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Mitigation strategies for enteric methane emission with special emphasis on biological approaches: A review

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ABSTRACT

Atmospheric concentration methane is almost 1782 ppb, which is 155% higher than its pre industrial concentration. Agricultural sector dispensing a consequential amount of methane (about 2/3rd of total anthropogenic); livestock rearing generates 7–12 Tg methane annually from the country. The methane generation from ruminants not only contributes significantly to global warming but also leads a loss of dietary energy (7–12% of GEI). This loss needs attention, where acute shortage of quality feeds prevails. Efforts were made in the past to control the enteric methane emission using customary feeding approaches including chemicals and methane analogues, but most of them dubitable with one or more critical limitations like few respond at high doses, toxicity to either host animal or inhabited synergistic microbes etc. Therefore, the search for suitable, effective and safe methane mitigating agent is still ongoing, and extensive screenings of newer strategies are on radar of animal nutritionist and microbiologists in association with biotechnologists for abatement of methane emission from ruminants. This review paper examines the biological approaches such as reductive acetogenesis, immunization, phage therapy, defaunation, disabling the protein bindings accountable for association of methanogens with other microorganisms in rumen. In conclusion, there is the foremost need to explore the potentialities of state-of-the-art biological approaches under *in vivo* and with topographical variations.

Key words: Immunization genomics, Livestock methane, Phage therapy, Protein genomics, Reductive acetogenesis

Since the middle of the 19th century, human agriculture and rapid industrialization have dispensed an enormous quantity of greenhouse gases into the atmosphere (Etheridge *et al*. 1998). Recently, Intergovernmental Panel on Climate Change (IPCC 2007) reported an increment of 38, 155 and 15% CO_2, CH_4 and NO, pre-industrial concentration. The current atmospheric concentration for the respective gases is about 385 ppm, 1782 ppb and 321 ppb. Methane is one of the major green house gases responsible for stratospheric ozone depletion (IPCC 1992) and second largest contributor to global warming after CO_2 as its comparative GWP to CO_2 is about 25 times over 100 years (Anonymous 2007). Human-related activities such as fossil fuel production, enteric fermentation, rice cultivation, biomass burning, and waste management is accountable for more than 60% of total global methane emission (CCSP 2006), which is about 330 Tg as reported by Houweling *et al*. (1999). About 2/3rd (205–245 Tg) of global methane emission from anthropogenic sources comes from agricultural sector alone (Watson *et al.* 1992, Duxbury *et al.* 1993) including enteric fermentation (80Tg), rice cultivation (60–100 Tg), biomass burning (40Tg) and animal wastes (25Tg). Among the agricultural sources, livestock emanate a considerable amount of methane in the tune of 75–80 Tg (28% of global emissions) as documented by EPA (2009) and Chhabra *et al.* (2009).

Indian scenario: In India where livestock rearing is an integral part of the agricultural system, possesses 485 millions livestock including 57 and 16% world's buffalo and cattle respectively. This gargantuan livestock in country dispense bountiful amount of methane to the total pool upon the enteric fermentation of low quality feeds in rumen (Malik 2007). Various agencies promulgate the annual methane emission from Indian livestock in the range of 7.2–12.9 Tg (Table 1). A summary of the approaches used by different agencies and proclaimed value is given in Table 1. Eightinth livestock census revealed the highest livestock population in Uttar Pradesh (39.98 million) followed by Andhra Pradesh (38.89 million) and Rajasthan (38.28 million) and these three states contribute 24.14% of the total livestock while the contribution from individual state is 8.24, 7.89 and 8.01%, respectively (DAHD, MOA, GOI 2007). Among the districts, Chhabra *et*

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Table 1. Estimates of methane emission from Indian livestock by various agencies

