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Invited Review Article

Exploring strategies to mitigate methane emissions in ruminants through 'direct-fed microbials'

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Abstract

Ruminal anaerobic fermentation is a key mechanism responsible for transforming carbon dioxide and hydrogen ions (H^+) into methane. Total GHG emissions from enteric fermentation are around 7186.3 Tg CO_2e worldwide and 214.5 Tg CO_2e in India, accounting for 2.98% of global emissions. Notably, methanogenic organisms like *Methanobrevibacter* and *Methanomicrobium* thrive in anaerobic conditions at an optimal temperature range of 39 to 40°C. They use H_2 to catalyze the conversion of carbon dioxide into methane through the well-defined methanogenesis pathway ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ and $HCO_3 + 4H_2 \rightarrow H^+ + CH_4 + 3H_2O$). The production of acetate and butyrate inside the rumen ecosystem results in the release of pure hydrogen, while the generation of propionate offers an alternative pathway for H^+ consumption within the rumen. Consequently, it becomes evident that metabolic pathways governing hydrogen synthesis and utilization represent pivotal elements requiring thorough investigation in the pursuit of solutions to mitigate ruminant methane emissions. We find several strategies in the published literature to mitigate ruminal production and enhance productivity of the ruminant livestock. In the present review, we will discuss the effect and mechanism of Direct-fed Microbials (DFMs) or probiotics to act as potential solutions to alleviate the problems of ruminal methane production. It is the need of the hour to identify specific strain/s of yeast or fungal culture and lactic acid bacteria to be used as potential probiotics for the reduction of methane in ruminants, and those strain/s should be commercially available for use by the farmers.

Keywords: Alleviate, Methane, Probiotics, Rumen, Yeast

Highlights

- Reduction of ruminal methane production could be done by several dietary manipulations including the use of probiotics or Direct-fed-microbials (DFMs).
- DFMs, yeast (*Sachharomyces cerevisiae*), and some potential lactic acid bacteria (LAB) may be used to reduce enteric methane production.
- However, the persistence of promising probiotics should be tested extensively in ruminants before recommendation for use.

INTRODUCTION

Methane, a key player in global warming, is produced through natural and anthropogenic activities. Anthropogenic activities caused by human beings are continuously damaging the ecology and environment. By 2050, the human population is expected to increase from 7.2 billion to 9.6 billion (UN, 2013). Although there is a 33% growth in population, the demand for agricultural goods will increase by nearly 70% over the same period as the global standard of living rises (FAO, 2011). The beef consumer demand is anticipated to increase by 80% by 2050, making the cattle sector essential for maintaining global food and nutrition security (Nadathur *et al.*, 2017). Since they provide 33% of the world's protein and 17% of its calorie needs, livestock products are a crucial agricultural input for food security worldwide (Rosegrant *et al*., 2009). The "livestock revolution" has been dubbed as a result of the sharp rise in demand for livestock products in developing nations (Thornton, 2010). However, feeding management in developing countries is generally based on grazing or roughage-based feeding systems, which are also responsible for increased greenhouse gas emissions from livestock.

Ruminants derive their sustenance from a plant-based diet rich in lignocellulose, a challenging-to-digest substance. This intricate dietary material undergoes a unique fermentation process within its specialized stomach, known as the "rumen", before proceeding with digestion. This process, termed "enteric fermentation", involves the enzymatic breakdown of carbohydrates by microbes into smaller molecules. Within this intricate ecosystem, ruminal anaerobic nutritional fermentation is a key mechanism responsible for transforming carbon

