Microbial ecosystem and methanogenesis in ruminants

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Ruminant production is under increased public scrutiny in terms of the importance of cattle and other ruminants as major producers of the greenhouse gas methane. Methanogenesis is performed by methanogenic archaea, a specialised group of microbes present in several anaerobic environments including the rumen. In the rumen, methanogens utilise predominantly H₂ and CO₂ as substrates to produce methane, filling an important functional niche in the ecosystem. However, in addition to methanogens, other microbes also have an influence on methane production either because they are involved in hydrogen (H₂) metabolism or because they affect the numbers of methanogens or other members of the microbiota. This study explores the relationship between some of these microbes and methanogenesis and highlights some functional groups that could play a role in decreasing methane emissions. Dihydrogen (‘H₂’ from this point on) is the key element that drives methane production in the rumen. Among H₂ producers, protozoa have a prominent position, which is strengthened by their close physical association with methanogens, which favours H₂ transfer from one to the other. A strong positive interaction was found between protozoal numbers and methane emissions, and because this group is possibly not essential for rumen function, protozoa might be a target for methane mitigation. An important function that is associated with production of H₂ is the degradation of fibrous plant material. However, not all members of the rumen fibrolytic community produce H₂. Increasing the proportion of non-H₂ producing fibrolytic microorganisms might decrease methane production without affecting forage degradability. Alternative pathways that use electron acceptors other than CO₂ to oxidise H₂ also exist in the rumen. Bacteria with this type of metabolism normally occupy a distinct ecological niche and are not dominant members of the microbiota; however, their numbers can increase if the right potential electron acceptor is present in the diet. Nitrate is an alternative electron sinks that can promote the growth of particular bacteria able to compete with methanogens. Because of the toxicity of the intermediate product, nitrite, the use of nitrate has not been fully explored, but in adapted animals, nitrite does not accumulate and nitrate supplementation may be an alternative under some dietary conditions that deserves to be further studied. In conclusion, methanogens in the rumen co-exist with other microbes, which have contrasting activities. A better understanding of these populations and the pathways that compete with methanogenesis may provide novel targets for emissions abatement in ruminant production.

Keywords: rumen fermentation, H₂ sinks, hydrogen producers, methanogens, methane

Implications

Ruminants, which sustain the livelihood of millions of people in the world, are capable of producing human food using fibrous feedstuffs that cannot be utilised directly by monogastric animals including man. A negative aspect is, however, the production in the rumen of the greenhouse gas methane. The methanogens, producers of methane, utilise the end products of fermentation from other rumen microbes, particularly H₂, as substrate. Available information indicates that decreasing the number of H₂ producers such as protozoa and some fibrolytic microbes and/or increasing the number and activity of non-methanogenic, H₂ utilisers are promising ways to reduce methane emissions.

Introduction

Livestock sustain the livelihood of millions of people in the world in both developing and developed countries. Up to 12% of the world’s population, in particular the rural poor, are highly dependent on domestic animals for their sustenance (Thornton et al., 2007; FAO Newsroom, 2009). The rapid economic growth observed in some regions of the world also brings increases in income for a large part of the population, and this translates into higher consumption of animal products per capita. This increase in consumption...
Rumen microbiota and methanogenesis

Methanogens belong to the domain archaea. The diversity of archaea found in the rumen have been recently reviewed by...
Janssen and Kirs (2008). Most archaea identified in the rumen belong to known methanogen clades with a predominance of *Methanobrevibacter* spp. The pooled data from several surveys show that the *Methanobrevibacter* clade accounts for nearly two-thirds of rumen archaea. The remaining one-third was composed of roughly equal parts by phylotypes belonging to *Methanomicrobium* and the rumen cluster C (Janssen and Kirs, 2008). However, in some studies the proportion of these different groups was inversed or differed greatly (Janssen and Kirs, 2008 and references therein), and it is not clear whether the major differences in archaeal distribution between studies are due to methodological differences or truly reflect differences due to animals and/or diets. Most rumen methanogens do not contain cytochromes and although they are less efficient at obtaining energy through the production of methane than their cytochrome-containing relatives of the order *Methanosarcinales* (Thauer et al., 2008), they are better adapted to the environmental conditions prevailing in the rumen. They have a lower threshold for H₂ partial pressure, a fast doubling time, that can be as short as 1 h, and they develop better at the mesophilic temperature and near neutral pH of the rumen (Thauer et al., 2008).

There are three major substrates used by methanogens to produce methane: CO₂, compounds containing a methyl group or acetate (Liu and Whitman, 2008). The contribution of these substrates to methanogenesis has not been measured, but it is likely to be small as the methanogens able to perform this conversion are not predominant members of the rumen methanogenic population (Janssen and Kirs, 2008). Methane is also produced from acetate via the aceticlastic pathway and this pathway appears to be limited to members of the order *Methanosaricarinales* (Liu and Whitman, 2008). Oppermann et al. (1961) measured the increase in radio-labelled methane following infusion of 14C-acetate in vivo and concluded that this pathway was not important in the rumen. *Methanosaricarina* grown on acetate had a very slow growth rate in vitro suggesting they would not thrive under normal rumen conditions and retention times (Stewart et al., 1997). This was confirmed by molecular biology based culture-independent methods that retrieved only low numbers of *Methanosaricarina* from the rumen (Janssen and Kirs, 2008).