Approach for estimation	Methane emission (Tg)	Estimate year	Reference
Methane emission measurement from cattle and buffalo using facemask technique and regression analysis	–	–	Krishna *et al*. (1978)
Default methane emission factors	10.40	1985	Ahuja (1990)
Using IPCC (1995) methodology	18.48	1990	ALGAS (1998)
In vitro dry matter digestibility evaluation of feed resources in different regions	9.02	1992	Singh (1998)
Emission estimates for enteric fermentation only and based on amount and quality of available feed resources	7.26–10.4	1995	Garg and Shukla (2002), EPA (1990)
Country-specific methane emission factors derived from Indian feed standards, IPCC energy equations and dry matter estimation	9.9	1994	MOEF (2004)
Dry matter intake approach under different agro-climatic regions	10.07	1994	Singhal *et al*. (2005)
Country-specific Indian feed standard-based methodology as a measure of gross energy intake and derived methane emission factors	9.00	1994	Swamy and Bhattacharya (2006)
National Livestock methane emission inventory with State/district level emission by using GIS approach	10.65	2003	Chhabra *et al*. (2009)

al. (2009) documented that Mednipur (West Bengal) hold the highest livestock population among the districts while Jhalawar (Rajasthan) secured the second position in total livestock population. Spatial analysis in GIS has identified Uttar Pradesh, Rajasthan and Madhya Pradesh as three highest methane emitters, with a contribution of 1.75, 1.07 and 1.0 Tg, respectively, while methane emission from all the districts in the North-East States, Sikkim, Goa, Himachal Pradesh, Kerala and Uttarakhand is very low lie in the range of < 0.02 Tg (Chhabra *et al.* 2009). The spatial analysis identified the districts with high methane emissions, viz. 0.025 Tg/yr in Bulandshar (Uttar Pradesh); 0.022 Tg/yr in Prakasham (Andhra Pradesh), Jaipur (Rajasthan), and 0.021 Tg/yr in Kolhapur (Maharashtra) and Mednipur (West Bengal). Goats are the dominant milching livestock in the country but dairy buffalo being the highest contributors to methane emission (1.78 Tg/yr) surpass the other dairy animals and the contribution of goat is only 0.14 Tg/yr (Chhabra *et al.* 2009). Chhabra *et al.* (2010) in an interesting study reported that dairy buffalo with highest CH_4 emission coefficient is the major livestock in Bulandshar, Prakasham, Kolhapur, Belgaum and Guntur, whereas goat and indigenous cattle are dominant in Jaipur and Mednipur, respectively.

Singh (1998) reported that among the livestock, cattle (76g/d and 5.50 Tg/y) and buffaloes (97g/d and 2.80Tg/y) are the noteworthy emitters while, sheep (0.19 Tg) and goat (0.40) contribute very diminutively (Singh 2001). Chhabra *et al*. (2009) enunciated that Northern, Eastern, Western, Southern and Union Territory region of the country contribute 25, 23, 34, 18 and 0.09% of the total enteric methane emission in the country, respectively.

Mitigation strategies to combat livestock methane emission

Many alternative approaches are available for the mitigation of enteric methane emission in the country, but the effectiveness of a particular strategy depends on the cost of the item, economic status of livestock keeper, toxicity to host animal or inhabiting microbes, persistency etc. Peoples from developed countries are blaming India for its high enteric methane emission and usually asked to reduce the number of livestock population but controlling enteric methane emission by this way is unrealistic as livestock play an important role in agricultural GDP and provide a source of supplementary income to the farmer's community in the country. Therefore, efforts have been made to control the enteric fermentation either by improving the digestibility of feeds, use of plant extracts, feed additives etc. which exert their anti-methanogenic effect by changing the fermentation pattern, direct inhibition of methanogens, indirect inhibition of methanogens via cutting the hydrogen supply etc. All the dietary approaches in the review is discussed in brief as many of the strategies suffers from one or more limitation therefore, animal nutritionists, focusing on the alternatives and one of the emerging and effective way is the use of biological agents

to control the enteric methane emission. In the paper various biological approaches that seem to have a scope in coping up the enteric methane problem are discussed in detail.

Dietary approaches: Dietary factors such as composition of diet, type of forage, processing, feeding frequency, nature of concentrate, type of starch (slowly versus rapidly degraded), level of intake influences methane emission from the rumen by altering surface area for microbial activity, ruminal pH, flow rate of digesta and fermentation pattern.

Methane emission from animals may be less on feeding leguminous forages than feeding of grasses (Varga *et al.*1985). Malik and Singhal (2008a) documented a significant reduction in methane production on the incorporation of limited quantity of lucerne fodder (30%) to the wheat straw based ration.

