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New aspects and strategies for methane mitigation from ruminants

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Abstract

The growing demand for sustainable animal production is compelling researchers to explore the potential approaches to reduce emissions of greenhouse gases from livestock that are mainly produced by enteric fermentation. Some potential solutions for instance, the use of chemical inhibitors to reduce methanogenesis are not feasible in routine use due to their toxicity to ruminants, inhibition of efficient rumen function or other transitory effects. Strategies, such as use of plant secondary metabolites and dietary manipulations have emerged to reduce the methane emission, but these still require extensive research before these can be recommended and deployed in the livestock industry sector. Furthermore, immunization vaccines for methanogens and phages are also under investigation for mitigation of enteric methanogenesis. The increasing knowledge of methanogenic diversity in rumen, DNA sequencing technologies and bioinformatics have paved the way for chemogenomic strategies by targeting methane producers. Chemogenomics will help in finding target enzymes and proteins, which will further assist in the screening of natural as well chemical inhibitors. The construction of a methanogenic gene catalogue through these approaches is an attainable objective. This will lead to understand the microbiome function, its relation with the host and feeds, and therefore, will form the basis of practically viable and eco-friendly methane mitigation approaches, while improving the ruminant productivity.

Keywords: Rumen; Methane Mitigation; Enteric Fermentation; Methanogens, Ruminants

Introduction

Greenhouse gas (GHGs) emission from ruminant production systems is of particular interest because of their consequences in changing the global climate. Methane comprises up to 16% of global GHGs emissions (Scheehle and Kruger 2006), and is mainly detrimental, as its warming potential is nearly 25 times greater than that of CO_2 (Zhou et al. 2011). Methane emissions from the agriculture sector represents 40% of total anthropogenic production (Key and Tallard, 2012), while enteric fermentation in ruminants makes the largest single (25%) contribution (Thorpe 2009). The emission of methane from ruminants also varies based on the geographical location (FAO 2010), feed composition and quality, feed intake, processing of feed and animal breed (Hook et al. 2010).

Apart from environmental issues, the methane emission also accounts for a 2 to 12% loss of ingested energy from the rumen (Moss et al. 2000). Such considerations have led to increased efforts in identification of newer and more effective practices to mitigate methane emissions from ruminants. Advances in understanding the gut microbial communities through genomics (Leahy et al. 2010, 2013; Attwood et al. 2011) and metagenomics (Brulc et al. 2009; Hess et al. 2011; Morgavi et al. 2013) have opened novel insights about the function of rumen ecosystem. This increased knowledge has also permitted the development of mitigation strategies to target the dominant methanogenic species directly. There have been reviews of methane abatement in recent times (Moss et al. 2000; Beauchemin et al. 2008; McAllister and Newbold 2008; Kumar et al. 2009; Eckard et al. 2010; Hook et al. 2010; Martin et al. 2010; Patra 2012; Wanapat et al. 2012), so this article will focus on the latest developments (phage therapy, immunization, chemogenomics approaches), possible future directions and challenges in mitigating enteric methane emissions from ruminants.

Mechanism of enteric methane production

Enteric methane (nearly 87%) is produced in rumen, the remainder being released from fermentation in the large intestine (Lascano and Cardenas 2010). Although many factors influence methane emissions from ruminants, the three major determinants are level of feed intake, type of carbohydrate fed, and manipulation of rumen microflora (Johnson and Johnson 1995). In rumen, the network of microbes act on feed particles to degrade plant polysaccharide and produce volatile fatty acids (VFAs; mainly acetate, propionate and butyrate) and gases (CO_2 and H_2) as main end products. The activity of hydrogen-utilizing methanogens in rumen reduces the end product inhibition of hydrogen, thereby allowing more rapid fermentation of feed. Even a small amount of hydrogen in rumen can limit the oxidation of sugar, VFAs conversion and hydrogenase activity, if alternative pathways for disposal are absent (McAllister and Newbold 2008). Two methods utilized for disposal of reducing equivalents are the production of more highly reduced VFAs and hydrogen by membrane-bound hydrogenases. However, these hydrogenases have an acute sensitivity to an increased partial pressure of hydrogen (Russell 2002).

Methane production in rumen is also affected by the passage rate of digesta in the gastrointestinal tract. The rumen residence time decreases with increased feed intake, thus reducing the extent of the rumen fermentation and shifting digestion from the rumen to the small intestine (Aluwong et al. 2011). As a consequence, methane production per unit of dry matter ingested declines, as feed intake increases

(Beauchemin and McGinn 2006a), although the total amount of methane produced is higher.

Strategies to reduce enteric methane emission

The strategies to reduce methane emission from enteric fermentation are classified into different categories and their respective mechanisms of action, problems associated with each and future prospects are shown in Table 1. The two main areas of intervention that will be reviewed here are the changes in the diet and the direct manipulation of the rumen ecosystem.

Dietary changes

Although there are many approaches to reduce methane formation in the rumen, only some of those that have been more intensively investigated during the last years will be treated here, including changes in nutrient composition, plant secondary compounds, lipid supplementation, organic acids and halogenated compounds. Other options, such as the use of ionophores, probiotics, acetogens and defaunation are listed in Table 1, but these are not described here.

Changing nutrient composition

By manipulating the nutrient composition of ruminants' diet, it is possible to reduce the enteric methane yield, the forage: concentrate ratio in the diet being one of the most studied dietary factors. A high proportion of concentrate in diet reduces rumen pH and consequently affects the protozoa population (Kumar et al. 2013a, b). Furthermore, it also reduces the acetate: propionate ratio and thus decreases the amount of methane produced per unit of feed intake (Beauchemin et al. 2008). However, the proportion of concentrates needed to bring about this effect may well be over 90% of the diet and such high levels are not desirable due to health concerns (i.e., acidosis, laminitis, liver abscesses etc.; Gandra et al. 2012). Moreover, feeding high amounts of concentrates is not always possible in the developing countries because of the economic constraints. Therefore, developing newer forages having high soluble carbohydrates can be a better option for reducing methane than feeding high-concentrate based diets. Niderkorn et al. (2011) reported that diets rich in certain grass varieties such as AberAvon (*Lolium perenne*) lead to significantly reduced methane production from *in vitro* fermentations than AberStar and AberMagic under the category of water soluble carbohydrates forages. Similarly, different grass and shrub species, such as *L. perenne* (Ludemann et al. 2013) *Gliricidia sepium*, *Brachiaria ruziziensis* (Meale et al. 2012) and *Acacia mangium* (Giraldo et al. 2007a), were able to reduce methane emissions. Therefore, grazing on these species has been proposed as a strategy to reduce methane emissions. Another approach would be the selection of better quality forages (low fibre and high soluble carbohydrates content), as increased quality should result in greater productivity at equivalent levels of intake and methane emissions (Clark et al. 2011).

Plants containing secondary compounds

Tannins, phenolic monomers and other plant secondary metabolites are toxic to ciliate protozoa, fibrolytic bacteria and methanogenic archaea, and thus may help in reducing methanogenesis (Goel et al. 2005; Bhatta et al. 2009; Patra and Saxena, 2009a,b; Jayanegara et al. 2011). It has been observed that condensed tannins (CT) containing temperate and tropical legumes reduce methanogenesis (Lascano and Cárdenas 2010; Guglielmelli et al. 2011; Calabrò et al. 2012; Cieslak et al. 2013). Tiemann et al. (2008) indicated that some tropical feeds with tannins have lower fibre digestibility and consequently, low hydrogen production and methane emissions. Moreover, binding of tannins to proteins also reduces degradation of plant protein in the rumen and lowers methanogenesis (Tavendale et al. 2005). The effects of tannin content of four different vegetative stages of *Onobrychis viciifolia* were evaluated by Guglielmelli et al. (2011), who found a negative correlation bordering on significance (r = -0.932; P = 0.068) between CTs content and methane production, indicating that methane production consistently declined as the CT content increased. The methane suppression effect of CT containing legumes, such as *Lotus pedunculatus* or *Acacia mearnsii,* relative to forages without tannins has been shown in sheep (Carulla et al. 2005; Ramirez-Restrepo and Barry 2005), Holstein cows (Woodward et al. 2001) and goats (Hess et al. 2006; Animut et al. 2008). The mechanism to decrease methanogenesis seems to vary with the nature of CT, as Bhatta et al. (2013) observed that *Ficus bengalensis* and *Autocarous integrifolis* reduced methane production due to defaunation, but *Azadirachta indica* reduced methanogenesis by a direct effect on methanogens. Overall, it seems that the effects of CT on rumen methanogenesis depend on the structure and concentration of CT.

Supplementation of lipids

Vegetables and animal lipids are originally used in ruminant rations to increase their energy density. These are also considered useful in terms of reduced rumen methanogenesis (Soliva et al. 2004; Beauchemin et al. 2007; Brask et al. 2013). Methane production has been consistently reduced by adding fat or fatty acids to ruminant diets, and it is estimated that fat can reduce methane emissions by 4-5% (g/Kg DMI) for every 1% increase in the fat content of the diet (Grainger and Beauchemin, 2011). However, the inclusion of lipids at levels above 6-7% of dry matter intake can reduce feed intake and fiber digestibility, resulting in lower milk yield or daily gain (Patra 2012).

