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Chemical markers for rumen methanogens and methanogenesis

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*The targeting of mcrA or 16S rRNA genes by quantitative PCR (qPCR) has become the dominant method for quantifying methanogens in rumen. There are considerable discrepancies between estimates based on different primer sets, and the literature is equivocal about the relationship with methane production. There are a number of problems with qPCR, including low primer specificity, multiple copies of genes and multiple genomes per cell. Accordingly, we have investigated alternative markers for methanogens, on the basis of the distinctive ether lipids of archaeal cell membranes. The membranes of Archaea contain dialkyl glycerol ethers such as 2,3-diphytanayl-O-*sn*-glycerol (archaeol), and glycerol dialkyl glycerol tetraethers (GDGTs) such as caldarchaeol (GDGT-0) in different proportions. The relationships between estimates of methanogen abundance using qPCR and archaeol measurements varied across primers. Studies in other ecosystems have identified environmental effects on the profile of ether lipids in Archaea. There is a long history of analysing easily accessible samples, such as faeces, urine and milk, to provide information about digestion and metabolism in livestock without the need for intrusive procedures. Purine derivatives in urine and odd-chain fatty acids in milk have been used to study rumen function. The association between volatile fatty acid proportions and methane production is probably the basis for empirical relationships between milk fatty acid profiles and methane production. However, these studies have not yet identified consistent predictors. We have evaluated the relationship between faecal archaeol concentration and methane production across a range of diets in studies on beef and dairy cattle. Faecal archaeol is diagnostic for ruminant faeces being below the limit of detection in faeces from non-ruminant herbivores. The relationship between faecal archaeol and methane production was significant when comparing treatment means across diets, but appears to be subject to considerable between-animal variation. This variation was also evident in the weak relationship between archaeol concentrations in rumen digesta and faeces. We speculate that variation in the distribution and kinetics of methanogens in the rumen may affect the survival and functioning of Archaea in the rumen and therefore contribute to genetic variation in methane production. Indeed, variation in the relationship between the numbers of micro-organisms present in the rumen and those leaving the rumen may explain variation in relationships between methane production and both milk fatty acid profiles and faecal archaeol. As a result, microbial markers in the faeces and milk are unlikely to relate well back to methanogenesis in the rumen. This work has also highlighted the need to describe methanogen abundance in all rumen fractions and this may explain the difficulty interpreting results on the basis of samples taken using stomach tubes or rumenocentesis.*

Keywords: archaea, archaeol, ether lipids, methane, polymerase chain reaction

Implications

Ether lipids provide an adjunct to quantitative PCR-based estimates of methanogen populations and kinetics in the ruminant digestive tract and, with further development, could provide information about the effects of the rumen environment on methanogens. Understanding the location and kinetics of rumen methanogens will be important both in developing sampling protocols, understanding responses to methane mitigation treatments, and for the development of other possible methanogen markers. It seems unlikely that markers based on microbial material leaving the rumen will provide robust predictions of processes, such as methanogenesis, that occur within the rumen.

Methods to estimate rumen methanogen abundance

Quantitative real-time PCR (qPCR)

In recent years, molecular biological methods have been used to study the abundance and diversity of methanogens in many ecosystems. qPCR has become the method of choice

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for quantification of methanogens. This involves the amplification of methanogenic target DNA, which is then quantified in comparison with reference genes. Methanogen abundance studies normally target the specific methanogenic 16S rRNA (***rrs***) gene, the methyl-coenzyme M reductase (*mcr*A) gene or both. It has been difficult to compare qPCR-based estimates of methanogen abundance in the ruminant gastrointestinal tract as studies used different methods for sample collection and processing, different primers, and results have been expressed in different ways (Table 1).

Nearly all qPCR studies that have compared methanogen abundance in the rumen with methane production failed to demonstrate a significant relationship (Guo *et al.*, 2008; Mao *et al.*, 2010; Mosoni *et al.*, 2011; Zhou *et al.*, 2011b; Liu *et al.*, 2012; Morgavi *et al.*, 2012), although Ding *et al.* (2012) found a strong positive relationship. This lack of consistent relationship may reflect the problems with sampling methods and choice of primers mentioned previously, as well as underlying problems with the qPCR technique. These problems include differences in the *rrs* gene copy number within a genome (Hook *et al.*, 2009) and differences in genome copy number within a cell (Hildenbrand *et al.*, 2011). There is increasing concern about bias in the PCR reaction (Tymensen and McAllister, 2012), and the small number of sequences related to known methanogens (Firkins and Yu, 2006) may also cause problems. Dehority and Tirabasso (1998) found no relationship between the concentration of celluloytic bacteria and digestion of cellulose in the rumen.