Introduction of fats, reduce methane emission without decreasing ruminal pH (Tyagi and Singhal 1999, Machmuller *et al.*2000, Bairagi and Mohini 2005). Addition of fats or oils to the diets of ruminants suppresses methane production by two mechanisms, first through indirect mechanism includes protozoal inhibition (Machmuller *et al.*1998), reduction of double bonds in unsaturated fatty acids, and increase propionate production, second mechanism through causing toxicity directly to methanogens (Machmüller *et al.* 2003) probably by changing their metabolic activity and composition. However, excessive dietary supplementation of fatty acids, oils or fats in ruminants reduces fibre digestion (McGinn *et al.* 2004), microbial protein synthesis (Dong *et al.* 1997) and may have adverse effect on animals. High cost and negative effect on milk fat concentration are other important constraints in the use of oil in the diet of ruminants for methane mitigation (Zheng *et al.* 2005).

Dietary inclusion of ionophores depresses methane emission by mixed rumen microbes (Moss *et al.* 2000, Barman *et al.* 2001, Beauchemin *et al.* 2008) through increasing feed conversion efficiency, shift in fermentation pattern, selective reduction in acetate production, inhibition of release of H_2 from formate and anti-protozoal effect. *In vitro* study by Fuller and Johnson (1981) indicated that monensin and lasalocid additions to the high grain substrate reduced CH_4 loss to as little as 53% of that in the control, however, Callaway *et al.* (1997) claimed the maximum methane inhibition by monensin as 18% under *in vitro* study. One of the major limitations in the use of ionophores is the inconsistency in results and usually animals turn back to normal value after with drawl of ionophores (Rumpler *et al.* 1986).

Dicarboxylic organic acids are also reported for exerting their anti-methanogenic action in number of studies (Iqbal *et al.* 2008, Sirohi *et al.* 2009) probably through increased propionate production (Bayaru *et al.* 2001), proliferation of cellulolytic bacteria (Asanuma *et al.* 1999). High malate content in fresh forages at early growth stage especially lucerne could lead to significant changes in rumen fermentation (Martin 1998) and 10% decrease in methane was reported by McCaughey *et al.* (1999) on replacing grasses with a mixture of lucerne (potent source of malate) and grasses (70:30). An exceptional decrease in methane production (75%) was reported by Wallace *et al.* (2006) with 10% encapsulated fumaric acid in the diet of sheep. On the other hand McGinn *et al.* (2004) did not observe any significant effect of fumeric acid on methane production in their study. Supplementation of fumaric acid (FA) as anti-methanogenic agent has the risk of acidosis and a consequent decrease in fibre breakdown and feed intake.

Extract of various plants from their leaves, fruits or roots are traditionally used as medicine since ages but their antimicrobial and anti-methanogenic properties attributed to secondary metabolites have been recognized recently (Alexander *et al.* 2008, Malik and Singhal 2008a). The exploration of bioactive photochemical in the extract has been of great interest among nutritionists and rumen microbiologists (Kamra *et al.* 2006, Sirohi *et al.* 2009) as an alternatives to chemical feed additives (Patra and Saxena 2009) to modify the rumen fermentation and inhibition of methanogenesis (Lila *et al.* 2005). The use of photochemical such as saponins, essential oils (Wallace *et al.* 2002), tannins as anti-methanogenic agent in the diet is generally safe, cheap, and easily available from a wide range of plants and therefore, can be employed to modify rumen microbial fermentation. Most of the trials with plant extracts have been conducted under *in vitro* and only few studies have taken *in vivo* shown highly variable response of these molecules on methanogenesis.

Tannins reportedly decreased methane production both *in vivo* (Berra *et al.* 2008) and *in vitro* (Bhatta *et al.* 2009). Soliva *et al.* (2008) from their study concluded that various plant materials, including accessions of *Acacia angustissima, Sesbania sesban* and *Cajanus cajan*, were promising to approach the goal of mitigating the methane emission from small ruminants. Tannins present in *Callindra calothyrus* reduced methane release per gram of organic matter degraded *in vitro* (Hess *et al.* 2003). Average reduction of 10–17% in methane production on the feeding of tannin rich forage was reported by Carulla *et al.* (2005) and Puchala *et al.* (2005) in Angora goats.