The addition of different oils (soya, coconut, canola, rapeseed, etc.) to ruminant diets have been shown to reduce methane production between 19 and 62% in Rusitec fermenters (Dohme et al. 2000), sheep (Ding et al. 2012), beef cattle (Machmüller and Kreuzer, 1999; Jordan et al 2006a b) and dairy cows (Odongo et al. 2007; Brask et al. 2013). The mechanism of methane inhibition by fat is likely to be a combination of bio-hydrogenation of unsaturated fatty acids and direct inhibition of activities of different microbes including methanogens (Beauchemin et al. 2007; Kong et al. 2010; Hook et al. 2010). Bio-hydrogenation acts as hydrogen sink and therefore decrease rumen methanogenesis, but is not the only mechanisms as there is no direct link between the methane reduction and the level of unsaturation (Dohme et al. 2000) or the length of the fatty acid. Medium-chain fatty acids may also reduce methanogenesis by directly acting on protozoa and/or methanogens. Thus, coconut oil decreased methane production and methanogens in both faunated and defaunated Rusitec fermenters, the inhibition of methanogenesis caused by coconut oil being similar to that produced by defaunation (Dohme et al. 1999). Comparison of the effects of different fatty acids revealed that lauric, myristic and linoleic acids were the most potent reducers of methanogenesis (Dohme et al. 2001; Jordan et al. 2006b; Ding et al. 2012), and the ability of lauric acid to decrease cell viability of M*ethanobrevibacter ruminantium* has been recently reported by Zhou et al. (2013).

In summary, increasing the dietary proportion of lipids may provide another feeding strategy for reducing rumen methanogenesis, but the appropriate lipid and dose for each dietary condition should be carefully chosen, as it has been shown that different lipid sources may have similar effects on methane production but variable effects on diet intake and digestion (Beauchemin et al. 2007). In the last years, the potential of essential oils as additives to manipulate rumen fermentation and decrease methane emissions has been extensively investigated and reviewed (Calsamiglia et al. 2007; Benchaar and Greathead, 2011; Bodas et al. 2012). A wide range of essential oils (derived from garlic, thyme, oregano, cinnamon, rhubarb, frangula, etc.) has been shown to decrease methane production *in vitro* in a dose dependent manner, but at high doses the decrease in methanogenesis was accompanied by adverse effects on fermentation such as reduction in VFA production and feed digestibility (Busquet et al., 2005; Patra and Yu, 2012). When used at low doses (≤300 mg/l), garlic oil and its organosulfur compounds consistently decreased methane production *in vitro* without negatively affecting feed fermentation (Busquet et al. 2005; Kamel et al. 2008; Kongmun et al. 2010; Mateos et al. 2013), but no effects of garlic oil or its compounds on methanogenesis have been observed in lactating cows (van Zijderveld et al. 2011), sheep (Patra et al. 2011) or fattening bulls (Staerfl et al. 2012). The lack of response *in vivo* is partly attributed to the adaptation of microbes (Bodas et al. 2012), but also to the use of lower doses compared to those in the *in vitro* experiments. The challenge now is to identify essential oils that selectively inhibit methanogenesis at concentrations that can be used in the practice, with lasting effects and without depressing feed digestion and animal productivity (Benchaar and Greathead, 2011).

Addition of organic acids

Inclusion of organic acids (i.e. malic and fumaric) or their sodium salts in diets, results in shifting rumen fermentation towards propionate and hence, less methane production. The addition of sodium fumarate consistently decreased methane production *in vitro* by 2.3 to 41% (Ungerfeld et al. 2007), and increased feed digestibility and VFAs production (García-Martínez et al. 2005; Giraldo et al. 2007b). Similarly, malate, that is converted to fumarate in the rumen, stimulated propionate formation and also inhibited methanogenesis in some *in vitro* studies (Carro and Ranilla 2003a; Tejido et al. 2005), although other studies have failed to find clear reductions of methanogenesis *in vitro* (Carro et al. 1999; Gómez et al. 2005; Ungerfeld and Forster 2011). *In vivo* effects of adding organic acids to the diet on methane mitigation are quite variable. Wood et al. (2009) noted 60 to 76% reductions in methane emissions by supplementing fumarate at 100 g/kg to growing lambs, while Foley et al. (2009a) observed the reductions of only 6 and 16%, when the diet of beef heifers was supplemented with malic acid at 37.5 and 75 g/kg, respectively. In contrast, no effects of fumaric or malic acid on methane emissions were observed in other studies (Beauchemin and McGinn 2006b; Foley et al. 2009b).

The effect of organic acids supplementation on methane reduction appears to be influenced by the forage to concentrate ratio and the type of cereal grain being fed in diet (Carro and Ranilla 2003a, b; Gómez et al. 2005; Tejido et al. 2005), although the number of studies conducted with different diets is too low to draw definitive conclusions. However, the high cost of purified organic acids makes supplementation of ruminant diets uneconomical at the doses required to be effective. Nevertheless, diet supplementation with plant tissues naturally rich in organic acids does have some potential and it may be possible to select forages with elevated levels of other acids. Studies on lucerne, Bermuda grass and tall fescue indicated that organic acids concentrations vary not only among species but also among cultivars of the same species (Callaway et al. 1997), although it is difficult to conclude whether differences in organic acid levels among forages and cultivars are sufficient enough to affect rumen methane emission. Overall, results of fumaric and malic acid supplementation *in vitro* and *in vivo* are heterogeneous, and the effectiveness of these additives seem to depend on their dose and nature of diet.

Use of halogenated compounds

Halogenated methane analogues, such as chloral hydrate, amichloral, bromochloromethane, nitroethane and 2-nitropropanol, are potential inhibitors of methane in ruminants (Nevel and Demeyer 1995). Bromochloromethane can inhibit methanogenesis by reacting with coenzyme B, which functions at the last step of the methanogenic pathway (McCrabb et al. 1997). Recently, Abecia et al. (2012) confirmed the methane reducing effects of bromochloromethane in lactating dairy goats and reported a 33% reduction with no effect on rumen bacteria, protozoa and methanogens. In contrast, Denman et al. (2007) reported that bromochloromethane decreased the number of methanogenic archaea in the rumen of cattle by 34% and reduced methane emission by 30%. Bromochloromethane is highly volatile but can be stabilized with cyclodextrin resulting in more effective reduction of enteric methane emission (May et al. 1995). When fed to Braham cattle at hourly intervals, it prevented all methane production (McCrabb et al. 1997) and when fed twice daily to cattle for 8 weeks, it not only reduced methane by 54% but also reduced feed intake. Some compounds, such a bromine analogue of coenzyme M were potent methane inhibitors in *in vitro* (Martin and Macy 1985), but the inhibition was not persistent *in vivo*, suggesting adaptation of methanogenic populations (Nevel and Demeyer 1995). An adaptation of methanogens to quaternary ammonium compounds has also been demonstrated (Tezel et al. 2006), but in contrast, no adaptation has been observed for chloroform, which decreased rumen methanogenesis and methanogens without altering rumen function over a 42 day period (Knight et al. 2011).

More recently, the effect of 3-nitrooxypropanol and ethyl-3-nitrooxypropanol on rumen fermentation and methane emission have been studied using Rusitec fermenters and in *in vivo* trials (Haisan et al. 2013; Martinez-Fernandez et al. 2013; Perez et al. 2013). Both additives showed promising potential as methane inhibitors in the rumen with no negative effects on rumen fermentation. Differences among methanogens regarding their resistance to chemical inhibitors should be considered, when designing strategies for inhibition of rumen methanogenesis, as selection of resistant species may result (Ungerfeld et al. 2004).

In summary, although dietary manipulation provides many viable options, there are significant variations in the effectiveness of these and currently there is not yet an approach that could be practically applied. Hence, there is a need to study the influence of plant components and essential oils on methane production in detail with standardized samples. Based on the limited information, it can be said that benefits associated with bioactive compounds *in vitro* do not always mimic *in vivo,* and *in vivo* effects are usually transient in nature due to microbial adaptation. Moreover, the different experimental conditions found *in vitro* and *in vivo* should be taken into account when comparing doses and results from experiments. Rumen dry matter content can vary with several factors, ranging from 10 to 25%, whereas dry matter content in most *in vitro* systems is usually much lower (Carro et al. 2006); therefore, doses are not directly comparable when expressed per diet dry matter. In addition, as pointed out by Beauchemin et al. (2008), most studies on reductions in methane production from ruminants due to diet management are short-term and focussed only on enteric emissions, but future research should investigate long-term impacts on methane emissions in the whole farm.

Microbial interventions

This section deals with the diversity of methanogens in rumen and also discusses the strategies such as usage of phages and vaccination that directly target methanogens and/ or their activities.

Methanogenic diversity

In order to target methanogens, knowledge of their population dynamics, physiology and diversity in the rumen is of utmost importance. Until recently, the rumen methanogens belonged to a few genera of the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*, within the phylum Euryarchaeota. However, based on 16S rRNA gene sequences, a novel group distantly related to the *Thermoplasmatales* (named as rumen Cluster C; previously described as rice cluster C Thermoplasmata) was found to be highly abundant in ruminants (Jannsen and Kirs, 2008; Poulsen et al. 2013).

