

Feed additives as a strategic approach to reduce enteric methane production in cattle: modes of action, effectiveness and safety

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Abstract. Increasing consumer concern in greenhouse-gas (GHG) contributions from cattle is pushing the livestock industry to continue to improve their sustainability goals. As populations increase, particularly in low-income countries, the demand for animal-sourced foods will place further pressure to reduce emission intensity. Enteric methane (CH₄) production contributes to most of the GHG from livestock; therefore, it is key to mitigating such emissions. Feed additives have primarily been used to increase animal productivity, but advances in understanding the rumen has resulted in their development to mitigate CH₄ emissions. The present study reviewed some of the main feed additives with a potential to reduce enteric CH₄ emissions, focusing on *in vivo* studies. Feed additives work by either inhibiting methanogenesis or modifying the rumen environment, such that CH₄ production (g/day) is reduced. Feed additives that inhibit methanogenesis or compete with substrate for methanogens include 3-nitroxypropanol (3NOP), nitrates, and halogenated compounds containing organisms such as macroalgae. Although 3NOP and macroalgae affect methyl-coenzyme M reductase enzyme that is necessary in CH₄ biosynthesis, the former is more specific to methanogens. In contrast, nitrates reduce CH₄ emissions by competing with methanogens for hydrogen. However, nitrite could accumulate in blood and be toxic to ruminants. Rumen modifiers do not act directly on methanogens but rather on the conditions that promote methanogenesis. These feed additives include lipids, plant secondary compounds and essential oils. The efficacy of lipids has been studied extensively, and although supplementation with medium-chain and polyunsaturated fatty acids has shown substantial reduction in enteric CH₄ production, the results have been variable. Similarly, secondary plant compounds and essential oils have shown inconsistent results, ranging from substantial reduction to modest increase in enteric CH₄ emissions. Due to continued interest in this area, research is expected to accelerate in developing feed additives that can provide options in mitigating enteric CH₄ emissions.

Keywords: greenhouse gases, methanogens, rumen function, ruminants.

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Introduction

The livestock sector is crucial for food and nutrition security globally, with a projected increase of 80% in consumer demand by 2050 for beef (Nadathur *et al.* 2017). Approximately 83% of global milk is produced from cattle (Visioli and Strata 2014) and, by the end of the decade, milk output is anticipated to have grown by 33% and 9% in developing and developed countries respectively (OECD/FAO 2018). Globally, beef is the third-most consumed meat, contributing 320 million tons of product to world food supply, representing 79% of total sourced meat (Opio *et al.* 2013; Ritchie and Roser 2019). Nutritional benefits from ruminants are pronounced as they have the ability to convert fibre-dense forages that are indigestible to humans into high-quality bioavailable nutrient sources. In fact, 86% of the feed

consumed by livestock worldwide is not considered edible for human consumption (Mottet *et al.* 2017). At the same time, ruminants occupy more land than do any other livestock species and their enteric methane (CH₄) emissions contribute to total anthropogenic greenhouse gases (GHG; Knapp *et al.* 2014). Enteric CH₄ is under increased scrutiny due to its heightened potency compared with carbon dioxide (CO₂) in the atmosphere, and the 39% it contributes to the sector's total emissions (Gerber *et al.* 2013; IPCC 2013).

Heightened attention on climate change by scientists, governments and consumers is challenging the livestock industry to reduce GHG emissions. Arguments for consumers to shift towards plant-based diets have gained traction; however, constructing diets on the basis of the level of GHG emissions will not necessarily have a positive

correlation with nutritional provision (Payne *et al.* 2016). Dietary manipulation has been studied over the past few decades as a strategy to reduce enteric CH₄ emissions and could be assimilated into management practices, notably through feed additives (Cottle *et al.* 2011). Feed additives are used in livestock diets to improve feed-use efficiency, quality of animal-source foods, and animal performance and health. These additives include vitamins, amino acids, fatty acids, minerals, pharmaceutical compounds, fungal products and steroidal compounds. Recent advances in understanding methanogenesis have led to the development and discovery of feed additives that can reduce CH₄ emissions to varying degrees. The present review aims to provide a concise summary of feed additives currently available, or in development, with some potential to reduce CH₄ emissions from ruminants. The secondary objective of the review is to summarise information on mode of action, efficacy, safety and readiness for adoption of anti-methanogenic feed additives. Although the focus is on feed additives tested *in vivo*, some *in vitro* studies are also discussed if there is paucity of *in vivo* trials for an additive or to help explain mode of action.

Rumen methanogenesis

Methane production can be substantial in ruminants, representing up to 12% of gross energy intake that could potentially be utilised for physiological processes, but, instead, is released into the atmosphere through eructation

(Beauchemin *et al.* 2009a). However, CH₄ synthesis represents a significant metabolic sink for reducing equivalents (hydrogen, H₂) that would otherwise accumulate in the rumen and create an unfavourable environment for fermentative digestion processes (Morgavi *et al.* 2010). Hydrogen itself does not accumulate due to methanogen activity, instead, methanogens participate in interspecies H₂ transfer, and dispose of the reducing equivalents from other metabolic processes (Bergman 1990; McAllister *et al.* 1996). Hydrogen synthesis is a self-limiting process that relies on separate and distinct reducing equivalent consumption pathways so as to continue production. Cellulose-degrading activity in both bacteria and fungi increases in the presence of methanogens, which contributes to the principle of rumen syntrophic relationships (Bauchop and Mountfort 1981; Sasaki *et al.* 2012).