Similarly, methane emission in ruminants may be reduced by using different materials having saponins as biomolecule and extracted from plant or herbs such as *Yucca schidigera, Quillaja saponaria, Enterolobium cyclocarpum, Sesbania sesban, Medicago sativa, Sapindus saponaria, Sapindus rarak and Sapindus mukorossi* (Hess *et al.* 2003, García-González *et al.*2008, Rejil *et al.* (2008). The anti-methanogenic action of saponin biomolecules is attributed to the inhibitory action of saponins on protozoa (Kamra *et al.*2000, Lila *et al.* 2003, Malik and Singhal 2008b), which are responsible for the interspecies hydrogen transfer to methanogens. Malik and Singhal (2008b) in their *in vitro*

study reported a reduction of 29% in methane production on the supplementation of 4% commercial grade saponins to wheat straw and concentrate based diet. However, Goel *et al.* (2008) found very little effect of fenugreek and sesbania addition on methane production despite the decrease in protozoal numbers, therefore, methane reduction and anti protozoal response of different saponins sources are transient and probably attributed to the variability in the chemical properties of saponins from different sources (Koenig *et al.* 2007).

Only few reports are available which inferred a direct inhibition of methanogens by essential oils. Evans and Martin (2000) in their *in vitro* study observed that essential oil thymol (400 mg/l), derived from thymus and origanum plants is a strong inhibitor of methane. Furthermore, a reduction of 69–74% in methane production on the addition of garlic oil and diallyl disulphide @300mg/l in ruminal fluid was reported by Busquet *et al.* (2005). McIntosh *et al.* (2003) perceived that the inhibition of methanogens depends on concentration of essential oil and found that the activity of *Methanobrevibacter smithii* did not inhibit up to 160 ppm, while inhibition was reported at 1000 ppm. On the other hand, archeal 16S rRNA analysis did not reveal any significant effect of cinnamaldehyde, garlic and juniper oil on methanogens (Ohene-Adjei *et al.* 2008). Rasmussem *et al.* (2005) found rosemary (*Rosmarinus officinalis*) as the potential inhibitor of the protozoa among the 19 essential oils screened for anti-protozoal action however, they also reported that the inhibition is concentration dependent.

Biological approaches: For mitigation of methane emission from enteric fermentation, nutritional strategies are– the primary target is identifying effective strategies and a wide range of specific agents aimed at suppressing methanogenesis. Such approaches are, however, often limited by a concomitantly impaired nutrient digestibility, reduced feed intake, expensive at high dose and transitory effect. The use of chemicals for methane mitigation from ruminants is always associated with the risk destined for human consumption. Similarly, novel plant compounds such as condensed tannins, saponins or essential oils may have merit in reducing methane emissions, but these responses may be through reduced digestibility of the diet. Therefore, the search for suitable, effective and safe methane mitigating agent is still ongoing, and extensive screenings of newer strategies are on radar of animal nutritionist for abatement of methane emission from ruminants.

This paper closely looks some biological strategies to reduce ruminal methanogenesis such as reductive acetogenesis, immunization, phage therapy and defaunation.

Reductive acetogenesis: Reductive acetogenesis is an alternative pathway for hydrogen disposal (Immig *et al.*1996) and therefore has the potential to control methanogenesis in rumen (Fig. 1). The first ruminal acetogen, *Eubacterium limosum* was isolated from a sheep fed on molasses-based

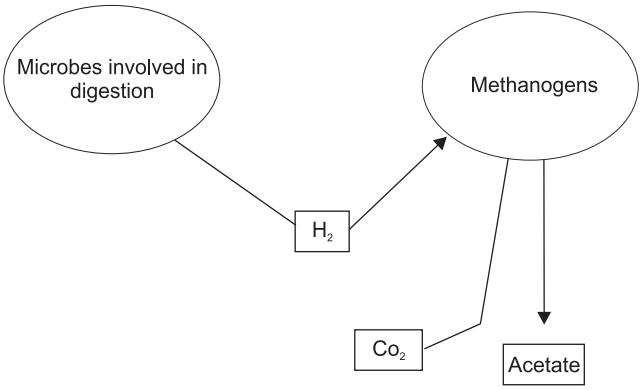

Fig. 1. Reductive acetogenesis, a possible alternative to ruminal methanogenesis (Joblin 1999)

