Analysis of archaeal ether lipids in bovine faeces

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ABSTRACT

This study evaluated concentrations of archaeol, a lipid biomarker for Archaea and, presumably, methanogens in cattle faeces. Twelve continental cross-bred steers were allocated at random to receive either a concentrate based diet or a grass silage based diet. Lipids were extracted from dried faeces, separated into specific compound classes and analysed by gas chromatography/mass spectrometry, and CH4 production was estimated using the sulphur hexafluoride (SF6) technique. Steers fed the grass silage based diet consumed less feed (9.15 versus 11.43 kg dry matter (DM)/d; SED = 0.824; P<0.05), emitted more CH4 (341 versus 174 g/d; SED = 60.5; P<0.05) and produced faeces with higher concentrations of archaeol (30.6 versus 5.1 mg/kg DM; SED = 5.42; P<0.001) than those fed the concentrate based diet. Other dialkyl glycerol lipids, such as hydroxyarchaeol, maccrocyclic archaeol and unsaturated archaeol analogues, were not detected. Since the feeds contained no archaeol, it must have been produced in the ruminant digestive tract, most likely by Archaea in the rumen. The relationship between CH4 emission and faecal archaeol concentration for individual animals was weak. Possible explanations include the inherent limitations of the SF6 technique, the sampling regime, selective retention of Archaea in the rumen and possible degradation of the archaeol during gut transit and post excretion. Despite these limitations, faecal lipid biomarker analysis shows potential as a new method to study ruminant methanogens.

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1. Introduction

Methanogens belong to the domain Archaea, and comprise less than 0.05 of rumen prokaryotes (Janssen and Kirs, 2008), but can lead to losses of up to 0.12 of gross energy intake by ruminants as CH4, which is a potent greenhouse gas. Bacterial membrane lipids are usually comprised of a phosphate head group attached to a glycerol backbone esterified to acyl chains in the sn-1,2 positions. In contrast, archaeal membrane lipids are comprised of a phosphate or phosphor–sugar head group attached to a glycerol backbone to which isoprenoid chains are ether–linked at the sn-2,3 positions forming dialkyl glycerol ether (DAGE) lipids. Archaeol (2,3-diphytanyl-O-sn-glycerol) is the simplest DAGE with two phytanyl chains and is

Abbreviations: CON, concentrate rich diet; DAGE, dialkyl glycerol ether; DM, dry matter; GS, grass silage rich diet; LW, live weight.

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Table 1
Ingredient (g/kg) and chemical composition (g/kg dry matter) of the concentrates used for the concentrate and grass silage diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentrate</th>
<th>Grass silage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley grain</td>
<td>820</td>
<td>460</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>100</td>
<td>460</td>
</tr>
<tr>
<td>Molasses</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral/vitamin premix(a)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Vegetable oil(b)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Dry matter, g/kg</td>
<td>866</td>
<td>860</td>
</tr>
<tr>
<td>Ash</td>
<td>56</td>
<td>73</td>
</tr>
<tr>
<td>Crude protein</td>
<td>159</td>
<td>297</td>
</tr>
<tr>
<td>aNDFom</td>
<td>157</td>
<td>138</td>
</tr>
<tr>
<td>Starch</td>
<td>485</td>
<td>235</td>
</tr>
</tbody>
</table>

\(a\) Supplying (per kg of concentrate) 10,000 IU vitamin A, 2000 IU vitamin D\(_3\), 50IU vitamin E, 0.50 mg Se and 10 mg Cu.

\(b\) Goldstar Lakeland Winter Blend 70; Goldstar Oils Ltd., Kilkenny, Ireland.

widespread among Archaea. Other DAGE lipids, including hydroxyarchaeol, macrocyclic archaeol and unsaturated analogues of archaeol are found in some Archaea (Koga and Morii, 2005).

