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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 H_2 and CO_2 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 (H_2) 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 ('H_2' from this point on) is the key element that drives methane production in the rumen. Among H_2 producers, protozoa have a prominent position, which is strengthened by their close physical association with methanogens, which favours H_2 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 H_2 is the degradation of fibrous plant material. However, not all members of the rumen fibrolytic community produce H_2. Increasing the proportion of non-H_2 producing fibrolytic microorganisms might decrease methane production without affecting forage degradability. Alternative pathways that use electron acceptors other than CO_2 to oxidise H_2 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, H_2 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 H_2, as substrate. Available information indicates that decreasing the number of H_2 producers such as protozoa and some fibrolytic microbes and/or increasing the number and activity of non-methanogenic, H_2 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

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is in addition to the normal estimated rate of increase in the world population. Indeed, global consumption of meat is expected to rise from 229 to 465 million tonnes and milk from 580 to 1043 million tonnes by 2050 (FAO, 2006). Much of this increase is projected to occur in China and India (Grigg, 1999; Delgado *et al*., 2001). This increased demand brings with it challenges in terms of global resource usage and food security (World Bank, 2008). Clearly, there is a need to develop sustainable systems of animal production that do not compete directly with mankind for foodstuffs. The efficiency of production of meat, milk and other products from ruminants depends on the basis on which efficiency is considered. Gill *et al*. (2010) estimated that only between 6% and 26% of dietary energy was recovered in ruminant products. However, when calculated as human-edible efficiency (human-edible energy contained in the product divided by human-edible inputs), these values increased to between 65% and 374% recovery dependent on the production system and reflect the ability of ruminants to utilise fibrous feedstuffs not readily utilised by monogastrics, including man. Gill *et al*. (2010) conclude, and we would concur, that when used to transform fibrous feedstuffs produced on land not suitable for primary cropping or by-products of the food industry, ruminants can be net contributors to the global supply of human-edible food. Unfortunately, however, ruminant production carries with it a significant environmental cost (Steinfeld *et al*., 2006) both at the local and global levels. Although locally this is mainly associated with intensive operations that contaminate the air, land or water with nitrogenous compounds and phosphorous, the global effect is predominantly due to the contribution to the emissions of greenhouse gases (GHGs), which occurs in both intensive and extensive systems. Global estimates of the contribution of livestock agriculture to GHG emissions vary between 9% and 18% of anthropogenic emissions, dependent on whether only direct emissions are accounted for or whether emissions associated with the production of feeds, fuel usage, land use change, transport of products, etc. are also included in the calculation (Steinfeld *et al*., 2006; IPPC, 2007). Methane emissions represent between 30% and 50% of the total GHG emitted from the livestock sector with enteric methane from ruminant production systems representing by far the most numerically important source being responsible for approximately 80% of the methane emissions from the sector (Gill *et al*., 2010).

Methane is produced in the gastrointestinal tract of ruminants and in particular within the rumen by a specialised group of microbes, the methanogenic archaea (Janssen and Kirs, 2008; Liu and Whitman, 2008). Because methanogens are ultimately responsible for methane production in ruminants, a considerable research effort is being directed to characterise these microbes (Attwood *et al*., 2008). However, in the complex microbial web of the gastrointestinal tract many other microorganisms also have an important influence on methane production, either by promoting an environment suitable for methanogen survival or by producing the substrates utilised by the methanogens. The aim of this paper is to highlight the relationship between methanogenesis and some of these latter microbial groups and bring attention to some particular functional groups that could play a role in developing strategies to decrease methane emissions from ruminant livestock.

Rumen microbiota

The microbial community inhabiting the rumen is extremely diverse. The majority of these microbes have not yet been cultivated; however, using molecular biology methods it has been estimated that, for the bacteria alone there are at least between 300 and 400 phylotypes (Edwards *et al*., 2004; Larue *et al*., 2005; Yu *et al*., 2006). In addition, protozoa, fungi, methanogenic archaea and bacteriophages also contribute to the diversity and functioning of the rumen microbial ecosystem. These microbial rumen symbionts, which have co-evolved with their animal hosts for millions of years, are highly specific and perform metabolic functions that are essential for the development, health and nutrition of the animal.