Acetogenesis

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \rightarrow \Delta\, Go' = -104.6 \text{ kJ}$$

Methanogenesis

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \rightarrow \Delta Go' = -135.6 \text{ kJ}$$

diet (Genthner *et al.*1981) while Greening and Leedle (1989) isolated the second acetogen i.e. *Acetitomaculum ruminis* from the rumen of cattle. The finding of acetogens from sheep and cattle's rumen opened new prospects for scavenging the H_2 through alternate disposal. Acetogens are the prominent hydrogen utilizers in the rumen of day-old lambs however, as lamb becomes mature, due to the greater affinity of methanogens for hydrogen the mission is now engross by methanogens which act as main hydrogen utilizers (Morvan *et al.* 1994). The threshold values of archea for hydrogen are almost 10–100 times lower than the acetogens (Fievez *et al.* 2001) so they normally out-compete the acetogens by keeping the H_2 concentration below the critical value needed to enable acetogens to persist (Cottle *et al.* 2011). The H_2 concentration may rise to critical level by inhibiting the methanogens which favor the growth of acetogens (Le Van *et al.* 1998).Phylogenic trees showing microbial evolution indicate that host animal factors under genetic control can inhibit the growth of methanogens or foster growth of acetogens (Hackstein 1997), but these mechanisms do not appear to exist normally in the rumen (Cottle *et al.* 2011). Nevertheless, at least 10 species of rumen bacteria have biochemical pathways that enable them to use H_2 and CO_2 to synthesise acetate by reductive acetogenesis (Joblin 1999). A variety of other microbes capable of RA have also been found recently (Henderson *et al.* 2010). They probably do not normally obtain their ATP for growth from CO_2 and H_2, but instead use other substrates (Mackie and Bryant 1994). Attempts have been made to induce reductive acetogenesis in rumen fluid from adult ruminants for searching the alternate way of H_2 disposal in rumen and also to improve the energetic gain (Fig. 1) to animal either by creating conditions thought suitable for the

establishment of acetogenic bacteria, such as addition of mucin or amino acids or by striving the inhibition of rival methanogens. Acetogen densities in rumen of beef cows fed on hay concentrate diet is found between 10^2 -10^5/ml (Leedle and Greening 1988), however in few studies acetogens numbers in cattle fed on various diet was reported $<10^2$/ml (Le Van *et al.*1998, Morvan *et al.*1996).

So far, at least 10 ruminal acetogens have been obtained and 4 of the 10 isolates were obtained from very young lambs (pre-ruminants) and rest were from ruminants fed mixed diets containing concentrates or conserved forages (Joblin 1999). Little is known about acetogens in forage-fed ruminants. If methanogenesis were able to be wholly replaced by acetogenesis in ruminants this would represent an energetic gain of between 4 and 15% to the animal (Nollet *et al.* 1997, Joblin 1996). If ruminal acetogens are to compete with methanogens for H_2 in the rumen, one important factor is likely to increase their capacity to grow by mixotrophy (Joblin 1999). The human-gut acetogen, *Ruminococcus hydrogenotrophicus*, has been shown to utilise glucose and H_2/CO_2 simultaneously during growth (Leclerc and Bernalier 1997), but as yet there are no reports of ruminal acetogens capable of mixotrophic growth on sugars and H_2/CO_2.

Reports are available which proclaimed that the methane production in Eastern grey kangaroos and tammar wallabies (von Engelhardt *et al.*1978) per unit of digestible DMI is less than ruminants even though they ferment fibrous feeds and generate VFA in a manner similar to ruminants (Dellow *et al.*1983). The less methane production in kangaroos is due to the appreciable number of acetogens with few methanogens in forestomach (Ouwerkerk *et al.* 2005), a situation similar to hind gut of pigs and rats (Joblin 1999), ostriches (Fievez *et al.* 2001), all of which have acetogens that apparently compete effectively with methanogens. Information on acetogens comes mainly from overseas studies on animals fed non forage diets and there is a shortage of information on ruminal acetogens in our conditions. Before the potential for reductive acetogenesis to lower ruminant methane emissions can be assessed, we need to understand the factors affecting their population levels and activities in the rumen. The challenge for the animal nutritionist and microbiologist is to induce the conditions in rumen which favor the growth to acetogens with a limited number of methanogens as in kangaroos.