DAGE lipids have been used as biomarkers in a wide range of matrices. Bai et al. (2000) determined the DAGE content of paddy rice soils, as well as phospholipid fatty acids and hydroxy fatty acids of lipopolysaccharides, and used the results to estimate the microbial biomass and community structure. Pancost et al. (2001) investigated the occurrence of DAGE lipids in two Eastern Mediterranean mud dome fields where archaeol was found to be the most abundant diether lipid. Moreover the \(\delta^{13}C\) results obtained for archaeol in all but two of the samples were depleted in \(^{13}C\) (−41.3 to −95.8 %), suggesting that archaeal progenitors were either directly or indirectly involved in \(\text{CH}_4\) production. Recent studies have demonstrated the occurrence of archaeol in faeces of herbivores with foregut (rumen) fermentation, but not in the faeces of other herbivores (Gill et al., 2010).

Our aim was to develop archaeol as a proxy for \(\text{CH}_4\) production in ruminants and perhaps overcome some of the limitations of current techniques of measuring \(\text{CH}_4\) production in ruminants. For example, respiration chambers (Blaxter and Clapperton, 1965) are laborious and cannot be used with grazing animals. The SF\(_6\) technique (Johnson et al., 1994) has been shown to be highly variable and may not be suitable for comparison of \(\text{CH}_4\) emissions among individuals (Pinares-Patiño and Clark, 2008). The first step in this research was to quantify archaeol in bovine faeces and compare variations in its concentration among animals fed the same, and different, diets. The objective was to determine if the concentration of archaeol was positively correlated with \(\text{CH}_4\) production.

2. Materials and methods

2.1. Animals and diets

Twelve continental cross-bred steers (initial live weight (LW) 541 ± 41.8 kg) were blocked according to LW and randomly allocated to one of two dietary treatments based of either grass silage or concentrate being: (1) a fixed allocation (1.25 kg DM/head daily in a single meal) of grass silage with ad libitum concentrates (diet CON); and (2) a fixed allocation (2.58 kg DM/head daily in a single meal) of concentrates with ad libitum grass silage (diet GS). The ingredient composition of the concentrates used for each diet is in Table 1. Samples of feeds were collected daily during the collection weeks and composited by collection week. Chemical analysis methods for feeds are described by McGeough et al. (2010).

2.2. Management of animals

Steers were housed and trained to receive their diet through Calan gates (American Calan Inc., Northwood, NH, USA) so that individual feed intake could be recorded. Steers were adapted to receive their allocated diets over the first two weeks of the study. Feed offered and refused was recorded daily. Steers were fed the same diet throughout the study. Measurements of exhaled/eructated \(\text{CH}_4\) were collected from three steers per treatment after 5 and 16 weeks of provision of their respective diets.

2.3. Animal measurements

Steers remained on the same dietary treatments, but were housed in individual stalls in a separate building for 10 d prior to \(\text{CH}_4\) measurements. The SF\(_6\) technique (Johnson et al., 1994; Hart et al., 2009) was used for individual measurements of \(\text{CH}_4\) production. Boluses containing SF\(_6\), of known release rate (3.1 ± 0.88 mg/day), were put into the rumen 10 d prior to collection of respiratory gases. Evacuated canisters were attached to the head halter and used to collect expired air over 24 h from each steer. A minimum of 3, and up to 5, separate daily samples were collected from each steer for estimation of daily \(\text{CH}_4\) emissions.
Faecal samples were collected once daily over 5 d during the week after CH₄ measurement. Rectal grab samples of faeces (~200 g) were obtained, stored at −20 °C, thawed, composited and dried at 60 °C for 48 h. Faecal samples were not collected at exactly the same time as the CH₄ emissions were made because of experimental constraints, and this may mask subtle relationships between CH₄ emitted and archaeol concentration, especially differences within individual steers fed the same diet.