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dioxide and hydrogen ions (H^+) into methane (Palangi *et al*., 2022). Livestock production is known to be a significant contributor to greenhouse gas (GHG) emissions, primarily through the release of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Giamouri *et al.*, 2023). Methane (CH_4) is a by-product of microbial fermentation that occurs in the rumen of ruminant animals. It is produced by methanogenic archaea during the breakdown of structural carbohydrates in feed (Patra, 2016). Fermentation is an oxidative process wherein reduced cofactors such as NADH, NADPH, and FADH are reoxidized to their counterparts (NAD+, NADP+, and FAD^+), releasing hydrogen in the process. This liberated hydrogen is subsequently utilized by methanogenic archaea present in the rumen ecosystem. The fermentation processes within the rumen generate hydrogen gas (H_2), which serves as the primary substrate for ruminal methanogenesis. Notably, methanogenic organisms like *Methanobrevibacter* and *Methanomicrobium* thrive in anaerobic conditions at an optimal temperature range of 39 to 40ºC. They use H_2 to catalyze the conversion of carbon dioxide into methane through the well-defined Wolfe cycle of methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ and $HCO_3 + 4H_2 \rightarrow H^+ + CH_4 + 3H_2O$) (Thauer *et al*., 2012). The microbial fermentation process in the rumen yields a variety of end products, leading to differences in hydrogen production efficiency. For instance, the creation of acetate and butyrate results in the release of pure hydrogen, while the generation of propionate offers an alternative pathway for H^+ consumption within the rumen. Consequently, it becomes evident that metabolic pathways governing hydrogen synthesis and utilization represent pivotal elements requiring thorough investigation in the pursuit of solutions to mitigate ruminant methane emissions. Additionally, understanding the composition of the methanogenic microbial community is of paramount importance. It is worth noting that ruminant methane emissions also pose a direct economic burden on farmers due to the loss of feed energy. According to the Food and Agriculture Organization (FAO), up to 12% of the energy contained in feed is often lost as a result of CH_4 generation, underscoring the multifaceted challenges posed by this issue (FAO, 2022).

Carbon dioxide (CO_2) and methane are the two most major greenhouse gases, and their concentrations in the atmosphere have risen from 350 to 410 ppm (a 28 percent increase) and from 1100 to 1875 ppb (a 70% increase), respectively, since 1950 (Black *et al.*, 2021). By absorbing energy and reducing the rate at which it escapes to space, they absorb solar heat and warm the atmosphere. This rise has major implications for humans, animals, and the environment (Moss *et al.*, 2000). Methane is the second most significant GHG after CO_2 in terms of energy absorption (Katari, 2015). In addition, CH_4 has a 28-fold larger global warming potential (GWP) than CO_2. Agriculture emits considerable volume of CO_2, CH_4, and N_2O to the atmosphere (IPCC, 2000). Gerber *et al.* (2013) estimate that the cattle sector provides 14.5% of worldwide GHG emissions. Ruminant livestock is predicted to release between 80 and 95 million tonnes of CH_4 per year around the world (Beauchemin *et al.*, 2008). Cattle and sheep production systems account for up to 18% of total global GHG emissions, largely in the form of enteric methane (Herrero and Thornton, 2013). Ruminant enteric CH_4 emissions are the most prevalent source of greenhouse gases, accounting for 46 percent of total CO_2e emissions from dairy and 55 percent of total CO_2e emissions from small ruminant production (Gerber *et al*., 2013). More than 90% of CH_4 emissions from livestock and 40% of agricultural GHG emissions are attributed to the enteric fermentation process (Tubiello *et al.*, 2013). According to the Intergovernmental Panel on Climate Change (IPCC) and the United Nations Food and Agriculture Organisation (UN-FAO), a fully matured cow can generate up to 500 liters of methane per day, accounting for around 3.7 percent of total greenhouse gas emissions (Lassen and Løvendahl, 2016).

In India, GHG emissions from the agriculture sector are 212.1, 2.44, 69.87, 43.4, and 6.61 million tonnes of CO_2e, respectively, from livestock, manure management, rice cultivation, soils, and crop residue burning (Sharma, 2020). Livestock accounted for 63.4% of agricultural GHG emissions and 11.1% of total GHG emissions in the country. The total methane emission by livestock in India was estimated to be around 9.37 Tg in 2003, with buffaloes contributing 3.8 Tg (40.0%), indigenous cattle contributing 3.75 Tg (40%), crossbred cattle contributing 0.71 Tg (8.0%), and sheep and goats contributing 0.96 Tg (10%) (Upadhyay *et al.*, 2008). Equines (horses, ponies, mules, and donkeys), pigs, yak, Mithun, and camels provided only 2% (0.15 Tg) of total livestock emissions. Ruminants, both small and large, were the primary sources of enteric methane emissions in India (98%). Total GHG emissions from enteric fermentation are around 7186.3 Tg CO_2e worldwide and 214.5 Tg CO_2e in India, accounting for 2.98% of global emissions (Sharmra, 2020). The methane emission from different sectors of livestock farming has been mentioned in Fig. 1. As a result, the task is to strike a balance between productivity, food security in the home, and environmental preservation (Wright *et al.*, 2012).