Methanobrevibacter is the most commonly encountered genus within *Methanobacteriales,* whereas *Methanobacterium,* that shares similar physiology as *Methanobrevibacter*, are rarely reported from rumen. Other *Methanobrevibacter* members of the order *Methanomicrobiales* have been also reported to be dominant in the rumen of buffalo (Tajima et al. 2001; Shin et al. 2004). In genus *Methanomicrobium*, *M. mobile* is mostly reported (Kumar et al. 2012) from ruminants, while other members of this genus had shown an abundance with culture independent methods, but are rarely detected/isolated with conventional approaches. The order *Methanosarcinales* comprises a group of physiologically distinct aceticlastic methanogens (Janssen, 2010), but their abundance in the rumen is low. Within this group, *Methanococcus* spp. and *Methanosarcina* spp. are the most commonly reported (Wedlock et al. 2013).

Apart from the microbial diversity analysis, functional and sequence based metagenomics have been evolved to uncover the diversity of enzymes and metabolic pathways in the rumen. This technique has been used to identify hydrolytic enzymes of industrial applications, particularly involved in plant polysaccharide degradation. Some researchers have employed this technique and identified enzymes from the rumen of cow, buffalo, camel, reindeer and yak (Zhao et al. 2010; Hess et al. 2011, Pope et al. 2012; Dai et al. 2012; Bhatt et al. 2013). This practice is based on the availability of suitable bioassays for the enzyme of interest and presently cellulose and hemicelluloses degradation is an area of interest for rumen microbiologists. Beside enzyme bioassays, heterologous complementation of host strains and mutants as well as induction of reporter genes are used for functional metagenomic screening (Leahy et al. 2013). Pope et al. (2010) reported unique bacterial lineages underpinning plant biomass conversion, and their distinct repertoire of glycoside hydrolases in Australian macropods. They also reported the abundance of polysaccharide utilization loci in Svalbard reindeer rumen micrbome, which is much similar to the microbiome of human gut (Pope et al. 2012). Similarly, Bhatt et al. (2013) highlighted the striking similarities and differences of dromedary camel *(Camelus dromedaries*) with other animal rumen ecosystem. Since, variations of microbial communities in ruminants is of great concern, Ross et al. (2012) suggested untargeted massive parallel sequencing (sequencing without target amplification of genes) approach for resolution of variation based rumen metagenome profiling.

Many developments in the exploration of gut microbial communities in different animal species have been made through sequence based metagenomics, and some recent examples follow. Dai et al. (2012) analyzed the fibrolytic microbiome in the rumen of yaks, and Brulc et al. (2009) used large sequence based studies to catalogue the genes involved in fiber degradation in the bovine rumen. Qi et al. (2011) applied metatranscriptomics to the study of rumen microbes function in muskox, and similar work has been carried out in sheep (Cammack et al. 2013), goats (Jakhesara et al. 2010) and Surti buffaloes (Singh et al. 2012a). Singh et al. (2012b) studied the virulence associated and antibiotic resistance genes of rumen microbes to facilitate the understanding of resistant gene transfer between and within habitats. However, researchers in this area have to explore the sequence based metagenomic into taxonomic perspective, as well as to link genomics and metagenomics to nutrition or other animal production parameters. For example, Li et al. (2012) characterized rumen microbiota of pre-ruminant calves and their metabolic potential so that the optimal early weaning nutritional strategies (like milk replacer) could be formulated, and recently Ross et al. (2013) analyzed the effect of methane mitigating diets on rumen microbiome.

Phage therapy

The lytic potential of phages and their genes make them an important tool for methane mitigation strategies. In contrast to nearly 300 phage genomes (Ackermann and Kropinski 2007), only six archaeal phages are sequenced and described, and just three of them are from methanogens: *Methanobacterium* phage psi M1, M2 and M100 (Pfister et al. 1998) and *Methanothermobacter* phage psi M100 (Luo et al. 2001). Little information is currently available on the genetic blueprint and gene functionality of archaeal, particularly methanogenic, phages but more are being discovered using electron microscopy (Ackermann 2007) and *in vitro* techniques (Stanton 2007). McAllister and Newbold (2008) reported siphophages that can infect methanogens (*Methanobacter*, *Methanobrevibacter* and *Methanococcus* spp.), although these phages have not been isolated from the rumen. A recent metagenomic study on phage-bacterial relationships showed ≤ 0.1% relative abundance of prophage in phylum *Euryarchaeota* (Berg Miller et al. 2012).

Metagenomic surveys are expected to reveal the presence of embedded prophages and phage-like elements that would have otherwise remain unnoticed. An unanticipated outcome from sequencing the *M. ruminantium* genome was the discovery of prophage φ-mru having 69 phage-related proteins (Leahy et al. 2010). A gene encoding a putative lytic enzyme was identified, expressed and shown to lyse *M. ruminantium*. Such lytic enzyme is potentially very useful biocontrol agents for manipulating of rumen methanogenic populations (Leahy et al. 2010). The genome sequence of *Methanobrevibacter* AbM4 and *Methanobrevibacter boviskoreani* strain JH1 revealed the presence of prophage/ phage like elements in strain JH1 while AbM4 is lacking in gene encoding prophage (Lee at al. 2013a and Leahy et al. 2013). Phages are host and even strain specific, so phage-based methane mitigation strategies could be developed without affecting other phylogenetically distinct microbes in the rumen. However, hosts and phages are also known to be involved in a rapid evolutionary race as the host changes to avoid infection and the phage changes to maintain infectivity.

In combination with the application of other phage enzymes and structural components, a rotation system can be envisioned that may overcome the rapid adaptation mechanisms of microbes to phage challenges. More methanogenic phages need to be identified, sequenced and characterized to identify and employ such phage-based strategies. However, high specificity of phages may also be a limiting factor in their effectiveness in reducing methane emissions, since there appears to be a high diversity of methanogens in rumen (Janssen and Kirs 2008). Finally, either mixture of phages or structural components of phages may prove useful against the greater diversity of methanogens in rumen.

Immunization

Host immunization commonly offers a diverse and ecofriendly solution to the problems especially associated with animal health. Therefore, developing vaccines against methanogens appears to be an alternative and attractive approach, which can avoid many of the issues mentioned above related to methane mitigation from ruminants. Wright et al. (2004) developed two vaccines, VF3 (based on three methanogenic strains 1Y, AK-87 and ZA-10) and VF7 (based on seven methanogens), that produced a 7.7% methane reduction in sheep (g per kg of dry matter intake); despite targeting only a minority (20%) of methanogens present within these host animals. They also created a vaccine based on five methanogens (*Methanobrevibacter s*pp. strains 1Y, AK-87, *M. millerae* ZA-10, *Methanomicrobium mobile* BP and *Methanosphaera stadtmanae* MCB-3) that was administered in three vaccinations to sheep (Williams et al. 2009). Surprisingly, immunization with this second vaccine caused methane output to increase by 18%, despite the fact that a larger proportion of the methanogenic population (52%) was targeted. Thus, further work is needed to optimize the individual components of these vaccines such that the most potent methanogens are specifically targeted.

Researchers believe that anti-methanogenic vaccines will only yield the short term reductions in methanogens and/ or methanogenesis, due to the possible proteolytic degradation and low persistence of host antibodies in rumen (Li et al. 2007; Cook et al.

2008; Lascano and Cárdenas 2010). Vaccination of sheep with entodinial or mixed protozoa antigens reduced protozoa and the released IgG antibodies against rumen protozoa remained active and continued to bind the target cells up to 8 hours (Williams et al. 2008). Vaccines targeting single surface antigens may not be effective, as methanogenic archaea differs largely based on their host, diet as well as geographical regions (Zhou et al. 2009). A new vaccine has been developed using sub-cellular fractions (cytoplasmic and cell wall derived protein) of *Methanobrevibacter ruminantium* M1 (Wedlock et al. 2010). Twenty sheep were vaccinated, then booster doses were given after three weeks, and the antisera were found to agglutinate and decrease the growth of archaeal methanogens and methane production *in vitro*.

In vivo efficacy of the vaccine on methanogens is yet to be evaluated. Based on liquid chromatography mass spectrometer, it was reported that most of the proteins were intracellular enzymes, particularly methyl-coenzyme M reductase, and these intracellular proteins would not be suitable as vaccine antigens owing to their inaccessibility for antibody binding. Since, there is the growing database for the genome sequences of rumen methanogens, the possibility of finding new target antigens/ proteins using comparative and pangenomics analysis have increased. The genome based reverse vaccine approach may also help in mining the new vaccine targets that might prove successful for efficacious vaccination against methanogens. Furthermore, extensive research is needed to identify adjuvants that stimulate high titer of antibody and are suitable for formulating with protein antigens to produce a low-cost and effective vaccine.

Overall, the genome sequencing will be leading to the better understandings toward methanogenic interactions with other microbes in the rumen suggesting some methane mitigation possibilities. The genomic techniques have provided positive clues for probable vaccine targets of methanogens in the rumen. Such approaches in future will optimistically lead to methane reducing practices for farm animals. However, the vaccine based inhibition method will have to pass the regulatory systems to guaranty animal health.