Archaeal membrane lipids as biomarkers for methanogens

The problems of using qPCR to quantify methanogens occur in other ecosystems, such as soils, and this led Huguet *et al.* (2010) to propose the use of chemical markers alongside qPCR. Accordingly, we have investigated alternative markers for methanogens, on the basis of the distinctive ether lipids of archaeal cell membranes. The membranes of Archaea contain dialkyl glycerol ethers (DAGE) that form bilayers, such as archaeol (2,3-diphytanyl-*O*-*sn*-glycerol), and glycerol dialkyl glycerol tetraethers (GDGTs), which form monolayers, such as caldarchaeol (GDGT-0), in different proportions.

There are four fundamental differences between the ester lipids of the bacteria and eukaryotes and the DAGE and GDGTs of the Archaea (Koga *et al.*, 1993; Figure 1). First, in archaeal membrane lipids the linkage between the glycerol and the hydrocarbon chains is an ether bond, not an ester bond. Second, the hydrocarbon chains are highly methyl-branched, saturated isoprenyl chains, not linear fatty acyl chains. Third, the stereochemical structure of the di-*O*-radyl glycerol moiety is *sn*-2,3-di-*O*-radyl glycerol for the Archaea and *sn*-1,2-di-*O*-radyl glycerol for the bacteria and eukaryotes. Finally, there is the presence of tetraether-type lipids, that is, GDGTs, although tetraethers can also occur in bacteria, *albeit* rarely (Koga *et al.*, 1998a).

Fritze *et al.* (1999) and Pancost *et al.* (2011) used archaeol as a proxy for methanogens in peat bogs. Pancost *et al.* (2011) showed increasing archaeol concentrations with increasing depth in bog profiles and this was thought to

Table 1 *Studies that have used quantitative real-time PCR to quantify total methanogens from the ruminant gastrointestinal tract*

Study	Sample type(s)	Primer target	Abundance
Guo *et al.* (2008)	Rumen fluid (*in vitro*)	qmcrA-F/qmcra-R	*mcrA* abundance relative to prokaryote DNA
Hook *et al.* (2009)	Rumen fluid	Met630F/Met803R	*rrs* copies /g wet weight
Frey *et al.* (2010)	Rumen, duodenum and ileal contents and faeces	S-P-March-0348-S-a-17 S-D-Arch-0786-A-a-20	*mcrA* abundance relative to prokaryote DNA
Mao *et al.* (2010)	Rumen fluid	qmcrA-F/qmcra-R	*mcrA* abundance relative to prokaryote DNA
Pei *et al.* (2010)	Liquid/solid/epithelium (bacterial pellet)	Met86F/Met1340R	*rrs* copies/g wet weight
Gu *et al.* (2011)	Rumen fluid/solid (bacterial pellet)	MET630F/MET803R	*rrs* copies/g wet weight
Hook *et al.* (2011)	Rumen fluid/solid and faeces	qmcrA-F/qmcra-R	methanogens/g wet weight
Morgavi *et al.* (2012)	Rumen fluid/solid	qmcrA-F/qmcra-R	*mcrA* abundance relative to prokaryote DNA
Mosoni *et al.* (2011)	Rumen fluid/solid	qmcrA-F/qmcra-R & Arch 896-915F/Arch1406-1389R	Log_{10} *mcrA*/*rrs* copies /g dry weight
Zhou *et al.* (2011b)	Rumen fluid	uniMet1-F/R	Log_{10} *rrs* copies /ml
Zhou *et al.* (2011a)	Rumen fluid	qmcrA-F/qmcra-R	*mcrA* abundance relative to prokaryote DNA
Ding *et al.* (2012)	Rumen fluid	qmcrA-F/qmcra-R	*mcrA* abundance relative to prokaryote DNA
Liu *et al.* (2012)	Rumen fluid and faeces	Met86F/Met1340R & uniMet1-F/R	*rrs* copies /ml

