STRATEGY PAPER 11

Rumen Microbiome and Amelioration of Methane Production

NATIONAL ACADEMY OF AGRICULTURAL SCIENCES, NEW DELHI

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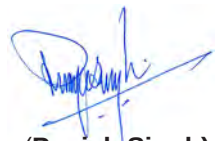
Preface

In the wake of growing concern over the contribution of ruminants to methane emission, there is an urgent need for enhancing the extraction of energy from lignified crop residues through rumen manipulation for a more profitable and green livestock production. According to a report from Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Govt. of India, the country is responsible for methane production to the tune of 14.55 Tg/year (13.27 Tg from enteric fermentation and 1.28 Tg from livestock waste management), out of which cattle (6.73 Tg/year) and buffalo (6.56 Tg/year) collectively are responsible for 91.3% of total methane emitted by the ruminants in India. A large number of feed additives like methane analogues, antibiotics, inophores, unsaturated fatty acids and inorganic terminal electron acceptors (sulphate, formate, nitrate etc.) have been reported with a potential to inhibit methane production in the rumen, but majority of them are either toxic to animals or to the microbes responsible for methanogenesis. Similarly, plant secondary metabolites are effective against methane emission and rumen protozoa, but some of them also have adverse effects on feed degradability and nutrient utilization by the ruminants. Inter-species/breed differences have been reported between the microbiome of the animals.

This strategy paper based on the presentations and discussions in a workshop organised by the Academy on “Rumen Microbiome and Amelioration of Methane Production” gives an overview of current status of research in the area of Rumen Microbiome and mitigation of methane production and future course of action to achieve the mission for effective utilization of lignified plants and production of animal protein with lesser effect on climate. Academy thanks all the eminent scientists and experts for their participation and in-depth interaction and deliberations. I especially compliment Convener Professor D.N. Kamra, and Co-convener Dr Raghavendra Bhatta, Director, NIANP for the initiative to organize the strategy consultation. The editorial support extended by Dr V.K. Bhatia and Dr Kusumakar Sharma is thankfully acknowledged.



(**Panjab Singh)**
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Rumen Microbiome and Amelioration of Methane Production

Introduction

More than 400 million tonnes of lignocellulosic crop residues are produced annually in India, which constitutes one of the major animal feed ingredients for milk, meat and wool production by the ruminants. These lignocellulosic by-products contain 60-70% gross energy primarily in the form of cellulose and hemicellulose, but due to the presence of anti-nutritional factors like lignin, tannins, silica etc. are only partially utilized by the ruminants. It is the rumen microbial ecosystem that enables the animal to extract more energy from such feeds. Therefore, the major focus is to enhance the extraction of dietary energy through rumen manipulation, and stimulation of microbes/enzymes accountable for fibre degradation. This might result in increasing livestock productivity with available feed resources and making livestock production more profitable in the country.

Further, the poor quality roughages fed to ruminants in India and other tropical countries, are responsible for higher enteric methane emission per unit of livestock production. Methane production largely depends upon nature of feed and its composition. Feeds such as straw and stovers produce maximum methane per unit of dry matter consumed; while concentrates produce comparatively lesser methane (Table 1).

Table 1: Methane production on different feeds

Feed	**g CH_4/kg DM uptake**
Hay	91.6
Straw	103.5
Pasture	82.6
Grass silage	86.9
Maize silage	82.2
Conc.	73.1

Methane emission by the ruminants is disadvantageous in two ways; firstly it reduces the feed conversion efficiency of the animals and secondly affects the environment adversely due to high global warming potential of methane. The present strategy paper aims at compiling available information on the rumen microbial diversity of fibre degrading

microbes (bacteria, fungi and ciliate protozoa) and methanogens and their correlation with fibre degradation and methanogenesis to ultimately workout an action plan for amelioration of methane production.

A complete in depth knowledge is a prerequisite to develop an effective technology for manipulation of any microenvironment. To fill the gap in knowledge on rumen microbes, some basic data has been generated on rumen microbiome in Indian domesticated (cattle, buffalo, sheep and goat) and some wild animals (nilgai, chinkara and gaur). The information generated is very superficial and still there is a need to bridge the gap in the knowledge on rumen microbes. However, the novel information on rumen microbiome will help in better understanding of the eco-system and formulating strategies for rumen microbial manipulation to improve livestock productivity. The precise measurement of greenhouse gases from livestock is absolutely necessary to explore the trend of emission, identifying hotspots of emissions and their potential contributors, devising effective mitigation strategies and evaluation of ameliorative measures.

Microbial Diversity (Conventional techniques)

The ruminants consume lignocellulosic feeds such as cereal straw and stovers, sugarcane based agricultural by-products and mature green fodders in India. The ruminants are not able to digest these feeds by themselves as none of the enzymes of animal origin have capability to degrade such fibrous feeds. Microbes such as bacteria, ciliate protozoa and fungi inhabiting in the gastro-intestinal tract help in digesting fibrous feeds and convert them into energy (volatile fatty acids) and nitrogen source (microbial protein). A representative biomass of various ruminal microbes is compiled in Table 2.