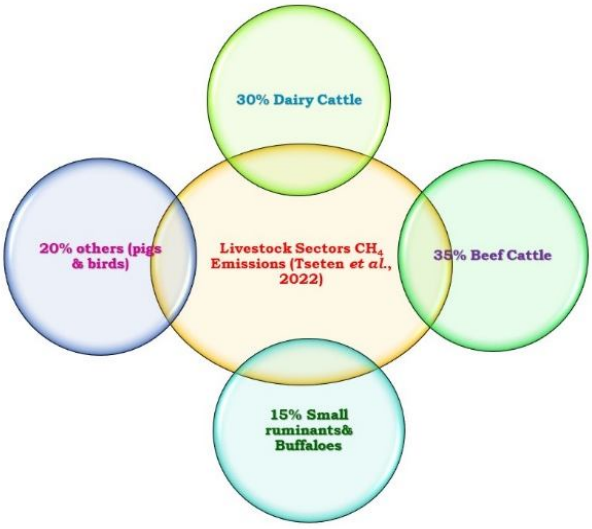

Fig. 1. Methane emission from the livestock sectors

Addressing environmental and energy-related issues is paramount, with particular attention to the role of rumen CH_4 in accounting for approximately 5% to 12% of dietary gross energy loss (Jeyanathan *et al*., 2014; FAO, 2022). In light of this concern, diligent research efforts are underway to explore avenues for mitigating rumen methanogenesis, as evidenced by studies conducted by Martin *et al.* (2010) and Buddle *et al.* (2011). One of the prominent strategies under scrutiny involves the alteration of rumen metabolic pathways to curtail CH_4 production. A noteworthy approach within this realm is the manipulation of Direct-fed Microbials (DFMs), which holds promise as a potential solution. So, this review is focussed on the efficacy of different probiotics or "Direct-fed-Microbials" on the mitigation of enteric methane production in ruminants.

Direct-fed microbial (DFM)

Direct-fed microbes have been defined as a source of live, naturally occurring microorganisms (Krehbiel *et al.*, 2003). They have been successfully used in ruminant production to increase productivity, prevent digestive disorders such as acidosis, and reduce pathogenic load in young animals (Adams *et al.*, 2008; McAllister *et al.*, 2011; Lettat *et al.*, 2013). They are a well-accepted alternative to the use of antibiotics and chemical compounds, which may result in the development of antibiotic resistance and residues in animal products. *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Bacillus*, and *Propionibacterium* species are extensively employed as probiotics for humans and monogastric animals, as well as inocula for dairy product processing (V´elez *et al*., 2007; Gareau *et al*., 2010; Taye *et al*., 2021). Other different bacterial species, including *Megasphaera elsdenii* and *Prevotella bryantii*, have been utilized as DFM to stabilize or improve rumen function. These bacterial DFM strains can be classed as making lactic acid, using lactic acid, or being other microorganisms. The rumen is the first organ DFM reaches after intake in ruminants. DFM grows in the rumen and changes the microbial environment and (or) fermentation properties. DFM may also live in the intestine. The synthesis and utilization of lactic acid in the rumen is linked to feeding efficiency and animal health. Most commercial yeasts contain *Saccharomyces* and *Aspergillus* species. However, there is little data to show that DFM is effective at controlling CH_4 generation in ruminants. To stabilize ruminal fermentation in high-producing cattle, Direct-fed Microbial (DFM) are extensively utilized in ruminant nutrition. Supplementation of *Saccharomyces Cerevisiae* may also results in an increased fibrolytic bacterial population in the rumen, enhanced propionate concentration, and stabilized rumen pH under high-concentrate feeding (Hristov *et al*., 2010).

