

Review

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[Abubeker Hassen](#)*, [Eyob Nangamso Tesfamariam](#), [Bulelani Nangamso Pepeta](#)*,
[Eyob Nangamso Tasfamariam](#)

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Review

The Potential of Strategies Used to Mitigate Enteric Methane Emissions in Nutrition Studies of Ruminants: A review

Abubeker Hassen *, Eyob Nangamso Tesfamariam, Bulelani Nangamso Pepeta * and Eyob Nangamso Tasfamariam

University of Pretoria

* Correspondence: abubeker.hassen@up.ac.za (A.H.); bulelani.pepeta@gmail.com (B.N.P.)

Abstract: This study explored techniques with enteric methane (CH₄) abating potential, ranging from chemical additives including 3-NOP, nitrate, and halogenated compounds to natural feed additives such as tannins, oils, seaweed extracts, and probiotics. Most of the evaluated strategies exert their influence either by promoting the growth of ruminal microbes that compete with methanogens for metabolic hydrogen as their resource or by directly consuming CH₄. Additionally, externally supplied probiotics, including propionate-forming bacteria and methane-oxidizing bacteria, are a promising tool. However, challenges persist in implementing these strategies effectively. High doses of chemical additives and plant based secondary metabolites often achieve significant CH₄ reduction, but they are associated with negative impact on dry matter intake and digestibility, thus compromising overall animal performance. On the other hand, using lower doses to maintain animal performance may not achieve beneficial CH₄ reduction. Furthermore, concerns about the impact of certain additives on human health have led to a shift towards more natural alternatives, but their efficacy and feasibility *in vivo* necessitate further evaluation. Comprehensive research that integrates data from diverse global experiments on ruminants through meta-analytical approaches is essential. Such research will help in identify strategies that either reduce methane emissions and/or enhance production performance, ensuring sustainable livestock farming practices. In conclusion, mitigating CH₄ emissions from ruminant livestock is a complex challenge with wide-ranging implication.

Keywords: ruminants methane grazing

Introduction

Current scenarios of global warming show strong evidence of more erratic fluctuations in weather patterns in the future as compared to the past and present, and this is attributable to the current agricultural and industrial practices that generate high levels of greenhouse gases into the atmosphere (Horrigan et al, 2022). About one-third of the total anthropogenic methane (CH₄) emissions in the atmosphere are from the microbial fermentation in ruminants (Akapali, 2018). Strategies with CH₄ reducing potential while at least maintaining or improving production performance necessitate assertion. Therefore, the focus of this review is to evaluate the overview of rumen ecology, ruminal microbiota in relation to strategies with CH₄ abatement potential (chemical additives including 3-NOP, nitrate, and halogenated compounds to natural feed additives such as tannins, oils, seaweed extracts, and probiotics), CH₄ modulation by such strategies and effect on animal productivity.

Overview of rumen ecology

The rumen ecosystem comprises a rich consortium microbiota exhibiting symbiotic and parasitic relationships. The isolation and analysis of ruminal microbiota showed the presence of bacteria

species (10^{10} to 10^{11} cells/ml, over 50 genera), anaerobic fungi (10^3 to 10^5 zoospores/ml, over five genera), and protozoa (10^4 to 10^6 cells/ml, over 25 genera) in rumen fluid sample (Cieslak et al., 2013; Adejoro, 2019) which are responsible for degradation of the digesta, and production of energy for the host animal from the volatile fatty acids (Mizhari and Jami, 2018). The rumen washout aids the translocation of microbes to the lower gut where they get digested and provide microbial protein to the host animal. The concentration and distribution of the various rumen microorganisms is hugely influenced by diet. Plant polysaccharides such as cellulose, hemicellulose, and starch are broken down by enzymes and ruminal microbes into principal components of utilization. By-products from hydrolysis of the pentose and hexose sugars are utilized by succinate, lactate and fumarate intermediates to generate volatile fatty acids (VFAs), which are then absorbed or deaminated in the ruminant system. Equally, protein and lipids go through a redox reaction in the rumen as depicted in **Figure 1** and discussed below.

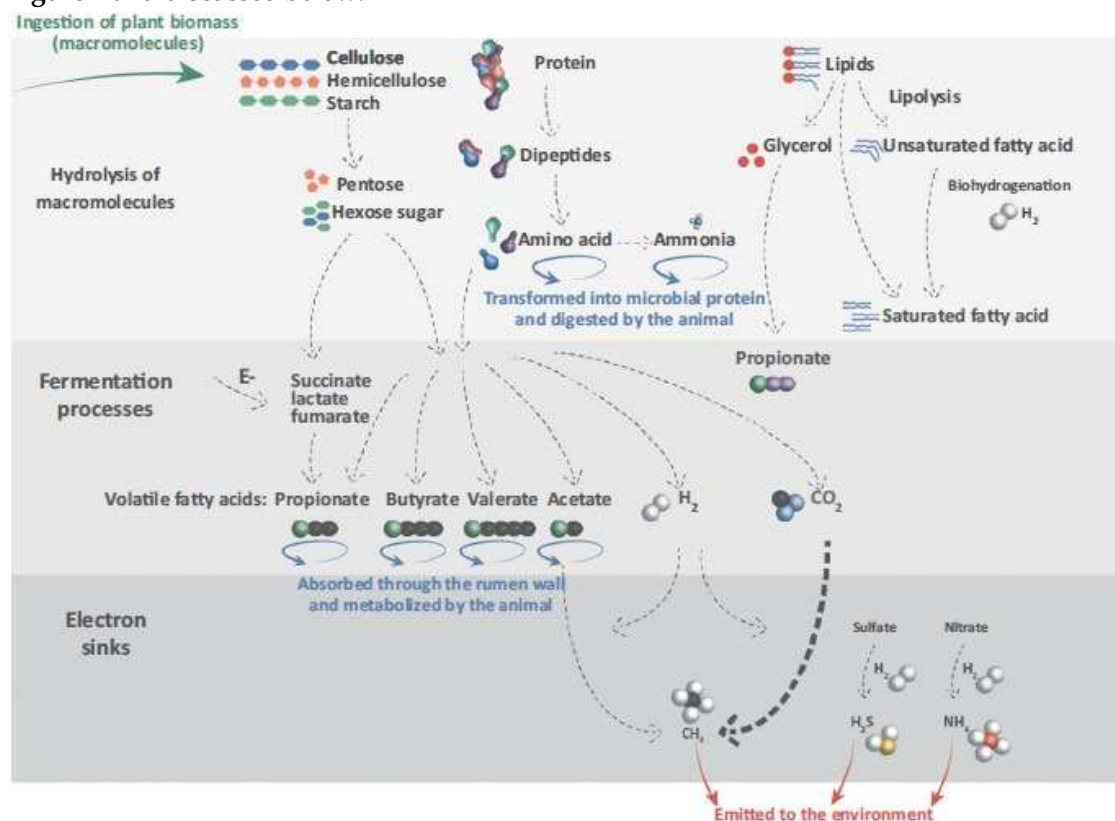


Figure 1. Fundamental pathways of the utilisation of consumed dietary sources by micro-organisms in the rumen to end products absorbed to the blood stream or emitted to the environment (Adopted from Morais and Mizrahi, 2019).