Methanogens are found associated with the rumen liquid and solid phases and also with the rumen epithelium. Information concerning the latter group is still scarce (Shin et al., 2004; Pei et al., 2010), but the rumen epithelium seems to sustain high concentration of methanogens and some novel phylotypes (Pei et al., 2010). It is not known whether this methanogenic community has a significant role in rumen methanogenesis but methanogens attached to the gut epithelium have been described in termites (Leadbetter and Breznak, 1996), and in such a microaerobic environment they are capable of producing methane and reducing oxygen at the same time (Tholen et al., 2007). Notwithstanding the possible contribution of epithelial methanogens, it is generally assumed that methanogens within the rumen milieu, be it in the free liquid, is associated with the solid digest or indeed attached to the rumen protozoa that make the major contribution to rumen methanogenesis.
Protozoa and methanogenesis

The rumen protozoa were first described in Gruby and Delafond (1843) and with their striking appearance it was assumed that they must be important for the welfare of their host. However, despite the fact that protozoa make up a large portion of the rumen biomass, their role in ruminal fermentation and their contribution to the metabolism and nutrition of the animal remains an area of considerable controversy (Williams and Coleman, 1992). Both flagellated and ciliated protozoa have been described in the rumen with ciliate protozoa by far the more prominent belonging to two orders: the Entodiniomorphida and the Vestibuliferida (Holotrich; Williams and Coleman, 1992). By classical morphological criteria, more than 250 species of ciliates have been described which live in the rumen of various feral and domesticated ruminants (Williams and Coleman, 1992).

Ciliated protozoa can account for a substantial fraction of both the biodiversity and the biomass in the rumen and up to 100 billion ciliates belonging to more than 20 species may populate the rumen of a single cow (Williams and Coleman, 1992). Rumen ciliated protozoa are metabolically very active, able to influence fermentation of feeds and other rumen microbial populations and, consequently, to affect the amount and proportion of the end products from rumen fermentation including methane (Williams and Coleman, 1992; Eugène et al., 2004). Protozoa engulf organic matter, particularly bacteria, into digestive vacuoles where hydrolysis and fermentation take place. The main volatile fatty acids produced are acetate and butyrate (Williams and Coleman, 1992; Hillman et al., 1995). Rumen protozoa are not essential to the animal to survive and defaunation (the removal of protozoa from the rumen using a wide variety of chemical and physical techniques) has been widely used to study the role of ciliate protozoa in rumen function (Williams and Coleman, 1992). However, the results obtained do need to be interpreted with some caution as defaunation also leads to major changes in the rumen microbial population (Williams and Coleman, 1992). In a meta-analysis of published data, Eugène et al. (2004) concluded that defaunation resulted in an increase in the molar proportion of propionate in the rumen (P < 0.05) and a decrease in concentrations of butyrate (P < 0.05) and acetate (P = 0.08). On the basis of stoichiometry, such a shift in rumen volatile fatty acid production should result in a decrease in methane production as less metabolic H₂ will be available as a substrate for methanogenesis (Demeyer, 1991). The effect of defaunation on methane production are less clear; Hegarty (1999) summarised published in vivo and in vitro trails and showed that the removal of protozoa from the rumen would result in a 13% decrease in methane production. We have repeated and updated this analysis using only in vivo studies (Table 1), again taken as an average over all studies defaunation resulted in a 10.5% decrease in methane emissions. However, a great deal of variability can be seen within the available data, with some studies recording increased emissions of methane following defaunation (Table 1). Although undoubtedly much of the variation can be explained by methodological differences including the method used to obtain protozoa-free animals, there is also evidence of a dietary effect and more study is required to help fully explain the relationship between defaunation and methanogenesis.

To further explore the relationship between methane production and rumen protozoa we analysed all available in vivo data in which measurements of methane emissions, intake and rumen protozoa numbers were recorded. A total of 17 publications and unpublished trials from our laboratory corresponding to 21 experiments fitted this criterion. The data set contained a range of dietary treatments that were tested for their modulating effect on methanogenesis. A total of 65 treatments were kept for analysis of which 21 were controls and 44 were experimental diets. The experimental factors tested in these diets were lipid supplementation (n = 23) of up to 7% of offered feed dry matter, plants or plant extracts rich in tannins or saponins (n = 16), and diets rich in starchy concentrates (30% to 50% of offered feed dry matter, n = 5). Cattle and small ruminants (sheep and goat) were equally represented (48% and 52%, respectively). The majority of the data (85%) were from production trials and the remaining were from trials in which animals were fed at maintenance. Methane production was measured in chambers (63% in open chambers or respiratory calorimeters) or with the SF₆ (sulphur hexafluoride) tracer method (37%) of Johnson et al. (1994).

