steers remained on the same diets and in  
162 the same pens as described above prior to entering the respiration chamber facility.  
163 The steers were allocated to six respiration chambers over a 12 week period using a  
164 4 (dietary treatment) x 6 (chamber) randomised block design which was replicated  
165 three times such that each dietary treatment was measured in each respiration



166 chamber three times over the 12 week period. Prior to entering the respiration  
167 chambers the steers were housed in training pens, identical in size and shape to the  
168 pens inside the chambers, for a period of one week, to adapt to individual housing.  
169 The steers were allocated to chambers to minimise variation in BW on entry into the  
170 respiration chambers between blocks; thus the heaviest steers for each treatment  
171 were included in the first block. The steers remained in the respiration chambers for  
172 3 days, during which time they were fed *ad libitum* once daily. Data for DMI during  
173 the 3 day chamber measurement period were averaged per animal. One chamber  
174 malfunctioned from weeks 1 to 6, which resulted in the requirement for a thirteenth  
175 week of chamber analysis to obtain measurements from each of 72 steers. Full  
176 details of the methods are described in Troy *et al.* (2015).

177

#### 178 *Rumen sampling and analysis*

179 Rumen fluid samples were taken to assess long term changes in rumen volatile fatty  
180 acid (VFA) molar proportions from each animal on five occasions: before adaptation  
181 to dietary treatments (day -42); during adaptation (day -28); pre-performance test  
182 (day -11); end of performance test (day 56); immediately after leaving respiration  
183 chambers. Samples, approximately 50 ml rumen liquid, were taken before fresh feed  
184 was offered, by inserting a stomach tube (16 × 2700 mm Equivet Stomach Tube,  
185 JørgenKruuse A/S, Langeskov, Denmark) nasally and aspirating manually. This  
186 liquid was filtered through two layers of muslin. A 5 mL sample of the filtered liquid  
187 was deproteinised by adding 1 mL metaphosphoric acid (215 g/l) and 0.5 ml  
188 methylvaleric acid (10 g/l) was added as an internal standard. These samples were  
189 stored at -20 °C between collection and analysis. Volatile fatty acid concentrations  
190 were determined by high performance liquid chromatography (Rooke *et al.* 1990).

191

192 *Calculations and statistical analyses*

193 Data from one steer from the 56 day test period was discarded as the steer was  
194 removed from the trial for health reasons unconnected to the diets and treatments  
195 imposed, leaving data from 79 steers available for analysis. Growth was modelled by  
196 linear regression of BW against test date, to obtain average daily gain (ADG), mid-  
197 test BW (mid-BW) and mid-test metabolic BW (mid-MBW,  $BW^{0.75}$ ). Mean DMI over  
198 the 56 day period was expressed as kg per day or as a proportion of mid-BW and  
199 mid-MBW. Feed conversion ratio (FCR) was calculated as average DMI (kg/day) /  
200 ADG. Residual feed intake (RFI) was calculated as deviation of actual DMI (kg/day)  
201 from DMI predicted based on linear regression of actual DMI on ADG, mid-MBW and  
202 FD1 (Basarab *et al.*, 2003).

203 Statistical analyses of performance data were conducted using the mixed  
204 procedure of SAS software (SAS 9.3 for Windows; SAS Inst. Inc., Cary, USA) with  
205 the fixed effects of breed, nitrate and lipid. In addition, in the analysis of FD1 and  
206 FD2, the deviation from the breed mean of FD0 was included as a covariate. In the  
207 analysis of the respiration chamber data, fixed effects were breed, nitrate and lipid,  
208 while the random effects were week of chamber measurement and chamber. The  
209 interactions, breed × nitrate, nitrate × lipid, breed × lipid and breed × nitrate × lipid  
210 were included as fixed effects in each model when these effects proved significant ( $P$   
211 < 0.05).