Table 2: Rumen microbial ecosystem

Microbe	Number/g rumen content	% of microbial mass
Bacteria	10^{10}-10^{11}	40-50
Protozoa	10^{4}-10^{6}	40-50
Archaea	10^{7}-10^{8}	2-3
Fungi	10^{3}-10^{5}	3-4
Bacteriophages	10^{8}-10^{9}	<0.1

These microbes in the rumen are present in billion, but only a small fraction (8-10% of total) is being cultured till now. The superficial knowledge on the specific substrate requirement, removal of metabolites, synergy with other microbes and precise culturing methodologies pose major hindrances in culture and characterization of ruminal microbes. There is a need

to determine their precise substrate requirement and culturing protocols to cultivate more number of microbes from rumen origin and broaden the list of cultured microbes. Unique features of ruminal microbes are presented below:

Bacteria

- Bacteria are mainly responsible for the synergistic effect on the production of volatile fatty acids and microbial proteins in the rumen.
- Majority of the bacteria are Gram-negative. The number of Gram-positive bacteria tend to increase on increasing high-energy diets in the ration.
- Most of the bacteria are obligate anaerobes and very sensitive to oxygen exposure that lead to death.
- Few of the rumen bacteria require a very low redox potential (indicating a high degree of anaerobiosis) and grow at a redox potential lower than –350 mV like methanogenic archaea.
- The optimum pH for the growth of rumen bacteria lies between 6.0 and 6.9 and temperature is 39ºC.
- The bacteria can tolerate a considerably higher level of organic acids without affecting adversely their metabolism.

The rumen bacteria present in the eco-system can be classified as lignocellulose degrading bacteria, hemicellulose, starch, protein, lipids, tannins, saponins, oxalate, nitrate and sulphate utilizing bacteria. By conventional cultivation techniques, the bacteria like *Fibrobacter succinogenes, Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens*, *Eubacterium*, *Clostridium* etc. have been identified as key fibre degrading bacteria in the rumen.

Protozoa

Ciliate protozoa play a vital role in the rumen fermentation. The protozoa perform dual function of contributing in feed fermentation and protecting easily fermentable carbohydrates (sugars and starch) from sugar/starch utilizing bacteria so that concentration of organic acids do not exceed the threshold. However, these sugars are released slowly to maintain a constant supply of energy for the animals in the form of short chain volatile fatty acids.

The number of ciliate protozoa in the rumen content of buffalo, cattle, sheep and goat varied between 11.35-28.13x10^4/ml, represented by 9, 12, 6 and 7 genera and 22, 38, 14 and 19 species, respectively (Baraka, 2012). However, these numbers might change as per chemical composition of diet, frequency of feeding, time of sampling, method of sampling and transportation of rumen liquor from animal sheds to the laboratory. The total number

of ciliate protozoa is lower in buffalo than that in cattle, but the ciliate protozoa represented in both the species included *Entodinium, Diplodinium, Eremoplastron, Eudiplodinium, Elytroplastron, Metadinium, Ostracodinium, Epidinium, Dasytricha* and *Isotricha*. The ciliate protozoa like *Polyplastron multivesculatum*, *Diplodinium, Epidinium caudatum* and *E. bicaudatum* are cellulose degraders. Eight genera of ciliate protozoa have been reported in the rumen of cattle and buffalo fed on wheat straw and concentrate mixture, e.g. *Isotricha, Dasytricha, Metadinium, Diplodinium, Eudiplodinium, Ophryoscolex, Entodinium*, and *Epidinium. Oscillospira guillermondii* while 22 genera of ciliates have been reported in buffalo rumen content.

The ciliate protozoa are affected by the diet composition and type of animals being studied. The protozoa, total holotrichs, dasytricha, total spirotrichs and small spirotrichs were significantly higher ($P < 0{\cdot}01$) on berseem feeding than those on oat feeding, while the numbers of *Isotricha* and large *spirotrichs* were unaffected by change of diet.

Fungi

Rumen fungi are efficient fibre degraders. Along with enzymatic degradation of plant tissue, they also act as biological cutters and provide more surface area for fibre degrading bacteria to attack on. The fibre degrading enzymes secreted by the rumen fungi are more active as compared to rumen bacteria. The number of fungi is quite low (10^2-10^4/ml) and reported to be very slow grower. Therefore; their contribution in fibre degradation is lesser than bacteria. So far only six genera of rumen anaerobic fungi have been identified namely; *Neocallimastix, Piromyces* (previously known as *Piromonas*), *Caecomyces* (previously known as *Sphaeromonas*), *Orpinomyces, Anaeromyces* (previously known as *Ruminomyces*) and *Cyllamyces*.

Piromyces sp. FNG5 (isolated from faeces of nilgai), FBB1 (*Anaeromyces* sp. isolated from faeces of blackbuck), FHD1 (*Piromyces* sp. isolated from faeces of hog deer), FS1 (*Orpinomyces* sp. isolated from rumen liquor of sheep) and FSD4 (*Piromyces* sp. isolated from the faeces of spotted deer) reported to be fibre degrading and tolerant to phenolic monomers. All the fungi reported so far are fibre degraders and make the substrate ready for degradation of these lignocellulosic feeds by other microbes.