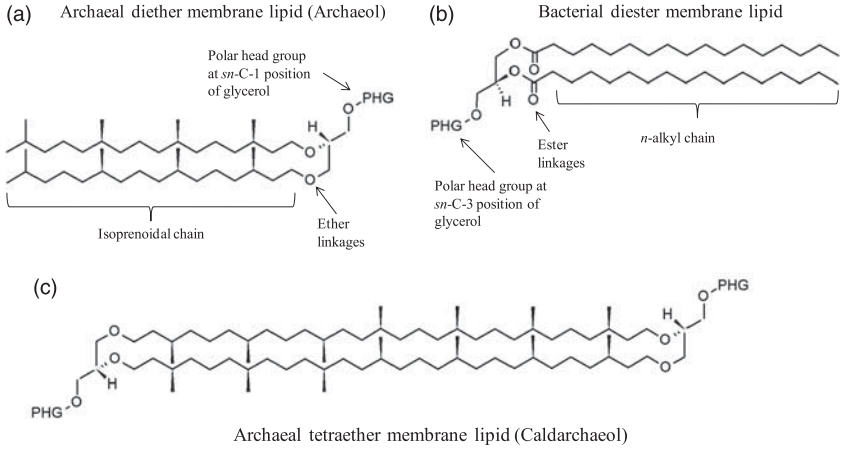

Figure 1 Structural differences in the membrane lipids of the Archaea (a) and the bacteria (b). Examples of the core membrane lipids found in the Archaea including archaeol (a) and caldarchaeol (c).

reflect increased methanogenesis in the progressively more anaerobic conditions. Fritze *et al.* (1999) showed some concordance between concentrations of archaeol extracted from peat and methane production measured *in vitro* in incubation flasks. The study of ether lipids has been particularly useful for palaeoclimatologists, who have developed two proxies based on these lipids. The TEX_{86} proxy for sea surface temperature (Schouten *et al.*, 2002) is based on GDGTs, which can contain varying numbers of 0 to 4 cyclopentyl moieties depending on temperature. The branched and isoprenoid tetraether index (Hopmans *et al.*, 2004) is used for reconstructing soil organic matter input into the ocean on the basis of the predominance of branched GDGTs in material from terrestrial environments and of crenarchaeol in material from marine and lacustrine environments. In addition, indices for air temperature and soil pH were developed in a study by Weijers *et al.* (2007), which is based on variations of cyclopentane moieties and extent methylation of branched GDGTs. A comprehensive review of these methods can be found in a study by Schouten *et al.* (2013).

Archaeal membrane lipids as biomarkers for rumen methanogens

Pancost *et al.* (2011) identified the presence of non-methanogenic Archaea as a potential complicating factor in their results with peat soils. It has been assumed that methanogens are the only Archaea present in the rumen (Sharp *et al.*, 1998; Janssen and Kirs, 2008), and Shin *et al.* (2004) and Lee *et al.* (2012) found virtually no non-methanogenic Archaea in the rumen (<0.05%). In contrast, the pyrosequencing study by Brulc *et al.* (2009) found around 20% of Archaea to be non-methanogenic, showing that more work is needed to confirm levels of non-methanogenic Archaea in the rumen.

McCartney (2012) compared estimates of methanogen populations in 12 rumen fluids using both archaeol analysis and qPCR with a number of primer sets (*rrs* for total methanogens and a range of methanogen species; *mcr*A targets). The relationships between the estimates of methanogen abundance using qPCR and archaeol measurements varied across primer sets. There were only weak correlations between archaeol and total methanogen estimates using *mcr*A ($P = 0.097$) and *rrs* ($P = 0.013$) genes, whereas the strongest relationship ($P < 0.001$) was with the *rrs* primer set that was specific to *Methanobrevibacter ruminantium.* This is further suggestive of problems with universal archaeal primer sets. The weaker relationship of archaeol with *Methanobrevibacter smithii* ($P = 0.068$) and *Methanosphaera stadtmanae* ($P = 0.046$) may be due to the lower abundance of these methanogens in the rumen (Janssen and Kirs, 2008).

Methods to estimate methane production in the rumen

The established methods for estimating methane production from individual animals, respiration chambers and the SF_6 technique have been reviewed extensively elsewhere, and their strengths and weaknesses are well recognised (Pinares-Patiño *et al.*, 2008). Accordingly, this review will consider less-intrusive approaches to estimating rumen methanogenesis, including those that can be used with free-grazing animals.