212 Changes in MetHb concentration were analysed using a repeated measures  
213 design where the fixed effects were breed, lipid and time and their interactions. As  
214 significant time × breed × lipid interactions were found, a two factor (breed × lipid)  
215 ANOVA was then performed for each time to characterise this interaction. Where a

216 significant breed x lipid interaction was detected, differences between individual  
217 treatments were characterized using LSD.

218 Molar proportions of VFA in rumen fluid when steers left the respiration  
219 chamber were analysed using fixed effects of breed, nitrate and lipid and their  
220 interactions with week of measurement as a random effect. For differences in the  
221 ratio, acetate to propionate, between samples taken at different stages of the  
222 experiment, a split plot ANOVA was used where sample was a split plot within steer  
223 and the effects of breed, nitrate, lipid and sample and their interactions were  
224 included in the model. Data for samples taken prior to introduction of dietary  
225 treatments (day -42), were included as a covariate in the model to control for pre-  
226 existing differences between steers in VFA pattern. For all analyses data are  
227 reported as means with their standard errors of the mean unless indicated otherwise.  
228 Probability values of  $P < 0.05$  were deemed to be significant, while probability values  
229  $P > 0.05$  and  $P < 0.1$  were deemed to indicate a tendency.

230

## 231 **Results**

### 232 *Met-haemoglobin response to dietary nitrate*

233 MetHb concentrations were low ( $< 1\%$  total haemoglobin (Hb), Figure 1) when nitrate  
234 was included at 25 and 50% of the maximum inclusion. Adding 100% nitrate  
235 increased MetHb concentrations ( $P < 0.001$ ); mean values were greater on day -6  
236 (7.9% total Hb) than on day -13 (2.9% total Hb) or day 1 (2.2% total Hb). There was  
237 a significant time x breed x nitrate interaction ( $P < 0.01$ ). On day -13, Met-Hb  
238 concentrations were greater ( $P < 0.01$ ) on the COMB than the NIT treatment.  
239 However, on day -6, AAx steers on treatment COMB had greater MetHb  
240 concentrations (4.1% SEM 0.65) than other treatments (1.6% SEM 0.25). For

241 individual steers, the highest values for MetHb concentration recorded were 13.0,  
242 20.5 and 7.6 % total Hb on days -13, -6 and 1 respectively. Clinical signs of toxicity  
243 are considered to become apparent at MetHb values of 30 to 40% total Hb (Bruning-  
244 Fann and Kaneene, 1993).

245

246 As there were no interactions between breed and dietary treatment for any  
247 measurement, for clarity, results in Tables 2 to 4 are presented as main effects of  
248 breed and dietary treatment.

249

#### 250 *Performance traits*

251 At the start of the experiment the treatments were balanced for age and BW. Thus,  
252 age at the start of the test period (AgeST) and Mid-BW did not differ across dietary  
253 treatments ( $P>0.05$ , Table 2). DMI was not affected by the inclusion of nitrate or lipid  
254 ( $P>0.05$ ). However, steers receiving dietary nitrate achieved poorer ADG throughout  
255 the 56 day test ( $P<0.01$ ). The inclusion of nitrate or lipid did not affect fat depth at the  
256 end of the 56 day test (FD1;  $P>0.05$ ). Steers receiving dietary nitrate were less  
257 efficient (greater FCR;  $P<0.05$ ) than those not receiving nitrate, although this  
258 difference was not observed for RFI. In contrast dietary lipid did not affect feed  
259 efficiency ( $P>0.05$ ). AgeST and Mid-BW did not differ between breeds ( $P > 0.05$ ).  
260 AAx steers achieved greater ADG compared to LIMx steers ( $P<0.01$ ). DMI was  
261 greater in AAx compared to LIMx steers, whether expressed daily ( $P<0.001$ ), or as a  
262 proportion of BW ( $P<0.001$ ). FD1 was greater in AAx compared to LIMx steers ( $P <$   
263  $0.05$ ). Due to the higher levels of DMI and FD1, AAx steers were less efficient with  
264 greater RFI scores than LIMx steers ( $P<0.01$ ).

