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Animal (2014), **8:2**, pp 250–261 © The Animal Consortium 2013
doi:10.1017/S1751731113002085

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The use of direct-fed microbials for mitigation of ruminant methane emissions: a review

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(Received 03 March 2013; Accepted 28 October 2013; First published online 25 November 2013)

Concerns about the environmental effect and the economic burden of methane (CH_4) emissions from ruminants are driving the search for ways to mitigate rumen methanogenesis. The use of direct-fed microbials (DFM) is one possible option to decrease CH_4 emission from ruminants. Direct-fed microbials are already used in ruminants mainly to increase productivity and to improve health, and are readily accepted by producers and consumers alike. However, studies on the use of DFM as rumen CH_4 mitigants are scarce. A few studies using Saccharomyces cerevisiae *have shown a CH_4-decreasing effect but, to date, there has not been a systematic exploration of DFM as modulators of rumen methanogenesis. In this review, we explored biochemical pathways competing with methanogenesis that, potentially, could be modulated by the use of DFM. Pathways involving the redirection of H_2 away from methanogenesis and pathways producing less H_2 during feed fermentation are the preferred options. Propionate formation is an example of the latter option that in addition to decrease CH_4 formation increases the retention of energy from the diet. Homoacetogenesis is a pathway using H_2 to produce acetate, however up to now no acetogen has been shown to efficiently compete with methanogens in the rumen. Nitrate and sulphate reduction are pathways competing with methanogenesis, but the availability of these substances in the rumen is limited. Although there were studies using nitrate and sulphate as chemical additives, use of DFM for improving these processes and decrease the accumulation of toxic metabolites needs to be explored more. There are some other pathways such as methanotrophy and capnophily or modes of action such as inhibition of methanogens that theoretically could be provided by DFM and affect methanogenesis. We conclude that DFM is a promising alternative for rumen methane mitigation that should be further explored for their practical usage.*

Keywords: direct-fed microbials, biochemical pathways, methane, rumen

Implications

Methane produced in the rumen contributes significantly to the global emission of greenhouse gases. Among the different strategies researched to reduce rumen methanogenesis, the use of direct-fed microbials (DFM) has received little attention so far. From a practical perspective, the DFM concept is well known to farmers as it is already utilised to increase animal productivity and to improve their health. This review explores the possibilities to modify some rumen biochemical pathways to decrease methanogenesis by using DFM.

Introduction

Methane (CH_4) is an important greenhouse gas (GHG) that has a global warming potential 25 times higher than that of carbon dioxide (CO_2; Intergovernmental Panel on Climate Change, 2007). Ruminants are the single largest source of CH_4 emission from agriculture, globally contributing about 40% of the emissions produced by human-related activities (Steinfeld *et al.*, 2006). Enteric CH_4 is produced in the rumen and to a lesser extent in the large intestine of ruminants. The rumen is the primary location for microbial fermentation of plant material in ruminants and it contains a microbial population made up of bacteria, archaea (methanogens), protozoa, fungi and phage. These rumen microorganisms function via complex interactions, which are essential to sustain their population and activity. The ingested feed is digested and fermented by bacteria, protozoa and fungi into short chain fatty acids (VFA), which are then used by the host as its energy source. Molecular hydrogen (H_2) is an important by-product of this fermentation and is used by rumen methanogens to reduce CO_2 into CH_4.

Concerns about the environment and energy economics (about 5% to 9% of dietary gross energy loss) of rumen CH_4

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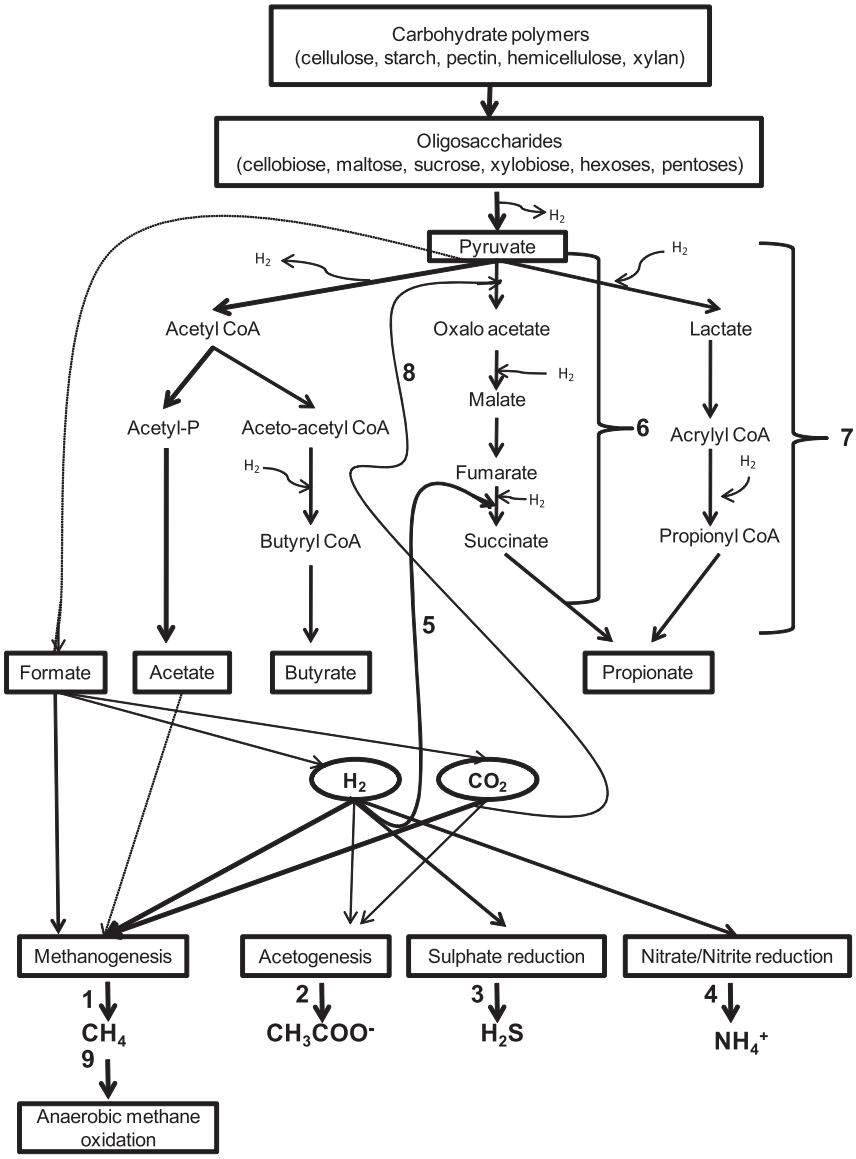