Non-invasive techniques to study rumen function

There is a long history of research on less- and non-invasive techniques to study aspects of rumen function. This has been driven partly by the desire to reduce the use of rumen-fistulated animals, both for animal welfare reasons, and because these techniques may be easier to apply in developing countries with free-ranging ruminants. The large numbers and high methane outputs from grazing ruminants make the latter objective of increased importance. Dewhurst *et al.* (2000) noted the potential for the application of non-invasive techniques for monitoring rumen function to guide feeding and other management decisions. Most pertinent now is the potential to use less- and non-invasive tools in breeding for increased feed efficiency and reduced methane production. Hegarty (2004) outlined the basis for possible genetic differences in methane production. The availability

of genomic tools means that our ability to select for these more complex traits is limited more by the availability of simple, rapid and low-cost phenotyping tools.

The urinary purine derivative (allantoin) technique provides a good example of what is possible in this area. Urinary purine derivatives have been used to predict microbial (protein) flow from the rumen on the basis of the strong relationship with the flow of purines from the rumen (Chen *et al.*, 1990). The technique has been used to identify effects of a wide range of dietary and other treatments on microbial protein yield from the rumen (Dewhurst *et al.*, 2000). There are a number of assumptions that limit the accuracy of the technique, but these are generally regarded as less problematic than the technical problems associated with post-rumen cannulation and marker techniques that are the other way to estimate microbial protein flow from the rumen. The technique depends on assumptions about the degradation of dietary purines, the purine content of rumen microbes, the endogenous contribution of purine derivatives from turnover within animal tissues and losses of purine derivatives through other routes (e.g. milk; Shingfield and Offer, 1998).

A key issue for the use of markers in faeces, urine or milk to describe processes occurring in the rumen is the relationship between what takes place in the rumen and what leaves the rumen. In the case of markers for rumen microbes, this means that relationships could be complicated by processes at the reticulo-omasal orifice – in particular, the selective retention of specific fractions of rumen digesta. This is not an issue for predicting microbial protein yield from the rumen, as both the target (microbial protein leaving the rumen) and the predictor (purine derivatives derived from purines leaving the rumen) have to leave the rumen. It may be more of an issue in the case of markers for rumen production of volatile fatty acid (VFA) and methane discussed later in this paper, as the products leave the rumen by substantially different routes (absorption across the rumen wall, eructation) than the markers.

Faecal archaeol and methane production

Archaeol is resistant to digestion in the intestines and can be measured in faeces of ruminants, but is not detectable in faeces from non-ruminant herbivores with hind gut fermentation using current methodologies (Gill *et al.*, 2010). A number of studies have now investigated the relationship between faecal archaeol and methane production. Gill *et al.* (2011) compared faecal archaeol and methane production from steers fed diets based primarily on grass silage or concentrates. Treatment differences in methane production mirrored differences in faecal archaeol concentrations. However, when considering individual animals within dietary treatments, there was no significant relationship.

Our own studies extended this analysis by comparing faecal archaeol concentration and CH_4 production across a range of diets in studies on beef and dairy cattle. Faecal archaeol was compared with methane measurements made by the sulphur hexafluoride (SF_6) tracer technique from animals consuming six dietary treatments with varying levels of concentrate (McCartney (2012) with samples taken from the study by McGeough *et al.* (2010)). A positive significant relationship ($P < 0.001$) was found between faecal archaeol and methane production; however, the relationship was again weak within dietary treatments.