Chemogenomics: An upcoming strategy

Genome sequencing of microbes is actually a useful technique that can provide information directly applicable to methane mitigation strategies from ruminants, based either on vaccines development or small molecule inhibitor practices. Further, it can help to identify methane inhibitors by predicting and/or determining specific enzyme structures. This can define the geometry of the enzyme's active site and help to design the molecules that fit exactly into the active site and hence, can inhibit/ block the enzyme's catalytic function. This approach identifies inhibitory molecules that can be tested for their effectiveness in animal trials.

For reaching to a realistic solution to the problem of methane emissions, the technologies for reducing enteric methane must effectively target all the rumen methanogens (major and minor groups), otherwise less abundant methanogens may occupy the vacated niches and lead to normal methanogenesis. Besides, they should not affect other microbes present in the rumen, so that rumen function would not be altered. For this, an understanding of the diversity and physiology of rumen methanogens is essentially required, that not only identifies the dominant methanogens in a particular geographical area, but also the conserved sequences that can be targeted. In this regard, more genome sequences of methanogens are required to validate that the targets are common and effective among all the methanogens in the rumen (Attwood et al. 2011).

The completed *M. ruminantium* genome and draft sequences from other rumen methanogenic species are paving the way for identification of the underlying cellular mechanisms that define these microbes, leading to a better understanding of their micro-ecology within the rumen. Aside from this, the genome sequence of *M. ruminantium*, draft genome sequence of *M. boviskoreani* strain JH1 from Korean native cattle (HanWoo) and AbM4 from abomasum's of sheep have been published (Lee et al. 2013a; Leahy et al. 2013). The strain AbM4 do not code for many adhesion-like proteins, which indicates that it invest less on the external interactions with its environment compared to strain JH1. Moreover, AbM4 has a broader repertoire of cofactors and coenzymes, which shows its lesser dependence on the other rumen microbes and CoM in the medium. On the contrary, strain JH1 showed very good growth in presence of both CoM and VFA in the medium, and had genes and enzymes for CO_2 plus hydrogen, as well as for formate utilization, so that these enzymes possibly can be targeted for inhibition of methanogenesis.

Another gene, which can be further targeted, is the membrane associated transpeptidase. The Mtr enzyme complex can also be used for the development of vaccines. The phylogenetic analysis using Maximum-Likelihood inference method (MEGA 5.1) with 1000 boot strapping, and genomic sequence shows that strain JH1 and AbM4 likely belong to the same species and is related to *M. wolinii*. At present, this research is mainly at exploratory stage but several promising leads for chemogenomic targets are being investigated as possible intervention points for the inhibition of rumen methanogens. The cellular studies indicate that many of the conserved enzyme targets are involved in energy generation via methanogenesis, while majority of the conserved surface protein targets are of unknown function.

Bioinformatic approaches used for the inhibitor prediction against F420 dependent NADP oxidoreductase enzyme that catalyzes an important electron transfer step in the methanogenesis from *Methanobrevibacter smithii* reveal that lovastatin and compactin had high affinity to the enzyme and can act as potential inhibitors (Sharma et al. 2011). Both *in silico* approaches and *in vitro* enzyme assays may be useful for screening chemical inhibitors of methanogenesis.

The analysis of more genome sequences of rumen methanogens would help to identify potential methane inhibitors. According to the recent report of Lee et al. (2013a, b), only very few rumen methanogens are cultured as pure isolates and 13 genome projects are completed as yet (Leahy et al. 2013; Morgavi et al. 2013). Most of these genome sequences are from genus *Methanobrevibacter*, which is considered to be dominating rumen methanogen, as per the global data set of rumen microbes (Jansen and Kirs, 2008; Jeyanathan et al. 2011; Cheng et al. 2009; Williams et al. 2009, Zhou et al. 2009). In near future, with the development of "Hungate1000", a catalogue of 1000 reference microbial genomes from the rumen (http://www.hungate1000.org.nz/), genomic dataset of rumen microbiome will be numerically high, thereby more targets for anti-methanogenic strategies can be identified and used for improving the animal health, productivity and beyond. Furthermore, single-cell isolation technique from the complex rumen community would provide more advantage, over isolation approach, and their whole genome sequencing can be accomplished later.

Researchers are currently investigating, whether animal variation in methane emission is controlled by a heritable characteristic. Although clear and persistent individual differences in methane emissions have been found among animals fed the same diet (Pinares-Patiño et al. 2003, 2011; Martínez et al. 2010), it has not been yet clearly established whether the low emission trait is associated with any unwanted side effects. Currently, it is not possible to say whether in future it will be possible to breed animals that produce lesser methane per unit of intake or not (Clark 2013). Overall, the chemogenomics allowed us to identify the key features of rumen methanogens that can be targeted to inhibit them and to mitigate enteric methane production, eventually reducing the release of anthropogenic GHGs in the environment.

Final remarks

Looking at the facts in a comprehensive manner, profiling of rumen methanogens seem to be an important tool for ensuring sustainability of ruminant based agriculture production systems. However, for successful methane mitigation strategies to be developed and adopted, a thorough understanding of the microbial ecology of rumen methanogens is essentially required. DNA-based microbial profiling to explore ruminant methane mitigation will support how the rumen microbes can be manipulated without hampering the animal's production potential. These approaches would identify the involved microbial species based on genome sequences. By comparing microbial profiles of animals, one can identify the microbial shifts in response to the methane mitigation strategies. Some of the dietary strategies used in different studies have produced changes in rumen microbial communities as revealed by profiling assays. The comparison of rumen microflora both in high and low methane producers will help in determining if the changes in the microbiota can be directly or indirectly linked to the reduced methane emissions (Ross et al. 2013). In addition, genetic improvement and management practices for increasing ruminant productivity and abating methane emissions, in conjunction with other strategies, can also play an important role in lowering enteric methane emissions globally. Finally, it must be taken into account that there should be some economic return to the producers, if strategies to reduce methane emissions are expected to be implemented at farm level, and that any adopted strategy should also ensure animal health, food security and environmental safety.

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New aspects and strategies for methane mitigation from ruminants

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Abstract

The growing demand for sustainable animal production is compelling researchers to explore the potential approaches to reduce emissions of greenhouse gases from livestock that are mainly produced by enteric fermentation. Some potential solutions for instance, the use of chemical inhibitors to reduce methanogenesis are not feasible in routine use due to their toxicity to ruminants, inhibition of efficient rumen function or other transitory effects. Strategies, such as use of plant secondary metabolites and dietary manipulations have emerged to reduce the methane emission, but these still require extensive research before these can be recommended and deployed in the livestock industry sector. Furthermore, immunization vaccines for methanogens and phages are also under investigation for mitigation of enteric methanogenesis. The increasing knowledge of methanogenic diversity in rumen, DNA sequencing technologies and bioinformatics have paved the way for chemogenomic strategies by targeting methane producers. Chemogenomics will help in finding target enzymes and proteins, which will further assist in the screening of natural as well chemical inhibitors. The construction of a methanogenic gene catalogue through these approaches is an attainable objective. This will lead to understand the microbiome function, its relation with the host and feeds, and therefore, will form the basis of practically viable and eco-friendly methane mitigation approaches, while improving the ruminant productivity.

Keywords: Rumen; Methane Mitigation; Enteric Fermentation; Methanogens, Ruminants

Introduction

Greenhouse gas (GHGs) emission from ruminant production systems is of particular interest because of their consequences in changing the global climate. Methane comprises up to 16% of global GHGs emissions (Scheehle and Kruger 2006), and is mainly detrimental, as its warming potential is nearly 25 times greater than that of CO_2 (Zhou et al. 2011). Methane emissions from the agriculture sector represents 40% of total anthropogenic production (Key and Tallard, 2012), while enteric fermentation in ruminants makes the largest single (25%) contribution (Thorpe 2009). The emission of methane from ruminants also varies based on the geographical location (FAO 2010), feed composition and quality, feed intake, processing of feed and animal breed (Hook et al. 2010).

Apart from environmental issues, the methane emission also accounts for a 2 to 12% loss of ingested energy from the rumen (Moss et al. 2000). Such considerations have led to increased efforts in identification of newer and more effective practices to mitigate methane emissions from ruminants. Advances in understanding the gut microbial communities through genomics (Leahy et al. 2010, 2013; Attwood et al. 2011) and metagenomics (Brulc et al. 2009; Hess et al. 2011; Morgavi et al. 2013) have opened novel insights about the function of rumen ecosystem. This increased knowledge has also permitted the development of mitigation strategies to target the dominant methanogenic species directly. There have been reviews of methane abatement in recent times (Moss et al. 2000; Beauchemin et al. 2008; McAllister and Newbold 2008; Kumar et al. 2009; Eckard et al. 2010; Hook et al. 2010; Martin et al. 2010; Patra 2012; Wanapat et al. 2012), so this article will focus on the latest developments (phage therapy, immunization, chemogenomics approaches), possible future directions and challenges in mitigating enteric methane emissions from ruminants.

