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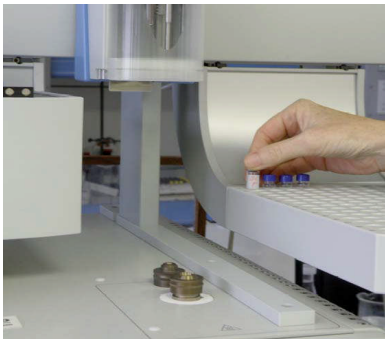
Ether lipids as biomarkers for methanogenic Archaea in the ruminant gastro-intestinal tract



Key external stakeholders:

Policymakers; dairy, beef and sheep farmers.

Practical implications for stakeholders:

Ether lipids are distinctive components of the membranes of methane-producing Archaea and can be used as markers in the following ways:

- To support molecular biology techniques used to estimate the population of methane-producing Archaea in the rumen, helping us to understand problems with some of the molecular methods.
- To provide broad estimates of methane yield (gCH_4/kg DM intake) for groups of animals. This approach does not provide good estimates for individual animals and we are exploring another possible marker technique to use with individuals.
- To provide information about the effect of the rumen environment on methanogens – in particular the ratio of diether to tetraether lipids appears to be indicative for the effect of rumen pH on methanogens and their methane production.

Main results:

There was a poor relationship between concentrations of archaeol and most qPCR-based estimates of total methanogens in rumen fluid. However, there was a highly significant relationship between archaeol and *Methanobrevibacter ruminantium* qPCR estimates, suggesting that the primer set based on this species may have provided the best estimate of methanogen numbers. There was a significant positive relationship between faecal archaeol concentration and methane yield (gCH_4/ kg DM intake), when considering treatment means – but the relationship was weak within dietary treatments. There was no overall relationship between faecal and rumen archaeol concentrations and evidence of differences between animals in the kinetics of methanogens (i.e. in the relationship between what is present in the rumen and what flows out of the rumen). Archaeol was useful as a marker for methanogens within the rumen and suggested differences in the time course of colonization by methanogens of grass and white clover. We have preliminary evidence of variation in the methanogen membrane lipid profiles in response to rumen environment (pH).

Opportunity / Benefit:

These ether lipid marker methods will be useful in allowing researchers to study the effects of dietary short- and long-term diet manipulations, as well as animal genotype, using large groups of animals. The key challenges in methane mitigation research are in understanding between-animal variation, whether due to genetics or management history, and understanding the way in which the rumen adapts to negate dietary treatments. Methanogen membrane lipids are of interest (1) to help in quantifying methanogens, and (2) in understanding how methanogens respond to adverse environments – such as the low pH conditions resulting from high levels of concentrate feeding.

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1. Project background:

Methane production by ruminants is a significant contributor to greenhouse gas emissions in animal production in Ireland, and a source of inefficiency in the conversion of feed energy into milk or meat. It is a particular problem for Ireland because of our large numbers of cattle and sheep. The high-forage diets that are typical for most production systems also lead to high methane emissions. Ireland is also committed to the EU target for a 20% reduction in greenhouse gas (GHG) emissions (from 1990 levels) by 2020. Enteric methane accounts for 13.2% of Ireland's total GHG emissions and 70% of total methane emissions. Progress in reducing enteric methane is essential if we are to meet projected global growth in demand for food products of animal origin. At the same time, reductions in methane output provide a direct benefit for energy supply to animals because methane can represent up to 12% of gross energy intake. Both respiration chambers and the SF_6 technique have been used to identify dietary effects on methane production. However, there remain technical concerns about the SF_6 technique, such as uncertainty about its fate after its release from permeation tubes, and these probably contribute to greater between-animal variation in estimated methane production. The new approach investigated in this project is based on identifying distinctive markers for Archaea, the organisms responsible for methane production, in the rumen. The membranes of Archaea contain distinctive ether lipids that have been used as biomarkers in a wide range of other sample types, including marine sediments, soils and peat. Recent studies at Bristol have demonstrated the occurrence of one of these biomarkers, 2, 3-diphytanyl-O-sn-glycerol (archaeol), in faeces taken from a range of ruminant and pseudo-ruminant species. This project explored variation in ether lipids in samples of intestinal digesta and faeces in order to address the questions listed below.

2. Questions addressed by the project:

- Can we use ether lipid biomarkers to study methanogenic Archaea in the rumen?
- Can we use ether lipids to investigate effects of diet and animal genetics on methanogens (and methane production)?
- What factors affect the relationship between production of ether lipids by methanogenic Archaea in the rumen and their concentration in faeces?
- Can we use ether lipids to identify the diversity and distribution of methanogens in the rumen?

3. The experimental studies:

A series of studies optimized the method for analysis of archaeol, developing more efficient methods for extraction, polar head-group cleavage and fractionation methods, as well as changes to the quantification of archaeol. Briefly, internal standard (1, 2-di-*O*-hexadecyl-*rac*-glycerol) was added to dried sample prior to a monophasic extraction procedure to obtain the total lipid extract (TLE). Removal of polar headgroups from archaeol was then achieved by acid methanolysis, and the TLE was then separated into 'apolar' and 'alcohol' fractions by column chromatography. The alcohol fraction was further trimethylsilylated and then run on the GC-MS. Archaeol was identified and then quantified against an archaeol calibration curve. The same alcohol fraction was filtered and then run on LC-MS for the detection of tetraethers.