One of the most used DFMs is the yeast *S. cerevisiae*, which has proven especially beneficial when the digestive microbiota is challenged, such as during feed transition or when diets are high in highly fermentable carbohydrates (McAllister *et al.*, 2011). Bacterial DFM has gotten less attention than yeast DFM, even though many possible bacterial DFM are naturally present in the rumen (Lettat *et al.*, 2013). Bacterial DFM has been recommended as a potential enteric methane (CH_4) mitigation supplement for ruminants due to its ability to control ruminal fermentations (Jeyanathan *et al.*, 2016). The main metabolic mechanisms for reducing CH_4 emissions from ruminants with DFM are the redirection of H_2 away from methanogenesis and the reduction of H_2 generation during feed fermentation.

Classification of probiotics

As probiotics, a variety of microorganisms are utilized, which can be categorized as follows: Bacterial vs non-bacterial probiotics. The majority of the microorganisms used are bacteria, except select yeast and fungal probiotics. Lactobacillus species are examples of bacterial probiotics (Yilma *et al.*, 2011). *Aspergillus oryzae* and *Candida pintolopesii* are two non-bacterial (yeast or fungal) probiotics (Das *et al.*, 2012). Probiotics that form spores versus those that do not form spores: Initially, non-spore-forming *Lactobacillus* and *Bifidobacterium* strains predominated; however, spore-forming bacteria such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* are

currently used (Eyassu, 2013).

Multi-species vs. single-species probiotics: The microbial composition of probiotic products can range from a single strain to multi-strain or species compositions. Examples of multi-species probiotics are Poultry Star ME (contains *Enterococcus faecium*, *Lactobacillus reuteri*, *L. salivarius*, and *Pediococcus acidilactici*) (Farnworth *et al.*, 2015); PrimaLac (contains *Lactobacillus* spp., *E. faecium* and *Bifidobacterium thermophilum* and Microguard (contains various species of *Lactobacillus*, *Bacillus*, *Streptococcus*, *Bifidobacterium*, and *Saccharomyces*).

Yeast (*Saccharomyces cerevisiae*)

Yeast cultures reduce methane production in four ways: 1) By increasing butyrate or propionate production, 2) By reducing protozoan numbers, 3) By improving animal productivity, 4) By promoting acetogenesis.

The above-mentioned effects may be related to yeast's ability to eliminate trace amounts of oxygen present in the rumen and/or to micronutrients found in the yeast itself (McAllister *et* al., 2011). Yeast works by competing for available sugar with lactate-producing bacteria (*Streptococcus bovis* and *Lactobacillus*) and supporting the growth of lactate-consuming bacteria (*Megasphaera elsdenii). M. elsdenii* is known to convert lactate into butyrate and propionate, resulting in less lactate build-up and higher rumen pH, which promotes microbial establishment and increases fiber decomposition (Chen *et al*., 2019).

Acetogenic bacteria, which generate acetate from CO_2 and H_2, appear to play a critical role in the re-utilization of fermentative hydrogen in various non-ruminant digestive environments. *Saccharomyces cerevisiae* yeast cultures may encourage acetogenic bacteria in the rumen, which consume H_2 to create acetate and so reduce CH_4 generation. It has been reported that extremely modest doses of aerobic viable yeasts or fungi (*Saccharomyces* or *Aspergillus* spp.) applied to the rumen can inhibit methanogenesis. *In vitro* investigations revealed that the yeast strain had interesting favorable effects on acetogenic bacteria growth and H_2 utilization, even in the presence of methanogens. In batch cultures with mixed rumen microflora, the results of investigations with different yeast strains have been rather contradictory, with either no effect or an increase in methane generation reported. However, the effects on CH_4 generation in the rumen appear to be strain-dependent and varied. More study is needed to test a large number of yeast strains to identify those with both a production benefit and strong CH_4 abatement potential (Umesh, 2012).

It is strongly proposed that yeast probiotics may promote acetogenic bacteria to compete with methanogens or co-metabolize H_2 as a result of which CH_4 synthesis is reduced (Chaucheyras *et al.*, 1995). After 48 hours of incubation, mixed rumen microorganisms including lucerne and a live yeast product produced 20% less CH_4 (Lynch *and* Martin, 2002). *Aspergillus oryzae* lowered CH_4 synthesis by 45% by reducing the protozoal population (Frumholtz *et al.*, 1989).