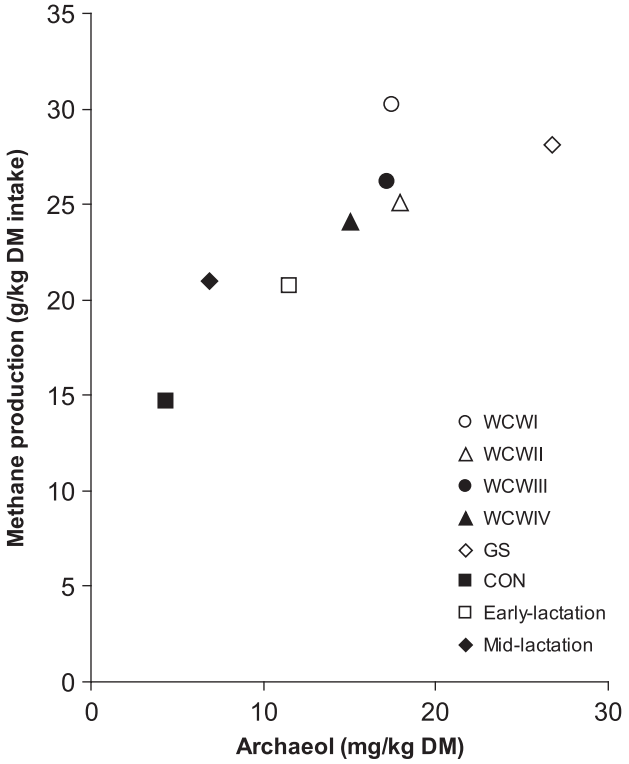

Figure 2 Relationship between faecal archaeol (mg/kg dry matter (DM)) and CH_4 production (g/kg DM intake). Average values for each dietary treatment are presented, which included grass silage (GS)[1], *ad libitum* concentrates (CON)[2] and whole-crop wheat (WCW)[3] silages. Early and mid lactation results from animals consuming a 70% concentrate diet are also presented. [1]GS plus 2.60 kg of supplemental concentrate DM. [2]CON plus 1.28 kg of GS DM. [3]WCW silage plus 2.60 kg concentrate DM. Grain to straw plus chaff: I = 11.89; II = 21 : 79; III=31 : 69; IV = 47 : 53.

One of the potential sources of variation in the studies mentioned above is problems with the SF_6 technique, and therefore a further study was conducted to investigate the relationship between faecal archaeol and methane measurements made using respiration chambers from animals consuming just one dietary treatment. Promisingly, a positive significant relationship ($P = 0.007$) was found within a dietary treatment. However, stage of lactation also affected the relationship ($P = 0.011$) and this may be related to higher dry matter (DM) intakes concurrent with more days in milk (McCartney *et al.*, 2013).

There was a good relationship between faecal archaeol concentration and methane production expressed per unit DM intake for treatment means across these three studies (Figure 2). However, as we have seen, there is considerable variability for individual values within treatment means. Further, the positive intercept for the relationship shown in Figure 2 suggests some selectivity in what flows out of the rumen. Selective retention of methanogens in the rumen

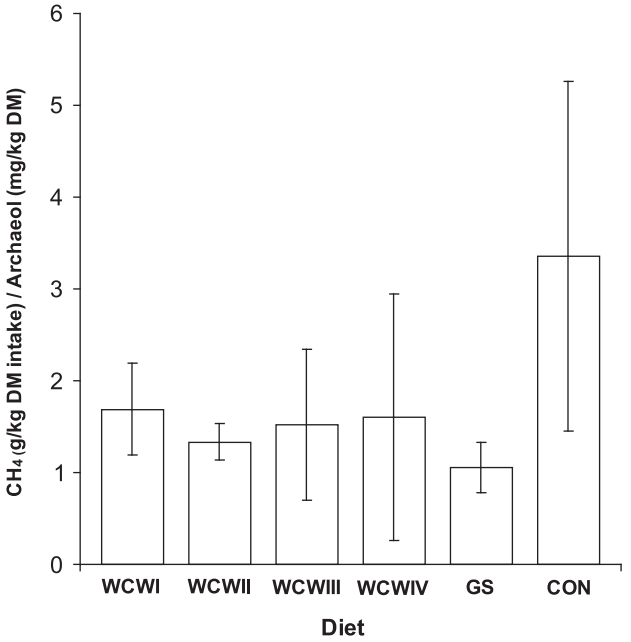

Figure 3 Effects of diet on CH_4 production per unit of faecal archaeol with error bars showing standard deviation. Dietary treatments included grass silage (GS)[1], concentrates (CON)[2] and whole-crop wheat (WCW)[3] silages. [1]GS plus 2.60 kg of supplemental concentrate DM. [2]CON plus 1.28 kg of GS dry matter (DM). [3]WCW silage plus 2.60 kg concentrate DM. Grain to straw plus chaff (DM basis): I = 11 : 89; II = 21 : 79; III = 31 : 69; IV = 47 : 53.