Bacteria is the dominant group in the ruminal microbiota and the major rumen fibre degrading (fibrolytic) bacteria species include, *Fibrobacter succinogens*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* etc. (Russel, 2002; Adejoro, 2019). Equally, structural carbohydrate degrading bacteria species such as *Butyrivibrios fibrisolvans*, and *Eubacterium cellulosolvans* have been reported in the rumen and contain extracellular cellulase with minute cellulolytic efficacy and these bacteria species should be attached to the substrate to carry out the cellulolytic activity owing to their enzymes being retained in their cell wall (Shreck, 2013). Protein degrading bacteria species include *Streptococcus bovis*, *Bacteroides amylophilus*, and *Bacteroides fibrisolvans*, whereas lipid associated species include *Anaerovibrio lypotica*. On the other hand, amylolytic bacteria species include *Bacteroides ruminicola*, and *Bacteroides amylophilus* (Krause et al., 2003). Homoacetogens are acetate-producing bacterial group with 23 diverse bacterial genera and are present in the rumen with concentration ranging from 10^7 to 10^8 cells/g (Drake et al., 2007). These can grow heterotrophically by utilising sugars and autotrophically by utilising metabolic hydrogen and carbon dioxide in the rumen (Fonty et al., 2007).

Autotrophic growth of these bacterial species is catalysed by a hydrogenase enzyme through the Wood-Ljungdhal pathway (Ragsdale and Pierce, 2008)

Anaerobic fungi make up about 10% of the microbiota (Rezaeina et al., 2004), and attach to the plant tissue where they degrade about 34% of the lignified fraction that is indigestible to other microorganisms in the rumen (Lowe et al., 1987). The persistency and abundance of anaerobic fungi is less than that of bacteria because the growth rate of fungi is much slower than the ruminal dilution rate (Krause et al., 2003). Anaerobic fungi are characterised by broad range of highly active enzymes, filamentous growth, and exo-acting cellulase activity. This class of ruminal microorganisms include *Neocallimastix frontalis*, *Caecomycens* (previously named *Sphaeromonas*) *communis*, and *Piromycens* (previously named *Priromonas*) *communis*. Moreover, *Neocallimastix frontalis* are the well-studied and notable exceedingly active fungal species against crystalline cellulose (Nagpal et al., 2004). However, the abundance and activity of anaerobic fungi is regulated to a certain extent by some bacteria and protozoa species in the rumen. The solubility of crystalline cellulose, release of endoglucanase, and production of short chain fatty acids was reduced when chitinolytic *Clostridium tertium* was co-cultured with anaerobic fungi (Hodrová et al., 1995). Also, when anaerobic fungi were incubated with protozoa, it was evident that protozoa are capable to prey and digest fungi (Morgavi et al., 1994). In this view, the presence of some protozoal and bacterial species limit fungi to exhibit their full lignified plant tissue degradation potential.

Of the two existing subclasses of protozoal population in the rumen, only the ciliates are found in the rumen contents of adult animals as the abundance of flagellates diminishes as animal grows. Rumen fluid collected from defaunated sheep revealed an increase in numbers of *Methanovibrio*, and *Selenomonas ruminantium* species (Newbold, 1996). Similarly, *Lactobacillus plantarum* vanished in the rumen due to protozoa predation (Sharp et al., 1994). Therefore, it is prudent to conclusively state that protozoa have the substantial capacity to engulf bacteria as their nitrogen source. Rumen fluid dilution rate is faster than the protozoal population outflow and this renders protozoa as microorganisms with adverse effect to the ruminant host animal due to the outflow of bacteria to the lower gut being reduced, thus a substantial setback in total nitrogen recycling. However, the authors (Devillard et al., 2003) postulated that even though not well understood, the protozoa group may have the capacity to digest fibrous fraction of plant tissue in the rumen.

Impacts of methane reducing strategies on ruminal microbiota

The diversity of microbiomes in the rumen is the key element making the ruminants to adapt in a vast array of dietary conditions (McCann et al., 2014). The research on the concept of host animal's individual microbiome has gained interest due to the demonstrated influence of the diversity and abundance of these communities on the rumen fermentation pathways associated with enteric CH₄ emission (Jami and Mizhari, 2012; Henderson et al., 2015; Weimer, 2015). To examine the rumen ecology, the fundamental use of 16S ribosomal RNA (16S rRNA) as a genetic marker is used, allowing for the identification and quantification of different microbial communities present in the rumen, given that 16S rRNA is a type of ribosomal RNA found in prokaryotic cells (bacteria and archaea). Furthermore, other techniques include but are not limited to; metagenomic sequencing, quantitative polymerase chain reaction, fluorescence in situ hybridisation, microbiota cutting, next generation gene sequencing and bioinformatics analysis.e.tc.

Dietary additives

Tannins

Tannins exert intricate and diverse effects on the microbial populations residing within the digestive systems of ruminants. These effects are depending upon the specific source of tannins or dietary components rich in tannins. For example, in a study of Min et al. (2014) involving goats, the consumption of pine bark (PB) powder led to an increased relative abundance of Clostridia populations compared to the control diet. Conversely, the population of Bacteroidetes, decreased under the same conditions. Smith et al. (2003) revealed that enhancing the oxidative stress response

in *Escherichia coli* allowed these bacteria to counteract the inhibitory effects of condensed tannins. Tabacco et al. (2006) demonstrated the complex nature of tannins, showing that chestnut tannins improved fermentation quality, proteolysis, and the degradation of protein in alfalfa silage. Beyond abundance shifts, tannins also exert influence on the diversity and composition of rumen microbial communities. Terré et al. (2016) found that the introduction of quebracho tannins into the diets of dairy cows resulted in a reduction in the diversity of rumen bacterial communities. Similarly, Patra and Saxena (2011) observed a reduction in the population of methanogenic archaea in sheep rumens when tannins were included in their diets. Furthermore, tannins play a role in regulating the activity and functions of microbial communities. A study by Min et al. (2018) demonstrated that the inclusion of tannins in the diets of goats led to a reduction in the activity of cellulolytic bacteria, resulting in decreased digestibility of fibre. In contrast, Wang et al. (2017) reported an increase in the activity of proteolytic bacteria in sheep rumens due to tannin inclusion, leading to improved utilization of protein and retention of nitrogen. The influence of tannins on rumen microbial communities is a complex phenomenon influenced by various factors such as the origin and concentration of tannins, dietary composition, and the specific microbial species present. This complexity highlights the need for a comprehensive understanding when considering the utilization of tannins to modulate microbial dynamics in the digestive systems of ruminants.