Immunization: One possible future way to reduce methane output is to immunize animals against their own methanogens. The methanogen cell envelope having pseudomurein serves as the interface between the organism and its rumen environment, and as such represents a key area for the identification of vaccine and drug targets (Leahy *et al.* 2010). The immunization of methanogens by vaccination indirectly affects the activity of archea as they have a commensal relationship with rumen protozoa.

Williams *et al*. (2009) evaluated a broader spectrum vaccine in 32 sheep, targeting 5 methanogen and the results from the study showed specific IgG titres in plasma, saliva and rumen fluid, but methane output and total number of rumen methanogens was unaltered. However, Wright *et al.* (2004) in their study reported a reduction of around 8% on the vaccination of Australian sheep against three selected methanogens. The main impediment in the use of vaccine to trigger the immune response is its effectiveness only for a selected archea and when tested with a different set of archeal species, it did not elicit a positive response (Wright *et al.* 2004, Clark *et al.* 2007). Furthermore same workers also enunciated that the effectiveness of vaccine also not tantamount in other geographical region and the immunization failure may be attributed to the highly diverse methanogenic community present in animals reared under different conditions (Wright *et al.* 2007). A more fundamental approach based on the genomic information of methanogens will hopefully identify common targets across species that could be used for the development of second generation vaccines (Attwood and McSweeney 2008). Metagenomic analysis, library construction and gene sequencing for the whole rumen microbial population enlightens only < 0.1% similarity with genomic sequence (Bath 2008), revealing a unique and complex community of ruminal microbes. Of the sequences, 1% showed homology to the *Archaeal* domain and a large range of different methanogens were identified, probably including previously unknown species. These results are being used as the basis for development of a rumen specific microarray aimed at improving FCR and reducing methane emission in dairy cattle.

More recently, passive immunization was also assayed using antibodies prepared from hen's eggs. Passive immunity is the transfer of *active* humoral immunity in the form of readymade antibodies, from one individual to another. Passive immunity can occur naturally, when maternal antibodies are transferred to the fetus through the placenta, and can also be induced artificially. Antibodies decreased methane production *in vitro* but the effect was short-lived (Cook *et al.* 2008).

More work is needed to make this technique effective, as there are multiple strains of archaea in the rumen and work done so far in developed countries is based on the selected strains of methanogens. If this proves successful, the vaccination would be a valuable tool for methane reductions as it could be applied to a whole ruminant population.

Phage therapy: In the rumen, bacterial viruses (bacteriophages or phages) are well known (Klieve and Bauchop 1988) having population density about $>10^9$ particles/ml fluid (Klieve and Swain 1993), and they attack a wide variety of bacterial hosts (Klieve *et al.* 1989). Although knowledge of the bacteriophages of rumen bacteria has progressed in recent years but it is still limited or rudimentary. Phage-like particles are known to infect *Methanobrevibacter smithii* and *Methanobacterium thermoautotrophicum* (Nagle

1989). *M. smithii* strain PS is the host for a virus that has a morphology identical to phages of the family *Siphoviridae* (Knox and Harris 1986). The morphological similarities between phages and archaeal viruses may have similar properties such as infectivity and lysogeny. Tarakanov (1994) has successfully used the phages of *S. bovis* as feed additives to suppress amylolytic bacteria and concluded that phages may be used as a biological agent for mitigating the enteric methane emission. However more studies on the genetic diversity and viral susceptibility of methanogens and the host range of archaeal viruses are required to assess their potential as biocontrol agents. Recently work has been initiated in Australia to control rumen methanogenesis using archeaphage therapy. The limited host range of phage CM1 of *M. thermoautotrophicum* to strain Marburg has already been suggested as a limitation to the use of this phage as a vector system for the genetic manipulation of methanogens (Leisinger and Meile 1990, McAllister *et al.* 1996). However, for a given strain there is evidence that phage may cause widespread infection with wide host range. Newbold *et al.* (1996) observed substantial lysis of *Methanobrevibacter* MF1 in cell-free rumen fluid, with lysis being avoided by prior autoclaving of the rumen fluid. Jiang *et al*. (1995) in their study specifically isolated phages of rumen acetogens for future use in the genetic manipulation of acetogens which keep the possibilities alive for using the archealphage therapy in controlling methanogenesis.