Mechanism of enteric methane production

Enteric methane (nearly 87%) is produced in rumen, the remainder being released from fermentation in the large intestine (Lascano and Cardenas 2010). Although many factors influence methane emissions from ruminants, the three major determinants are level of feed intake, type of carbohydrate fed, and manipulation of rumen microflora (Johnson and Johnson 1995). In rumen, the network of microbes act on feed particles to degrade plant polysaccharide and produce volatile fatty acids (VFAs; mainly acetate, propionate and butyrate) and gases (CO_2 and H_2) as main end products. The activity of hydrogen-utilizing methanogens in rumen reduces the end product inhibition of hydrogen, thereby allowing more rapid fermentation of feed. Even a small amount of hydrogen in rumen can limit the oxidation of sugar, VFAs conversion and hydrogenase activity, if alternative pathways for disposal are absent (McAllister and Newbold 2008). Two methods utilized for disposal of reducing equivalents are the production of more highly reduced VFAs and hydrogen by membrane-bound hydrogenases. However, these hydrogenases have an acute sensitivity to an increased partial pressure of hydrogen (Russell 2002).

Methane production in rumen is also affected by the passage rate of digesta in the gastrointestinal tract. The rumen residence time decreases with increased feed intake, thus reducing the extent of the rumen fermentation and shifting digestion from the rumen to the small intestine (Aluwong et al. 2011). As a consequence, methane production per unit of dry matter ingested declines, as feed intake increases

(Beauchemin and McGinn 2006a), although the total amount of methane produced is higher.

Strategies to reduce enteric methane emission

The strategies to reduce methane emission from enteric fermentation are classified into different categories and their respective mechanisms of action, problems associated with each and future prospects are shown in Table 1. The two main areas of intervention that will be reviewed here are the changes in the diet and the direct manipulation of the rumen ecosystem.

Dietary changes

Although there are many approaches to reduce methane formation in the rumen, only some of those that have been more intensively investigated during the last years will be treated here, including changes in nutrient composition, plant secondary compounds, lipid supplementation, organic acids and halogenated compounds. Other options, such as the use of ionophores, probiotics, acetogens and defaunation are listed in Table 1, but these are not described here.

Changing nutrient composition

By manipulating the nutrient composition of ruminants' diet, it is possible to reduce the enteric methane yield, the forage: concentrate ratio in the diet being one of the most studied dietary factors. A high proportion of concentrate in diet reduces rumen pH and consequently affects the protozoa population (Kumar et al. 2013a, b). Furthermore, it also reduces the acetate: propionate ratio and thus decreases the amount of methane produced per unit of feed intake (Beauchemin et al. 2008). However, the proportion of concentrates needed to bring about this effect may well be over 90% of the diet and such high levels are not desirable due to health concerns (i.e., acidosis, laminitis, liver abscesses etc.; Gandra et al. 2012). Moreover, feeding high amounts of concentrates is not always possible in the developing countries because of the economic constraints. Therefore, developing newer forages having high soluble carbohydrates can be a better option for reducing methane than feeding high-concentrate based diets. Niderkorn et al. (2011) reported that diets rich in certain grass varieties such as AberAvon (*Lolium perenne*) lead to significantly reduced methane production from *in vitro* fermentations than AberStar and AberMagic under the category of water soluble carbohydrates forages. Similarly, different grass and shrub species, such as *L. perenne* (Ludemann et al. 2013) *Gliricidia sepium*, *Brachiaria ruziziensis* (Meale et al. 2012) and *Acacia mangium* (Giraldo et al. 2007a), were able to reduce methane emissions. Therefore, grazing on these species has been proposed as a strategy to reduce methane emissions. Another approach would be the selection of better quality forages (low fibre and high soluble carbohydrates content), as increased quality should result in greater productivity at equivalent levels of intake and methane emissions (Clark et al. 2011).

Plants containing secondary compounds

Tannins, phenolic monomers and other plant secondary metabolites are toxic to ciliate protozoa, fibrolytic bacteria and methanogenic archaea, and thus may help in reducing methanogenesis (Goel et al. 2005; Bhatta et al. 2009; Patra and Saxena, 2009a,b; Jayanegara et al. 2011). It has been observed that condensed tannins (CT) containing temperate and tropical legumes reduce methanogenesis (Lascano and Cárdenas 2010; Guglielmelli et al. 2011; Calabrò et al. 2012; Cieslak et al. 2013). Tiemann et al. (2008) indicated that some tropical feeds with tannins have lower fibre digestibility and consequently, low hydrogen production and methane emissions. Moreover, binding of tannins to proteins also reduces degradation of plant protein in the rumen and lowers methanogenesis (Tavendale et al. 2005). The effects of tannin content of four different vegetative stages of *Onobrychis viciifolia* were evaluated by Guglielmelli et al. (2011), who found a negative correlation bordering on significance (r = -0.932; P = 0.068) between CTs content and methane production, indicating that methane production consistently declined as the CT content increased. The methane suppression effect of CT containing legumes, such as *Lotus pedunculatus* or *Acacia mearnsii,* relative to forages without tannins has been shown in sheep (Carulla et al. 2005; Ramirez-Restrepo and Barry 2005), Holstein cows (Woodward et al. 2001) and goats (Hess et al. 2006; Animut et al. 2008). The mechanism to decrease methanogenesis seems to vary with the nature of CT, as Bhatta et al. (2013) observed that *Ficus bengalensis* and *Autocarous integrifolis* reduced methane production due to defaunation, but *Azadirachta indica* reduced methanogenesis by a direct effect on methanogens. Overall, it seems that the effects of CT on rumen methanogenesis depend on the structure and concentration of CT.

Supplementation of lipids

Vegetables and animal lipids are originally used in ruminant rations to increase their energy density. These are also considered useful in terms of reduced rumen methanogenesis (Soliva et al. 2004; Beauchemin et al. 2007; Brask et al. 2013). Methane production has been consistently reduced by adding fat or fatty acids to ruminant diets, and it is estimated that fat can reduce methane emissions by 4-5% (g/Kg DMI) for every 1% increase in the fat content of the diet (Grainger and Beauchemin, 2011). However, the inclusion of lipids at levels above 6-7% of dry matter intake can reduce feed intake and fiber digestibility, resulting in lower milk yield or daily gain (Patra 2012).

The addition of different oils (soya, coconut, canola, rapeseed, etc.) to ruminant diets have been shown to reduce methane production between 19 and 62% in Rusitec fermenters (Dohme et al. 2000), sheep (Ding et al. 2012), beef cattle (Machmüller and Kreuzer, 1999; Jordan et al 2006a b) and dairy cows (Odongo et al. 2007; Brask et al. 2013). The mechanism of methane inhibition by fat is likely to be a combination of bio-hydrogenation of unsaturated fatty acids and direct inhibition of activities of different microbes including methanogens (Beauchemin et al. 2007; Kong et al. 2010; Hook et al. 2010). Bio-hydrogenation acts as hydrogen sink and therefore decrease rumen methanogenesis, but is not the only mechanisms as there is no direct link between the methane reduction and the level of unsaturation (Dohme et al. 2000) or the length of the fatty acid. Medium-chain fatty acids may also reduce methanogenesis by directly acting on protozoa and/or methanogens. Thus, coconut oil decreased methane production and methanogens in both faunated and defaunated Rusitec fermenters, the inhibition of methanogenesis caused by coconut oil being similar to that produced by defaunation (Dohme et al. 1999). Comparison of the effects of different fatty acids revealed that lauric, myristic and linoleic acids were the most potent reducers of methanogenesis (Dohme et al. 2001; Jordan et al. 2006b; Ding et al. 2012), and the ability of lauric acid to decrease cell viability of M*ethanobrevibacter ruminantium* has been recently reported by Zhou et al. (2013).

In summary, increasing the dietary proportion of lipids may provide another feeding strategy for reducing rumen methanogenesis, but the appropriate lipid and dose for each dietary condition should be carefully chosen, as it has been shown that different lipid sources may have similar effects on methane production but variable effects on diet intake and digestion (Beauchemin et al. 2007). In the last years, the potential of essential oils as additives to manipulate rumen fermentation and decrease methane emissions has been extensively investigated and reviewed (Calsamiglia et al. 2007; Benchaar and Greathead, 2011; Bodas et al. 2012). A wide range of essential oils (derived from garlic, thyme, oregano, cinnamon, rhubarb, frangula, etc.) has been shown to decrease methane production *in vitro* in a dose dependent manner, but at high doses the decrease in methanogenesis was accompanied by adverse effects on fermentation such as reduction in VFA production and feed digestibility (Busquet et al., 2005; Patra and Yu, 2012). When used at low doses (≤300 mg/l), garlic oil and its organosulfur compounds consistently decreased methane production *in vitro* without negatively affecting feed fermentation (Busquet et al. 2005; Kamel et al. 2008; Kongmun et al. 2010; Mateos et al. 2013), but no effects of garlic oil or its compounds on methanogenesis have been observed in lactating cows (van Zijderveld et al. 2011), sheep (Patra et al. 2011) or fattening bulls (Staerfl et al. 2012). The lack of response *in vivo* is partly attributed to the adaptation of microbes (Bodas et al. 2012), but also to the use of lower doses compared to those in the *in vitro* experiments. The challenge now is to identify essential oils that selectively inhibit methanogenesis at concentrations that can be used in the practice, with lasting effects and without depressing feed digestion and animal productivity (Benchaar and Greathead, 2011).