Figure 1 Rumen biochemical pathways that could be modulated by direct-fed microbials to decrease CH_4 production. 1. Methanogenesis, 2. homoacetogenesis, 3. sulphate reduction, 4. nitrate/nitrite reduction, 5. fumarate reduction, 6. propionate production (succinate/randomizing pathway), 7. propionate production (acrylate pathway) 8. capnophily (CO_2 fixation), 9. methane oxidation (methanotrophy).

emission compel researchers to look for ways to decrease rumen methanogenesis (Martin *et al.*, 2010; Buddle *et al.*, 2011). Among the different strategies studied, one promising method is the manipulation of biochemical pathways existing in the rumen to produce less CH_4. Use of direct-fed microbials (DFM) for this manipulation is one possible option. Direct-fed microbials have been defined as a 'source of live, naturally occurring microorganisms' (Krehbiel *et al.*, 2003) and, they have been successfully used in ruminant production to increase productivity, to prevent digestive disorders like acidosis and to decrease pathogenic load in young animals (Adams *et al.*, 2008; McAllister *et al.*, 2011; Lettat *et al.*, 2012b). They are an accepted alternative to the use of antibiotics and chemical substances that may induce a risk of antibiotic resistance and residues in animal products. However, to date there is little evidence to suggest the efficacy of DFM to control the production of CH_4 in ruminants.

The majority of rumen methanogens use H_2 to reduce CO_2 to CH_4. Some rumen methanogens can utilise formate or methyl group containing compounds such as methanol and methylamine (Janssen and Kirs, 2008). Carbon dioxide constitutes up to 65% of total gas in the rumen (Ellis *et al.*, 1991) and it is not a limiting substrate of rumen methanogenesis. Therefore, H_2 is a key compound for controlling CH_4 production. The major biochemical pathways explored in this review to decrease CH_4 emissions from ruminants by using DFM are the redirection of H_2 away from methanogenesis and decreased production of H_2 during feed fermentation. Other potential pathways are also briefly explored in this review. The different pathways that could be modulated to decrease rumen methanogenesis by providing DFM are shown in Figure 1. Biohydrogenation of

unsaturated fatty acids is another H_2-utilising pathway present in the rumen. However, biohydrogenation accounts only for 1% to 2% of the H_2 consumed (Nagaraja *et al*., 1997). As such, this pathway is not discussed in this review.

Yeast (*Saccharomyces cerevisiae*)

Saccharomyces cerevisiae is the most commonly used DFM in ruminant production and not surprisingly is the DFM that has been more extensively studied for its effect on rumen methanogenesis. However, results from both *in vitro* (Table 1) and *in vivo* (Table 2) studies are inconsistent. These variations can be partly explained by the differences in experimental conditions (yeast strains and formats (live culture or freezed-dried preparation), dose, animal species, physiological state of animals and diets) but also because up to now no strain has been selected based on effects on methanogenesis. The effects and modes of action of yeast on rumen fermentation have been extensively studied (Newbold *et al*., 1996; Chaucheyras-Durand *et al*., 2008). Chaucheyras-Durand *et al*. (2008) in their review identified three main effects of yeast on rumen development: improvement of rumen maturity by favouring microbial establishment, stabilising rumen pH and increasing fibre degradation. Live yeast also showed beneficial effects on the growth and H_2-utilisation of acetogenic bacteria *in vitro* (Chaucheyras-Durand *et al*., 1995). Being an aerobic organism, the above mentioned effects may be due to the ability of yeast to remove the trace amounts of oxygen present in the rumen and/or due to the micronutrients present in the yeast itself (McAllister *et al*., 2011).

Propionate-forming bacteria

The major VFA produced in the rumen are acetic, propionic and butyric acids and their proportion mainly depends on the diet offered to the animal. Ruminants fed a concentrate-based diet produce proportionally more propionate than those fed a high forage diet, which produces more acetate. Propionate formation consumes reducing equivalents, pyruvate is reduced to propionate, therefore it is considered as H_2-utilisation pathway, while in H_2 formation, protons (H^+) are reduced to H_2 (Baldwin *et al*., 1963). As H_2 is the main precursor for CH_4 production, increase in propionate formation is stoichiometrically associated with decrease in CH_4 production. In the rumen, propionate is produced through two pathways; succinate pathway and acrylate pathway (Figure 1). Succinate pathway is the major pathway in the rumen and along this pathway intermediate products such as malate, fumarate and succinate are formed. Moreover, this pathway involves a mixture of bacteria such as lactate producers (e.g. *Streptococcus bovis*), lactate utilisers (e.g. *Selenomonas ruminantium*), fumarate reducers (e.g. *Wolinella succinogenes*), succinate producers (*Fibrobacter succinogenes*) and succinate utilisers (e.g. *S. ruminantium*).