2.4. Laboratory analysis

An extract of total lipids was obtained from samples (0.5–1 g) of dried, ground (1 mm screen) faeces and feeds through solvent extraction in 200 ml of dichloromethane:acetone (9:1 v/v) for 24 h using a Soxhlet apparatus. Internal standards (22.5 μg each of preg-5-en-3β-ol and hyocholic acid) were added to the extract. An aliquot of the total lipid extract was saponified and initially separated into two fractions as described by Bull et al. (2003). The second of these fractions was further separated by column chromatography using a slightly modified version of the procedures of Bull et al. (1999). The flash column chromatography step was shortened by reducing extraction to a three solvent system being: dichloromethane, dichloromethane:methanol 1:1 and methanol, which were applied in order of polarity to give three fractions identified as: “apolar”, “alcohol” and “polar”.

Analytes in the alcohol fraction were derivatised to their respective trimethylsilyl ethers by adding 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide containing 0.01 trimethylchlorosilane, and 50 μL pyridine to the sample and heating at 70 °C for 1 h. The samples were dissolved in ethyl acetate prior to analysis by gas chromatography (Carlo Erba HRGC 5300 equipped with a non-polar fused silica capillary column, CPSil-5CB, 50 m × 0.32 mm × 0.12 μm, Varian Chrompack, Oxford, UK). Gas chromatography mass spectrometry was conducted using a ThermoQuest TraceMS GC-MS equipped with a column identical to that used for GC analyses. In both cases, the temperature program used was: initial temperature 70 °C, rising to 130 °C at 20 °C/min, then to 300 °C at 4 °C/min, with a final hold at 300 °C for 25 min. Archaeol was identified on the basis of its characteristic mass spectrum (Fig. 1), featuring prominent fragment ions with a mass to charge ratio (m/z) of 130, 278, 412 and 426 (Teixidor and Grimalt, 1992) and quantified by comparison to the internal standard.

Methane and SF₆ were measured in gas samples using gas chromatography (Hart et al., 2009) using a gas chromatograph equipped with both flame ionization and electrochemical detectors.

2.5. Statistical analysis

Effects of dietary treatments were analysed using analysis of variance (Genstat, 2007) with ‘diet’ as a treatment factor and blocking according to measurement week.
3. Results

The chemical composition of the concentrates used for diets CON and CS is in Table 1. The grass silage had a pH of 3.81 and contained 252 g DM/kg and, on a DM basis, 100 g ash/kg, 138 g CP/kg, 511 g aNDFom/kg, 106 g lactic acid/kg, 26.6 g acetic acid/kg, 11.9 g butyric acid/kg and 18.6 g ethanol/kg.

Feed intake, CH₄ emissions and faecal archaeol concentrations are in Table 2. Feed intake was higher (P<0.05) for the ad libitum concentrate diet (11.43 kg/d) compared to the ad libitum grass silage diet (9.15 kg/d). Methane production was higher for the grass silage diet than the concentrate diet, whether expressed on a daily basis (341 g/d versus 174 g/d) or relative to DM intake (37.4 g/kg DM intake versus 15.0 g/kg DM intake). The concentrate diet also resulted in lower (P<0.001) faecal archaeol concentrations compared to the grass silage diet (5.1 mg/kg DM versus 30.6 mg/kg DM; Fig. 2). The relative difference in faecal archaeol concentration (x6) between the silage and concentrate diet was much bigger than the difference in CH₄ production (x2), indicating a possible positive non-linear relationship between them. There was no difference in the relationship between faecal archaeol concentration (mg/kg DM) and CH₄ production (g/kg DM intake) for measurements made in week 5 and 16 of the experiment (Fig. 2). We did not detect any of the other DAGE lipids (i.e., hydroxyarchaeol, macrocyclic archaeol, unsaturated analogues of archaeol), commonly observed in unrelated samples analysed routinely by this laboratory, in feeds or faeces.

