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A complete review on: Methanogens methane producers of rumen and abatement strategies- biotechnology and microbiological strategies review

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Abstract

Emerging research focuses on ruminant methanogens due to their emission of methane globally. Methane emission with annual global contribution of 14.5 per cent. Livestock is one of the most important sources of greenhouse gases from agriculture, contributing more than 25% of global greenhouse gases emission. The trend and legal obligation of mitigating greenhouse gas emissions will likely to directly influence improved efficiency of livestock systems, including animal nutrition, productivity, handling and management. The development of mitigation strategies and the viability of their practical applications have been researched around the world. In the current review, Microbiology and biotechnological strategies such as emphasizing on animal breeding, genetic merit, unproductive animals, vaccination, immunisation and biological control, chemical defaunation that can be leads to decreasing methane production from ruminant and thereby improving animal production are discussed.

Keywords: Carcass characteristics, weaner pigs, meat quality, palm kernel cake

1. Introduction

Climate change is a subject of global environmental concern. Globally, the Livestock farming is one of the most prominent anthropogenic sources of greenhouse gases. The total global greenhouse gases emission from livestock is 7.1 gigatonnes CO₂e year⁻¹, which accounts for 14.5% of all anthropogenic emissions (IPCC, 2014) [29]. India has the world's largest livestock population at 500 million heads, and this emits huge amounts of methane, a greenhouse gas responsible for global warming. Methane is a more potent greenhouse gas, having 28 folds greater global warming potential than carbon dioxide (Sirohi *et al.*, 2013, IPCC 2013) [29, 55]. The estimation of CH₄ emission from four different livestock categories, cattle, buffalo, goat, and sheep, in India are evaluated at districts, state, and national level. Ruminant animals (particularly cattle, buffalo, sheep, goat and camels) produce significant amounts of CH₄ under the anaerobic conditions of the digestive processes (Sejian *et al.*, 2011) [54]. About 81 to 92 MT methane produce per annum globally (IPCC, 2007; Patra *et al.*, 2012) [28, 46]. India has livestock wealth of 192.49 million cattle, 109.85 million buffaloes, 74.26 million sheep and 148.8 million goats which produce large amounts of CH₄ (GOI, livestock census 2019) [20]. Ruminants typically lose 2-12% of their gross energy intake as emissions of methane (Kamra *et al.*, 2008) [32]. Increased anthropogenic Greenhouse Gas (GHG) emissions have increased the global temperature the last 100 to 200 years (Mirzaei-Aghsaghali and Maheri-Sis 2011) [45]. Methane production in ruminants has received global attention in relation to its contribution to the greenhouse gas effect and global warming. Much research has been directed toward methane abatement strategies in ruminants. Mitigation strategies are often limited by the diet fed, the management conditions, physiological state and use of the animal. To this end, the aim of this paper we review to provide background on methane and methanogens, as well as alternatives to reduce CH₄ emissions from ruminants through Microbiology and biotechnology strategies.

2. Methane and Methanogen

Methanogens are the only group of microorganisms on earth producing significant amounts of methane. Cattle produce methane from enteric fermentation (85 to 90%) and fecal excretion. A total of 95% of rumen methane is excreted via eructation through mouth and from the

intestines, and around 5% excreted via the anus (Murray *et al.*, 1976) [46]. Methane from enteric fermentation represents 25% of anthropogenic methane emissions (Wuebbles and Hayhoe, 2002) [68]. Enteric methane, responsible for 15% of global warming, is directly related with rumen fermentation efficiency because of the loss of carbon and consequent loss of energy, which affects animal performance (Bell *et al.*, 2011) [6]. Methanogens belong to the domain Archaea and the phylum Euryarchaeota. Unlike Bacteria, methanogens lack peptidoglycan in the cell wall, replaced by pseudomurein in *Methanobrevibacter* and *Methanobacterium*, heteropolysaccharide in *Methanosarcina*, and protein in *Methanomicrobium* (Balch *et al.*, 1979) [4]. Methanogens are usually either coccoid (spherical) or bacilli (rod shaped) and required redox potential -250Mv to -450Mv. Methanogens are having 31 genera and 113 species (Liu and Whitman, 2008) [36] out of that seven species are culturable from the rumen (Janssen and Kirs, 2008) [30]. Between the years 2008 and 2012 another two orders of methanogens, namely Methanocellales (Sakai *et al.*, 2008) [52] and Methanomassiliicoccales (Dridi *et al.*, 2012; Iino *et al.*, 2013) [15, 27], were added to the phylum Euryarchaeota. Methanogens are a relative diverse group of archaea and can be found in various anoxic habitats (Garcia *et al.*, 2000) [18]. For example, they can be cultured from extreme environments such as hydrothermal vents or saline lakes, paddy fields. Extensive recent metagenomic analyses suggested that methanogens may no longer restrict to the Euryarchaeota. Two new phyla, namely the Bathyarchaeota (Evans *et al.*, 2015) [17] and the