The acrylate pathway is also an important propionate-producing pathway in the rumen. Lactate-utilising *Megasphaera*

Table 1 *Use of* Saccharomyces cerevisiae *as a DFM for reducing rumen methanogenesis (summary of published* in vitro *studies)*

Yeast preparation[1]	*In vitro* system	Substrate used	Effect on CH_4 production	Reference
Commercial live product-Yea-sacc 1026 (5×10^7 CFU/ l medium)	Batch	Barley-based beef ration	Decreased by 10% at 12 h but not sustained over a long period.	Mutsvangwa *et al.* (1992)
Live cell product (15 mg/g DM feed)	Rusitec technique	Low (30%), medium (50%) and high (70%) concentrate diets	No effect	Carro *et al.* (1992)
Commercial live culture- XP yeast (4.1×10^3 and 8.5×10^3 CFU/l medium)	Batch	Ground corn, maltose, alfalfa hay, Bermuda grass hay and lactate were tested individually	14% increase with alfalfa hay after 48 h with both concentration 34% increase with Bermuda grass hay after 48 h with high concentration	Sullivan and Martin (1999)
Commercial live culture- XP yeast (4.1×10^3 and 8.5×10^3 CFU/l medium) and live cell- PMX70SBK (4.9×10^6 and 1.0×10^7 CFU/l medium)	Batch	Ground corn, soluble starch, alfalfa hay and Bermuda grass hay were tested individually	20% decrease with alfalfa hay after 24 h with of live cell-PMX70SBK	Lynch and Martin (2002)
Mixture of two strains (8417 and 1026) live cell products (0, 1.7×10^9, 3.3×10^9, 5.0×10^9 and 6.6×10^9 CFU/l medium)	Batch	Corn starch, soluble potato starch and the mixture of sudan grass hay (60.5%) and concentrate (39.5%) were tested individually	6%, 8%, 10% and 10% decrease with increasing yeast concentration with sudan grass hay and concentrate combination	Lila *et al.* (2004)

DFM = direct-fed microbial.
[1]commercial producers: Yea-sacc-Alltech Biotechnology centre, XP yeast-Diamond V Mills, PMX70SBK-Saf Agri, Twin strain (8417 and 1026)-Bussan Biotech. Co. Ltd. When available, doses are given as colony forming units (CFU) per liter medium

Table 2 *Use of* Saccharomyces cerevisiae *as a DFM for reducing rumen methanogenesis (summary of published* in vivo *studies)*

Yeast preparation[1]	Animal species	Diet	Effect on CH_4 production	Reference
Strain CNCM 1-1096 (1.38×10^7 CFU/kg LBW)	Sheep (defaunated and refaunated)	Timothy grass hay (44.5%), barley (44.5%) and soya bean meal (11%)	No effect	Mathieu *et al.* (1996)
Strain CNCM 1-1077 (4.67×10^5 CFU/kg LBW)	Lactating dairy cows	Corn silage (60%), concentrate (40%)	No effect	Doreau and Jouany (1998)
Two commercial products Procreatin-7 and Levucell sc (CNCM 1-1077) (1.92×10^8 and 5.77×10^7 CFU/kg LBW)	Dairy steers	Barley silage (75%), barley (19%) and supplement (6%)	3% decrease per kg DM intake with Procreatin-7 No effect with Levucell	McGinn *et al.* (2004)
Trichosporom sericeum (1.1×10^6 CFU/kg LBW)	Sheep	Timothy hay (40%), alfalfa hay (30%) and concentrate (30%)	7% decrease per kg DM intake	Mwenya *et al.* (2004)
Two commercial products Levucell sc and a novel strain (1.32×10^7 CFU/kg LBW)	Dry dairy cows (non-lactating cow)	Barley silage (50%), steam-rolled barley grain (19.5%) and pellet supplement (30.5%)	7% decrease per kg DM and gross energy intake with novel strain	Chung *et al.* (2011)

DFM = direct-fed microbial; LBW = live body weight; DM = dry matter.
Doses are given as colony forming units (CFU) per kg LBW.
[1]Commercial producers: CNCM 1-1096 and CNCM 1-1077 – Institut Pasteur, France, Procreatin-7 -Saf Agri, Levucell sc-Lallemand Inc.

elsdenii is the major rumen bacteria involved in this pathway (Russell and Wallace, 1997), which requires the presence of lactate. In the absence of lactate, *M. elsdenii* utilise glucose and produce acetate and butyrate but not propionate (Hino *et al.*, 1994). Lactate-producing bacteria such as *Streptococcus bovis* play therefore a regulatory role in this pathway. There are other bacterial spp. in the rumen that can utilise lactate (e.g. *S. ruminantium and Propionibacterium* spp.) but *in vitro* (Counotte *et al.*, 1981) and *in vivo* (Klieve *et al.*, 2003) studies have shown that *M. elsdenii* is the key species performing this function. It has been shown that in cows supplemented with a *M. elsdenii* DFM, the pattern of rumen fermentation was altered in favour of propionate with potential benefits on energy balance and animal productivity (Henning *et al.*, 2010; Aikman *et al.*, 2011). Hino *et al.* (1994) have also shown that combining lactate-producing bacteria with *M. elsdenii* is effective for increasing propionate production. Some strains of the rumen bacterium *Prevotella ruminicola* can also form propionate via the acrylate pathway, but the amount of propionate formed by these strains is not significant in the rumen (Wallnofer and Baldwin, 1967).