The greatest limitation in the use of rumen phages as biocontrol agent is probably lack of universal infectivity. If, specific strains or groups of genetically similar strains of methanogens are to be targeted, viruses could be suitable as biocontrol agents and have a significant impact on ruminal methane production, but if genetically heterogenous groups, as with the ruminal bacteria *P. ruminicola* and *Butyrivibrio fibrisolvens* (Hudman and Gregg 1989), are to be targeted, it may be difficult to locate the same.

Defaunation: Protozoa provide a habitat for up to 20% of rumen methanogens (Stumm *et al.* 1982), and rumen fluid with high numbers of protozoa tends to produce more enteric methane (Krumholz *et al.* 1983). Moreover, removal of protozoa and feed particles from rumen contents by centrifugation also removed 76% of the methanogens present (Newbold *et al.*1995). Rumen ciliate protozoa are known to be endosymbiotically associated with methanogens (Finlay *et al.*1994). These protozoa-associated methanogens are accounted up to 37% of total rumen methane emissions. Defaunated animals produce less methane than produced by faunated animals (Williams and Coleman 1992), it is attributed to the shift of digestion from rumen to hind gut (Van Nevel and Demeyer 1996) or the loss of methanogens associated with protozoa (Hegarty 1999). Elimination of rumen protozoa therefore offers an opportunity to reduce enteric methane emissions up to a meaningful extent (Hegarty 1999). Although wide range of dietary regimes and synthetic chemicals are well known for their antiprotozoal action (Hegarty 1999), however, no systematic assessment of the use of organisms in biologically suppression of rumen protozoa has been done. Several methods are available for experimental purposes, mostly based on the use of surface-active agents that are toxic to protozoa (Newbold and Chamberlain 1988). Unfortunately, none of the methods has been without a negative effect either on the rest of the rumen microbes or on the host itself (Williams and Coleman 1997). Defaunation can also be achieved by emptying the rumen, carefully washing the rumen mucosa and treating the digesta by freezing before re-introducing it into the rumen (Jouany and Senaud 1979), but obviously this is not a practical on-farm approach. Recently, interest has increased in the use of tropical plants particularly saponin containing plants a possible means of suppressing or eliminating protozoa (Malik and Singhal 2008b) beyond nutritional value (Teferedegne 2000). In ruminants saponins are differentially toxic to rumen protozoa which may be explained by the presence of cholesterol in eukaryotic membranes but not in prokaryotic cells (Kilta *et al.* 1996). Saponins are degraded in batch cultures of rumen fluid *in vitro* (Makkar and Becker 1997), although apparently the resultant sapogenins are more resistant to degradation (Wang *et al.* 1998).

A successful defaunation treatment for use in feeding practice would be one that could be applied continuously throughout the desired period and be safe to the rest of rumen microbes and host. The majority of protozoan- associated archaea is placed under 3 genera namely *Methanobrevibacter, Methanomicrobium* and RCC clade (Janssen and Kirs 2008) however, notable variations in the abundance of different groups of protozoan-associated archaea are reported in different studies. Members of the *M. ruminantium* clade were reported in abundancy (62%) by Chagan *et al.* (1999) while Regensbogenova *et al.* (2004) reported *Methanomicrobium* spp. as dominating one. On the other hand, Ohene-Adjei *et al.* (2007) observed members of RCC clade as major archeal associated protozoa. Elimination of protozoa from the rumen of sheep resulted in changes in the archaeal community (Morgavi *et al*. 2006), but it was not clear if this was due to the disappearance of uniquely protozoan-associated methanogens or due to broader changes in rumen function as a result of defaunation. The true significance of different clades of protozoan-associated archaea could be determined by careful analysis of the two groups in fractionated rumen samples and by using rRNA targeted FISH methods to observe the localization of cells of different archaeal groups in total rumen samples. It has been shown that defaunation may depress fiber digestion, thus complete elimination of protozoa (rather than selective defaunation) is not recommended as a method for reducing methane (Itabashi 2001). On the other hand, protozoa have been reported to negatively affect ruminal protein metabolism through predation of bacteria, which reduces the flow of microbial

protein leaving the rumen (Koenig *et al.* 2000). Therefore, the use of defaunation to mitigate methane production from ruminants should be weighed against its possible impact on the efficiency of the whole ruminal system (Boadi *et al.* 2004). Further work is needed in this area to develop commercial means of controlling rumen protozoa (Klieve and Hegarty 1999).