Addition of organic acids

Inclusion of organic acids (i.e. malic and fumaric) or their sodium salts in diets, results in shifting rumen fermentation towards propionate and hence, less methane production. The addition of sodium fumarate consistently decreased methane production *in vitro* by 2.3 to 41% (Ungerfeld et al. 2007), and increased feed digestibility and VFAs production (García-Martínez et al. 2005; Giraldo et al. 2007b). Similarly, malate, that is converted to fumarate in the rumen, stimulated propionate formation and also inhibited methanogenesis in some *in vitro* studies (Carro and Ranilla 2003a; Tejido et al. 2005), although other studies have failed to find clear reductions of methanogenesis *in vitro* (Carro et al. 1999; Gómez et al. 2005; Ungerfeld and Forster 2011). *In vivo* effects of adding organic acids to the diet on methane mitigation are quite variable. Wood et al. (2009) noted 60 to 76% reductions in methane emissions by supplementing fumarate at 100 g/kg to growing lambs, while Foley et al. (2009a) observed the reductions of only 6 and 16%, when the diet of beef heifers was supplemented with malic acid at 37.5 and 75 g/kg, respectively. In contrast, no effects of fumaric or malic acid on methane emissions were observed in other studies (Beauchemin and McGinn 2006b; Foley et al. 2009b).

The effect of organic acids supplementation on methane reduction appears to be influenced by the forage to concentrate ratio and the type of cereal grain being fed in diet (Carro and Ranilla 2003a, b; Gómez et al. 2005; Tejido et al. 2005), although the number of studies conducted with different diets is too low to draw definitive conclusions. However, the high cost of purified organic acids makes supplementation of ruminant diets uneconomical at the doses required to be effective. Nevertheless, diet supplementation with plant tissues naturally rich in organic acids does have some potential and it may be possible to select forages with elevated levels of other acids. Studies on lucerne, Bermuda grass and tall fescue indicated that organic acids concentrations vary not only among species but also among cultivars of the same species (Callaway et al. 1997), although it is difficult to conclude whether differences in organic acid levels among forages and cultivars are sufficient enough to affect rumen methane emission. Overall, results of fumaric and malic acid supplementation *in vitro* and *in vivo* are heterogeneous, and the effectiveness of these additives seem to depend on their dose and nature of diet.

Use of halogenated compounds

Halogenated methane analogues, such as chloral hydrate, amichloral, bromochloromethane, nitroethane and 2-nitropropanol, are potential inhibitors of methane in ruminants (Nevel and Demeyer 1995). Bromochloromethane can inhibit methanogenesis by reacting with coenzyme B, which functions at the last step of the methanogenic pathway (McCrabb et al. 1997). Recently, Abecia et al. (2012) confirmed the methane reducing effects of bromochloromethane in lactating dairy goats and reported a 33% reduction with no effect on rumen bacteria, protozoa and methanogens. In contrast, Denman et al. (2007) reported that bromochloromethane decreased the number of methanogenic archaea in the rumen of cattle by 34% and reduced methane emission by 30%. Bromochloromethane is highly volatile but can be stabilized with cyclodextrin resulting in more effective reduction of enteric methane emission (May et al. 1995). When fed to Braham cattle at hourly intervals, it prevented all methane production (McCrabb et al. 1997) and when fed twice daily to cattle for 8 weeks, it not only reduced methane by 54% but also reduced feed intake. Some compounds, such a bromine analogue of coenzyme M were potent methane inhibitors in *in vitro* (Martin and Macy 1985), but the inhibition was not persistent *in vivo*, suggesting adaptation of methanogenic populations (Nevel and Demeyer 1995). An adaptation of methanogens to quaternary ammonium compounds has also been demonstrated (Tezel et al. 2006), but in contrast, no adaptation has been observed for chloroform, which decreased rumen methanogenesis and methanogens without altering rumen function over a 42 day period (Knight et al. 2011).

More recently, the effect of 3-nitrooxypropanol and ethyl-3-nitrooxypropanol on rumen fermentation and methane emission have been studied using Rusitec fermenters and in *in vivo* trials (Haisan et al. 2013; Martinez-Fernandez et al. 2013; Perez et al. 2013). Both additives showed promising potential as methane inhibitors in the rumen with no negative effects on rumen fermentation. Differences among methanogens regarding their resistance to chemical inhibitors should be considered, when designing strategies for inhibition of rumen methanogenesis, as selection of resistant species may result (Ungerfeld et al. 2004).

In summary, although dietary manipulation provides many viable options, there are significant variations in the effectiveness of these and currently there is not yet an approach that could be practically applied. Hence, there is a need to study the influence of plant components and essential oils on methane production in detail with standardized samples. Based on the limited information, it can be said that benefits associated with bioactive compounds *in vitro* do not always mimic *in vivo,* and *in vivo* effects are usually transient in nature due to microbial adaptation. Moreover, the different experimental conditions found *in vitro* and *in vivo* should be taken into account when comparing doses and results from experiments. Rumen dry matter content can vary with several factors, ranging from 10 to 25%, whereas dry matter content in most *in vitro* systems is usually much lower (Carro et al. 2006); therefore, doses are not directly comparable when expressed per diet dry matter. In addition, as pointed out by Beauchemin et al. (2008), most studies on reductions in methane production from ruminants due to diet management are short-term and focussed only on enteric emissions, but future research should investigate long-term impacts on methane emissions in the whole farm.

Microbial interventions

This section deals with the diversity of methanogens in rumen and also discusses the strategies such as usage of phages and vaccination that directly target methanogens and/ or their activities.

Methanogenic diversity

In order to target methanogens, knowledge of their population dynamics, physiology and diversity in the rumen is of utmost importance. Until recently, the rumen methanogens belonged to a few genera of the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*, within the phylum Euryarchaeota. However, based on 16S rRNA gene sequences, a novel group distantly related to the *Thermoplasmatales* (named as rumen Cluster C; previously described as rice cluster C Thermoplasmata) was found to be highly abundant in ruminants (Jannsen and Kirs, 2008; Poulsen et al. 2013).

Methanobrevibacter is the most commonly encountered genus within *Methanobacteriales,* whereas *Methanobacterium,* that shares similar physiology as *Methanobrevibacter*, are rarely reported from rumen. Other *Methanobrevibacter* members of the order *Methanomicrobiales* have been also reported to be dominant in the rumen of buffalo (Tajima et al. 2001; Shin et al. 2004). In genus *Methanomicrobium*, *M. mobile* is mostly reported (Kumar et al. 2012) from ruminants, while other members of this genus had shown an abundance with culture independent methods, but are rarely detected/isolated with conventional approaches. The order *Methanosarcinales* comprises a group of physiologically distinct aceticlastic methanogens (Janssen, 2010), but their abundance in the rumen is low. Within this group, *Methanococcus* spp. and *Methanosarcina* spp. are the most commonly reported (Wedlock et al. 2013).

Apart from the microbial diversity analysis, functional and sequence based metagenomics have been evolved to uncover the diversity of enzymes and metabolic pathways in the rumen. This technique has been used to identify hydrolytic enzymes of industrial applications, particularly involved in plant polysaccharide degradation. Some researchers have employed this technique and identified enzymes from the rumen of cow, buffalo, camel, reindeer and yak (Zhao et al. 2010; Hess et al. 2011, Pope et al. 2012; Dai et al. 2012; Bhatt et al. 2013). This practice is based on the availability of suitable bioassays for the enzyme of interest and presently cellulose and hemicelluloses degradation is an area of interest for rumen microbiologists. Beside enzyme bioassays, heterologous complementation of host strains and mutants as well as induction of reporter genes are used for functional metagenomic screening (Leahy et al. 2013). Pope et al. (2010) reported unique bacterial lineages underpinning plant biomass conversion, and their distinct repertoire of glycoside hydrolases in Australian macropods. They also reported the abundance of polysaccharide utilization loci in Svalbard reindeer rumen micrbome, which is much similar to the microbiome of human gut (Pope et al. 2012). Similarly, Bhatt et al. (2013) highlighted the striking similarities and differences of dromedary camel *(Camelus dromedaries*) with other animal rumen ecosystem. Since, variations of microbial communities in ruminants is of great concern, Ross et al. (2012) suggested untargeted massive parallel sequencing (sequencing without target amplification of genes) approach for resolution of variation based rumen metagenome profiling.

Many developments in the exploration of gut microbial communities in different animal species have been made through sequence based metagenomics, and some recent examples follow. Dai et al. (2012) analyzed the fibrolytic microbiome in the rumen of yaks, and Brulc et al. (2009) used large sequence based studies to catalogue the genes involved in fiber degradation in the bovine rumen. Qi et al. (2011) applied metatranscriptomics to the study of rumen microbes function in muskox, and similar work has been carried out in sheep (Cammack et al. 2013), goats (Jakhesara et al. 2010) and Surti buffaloes (Singh et al. 2012a). Singh et al. (2012b) studied the virulence associated and antibiotic resistance genes of rumen microbes to facilitate the understanding of resistant gene transfer between and within habitats. However, researchers in this area have to explore the sequence based metagenomic into taxonomic perspective, as well as to link genomics and metagenomics to nutrition or other animal production parameters. For example, Li et al. (2012) characterized rumen microbiota of pre-ruminant calves and their metabolic potential so that the optimal early weaning nutritional strategies (like milk replacer) could be formulated, and recently Ross et al. (2013) analyzed the effect of methane mitigating diets on rumen microbiome.