Verstraetearchaeota (Vanwonterghem *et al.* 2016) [61] were postulated. Genome sequences from both phyla indicate a methylotrophic methane metabolism in these as of yet uncultivated- potential methanogens. Methanogenesis or biomethanation is the formation of methane by methanogens. Methanogenesis is in the form of anaerobic respiration. The terminal electron acceptor in methanogenesis is not oxygen, but carbon. Various substrates are used for methane formation such as acetate, formate, methanol etc. Methane formation from acetate, called acetoclastic methanogenesis, can be found only in the order Methanosarcinales. In contrast to that, methylotrophic methanogenesis, which is the methane formation from different methylated compounds such as methanol, methylamines or methylated thiols, is found in the orders Methanomassiliicoccales, Methanobacteriales and Methanosarcinales (Evans *et al.*, 2015) [17]. Hydrogenotrophic methanogenesis from H₂ and CO₂ is found in almost all methanogenic orders with the exception of the Methanomassiliicoccales. Due to its broad distribution it is postulated that this type of methanogenesis is the ancestral form of methane production (Baptiste *et al.*, 2005) [5].

Enteric methane responsible for 15% of global warming, is directly related with rumen fermentation efficiency because of the loss of carbon (Bell *et al.*, 2011) [6] and consequently leads to loss of gross energy 2-12% (Kamra, 2008) [32], which affects animal performance.

- CH₄ emission - Enteric fermentation is estimated to be 14.20 Tg/Yr. (livestock census, 2014) [20]

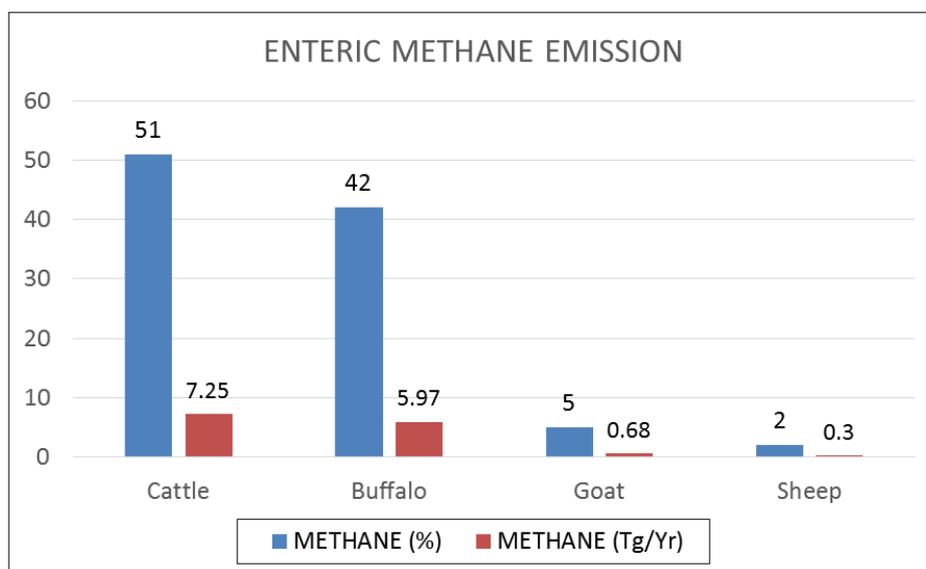


Fig 1: Development of mitigation strategies and their viability have been researched around the world (Thornton, 2010) [57].