Limitations of yeast probiotics: Variations in the yeast strain employed, its viability (for example, survival during pelleting), animal status, and the composition of the diet contribute to the heterogeneity in the response to yeast supplementation. It has been established that after ingestion of the yeast product, the same concentration of live cells can remain in the rumen for up to 24-30 hours without considerable development. If the distribution is not renewed, the yeast concentration will fall to undetectable levels in 4-5 days. The effects of *S. cerevisiae* on ruminal fermentations vary depending on the strain. Organic acids and vitamins generated by yeast activity, as well as yeast components such as peptides and amino acids, play a role in the impacts reported on rumen microbiota (Chaucheyras Durand *et al*., 2012). Another key consideration is the inherent variance among animals in the same herd. Feeding behavior parameters such as rate of feed and water intake, as well as physiological factors such as ruminal content turnover and saliva volume generation, vary from animal to animal, which may explain variations in the results reported from different studies.

Lactic acid bacteria

Lactic acid bacteria are normally present in the gastrointestinal tract; however, in ruminants, these commensal bacteria are seen only in young ruminants before the rumen has fully developed (Stewart *et al*., 1988). *Lactobacillus ruminis* and *Streptococcus equinus* (formerly *S. bovis*) are considered natural rumen inhabitants, while others (*L. plantarum* and *L. lactis*) are generally introduced with the feed (Stewart, 1992). However, the studies are very scanty in order to prove the efficacy of these LABs on the reduction of ruminal methane production.

How could LAB reduce ruminant CH_4 production?: It is hypothesized that LAB could influence ruminal methanogenesis in three ways: (1) use of LAB or their metabolites to modify rumen fermentation, resulting reduction in CH_4 production, (2) use of LAB or their

metabolic products to directly reduce rumen methanogens, and (3) use of LAB or their metabolites to slow down specific rumen bacteria that produce H_2 or methyl-containing compounds that are methanogenesis substrates (Doyle *et al*., 2019). Little research was done on the topic, and solid data from animal trials to support this hypothesis is absent. Jeyanathan *et al.* (2016) tested 45 bacteria, including LAB, *Bifidobacteria*, and *Propionibacteria* strains, for their capacity to inhibit methanogenesis in 24h rumen *in vitro* batch incubations. Three strains were chosen for *in vivo* experiments in sheep (n = 12), and one strain (*Lactobacillus pentosus* D31) reduced CH_4 generation (g CH_4/kg/DMI) by 13% over four weeks when dosed at 6×10^{10}cfu/animal/day. In this investigation, the mechanism of action was not mentioned, but the ability of introduced bacterial strains to remain in the rumen environment was emphasized as an essential component. Using the same strains, (Jeyanathan *et al.*, 2019) found no ability to lower CH_4 emissions in dairy cows. Very few research works was done on bacteriocins and their capacity to inhibit ruminal CH_4 generation. The few bacteriocins and preparations derived from bacteriocin-producing lactic acid bacteria tested positive *in vitro* and *in vivo*. Callaway *et al.* (1997) investigated the effect of *Lactococcus lactis* on rumen fermentation *in vitro* and reported that it reduced CH_4 generation by 36%.

Future perspective: There are very few studies on the use of LAB to reduce CH_4 generation in ruminants. *In vitro* studies show that LAB can efficiently lower CH_4 generation. The effect is definitely strain-dependent, and it is unclear whether the LAB or their metabolites affect the methanogens or the other rumen microorganisms that provide substrates for methanogenesis. Rigorous long-term animal experimentations are required to prove the efficiency of promising LAB on the reduction of ruminal methane production. The mechanisms underlying the usage of LAB as rumen modifiers to reduce methane production may only be explored (Doyle *et al*., 2019). Tseten *et al*. (2022) described different pathways to reduce enteric methane production by direct-fed-microbials (DFMs) (Fig. 2).