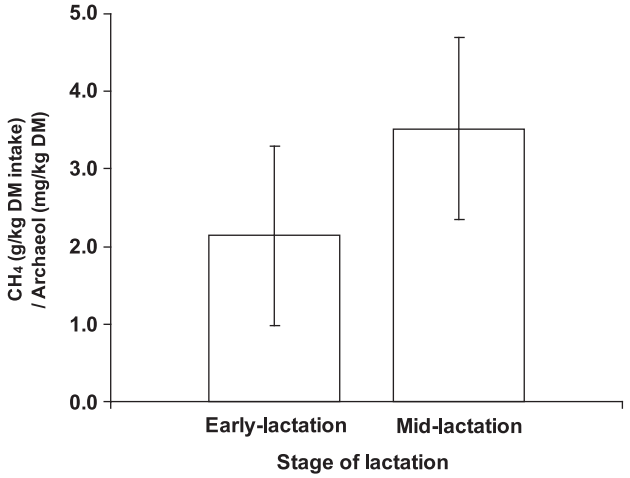

Figure 4 Significant effects of stage of lactation on CH_4 production per unit of faecal archaeol with error bars showing standard deviations.

means that, although methane is still being produced in the rumen by methanogens, their retention in the rumen means that they cannot be detected (as archaeol) in the faeces.

Selective retention of methanogens in the rumen is also evident in our recent study that found no significant relationship between concentrations of archaeol in whole rumen digesta and corresponding samples of faeces, although there were trends ($P < 0.1$) for effects of diet and experimental period on the relationship (McCartney, 2012). Differences in the methane : faecal archaeol ratio (CH_4 : A) reflect diet and physiological effects on the extent of selective retention, with a higher CH_4 : A ratio, suggesting greater retention of methanogens in the rumen. The higher CH_4 : A ratio for animals offered a high concentrate diet (Figure 3; McCartney, 2012) that may result from increased retention of methanogens associated with protozoa, which are selectively retained in the rumen (Leng *et al.*, 1981). The higher CH_4 : A for cows in mid lactation in comparison with early lactation (Figure 4; McCartney *et al.*, 2013) was probably related to the increased DM intake affecting digesta kinetics.

The growth rates of methanogens may cause additional variation in the relationship between faecal archaeol concentrations and methane production. This is because methanogenesis is linked to ATP production (Thauer, 1998), and the maintenance energy requirement and growth rate of a microbe can affect the molar growth yield per mole of ATP (g microbial DM/mol ATP; Y_{ATP}; Stouthamer and Bettenhaussen, 1973). Rapidly growing microbes have a higher Y_{ATP}, as less energy is devoted to maintenance processes, whereas microbes with lower maintenance energy requirements have higher Y_{ATP} values as more energy can be devoted to growth (Stouthamer and Bettenhaussen, 1973).

It is likely that methanogens associated with the liquid phase grow faster in order to avoid 'wash out' from the rumen owing to faster passage rate of this fraction and this would increase Y_{ATP}. Methanogens may also conserve energy by altering their membrane permeability in order to reduce the inadvertent loss of ions that maintain the chemiosmotic potential (Valentine, 2007). Reducing the permeability of membranes can be achieved by increasing the proportion of GDGTs (Mathai *et al.*, 2001) and/or increasing the proportion of phosphoglycolipids with two or more sugar head groups (Shimada *et al.*, 2008). Of course, altering the archaeol : GDGT ratio in membranes will cause further variation in the relationship between archaeol and methane production.

The advantages of using the faecal archaeol method for estimating methane emissions in comparison with traditional techniques include limited/no contact with the animals, relatively simple sampling/processing methods, relatively low cost and the opportunity to pool samples together or to take individual measurements. However, the substantial variation in the relationship between faecal archaeol concentrations and methane production limits the value of faecal archaeol to predict methanogenesis in the rumen. Thus, in future studies, it may be more sensible to focus on the potential of archaeol as a general methanogen biomarker in the ruminant gastrointestinal tract. In addition, the role of GDGTs in the rumen methanogen membrane should be further investigated. It would be much easier if a non-invasive predictor of methane production could be based on the use of milk samples, which are much easier to collect and potentially analyse online than faecal samples. Possible approaches based on milk samples are discussed in the next section.

Odd- and branched-chain fatty acids (OBCFAs) in milk

Milk is the most accessible sample from cows and has the advantage of being a composite sample over the entire

milking interval. When it became clear that milk allantoin was not a useful predictor for microbial protein yield from the rumen, attention turned to the study of OBCFAs, which are components of rumen microbes that are transferred to milk at a much higher level. OBCFAs are not normally present in plant-derived feeds (Diedrich and Henschel, 1990), though some forages can have modest levels of the anteiso-$C_{15:0}$ homologue (Kim *et al.*, 2005). They have long been recognised as a signature of ruminant fats (e.g. Polidori *et al.*, 1993; Jenkins, 1995). Although there are complicating issues with OBCFA synthesis and chain elongation within animal tissues (Vlaeminck *et al.*, 2006; Dewhurst *et al.*, 2007), there has been some success in establishing relationships between milk OBCFA and aspects of rumen function (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012).