265

266 *Methane and hydrogen emissions*

267 Steers receiving treatments which included nitrate produced less CH<sub>4</sub> and more  
268 hydrogen (Table 3) than those treatments without added nitrate, expressed on either  
269 a daily or on a DMI basis ( $P<0.001$ ). Increasing the lipid content of the diets had no  
270 effect on CH<sub>4</sub> or H<sub>2</sub> emissions. When expressed on a GE basis, CH<sub>4</sub> emissions  
271 (kJ/MJ GE intake) were reduced when lipid was included in the diet. There were no  
272 significant interactions between inclusion of nitrate and lipid on CH<sub>4</sub> emissions on  
273 either a daily ( $P=0.59$ ) or DMI ( $P=0.82$ ) basis. **There were no differences in CH<sub>4</sub>**  
274 **emissions when calculated /kg ADG for any nutritional treatment.** AAx steers were  
275 heavier than the LIMx steers ( $P<0.01$ ) and had a higher DMI during the chamber  
276 period ( $P<0.001$ ). Therefore, they produced more CH<sub>4</sub> on a daily basis ( $P<0.001$ ). In  
277 contrast, the LIMx steers produced more CH<sub>4</sub> on a DMI basis ( $P<0.05$ ). Breed had  
278 no effect on H<sub>2</sub> emissions on either a daily or DMI basis.

279

280 *Volatile fatty acid molar proportions*

281 When nitrate was included in the diets, acetate molar proportions were greater  
282 ( $P<0.001$ ), those of propionate less ( $P<0.01$ ) and therefore acetate to propionate  
283 ratio (APR) greater ( $P<0.001$ ) in rumen samples taken when steers left the  
284 respiration chambers (Table 4). Increasing the lipid concentration of the diet also  
285 increased ( $P < 0.05$ ) the molar proportions of acetate. There were greater molar  
286 proportions of acetate ( $P<0.01$ ) and lesser propionate proportions ( $P<0.05$ ) in LIMx  
287 than AAx steers. The APR ratio differed ( $P<0.001$ ) in samples taken at different  
288 times during the experiment (Figure 2), increasing as the experiment progressed.  
289 APR in samples taken prior to introduction of dietary treatments was a significant  
290 covariate ( $P<0.01$ ) in the model indicating that individual steer VFA pattern prior to

291 inclusion of treatments influenced VFA pattern throughout the experiment. The main  
292 effects of breed and treatment across the experiment were consistent with samples  
293 taken when steers left the respiration chambers (Table 4). Thus, LIMx steers had  
294 greater APR than AAx steers ( $P<0.05$ ) and inclusion of both nitrate ( $P<0.001$ ) and  
295 lipid ( $P<0.05$ ) increased APR.

296

297

## 298 **Discussion**

299 This study extended those of Troy *et al.* (2015) and Duthie *et al.* (2016) by using a  
300 factorial design to investigate whether the effects of individual treatments to reduce  
301 CH<sub>4</sub> emissions were additive and to characterize the consequences for animal  
302 performance. The diets were formulated from feedingstuffs practical for use in beef  
303 cattle systems. To maintain CP constant, MDDG and nitrate replaced dietary  
304 rapeseed meal. Thus the increase in dietary lipid concentration achieved with MDDG  
305 was modest. However, this was representative of what is practically achievable using  
306 by-product feeds. To achieve higher lipid concentrations, it would have been  
307 necessary to use materials from which oil could have been extracted for human food  
308 consumption. Since a main objective was to investigate the combined effects of lipid  
309 and nitrate, the COMB treatment inevitably contained a higher CP concentration than  
310 the other treatments. However, there were no interactions for any performance  
311 measurement between lipid and nitrate and thus no adverse effects of the higher CP.