The rumen is an anaerobic, methanogenic environment where CO_2 and H_2 produced from the fermentation of feeds, which are the main electron acceptor and donor, respectively, within the system (Figure 1). In this type of habitat, the degradation of organic matter is accomplished by the concerted cooperation of different groups of anaerobes. Plant structural carbohydrates, proteins and other organic polymers contained in feeds ingested by the animal are degraded to their monomer components by primary anaerobic fermenters. These monomers are then further converted into volatile fatty acids, CO_2 and H_2 by both the primary fermenters and other microbes that do not have the capacity to hydrolyse complex polymers by themselves (secondary fermenters). Methanogens are at the bottom of this trophic chain and use the end products of fermentation as substrates (Figure 1). The synthesis of methane contributes to the efficiency of the system in that it avoids increases in the partial pressure of H_2 to levels that might inhibit the normal functioning of microbial enzymes involved in electron transfer reactions, particularly NADH dehydrogenase, resulting in NADH accumulation, and ultimately reduce rumen fermentation. The capturing of the H_2 produced by one microbial species by another is normally referred as interspecies H_2 transfer (Wolin *et al*., 1997) and is a process that in many cases involves a syntrophic relationship between two microbes.

As discussed below the type of diet and the presence of electron acceptors other than CO_2 in the rumen will have a major effect on the presence and activity of H_2 producers and utilisers. This is because pathways other than methanogenesis can also consume H_2 and thus potentially compete with and decrease methanogenesis in the rumen.

Rumen methanogens

Methanogens belong to the domain archaea. The diversity of archaea found in the rumen have been recently reviewed by

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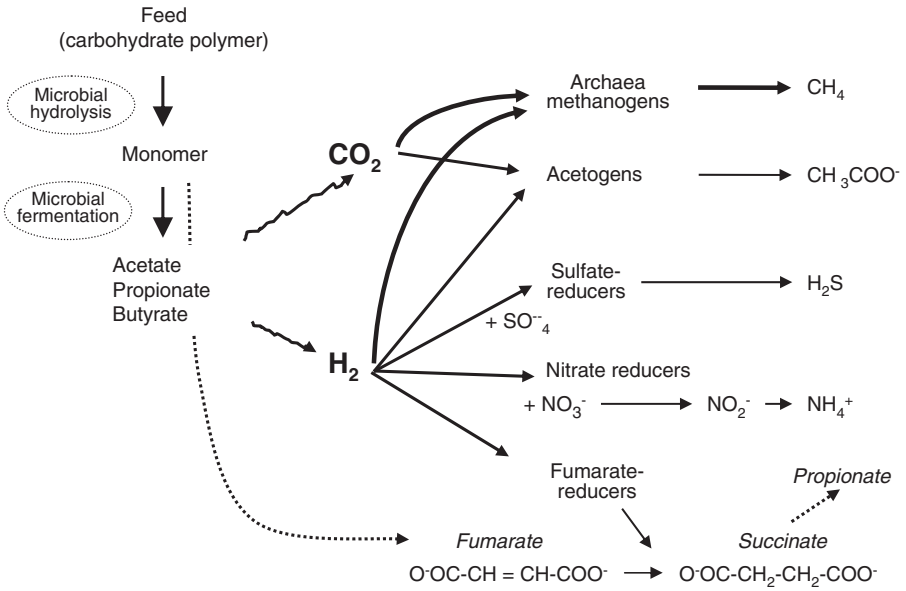


Figure 1 Schematic microbial fermentation of feed polysaccharides and H_2 reduction pathways in the rumen.

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 H_2 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: CO_2, compounds containing a methyl group or acetate (Liu and Whitman, 2008). In the rumen, the predominant pathway is the hydrogenotrophic using CO_2 as the carbon source and H_2 as the main electron donor (Hungate, 1967). Formate is also an important electron donor used by many rumen hydrogenotrophic methanogens and may account for up to 18% of the methane produced in the rumen (Hungate *et al*., 1970). Methylamines and methanol produced in the rumen can also be used by methylotrophic methanogens of the order Methanosarcinales and *Methanosphaera* spp. from the order Methanobacteriales (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 Methanosarcinales (Liu and Whitman, 2008). Oppermann *et al*. (1961) measured the increase in radio-labelled methane following infusion of ^{14}C-acetate *in vivo* and concluded that this pathway was not important in the rumen. *Methanosarcina* 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 *Methanosarcina* 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.