Data obtained over this broad range of conditions were analysed using the general linear procedure included in Minitab Version 14 (Minitab Inc., State college, PA, USA) to evaluate the relationship between methane yield (g/kg dry matter intake (DMI)) and concentration of protozoa (10⁵/ml of rumen liquid; Figure 2). The linear model included the effect of the study and of the dietary treatment as fixed effect (Sauvant et al., 2008). We observed that the number of protozoa explained 47% of the variability in methane emissions (P < 0.0001) suggesting that protozoa played a catalytic role in rumen methanogenesis under the experimental conditions studied. The extent of the decrease in methane yield averaged 0.6 g methane/kg DMI per reduction of 10⁵ protozoal cell/ml and does not appear to be affected by the type of dietary treatment studied (P = 0.14), but methane yield indicated by the intercept of the regressions (not shown) differed slightly between the treatments (P = 0.07). This difference is probably due to the nature of the dietary treatments and corresponding controls and to the different experimental conditions associated with each one of them, for example, mainly small ruminants for plant extracts and only productive cattle for starch-rich concentrates. Within the data set there are a few contrasting results that are not large enough to affect the tendency, but that should be noted. Guan et al. (2006) observed a significant decrease in protozoal numbers for a high-concentrate diet compared with a low-concentrate diet without any modification in the amount of methane emitted. A large variation in methane emission without any effect on...
## Table 1 The effect of defaunation on methane production as measured in in vivo experiments

<table>
<thead>
<tr>
<th>Methane (g/animal per day)</th>
<th>With protozoa</th>
<th>Defaunated</th>
<th>Response (% change)</th>
<th>Animal sp.</th>
<th>Diet</th>
<th>Notes</th>
<th>Reference</th>
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<tr>
<td>13.4</td>
<td>14.9</td>
<td>11.1</td>
<td>Sheep</td>
<td>Dried lucerne</td>
<td>Defaunated using a detergent, methane measured in a chamber after 10 weeks of defaunation</td>
<td>Bird et al. (2008)</td>
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</tr>
<tr>
<td>13.9</td>
<td>15.1</td>
<td>8.6</td>
<td></td>
<td>Native starch</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
<td>Kreuzer et al. (1986)</td>
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<tr>
<td>15.7</td>
<td>14.0</td>
<td>-10.8</td>
<td>Sheep</td>
<td>Cellulose based</td>
<td>25 weeks after defaunation</td>
<td></td>
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<tr>
<td>8.2</td>
<td>9.1</td>
<td>11.1</td>
<td>Sheep</td>
<td>Steamflaked starch based</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
<td>Chandramoni et al. (2002)</td>
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<tr>
<td>12.0</td>
<td>5.7</td>
<td>-47.5</td>
<td>Sheep</td>
<td>Roughage and concentrate (3 : 7)</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
<td>Hegarty et al. (2008)</td>
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<tr>
<td>13.4</td>
<td>9.4</td>
<td>-29.9**</td>
<td>Sheep</td>
<td>Roughage and concentrate (2 : 8)</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
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<tr>
<td>12.6</td>
<td>13.1</td>
<td>4.0</td>
<td>Sheep</td>
<td>Roughage and concentrate (2 : 8)</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
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<tr>
<td>15.6</td>
<td>16.1</td>
<td>3.2</td>
<td>Sheep</td>
<td>Roughage</td>
<td>Isolated at birth, methane measured in a chamber</td>
<td>Yanez-Ruiz et al. (2007)</td>
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<tr>
<td>25.1</td>
<td>18.6</td>
<td>-25.9*</td>
<td>Sheep</td>
<td>Roughage and concentrate (1 : 1)</td>
<td>Defaunated by rumen washing, methane measured using SF6.</td>
<td>Morgavi et al. (2008)</td>
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<tr>
<td>31.5</td>
<td>23.9</td>
<td>-24.1*</td>
<td>Sheep</td>
<td>Roughage and concentrate (3 : 1)</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
<td>Whitelaw et al. (1984)</td>
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<tr>
<td>129.6</td>
<td>64.1</td>
<td>-49.6**</td>
<td>Cattle</td>
<td>Barley with a protein supplement</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
<td>Machmüller et al. (2003b)</td>
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<tr>
<td>16.6</td>
<td>18.2</td>
<td>9.6</td>
<td>Sheep</td>
<td>Maize silage concentrate diet supplemented with protected fat</td>
<td>Defaunated using a detergent, methane measured in a chamber</td>
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<tr>
<td>14.3</td>
<td>14.6</td>
<td>2.1</td>
<td>Sheep</td>
<td>Hay</td>
<td>Isolated at birth methane measured in a chamber</td>
<td>Itabashi et al. (1984)</td>
<td></td>
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<tr>
<td>14.1</td>
<td>14.6</td>
<td>3.5</td>
<td>Goat</td>
<td>Hay</td>
<td>Average response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.9</td>
<td>17.9</td>
<td>-21.8**</td>
<td>Hay plus concentrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.6</td>
<td>16.4</td>
<td>-11.8</td>
<td>Hay plus concentrate plus monensin</td>
<td></td>
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* ** Indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as reported in the original reference.
 protozoal numbers was observed in some trials using condensed tannins (Animut et al., 2008), starch-rich concentrates (Martin et al., 2007a and unpublished data) and lipids (Machmüller and Kreuzer, 1999, Machmüller et al., 2003a). This analysis confirms the important influence that rumen protozoa have on methanogenesis under certain conditions. Where possible we suggest that protozoal measurements should be done in all animal trials in which methane mitigation options are tested to corroborate this finding. Protozoa may also be important for the success of other antimethanogenic strategies. For instance, the temporary mitigation effect of ionophores in a cattle trial has been directly associated with the recovery of protozoal numbers in animals following 4 to 6 weeks of continuous treatment (Guan et al., 2006).