312

### 313 *Diet effects*

314 *Nitrate.* Addition of nitrate to the diet reduced CH<sub>4</sub> emissions, a consistent finding  
315 across many studies (see review by Lee and Beauchemin, 2014 and more recent

316 studies including: Newbold *et al.*, 2014; Guyader *et al.*, 2015, 2016; Lee *et al.*, 2015;  
317 Troy *et al.*, 2015; Veneman *et al.*, 2015; Klop *et al.*, 2016). Unexpectedly, the  
318 reduction in CH<sub>4</sub> from added nitrate was only 10% or 2.2 g/kg DM less than for diets  
319 not containing nitrate. As H<sub>2</sub> consumed in reduction of 1 mole nitrate is equivalent to  
320 that used in formation of 1 mole CH<sub>4</sub>, the reduction in CH<sub>4</sub> was only 45% of the  
321 theoretical maximum possible for the dietary nitrate inclusion. A 17% (4.4 g/kg DMI)  
322 reduction in CH<sub>4</sub> yield (80% of theoretical maximum) was reported by Troy *et al.*  
323 (2015) who used very similar experimental conditions to the present experiment. The  
324 meta-analysis of Lee and Beauchemin (2014) predicted that the amount of nitrate  
325 used would have reduced CH<sub>4</sub> emissions by 18%. A more recent analysis of the  
326 efficacy of nitrate, including the studies cited above (Rooke *et al.*, 2016) found that a  
327 mean inclusion of 21g nitrate / kg DMI, reduced mean CH<sub>4</sub> (g/kg DMI) by 21%.

328 The lower than expected reduction in CH<sub>4</sub> by nitrate was accompanied by  
329 reduced FCR in nitrate-fed animals. Again, the reduction in animal performance was  
330 unexpected as in none of the studies reviewed by Lee and Beauchemin (2014) or  
331 most recent studies (Li *et al.*, 2013; de Raphelis-Soissan *et al.*, 2014; Lee *et al.*,  
332 2015; Veneman *et al.*, 2015; Duthie *et al.*, 2016; Klop *et al.*, 2016) has animal  
333 performance been compromised by inclusion of nitrate. However, Guyader *et al.*  
334 (2016) reported reduced fat and protein corrected milk yield when both nitrate and  
335 extruded linseed were added to the diet and Hegarty *et al.* (2016) in a feedlot study  
336 using high grain diets (700 g / kg DM) reported reduced DMI, ADG and FCR when  
337 nitrate replaced urea. In the current experiment, since DMI was not changed when  
338 nitrate was added to the diet, then the poorer FCR must have been due to alterations  
339 in nutrient supply or utilization.

340 Although MetHb concentrations of greater than 15% total Hb were observed  
341 during adaptation to nitrate, these were isolated occurrences and were substantially  
342 less than the 30 to 40% total Hb considered to lead to clinical toxicity (Bruning-Fann  
343 and Kaneene, 1993). As reduced performance is the most likely response to mild or  
344 subclinical toxicity, then this cannot be excluded as a reason for the poorer FCR in  
345 nitrate-fed animals. Another possibility is that rumen microbial protein synthesis and  
346 therefore host animal amino acid supply may have been less than expected. The  
347 reduction in CH<sub>4</sub> attributed to nitrate was only 45% of the theoretical maximum. This  
348 implies that there was a corresponding reduction in the conversion of nitrate to  
349 ammonia. When the degradable protein supply to the rumen (ERDP) was estimated  
350 according to AFRC (1993) for diets CTL and NIT, ERDP supply was greater than  
351 requirement (1.14 and 1.18-fold respectively; 9.9 and 10.6 g ERDP/MJ fermentable  
352 ME, FME). When the ERDP supply from nitrate was reduced to 0.45 of that supplied  
353 by nitrate on diet NIT, ERDP supply was reduced to 0.99 of requirement or 9.0 g  
354 ERDP/MJ FME. However, estimated metabolisable protein supply to the animal was  
355 in excess of requirement (1.40 (CTL), 1.35 (NIT) and 1.30 fold (NIT with reduced  
356 ERDP supply) and therefore overall no reduction in performance as a result of  
357 reduced ERDP supply would have been expected as a consequence of a reduction  
358 in conversion of nitrate to ammonia. A further possibility is that as nitrate, a non-  
359 protein nitrogen source of ERDP replaced rapeseed meal in the diet then protein  
360 supply may have been impaired. However, this is unlikely as the reduction in FCR  
361 was a main effect of nitrate and so was also observed in diet COMB where both  
362 nitrate and MDDG were included in the diet and protein supply to both rumen and  
363 animal would have been in excess.