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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 H_2 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 SF_6 (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 (10^5/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 10^5 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

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Table 1 *The effect of defaunation on methane production as measured in* in vivo *experiments*

Methane (g/animal per day)						
With protozoa	Defaunated	Response (% change)	Animal sp.	Diet	Notes	Reference
13.4	14.9	11.1	Sheep	Dried lucerne	Defaunated using a detergent, methane measured in a chamber after 10 weeks of defaunation	Bird *et al.* (2008)
13.9	15.1	8.6			25 weeks after defaunation	
15.7	14.0	−10.8	Sheep	Cellulose based	Defaunated using a detergent, methane measured in a chamber	Kreuzer *et al.* (1986)
8.2	9.1	11.1		Native starch		
12.0	5.7	−47.5		Steamflaked starch based		
13.4	9.4	−29.9**	Sheep	Roughage and concentrate (3 : 7)	Defaunated using a detergent, methane measured in a chamber	Chandramoni *et al.* (2002)
12.6	13.1	4.0	Sheep	Roughage and concentrate (2 : 8)	Defaunated using a detergent, methane measured in a chamber	Hegarty *et al.* (2008)
15.6	16.1	3.2		Roughage		
25.1	18.6	−25.9*	Sheep	Roughage and concentrate (1 : 1)	Isolated at birth, methane measured in a chamber	Yanez-Ruiz *et al.* (2007)
31.5	23.9	−24.1*	Sheep	Roughage and concentrate (3 : 1)	Defaunated by rumen washing, methane measured using SF6.	Morgavi *et al.* (2008)
129.6	64.1	−49.6**	Cattle	Barley with a protein supplement	Defaunated using a detergent, methane measured in a chamber	Whitelaw *et al.* (1984)
16.6	18.2	9.6	Sheep	Maize silage concentrate diet supplemented with protected fat	Defaunated using a detergent, methane measured in a chamber	Machmüller *et al.* (2003b)
14.3	14.6	2.1		Supplemented with coconut oil		
14.1	14.6	3.5	Goat	Hay	Isolated at birth methane measured in a chamber	Itabashi *et al.* (1984)
22.9	17.9	−21.8**		Hay plus concentrate		
18.6	16.4	−11.8		Hay plus concentrate plus monensin		
		−10.5				Average response

*, ** Indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as reported in the original reference.

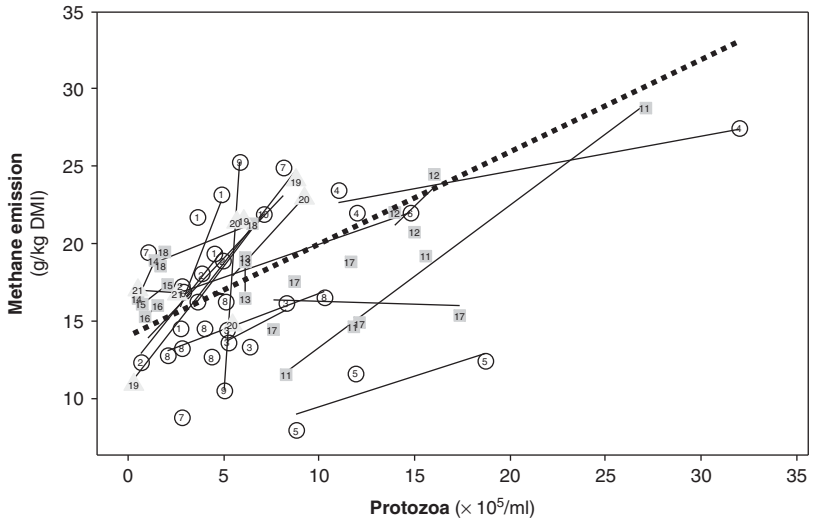


Figure 2 Methane emissions and rumen protozoa concentration in ruminants receiving diets rich in starchy concentrates (△), lipids (○) and plant extracts (□): review of available literature. Data from Machmüller and Kreuzer, 1999 (7); Machmüller *et al.*, 2000 (8); Sliwinski *et al.*, 2002 (17); Lovett *et al.*, 2003 (6, 20); Machmüller *et al.*, 2003a and 2003b (9, 10); Hess *et al.*, 2004 (14, 15, 16); Lila *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^{-5} (s.e. = 0.11) × protozoa (10^5/ml); (R^2 = 0.65; R^2 adjusted = 0.47; $P < 0.001$).

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 rumen 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 rumen 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 phylotypes 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 Entodiniomorphs seem able to carry methanogens on their surface, but no episymbiotic 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

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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 H_2/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 H_2 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 cellulolytic protozoan *Polyplastron* was a weak producer, *Epidinium 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 emissions (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) indicated 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 H_2 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, CO_2 and H_2. They are thus considered as a major microbial functional group in the rumen with a population estimated at $\approx 10^9$ cells/ml digesta either by culture or by molecular methods (Mosoni *et al.*, 2007). Most members of this group produce H_2 as a main end product of fermentation, which under normal physiological conditions, is in turn rapidly used by methanogens. This interspecies H_2 transfer between H_2 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 H_2 in the milieu inhibits the reoxidation of co-enzymes involved in redox reactions within bacterial cells, ultimately depressing the fermentation processes. This is the reason why methanogenesis is intimately linked to degradation of plant fibre in the rumen.