A number of mechanisms by which protozoa could enhance methanogenesis are possible in which protozoa produce $H_2$, serve as hosts for methanogens and also protect them from oxygen toxicity. $H_2$ is a fermentation by-product that is produced in large quantities by the protozoa in a specialised organelle equivalent to the mitochondria of aerobic eukaryotes: the hydrogenosome. This $H_2$ is utilised by methanogens that are found inside (Finlay et al., 1994) or in close association with protozoal cells (Stumm et al., 1982). This interaction is a typical example of interspecies $H_2$ transfer that favours both the methanogens and the protozoa (Hillman et al., 1988; Ushida et al., 1997). Removal of $H_2$ allows the fermentation of organic matter to proceed mainly to acetate and $CO_2$ at the expense of butyrate and lactate production resulting in more efficient ATP production by the protozoan host.

Intracellular methanogens are found inside the most common protozoal species accounting for 1% to 2% of the host cell volume (Finlay et al., 1994). It has been shown that in other environments including freshwater ecosystems and also in the gastrointestinal tract of invertebrates and amphibians, the endosymbiont methanogens within protozoa are protozoan genera-specific (van Hoek et al., 2000). However, there is still no solid evidence that the same phenomenon occurs in rumin ciliates (Janssen and Kirs, 2008). Tóthová et al. (2008) have found that a specific methanogen, which differed from other rumen methanogens, could be isolated from in vitro cultures of the rumin ciliate Entodinium caudatum, but it is not clear whether this is an artefact of the culture technique. Others (Sharp et al., 1998; Ohene-Adjei et al., 2007) have detected changes in the methanogenic phytypes retrieved in the presence or absence of protozoa, but again it is not clear to what extent this could be due to a specific association of methanogens with individual protozoal genera and to what extent it reflects the wider changes in rumen metabolism associated with defaunation and selective refaunation (Williams and Coleman, 1992).

In contrast to intracellular methanogens, the methanogens attached to the cell surface appear to be less numerous and less ubiquitous, as at a given time only 30% to 50% of protozoal cells being observed do have extracellular-associated methanogens (Vogels et al., 1980; Finlay et al., 1994). The extent of the association is influenced by diet and time of feeding (Stumm et al., 1982; Tokura et al., 1997). Adherence of methanogens to protozoa is low after feeding and appears to be related to the relative abundance of $H_2$ in the medium (Stumm et al., 1982). Most Entodinimorphs seem able to carry methanogens on their surface, but no epysymbiotic methanogens were observed associated with Holotrichs protozoa (Vogels et al., 1980). Nevertheless, the Holotrich Isotricha prostoma isolated from the rumen produced large amount of methane indicating that extracellular, as

**Figure 2** Methane emissions and rumen protozoa concentration in ruminants receiving diets rich in starchy concentrates (●) review of available literature. Data from Machmüller and Kreuzer, 1999 (7); Machmüller et al., 2000 (8); Slivinski et al., 2002 (17); Lovett et al., 2003 (6, 20); Machmüller et al., 2003a and 2003b (9, 10); Hess et al., 2004 (14, 15, 16); Ushida et al., 2005 (18); Guan et al., 2006 (21); Jordan et al., 2006a (5); Jordan et al., 2006b (4); Martin et al., 2007a and unpublished data (19); Martin et al., 2007b and unpublished data (1); Pen et al., 2007 (12); Animut et al., 2008b (11), Beauchemin et al., 2009 (3); Holtshausen et al., 2009 (13); Martin et al. personal communication (2). The black dashed line represents the regression after taking into account the effect of study: methane (g/kg dry matter intake) = 13.95 (s.e. = 0.87) + 0.6×10⁻³ (s.e. = 0.11) × protozoa (10⁵/ml); ($R² = 0.65$; $R²$ adjusted = 0.47; $P < 0.001$).
opposed to intracellular, methanogens are not crucial (Ushida and Jouany, 1996). Tokura et al. (1997) used mixed protozoa isolated from the rumen at different times of the day and reported that the number of both intra- and epi-associated methanogens per protozoal cell was less than three per cell before feeding and increasing to 100 to 1000 per cell after 1 h of feeding. Taken together, these data suggest that intracellular methanogens are more important both in number and activity than the externally associated methanogens.