3. Strategies Involved in Methane Reduction

Methane mitigation strategies based on the relationship that methanogens have with other microorganisms present in the rumen. Methane mitigation is effective in one of two ways: either a direct effect on the methanogens caused by the impact of the strategy on substrate availability for methanogenesis, or an indirect effect, usually through an effect on the other microbes of the rumen. Methane mitigation strategies solutions can be discussed in four topics including: Management strategies, Nutritional strategies and Biotechnology and Microbiological strategies. In this paper we review alternatives to reduce CH₄ emissions from ruminants through Microbiology and biotechnology strategies.

Review alternatives to reduce CH₄ emissions from ruminants through Microbiology and biotechnology strategies

3.1 Animal breeding

Animal breeding has focused on production traits, such as milk production and growth rate, since these are relatively easy to measure in the national herd or flock. One of the key challenges for the industry, and opportunities for bioscience, is the ability to incorporate a much wider range of traits into breeding indices. One study was existing on validations of the SF₆ technique and found SF₆ estimates were 93 to 95% of chamber-based measurements of methane emissions and showed that variation between days was attributable to animals (sheep or cattle fed forages) even though intakes and

composition of each diet were relatively constant (Ulyatt *et al.* 1999) [59]. Robertson *et al.* 2002 [49] studied that Overseas genotypes produced 8-11% less methane, as a percentage of gross energy (GE) intake, compared to New Zealand genotypes and total mixed rations (TMR) resulted in similar energy losses to methane % of GE as pasture. Genotype differences had disappeared by day 240. The result of this experiment demonstrates a persistent high or low methanogenesis for some animals, but not all cows fed pasture. A similar variation between individuals was evident for total mixed ration diets fed to cows (Robertson and Waghorn 2002) [50]. Sheep with high CH₄ yields had larger rumen volumes, a slower passage outflow rate, higher fiber digestibility and longer retention times than sheep with low CH₄ per kg of dry matter intake. Methane yield is best predicted as a function of particulate fractional outflow rate, organic matter intake (g/kg BW^{0.75}) and molar proportion of butyrate. A number of experiments have reported variation between animals in CH₄ emissions per unit of feed intake. Clark *et al.* 2005 [12] conducted a trial involving 302 grazing dairy cows showed that mean CH₄ emissions of 19.3 ± 2.9 g/kg DMI were reported the 15% variance suggesting heritable differences in methanogenesis. Eckard *et al.* 2010 [16] reported similar study were in sheep on an unlimited pasture diet they reported that breeding for reduced methanogenesis is unlikely to be compatible with other competing breeding objectives. In contrast, breeding for improved feed conversion efficiency (lower net feed intake) should be compatible with existing breeding objectives and likely to both reduce CH₄ and the ratio of CH₄ per unit of product produced. Hegarty *et al.* 2007 [23] conducted a study showed that residual feed intake explained only a small proportion of the observed variation in methane production. A genotype with nutrition interaction can be anticipated, and the methane production ratio with residual feed intake that is (estimated breeding value) relationship will need to be defined over a range of diet types. Waghorn *et al.* 2006 [63] suggested that animal breeding could achieve a 10 to 20% reduction in CH₄ losses from DM during digestion. Pickering *et al.*, 2015 [48] studied heritabilities of CH₄ production on an absolute emission basis (g CH₄/day) are moderate and estimated at 0.29 and 0.40 in sheep and cattle, respectively, but much lower at 0.13 and 0.19, respectively, on a yield basis (g CH₄/kg) dry matter intake. Lovendahl *et al.*, 2018 [37] observed that incorporating CH₄ production in a genetic selection program represents a major challenge because of the difficulty of measuring CH₄ in a manner that reflects the long-term CH₄ phenotype of the animal. Some animal breeding programs use a 'sniffer' technique to measure breath CH₄ concentration at a feeder or during milking (source-sampling distance, air turbulence, cow's head movement; Wu *et al.*, 2018) [67], it has been shown to be correlated (r = 0.75) to flux methods when used by skilled researchers (Difford *et al.*, 2019) [14]. Breider *et al.* (2019) [10] recently showed genetic correlations of 0.49 to 0.54 between CH₄ production and milk yield indicating that genetically selecting for lower CH₄ production may decrease productivity.