Archaea (Methanogens)

The archaea are the most important group of microbes that act as major hydrogen sink in the rumen by reducing carbon dioxide into methane. This conversion helps in maintaining partial pressure of hydrogen in the rumen. The methanogens' population in the rumen ranges from 10^6-10^8/ml contributing to 2-3% of total biomass and represented by eight genera. Unfortunately, very limited number of methanogens has been cultured so far,

which could be explained by lack of information about their precise substrate requirement and culturing methodologies. Therefore, at present the cultivable methanogens are very few in numbers and restricted to *Methanobrevibacter ruminantium, Methanobacterium formicicum, Methanosarcina barkeri* and *Methanomicrobium mobile*.

Association of methanogens with anaerobic fungi has been well established. The rumen fungus was collected from the goat rumen and sub-cultured to obtain uniform colonies. The methanogens associated with fungal cultures belonged to novel RCC cluster and identified as *Candidatus* and *Methanomethylophilus alvus*. This small group of methanogens is distantly related to Thermoplasmatales, hence, a new order Methanoplasmatales was proposed. Poulsen *et al*., (2013) observed a decrease in methylotrophic methanogens population by the supplementation of rapeseed oil in lactating cows. These methylotrophic methanogens degrade methylamines. Thermoplasmatales are also reported in other microenvironments such as gastrointestinal tract of termites and mammals, soil and marine habitats. These methanogens can survive with rumen fungi and strictly utilize hydrogen to reduce methanol and methylamines into methane. The RCC cluster comprised of uncultured rumen archaea, which constitute more than 90% archaea in the rumen (Janssen and Kirs, 2008). Therefore, more work is to be done to explore the information on archael community composition and their characterization.

Metagenomics

Under Indian conditions, unraveling the mechanism of fibre degradation and methanogenesis are important. Metagenomic studies have been confined to mainly these two pathways. The study of genetic material of environmental samples is known as eco-genomics or community genomics. This work revealed that the vast majority of microbial diversity had been missed by cultivation-based or nucleic acid based procedures. Metagenomics allow microbial ecology to be investigated at a greater depth. The metagenomic studies can be conducted using high-throughput sequencing (454 pyrosequening 400 bp reads), Ion Torrent Personal Genome (400 bp reads), Illumina Mi sec. and Hi. sec. (400-700 bp reads).

The microbiome of rumen studied in detail by rRNA sequencing, consisted of several thousand microbes that belong to three different domains like Bacteria, Archaea, and Eukaryota (fungi and protozoa). Bacteria are the most diverse domain and constitute about 95% of total microbes. *Prevotella* is the predominant bacteria representing about 30 per cent of rumen bacteria effective for cellulose degradation. The known important key fibrolytic bacteria represented only ~2% of rumen bacterial 16S rRNA. Fourteen Holstein cows of similar age, had the most abundant phyla in decreasing order as Bacteroidetes (49.42%), Firmicutes (39.32%), Proteobacteria (5.67%) and Tenericutes

(2.17%) and the most abundant genera included Prevotella (40.15%), Butyrivibrio (2.38%), Ruminococcus (2.35%), Coprococcus (2.29%), and Succiniclasticum (2.28%) (Jewell *et al*., 2015). In Murrah buffaloes, *Prevotella* was the predominant bacterium representing 30 per cent of the total rumen bacteria. A metagenomic study revealed that key fibre degrading bacteria; *Ruminococcus* and *Fibrobacter* represent only 2-3 per cent of the total bacterial community in buffaloes (Kala *et al.,* 2017). Lim *et al*., (2013) also reported the abundance of key fibre degrading bacteria less than 10% of the total population. Presence of polysaccharide utilizing loci (PUL), a system of lignocellulosic feed utilization other than extracellular and cell bound cellulosomes, has been reported in *Bacteroides* which also exhibit celluloytic activity. The findings strengthen the hypothesis that there are other microbes too that contribute significantly to the fibre degradation. The population density of *Bacteroides* has been reported as the second most abundant genus after *Prevotella.* No change in abundance of the major phyla in the metagenomic libraries of rumen microbiome of Surti buffaloes fed on four different diets was observed (Singh *et al.,* 2011). The analysis of metagenomic libraries from the rumen microbiome of goat fed on different diets revealed that the population size of some fibre, protein and fat digesting bacteria change according to the diet, while others remain constant (Liu *et al*., 2017).

The microbial and enzyme profile are difficult to explore in its entirety because of the limitations of conventional techniques of cultivation of rumen microbes, but invention of molecular techniques like real time PCR and next generation sequencing have made the job a bit easier. Recent progress in metatranscriptomic genomic studies has discovered the richness of genetic resources and enzyme pool (Carbohydrate-Active enzymes Database, CAZy) in the rumen that had not been previously even imagined. Although large number of fibrolytic genes and gene clusters have been identified from the rumen microbiome, but still it is not known that how expression of these genes are regulated in an efficient manner. Like glycoside hydrolase 48 (GH48), supposed to be one of the major proteins for plant cell wall polysaccharide (PCWPs) degradation, is poorly represented in many of the rumen microbiome. Therefore, it is difficult to explain how PCWPs degradation is taking place in the rumen. The alternate possibility might be that there are some additional GH proteins closely associated with cellulosome for PCWPs degradation (Dai *et al.,* 2015). The number of GH families, except GH48, have been reported in the metatranscriptome libraries of buffaloes, fed three different diets varying in total digestible nutrients (Kala *et al.,* 2017).