Oils

The study by Gruninger et al. (2021) reported a decrease in both the amplified sequence variants and phylogenetic diversity in the rumen fluid collected at and analysed for different time intervals in response to oil inclusion in the diets of Angus beef heifers. Chestnut oil inclusion in the diet of sheep reduced protozoa microbial populations (Liu et al., 2011). Linseed reduced the abundance of *Ruminococcaceae* in a study of Popova et al. (2019). The study of Guyander et al. (2015) reported an increase in *Selenomonas* abundance in dairy cows fed linseed. These studies agree with the notion stating that fatty acids are toxic to cellulolytic microbes while in favour of succinate reducing microbes to aid the formation of propionate in ruminants (Zened et al., 2012; Maia et al., 2007). This is an indication that the addition of oil treatment into the diet of ruminants modulates the rumen microbiota favouring the proliferation of members of the order *Selenomonadales* (propionate producers; metabolic hydrogen consumers) while reducing cellulolytic *Ruminococcaceae* (hydrogen producers). Therefore, the supplementation of oils facilitates the diversion of hydrogen from the methane formation pathways to the pathway forming propionate the precursor of glucose in ruminants.

Saponin

The findings of Patra and Yu (2013) revealed *in vitro* potency of Quillaja saponin in reducing protozoal counts. These findings from Patra and Yu (2013) are in accord with other studies including an *in vitro* study of Guo et al., (2010), and *in vivo* studies in sheep (Zhou et al., 2011) and goats (Mao et al., 2010) where tea seed saponin inhibited protozoal growth. Similarly, the use of *Yucca shidigera* saponins had the inhibitory effect in ruminal ciliate protozoa *in vitro* (Pen et al., 2006), sheep (Pen et al., 2007), dairy cattle (Lovett et al., 2006), and beef cattle (Lila et al., 2005). However, other studies reported no effect of *Yucca shidigera* saponins on protozoal count *in vivo* (Singer et al., 2008; Holtshausen et al., 2009). The study of Ramírez-Restrepo et al. (2016) reported a decrease in abundance of *R. flavefaciens*, whereas *F. succinogens* and *R. albus* increased in steers fed tea seed saponin. The effect of saponin on the ruminal microbiome from the currently reviewed studies represent a complex picture as there are conflicting results. However, it is clear that fundamentally saponins reduce protozoal counts and their effects are influenced by type of saponin, animal species and microbial species targeted.

Nitrate

When nitrate was added to diets of nitrate-adapted steers there was an increase in abundance of nitrate reducing bacteria including *C. fetus*, *S. ruminantium*, and *M. succiniproducens* (Zhao et al., 2015). Similarly, Lin et al. (2013) reported high abundance of *S. ruminantium* even though it was 0.55% higher than the study of Zhao et al. (2015), which could be attributed to dietary and animal differences between the studies. The supplementation of nitrate increased the abundance of orders *Coriobacteriales* and *Burkholderiales* (Ashida et al., 2010). These orders contain taxa which is known to exhibit nitrate reducing capacity and these are in line with high nitrite concentration in nitrate-fed dairy cows in the study of Guyader et al. (2015). The supply of nitrate to ruminants exerts its influence on microbiota through being an alternative hydrogen sink. This modulates methanogenesis and/or dihydride which are energetically costly to the animal towards production pertaining metabolic hydrogen reductants. The translocation of metabolic hydrogen towards the reduction of nitrate is beneficial for ruminants, but the process is regulated by the ruminal conditions including microbiota abundance and composition, to name yet a few.

3-. Nitroxy propanol

There were no changes on the population of methanogens in response to supplementation of 3-NOP to diets of sheep (Martínez-Fernández et al., 2014) and beef cattle (Romero-Perez et al., 2014). However, the compound decreased methanogen population in dairy cows (Haisan et al., 2014). Also, the abundance of *Methanobrevibacter* genus (hydrogenotropic methanogens) and *Methanomassiliicoccaceae* family (methylootropic methanogens) were reduced by 3-NOP in a study of Martinez-Fernandez et al. (2018). Furthermore, in a similar study the ratio of *Methanobrevibacter* genus to both *Methanomassiliicoccaceae* and *Methanospaera* was reduced in response to the dose supply of 3-NOP to beef cattle, suggesting a greater effect of 3-NOP in hydrogenotropic methanogens. The amount of metabolic hydrogen released does not increase in 3-NOP treated groups compared to the control groups (Haisan et al., 2014; Martinez-Fernandez et al., 2018). This could be an indication of that the substantial concentration of hydrogen in the rumen of animals subjected to 3-NOP is redirected to other reduced end products other than CH₄ and H₂, such as microbial cell mass (Ungerfeld, 2015).

Seaweed

There is scanty literature reporting the use of genomics and metagenomics approaches to examine the effects of seaweed on the composition, abundance, and function of the ruminal microbiome (Abbott et al., 2020). When *Ascophyllum nodosum* was added to the Rusitec *in vitro* fermentation system there were noticeable decrease in growth of *Fibrobacter succinogens* while *Prevotella bryantii* increased (Wang et al., 2009). The abundance of *Methanobrevibacter* reduced in a Rusitec *in vitro* fermentation system in a study of Abbott et al. (2020) and there was a shift in dominance towards *Methanomicrobium* on day 13 of the *in vitro* trial evaluating the effect of seaweed on ruminal microbiome. Dietary administration of seaweed led to a decrease in the levels of *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, and *Prevotella ruminicola*, while *Anaerovibrio lipolytica* increased in abundance (Choi et al., 2021). Roque et al. (2019) reported that *Asparagopsis taxiformis* and other studies evaluating other seaweeds reported similar trend of modulating the metabolic functionality of the methanogens and abundance. Since knowledge about the ruminal microbiome's response to seaweeds is still in its early stages, metagenomic studies are imperative to gain insight into whether the effects of these seaweeds could be manipulated to improve the productivity and health of ruminants.

Dietary manipulation

The 16S rRNA gene libraries in the study of Fernando et al. (2010) revealed two distinct microbial populations in beef steers: one group fed roughage-based diet alone, and the other supplemented with concentrate diet. Furthermore, the researchers reported only 24 common operational taxonomic units (OUTs) found between the rumen contents of steers assigned to two diets out of 398 and 315

OUTs in roughage based and concentrate-supplemented steers, respectively. Consequently, steers fed roughage-based diet had a higher abundance of Fibrobacteres, while concentrate-supplemented steers had a higher number of Bacteroidetes. Metzler-Zebel et al. (2013) found that concentrate supplementation of up to 60% DM in the diet fed to goats reduced the abundance of Prevotella and Fibrobacter succinogens, while the abundance of Lactobacillus species increased.