Propionate-forming bacteria

Propionate production consumes reducing equivalents since pyruvate is reduced to propionate. Hence, it is classified as an H_2-utilisation pathway, whereas H_2 formation involves the reduction of protons (H^+) to H_2 (Baldwin *et al*., 1963). Because H_2 is the primary precursor for the creation of CH_4, an increase in propionate formation is stoichiometrically related to a decrease in CH_4 production. Propionate is synthesized in the rumen via two pathways: the succinate pathway and the acrylate pathway. The succinate pathway is the main one in the rumen, and it produces intermediate products such as malate, fumarate, and succinate. Furthermore, bacteria such as lactate producers (e.g. *Streptococcus bovis*), lactate users (e.g. *Selenomonas ruminantium*), fumarate reducers (e.g. *Wolinella succinogenes*), succinate producers *(Fibrobacter succinogenes*), and succinate users (e.g. *S. ruminantium*) are involved in this route (Jeyanathan *et al*., 2014). In the rumen, the acrylate pathway is also critical for propionate production. The lactate-utilizing *Megasphaera elsdenii* is the primary rumen bacteria engaged in this process, which needs the presence of lactate (Russell and Wallace, 1997). Several researches have been undertaken to improve propionate production in the rumen to increase animal productivity using dietary probiotics (Seo *et al.*, 2010), which are indirectly related to methane mitigation strategies. Lactating dairy cows fed a mixed *Propionibacterium jensenii - Lactobacillus* spp. direct fed microbial produced less CH_4 (Berger *et al.*, 2014). According to (Mamuad *et. al.*, 2014), fumarate-reducing bacteria affect the rumen microbiome, aiding ruminal fermentation and decreasing CH_4 generation *in vitro*. In a recent *in vitro* experiment, CH_4 synthesis was lowered by supplementing fumarate reductase-producing Enterococci *(Enterococcus faecium* SROD) with an increase in total VFAs.

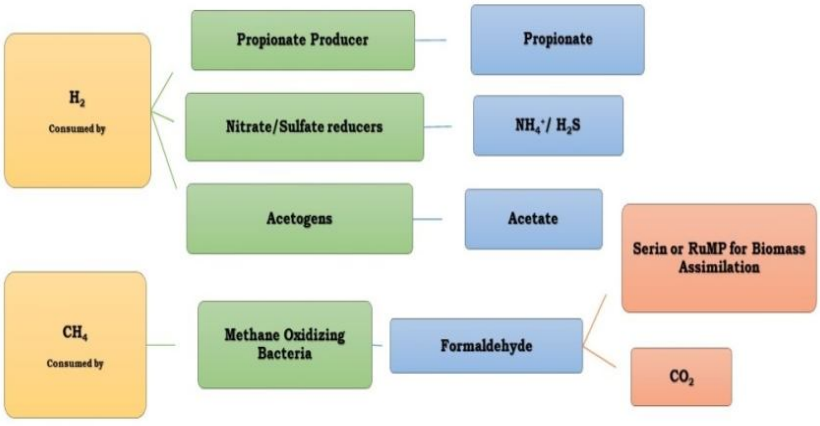

Fig. 2. Pathways to reduce enteric methane by probiotics

Nitrate/nitrite-reducing bacteria

Nitrate, a different H_2 sink to CO_2 in the rumen, inhibits rumen methanogenesis and the toxicity of the intermediate product nitrite during their metabolism (Jeyanathan *et al*., 2014). Rumen bacteria rapidly convert nitrate to nitrite, whereas the conversion of nitrite to ammonia is slower. This can result in nitrate buildup in the rumen and methemoglobinemia in the blood (Iwamoto *et al.*, 1999). Methemoglobinemia reduces the capacity of the blood to deliver oxygen to tissues, resulting in poor performance or, in severe cases, death of animals (Morris *et al.*, 1958). One potential way to avoid this toxicity is the use of nitrate and/or nitrite-reducing microorganisms as probiotics (Anderson and Rasmussen, 1998). The bacteria can decrease nitrate, nitrite, or both molecules that are already present in the rumen, but their concentration is lower than that of their methanogen counterparts (Asanuma *et al.*, 2002). *W. succinogenes* and *S. ruminantium* are the primary rumen nitrate-reducing bacteria (Yoshii *et al.*, 2003). To reduce methanogenesis, increasing the number and/or activity of nitrate- and/or nitrite-reducing bacteria in the rumen may be advantageous. Nitrate supplementation enhanced the number of nitrate-reducing bacteria *(W. succinogenes* and *Veillonella parvula*) *in vitro*, although this may be insufficient to compete with methanogenesis (Yoshii *et al.*, 2003). Giving nitrate and/or nitrite-reducing bacteria as probiotics in addition to nitrate may thus accelerate the nitrate reduction process and, as a result, avoid nitrite toxicity. Along with nitrate, *Denitrobacterium detoxifications* strain NPOH1 reduced CH_4 generation by 95% (Anderson and Rasmussen, 1998), while *W. succinogenes*, *S. ruminantium*, or *V. parvula* reduced it by more than 70% (Yoshii *et al*., 2003). On the other hand, there is a scarcity of *in vivo* evidence on this subject.