Disabling the protein binding: Another potential target for the inhibition of methane lies on the outside of methanogens because they themselves are associated with other ruminal organisms via specific surface proteins. Disabling these proteins or preventing their binding could disrupt these interactions and upset their normal behaviour. The whole genome sequencing of the *Methanobrevibacter ruminantium* by Leahy *et al.* (2010) identified several key genes (2,200 genes and almost 3 million base pairs) and enzymes which may be potential targets involved in the methanogensis pathway within the cell. Targeting the methanogens is a very strategic option due to the uniqueness and commonness of identified enzymes in all methanogens species. Additional cell-surface protein targets were also identified through genome sequencing of *Methanobacterium ruminantium*, which may be used to design peptide antigens for testing against methanogens and in developing vaccines. Leahy and associates (2010) reported that *M. ruminantium* genome encodes 25 very large surface proteins that contain repeat sequences similar to those found in proteins from other gut methanogens, *Methanosphaera stadtmanae* and *Methano-brevibacter smithii*. These proteins are predicted to be membrane-anchored at both ends with the central portion external to the cell. Many of these externally exposed protein regions contain DUF11 (domain of unknown function) or C-POMP (chlamydial polymorphic outer membrane protein) repeat domains which are believed to be involved in cell adhesion and in some cases act as virulence determinants. This suggests that *M. ruminantium* has the capability to attach to surfaces in the rumen, possibly on protozoa or bacteria. Interrupting the association of methanogens with other organisms or surfaces in the rumen is likely to interfere with their normal function, therefore these distinctive cell surface proteins are candidates as possible vaccine targets. Furthermore, *M. ruminantium* represents only one species of a larger group methanogens known to inhabit the rumen. Therefore, much remains to be learnt about the role of *M. ruminantium* and other methanogens in methane formation in the rumen. The function assigned to genes need to be investigated using microarrays technique for gene expression. Microarray is being used to quantify gene expression during coculture with H_2–forming rumen microbes to identify methanogen genes involved in the transfer and utilization of H_2. This process is central to methanogen growth in the rumen so understanding the function of the genes involved, may explore new options for methanogen inhibition.

Future line of work

It seems unrealistic to mitigate the enteric methane emission using customary approaches as most of them dubitable with one or more critical limitations, exasperated by the type of feeds and fodders available in the country. However, novel plant secondary metabolites such as tannin, saponin, essential oil etc. show propitious result in abatement of methane emission, but the responses may often be obtained through reduced digestibility of the diet. Moreover, the paucity of information about dose appropriation for different category of livestock and other adverse effect, questions the viability of these compounds to use as methane mitigating agent. Therefore, the search for suitable, effective and safe methane mitigating agent is still ongoing, and extensive screenings of newer strategies are on radar of animal nutritionist and microbiologists in association with biotechnologists for abatement of methane emission from ruminants. The ongoing preliminary work in developed countries delves the possibilities for using biological agent in enteric methane mitigation. As diminutive work has been initiated so far and that only under *in vitro* conditions, therefore, there is the foremost need to explore the potentialities of state-of-the-art biological approaches under *in vivo* and with topographical variations. Animal nutritionist in collaboration with rumen microbiologists and biotechnologists must give priority to work on improving the H_2 utilization efficiency of acetogens, exploring the archeal diversity within rumen for developing the second generation vaccine that may be effective against all prevailing and infinitesimal methanogens, probing the new H_2 utilizing species that can offer a better alternative than previously tested acetogens in rumen, modeling of enzyme structures and designing of small chemical molecules that can block the active site of methanogens enzyme using chemi-genomics. Furthermore, the research on universal infectivity aspect of rumen phages along with protein genomics for disarming the adhering of methanogens with other microbes in rumen is also need to be examined thoroughly.

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