Archaea

Henderson *et al.,* (2015) studied the rumen archaeal diversity in the samples collected from 35 countries and explored the impact of diet, species and geography on methanogens

diversity. Intuitively, archaea should be the microbial group most closely correlated with methane emissions, and controlling prominent methanogens species would help in reducing methane emission effectively. However, some studies have shown no such correlation with their overall abundance while in others the correlation has been weak. By meta-transgenomics, 18 genera (instead of eight by conventional techniques) of methanogens/ hydrogen utilizers have been reported i.e. *Methanobrevibacter, Methanothermobacter, Methanoplanus, Sulfolobus, Methanosarcina, Methanospirillum, Pyrococcus, Methanoculleus, Aciduliprofundum, Methanoregula, Methanosphaera, Methanosphaerula, Methanococcoides, Methanocaldococcus, Methanocorpusculum, Thermoplasma, Methanococcus, Methanobacterium etc.* in buffalo rumen (Kala *et al.,* 2017).

The metagenomic studies revealed that variation in methane production among breeds was not due to total number of methanogens. Although methanogenic archaea have been found to be solely responsible for methane emission in the rumen by reduction of carbon dioxide with hydrogen, but there is very poor correlation of the number of methanogens and methane emission from individual animals (Kamra *et al*., 2017). Comparative study of Indian (Gir and Kankrej) and exotic (Holstein and Jersey) cattle revealed that the enzymes associated with methanogenesis were contributed by Methanobacteriales in exotic cattle, whereas, in Indian cattle the major source was Methanomicrobiales. The abundance of the individual methanogen groups and the genes involved in conversion of formate to methane and acetate to methane also varied in the two groups (Parmar *et. al.,* 2017). It has been observed that archaeal genes, which were directly or indirectly involved in production of methane, were 2.7 fold higher in high methane emitters as compared to low emitters and abundance of these archaeal genes was influenced by host physiology. Formate to methane producing enzymes along with shared enzymes contributed by Methanobacteriales were more in Australian Holstein cattle and Jersey cattle as compared to Indian Gir and Kankrej cattle. Whereas, the formate to methane producing enzymes along with shared enzymes contributed by Methanomicrobiales were more in Indian origin cattle breeds as compared to Holstein and Jersey breeds. Acetate to methane producing enzymes contributed by Methanosarcinales group of organisms showed more representation in Indian cattle and Jersey cattle as compared to Holstein cattle. The study by Parmar *et al.,* (2015) revealed that the M50GL group harboured more Proteobacteria than the M100GL group, which harboured more Bacteroidetes. The classes of Proteobacteria (methanotrophs) differed significantly in response to the change in diet. α-Proteobacteria and β-proteobacteria were found to be significantly ($p<0.05$) higher in the M100GL group, whereas γ-proteobacteria were significantly more abundant in the M50GL group than in the M100GL group. Different species of methanogens were more abundant in the M100GL group than in the M50GL group.

Enteric Methane Emission by Indian Livestock

The enteric methane emission from Indian livestock has been worked out, but these estimates are quite variable and suggest annual emission in the range of 7.25-18.4 Tg (Bhatta *et al.,* 2016). The large disparity in quantifying the enteric methane emission from Indian livestock might be due to lack of suitable and validated methodologies. According to Department of Animal Husbandry, Dairying and Fisheries (DAHDF), Ministry of Agriculture, Govt. of India, the livestock are emitting 13.27 Tg enteric methane per year. DAHDF data further revealed that cattle contribute 6.73 Tg to the total enteric methane emission in the country; while the contribution of buffalo is 6.56 Tg. Cattle and buffalo collectively emit 91.3% of total methane emission in the country; while rest 8.7% come from other species such as goat, sheep, yak, mithun, horse, donkey, mules, pig etc. (Kamra *et al.,* 2011). However, National Institute of Animal Nutrition and Physiology (NIANP), Bengaluru estimate revealed less annual methane emission from cattle (4.92) and buffalo (2.91). The NIANP has developed state wise enteric methane inventory (Fig. 1).

The factors such as livestock population (19th census), sex, different categories, physiological stage, seasonal variation in feed resource availabilities and feeding practices are taken into consideration for the development of methane inventory. The NIANP database relies upon the primary data on methane production potential rather using IPCC tier system to quantify the emission using single equation for the whole country. Based on methane production potential, the feeds have been categorized and presented in Table 3.