364 *Lipid.* Because inclusion of MDDG in diets was limited by the need to avoid excess  
365 CP intake, the increase in lipid content of the diet was modest (12 g/kg DMI) and the  
366 overall reduction in CH<sub>4</sub> emissions was not significant (P=0.12) on a g/kg DMI basis  
367 and only became significant on a kJ/MJ basis. However, the numerical decrease in  
368 CH<sub>4</sub> emissions (g/kg DMI) of 4% (3% for a 10 g/kg diet DM increase in lipid) was  
369 consistent with the meta-analysis of Martin *et al.* (2010) of a reduction in CH<sub>4</sub> (g/kg  
370 DMI) of 3.8% for a 10 g/kg DM addition of supplementary fat.

371 *Nitrate and lipid.* A primary aim of the experiment was to investigate whether the  
372 effectiveness of different nutritional methods for reducing CH<sub>4</sub> were additive. Since  
373 the interaction between treatments was not significant (P=0.82) for CH<sub>4</sub> yield (g/kg  
374 DMI) then there was no evidence that the effects of nitrate and lipid were not  
375 additive. Most *in vivo* studies investigating different strategies for reducing CH<sub>4</sub> have  
376 either compared different treatments (e.g. van Zijderveld *et al.*, 2011a; El-Zaiat *et al.*,  
377 2014) or the combined effects of treatments (van Zijderveld *et al.*, 2011b; Li *et al.*,  
378 2013; Caetano *et al.*, 2016) but have not adopted the factorial design necessary to  
379 quantify interactions between treatments. Van Zijderveld *et al.* (2010) compared  
380 nitrate and sulphate when fed to sheep and found the effects of treatments on CH<sub>4</sub>  
381 emissions were additive. Similarly de Raphelis-Soissan *et al.* (2014) fed nitrate and  
382 *Propionibacterium acidipropionici* to sheep and Klop *et al.* (2016) nitrate and  
383 docosahexaenoic acid to dairy cows and again treatment effects on CH<sub>4</sub> emissions  
384 were additive. Thus in agreement with the current study, there is no evidence that  
385 the effects of nutritional mitigation strategies for reducing CH<sub>4</sub> are not additive.

386 The additive nature of mitigation treatments is of practical importance. The  
387 extent to which lipid-containing feeds can be incorporated into diets is limited by the  
388 need to avoid reductions in fibre digestion when lipid concentrations are greater than

389 70 g/kg DM (Patra 2013). During adaptation to nitrate, in both the current experiment  
390 and Duthie *et al.* (2016), blood Met-Hb concentrations did not increase until 15 g  
391 nitrate / kg diet DM was included in the diet. This is likely because the apparent  
392 efficiency of CH<sub>4</sub> reduction by nitrate decreases as nitrate inclusion increases (Leng  
393 2014). Thus, potential adverse effects of mitigation strategies such as toxicity and  
394 impaired animal performance could be avoided by feeding lesser amounts of nitrate  
395 than in the present experiment.