Phage therapy

The lytic potential of phages and their genes make them an important tool for methane mitigation strategies. In contrast to nearly 300 phage genomes (Ackermann and Kropinski 2007), only six archaeal phages are sequenced and described, and just three of them are from methanogens: *Methanobacterium* phage psi M1, M2 and M100 (Pfister et al. 1998) and *Methanothermobacter* phage psi M100 (Luo et al. 2001). Little information is currently available on the genetic blueprint and gene functionality of archaeal, particularly methanogenic, phages but more are being discovered using electron microscopy (Ackermann 2007) and *in vitro* techniques (Stanton 2007). McAllister and Newbold (2008) reported siphophages that can infect methanogens (*Methanobacter*, *Methanobrevibacter* and *Methanococcus* spp.), although these phages have not been isolated from the rumen. A recent metagenomic study on phage-bacterial relationships showed ≤ 0.1% relative abundance of prophage in phylum *Euryarchaeota* (Berg Miller et al. 2012).

Metagenomic surveys are expected to reveal the presence of embedded prophages and phage-like elements that would have otherwise remain unnoticed. An unanticipated outcome from sequencing the *M. ruminantium* genome was the discovery of prophage φ-mru having 69 phage-related proteins (Leahy et al. 2010). A gene encoding a putative lytic enzyme was identified, expressed and shown to lyse *M. ruminantium*. Such lytic enzyme is potentially very useful biocontrol agents for manipulating of rumen methanogenic populations (Leahy et al. 2010). The genome sequence of *Methanobrevibacter* AbM4 and *Methanobrevibacter boviskoreani* strain JH1 revealed the presence of prophage/ phage like elements in strain JH1 while AbM4 is lacking in gene encoding prophage (Lee at al. 2013a and Leahy et al. 2013). Phages are host and even strain specific, so phage-based methane mitigation strategies could be developed without affecting other phylogenetically distinct microbes in the rumen. However, hosts and phages are also known to be involved in a rapid evolutionary race as the host changes to avoid infection and the phage changes to maintain infectivity.

In combination with the application of other phage enzymes and structural components, a rotation system can be envisioned that may overcome the rapid adaptation mechanisms of microbes to phage challenges. More methanogenic phages need to be identified, sequenced and characterized to identify and employ such phage-based strategies. However, high specificity of phages may also be a limiting factor in their effectiveness in reducing methane emissions, since there appears to be a high diversity of methanogens in rumen (Janssen and Kirs 2008). Finally, either mixture of phages or structural components of phages may prove useful against the greater diversity of methanogens in rumen.

Immunization

Host immunization commonly offers a diverse and ecofriendly solution to the problems especially associated with animal health. Therefore, developing vaccines against methanogens appears to be an alternative and attractive approach, which can avoid many of the issues mentioned above related to methane mitigation from ruminants. Wright et al. (2004) developed two vaccines, VF3 (based on three methanogenic strains 1Y, AK-87 and ZA-10) and VF7 (based on seven methanogens), that produced a 7.7% methane reduction in sheep (g per kg of dry matter intake); despite targeting only a minority (20%) of methanogens present within these host animals. They also created a vaccine based on five methanogens (*Methanobrevibacter s*pp. strains 1Y, AK-87, *M. millerae* ZA-10, *Methanomicrobium mobile* BP and *Methanosphaera stadtmanae* MCB-3) that was administered in three vaccinations to sheep (Williams et al. 2009). Surprisingly, immunization with this second vaccine caused methane output to increase by 18%, despite the fact that a larger proportion of the methanogenic population (52%) was targeted. Thus, further work is needed to optimize the individual components of these vaccines such that the most potent methanogens are specifically targeted.

Researchers believe that anti-methanogenic vaccines will only yield the short term reductions in methanogens and/ or methanogenesis, due to the possible proteolytic degradation and low persistence of host antibodies in rumen (Li et al. 2007; Cook et al.

2008; Lascano and Cárdenas 2010). Vaccination of sheep with entodinial or mixed protozoa antigens reduced protozoa and the released IgG antibodies against rumen protozoa remained active and continued to bind the target cells up to 8 hours (Williams et al. 2008). Vaccines targeting single surface antigens may not be effective, as methanogenic archaea differs largely based on their host, diet as well as geographical regions (Zhou et al. 2009). A new vaccine has been developed using sub-cellular fractions (cytoplasmic and cell wall derived protein) of *Methanobrevibacter ruminantium* M1 (Wedlock et al. 2010). Twenty sheep were vaccinated, then booster doses were given after three weeks, and the antisera were found to agglutinate and decrease the growth of archaeal methanogens and methane production *in vitro*.

In vivo efficacy of the vaccine on methanogens is yet to be evaluated. Based on liquid chromatography mass spectrometer, it was reported that most of the proteins were intracellular enzymes, particularly methyl-coenzyme M reductase, and these intracellular proteins would not be suitable as vaccine antigens owing to their inaccessibility for antibody binding. Since, there is the growing database for the genome sequences of rumen methanogens, the possibility of finding new target antigens/ proteins using comparative and pangenomics analysis have increased. The genome based reverse vaccine approach may also help in mining the new vaccine targets that might prove successful for efficacious vaccination against methanogens. Furthermore, extensive research is needed to identify adjuvants that stimulate high titer of antibody and are suitable for formulating with protein antigens to produce a low-cost and effective vaccine.

Overall, the genome sequencing will be leading to the better understandings toward methanogenic interactions with other microbes in the rumen suggesting some methane mitigation possibilities. The genomic techniques have provided positive clues for probable vaccine targets of methanogens in the rumen. Such approaches in future will optimistically lead to methane reducing practices for farm animals. However, the vaccine based inhibition method will have to pass the regulatory systems to guaranty animal health.

Chemogenomics: An upcoming strategy

Genome sequencing of microbes is actually a useful technique that can provide information directly applicable to methane mitigation strategies from ruminants, based either on vaccines development or small molecule inhibitor practices. Further, it can help to identify methane inhibitors by predicting and/or determining specific enzyme structures. This can define the geometry of the enzyme's active site and help to design the molecules that fit exactly into the active site and hence, can inhibit/ block the enzyme's catalytic function. This approach identifies inhibitory molecules that can be tested for their effectiveness in animal trials.

For reaching to a realistic solution to the problem of methane emissions, the technologies for reducing enteric methane must effectively target all the rumen methanogens (major and minor groups), otherwise less abundant methanogens may occupy the vacated niches and lead to normal methanogenesis. Besides, they should not affect other microbes present in the rumen, so that rumen function would not be altered. For this, an understanding of the diversity and physiology of rumen methanogens is essentially required, that not only identifies the dominant methanogens in a particular geographical area, but also the conserved sequences that can be targeted. In this regard, more genome sequences of methanogens are required to validate that the targets are common and effective among all the methanogens in the rumen (Attwood et al. 2011).

The completed *M. ruminantium* genome and draft sequences from other rumen methanogenic species are paving the way for identification of the underlying cellular mechanisms that define these microbes, leading to a better understanding of their micro-ecology within the rumen. Aside from this, the genome sequence of *M. ruminantium*, draft genome sequence of *M. boviskoreani* strain JH1 from Korean native cattle (HanWoo) and AbM4 from abomasum's of sheep have been published (Lee et al. 2013a; Leahy et al. 2013). The strain AbM4 do not code for many adhesion-like proteins, which indicates that it invest less on the external interactions with its environment compared to strain JH1. Moreover, AbM4 has a broader repertoire of cofactors and coenzymes, which shows its lesser dependence on the other rumen microbes and CoM in the medium. On the contrary, strain JH1 showed very good growth in presence of both CoM and VFA in the medium, and had genes and enzymes for CO_2 plus hydrogen, as well as for formate utilization, so that these enzymes possibly can be targeted for inhibition of methanogenesis.

Another gene, which can be further targeted, is the membrane associated transpeptidase. The Mtr enzyme complex can also be used for the development of vaccines. The phylogenetic analysis using Maximum-Likelihood inference method (MEGA 5.1) with 1000 boot strapping, and genomic sequence shows that strain JH1 and AbM4 likely belong to the same species and is related to *M. wolinii*. At present, this research is mainly at exploratory stage but several promising leads for chemogenomic targets are being investigated as possible intervention points for the inhibition of rumen methanogens. The cellular studies indicate that many of the conserved enzyme targets are involved in energy generation via methanogenesis, while majority of the conserved surface protein targets are of unknown function.

Bioinformatic approaches used for the inhibitor prediction against F420 dependent NADP oxidoreductase enzyme that catalyzes an important electron transfer step in the methanogenesis from *Methanobrevibacter smithii* reveal that lovastatin and compactin had high affinity to the enzyme and can act as potential inhibitors (Sharma et al. 2011). Both *in silico* approaches and *in vitro* enzyme assays may be useful for screening chemical inhibitors of methanogenesis.