Vlaeminck *et al.* (2006) established that microbial OBCFA profiles depend more on the fatty acid synthase activities of the rumen microbes than on the substrates that are available. A number of workers have shown higher levels of iso-branched-chain fatty acids (iso-$C_{14:0}$ and iso-$C_{16:0}$) in rumen solid-associated bacteria in comparison with liquid-associated bacteria (Vlaeminck *et al.*, 2006; Bessa *et al.*, 2009), and this is probably related to the higher proportions of these fatty acids in some of the main celluloytic bacteria (data summarised by Vlaeminck *et al.*, 2006).

Milk OBCFAs have been used to predict rumen VFA proportions (Vlaeminck and Fievez, 2005; Vlaeminck *et al.*, 2006; Bhagwat *et al.*, 2012; Fievez *et al.*, 2012). The most recent rational models based on 224 observations gave Q^2 values (analogous to R^2 values) of 0.74 (acetate molar proportion), 0.67 (propionate molar proportion) and 0.65 (butyrate molar proportion; Bhagwat *et al.*, 2012). A higher R^2 was obtained in a validation study (Fievez *et al.*, 2012) and this may be the consequence of making comparisons with a single study. Indeed, experiment appears as a significant term in models relating milk OBCFA to rumen VFA and methane production and it is not yet clear why this is the case. One possible explanation is animal or environmental (e.g. ambient temperature) effects on rumen digesta kinetics, which may affect the relationship between rumen processes and the flow of microbial OBCFA from the rumen. The iso-branched-chain fatty acids mentioned above have a significant relationship with rumen VFA proportions that is consistent with their association with the solid-phase and celluloytic bacteria – iso-$C_{14:0}$ was positively related to acetate molar proportion and negatively related to propionate and butyrate molar proportion, whereas iso-$C_{15:0}$ was positively related to acetate molar proportion.

The most recent interest has been to relate milk OBCFA and other fatty acids to methane production, and a number of studies that have done this are summarised in Table 2. It is interesting to note that two of the OBCFAs, iso-$C_{14:0}$ and iso-$C_{16:0}$, are consistently related to methane production and this may be related to the previously mentioned associations of these fatty acids with rumen VFA proportions and celluloytic bacteria. Castro-Montoya *et al.* (2011) showed a negative relationship between methane production and both $C_{15:0}$ and $C_{17:0}$, which are positively correlated with propionate and negatively correlated with acetate. Although their study involved only a single dietary treatment (myristic acid), Odongo *et al.* (2007) also noted a decrease in iso-$C_{14:0}$ and iso-$C_{16:0}$ with decreased methane production. Unfortunately, all of these studies have limitations – either in terms of the number and range of dietary treatments involved or in the technique used to estimate methane production – and full interpretation awaits more complete data sets.

Many of the studies have used supplements based on fatty acid or oils, as these are among the most effective diet supplements for reducing methane production. However, adding high levels of fatty acids inevitably alters the milk fatty acid profile and it becomes difficult to identify relationships that are of more general applicability. For example, Chilliard *et al.* (2009) obtained excellent predictions of methane output when supplementing diets with linseed products. However, the predictions are dominated by isomers of the $C_{18:2}$ and $C_{18:1}$ homologues that would derive from, or be affected by, the linseed fatty acids, and thus it is not clear that these relationships would have general applicability.

Mohammed *et al.* (2011) also obtained significant relationships between methane production and milk fatty acid profiles. In this case, relationships were best within dietary treatment. They also showed that equations to predict methane production based on earlier work overestimated methane production and that the relationship between milk fatty acids and methane production was affected by the rumen protozoal population. This latter effect could be related to selective retention of microorganisms in the rumen as protozoa are preferentially retained (Leng *et al.*, 1981).