3.2 Genetic merits

Improving the genetic merit of dairy cows has escalated in the last decade with the import of Holstein genetic material on the native dairy breeds. As a result, average national yields have increased. Kirchgessner *et al.*, 1995 [50] suggested that increasing milk production of dairy cows from 5000 to 10000

liters milk annually would only increase methane production by 5% (i.e. from 110 to 135 kg methane per year).

The key microbiota Archea is a very small population and it emits large portion of methane in rumen. Molecular analysis provided that methyl coenzyme-M reductase gene is a genetic marker common for the Methanogenic population (Martino *et al.*, 2013) [70]. De Haas *et al.*, 2011 [69] analyzed the association between cumulate enteric methane emission and Genome wide Single Nucleotide Polymorphism. Though SNP effect could be identified, no large regions were significantly associated. The cows with lower residual feed intake have lower predicted methane emission grams/day. Hence, it is possible to reduce methane emission. Genetic variation suggests that 11 to 26% methane mitigation in 10 years could be more in a genetic selection program.

3.3 Unproductive animals

Reducing the number of unproductive animals on farm has potential to both improve profitability and reduce CH₄. Trapnell and Malcolm 2006 [58] studied Methane mitigation strategies like lengthening lactation period in dairying, where cows calve every 18 months rather than annually, reduce herd energy demand by 10.4% and thus potentially reduce CH₄ emissions by a similar amount. Through earlier finishing of beef cattle in feedlots, slaughter weights are achieved at a younger age, with reduced lifetime emissions per animal and thus proportionately less animals producing CH₄ (Eckard *et al.* 2010) [16].

3.4 Immunization/vaccination

Two vaccines were developed, named VF3 (based on three methanogen strains) and VF7 (based on seven methanogen strains), produced a 7.7% reduction/kg DM in methane emissions from sheep despite only one antigen being effective against the methanogenic species in the sheep. The vaccine was much more effective than the seven methanogen mix tested previously and was able to increase saliva and plasma antibody titres by four to nine folds over the seven methanogen mixture. Vaccination against rumen methanogens has the potential to reduce methane emissions from livestock, which is a major contributor to agricultural greenhouse gases (Wright *et al.*, 2004; Buddle *et al.*, 2010; Williams *et al.*, 2009) [11, 65, 66]. Williams *et al.* (2009) [65] studied methane output levels corrected for dry-matter intake for the control and treatment groups were not significantly different, and real-time PCR data also indicated that methanogen numbers were not significantly different for the two groups after the second vaccination. However, clone library data indicated that methanogen diversity was significantly greater in sheep receiving the antimethanogen vaccine and that the vaccine may have altered the composition of the methanogen population (Williams *et al.* 2009) [65]. Passive immunization was also assayed using antibodies prepared from hen's eggs. Antibodies decreased methane production *in vitro* but the effect was short-lived (Cook *et al.* 2008) [13]. An additional vaccine has recently been developed using sub cellular fractions of *Methanobrevibacter ruminantium* M1. Twenty sheep were vaccinated and revaccinated three weeks later and the antisera was found to cause agglutination of methanogens and decrease growth and methane production *in vitro* (Wedlock *et al.*, 2010) [64]. Subharat *et al.*, 2015 [56] studied an experiment on sheep were vaccinated twice at a 3-week interval with a model methanogen antigen, recombinant glycosyl transferase protein (rGT2) formulated with one off our adjuvants: saponin, Montanide ISA61, a chitosan

thermogel, or a lipid nano particle/cationic liposome adjuvant (n=6/formulation). A control group of sheep (n=6) was not vaccinated. The highest antigen-specific IgA and IgG responses in both saliva and serum were observed with Montanide ISA61, which promoted levels of salivary antibodies that were five-fold higher than the second most potent adjuvant saponin. ArGT2-specific IgG standard was used to determine the level of rGT2-specific IgG in serum and saliva. Vaccination with GT2/Montanide ISA61 produced a peak antibody concentration of 7×10^{16} molecules of antigen-specific IgG per litre of saliva, and it was estimated that in the rumen there would be more than 10^4 molecules of antigen-specific IgG for each methanogen cell. Both IgG and IgA in saliva were shown to be relatively stable in the rumen. 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 (Cook *et al.* 2008; Lascano and Cárdenas 2010)^[13, 33].