A single protozoan may produce between 0.5 and 4.5 nmol H2/day (Williams and Coleman, 1992; Finlay et al., 1994; Ushida and Jouany, 1996) with a production of methane that ranges from trace amounts up to 3 nmol/day (Ushida and Jouany, 1996; Tokura et al., 1997). There is some discrepancy in these figures as using stoichiometric calculations, the H2 produced per protozoan is not enough to synthesise all the methane emitted. Protozoa also produce considerable amounts of formate (Tokura et al., 1997), which can be partly used as a substrate for methane production. In addition, the methodology used in these different works might have contributed to this variation. Not all protozoal genera will have the same role in methanogenesis and despite their importance there is still scant information on the individual contribution to methane emissions. The cel-

lulolytic protozoan Polyplastron was a weak producer, Epi-
dinium caudatum was intermediate and I. prostoma and Entodinium caudatum were high producers (Newbold et al., 1995; Ushida and Jouany, 1996; Ranilla et al., 2007). When single protozoa were incubated at densities normally found in the rumen Entodinium sp. was identified as the most important protozoal genera contributing to methane emis-
sions (Ranilla et al., 2007). However, these results were obtained in vitro and need to be confirmed in animal trials.

As mentioned at the beginning of this section, rumen protozoa are metabolically very active and influence rumen function in several ways. In addition to their role in methane emissions, the presence of protozoa is positively related to fibre digestion and is negatively associated with the outflow of microbial protein from the rumen (Williams and Coleman, 1992; Eugène et al., 2004). All these aspects should be considered in any strategy aiming to control the protozoal population in the rumen. Williams and Withers (1993) indi-
cated that when the numbers of protozoa were low their effect on fibre hydrolysis was still positive but microbial protein synthesis seemed not to be affected. Incidentally, the results presented in Figure 2 also indicate the benefits of low protozoal populations on methane emissions. Methods, other than dietary, are needed to control the protozoal load and evaluate the effect of reduced protozoal populations on emissions and animal performances.

Plant fibre degradation and H2 production

Fibrolytic microorganisms play a pivotal role in the rumen ecosystem. Indeed, they are at the first level of the microbial trophic chain transforming plant cell wall polysaccharides from feeds into volatile fatty acids, CO2 and H2. They are thus

considered as a major microbial functional group in the rumen with a population estimated at ≈109 cells/ml digesta either by culture or by molecular methods (Mosoni et al., 2007). Most members of this group produce H2 as a main end product of fermentation, which under normal physiological conditions, is in turn rapidly used by methanogens. This interspecies H2 transfer between H2 producers and utilisers is fundamental in the functioning of anaerobic ecosystems including the rumen (Wolin et al., 1997). If this transfer is affected, the build-up of H2 in the milieu inhibits the reoxi-
idation of co-enzymes involved in redox reactions within bacterial cells, ultimately depressing the fermentation pro-
cesses. This is the reason why methanogenesis is intimately linked to degradation of plant fibre in the rumen.

Interspecies H2 transfer has been well described in vitro, especially between cellulolytics and methanogens (Wolin et al., 1997). One of the consequences of this interaction is a shift in fermentative metabolic pathways of the H2 produ-
cers. For example, the fibrolytic bacterium Ruminococcus albus produces ethanol, acetate, H2 and CO2 in monoculture in vitro, whereas it does not produce ethanol when co-cul-
tured with a methanogen (Wolin et al., 1997). When R. albus is co-cultivated with a methanogen, H2 never accumulates in the culture as it is transformed into methane, and acetate is the main metabolite produced. In the presence of metha-
nogens, as in the rumen, R. albus will thus mainly produce acetate from cellulose, and cellulose degradation is increased in vitro (Pavlostathis et al., 1990). Other bacterial species such as Ruminococcus flavefaciens and all the rumen fungi and protozoa produce H2 and have been shown to interact positively with methanogens (Latham and Wolin, 1977; Joblin et al., 1990; Williams et al., 1994). As described above for R. albus, in addition to changing the end products of fermentation, an increase in cellululysis was also observed when anaerobic fungi are cultured in association with methanogens (Latham and Wolin, 1977; Joblin et al., 1990). This synergic effect was also measured in vivo, in the rumen of gnotobiotic lambs with controlled microbiota (Fonty et al., 1997); the establishment of a methanogenic strain in the rumen of lambs (previously lacking methanogens) induced an increase in the population of R. flavefaciens, as well as in straw degradation and glycoside hydrolyase activity.