#### 396 *Breed effects*

397 Total CH<sub>4</sub> emissions were greater in AAx than LIMx steers as noted before (Rooke  
398 *et al.*, 2014) primarily because of greater DMI. However unlike Rooke *et al.* (2014),  
399 CH<sub>4</sub> yield (g/kg DMI) was lower in AAx than LIMx steers. This was probably because  
400 of a faster rumen turnover rate in the AAx steers, an effect that has been well  
401 documented and included in empirical prediction equations for CH<sub>4</sub> yield (Sauvant  
402 and Giger-Reverdin 2009; Ramin and Huhtanen 2013). Similarly the smaller  
403 proportion of acetate in rumen fluid samples from AAx steers was consistent with the  
404 meta-analysis of Nozière *et al.* (2011). In the performance trial, the greater DMI of  
405 AAx steers noticed when CH<sub>4</sub> was measured was also evident and resulted in  
406 greater ADG in AAx steers. However when the RFI of the steers was assessed, the  
407 LIMx steers were more efficient (smaller RFI) than the AAx steers. The lower CH<sub>4</sub>  
408 emissions (g/kg DMI) and higher propionic molar proportion achieved with the AAx  
409 steers is not consistent with the differences in RFI. There is no obvious reason for  
410 these discrepancies but it should be noted that the performance and respiration  
411 chambers measurements were made sequentially.

412 In conclusion, inclusion of nitrate in the diet reduced CH<sub>4</sub> emissions in  
413 growing beef cattle although the efficacy of nitrate was less than in previous work

414 (Troy *et al.*, 2015). Whereas Duthie *et al.* (2016) recorded no changes in animal  
415 performance when nitrate was fed, in the present experiment, growth rate and feed  
416 conversion ratio were poorer when nitrate was included in the diet. However, when  
417 both increased dietary lipid and nitrate inclusion were combined there was no  
418 evidence of any interaction between treatments in CH<sub>4</sub> emissions or performance  
419 traits. Therefore, combining different nutritional treatments to mitigate CH<sub>4</sub> emissions  
420 could be a useful means of achieving reductions in CH<sub>4</sub> emissions without adverse  
421 effects.

422

423

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**Table 1.** Ingredient and chemical composition (g/kg DM) of the experimental diets

Treatment	Control	Nitrate	MDDG	Combined
Ingredient Composition				
Barley	336	388	289	263
Grass silage	210	211	209	210
Whole crop barley silage	347	347	346	346
Rapeseed meal	79	0	0	0
Calcinit <sup>1</sup>	0	25	0	24
Maize distiller's dark grains	0	0	128	127
Molasses	19	20	19	19
Minerals <sup>2</sup>	9	9	9	9
Chemical Composition				
DM, g/kg	533	531	533	533
Ash	52	48	51	51
CP	135	141	136	162
ADF	184	166	184	183
NDF	308	295	317	313
Starch	281	308	264	250
Ether extract	25.0	23.4	36.7	35.9
GE, MJ/kg DM	18.1	17.6	18.5	18.0
Estimated ME, MJ/kg DM	11.7	11.5	12.0	11.7

<sup>1</sup>Contained (g/kg DM): nitrate, 757; Ca, 225.

<sup>2</sup>Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; (µg/kg): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500.



**Table 2.** Effect of breed and dietary treatment on growth, fat depth, feed intake and feed efficiency of Aberdeen Angus- (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB)

Treatment	Breed		Significance <sup>2</sup>	Treatment				SEM <sup>1</sup>	Significance <sup>2</sup>	
	AAx	LIMx		CTL	NIT	MDDG	COMB		Nitrate	Lipid
AgeST (days)	417	411	NS	414	414	413	415	5.3		
Mid-BW (kg)	542	539	NS	547	543	538	534	17.5		
Mid-MBW (kg <sup>0.75</sup> )	112	112	NS	113	112	112	112	2.7		
ADG (kg/day)	1.75	1.56	**	1.74	1.54	1.72	1.63	0.076	**	
DMI (kg/day)	12.15	11.07	***	11.78	11.43	11.76	11.47	0.425		
DMI/BW(g/kg)	22.44	20.58	***	21.60	21.08	21.90	21.47	0.483		
DMI/MBW(g/kg <sup>0.75</sup> )	108.1	99.1	***	104.3	101.6	105.3	103.1	2.31		
FCR (kg, kg)	7.02	7.21	NS	6.85	7.52	6.90	7.18	0.269	*	
RFI (kg)	0.24	-0.24	**	-0.08	0.06	-0.02	0.04	0.231		
FD1 (mm) <sup>3</sup>	9.14	8.05	**	8.40	8.86	8.81	8.31	0.663		

<sup>1</sup>SEM for 10 observations.