Mechanism of action of vaccination on methane abatement

The concept is to induce salivary anti-methanogen antibodies which are delivered to the rumen and reduce the activity of methane-producing methanogens. Vaccinating sheep and cattle against the rumen dwelling organisms *Streptococcus bovis* and *Lactobacillus* species, the major etiological microbes responsible for acute ruminal acidosis, has shown that antibodies can translocate to the rumen via saliva and protect against lactic acidosis.

3.5 Phage therapy

The phages have lytic potential and their genes make them an important tool for methane mitigation strategies. In contrast to nearly 300 phage genomes (Ackermann and Kropinski 2007)^[1], 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)^[47] and *Methanothermobacter* phage psi M100 (Luo *et al.*, 2001)^[38]. McAllister and Newbold (2008)^[41] reported siphophages that can infect methanogens (*Methanobacter*, *Methanobrevibacter* and *Methanococcus* spp.), although these phages have not been isolated from the rumen. A metagenomic study on phage-bacterial relationships showed ≤ 0.1 % relative abundance of prophage in phylum Euryarchaeota (Berg Miller *et al.*, 2012)^[7]. 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)^[34]. 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)^[34]. 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 *et al.* 2013; Leahy *et al.* 2013)^[35].

Mechanism of action of phage

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. More methanogenic phages need to be identified, sequenced and characterized to identify and employ such phage-based strategies. Finally, either mixture of phages or structural components of phages may prove useful against the greater diversity of methanogens in rumen.

3.6 Chemical Defaunation

The removal of protozoa from the rumen (defaunation) has been shown to reduce CH₄ production by up to 13%-50% but the frequency of reduction varied with diet. The greatest reduction in methane production with defaunation was measured on a high-concentrate diet, likely because protozoa are the predominant source of hydrogen for methanogenesis on starch based diets (Hegarty *et al.*, 1999)^[24]. McAllister and Newbold 2008^[41] studied the effect of rumen protozoa on methane production and on methanogens have been investigated by molecular biology. The decrease in methane production of 26% per kg DM intake in protozoa-free lambs was related to a decrease in the proportion of methanogens in the total bacterial population of the whole ruminal content.

Mechanism of action

Rumen protozoa share a symbiotic relationship with methanogens, participating in interspecies hydrogen transfer, which provides methanogens with the hydrogen they require to reduce carbon dioxide to methane for this reason, treatments that decrease the protozoal population of the rumen, may also decrease the protozoa-associated methanogen population and therefore, decrease the methane production within the rumen (Machmuller *et al.*, 2003; Newbold *et al.*, 1995)^[40]. Treatments that have been used include copper sulphate, acids, surface-active chemicals, triazine, lipids, tannins, ionophores, and saponins (Hobson *et al.*, 1997)^[25]. a study of ionophore supplementation by Guan *et al.*, (2006)^[22] studied ionophore supplementation in ruminants found that reductions in rumen methanogenesis were short-lived and hypothesized this was due to adaptation of ciliate protozoa.

Table 1: A wide range of chemicals are available that will reduce rumen methanogenesis

Action	Chemicals	Reference
Alternative hydrogen acceptors	fumarate, sulphate/sulphite, nitrate/nitrite and unsaturated fatty acids	Gerald Wischer <i>et al.</i> , 2012; Hegarty <i>et al.</i> , 2007 ^[23] .
Halogenated methane analogues	chloroform, carbon tetrachloride, chloral hydrate, bromo-chloromethane and bromo-ethanesulphonic acid	(McCrabb <i>et al.</i> 1997; Mathison <i>et al.</i> 1998) ^[25] .
Ionophores	monensin and lasalocid	(Johnson and Johnson 1995) ^[31] .
Defaunating agents	manoxol, teric, alkanate 3SL3 and sulpho succinate	(Mathison <i>et al.</i> 1998) ^[25] .