Propionate formation is the main rumen biochemical pathway explored and exploited by bacterial DFM in ruminant production (Seo *et al.*, 2010). *Propionibacterium* spp. and/or *Lactobacillus* spp. were used to increase animal productivity (Ghorbani *et al.*, 2002; Adams *et al.*, 2008), *M. elsdenii*, *Propionibacterium* spp. and/or *Lactobacillus* spp. were used to prevent rumen acidosis in concentrate-fed animals (Aikman *et al.*, 2011; Lettat *et al.*, 2012b) and *Lactobacillus* spp. were used to decrease pathogenic load in young animals (McAllister *et al.*, 2011) but CH_4 production was not measured in any of these studies. Decrease in CH_4 emission was recently observed in lactating dairy cows receiving a mixed *Propionibacterium jensenii – Lactobacillus spp.* DFM (Lettat *et al.*, 2012a) showing the potential of this approach to mitigate rumen CH_4 emission.

Nitrate/nitrite-reducing bacteria

Nitrate can act as an alternative H_2 sink to CO_2 in the rumen. The predominant pathway of nitrate metabolism in the rumen has been assumed to be dissimilatory nitrate reduction in which nitrate is reduced to ammonia in two-step processes: nitrate to nitrite and nitrite to ammonia. The potential use of nitrate to decrease rumen methanogenesis has been hindered by the toxicity of the intermediate product nitrite. Rumen microbes rapidly reduce the nitrate into nitrite, but the rate of reduction of nitrite into ammonia is slower, which can cause nitrite accumulation in the rumen (Iwamoto *et al.*, 1999). When nitrite is absorbed from the rumen into the blood it converts blood haemoglobin into methaemoglobin and if its concentration is high it causes the condition called methemoglobinemia. Methemoglobinemia decreases the blood's capacity to transport oxygen to tissues, resulting in depressed performances and, in severe cases, death of the animal (Morris *et al.*, 1958). Many studies on nitrate supplementation were focused on avoiding this nitrite toxicity. As a result, several

solutions were proposed to avoid this problem (Alaboudi and Jones, 1985; Takahashi and Young, 1991; van Zijderveld *et al.*, 2010). Among them, the use of nitrite-reducing bacteria as DFM was tested (Anderson and Rasmussen, 1998).

Bacteria with the ability to reduce nitrate, nitrite or both compounds are already present in the rumen. The major nitrate-reducing bacteria *W. succinogenes* and *S. ruminantium* are both present at a concentration of 10^6 cells/ml of rumen fluid (Asanuma *et al.*, 2002; Yoshii *et al.*, 2003). However, to compete with methanogens present at about 10^9 cells/ml (Jeyanathan *et al.*, 2011) it may be advantageous to increase the number and/or the activity of nitrate- and/or nitrite-reducing bacteria in the rumen if nitrate is going to be a strategy to decrease methanogenesis. Bacteria that have the ability to reduce nitrate or/and nitrite are more active when nitrate is included in the diet. Iwamoto *et al.* (2002) showed that addition of nitrate increased the number of nitrate-reducing bacteria such as *W. succinogenes* and *Veillonella parvula in vitro*. But, this increase may not be sufficient to compete with methanogenesis. Therefore, providing nitrate- and/or nitrite-reducing bacteria as DFM along with nitrate may improve the nitrate reduction process and avoid nitrite toxicity.

Denitrification is another nitrate/nitrite-reduction pathway in which nitrate or nitrite are reduced via gaseous nitrogen-oxides (NO and N_2O) to N_2. Denitrification is prominent in the soil ecosystem (McKenney *et al.*, 1982; Raciti *et al.*, 2011). The presence of more organic carbon in the soil is thought to be the reason for this high denitrification activity (Tiedje *et al.*, 1982). Although the rumen has considerable organic carbon and has the possibility of inoculation of denitrifiers from soil through feed, denitrification process has not been reported in the rumen. Short turnover time of the rumen contents may be the reason for this observation (Ao, 2008). However, traces of N_2O were observed when rumen liquor from cattle was incubated with nitrate *in vitro* (Kaspar and Tiedje, 1981). This N_2O was thought to be the by-product of dissimilatory nitrate reduction by rumen bacteria. Kaspar (1982) tested five *Propionibacterium* species for their ability to reduce nitrate and found all five species reduced nitrate to N_2O, but not to N_2. Reduction of nitrate to N_2O by *Propionibacterium* spp. was considered to be a detoxification mechanism rather than a part of an energy transformation reaction. As *Propionibacterium* spp. are present in the rumen N_2O production occurs in the rumen and N_2O is present in rumen gases, although in trace amounts.

There have been only few published studies on the use of nitrate/nitrite-reducing bacteria as DFM to decrease rumen methanogenesis. In an *in vitro* trial, Anderson and Rasmussen (1998) inoculated the nitrate-reducing rumen bacterium *Denitrobacterium detoxificans* strain NPOH1 along with added nitrate (10 μmol/ml) and observed up to 95% decrease in the CH_4 production without nitrite accumulation. In the absence of *D. detoxificans* the decrease in CH_4 production was only of 25% and nitrite accumulation was observed. In another *in vitro* study addition of nitrate-reducing-bacteria *W. succinogenes*, *S. ruminantium* or *V. parvula* to mixed methanogens in the presence of nitrate (5 mM) drastically decreased methanogenesis (>70% decrease). The highest decrease was observed with *W. succinogenes*, with low nitrite accumulated in the culture media compared with the other two nitrate-reducing bacteria (Iwamoto *et al.*, 2002). Nitrite-reducing *Escherichia coli* strains decreased nitrite accumulation *in vitro* as well as *in vivo* when added with nitrate (Sar *et al.*, 2005b and 2005c). *Propionibacterium acidipropionici*, which has the ability to reduce nitrite into N_2O (Rehberger and Hibberd, 2000) is commercially available to avoid nitrite toxicity in cows fed high nitrate forages. For the bacterial species mentioned above, however, there is still scarce *in vivo* data on their ability to decrease CH_4 production and/or avoid nitrite toxicity particularly when nitrate is used as feed additive.