AgeST, Age at start of test; Mid-BW, mid-test BW; Mid-MBW, mid-test metabolic BW; ADG, average daily gain at the end of the 56 day test; FCR, feed conversion ratio; RFI, residual feed intake; FD1, fat depth at the 12/13<sup>th</sup> rib at the end of the 56 day test.

<sup>2</sup> There were no significant ( $P > 0.05$ ) interactions between breed and dietary treatment or between nitrate and lipid.

<sup>3</sup> Deviation from breed mean of FD0 (measured at start of 56 day performance test) fitted as covariate.

\* $P < 0.05$ ; \*\* $P < 0.01$ ;  $P < 0.001$

**Table 3.** Effect of breed and dietary treatment on methane and hydrogen emissions of Aberdeen Angus-sired (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB)

	Breed		Significance	Treatment				SEM <sup>1</sup>	Significance <sup>2</sup>	
	AAx	Limx		CTL	NIT	MDDG	COMB		Nitrate	Lipid
BW (kg)	669	648	**	677	650	652	655	9.5		
DMI										
kg/day	11.0	9.3	***	10.3	9.8	10.2	10.2	0.51		
g/kg BW	16.4	14.3	***	15.3	15.0	15.6	15.6	0.65		
Methane										
g/day	241	214	***	246	219	238	210	12.2	***	
g/kg DMI	22.0	23.2	*	24.0	22.1	23.4	20.9	0.94	***	
g/kg ADG <sup>3</sup>	154	163		161	163	161	147	7.9		
kJ/MJ GEI	67.5	71.1	*	73.3	69.5	70.2	64.2	2.9	**	*
Hydrogen										
g/day	0.86	0.67		0.45	0.99	0.40	1.04	0.095	***	
g/kg DMI	0.06	0.07		0.04	0.10	0.04	0.10	0.009	***	
kJ/MJ GEI	0.56	0.58		0.35	0.81	0.30	0.82	0.073	***	
H <sub>2</sub> :CH <sub>4</sub> molar ratio	0.025	0.025		0.015	0.035	0.01	0.04	0.003	***	

DMI, DM intake; GEI, Gross Energy intake;

<sup>1</sup> SEM for 9 observations.

<sup>2</sup> There were no significant ( $P > 0.05$ ) interactions between breed and dietary treatments or between nitrate and lipid.

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$

<sup>3</sup> Calculated from methane (g/kg DMI) and DMI and ADG from Table 2.

**Table 4.** Effect of breed and dietary treatment on VFA (mmol/mol) molar proportions in rumen fluid from Aberdeen Angus-sired (AAx) and Limousin-sired (LIMx) steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB). Rumen samples taken when steers left respiration chambers

Treatment	Breed			Treatment				SEM <sup>1</sup>	Significance <sup>2</sup>	
	AAx	Limx	Significance	CTL	NIT	MDDG	COMB		Nitrate	Lipid
Acetate	672	689	***	664	685	676	696	7.4	***	*
Propionate	167	155	*	175	154	164	150	7.3	**	
Butyrate	126	123		124	127	125	123	5.8		
Valerate	13	11	†	13	12	12	10	1.0	†	†
Branched chain	34	35		40	34	29	34	2.8		**
Acetate: Propionate Ratio	4.0	4.5	**	3.9	4.5	4.2	4.7	0.33	***	

<sup>1</sup>SEM given for 9 observations.

<sup>2</sup>There were no significant interactions ( $P > 0.05$ ) between breed and treatments

†,  $P < 0.1$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

## Legends for Figures

Figure 1. Changes in blood met-haemoglobin (% total haemoglobin) during adaptation to nitrate-containing diets. Samples were obtained from cross bred Aberdeen Angus (AAx) or Limousin (LimX) steers offered diets containing nitrate alone (NIT) or nitrate and maize distillers dark grains (COMB). Blood samples were taken when 25% (day -34, where day 0 was the start of the performance test), 50% (day -27) and 100% (days -13, -6 and 1) of the dietary nitrate (100% = 21.5 g nitrate / kg DM) inclusion was offered.