3.7 Antibiotics

Streptomyces cinnamomens is secondary metabolite known as monensin. The effect of monensin on lowering CH₄ emission is dose-dependent: at lower doses (10 to 15 ppm), it results in

the production of profitable milk, but has no effect on CH₄ (Grainger *et al.*, 2008; Waghorn *et al.*, 2008)^[21, 62]; but at higher doses (24 to 35 ppm), it reduces CH₄ production by up to 10% (Mc Ginn *et al.*, 2004; Sauer *et al.*, 1998; Van Vugt *et*

al., 2005)^[43, 53, 60]. However, there have been unanswered questions over the perseverance of CH₄ suppression (Johnson and Johnson, 1995; Eckard *et al* 2010)^[16, 31]. Monensin selects for gram-negative microorganisms, which causes a shift towards propionate production in the rumen for this reason, it is hypothesized that monensin does not affect methane production by inhibiting methanogens, but instead inhibits the growth of the bacteria, and protozoa, providing a substrate for methanogenesis. This statement is strengthened by the fact that when rumen fluid was dosed with monensin *in vitro*, methane production decreased until a supply of hydrogen was given (Russell *et al.*, 1989)^[51].

Nisin is thought to act indirectly, affecting hydrogen producing microbes in a similar way to that of the ionophore antibiotic monensin. Nisine, a bacteriocin produced by *Lactococcus lactis*, has been studied as a tool for mitigating methane. Sar *et al.*, (2005)^[71] evaluated the effects of different concentrations of nisine on methane production *in vitro* in a continuous culture system. As its concentration increased from 5 to 30 µmol/L, methane production was reduced from 14 to 40%. Cattle HC5 bacteriocin, produced by *Streptococcus bovis*, inhibited *in vitro* methanogenesis up to 50%.

Mechanism of action

Monensin inhibits the gram positive bacteria, which is responsible for supplying substrate to methanogens. Monensin acts on the cell wall of the gram positive bacteria. It interferes with ion flux and decreases the acetate: propionate ratio in the rumen thereby effectively decreasing CH₄ production.

3.8 Bacteriophages and bacteriocins

Biological control strategies, such as the use of bacteriophages and bacteriocins, can be effective to directly inhibit Archaea methanogens and redirect H₂ to reductive rumen bacteria that may be propiogenic or acetogenic (McAllister *et al.*, 2008)^[41].

Bacteriophages are present in all biological ecosystems and have the ability to penetrate and consequently cause lysis in the host cell. This effect of bacteriophages and their genes can be a potential strategy to mitigate methane (Buddle *et al.*, 2010)^[11]. Bacteriophages are host-specific, which is another limiting factor for using this strategy to reduce methane due to the high number of methanogen species in the rumen (Janssen and Kirs, 2008; McAllister *et al.*, 2008)^[30, 41]. Bacteriocins, bactericidal peptides produced by bacteria, could also be used (McAllister *et al.*, 2011)^[42]. However, there is scarce information on their effects on methanogenesis. Nisine, a bacteriocin produced by *Lactococcus lactis*, has been studied as a tool for mitigating methane. Identification of stable bacteriocins in the rumen environment and specific to methanogenic bacteria is an area for future research. *In vivo* studies are necessary to establish the lasting adaptability and effectiveness to use bacteriocins as a feed additive (Boadi *et al.*, 2004; McAllister *et al.*, 2008)^[9, 41].

4. Conclusion

Livestock, produced throughout the world, are an important agricultural product in virtually every country. Methane is the most effective global warming greenhouse gas and methanogens are the key microbiota in methane emission. Ruminant animals (particularly cattle, buffalo, sheep, goat and camels) produce significant amounts of CH₄ under the anaerobic conditions of the digestive processes. Various

strategies have been implemented to mitigate methane such as by changing diet, especially by providing diet rich in oil seed or proteins rather than carbohydrates. It is notable that, other than nutritional and managemental related strategies, two important strategies including integral microbiology and biotechnology strategies in ruminant production such as animal breeding, genetic merit, unproductive animals, vaccination, immunization and biological control (bacteriophage, acetogenesis reductive), chemical defaunation; can be results in mitigating methane production.

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