Recent in vitro work investigated the effects of metha-
nogen inhibition on fibrolytic populations in complex rumen microbiota (Table 2). Inhibitors of the methane pathway, bromochloromethane (BCM) and bromothanesulfonate (BES) led to a disappearance of the methanogen populations and to a strong decrease in methane production. BCM led to a decrease in R. flavefaciens populations with a concomitant proliferation of Fibrobacter and fungi with no effect on total bacteria and protozoa (Goel et al., 2009). In contrast, BES had no effect on R. flavefaciens and decreased significantly the fungal population while increasing Fibrobacter (Guo et al., 2007). These different effects of BCM and BES on the ruminococcal and fungal populations are not easily explained, and could be due to different experimental conditions. The addition of different saponins, used as defaunating
agents, led to a marked decrease in protozoa, as expected, but sometimes also in fungi and methanogens (Table 2). In Guo et al. (2008), the methanogen populations were not affected by the addition of tea saponins, but the methanogenic activity, estimated by quantification of the expression of the mcrA gene (coding for the enzyme of the terminal step of the methanogenesis pathway), clearly decreased. Methane production was slightly affected by saponins (<10% decrease), which correlates with in vivo data (see the previous section: Protozoa and methanogenesis). The Fibrobacter population tend to increase (Table 2; Wina et al., 2005; Goel et al., 2008; Guo et al., 2008). To summarise the results of these in vitro experiments, the inhibition of methanogens was correlated to a decrease in methane synthesis, but it also led to an inhibition of the Ruminococci and the fungi in some of them. The inhibition of methanogenesis is expected to increase the partial pressure of H2, leading to an inhibition of H2-producing microorganisms such as the Ruminococci and the fungi. The data collected in Table 2 suggest that in the complex rumen microbiota the mechanisms involved in H2 balance are not straightforward. Nevertheless, fibre digestibility was not affected in these experiments (Table 2), except in Wina et al. (2005). The increase in Fibrobacter populations observed in these studies may have allowed an efficient fibre degradation to be maintained. Thus, promoting non-H2-producing fibrolytic organisms such as Fibrobacter succinogenes might be an alternative way to decreasing methane emissions in the rumen without impairing fibre digestibility.

The effect of the composition of the fibrolytic community on methane production has also been investigated. In vitro incubation of rumen content from gnotobiotic lambs harbouring H2-producing (Ruminococci and fungi) or non-H2 producing (Fibrobacter) fibrolytic microorganisms and methanogens indicated that methane was produced in higher quantities by the inoculum containing the H2-producing cellulolytic microbiota (Chaucheyras-Durand et al., 2008). These results suggest that the composition of the fibrolytic community (producers or non-producers of H2) may have an impact on H2 accumulation and subsequent methane production. However, in an in vivo study with gnotobiotic lambs, inoculation of a methanogenic strain increased both the Ruminococci and the Fibrobacter populations (Mosoni et al., 2008).

Earlier study has shown that numbers of fibrolytic bacteria were positively correlated to that of methanogens in the rumen of various animals, including cattle, sheep, deer and llama, and in the horse caecum (Morvan et al., 1996). This correlation was explained because the major fibrolytic species such as R. albus and R. flavífaciens produce H2 as a main fermentation end product. In contrast, the same authors reported that in the rumen of buffalo the numbers of methanogens were comparatively lower than that of fibrolytic bacteria. This may be because Fibrobacter, a bacterium that does not produce H2, was the dominant fibrolytic bacterium in the rumen of buffalos (Morvan et al., 1994). Unfortunately, methane emissions were not measured in the study. Ionophores such as monensin have been shown to cause a moderate inhibition of methane production, and a similar mechanism was proposed because monensin is known to inhibit the Ruminococci without affecting F. succinogenes (Chen and Wolin, 1979). The relationship between cellulolytic microorganisms and methane production thus merits further investigation as promoting non-H2-producing fibrolytic microbes could be a way to decrease H2 — and consequently methane— production while keeping active rumen fibre degradation.

In conclusion, although some in vitro experiments tend to show a relationship between methanogens or methane production and the ratio of H2-producing/non-H2-producing fibrolytic bacteria, which could be explained on the basis of interspecies H2 transfer, in vivo experiments are needed to explore more deeply these relationships in the rumen.

### Reductive acetogenesis

The conversion of H2 and CO2 into acetate through reductive acetogenesis occurs in many microbial anaerobic ecosystems including the gastrointestinal tract of non-ruminant animals, where it can co-exist with methanogenesis (Klieve and

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### Table 2: Effect of several additives on methane production, microbial populations, fibre digestibility and acetate/propionate ratio in rumen incubations in vitro*  

<table>
<thead>
<tr>
<th>Additive</th>
<th>CH4</th>
<th>Methanogens</th>
<th>RfI</th>
<th>Ral</th>
<th>Fsu</th>
<th>Fungi</th>
<th>Protozoa</th>
<th>Fibre digestibility</th>
<th>Acetate/propionate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCM</td>
<td>-90%*</td>
<td>-100%*</td>
<td>-48%*</td>
<td>+68%*</td>
<td>+30%</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Goel et al. (2009)</td>
</tr>
<tr>
<td>BCM 2</td>
<td>-90%*</td>
<td>-100%*</td>
<td>-66%*</td>
<td>+8%</td>
<td>-62%*</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Goel et al. (2009)</td>
</tr>
<tr>
<td>BES</td>
<td>-86%**</td>
<td>-90%**</td>
<td>No effect</td>
<td>+50%**</td>
<td>-60%**</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Goel et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>-8%**</td>
<td>No effect</td>
<td>No effect</td>
<td>+41%**</td>
<td>-79%**</td>
<td>-50%*</td>
<td>No effect</td>
<td>Goel et al. (2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>-16%*</td>
<td>-80%</td>
<td>-90%</td>
<td>No effect</td>
<td>-70%*</td>
<td>-50%</td>
<td>-25%*</td>
<td>No effect</td>
<td>Wina et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>-6%</td>
<td>-78%</td>
<td>+30%</td>
<td>+40%</td>
<td>-40%</td>
<td>-39%</td>
<td>No effect</td>
<td>No effect</td>
<td>Goel et al. (2008)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