Archaeol in rumen fluid was assessed as a total methanogen marker in comparison to the well-established quantitative real-time PCR (qPCR) technique. Genomic DNA was isolated from the ruminal fluid using the established bead beating method. Specific primer sets and probes were used to detect dominant methanogen species: *M. ruminantium, M. smithii, M. stadtmanae* and total methanogen populations, along with a prokaryote reference gene. qPCR was performed using either SYBR green chemistry or FAM dye. Methanogens were quantified relative to the prokaryote *rrs* gene using the equation: $\Delta Ct = 2^{-(Ct\ methanogen - Ct\ reference)} \times 10^6$. ΔCt values were then expressed on a sample DM basis. Relationships between the various estimates of methanogen abundance (based on qPCR and archaeol) were made using simple linear regression (one outlier was identified on the basis of Cook's test and excluded from subsequent

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analysis).

Samples were obtained from three previous animal studies to allow comparison of archaeol concentrations with measured methane production. Firstly, faecal archaeol was compared to CH_4 measurements made using the sulfur hexafluoride (SF_6) tracer technique from beef cattle consuming 6 dietary treatments with varying levels of concentrate. Secondly, faecal archaeol was compared to CH_4 measurements made with dairy cows using respiration chambers from animals consuming a single dietary treatment. Finally, archaeol was assessed in faeces, total rumen contents and solid- and liquid-associated microbial fractions from dairy cows grazing either white clover or perennial ryegrass, with rumen samples taken in the morning and afternoon. Tetraethers were also analysed in some of these samples.

4. Main results:

There was only a weak relationship between archaeol concentrations and total methanogen qPCR estimates, but unexpectedly a very significant ($P < 0.001$) relationship was found between archaeol and *Methanobrevibacter ruminantium* qPCR estimates. Amplification bias with some of the qPCR primers and the dominance of *M. ruminantium* in the rumen may explain the strong relationship with this species.

There was a significant positive relationship ($P < 0.001$) between faecal archaeol and CH_4 production in the SF_6 study; however, the relationship was weak within dietary treatments. To further investigate the relationship, faecal archaeol was then compared to CH_4 measurements made using respiration chambers from animals offered just one dietary treatment. There was a significant positive relationship ($P = 0.007$) between faecal archaeol concentration and methane yield, though stage of lactation also affected the relationship ($P = 0.011$) and this may have been the result of higher dry matter intakes in mid-lactation.

There was a significant 'diet' × 'time' interaction for archaeol concentration in total rumen contents, which may be the consequence of the lower pH, lower NDF, or higher rumen passage rates when cows grazed white clover rather than perennial ryegrass. However, there was no relationship between archaeol concentrations in rumen and faecal samples, probably as a result of differences between cows in the selective retention of methanogens in the rumen.

There was a higher tetraether to diether ratio in the faecal samples from animals consuming a high concentrate diet (in comparison to those consuming a grass silage diet). An increase in the proportion of GDGT-0 may help methanogens under conditions of low pH by reducing the permeability of the cell membrane and thus conserving energy for growth. It will be important to consider both diether and tetraether membrane lipids when looking for relationships with methanogens and methanogenesis.

Overall, it seems the use of archaeol as a marker for methanogenesis is complicated by the issues of selective retention of digesta in the rumen, the wide ranging effects of diet, stage of lactation and individual differences of cattle. However, in comparison to qPCR, this method may be more robust in quantifying the total methanogen populations in the rumen.

5. Opportunity/Benefit:

The primary opportunities from the research are for companies developing anti-methanogen strategies, including dietary or other manipulations. These techniques will be of value in understanding effects on methanogen numbers, as well as the way in which the methanogens respond to nutritional or other challenges. In the longer-term, we expect that the work will contribute to selection strategies for methane production, most likely through indirect routes (i.e. identification of high- or low-emitting animals for use in detailed studies to understand the physiological basis of the phenotype).

6. Dissemination:

Results from this project formed the basis of an invited conference paper at the Greenhouse Gases and Animal Agriculture Conference in UCD, June 2013. Results have also been presented at scientific meetings, including the 8th International Symposium on Herbivore Nutrition and the British Society of Animal Science and Agricultural Research Forum annual meetings.

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Main publications:

McCartney, C.A., Bull, I.D. and Dewhurst, R.J. (2013) 'Chemical markers for rumen methanogens and methanogenesis' *Animal*, 7 (s2): 409-417.

McCartney, C.A., Bull, I.D., Yan, T. and Dewhurst, R.J. (2013) 'Assessment of archaeol as a molecular proxy for methane production in cattle' *Journal of Dairy Science*, 96: 1211-1217.

Popular publications:

McCartney, C.A., Bull, I.D. and Dewhurst, R.J. (2013) 'Comparison of biomarker and molecular biological methods for estimating methanogen abundance' *Proceedings of the Greenhouse Gases and Animal Agriculture Conference, Dublin, Ireland. Advances in Animal Biosciences,* 4: 555.

McCartney, C.A., Bull, I.D., Van Rooyen, L. and Dewhurst, R.J. (2013) 'Changes in the ratio of tetraether to diether lipids in cattle faeces in response to altered dietary ratio of grass silage and concentrates' *Proceedings of the Greenhouse Gases and Animal Agriculture Conference, Dublin, Ireland. Advances in Animal Biosciences,* 4: 563.

McCartney, C.A., Bull, I.D. and Dewhurst, R.J. (2011) 'Analysis of total methanogens in rumen fluid using quantitative real-time PCR and gas chromatography/mass spectrometry' *Proceedings of the 8th International Symposium on Herbivore Nutrition, Aberystwyth, UK. Advances in Animal Biosciences,* 2: 486.

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