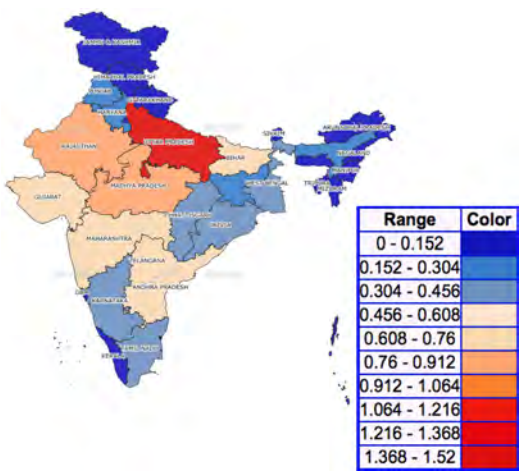

Fig. 1: Methane emission (Tg) by ruminants in different states of India

Bhatta *et al.,* (2016)

Table 3: Categorization of feed based on their methane production potential

Category of feed	MPP (CH_4 ml/100 mg truly digested substrate)	Energy loss as methane (KJ per kg digested substrate)
Tree leaves	1.34	0.53
Cereal grains	2.44	0.96
De-oiled cake	2.47	0.98
Cultivated fodder	2.83	1.12
Compound feed (KMF)	4.56	1.80
Local grass (uncultivated)	4.67	1.85
Cereal by-products	5.92	2.34
Dry straw	6.01	2.38

Bhatta *et al*., 2015

Among the states, Uttar Pradesh contributes the highest (1.52 Tg) to the total enteric methane emission in the country. Other states that are potential contributors to the total enteric methane emission include Rajasthan (8.75%), Madhya Pradesh (8.54%), undivided Andhra Pradesh (7.87%), Maharshtra (7.57%), Gujarat (7.35%), Bihar (6.92%) and West Bengal (4.88%). NIANP, Bengaluru also worked out the contribution of livestock from different zone to enteric methane emission (Bhatta *et al*., 2016). It was revealed that the livestock from North Indian states contribute maximum (25.2%), followed by Western states (23.7%), Eastern states (19.0%) and Southern states (16.2%); whilst the contribution from Northeast region is only 3.58%.

Methane Amelioration

India is determined to reduce greenhouse gas emission from the livestock within the resources it has at its disposal. The country has the maximum number of ruminants fed on poor quality lignocellulosic feeds. Exhaustive work has been done to mitigate/ameliorate methane production by using different feeds, chemical, herbal and microbial feed additives, plant secondary metabolites and ration balancing. The plant secondary metabolites appear to be the most preferred source of feed additives which are natural occurring compounds with the least adverse effect on the animal performance and are also socially acceptable. The alternatively inorganic terminal electron acceptors like sulphate, formate, nitrate etc. have also being explored as feed additives. A combination of above feed additives might have a synergistic reducing effect on methane emission (upto an extent of 25-30% inhibition) and therefore can be used successfully for the control of methane emission.

Different chemical and microbiological techniques have been standardized for reducing methane emission, but majority of them are linked with depression in feed utilization.

Therefore, there are limitations for their use in practical livestock production. Basic work on plants containing secondary metabolites has been done in different laboratories of the world and their compiled information (as done in the present strategy paper) can lead to development of herbal feed additives which can be used for improving livestock production and protecting climate by lower production of green house gases.

Inhibition of Methane Production

More than 100 plants/plant parts and their extracts have been screened for their anti-methanogenic activity and effect on feed degradability at IVRI, Izatnagar. The extracts of some plant parts with different solvents were found effective in inhibiting methanogenesis (Table 4a).

Table 4a. Effect of plant extracts on inhibition of *in vitro* methanogenesis

Plant	Common name	Plant part	*In vitro* inhibition of methanogenesis (%)		
			Ethanol extract	Methanol extract	Water extract
Acacia concinna	Shikakai	Seed pulp	5.32	17.60	19.61
Allium cepa	Onion	Bulb	8.76	16.38	32.64
Allium sativum	Garlic	Bulb	61.31	69.73	19.88
Azadirachta indica	Neem	Seed cake	34.59	21.89	–14.80
Canabis indica	Bhang	Leaves	34.42	30.67	3.33
Citrus limonum	Lemon	Peel extract	12.90	8.67	11.84
Emblica officinalis	Amla	Seed pulp	19.51	27.68	–26.69
Eugenia jambolana	Jamun	Leaves	5.61	24.27	5.66
Foeniculum vulgare	Fennel	Seed	39.42	70.72	–14.54
Mangifera indica	Mango	Leaves	23.17	35.67	9.15
Populus deltoides	Poplar	Leaves	8.49	85.86	–7.72
Psidium guajava	Guava	Leaves	81.79	9.29	9.44
Sapindus mukorossi	Soapnut	Seed pulp	95.80	20.18	39.40
Syzygium aromaticum	Clove	Flower bud	46.96	85.61	2.37
Terminalia belerica	Baheda	Seed pulp	5.54	28.11	13.18
Terminalia chebula	Harad	Seed pulp	58.54	99.79	6.43
Trachyspermum ammi	Ajwain	Seed	42.28	–2.68	–11.35

Patra *et al.,* (2006), Kamra *et al., (2006,* 2008), Kreuzer *et al*., (2009), Inamdar *et al*., (2015)

Similarly, NIANP also screened more than 300 phyto-sources for their anti-methanogenic activity. The promising sources that lead to significant reduction in methane production are compiled in Table 4b.