Mitigating enteric methane emission

There is a global interest on finding effective solutions to ameliorate enteric CH₄ emission arising from ruminant livestock. Hence, extensive research has been conducted to evaluate various approaches to mitigate enteric methane emissions. Notwithstanding numerous strategies previously demonstrating successful evidence of efficacy towards reducing enteric CH₄ emission in conjunction with improved animal productivity, most of these strategies are allied with costs and pose both environmental and human health risks. Therefore, it is imperative to understand existing strategies to facilitate creation of better and efficient solutions towards the curb of CH₄ emission from ruminants.

Plant-based antimethanogenic compounds

Tannins

Tannins are plant secondary metabolites having pH-dependent binding effects with the substrate (proteins) in the rumen (Poudel et al., 2023). There are two forms of tannins based on the molecular weight, which are hydrolysable (HT), and condensed tannins (CT). In most forage species commonly fed to ruminants, HT are less common compared to CT (Aboagye and Beauchemin, 2019). The source, molecular weight and structure of the tannins are factors influencing their reactivity and impact on digestibility in ruminants, which are extensively reviewed elsewhere (Naumann et al., 2017). **Table 1** shows summarised effects of HT and CT from various plats including *Schinopsis lorentzii*, *Schinopsis balansae*, *Prosopis cineraria*, *Quercus robur*, *Ficus infectoria*, *Psidium guajava*, *Acacia mearnsii*, *Castanea sativa*, *Vitis vinifera*, *Onobrychis viciifolia*, *Schinopsis balansae*, and Tea waste on dry matter digestibility, nitrogen balance and CH₄ production in ruminant livestock.

Table 1. Effect of tannins on dry matter digestibility, nitrogen balance parameters and methane production in ruminants.

Tannin source	Tannin type	Inclusion level (g/kg)	Animal species	Diet CP (g/kg)	F: C ratio	Changes relative to the control treatment (%)					Reference
						Digestibility	Urinary N	Faecal N	Retained N	CH ₄	
<i>Schinopsis lorentzii</i>	CT	10	Dairy heifers	265	50/50	-1.20	-15.87	+6.90	+190	n.d	Ahnert et al., 2015
<i>Prosopis cineraria</i>	HT	18.40	Lambs	177	TMR	-3.57	-27.59	+3.33	-1.59	-22	Bhatt et al., 2020
<i>Schinopsis balansae</i>	CT	40	Castrate goats	150	51/49	-6.92	-35.26	+35.13	+22.85	n.d	Al-Kindi et al., 2016
<i>Schinopsis balansae</i>	CT	10	Beef steers	140	TMR	-6.28	-9.76	+32.14	-1.82	+8.62	Ebert et al., 2017
<i>Quercus robur</i>	CT	8.70	Dairy cows	131	TMR	-0.59	-12.15	+3.29	+6.67	-1.06	Focant et al., 2019
<i>Ficus infectoria</i>	CT	15	Lambs	n.d	70/30	-1.55	-20.41	+30.25	+82.07	-14.34	Pathak et al., 2017

and *Psidium guajava*
(mixture of 1:1 ratio)

<i>Acacia mearnsii</i>	CT	40	Wethers	146	50/50	-20.25	-47.98	41.27	-5.88	-31.83	Adejoro et al., 2019
<i>Castanea sativa</i>	HT	15	Beef steers	171	50/50	n.d	-6.74	n.d	n.d	+3.24	Aboagye et al., 2018
<i>Vitis vinifera</i>	CT	15.5	Goat kids	127	55/45	+4.92	-17.53	+52.09	-26.94	+1.19	Sinz et al., 2021
Tea waste	CT	15	Dairy goats	164	TMR	-2.04	-32.94	+5.56	+0.7	+10.02	Sundod et al., 2023
<i>Onobrychis viciifolia</i>	CT	8.8	Dairy cows	172	TMR	-5.45	+3.10	+15.13	+145.45	-2.71	Huyeen et al., 2016
<i>Schinopsis balansae</i>	CT	40	Beef heifers	103	TMR	-19.10	n.d	n.d	n.d	-56.26	Piñeiro-Vázquez et al., 2018

N; nitrogen, F:C; forage to concentrate, CH₄; methane, CT; condensed tannins, HT; hydrolysable tannin, n.d; no data, TMR; total mixed ration; negative and positive values respectively imply either a decrease or an increase in digestibility, nitrogen balance parameters and methane production in response to tannin supply treatments compared to the control groups of ruminant livestock.

Tannin-protein binding complexes reduce ruminal digestion of protein thereby improving protein bypass in the host animal. Yanza et al. (2021) postulated that the bypass protein is not absorbed in the small intestine because protein-tannin molecule bonds are resistant to enzymes in the small intestines and therefore it is directly excreted in faeces. In consistency with such, in the study by Dschaak et al. (2010), milk urea nitrogen (N) was reduced, whereas the concentration of faecal N increased, which is partly explained by the poor absorption of protein in the small intestine due to tannin supply in dairy cows. Moreover, Orlandi et al. (2015) reported a linear increase in N retention, and faecal N concentrations while ammonia concentrations in the rumen decreased in steers supplemented with *Acarcia mearnsi*. Similarly, the findings from Wischer et al. (2014) revealed an increase in faecal N concentration in sheep supplemented with tannin from valonia and chestnut. These studies are in congruent with the observation of increased faecal N content in relation to tannin supply among studies summarized in **Table 1**. Also, methane and ammonia production were reduced in response to tannin inclusion in diets fed to Polish Holstein-Frisian dairy cows in a study by Cieslak et al. (2012). Therefore, it is noticeable with caution underlying the low dose, that tannins improve nitrogen efficiency and alleviates ammonia toxicity incidents in ruminants. Authors (Silanikove et al., 2001; Woodward et al., 2004; Lima et al., 2019) have reported that tannins have a toxic effect on ruminal microorganism, particularly fibrolytic bacterial species thereby adversely affecting fibre degradation in the rumen which is evident in reduced dry matter digestibility in **Table 1**. In view of tannin type (CT and HT), the CT have the greatest capacity to reduce ruminal degradability compared to HT owing to CT having strong affinity for particulate phase of digesta and is inherently resistant to hydrolysis, whereas HT is easily degraded by the ruminal microbes (Yanza et al., 2021). Howsoever, protein bypass, and the reduction of enteric gaseous emissions to the environment in response to tannin supply are noticeable benefits in ruminants (Hassanpour et al., 2011).