In addition to the bacteria mentioned above, other isolates have also shown the ability to decrease CH_4 emission in the presence of nitrate *in vitro*. Asanuma *et al.* (2003) isolated a *Clostridium* sp. from dog faeces with high nitrite-reducing activity and Sakthivel *et al.* (2012) isolated a bacterium from a buffalo rumen. Both bacteria alleviate nitrite toxicity, the buffalo isolate in particular nearly inhibited methanogenesis when combined with 10 mM nitrate without a negative effect on feed digestibility. In contrast, nitrate alone, although it decreased CH_4 production by 69%, decreased feed digestibility by 14%. These two bacteria need to be fully characterised and, more importantly, their effect on the decrease in CH_4 emission has to be confirmed *in vivo*.

Nitrate as feed additive can decrease rumen methanogenesis in different ruminant species and production conditions (van Zijderveld *et al.*, 2011; Hulshof *et al.*, 2012). It is noted that nitrate could replace urea used as a nitrogen source for rumen microbial protein synthesis in diets with low N. In an *in vivo* study Li *et al.* (2012) replaced 1.5% urea by 3% calcium nitrate with a decreasing effect on CH_4 emission. However, the possible negative impact of long term supplementation of nitrate on animal health and on the environment has to be explored more. As mentioned earlier, DFM can be used to avoid these negative effects (Perdok *et al.*, 2011).

Sulphate-reducing bacteria

Competitive and co-operative relationships between methanogens and sulphate-reducing bacteria (SRB) have been described in anaerobic environments including in the rumen. In anaerobic environments, in which sulphate is not limiting, SRB compete with methanogens for common substrates (e.g. H_2, formate and acetate). As the energetic of sulphate reduction is slightly more favourable than methanogenesis (−152 kJ *v.* −131 kJ/mol; Gibson *et al.*, 1993), encouraging competition between these two groups theoretically decreases methanogenesis. The co-operative relationship between methanogens and SRB is another example of interspecies H_2 transfer. In sulphate-depleted environments they grow in syntrophy with methanogens by producing H_2. Limited sulphate availability in the rumen likely makes SRB as net producers of H_2 (Bryant *et al.*, 1977).

The population of SRB in the rumen is low (10^5 to 10^6 cells/ml) and mainly from the genus *Desulfovibrio* and *Desulfotomaculum*

(Campbell and Postgate, 1965; Huisingh *et al*., 1974). Recently, a sulphate-reducing bacterium belonging to the genus *Fusobacterium* was isolated from buffalo (Paul *et al*., 2011) suggesting that there may be other not-yet-cultured SRB in the rumen. The ability of SRB to compete with methanogens is largely determined by the introduction of sulphate into the rumen. In an experiment with steers fed high-sulphate diet no significant increase in the numbers of SRB was observed. Instead their sulphate reducing capacity was enhanced (Cummings *et al*., 1995). As such, sulphate reduction may be facilitated by introducing SRB when sulphate is used as an additive to decrease methanogenesis. However, there were only few studies on effect of sulphate supplementation in rumen methanogenesis (Morvan *et al*., 1996; van Zijderveld *et al*., 2010). The toxic end product (H_2S) resulting from the sulphate reduction is the major reason for the lack of studies on this option.

Using sulphate alone as an additive cannot be an alternative for reducing rumen methanogenesis due to the sulphide toxicity. However, SRB are versatile organisms and published information indicates that they may possess some characteristics favouring rumen CH_4 mitigation. For instance, a decrease in CH_4 emission was observed in an *in vitro* study using the newly identified SRB, *Fusobacterium* sp., as a DFM with a high sulphate diet. The CH_4 production at 72 h was decreased from 2.66 to 1.64 mmol/g digested dry matter (DM) without H_2S accumulation (Paul *et al*., 2011). Fibre digestion and number of cellulolytic bacteria were also increased. Absence of sulphide accumulation in this study may be due to its rapid utilisation by other microbes such as cellulolytic bacteria for synthesis of sulphur-containing amino acids (Bryant, 1973) or *Fusobacterium* sp. itself might be able to oxidise sulphide into sulphate as described in the termite gut (Droge *et al*., 2005). The full characterisation of the isolated *Fusobacterium* sp. as well as additional studies are needed to gain a better understanding of this effect.

Major interactions were observed between nitrate and sulphate metabolism in microorganisms that are present in diverse anaerobic ecosystems (Garcia-de-Lomas *et al*., 2007; Hubert and Voordouw, 2007; van Zijderveld *et al*., 2010). Importantly, many SRB appear to have dual roles, that is, they reduce inorganic and organic sulphur, and the majority of them can reduce nitrite to ammonia. Their ability to reduce nitrite was explored to avoid the potential toxicity problem encountered when supplementing with nitrate alone (Takahashi *et al*., 1998; Perdok *et al*., 2011). Moreover, an additive effect was observed on decrease in CH_4 emission *in vivo* when sulphate (2.6% of DM) and nitrate (2.6% of DM) were used together (van Zijderveld *et al*., 2010). In anaerobic environments, H_2S can act as an electron donor for nitrite ammonification in nitrate-reducing-sulphide-oxidising bacteria (NR-SOB) (Garcia-de-Lomas *et al*., 2007; Hubert and Voordouw, 2007). These bacteria reduce nitrate to nitrite and oxidise sulphide to sulphate when further reducing nitrite to ammonia. The low concentration of H_2S certainly limits the abundance of this group of bacteria in the rumen. However, physiological similarities between *W. succinogenes* and NR-SOB *Sulphurospirillium deleyianum,* suggest that this function is present in the rumen (Simon, 2002). Indeed, *W. succinogenes* can grow using sulphide as an electron donor and fumarate as an electron acceptor (Macy *et al*., 1978). The possibility of preventing nitrite toxicity by provision of sulphate further suggests that some nitrate reducing bacteria present in the rumen may possess the characteristic of NR-SOB (Ao, 2008).