Mid IR reflectance (MIR) spectroscopy of milk

Although milk is an easier sample to collect than faeces, it would be preferable if a rapid method for prediction of methane production could be adopted as part of routine milk testing. A recent study by Dehareng *et al.* (2012) identified the potential to predict methane production on the basis of mid MIR spectroscopy analysis of milk. It seems likely that a significant proportion of this relationship is driven by the association between methanogenesis and milk fatty acids with all its limitations described above. However, Dehareng *et al.* (2012) also suggest that some of the variation is explained by spectral information coming from other milk components.

Conclusions and recommendations for future work

qPCR-based approaches to quantifying methanogens have a number of problems and the use of ether lipids as biomarkers has the potential to quantify rumen methanogen populations and also to help understand qPCR results. More attention must be paid to the distribution of methanogens in different locations and digesta fractions within the rumen. These have important implications both for the energetic efficiency of methanogenesis and the relationships between the rumen methanogen population, methanogenesis and the rumen outflow of methanogens. Improved understanding of

Table 2 *Use of milk fatty acids to predict methane production*

Reference	Number of observations (number of studies)	Method for measuring/ estimating methane production	Treatments	Percentage of variance in methane production explained by best combination of milk fatty acids	Individual milk fatty acids related to methane production:	
					Positive relationship	Negative relationship
Chilliard *et al.* (2009)	32 (1)	SF_6 tracer technique	Maize/grass silage and concentrates with different linseed products	95 (within study)	4:0 6:0 8:0 9:0 10:0 10:1 11:0 12:0 12:1 14:0 15:0 17:0 20:4	18:1 *trans*-16 + *cis*-14 18:2 *cis*-9, *trans*-13 16:1 *trans*-11 18:1 *trans*-12 18:1 *cis*-13 18:1 *trans*-13 + 14 18:1 *trans*-6,7,8 18:1 *cis*-15 + *trans*-17 18:2 *trans*-11,*cis*-15 18:1 *cis*-9 18:1 *cis*-10 18:1 *trans*-10
Mohammed *et al.* (2011)	16 (1)	Respiration chambers	Barley silage-based TMR with different crushed oilseeds	83 (within study)	8:0 Iso-16:0*	17:1 *cis*-9 18:1 *cis*-11 18:1 *cis*-13 18:1 *trans*-6,7,8 18:2 Iso-17:0/16:1 *trans*-6,7,8 18:2 *cis*-9,*trans*-13/*trans*-8,*cis*-12 18:3
Dijkstra *et al.* (2011)	50 (10)	Respiration chambers	TMR based on grass and maize silages with a range of supplements (fumarate, diallyldisulphide, yucca powder, fatty acids, linseed products)	73 (within study)	Iso-14:0 Iso-15:0 Anteiso-7:0	17:1 *cis*-9 18:1 *trans*-10 + 11 18:1 *cis*-11
Casto Montoya *et al.* (2011) (only considered odd- and branched-chain fatty acids)	224 (13)	Calculation based on volatile fatty acid proportions	Wide range of forages and forage/concentrate ratios	66 (cross-validation)	Iso-14:0 Iso-15:0 Iso-16:0	15:0 17:0 + 17:1 *cis*-9

TMR = total mixed rations.
*$P = 0.07$.

the location of the methanogens in the rumen could lead to more informed rumen sampling protocols.

The DAGE : GDGT ratio and/or proportion of phosphoglycolipids should be investigated owing to potential effects on maintenance energy requirements. It is hypothesised that the methanogen membrane adapts to contain more GDGT/phosphoglycolipids in challenging rumen conditions such as low rumen pH, low structural carbohydrate availability and/or high passage rates to reduce permeability of the membrane, and thus increase efficiency of the methanogen cell. If there was a change in the GDGT : DAGE ratio or phosphoglycolipid proportion, the next step would be to see whether this was because of a shift of the methanogen community, or whether the methanogen community already present in the rumen adapts its membranes.

Overall, the use of both milk fatty acids and faecal archaeol as markers for *methanogenesis* is complicated by the issues of selective retention of digesta in the rumen. Although these techniques explain some of the variation in methane production, particularly when comparing treatment means, there remains potential for significant mismatch between processes occurring in the rumen (VFA production or methanogenesis) and flows from the rumen (bacteria and their OBCFA; Archaea and their ether lipids).

Although these problems limit the application of marker approaches on the basis of analysis of milk or faeces, the use of archaeol as a general marker for *methanogen abundance* in the digestive tract shows promise. Indeed, the use of a chemical marker to support qPCR approaches will be helpful to understand the location and kinetics of methanogens in the rumen that may be the cause of some of this variation.

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