*RfI = Ruminococcus flavefaciens; Ral = Ruminococcus albus; Fsu = Fibrobacter succinogenes; BCM = bromochloromethane; BES = bromoethanesulfonate.

*All the additives were added to batch mixed cultures in vitro except BCM 2 that was added to continuous cultures.

<table>
<thead>
<tr>
<th>Additions to rumen content</th>
<th>Methanogens</th>
<th>H2</th>
<th>CO2</th>
<th>Acetate</th>
<th>Propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromochloromethane</td>
<td>90%**</td>
<td>No effect</td>
<td>40%</td>
<td>30%</td>
<td>60%**</td>
</tr>
<tr>
<td>Bromoethanesulfonate</td>
<td>80%</td>
<td>No effect</td>
<td>50%</td>
<td>10%</td>
<td>60%**</td>
</tr>
</tbody>
</table>

**Indicate significant differences (P < 0.05 and P < 0.01, respectively) as reported in the original reference.
Ouwerkerk, 2007). A decreased emission of methane associated with an increase in acetate available to the host animal is a desirable condition that, combined with the observation that reductive acetogenesis is the main electron sink observed in many non-ruminant herbivores (Klieve and Ouwerkerk, 2007), has stimulated the study to promote this pathway in the rumen (Lopez et al., 1999). Active homoacetogens become established in the rumen of newborn animals as early as 20 h after birth (Morvan et al., 1994). Methanogens became established soon after, as they are detected within 1 to 3 days after birth, but when methanogens’ numbers increase, before reaching adult concentrations at 3 weeks of age in lambs, the concentration of homoacetogens decrease (Morvan et al., 1994; Skillman et al., 2004). In adult animals, densities of homoacetogens range from $10^3$ to $10^7$/g (Le Van et al., 1998; Fonty et al., 2007). Numbers are affected by diet and also by the technique used to cultivate and enumerate these bacteria, but they are at least 10-fold less numerous than the methanogens (Le Van et al., 1998; Fonty et al., 2007). In lambs isolated at birth with a controlled rumen microbiota not harbouring methanogens, homoacetogen numbers were $10^7$ to $10^9$/g and reductive acetogenesis represented up to 25% of the total fermentation output (Fonty et al., 2007). However, the H$_2$ recovery calculated from the stoichiometry of VFA production was less than 50%, indicating a probable loss of H$_2$ as gas. Introduction of methanogens into the rumen of these animals eliminated reductive acetogenesis altogether and restored H$_2$ recovery to normal values, near 100% (Fonty et al., 2007). Homoacetogens are outcompeted by methanogens because they are less efficient at obtaining energy from the oxidation of H$_2$ and have both a lower affinity and a higher threshold for the substrate (Hoehler et al., 1998; Le Van et al., 1998; Weimer, 1998). The partial pressure of H$_2$ in the rumen is less than 50%, indicating a probable loss of H$_2$ as gas. Introduction of methanogens into the rumen of these animals eliminated reductive acetogenesis altogether and restored H$_2$ recovery to normal values, near 100% (Fonty et al., 2007). Homoacetogens are outcompeted by methanogens because they are less efficient at obtaining energy from the oxidation of H$_2$ and have both a lower affinity and a higher threshold for the substrate (Hoehler et al., 1998; Le Van et al., 1998; Weimer, 1998). The partial pressure of H$_2$ in the rumen is normally lower than the threshold of reductive acetogenesis. Isolates from the rumin, in addition to being autotrophic are also heterotrophic and it has been proposed that they rely mainly on organic substrates for their survival in the rumen ecosystem (Le Van et al., 1998). Attempts to boost reductive acetogenesis by increasing the numbers of natural rumen homoacetogens or by addition of non-rumen isolates have not been successful in several in vitro and in vivo trials (Demeyer et al., 1996; Immig et al., 1996; Nollet et al., 1997 and 1998; Le Van et al., 1998; Lopez et al., 1999).