Homoacetogens

Homoacetogens are present in diverse environments including rumen, and have the ability to produce acetate via heterotrophic and autotrophic growth. They grow heterotrophically by utilising sugars and autotrophically by utilising formate, CO and H_2/CO_2. Promoting the autotrophic growth of homoacetogens (Wood-Ljungdahl pathway) is thought to be a competitive pathway to methanogenesis as the same substrates are used. Acetate is a beneficial nutrient for the host and for other microbes within the rumen community.

The population of homoacetogens in the rumen highly varies (undetectable to 10^7 cells/ml) depending on diet, age of the animal and time of sampling (Leedle and Greening, 1988; Fonty *et al*., 2007). They are among the first species to colonise the rumen but their numbers decrease when methanogens appear at 30 h after birth (Morvan *et al*., 1994). In a study using lambs placed in a sterile isolator 17 h after birth, the acetogen population was increased up to 5×10^8 cells/ml at 150 days before methanogens inoculation (Gagen *et al*., 2012). A similar observation was reported previously by Fonty *et al.* (2007) in their study with methanogen-free lambs. A negative correlation between numbers of homoacetogenic bacteria and methanogens has been described in the rumen of adult ruminants (Doré *et al*., 1995). Notwithstanding, using acetogens as a DFM to decrease rumen methanogenesis has some limitations. Methanogens have a lower threshold for H_2 than acetogens and the energy yield from methanogenesis is greater than that from acetogenesis (Thauer *et al*., 1977). As such, at the low H_2 concentrations prevailing in the rumen, methanogens out-compete acetogens. In addition, acetogens can grow heterotrophically by utilising sugars and other substrates (e.g. alcohols, organic acids; Ragsdale and Pierce, 2008).

Several attempts to increase the reductive acetogenesis process in the rumen by supplying DFM containing homoacetogens of rumen and non-rumen origin were unsuccessful. Lopez *et al.* (1999) tested six acetogenic bacteria for their effect on rumen CH_4 emission *in vitro* and found that only two of them slightly decreased methanogenesis. *Peptostreptococcus productus*, an acetogen isolated from an anaerobic sludge digester promoted acetogenesis *in vitro* only when methanogenesis was selectively inhibited (Nollet *et al*., 1997 and 1998). The same acetogen administered in association with spent *Lactobacillus plantarum* culture media failed to sustain the antimethanogenic effect for long term in sheep (Nollet *et al*., 1998). In another *in vitro* study, a combination of an acetogen (isolated from a lamb) and yeast stimulated acetogenesis significantly in the presence of

methanogens (Chaucheyras *et al*., 1995). Further confirmations of above observations by *in vitro* or *in vivo* studies were not reported so far.

Homoacetogens have been shown to sustain a functional rumen in methanogen-free lambs (Fonty *et al*., 2007) and studies have already shown that by inhibiting methanogenesis, the acetogenesis can be stimulated (Boccazzi and Patterson, 1996; Nollet *et al*., 1997). However, acetogens were less efficient in H_2 capture from fermentation (28% to 46%) than methanogens (>90%; Fonty *et al*., 2007; Gagen *et al*., 2012) and this may affect the overall fermentation process in the rumen. Identifying homoacetogens that are competitive to methanogens in the rumen is required. Recently, in an attempt to isolate rumen acetogenic bacteria able to grow on low threshold concentrations of H_2, an isolate was successfully obtained using H_2-limited continuous cultures (Boccazzi and Patterson, 2011). Such acetogens could compete with methanogens in the rumen. Homoacetogenesis is competitive to methanogenesis in the gut microbial ecosystem of humans, rodents, macropods, and wood-digesting termites (Breznak and Switzer, 1986). The Tammar wallaby (*Macropus eugenii*) is a foregut fermenter but it is a low CH_4 emitter. The homoacetogen population in Tammar wallaby forestomach is different compared with that found in ruminants (Gagen *et al*., 2010), which can be one of the reasons for lower CH_4 emission. The acetogens in the Tammer Wallaby may be more effective hydrogenotrophs than those in the rumen and possibly better competing with methanogens. If this is true, these animals may act as a source of novel acetogens to be used as DFM. The success of such option mainly depends on the ability of those acetogens to be active under rumen conditions.

Methylotrophs

Methylotrophs are microorganisms able to utilise one-carbon organic compounds such as methanol and methylamine as carbon sources and thus competing for substrates with methanogens. Some methylotrophs, the methanotrophs are also capable to use CH_4 avoiding its release into the atmosphere. Understanding the pathways involved in the metabolism of these compounds may provide a novel biological control agent in mitigating rumen CH_4 emission.