Terpenes

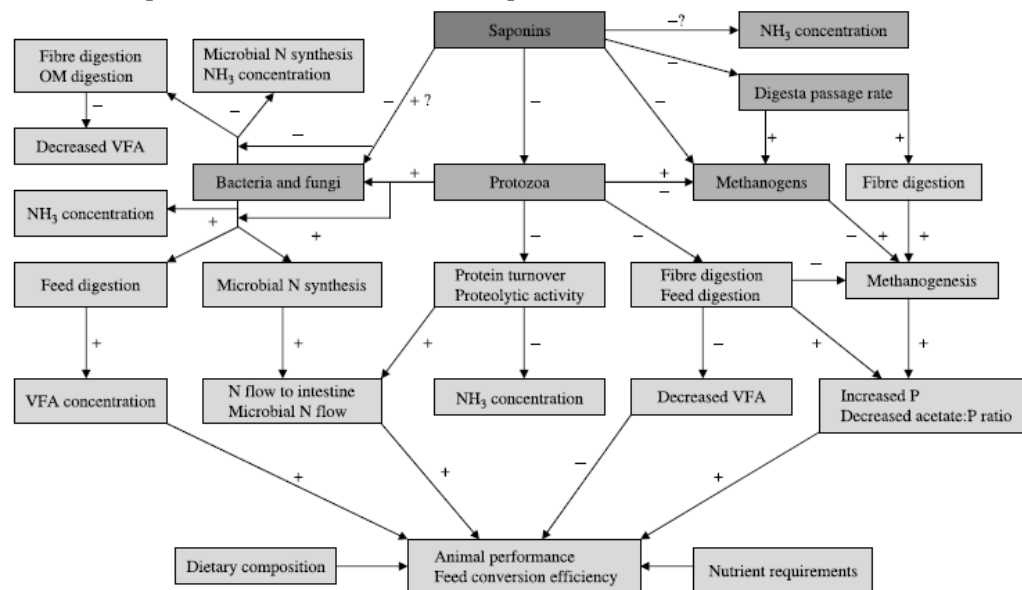
The terpene group is one of the most diverse natural products (Tiwari and Rana 2015). The group has a 5-carbon chain length with the biological activity in ruminants ranging from herbivory tolerance to ruminal microbiota suppression. The ethnoveterinary benefits of terpenes include the control of infections caused by fungi, bacteria arthropods, and gastrointestinal nematodes (Silver et al., 2020). Ghamkhar et al. (2018) reported that terpenes reduce enteric methane emission *in vitro*. Equally, the inclusion of terpene-based *Pinus densiflora* (50 mg/L) and *Mentha canadensis* (70 mg/L) extracts in diets evaluated for methane emission *in vitro* using rumen fluid from steers offered Timothy hay and commercial concentrate at 60:40 forage to concentrate ratio, showed a decrease on CH₄ production (Lee et al. 2020). Furthermore, it was conclusively advised that the inclusion level threshold of terpenes on ruminant diets should be 30 mg/L (Lee et al., 2020). However, *in vivo* studies using terpenes as CH₄ mitigating strategy is scarce. Therefore, *In vivo* studies of terpenes need to expand to realize the potential of terpenes in alleviating CH₄ emissions in ruminants.

Oils

The study of Chillard et al. (2009) show a reduction in dry matter intake (DMI) in response to linseed oil inclusion in lactating dairy cows. Moreover, such was reflected on the suppression of milk yield by 15% in the linseed compared to the control treatment. Also, milk fat content was lower for a diet containing the linseed oil compared to other treatments, such would be due to the oils suppressing DMI owing to reduced rumen degradability and passage rate. The reduction in ruminal degradability could be possibly attributed to oils coating feed particles and creating a physical barrier that reduces microbial attachment to the digesta in the rumen. Such ultimately influences the rumen fill which is the physical distension of the rumen and is implicated to regulate dry matter intake. Therefore, it could be possible that dry matter intake was suppressed through the slow passage clearance owing to reduced degradability. In a study with non-lactating Holstein dairy cows, linseed oil inclusion reduced CH₄ emission by 22% compared to the control treatment and by 5% more than the nitrate fed group. When Sunflower oil was added to high versus low roughage-based rations of dairy cattle in the study by Bayat et al. (2017), the methane emission (g/d and g/kg) was reduced, respectively. Also, the dry matter intake was noticeable lower in lactating dairy cows fed high roughage-based diet compared to the other group (Bayat et al., 2017). The observed reduced dry matter intake in lactating dairy cows fed low concentrate diet could be attributed to interacting factors which are prolonged retention times of digesta associated with the high fibre content (carbon: nitrogen ratio) found in roughage-based diet compared to high concentrate diets and the physical barrier created by the oils in the digesta preventing access by the ruminal microbes for microbial fermentation. Chestnut oil inclusion in the diet of sheep reduced CH₄ emission with no adverse effects on the growth performance of Rideau Arcott sheep (Liu et al., 2011). Origanum oil as an additive in diets formulated for sheep (small Tailed Han × Dorper male castrate sheep) resulted in linear decrease of CH₄ production. Therefore, inclusion of oils in ruminant diets have both the positive and negative feedback, there is variability in response by ruminants based on the inclusion levels and the source of the oils (Giannenas et al., 2011). The use of oils in ruminant diets suppresses dry matter intake at higher doses while reducing or at least maintaining methane emission.

Saponin

Saponins are glucosides with foaming characteristics and defaunating capacity directly targeting ciliate protozoa in the rumen which have positive symbiotic relationship with methanogens, lowering the abundance of methanogens associated with protozoa, thus exerting their influence on reducing enteric methane production (Benchaar et al., 2007). In an *in vitro* study by Patra and Yu (2013), in response to a dose rate of 0.6 and 1.2 g/L of quillaja saponin they reported a 12% and 25% reduction in methane production, respectively. However, Goel et al. (2008) reported no change in methane production even though the protozoal counts were adversely affected. Notable, the relationship between the methanogens and protozoa is not unidirectional which is reflected by the lack of correlation in reduction of protozoal count and methanogenesis. Tea saponin reduced *in vitro* CH₄ production whereas it increased (+14%) CH₄ yield (g/kg DMI) in lactating dairy cows *in vivo*