Table 4b: Phyto-leaves showing moderate to high anti-methanogenic activities

Common name	Botanical name	Methane reduction (%)
Palmorosa grass	*Cymbopogon martini*	12.07
Fennel leaves	*Foeniculum vulgare*	10.07
Origanum leaves	*Origanum vulgare*	22.92
Citronella grass	*Cymbopogon winterianus*	18.05
Curry leaves	*Murraya koenigii*	24.41
Adathoda leaves	*Adhatoda zeylanica*	10.32
Worm wood leaves	*Artemisia absinthum*	14.31
Sweet worm wood	*Artemisa annua*	13.13
Indian privet	*Vitex negundo*	27.37
Sikakai (Seege) leaves	*Acacia sinuate*	18.10
Neem leaves	*Azardirachta indica*	17.87
Bergamont mint leaves	*Mentha citrata*	49.46
Mehandi leaves	*Lawsonia inermis*	47.45
Rosemary leaves	*Rosmarinus officinalis*	42.24
Cinnamon leaves	*Cinnamomum verum*	33.45
Nutmeg fruit	*Myristica frograns*	30.62
Jatropha leaves	*Jatropha curcus*	32.0
Jack leaves	*Autocarpus integrifolis*	31.5
Agasse leaves	*Sesbania grandiflora*	25.0
Banyan leavs	*Ficus bengalensis*	36.0
Neeligida leaves	*Indigofera tinctoria*	32.94
Alfalfa fodder	*Medicago sativa*	30.41
Selastras paniculatus	*Selastras paniculatus*	30.34
Indian birthwort leaves	*Aristolochia indica*	30.70
Chebulic myrobalan	*Terminalia chebula*	37.94
Prime rose leaves	*Oenothera lamarckiana*	47.3

Bhatta *et al.,* (2012, 2015, 2016)

Results from *in vitro* studies unequivocally established that tree leaves such as *Autocarpus integrifolis (Jack leaves), Jatropha curcus* (Jatropha leaves), *Azardirachta indica* (Neem leaves) and *Sesbania grandiflora* (Agasse leaves), *Ficus bengalensis* (Banyan leaves) that contain appreciable amount of tannins can be used in diet to suppress rumen methanogenesis by 25-30 per cent. Pal *et al.,* (2015) and Baruah *et al.,* (2018) have screened about fifty tree leaves in *in vitro* system, out of which few of them exhibited very promising results. Feeding of *Artocarpus heterophyllus* (kathal) leaves to goats resulted in 15.4% reduction in methane production (ml/kg DDMI) (Gangwar, 2015). Essential oils

are also well documented for their anti-methanogenic activity (Pawar *et al.,* 2014, Josch *et al.,* 2016). The feeding trials using some additives have shown promising *in vivo* results in terms of methane inhibition (Table 5), which can be explored further to get a transferable technology.

Table 5. Effect of plant extracts on inhibition of *in vivo* methane production (l/kg DDMI)

Plant	Inhibition (%)	Body weight gain (%)	Animal	References
Anti-methane	32	-	Buffalo	Kamra *et al.*, 2011
Methane-Suppressor	23	16	Buffalo	Kamra *et al.*, 2012
BEO (Blend of essential oils)	14.6	7.4	Buffalo	Yatoo *et al*., 2018
Terminella chebula	24.6	-	Sheep	Patra *et al*., 2011
Ajwain oil and lemon grass oil in 1 : 1 ratio	16.7	No effect	Buffalo	Samal *et al*., 2016
Garlic and soapnut in 2 : 1 ratio	12.9	No effect	Buffalo	
Garlic, soapnut, harad andajwain in 2 : 1 : 1 : 1 ratio	8.4	No effect	Buffalo	
Ficus benghalensis leaves	21.8	-	Sheep	Malik *et al.,* 2017
Artocarpus heterophyllus leaves	20.6	-	Sheep	
Azadirachta indica leaves	24.07	-	Sheep	

Malik *et al*., (2019) screened sixteen leaves from Uttarakhand for their anti-methanogenic properties and reported that kilmoda (*Berberis Lycium)*, satavar (*Asparagus racemosus)*, akrot (*Juglans regia)* and timoor (*Zanthoxylum alatum)* has tremendous potential to reduce methane emission. For selection of low methane emitting feed ingredients, *in vitro* screening of various oil cakes, chunnies, forages, straws, shrubs etc. was performed to compare the methane production ability of feed ingredients in different Indian loborateries. However, there are very few *in vivo* studies conducted so far. From a recent *in vivo* study in sheep, Baruah *et al*., (2019) concluded that tanniferous leaves *Syzygium cumini* and *Machilus bombycina* inclusion in diet at 10% level decreased enteric methane emission by 15-18%. Tamarind seed husk, an agricultural waste from starch industry was found very effective in reducing *in vitro* and *in vivo* enteric methane emission by 17-20% when incorporated at 5% level in complete diet (Malik *et al*., 2017).