Up to now, there is little evidence to suggest that methanotrophy is important in the rumen. Only one study reported possible, albeit low 0.2% to 0.5%, methanotrophy in rumen fluid (Kajikawa *et al*., 2003). In another study bacterial clones closely related to *Nitrosomonas* spp. were identified from a clone library constructed from samples of bacterial communities attached to the rumen epithelium (Mitsumori *et al*., 2002). Members of the genus *Nitrosomonas* are ammonia-oxidising bacteria, which have the ability to oxidise CH_4 under some conditions (Hyman and Wood, 1983; Jiang and Bakken, 1999). Close to the rumen wall there is always ammonia as urea coming from blood, which is rapidly degraded by ureolytic bacteria attached to the rumen wall (Cheng and Wallace, 1979). Therefore, there is a possibility that *Nitrosomonas* spp. in the rumen wall may be involved in CH_4 oxidation. However, their presence in the rumen epithelium was not always observed suggesting that they are minor or occasional members (Sadet-Bourgeteau *et al*., 2010; Li *et al*., 2011).

Several clone libraries of rumen samples reported significant numbers of bacteria belonging to the phylum *Verrucomicrobia* (Romero-Perez *et al*., 2011; Godoy-Vitorino *et al*., 2012). Although their role in the rumen is not well understood, some members of the *Verrucomicrobia* have been found to oxidise CH_4 as the sole source of carbon and energy in non-rumen environments (Hou *et al*., 2008). Information on whether *Verrucomicrobia* are capable of this function in the rumen is still missing. Also, Klieve *et al.* (2012) identified clones related to CH_4-oxidising archaea in the rumen of cows. Methane-oxidising archaea have been described as having an important role in aquatic ecosystems (Hallam *et al*., 2003; Knittel *et al*., 2005). Although the rumen conditions with a nutrient-rich environment and a high turnover rate does not favour the activity of methane-oxidising archaea, their importance remains to be assessed.

Other than methanotrophs, methyloptrophs that can utilise methanol and/or methylamine could also be helpful in reducing methanogenesis. Methanol and methylamine are substrates, in some cases the unique substrate, for some rumen methanogens. For example, methanogens belonging to the genus *Methanosphaera* absolutely require methanol (Miller and Wolin, 1985) while genus *Methanomicrococcus* require methanol or methylamine (Sprenger *et al*., 2000) for their growth. The *Methanosarcina* spp. can also utilise methanol and methylamine as substrates, although they are not their obligatory requirements (Jarvis *et al*., 2000). Recent reports on a human methanogen isolate and on termite gut enrichment cultures suggested that methanol may be an obligatory requirement for rumen cluster C group (Dridi *et al*., 2012; Paul *et al*., 2012).

Among the methylotrophic methanogens, *Methanosphaera* spp. are common inhabitants of the rumen and found to be an important group in forage-fed cow, sheep and red deer (Jeyanathan *et al*., 2011). Although they are categorised as a minor group based on the meta-analysis of partial 16S rRNA sequences by Janssen and Kirs (2008), recent studies showed that they are ubiquitous members of the rumen in different ruminant species, fed different diets and at various geographical locations (Franzolin *et al*., 2012; St-Pierre and Wright, 2012). *Methanomicrococcus* and *Methanosarcina* were also retrieved from several rumen clone libraries (Whitford *et al*., 2001; Sundset *et al*., 2009), but not in significant numbers. Instead, the rumen cluster C group is one of the major methanogen groups (Janssen and Kirs, 2008) sometimes contributing up to 80% of the total clones analysed (Wright *et al*., 2006). If their substrate requirement is similar to the human and termite representatives, methanol could be an important substrate for rumen methanogenesis. Methanol is formed by enzymatic cleavage of pectin methyl esters by anaerobic pectinolytic bacteria in the rumen. Utilisation of

these substrates (methanol and/or methylamine) by organisms other than methanogens theoretically could decrease methanogenesis. However, the affinity of these substrates to methanogens and other organisms has to be considered. Methanol can also be utilised by homoacetogens (Lopez *et al*., 1999). Other than acetogens, there are no other methylotrophic bacteria reported in the rumen. Understanding methylotrophy especially methanotrophy in other environments such as soil may help to identify potential DFM, which could decrease rumen methanogenesis.

Capnophiles

Capnophiles are microorganisms that require high levels of CO_2 for their growth. The rumen is an anaerobic environment where CO_2 is the major gas. The presence of capnophiles in the rumen is therefore expected but their use as scavengers of CO_2 to mitigate methanogenesis is questionable as this gas is not a limiting substrate for rumen methanogens. Dehority (1971) suggested two types of CO_2 requirement among rumen bacteria: biosynthesis type in which CO_2 is required for cell growth (e.g. *S. bovis*) and the second type includes bacteria that are forming succinate in addition to biosynthesis (e.g. *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *succinivibrio dextrinosolvens*). During succinate production, CO_2 is attached to the three-carbon phosphoenolpyruvate, an end product in glycolysis, to generate the four-carbon compound, oxaloacetate. Oxaloacetate accepts two pairs of electrons, when reduced into succinate. As such, both CO_2 and H_2 are used during this succinate formation and may have an impact on rumen methanogenesis. Succinate is also an important intermediate product during the propionate production.

The Tammer wallaby produces only one-fifth of the amount of CH_4 produced by ruminants per unit of digestible energy intake (Kempton *et al*., 1976). A physiological difference such as shorter retention time of feed in the foregut partially explains this. The presence of a novel group of acetogenic bacteria may be the reason for this lower CH_4 emission (Gagen *et al*., 2010). Presence of capnophiles may also be contributing to this observation. In a clone library constructed from foregut samples of Tammer wallaby, a large proportion of novel capnophiles (9% of all sequences recovered in the 16S rRNA clone library) was observed (Pope *et al*., 2011). These clones were assigned to a group within the family *Succinivibrionaceae*. A member from this group was isolated and the genome sequence of this isolate proved that it is a capnophile dependent on CO_2 to support its metabolism via succinate biosynthesis (Pope *et al*., 2011). A clear understanding of their pathway in the rumen and other similar environment is needed to assess their potential as rumen CH_4 mitigants.