Only a few research works on the use of nitrate/nitrite-reducing bacteria as DFM to reduce rumen methanogenesis have been published. Anderson and Rasmussen (1998) observed a 95% reduction in CH_4 synthesis without nitrite accumulation in an *in vitro* assay using the nitrate-reducing rumen bacterium *Denitrobacterium detoxificans* strain NPOH1 and additional nitrate (10 mol/mL). In the absence of *D. detoxificans*, there was only a 25% decrease in CH_4 generation and nitrite accumulation. Another *in vitro* investigation found that adding nitrate-reducing bacteria *W. succinogenes*, *S. ruminantium*, *or V. parvula* to mixed methanogens in the presence of nitrate (5 mM) significantly reduced methanogenesis (by more than 70%) (Jeyanathan *et al*., 2014).

Sulphate-reducing bacteria

The ability of sulfate-reducing bacteria (SRB) to compete with methanogens is heavily influenced by sulfate addition to the rumen. SRB competes with methanogens for common substrates such as H_2, formate, and acetate in anaerobic conditions where sulfate is limitless (Jeyanathan *et al*., 2014). The rumen SRB population is small (10^5 to 10^6 cells/mL) and mostly composed of *Desulfovibrio* and *Desulfotomaculum* (Campbell and Postgate, 1965). Another SRB from the genus *Fusobacterium* was recently isolated from buffalo (Paul *et al.*, 2011). Due to the hazardous end product hydrogen sulfide (H_2S), only a few studies have been undertaken on the effect of sulfate supplementation alone in rumen methanogenesis (Morvan *et al.*, 1996). As a result, SRB may only aid sulfate reduction due to decreased methanogenesis when sulfate is provided as a feed supplement. For example, in an *in vitro* trial using *Fusobacterium* sp. as a probiotic with a high sulfate diet, CH_4 generation at 72 hours was reduced from 2.66 to 1.64 mmol/g digested dry matter (DM) without H_2S accumulation (Paul *et al.*, 2011).

Homoacetogens

In the rumen, reductive acetogenesis coexists with methanogenesis (Mackie and Bryant, 1994). Because the same substrates are employed, promoting the autotrophic growth of homoacetogens (Wood-Ljungdahl pathway) is assumed to be a competitive pathway to methanogenesis (Jeyanathan *et al*., 2014). Acetate, the end product of this reaction, has the added benefit of providing energy to the animal. However, acetogens are less common in the rumen environment and less efficient in competing for reducing equivalents than methanogens. As a result of CH_4 reduction, it is necessary to increase the number of acetogenic bacteria in the rumen, which compete for hydrogen with methanogens. Martin *et al.* (2010) and Kim *et al.* (2018) found that food supplementation with acetogen probiotics reduced CH_4 generation in ruminants. Several attempts to boost the rumen's reductive acetogenesis process by providing DFM-containing rumen and non-rumen homoacetogens were futile. Lopez *et al.* (1999) investigated the effect of six acetogenic bacteria on rumen CH_4 emission *in vitro* and discovered that only two of them reduced methanogenesis somewhat.