There was a significant reduction in *in vitro* methane production associated with decreased protozoa population by inclusion of extracts of soapnut (Agarwal *et al*., 2006). A significant reduction in methane emission with saponins supplementation was also reported by others both *in vitro* (Malik and Singhal, 2008) and *in vivo* (Malik *et al*., 2009). After compiling the data of a series of experiments, it was found that methane inhibition is not essentially

associated with reduction in methanogens. However, recently Poornachandra *et al*., (2019) did not find any effect of individual supplementation of soapnut on methane emission in cattle. They reported that combined supplementation of tamarind seed husk and soapnut in 60:40 at 5.1% level of diet was effective in achieving 17% reduction in methane emission.

Forages containing condensed tannins have been shown to decrease methane production by ruminants. Phyto-sources such as *Bergenia crassifolia, Emblica officinalis, Peltiphyllum peltatum, Populus deltoides, Quercus incana, Rheum undulatum, Terminalia belerica, Terminalia chebula* and *Vaccinium vitis-idaea* contain high tannins content and have potential to inhibit methane emission (Bhatta *et al*., 2011, 2013). *Allium sativum, Coriandrum sativum, Eucalyptus globulus, Foeniculum vulgare, Mentha piperita, Ocimum sanctum, Populus deltoides* and *Syzygium aromaticum* are some of the plants which contain high concentration of essential oils and are effective against methane emission and protozoa growth in the rumen, but some of them also have adverse effects on degradability of feed and nutrient utilization by the animals (Kamra *et al*., 2008, 2009; Pawar *et al.,* 2014, Yatoo *et al*., 2017). There was a linear decrease (P<0.05) in *in vitro* methane emission (ml/g DDM) with increasing levels of peppermint oil. The methanogenesis was inhibited to the extent of 19.9, 46.0 and 75.6% at a concentration of 0.33, 1.0 and 2.0 ìl peppermint oil per ml of reaction mixture, respectively.

It is well documented that in spite of initial reduction in methane emission with mitigating agents, the animals after sometime may get back to their previous level of emission. This is due to the adaptation of rumen methanogens to mitigating agents during long-term supplementation. However, no systematic and long-term studies have been conducted to prove this aspect. Recently, NIANP has conducted six month long studies in sheep and cattle with silkworm pupae oil and compared the emission in short and long-term. From these studies, it was concluded that silkworm pupae oil at 2% level was quite effective in reducing methane emission by 12-15% in both short and long-term.

Based on the decade long exhaustive *in vitro* and *in vivo* studies, NIANP has developed two farmers' friendly anti-methanogenic products namely *Harit Dhara* and *Tamarin Plus*. These products can reduce methane emission by 15-20%. The products are inexpensive and require minimum inputs for the formulation.

Relation between Hydrogen Producers and Methanogens

Yak is a lower methane producer than cattle, in spite of the fact that both the animals are fed similar diets and there are only small variations between the microbiomes of both the animals. The methane and hydrogen yields in yak *vs* cattle are 0.26 *vs* 0.33 mmol methane/g dry matter intake and 0.28 *vs* 0.86 mmol/d hydrogen generation. Hydrogen recovery from cattle was significantly higher than that from yak (Mi *et al*., 2017). The relative abundance of

methanogens was not different between the two animal species. It was hypothesized that more H_2 production is the reason for the higher methane emission in cattle as compared to yak. Kittlemann *et al.,* (2013) was of the view that abundance of fibrolytic bacteria (major hydrogen producers) is related with the methanogen communities and consequently with methane production. Therefore, abundance of methanogens do not have direct correlation with methane production, but the partial pressure of hydrogen is more important. Minimizing metabolic H_2 production in the rumen might reduce the availability of H_2 to methanogens. Suppression in ruminal H_2 producers is usually accompanied with concurrent decrease in feed fermentation. This can be achieved by the intensification of propiogenesis, rumen biohydrogenation or promoting reductive acetogenesis in the rumen. Targeting H_2 utilizing protozoa or other microbes accountable for interspecies H_2 transfer to the methanogen can be a fruitful strategy to reduce methane emissions.

Tammar wallaby (*Macropus eugenii*) harbors unique gut bacteria and produces 20% of the amount of methane produced by ruminants per unit of digestible energy intake. Pope *et al*., (2011) isolated a dominant bacterial species (WG-1) from wallaby, which was affiliated to the family Succinivibrionaceae and implicated the lower methane emission from starch-containing diets. Pure-culture studies confirm that the bacterium is capnophilic and produces succinate, further explaining a microbiological basis for lower methane emission from macropodids. The abundance of WG-1 is variable in samples collected from animals in winter and spring; their results show that these bacteria will be numerically dominant when the plane of nutrition is rich in starch and soluble sugars.