Other possible bacterial DFM

Bacterial cellulolytic populations in the rumen are the major contributors to fibre degradation. Some like *Ruminococcus* spp. primarily produce acetate, which leads to more H_2 production. However, the major cellulolytic bacterium, *F. succinogenes,* primarily produces succinate that leads to propionate production with less H_2 formation. Using bacteria which produce less H_2 as DFM may help to decrease methanogenesis without impairing fibre degradation especially in forage-fed animals. Less H_2 production was observed in gnotobiotically reared lambs inoculated with *F. succinogenes* as the only cellulolytic microbe compared to lambs inoculated with *Ruminococcus* species. Also, the rumen contents from *F. succinogenes*-containing-lambs produced less CH_4 *in vitro* (Chaucheyras-Durand *et al*., 2010). However, cellulolytic organisms such as *F. succinogenes* are already present in the rumen in high numbers and studies using fibrolytic bacteria as DFM did not demonstrate any improvement in cellulose digestion (Dehority and Triabasso, 1998; Krause *et al*., 2001).

Some bacteria have demonstrated inhibitory activity against methanogens. For example, some lactic acid-producing bacteria produce bacteriocins that can act against methanogens (Nollet *et al*., 1998; Lee *et al*., 2002b; Asa *et al*., 2010). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* is widely utilised in the food industry for controlling pathogenic bacteria. *In vitro* experiments have shown that nisin suppresses methanogenesis as much as 20% without negative impact on VFA production (Callaway *et al*., 1997; Sar *et al*., 2005a). However, nisin is susceptible to rumen proteases (Lee *et al*., 2002a) limiting its utilisation *in vivo*. The spent media of *L. plantarum* decreased methanogenesis by 18% *in vitro* (Nollet *et al*., 1998) while compounds produced by another *L. plantarum* strain decreased methanogenesis by 90% (Asa *et al*., 2010). Bovicin HC5, a bacteriocin produced by *S. bovis* HC5, inhibited CH_4 production by 53% *in vitro* (Lee *et al*., 2002b). In contrast, there is less information on the effect of these bacteriocins *in vivo*. Nollet *et al.* (1998) tested the effect of spent *L. Plantarum* media with an acetogen DFM on sheep and found only a short-term effect (80% decrease after 3 days of treatment). Although there is a possibility to use bacteriocins as additives to decrease rumen methanogenesis, the administration of DFM-producing bacteriocins *in vivo* may not be possible. The requirement of large population densities to induce bacteriocin production could make this option physiologically unsuitable and, certainly, economically not possible.

Formate is an important substrate used by rumen methanogens (Hungate *et al*., 1970). It is known that SRB and nitrate-reducing bacteria can utilise formate as H_2 donors. Nitrate-reducing bacteria probably out-compete the methanogens in formate utilisation because of their high affinity (Asanuma *et al*., 2002). But limited availability of nitrate and sulphate prevents them to utilise formate. Rumen bacteria such as *F. succinogenes* and *Anaerovibrio lipolytica* also can consume formate (Asanuma *et al*., 1998). These bacteria are already present in the rumen in high numbers. As such, their ability to utilise formate may be less efficient than that of methanogens. Identifying and isolating formate-utilising bacteria, which can compete with methanogens may be one possibility to decrease CH_4 emission from ruminants.

Concluding remarks

Many of the bacterial strains, mentioned in this review as potential DFM for reducing rumen methanogenesis, are isolates that can be or are actually grown industrially. However, relatively few of them have been tested for their anti-methanogenic activity. In addition to these already available microorganisms, the rumen as well as other environments are rich sources of potential DFM as the vast majority of microorganisms have not been yet cultured. Developments in genomics will help to identify microbes that could have the ability to decrease methanogenesis in the rumen.

The most promising species/strains studied so far have more than one feature considered to be beneficial in reducing methanogenesis. For example, some of the lactic acid bacteria produce antimethanogenic substances (e.g. *L. lactis*), nitrate and sulphate-reducing bacteria can utilise formate as substrate and some of the propionate-producing bacteria can reduce nitrate (*W. succinogenes*). This metabolic multiplicity seems important and should be sought when selecting candidates to be used as DFM. For the same reason, combination of different groups of bacteria could increase the efficacy of antimethanogenic DFM. If the DFM has particular nutrient requirements, incorporating them in the preparation as prebiotics might also help them to function efficiently in the rumen.

There are also technological aspects that should be considered for the development of DFM. All the bacteria presently used as DFM in ruminant production are facultative anaerobes that were mainly isolated from dairy products. Industrial preparation of anaerobic bacteria is technically difficult and costly as compared with standard production in the presence of oxygen. This constrain limits, for the time being, the type of DFM that can be developed for in-farm use. In addition, keeping obligate anaerobic bacteria viable for long periods after production is a challenge. Development of encapsulation methods for strictly anaerobic bacteria may help to overcome this problem. Technical advancements for the preparation of anaerobic bacteria are necessary to increase the scope of antimethanogenic DFM.

For newly developed DFM, *in vivo* trials are particularly important as DFM might both influence the rumen environment and induce shifts in the microbiota that will not occur *in vitro*. Such microbial changes have to be assessed to avoid any negative impacts on animals, the environment as well as consumers. Another aspect to consider is the persistency of the antimethanogenic effect that should be assessed in long-term animal trials. As for other mitigation strategies using feed additives and supplements, DFM have to be administered daily to be efficacious.

Acknowledgements

The financial support by Danone for this study is highly appreciated.

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