Ether extract

Halogenated methane analogue

Seaweeds (e.g., *A. Armata* and *A. taxiforms*) contain bromochloromethane (BCM), a compound that inhibits cobamide-dependent methyl transferase step in enteric methane formation pathway in the rumen. This compound has been reported to reduce CH₄ yield and improve feed efficiency in sheep and cattle (May et al., 1995; Goel et al., 2009). In contrary to this view, results from studies

conducted in beef cattle are consistent in dose-dependent reduction of DMI (-7.4% to -11.3%) and CH₄ yield (-90.6% to -95.2%) in response to BCM (Johnson et al., 1972; McCrabb et al., 1997). However, BCM in sheep and goats had little (-0.5%) to no detrimental effect on DMI while CH₄ yield was reduced by 33.6% to 86.9% (Sawyer et al., 1974; Abecia et al., 2012). Methane mitigating potential of seaweed (containing BCM) has been evaluated in beef steers (Kinley et al., 2020), dairy cows (Roque et al., 2019) and sheep (Li et al., 2018) with consistent CH₄ reduction. When *A. Armata* was used in dairy cows at the dose rate of 1% in dry matter basis there was a 37% and 50% decrease in dry matter intake (DMI) and CH₄ yield (g/ kg DMI), respectively (Roque et al., 2019). In a study by Kinley et al. (2020) beef steers in a feedlot subjected to *A. taxiforms* had a linear decrease in DMI and CH₄ yield by -0.4% and -98%, respectively. However, in a study by Li et al. (2018) when *A. taxiforms* was used in sheep at higher dosage (3% vs 0.2%) compared to beef steers in a study by Kinley et al. (2020), there were no changes in DMI whereas CH₄ yield reduction was lower in magnitude (80.7% vs 98%). Beside the study of Kinley et al. (2020) the results from studies evaluating the efficacy of halogenated CH₄ analogues on enteric CH₄ from ruminant livestock are consistent in reducing CH₄.

Chemical Antimethanogenic Feed Additives

3-. nitrooxy-propanol

3-nitroxy-propanol (3-NOP) is an analogue of enzyme methyl-coenzyme-M, known for its inhibitory role for enzyme methyl-coenzyme-M reductase (Duval and Kinderman, 2012; Pitta et al. 2018). The Methyl-coenzyme M is a cofactor involved in the transfer of methyl group to methyl-coenzyme M reductase (MCR) at the end step of methane formation in the rumen. The MCR in the tetrapyrrole derivative known as cofactor F₄₃₀ contains nickel and for it to be in the active state it should be in a form of nickel (Ni⁺) oxidation state (Jayanegara et al., 2017). The active site of MCR is directly inactivated through oxidation by 3-nitroxy-propanol (3-NOP), forming nitrate, nitrite, and 1,3-propane-diol (Duin et al., 2016; Jayanegara et al., 2017). There are two major metabolic stages of 3-NOP *in vivo*, first step is the oxidation of the primary alcohol to a carboxylic group (i.e., 3-nitroxy-propionic acid; NOPA) and lastly the nitrate ester cleavage to form 3-hydroxy-propionic acid; HPA (Figure 2).

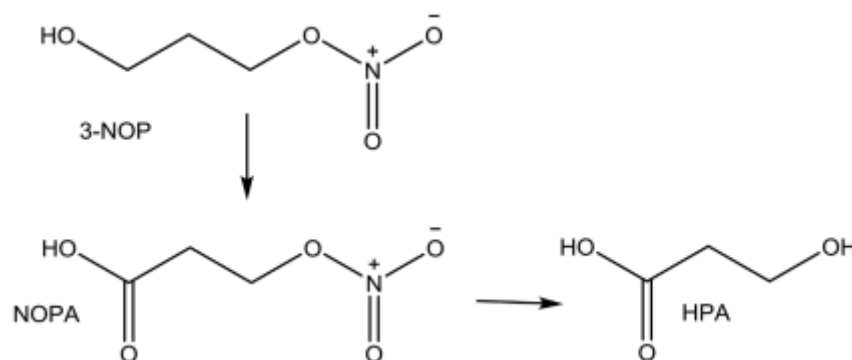


Figure 2. The chemical structures of 3-NOP and the metabolites (NOP and HPA) in the blood plasma (Adapted from Thiel et al., 2019).

In a study by Garcia et al. (2022), lactating Holstein Friesian dairy cows had a 54% decrease in CH₄ emission per digestible organic matter in response to 3-NOP at the supplementation rate of 100 mg/kg DM. Short-term supply of 3-NOP on DMI in lactating dairy cows had no effects even at the highest inclusion level of 120 mg/kg MD (Melgar et al., 2020). There was a linear dose-dependent decrease in CH₄ corrected for DMI in Angus heifers fed high forage diet supplemented with incremental levels of 3-NOP, and at the highest inclusion level there was a 33% reduction of CH₄ g/kg DMI (Romero-Perez et al. 2014). The 3-nitroxy-propanol has never manifested any toxicity in ruminants when used as CH₄ abatement strategy in sheep (Martinez-Fernandez et al., 2013), dairy cattle (Reynolds et al., 2014; Garcia et al., 2022), and beef cattle (Romero-Perez et al., 2014). In a meta-

analysis by Jayanegara et al. (2017) it was predicted that the inclusion of 3-NOP at 200 mg/kg DM may reduce CH₄ emissions by 33.4-42.1% in ruminants. The use of 3-NOP at the threshold of 200 mg/kg DM and beyond warrants further studies both *in vitro* and *in vivo* on reducing CH₄ emissions and to facilitate the adoption of this dose it should not induce reduced *in vitro* and *in vivo* dry matter digestibility and intake in ruminants (Vyas et al., 2016).

Nitrate

Nitrate (NO₃⁻) provides non-protein nitrogen to the ruminal microbiota and outcompetes the methanogenesis pathway for metabolic hydrogen (H₂). Nitrate has a strong affinity for H₂ than carbon dioxide (CO₂) in the rumen system (Latham et al., 2016; Almeida et al., 2021). Hence, the energy from the consumed substrate by the ruminant usually lost in methanogenesis is redirected to microbial growth in the rumen system. However, the use of nitrate in ruminants as antimethanogenic agent has limited application due to toxicity through methemoglobinemia. Nitrite is formed post-ingestive of NO₃⁻ and when absorbed into the bloodstream of the ruminant it oxidizes the iron (Fe²⁺) in haemoglobin forming methemoglobin (Met-Hb). However, to circumvent such, animals are gradually introduced to NO₃⁻ to attain persistency. Also, nitrite as an intermediate of nitrate reduction has a direct toxic effect to methanogens (van Zijderveld et al., 2010 and Zhou et al., 2012). When the dose rate of 0.9 g NO₃⁻/kg^{0.75}/d was evaluated in a growth trial of Thai native × Angola-Nubian goat kids either fed rice straw or Pongola grass there was an overall increase in DMI by 3% (Yuangklang et al., 2022). In the study by Adejoro et al. (2020) there was a 20% improvement in average daily gain with 17% reduction in methane (g/kg DM intake) of lambs supplied with nitrate. The authors concluded that the energy usually lost in methane formation could have shifted towards growth performance in lambs receiving nitrate-based diets. Other studies evaluating nitrate supplementation reported consistent reduction in methane production in beef cattle (Hulshof et al., 2012; Granja-Salcedo et al., 2019), dairy cows (van Zijderveld et al., 2011; Olijhoek et al., 2016), and sheep (Nolan et al., 2010; Guyader et al., 2016). The variability in the magnitude of reduction in methane production could be attributed to quite several possible factors which include but are not limited to; differences in animal species, age of trial animals, basal diets, and NO₃⁻ dose rate.