Hydrogen produced during fermentation of feed is responsible for methane production in the rumen; the minimized metabolic H_2 production during enteric fermentation and diversion of H_2 away from the methanogenesis might result into useful energy rich metabolites. The most important process of methane reduction may be to reduce number of unproductive animals. However, this approach is neither ethical nor possible in India where the slaughter of cattle is not permissible in most of the states of the country. Minimizing metabolic H_2 production in the rumen might reduce the availability of H_2 to methanogens. Suppression in ruminal H_2 producers is usually accompanied with concurrent decrease in feed fermentation and diversion of metabolic H_2 away from the methanogenesis. This can be achieved by the intensification of propiogenesis, rumen biohydrogenation or promoting reductive acetogenesis in the rumen. Targeting H_2 utilizing protozoa or other microbes accountable for interspecies H_2 transfer to the methanogen, which could be a good alternate for eradicating enteric methane emission. However, significant reduction in rumen ciliates might lead to reduce fibre degradation. Another important way to tackle the emission of methane may be to directly target rumen archaea through various approaches. By doing so, the enteric methane emission will decrease and additional H_2 will also be adequate to stimulate alternate hydrogenotrophic pathways i.e reductive acetogenesis.

Future Strategies

- There is a need for an expanded research agenda to make economic exploitation of enormous diversity of rumen microbes for improvement in health and productivity of animals and to bridge the gap between the meta-genomic data generated for various livestock species and its functional application for better feed conversion by the animals.
- Research on rumen microbiome and mitigation of methane production must contribute to the mission for effective utilization of lignified plants and production of animal protein with lesser effect on climate.
- Long term feeding trials using large number of animals with established mitigating agents/products should be conducted. It should be ensured that the rumen modifiers should not adversely affect animal productivity and cause any toxicity to consumers.
- There is a need to test and validate the developed technologies at farmers' doorstep so that the frontline demonstration help in adoption of technologies in the field.
- Studies on detailed rumen microbiome diversity and dynamics of indigenous animals using various latest molecular tools may be undertaken to understand the microenvironment of the rumen.
- Rumen microbiome does not work in isolation and largely dependent on feed resources, climate and genetic make- up of the animal. Therefore, a standard operating procedure needs to be developed to conduct experiments and compare database.
- The methane production figures/data from different sources need to be harmonized to respective population size so that a correct value is uniformly arrived at each time.
- Considering the regional and seasonal availability of feed resources including anti-methanogenic agents, there is a need to develop region and season specific methane mitigating strategies.
- Feeding balanced ration to animals is helpful in reducing the production of methane in the rumen and also helps in optimising the feed utilisation and productivity of milk, meat and wool. More efforts need to be directed to bridge the gap in supply and demand of desired animal feeds to the farmers.
- Studies on environmental changes are being encouraged by a large number of international funding agencies. India should tap such resources by taking up collaborative projects with the international community. With highest numbers of livestock in the country, our efforts should not remain restricted to isolation. India should join global agencies and teams like Hungate 1000, Rumen Microbial Genomics Network and Global Rumen Census, and others.

Actionable Points

- To address the issue of methane mitigation, the laboratories working on the area should be strengthened financially and technically. Facilities for estimation of methane emission should be created in various laboratories to maintain methane inventory at different locations.
- To broaden the list of cultured microbes, culturing of ruminal microbes including methanogens should be one of the thrust areas of research.
- The work on rumen microbiome of domesticated and wild ruminants should be carried out in multi locational project (represented by the experts of nutrition, microbiology, biochemistry, biotechnology, bioinformatics, statisticians etc.) to understand the mechanism of feed degradation and methane production.
- *In vivo* methane production trials should be carried out in different age groups, on different feeds and fodders in large number of animals for a longer duration to quantify methane emission in the country and result in economic livestock production. It needs to be confirmed whether methane production is related to net energy or metabolizable energy of feed.
- Funds from international funding agencies may be tapped by taking up collaborative projects with the international community.
- To study the complexity of the rumen microbiome, metagenomics and bioinformatics are the much-needed tools therefore, should be the integral part of the curriculum of graduate and postgraduate students of animal nutrition.
- Methane production should be used as a parameter for breeding of ruminants. If the genetics of the host animal has a significant role in determining the key activities of the microbiota, then breeding would be a cost effective tool to reduce methane emissions and improve the feed efficiency.
- Low methane producing feeds and selected feed additives should be recommended to farmers for improved livestock productivity.
- Tree leaves, herbal extracts, tannins and saponins etc. have been tried and found promising in reducing the methane generation by 20-30%. The *in vivo* efficacy of such agents needs to be verified through feeding trials on large scale and *in vivo* measurement of gas production. The availability and cost of such agents are very important for making the technology sustainable under field conditions. A patented product named Harit-Dhara has been released by NAINP to ameliorate methane production. The efficacy and sustainability of such product(s) needs to be studied through multi-locational trials at organised farms.
- One World - One Health Concept: A broader understanding of health and disease demands a unity of approach achievable only through a consilience of Plants-Animals-

Human beings-Environment-Wildlife health, their food supplies and economies, and the biodiversity essential to maintaining the healthy environments and functioning ecosystems we all require. There is a need to devise adaptive, forward-looking and multidisciplinary solutions to the challenges that undoubtedly lie ahead.

- Actionable points may be carried out at ICAR-IVRI, Izatnagar, ICAR-NIANP, Bengaluru, Anand Agriculture University, Anand, ICAR-NDRI, Karnal, GADVASU, Ludhiana in collaborative mode.

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