The analysis of more genome sequences of rumen methanogens would help to identify potential methane inhibitors. According to the recent report of Lee et al. (2013a, b), only very few rumen methanogens are cultured as pure isolates and 13 genome projects are completed as yet (Leahy et al. 2013; Morgavi et al. 2013). Most of these genome sequences are from genus *Methanobrevibacter*, which is considered to be dominating rumen methanogen, as per the global data set of rumen microbes (Jansen and Kirs, 2008; Jeyanathan et al. 2011; Cheng et al. 2009; Williams et al. 2009, Zhou et al. 2009). In near future, with the development of "Hungate1000", a catalogue of 1000 reference microbial genomes from the rumen (http://www.hungate1000.org.nz/), genomic dataset of rumen microbiome will be numerically high, thereby more targets for anti-methanogenic strategies can be identified and used for improving the animal health, productivity and beyond. Furthermore, single-cell isolation technique from the complex rumen community would provide more advantage, over isolation approach, and their whole genome sequencing can be accomplished later.

Researchers are currently investigating, whether animal variation in methane emission is controlled by a heritable characteristic. Although clear and persistent individual differences in methane emissions have been found among animals fed the same diet (Pinares-Patiño et al. 2003, 2011; Martínez et al. 2010), it has not been yet clearly established whether the low emission trait is associated with any unwanted side effects. Currently, it is not possible to say whether in future it will be possible to breed animals that produce lesser methane per unit of intake or not (Clark 2013). Overall, the chemogenomics allowed us to identify the key features of rumen methanogens that can be targeted to inhibit them and to mitigate enteric methane production, eventually reducing the release of anthropogenic GHGs in the environment.

Final remarks

Looking at the facts in a comprehensive manner, profiling of rumen methanogens seem to be an important tool for ensuring sustainability of ruminant based agriculture production systems. However, for successful methane mitigation strategies to be developed and adopted, a thorough understanding of the microbial ecology of rumen methanogens is essentially required. DNA-based microbial profiling to explore ruminant methane mitigation will support how the rumen microbes can be manipulated without hampering the animal's production potential. These approaches would identify the involved microbial species based on genome sequences. By comparing microbial profiles of animals, one can identify the microbial shifts in response to the methane mitigation strategies. Some of the dietary strategies used in different studies have produced changes in rumen microbial communities as revealed by profiling assays. The comparison of rumen microflora both in high and low methane producers will help in determining if the changes in the microbiota can be directly or indirectly linked to the reduced methane emissions (Ross et al. 2013). In addition, genetic improvement and management practices for increasing ruminant productivity and abating methane emissions, in conjunction with other strategies, can also play an important role in lowering enteric methane emissions globally. Finally, it must be taken into account that there should be some economic return to the producers, if strategies to reduce methane emissions are expected to be implemented at farm level, and that any adopted strategy should also ensure animal health, food security and environmental safety.

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Table 1: Methane mitigation categories, mechanism of mitigation, problems associated and future directions

Mitigation categories	Subgroups	Example(s)	Mitigation mechanism (s)	Difficulties associated	Benefits and prospects	Reference(s)
i) Animal dietary manipulation	Manipulating animal diet composition	Shifting towards concentrate diets, use of newer forages	Improved passage rate of feed; increased propionate: acetate ratio; reduced rumen pH and protozoa counts	Shifting of methanogenesis towards hindgut; threat of rumen acidosis; economic losses in developing world	*In vivo* trails along with other strategies are essential	Niderkorn et al. (2011); Meale et al. (2012)
	Feed additives having secondary compounds	Condensed tannins, phenolic monomers, saponins, etc.	Inhibition of protozoa activity, fibre degraders and methanogenic archaea; decreased hydrogen availability	A few tannins lowers fibre digestibility; reduce palatability/ intake, performance, and change milk composition	Natural products; *In vivo* trials are needed for optimizing dose	Woodward et al. (2001); Carulla et al. (2005); Ramirez-Restrepo and Barry (2005); Tavendale et al. (2005); Hess et al. (2006); Animut et al. (2008); Tiemann et al. (2008); Lascano and Cárdenas (2010); Kamra et al. (2012)
	Feeding oils	Chain length (C_8-C_{16}) fatty acids	Having antimicrobial activities against methanogens and protozoa; biohydrogenation as a hydrogen sink; increased propionate: acetate ratio	Low palatability/ intake, animal performance, and changed milk composition; dose varies with diet and type of ruminant species	Continuing studies are must before making any conclusion	Machmuller and Kreuzer (1999); Dohme et al. (2000); Dohme et al. (2001); Soliva et al. (2004); Jordan et al. (2006a); Jordan et al. (2006b); Calsamiglia et al., (2007); Hook et al. (2010); Ding et al. (2012); Lunsin et al. (2012); Patra and Yu (2012)
	Addition of organic acids	Mainly fumaric and malic acids	Act as hydrogen sinks; shifting of rumen fermentation towards propionate formation	Contradictory reports; addition is affected by the type of diet; may increase rumen acidity; high cost	Screening of forages with higher organic acids content is desirable	Martin and Streeter (1995); Callaway et al. (1997); Carro et al. (1999); Carro and Ranilla (2003a,b) ; Ungerfeld et al. (2007); Wood et al. (2009); Foley et al. (2009a,b)

	Use of halogenated compounds	Chemicals like bromo alkyl sulphonates, lumazine, ethyl-2-butynoate, amichloral, cyclodextrin, lovatstain anthraquinone, etc. may be used	Inhibit protozoa, Gram-positive bacteria and methanogens; decrease substrate (s) for methanogenesis	Mostly *in vitro* reports; toxicity proven; transient effect due to adaptation of microflora and changes in diets	Combined administration is needed for constant results	Nevel and Demeyer (1995); May et al. (1995); Van Nevel and Demeyer (1996); McCrabb et al. (1997); Abecia et al. (2012)
	Role of ionophores	Mainly monensin, lasalocid, salinomycin, avoparcin, etc.	Eradicate methanogens; decrease substrate (s) for methanogenesis; inhibits protozoa and Gram-positive bacteria	No lasting effect; problem of absorption in rumen and reaching both in milk and meat; reduces intake	Further research required for a concrete solution	O'Kelly and Spiers (1992); Van Nevel and Demeyer (1996); Odongo et al. (2007); Hook et al. (2009)
ii) Rumen controls	By using bacteriocins	Bovicin HC5, Nisin	Aims at biological mitigation and hydrogen producers	Bacteriocins degradation; adaptation of rumen microbes; not target specific	More extensive research is required	Callaway et al. (1997); Teather and Forster (1998);
	Application of phage therapy	Appropriate phages for different groups of microbes	Should targets methanogens specifically	Host changes to avoid infections; specific phages	Regular change of phages and combination of phages can be tested	Pfister et al. (1998); Luo et al. (2001); Ackermann (2007); Ackermann and Kropinski (2007); Stanton, (2007); Attwood and McSweeney (2008); McAllister and Newbold (2008); Janssen and Kirs (2008)
	Through immunization	Development of relevant vaccines	Activates immune response of hosts against methanogens	Inadequate targets of vaccine due to differences in dietary regimen	Require genomic data for identifying universal immunization targets; *in vivo* efficacy tests yet not done	Wright et al (2004); Li et al. (2007); Cook et al. (2008); Williams et al. (2009); Zhou et al. (2009); Lascano and Cárdenas (2010); Wedlock et al. (2010)

	Via defaunation	Using chemical inhibitors; plant extracts, vaccines for protozoa	Reduces hydrogen	Transient effects; toxicity; reduced feed digestibility	Needs a validation before field application	Moss et al. (2000); Holtshausen et al. (2008); Morgavi et al. (2008);Williams et al. (2008); Patra and Saxena (2009); Bird et al. (2010); Hegarty et al. (2010)
	Using acetogens	Acetogenic microflora	Provide an alternative hydrogen sink	Needs high levels of hydrogen to grow at the rate of methanogens; unsure sustenance in rumen	Increases acetate, feeding experiments are must	Joblin (1999); Joblin (2005); Fonty et al. (2007)
	Feeding probiotics or direct-fed microbials	Yeasts (*Saccharomyces cerevisiae*); fungi (*Aspergillus oryzae*, *Trichosporonsericeum*, etc.)	Increases butyrate or propionate and decreases protozoa; Enhances acetogenesis	Lowers rumen efficiency; *in vitro* reports for strain selection are erratic; Scanty *in vivo* experiments	Better hydrogen utilizing bacteria followed by *in vivo* study is desirable	Martin and Nisbet (1990); Carro et al. (1992); Mathieu et al. (1996); Newbold et al. (1998); Lynch and Martin (2002)
iii) Other systematic changes	Animal breed selection and intensiveness of production	Low producers of methane	Genetic and retention time based differences of animals	Affects digestibility; environmental impacts	Increased animal production	Pinares-Patiño et al. (2003); Pinares-patiño et al. (2011); Waghorn et al. (2006); Kumar et al. (2009); Lascano and Cárdenas (2010); Attwood et al. (2011)