Diet Manipulation

Dry matter intake is the principal variable influencing enteric methane emissions in ruminants (Ellis et al., 2007; Hristov et al., 2013) and grazing is the most common and economical system of feeding for herbivores (Pepeta et al., 2022). However, this system is unavoidable subject to seasonal variation of quantity and quality of pastures, which in turn affect production performance and carbon footprint of animals reared under this system. During the seasons where forage availability is low with poor quality such as in dry winter season in the tropics, supplementation is of paramount importance to at least maintain production performance of ruminants in these regions. When 0 vs 5 kg/head of cow per day was supplemented to grazing Holstein-Frisian lactating dairy cows in a study by Muñoz et al. (2015) there were no changes in CH₄ g/kg DMI (CH₄ yield) and CH₄ g/kg milk yield (CH₄ intensity). This could be attributed to the fact that in the study by Muñoz et al. (2015) animals were grazing pastures with temperate grass (*Lolium perenne*) which is of good quality. Therefore, supplementation of concentrate increased both the milk yield and CH₄ through improved dry matter intake, such that the CH₄ g/kg milk yield remained unchanged. Conversely, in lactating Jersey dairy cows grazing “medium quality” *Pennisetum clandestinum*-dominant pastures in summer in the tropics, CH₄ yield, and intensity decreased linearly while CH₄ g/day (production) increased with incremental levels of concentrate supplementation (van Wyngaard et al., 2018). Similarly, Jiao et al. (2014) reported congruent results in lactating Holstein-Frisian dairy cows grazing *Lolium perenne* and supplemented with incremental levels of concentrate diets. Both studies by the authors (Jiao et al., 2014 and van Wyngaard et al., 201), reported induced increase in total dry matter intake with the proportion of forage consumed decreasing when concentrate supplementation levels were increasing. In this view, the addition of concentrate diets in dairy rations shifted the concentration of neutral detergent fibre (NDF) to starch ratio to a lower proportion resulting in reduced CH₄

production (Knapp et al., 2014). Also, the controversy of reduced CH₄ yield and intensity with increased CH₄ production in a study of van Wyngaard et al. (2018) could be attributed to the shift in the rumen system caused by the increase in starch to NDF ratio not being enough for propionate production rather favored the production of butyrate. Medium-high quality forages are characterized with higher amounts of starch and carbohydrates, altering the abundance and functionality of the microbiota in the rumen. From such, there is a noticeable increase in proteobacteria phylum and *Succini vibrionaceae* family which are hydrogen-consumers to form succinate a propionate precursor, thus reducing enteric CH₄ production (Karekar et al., 2022). Brazilian semi-arid non descriptive goats had reduced CH₄ production and yield with increasing total dry matter intake and average daily gain in response to increasing levels of concentrate supplementation (Barbosa et al., 2017). Conversely, goats and sika deer had increased CH₄ production and yield in response to increasing levels of concentrate supplementation in consumed diets (Na et al., 2017). Goats and sika deer are intermediate and concentrate eaters, respectively, thus they have low stomach capacity, large opening with fast passage rate of digesta (Na et al., 2017). Therefore, the indigested material in the rumen of concentrate and intermediate feeders passes to the omasum at a faster rate for complete methanogenesis to occur due to short retention time. High residual feed intake groups of Holstein-Friesian and Jersey cows when both were fed high concentrate diet (61% of total diet, DM basis) compared to high fibre diet (68% of total diet, DM basis) had lower CH₄ (L/day) of 540 vs 635 and 493 vs 499, respectively (Olijhoek et al., 2017). Reduction in forage to concentrate ratio in diets of ruminants (grazers) is noted to reduce CH₄ production without adversely affecting production performance and it is advisable that this strategy should be applied with caution to avoid acidosis occurrence especially in animals fed medium-high quality forages.

Exogenous Supply of Microbes

Exogenous supply of microbes commonly referred to as direct fed microbials are living organisms, either alone or in a mixture, that encourage the growth of beneficial microorganisms in the rumen and offer positive effects when given to animals (Tseten et al., 2022). Several rumen bacteria are believed to engage in competition with methanogens for the available hydrogen supply by stimulating processes like propionogenesis, acetogenesis, and the reduction of nitrate/nitrite or sulfate, which can serve as an alternative way to consume metabolic hydrogen (Ungerfeld, 2015). Possible ruminal pathways modulated by exogenously supplied microbes is depicted in **Figure 3**.