Interspecies H_2 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 H_2 producers. For example, the fibrolytic bacterium *Ruminococcus albus* produces ethanol, acetate, H_2 and CO_2 in monoculture *in vitro*, whereas it does not produce ethanol when co-cultured with a methanogen (Wolin *et al.*, 1997). When *R. albus* is co-cultivated with a methanogen, H_2 never accumulates in the culture as it is transformed into methane, and acetate is the main metabolite produced. In the presence of methanogens, 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 H_2 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 cellulolysis 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 hydrolase activity.

Recent *in vitro* work investigated the effects of methanogen inhibition on fibrolytic populations in complex rumen microbiota (Table 2). Inhibitors of the methane pathway, bromochloromethane (BCM) and bromoethanesulfonate (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

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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[a]

Additive	CH_4	Methanogens	Rfl	Ral	Fsu	Fungi	Protozoa	Fibre digestibility	Acetate/propionate	Reference
BCM	−90%*	−100%*	−48%*		+68%*	+30%	No effect	No effect	No effect	Goel *et al.* (2009)
BCM 2	−90%*	−100%*	−66%*		+8%	+62%*	No effect	No effect	No effect	Goel *et al.* (2009)
BES	−86%**	−90%**	No effect		+50%**	−60%**	No effect	No effect	No effect	Guo *et al.* (2007)
Saponins	−8%**	No effect	No effect		+41%**	−79%**	−50%*		No effect	Guo *et al.* (2008)
Saponins		−16%*	−80%	−90%	No effect	−70%*	−50%	−25%*	No effect	Wina *et al.* (2005)
Saponins	−6%	−78%	+30%		+40%	−40%	−39%	No effect	No effect	Goel *et al.* (2008)

Rfl = *Ruminococcus flavefaciens*; Ral = *Ruminococcus albus*; Fsu = *Fibrobacter succinogenes*; BCM = bromochloromethane; BES = bromoethanesulfonate.
[a]All the additives were added to batch mixed cultures *in vitro* except BCM 2 that was added to continuous cultures.
Cellulolytic bacterial species, methanogens and fungi were estimated by quantification of their *rrs* gene by relative quantitative PCR, except in the work of Wina *et al.* (2005) in which they were estimated by quantification of 16S RNA using dot blots, and in Guo *et al.* (2008), in which methanogens were estimated by PCR quantification of the *mcrA* gene.
Protozoa were quantified by cell counting or by *rrs* gene PCR quantification (only in Guo *et al.*, 2008).
*, ** Indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) as reported in the original reference.

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 H_2 leading to an inhibition of H_2-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 H_2 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-H_2-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 H_2-producing (*Ruminococci* and fungi) or non-H_2 producing (*Fibrobacter*) fibrolytic microorganisms and methanogens indicated that methane was produced in higher quantities by the inoculum containing the H_2-producing cellulolytic microbiota (Chaucheyras-Durand *et al.*, 2008). These results suggest that the composition of the fibrolytic community (producers or non-producers of H_2) may have an impact on H_2 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. flavefaciens* produce H_2 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 H_2, 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-H_2-producing fibrolytic microbes could be a way to decrease H_2 – 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 H_2-producing/non-H_2-producing fibrolytic bacteria, which could be explained on the basis of interspecies H_2 transfer, *in vivo* experiments are needed to explore more deeply these relationships in the rumen.

Reductive acetogenesis

The conversion of H_2 and CO_2 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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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 10^8/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 normally lower than the threshold of reductive acetogenesis. Isolates from the rumen, 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, *Denitribacterium 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 (Sar *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, is also present in the rumen with many rumen isolates from common bacterial species such as *Prevotella, Fibrobacter, Ruminococcus, Lachnospira* and *Succinomonas* requiring CO_2 to grow (Dehority, 1971). Capnophilic bacteria also use H_2 to produce organic acids, particularly succinic acid as final products, but the influence of this metabolic conversion on H_2 balance is not known. *Mannheimia succiniciproducens*, a rumen bacterium whose genome has been sequenced, produces succinic acid as the main metabolic product and consumes CO_2 and H_2 in the process (Hong *et al*., 2004). This and other similar bacteria may have an effect on net H_2 and methane balance if their numbers and activity are sufficient in the rumen.

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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 H_2. 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 H_2 producers and non-H_2 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-H_2 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 H_2-consuming and releasing processes, and will help the scientific community to find new targets for mitigation.

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