Figure 2 Changes in the ratio (mol /mol) of acetate to propionate during experiment in rumen fluid from steers fed four dietary treatments in which rapeseed meal (CTL) was replaced by nitrate (NIT) or lipid (MDDG) alone or in combination (COMB). Samples were taken during adaptation to basal diet (Prelim, day -42); during introduction of experimental treatments (Adapt, day -28); prior to the start (Start, day -11) and at the end (End, day 56) of performance measurement and when steers left respiration chambers (Chamber).

Figure 1. Changes in blood met-haemoglobin (% total haemoglobin) during adaptation to nitrate-containing diets. Samples were obtained from cross bred Aberdeen Angus (AAx) or Limousin (LimX) steers offered diets containing nitrate alone (NIT) or nitrate and maize distillers dark grains (COMB). Blood samples were taken when 25% (day -34, where day 0 was the start of the performance test), 50% (day -27) and 100% (days -13, -6 and 1) of the dietary nitrate (100% = 21.5 g nitrate / kg DM) inclusion was offered.

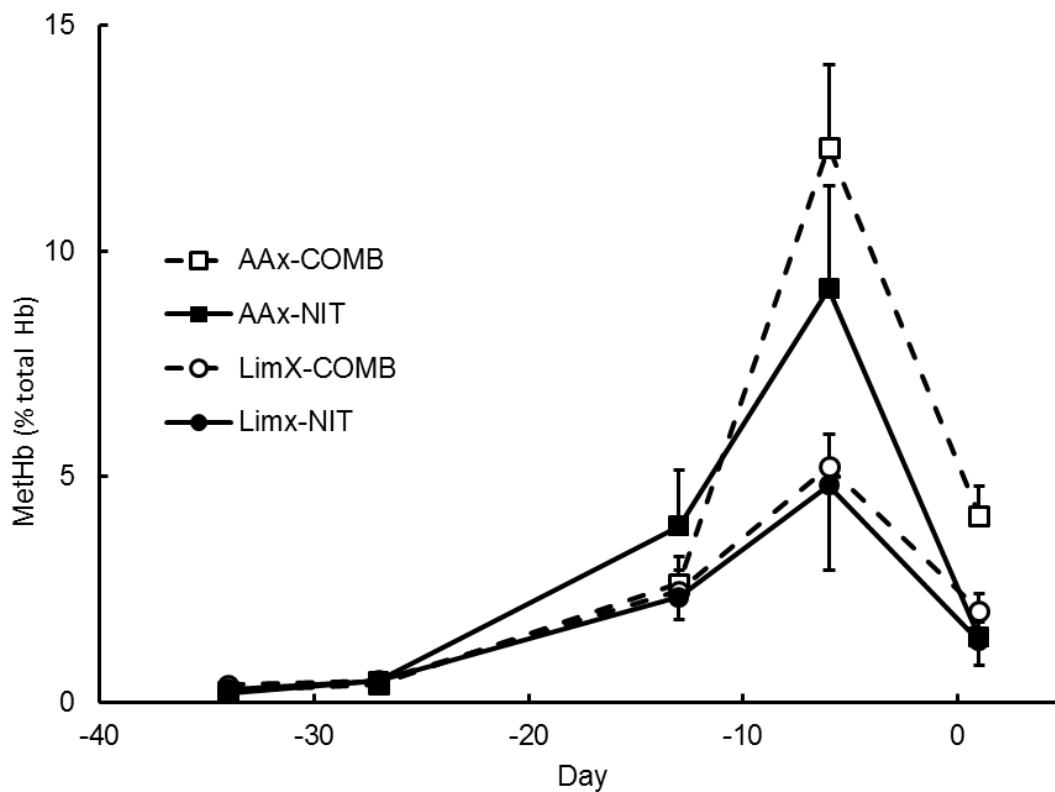


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Department