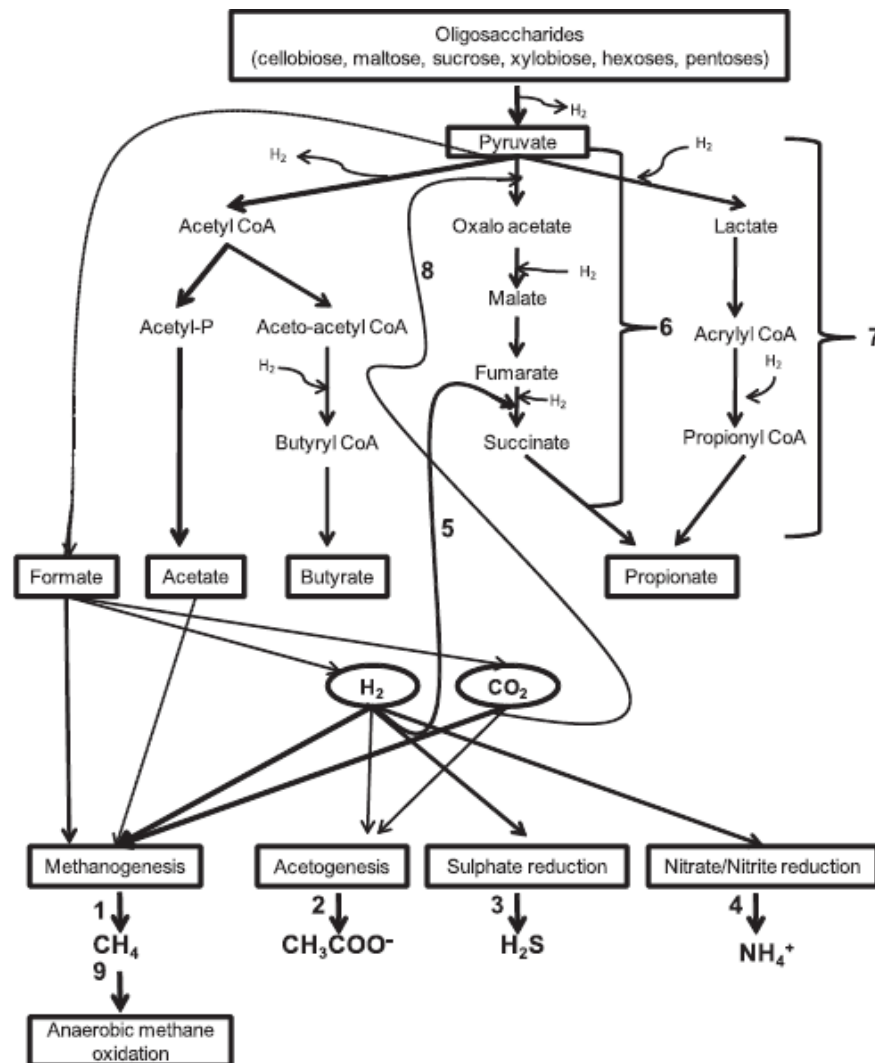


Figure 3. Modulated pathways of ruminal fermentation due to exogenously supplied bacteria (adopted from Jeyanathan et al., 2013).

Acetogens

Several attempts have been made to isolate homoacetogens from the rumen and analyze their role as an alternative hydrogen sink, including *Acetitomaculum ruminis*, *Eubacterium limosum*, *Blautia schinkii*, and *Blautia producta* (Henderson et al., 2010). Furthermore, *in vitro* studies have also suggested that acetogenesis could serve as an alternative to methanogenesis in eliminating metabolic hydrogen [H] from the rumen (Morvan et al., 1996). However, their abundance and affinity towards [H] are generally lower than hydrogenotrophic methanogens (Le Van et al., 1998; Lopez et al., 1999). As Lopez et al. (1999) have concluded, high concentrations of acetogenic bacteria cannot compete against methanogens for [H] disposal, making it unclear whether homoacetogens could play a pivotal role in the ruminal ecosystem (Henderson et al., 2010). Homoacetogens, found in diverse environments including the rumen, possess the ability to produce metabolic acetate through heterotrophic and autotrophic growth. Stimulating their autotrophic growth is considered a competitive strategy against methanogenesis, as both pathways share common substrates. While they exhibit variability in abundance based on factors like diet and animal age, a negative correlation between homoacetogens and methanogens in the rumen of adult ruminants has been observed (Henderson et al., 2010). Efforts to utilize homoacetogens to mitigate ruminal methane production as direct fed microbials (DFMs) have encountered limitations. Methanogens hold advantages in terms of [H] threshold and energy yield. Furthermore, the ability of acetogens to grow heterotrophically

affects their potential as effective methane reducers. However, the feasibility of utilizing these acetogens as DFMs depends on their performance under rumen conditions.

Propionate-forming bacteria

Methane emission (g/kg DMI) was reduced in a study of Vyas et al. (2014) using a combination of *Propionibacterium jensenii* P54 with P169 and P5 strains of *Propionibacterium acidipropionic* in steers fed roughage-based diets. However, when the same strains were used in beef heifers fed grain rich diet they were ineffective on CH₄ emission (Vyas et al., 2014). In the study of Philippeau et al. (2017) *Propionibacteria* failed to elicit changes on CH₄ emission in dairy cows receiving high-grain diet as opposed to high fibre diet where there was a decrease on CH₄ emission. These studies suggest that the effect of *Propionibacteria* is masked or negated by the general high production of propionate in ruminants fed high grain-based diets. However, this strategy has a great potential of utilisation in ruminants feeding on roughage-based diets including grazing ruminants.

Methylobacteria

Methanotrophs are methylobacteria (microorganism utilising organic mono-carbon compounds as energy source) that classically utilise CH₄ thereby preventing its emission from ruminants to the environment. Generally, methylobacteria utilise compounds such as methanol (CH₃OH) and methylamine (CH₃NH₂) as their carbon sources rendering them suitable competitors with methanogens for substrate. Attainment of meticulous better apprehension of biological pathways entailed in the metabolism of methylobacteria may provide novelty in biological control of enteric CH₄. There is dearth of literature regarding the use of these compounds as probiotics in ruminants. Methanotrophs utilize monooxygenase as their enzyme that converts CH₄ to CH₃OH through oxidation (Tseten et al., 2022). The latter is then further oxidized to formaldehyde and the enzyme involved as a catalyst is CH₃OH dehydrogenase. Thereafter, formaldehyde is assimilated into either serine or ribulose-5-monophosphate pathway aiding biomass synthesis (Kalyuzhnhaya et al., 2015). However, there is only one study by Kajikawa et al. (2003) reporting methanotrophy and it was an *in vitro* study with minute evidence of between 0.2-0.5%. Other methylobacteria such as *Methanospira* spp. are considered important in ruminants fed forage-based diets (Jayanathan et al., 2011). However, the literature reporting *in vivo* studies of methanotrophy remains scanty, therefore there is a need to develop isolation, screening and *in vivo* validation studies to meticulously understand the potential of methylobacteria in lessening enteric CH₄ emission.

Conclusions

Chemical additives like 3-NOP, nitrate, and halogenated compounds have demonstrated significant methane reduction in both *in vitro* and *in vivo* experiments. Such is achieved by either fostering the growth of microbes that compete for resources with methane producers or by directly inhibiting methane production. The focus has shifted towards natural feed additives such as oils, seaweed extracts, and other plant-derived compounds due to human health concerns. Additionally, the use of probiotics as animal dietary supplements has gained importance. One of the approaches is introducing microbes that compete for hydrogen with methane-producing organisms, like propionate-forming bacteria and acetogens. The other relies on methylobacteria, particularly methane-oxidizing bacteria, which directly consume methane produced during rumen fermentation. Developing efficient strategies to mitigate methane emissions while enhancing animal performance is crucial for agricultural sustainability. However, there are conflicting observations regarding the use of organic and inorganic strategies in ruminants to reduce methane. In many cases, when used in high doses to achieve significant methane reduction, these strategies negatively impact dry matter intake and digestibility, potentially reducing overall production performance. Conversely, when used at lower doses to maintain intake, digestibility, and performance, methane reduction is often insufficient. Incorporating data from various global experiments conducted in ruminants using meta-

analytical approaches to identify potential benefits of these strategies in mitigating methane emissions and improving production performance, is mandatory and needs urgent attention.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, X.X. and Y.Y.; methodology, X.X.; software, X.X.; validation, X.X., Y.Y. and Z.Z.; formal analysis, X.X.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.”, please turn to the [CRediT taxonomy](#) for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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