Bacteria using alternative H$_2$ sinks

Some bacteria present in the rumen can respire anaerobically. These bacteria are capable of using electron acceptors other than CO$_2$ to oxidise H$_2$, hence decreasing its availability to form methane (Figure 1). Sulphate-reducing bacteria, Desulfibacterium detoxificans and Wolinella succinogenes utilise sulphate, nitrocompounds and nitrate, respectively, as electron acceptors (Weimer, 1998; Anderson et al., 2000; Simon, 2002). Normally these bacteria are not dominant in the ecosystem, but their numbers can increase if the right potential electron acceptor is provided in the diet (Figure 1). The reduction of sulphate is not desirable as the end product, H$_2$ sulphide, is toxic to the host animal (Gould et al., 1997). Nitrocompounds have been shown to reduce methanogenesis (Anderson et al., 2006). However, the main mechanism of action of nitrocompounds seems to be mediated by the inhibition of formate dehydrogenases and/or H$_2$ dehydrogenases (Anderson et al., 2008) and, paradoxically, increases in the numbers of D. detoxificans, which uses H$_2$ to reduce these compounds, may reduce the abating potential of nitrocompounds (Anderson et al., 2006, Gutierrez-Banuelos et al., 2007). In contrast, one strain of D. detoxificans was able to utilise nitrate and reduced methane production in vitro when this substrate was added (Anderson and Rasmussen, 1998).

Nitrate, like sulphur, can be considered as an alternative electron sink. The thermodynamics of the conversion of nitrate into NH$_3$ is more favourable than the formation of methane and can effectively replace methanogenesis in the rumen if nitrate is available. However, in the rumen nitrate is rapidly reduced into nitrite and the rate of reduction of nitrite into NH$_3$ is slower (Iwamoto et al., 1999). Nitrite accumulation is undesirable as it is extremely toxic to the host animal. In spite of this, increases in the number of bacteria capable of reducing nitrite have been shown to reduce both methane production and nitrite toxicity. Wolinella succinogenes, a rumen bacterium that grows by respiratory nitrate ammonification (Simon, 2002), has the ability to reduce nitrate into NH$_3$ with little accumulation of nitrites. Wolinella was shown to reduce methane production in vitro but only if nitrate was added to the incubation media (Iwamoto et al., 2002). These effects were also demonstrated in vivo using strains of Escherichia coli with high nitrate/nitrite-reducing activity (Sa et al., 2005a and 2005b).

Methanotrophy, that is, the oxidation of methane, was reported to account for <0.5% of rumen methane production in vitro (Kajikawa et al., 2003). This oxidation was found to be anaerobic and associated with sulphate reduction. In contrast, putative methane-oxidising bacteria, evidenced by PCR amplification with specific primers, were found attached to the rumen epithelium (Mitsumori et al., 2002) where conditions may favour aerobic oxidation of methane. This metabolic pathway has yet to be quantified in vivo. In terms of benefit to the host, the final product of this pathway, CO$_2$, does not supply any energy, and the nitrogen and energy contained in the biomass of methanotrophs would be negligible. Capnophily, that is, the ability to use CO$_2$ as the final product of the reductive acetogenesis is mediated by the inhibition of formate dehydrogenases and/or H$_2$ dehydrogenases (Anderson et al., 2008) and, paradoxically, increases in the numbers of D. detoxificans, which uses H$_2$ to reduce these compounds, may reduce the abating potential of nitrocompounds (Anderson et al., 2006, Gutierrez-Banuelos et al., 2007). In contrast, one strain of D. detoxificans was able to utilise nitrate and reduced methane production in vitro when this substrate was added (Anderson and Rasmussen, 1998).
Concluding remarks

The microbiota and the host animal have co-evolved for millions of years and the production of methane is a mechanism that improves the fermentation process. There is not a clear relationship between the number of methanogens and methanogenesis in the rumen (Nollet et al., 1998; Machmüller et al., 2003a and 2003b; Yanez-Ruiz et al., 2008; Mosoni et al. personal communication), except for trials when specific inhibitors of methanogenesis were used (i.e. BSM; Denman et al., 2007). It is reasonable to think that the reduction in methane emissions without an associated change in the number of methanogens must be attributed to changes in the rumen microbiota and the availability of H2. In this study, we show that there is direct correlation between the number of protozoa and methane production. Indeed, some of the most successful strategies to reduce emissions have to some extent a negative effect upon protozoa. The balance between H2 producers and non-H2 producers in the fibrolytic community can be shifted under experimental conditions to reduce methanogenesis without altering their fibre-degrading capability. Communities predominantly composed of non-H2 producers have been observed in some ruminant species under natural conditions indicating the viability of the approach. Finally, the use of an alternative electron sink such as nitrate needs to be revisited and tested under long periods of supplementation to allow increases in the number of nitrate utilisers in the rumen and avoid toxicity. Nitrate supplementation could be a feasible alternative in N-poor diets.

Decreasing rumen methanogenesis is possible, with some strategies already available and many others that are being tested. It is possible to modify the microbial ecosystem to decrease its production of methane, but the manipulation of microbial components of the system has to be considered in an integrated way. In the next few years, advances brought about through rumen metagenomic projects and the utilisation of new ‘omics’ technologies will broaden our understanding of the mechanisms involved in methanogenesis and other metabolic H2-consuming and releasing processes, and will help the scientific community to find new targets for mitigation.

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Rumen microbiota and methanogenesis


Morgavi, Forano, Martin and Newbold