4. Discussion

4.1. Methane production

Treatment means for CH₄ production (g/kg DM intake) corresponded closely to the lowest and highest treatment means in the studies reviewed by Beauchemin et al. (2008). Animals fed a predominantly grass silage based diet produced more CH₄ than those fed a predominantly concentrate based diet. The relationship between forage:concentrate ratio and CH₄ production is well recognized in cattle (e.g., Johnson and Johnson, 1995). Methane emissions are lower on high concentrate diets due to several factors, including higher ruminal passage rates, an increase in the flow of reducing equivalents to propionate.

![Fig. 2. Archaeol concentrations in bovine faeces from steers fed concentrate or grass silage diets ad libitum versus methane production as estimated by the SF₆ technique.](image-url)
(Johnson and Johnson, 1995) and direct inhibition of methanogens at low pH (Van Kessel and Russell, 1996). Recent studies suggest that variation in CH₄ production may not be correlated with the population size of rumen methanogens (Morgavi et al., 2010), although this is equivocal since estimates of archaeal populations are likely to have large experimental errors. Another possible source of variation in the relationship between archaeal and CH₄ production is that not all Archaea in the rumen are methanogens. Whilst most rumen Archaea are known to be methanogens, this has not yet been confirmed for Archaea belonging to Rumen Cluster C which are abundant under some dietary conditions (Janssen and Kirs, 2008).

4.2. Faecal dialkyl glycerol ether lipids

This study confirmed that archaeol is generated during passage through the digestive tract, since none was detected in the diets. Several observations indicate that the rumen is the most probable source of archaeol. For example, the population of prokaryotes in the rumen is at least an order of magnitude higher than populations in the hind gut or faeces (Mould et al., 2005), whilst analysis of the 16S RNA gene provided no evidence for a higher proportion of Archaea in the hind gut or faeces compared with the rumen (Lin et al., 1997). The evidence for a rumen origin is implicit in the absence of detectable levels of archaeol derived from the archaeal populations in faeces in non-ruminant herbivores such as horses, zebra, elephant and rhinoceros.

Non-ruminant gastric systems produce CH₄ and there will almost certainly be archaeol that is derived from methanogenic Archaea in these systems, albeit below the limit of detection using the methods employed by this and previous research (Gill et al., 2010). Nonetheless, further studies are required to elucidate the relationships between concentrations of archaeol in faeces, the size of the methanogen population in the rumen, and CH₄ output. The unknown elements of this relationship include selective retention of Archaea in the rumen, differences in the species composition of the methanogen community, which might differ in archaeol concentration per cell, differences in the post-rumen digestibility of DAGE, and the production of archaeol by non-methanogenic Archaea.

There was only a weak relationship between faecal archaeol concentration and CH₄ production/kg DM intake (r = 0.55; P<0.05), despite the large effect of dietary treatment. This may be related to uncertainties about faecal archaeol outlined above, as well as experimental error with the SF₆ technique (Pinares-Patiño and Clark, 2008).

The absence of hydroxyarchaeol, macrocyclic archaeol and unsaturated analogues of archaeol in bovine faeces is consistent with other information about the family level composition of ruminant Archaea (Janssen and Kirs, 2008) and their membrane lipids (Koga and Morii, 2005). The review by Janssen and Kirs (2008) suggested that most rumen Archaea belong to the families Methanobacteriaceae and Methanomicrobiaceae, which do not contain hydroxyarchaeol, macroyclic archaeol or unsaturated archaeol. Some members of the family Methanosarcinaceae contain hydroxyarchaeol and unsaturated archaeol, but are rare in the rumen (0.02–0.03 of the archaeal population). There is some uncertainty about the presence of members of the family Methanococcaceae, which contain hydroxyarchaeol, in the rumen, but these results suggest that they were not present in substantial numbers.

5. Conclusions

Archaeol is produced in the ruminant digestive tract, presumably by methanogenic Archaea within the rumen (Gill et al., 2010). Large differences in faecal archaeol concentration between cattle fed diets with different forage/concentrate ratio mirrored effects on CH₄ production, but further research is needed to before this approach can be used as a direct indicator or CH₄ emissions in ruminants.

Conflict of interest

None.

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