Methylotrophs

Methylotrophs are microorganisms that can use one-carbon organic molecules like methanol and methylamine as carbon sources, competing with methanogens for substrates. Some methylotrophs and methanotrophs can use CH_4 and avoid releasing it into the environment (Jeyanathan *et al*., 2014). Understanding the metabolic routes of these chemicals may lead to the development of a novel biological

control agent for reducing rumen CH_4 emissions. There has been no evidence to show that methanotrophy is essential in the rumen up to this point. Only one study showed the possibility of methanotrophy in rumen fluid, albeit at a low level of 0.2% to 0.5% (Kajikawa *et al.*, 2003). In another investigation, bacterial clones closely related to *Nitrosomonas* spp. were found using a clone library built from samples of rumen epithelial bacterial populations (Mitsumori *et al.*, 2002). Nitrosomonas members are ammonia-oxidizing bacteria that may oxidize CH_4 under certain conditions (Jiang and Bakken, 1999). There is constant ammonia close to the rumen wall as urea from the blood, which is quickly destroyed by ureolytic bacteria attached to the rumen wall (Cheng and Wallace, 1979). As a result, *Nitrosomonas* spp. in the rumen wall could be implicated in CH_4 oxidation.

Capnophiles

Capnophiles are microorganisms that need a lot of CO_2 to grow (Jeyanathan *et al.*, 2014). The rumen is an anaerobic environment with a high concentration of CO_2. Capnophiles are thus expected in the rumen, but their use as CO_2 scavengers to reduce methanogenesis is debatable because this gas is not a limiting substrate for rumen methanogens. Dehority (1971) proposed two types of CO_2 requirement among rumen bacteria: biosynthesis type, in which CO_2 is required for cell growth (e.g. *S. bovis*), and succinate forming bacteria (e.g. *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *succinivibrio dextrinosolvens*). CO_2 is coupled to the three-carbon phosphoenol pyruvate, an end product of glycolysis, during succinate synthesis to form the four-carbon molecule oxaloacetate. When oxidoacetate is converted to succinate, it receives two pairs of electrons. As a result, both CO_2 and H_2 are consumed in the synthesis of succinate, which may affect rumen methanogenesis. Succinate is another significant intermediate product in the synthesis of propionate (Jeyanathan *et al.*, 2014). The Tammar wallaby generates barely one-fifth of what ruminants do per unit of digestible energy intake (Kempton *et al.*, 1976). A physiological variation, such as a shorter meal retention time in the foregut, explains part of this. The presence of a new genus of acetogenic bacteria could explain the reduced CH_4 emission (Gagen *et al.*, 2010). This observation may potentially be influenced by the presence of capnophiles.

Conclusions

Reduction of ruminal methane production is a real challenge for production scientists without compromising the production potential of high-yielding animals. Many of the bacterial strains identified as possible DFM for lowering rumen methanogenesis are isolates that can be or are being produced industrially. However, only a few of them have been studied for anti-methanogenic action. In addition to these previously available microorganisms, the rumen and other habitats are significant sources of prospective DFM because the vast majority of microorganisms have yet to be cultured. Advances in genomics studies will aid in the identification of bacteria strains that may be capable of reducing methanogenesis in the rumen ecosystem. The most promising species/strains tested thus far have more than one characteristic thought to be useful in lowering methanogenesis.

This metabolic diversity appears to be relevant and should be sought when selecting candidates for DFM use. For the same reason, combining different types of bacteria could improve the efficiency of anti-methanogenic DFM. If the DFM has specific nutrient requirements, including them in the preparation as prebiotics may also assist them in working well in the rumen. Technological breakthroughs in the manufacture of anaerobic bacteria are required to broaden the scope of anti-methanogenic DFM. *In vivo* studies are especially crucial for newly developed DFM since DFM may alter the rumen environment and create microbial modifications that will not occur *in vitro*. Such microbial changes must be evaluated to minimize detrimental effects on animals, the environment, and consumers. Another factor to examine is the persistence of the anti-methanogenic action, which should be tested in long-term animal trials. In contrast to other mitigation treatments including feed additives and supplements, DFM must be delivered regularly to be effective. It is the need of the hour to identify specific strain/s of yeast or fungal culture and lactic acid bacteria to be used as potential probiotics for the reduction of methane in ruminants, and those strain/s should be commercially available for use by the farmers.

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