Rumen methanogenesis is performed strictly by archaea (Hook *et al.* 2010). A methanogenesis pathway is presented in a simplified diagram (Fig. 1), which includes the convergence of pathways known to occur in a *Methanosarcina* spp. Lambie *et al.* (2015) categorised methanogens on the basis of their metabolic pathways, as follows: hydrogenotrophic, acetoclastic and methylotrophic that can yield CH₄ in the rumen from *Methanosarcina* spp. Methanogens reduce CO₂ with H₂ (hydrogenotrophic), source a methyl group from acetate (acetoclastic), or a methyl group from compounds such as methanol, methylthiol, dimethylamine, and mono-, di-, tri- methylamine (methylotroph). Formate contributes to methanogenesis as an electron donor within the

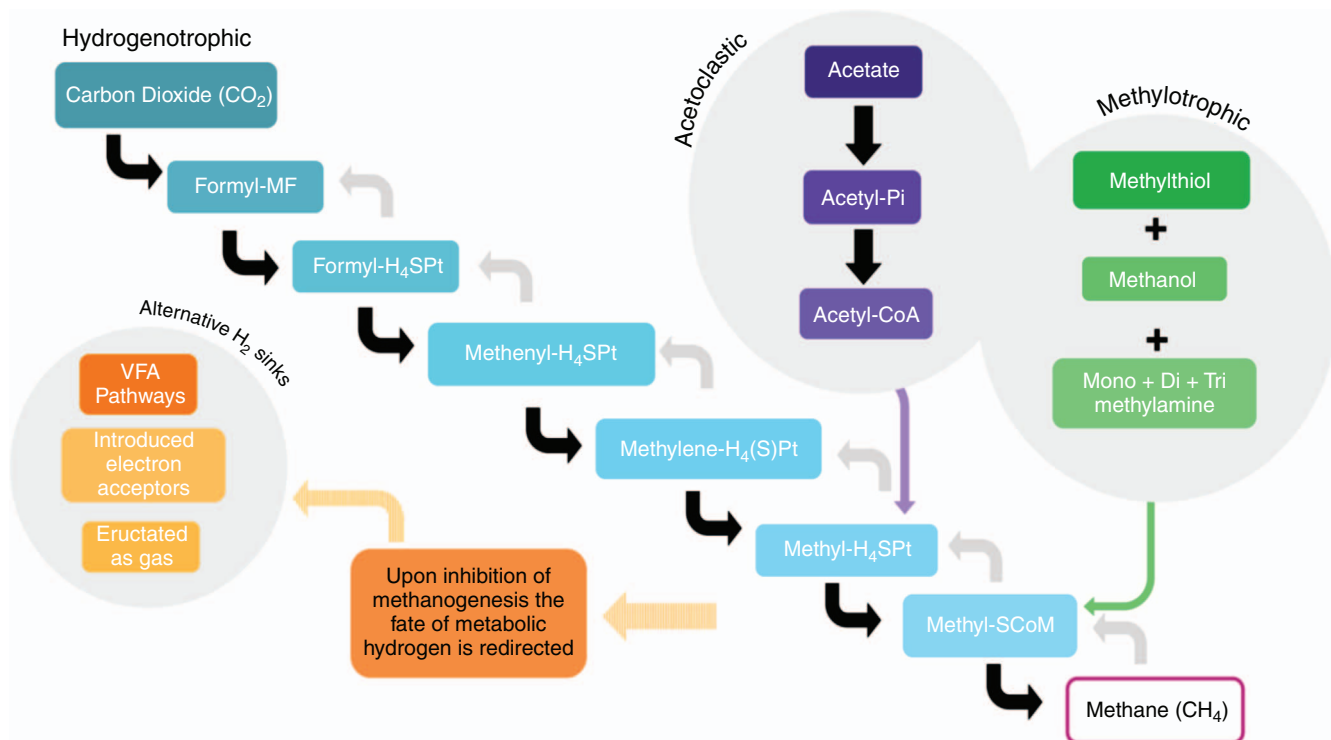


Fig. 1. Simplified methanogenesis pathway from *Methanosarcina barkeri* CM₁, adapted from Lambie *et al.* (2015). The three pathways depicted include hydrogenotrophic (carbon dioxide utiliser), acetoclastic (acetate utiliser) and methylotroph (methyl-group utiliser), which all have the potential to donate a methyl group and form methane.

hydrogenotrophic pathway, representing ~16–18% of CH₄ in batch- and continuous-culture experiments (Seedorf *et al.* 2014; Ungerfeld 2015; Hungate *et al.* 1970). Coenzyme M requires a methyl group for the reduction to CH₄, which is provided through each of these pathways. Methane mitigation could be achieved by directly targeting methanogens or modifying the rumen environment to shift the metabolic pathways away from methanogenesis or reduce substrates for the archaea.

Rumen inhibitors

Feed additives classified as CH₄ inhibitors directly act on the methanogenesis pathway (Fig. 1) in a way that can disrupt the process and reduce CH₄ production (g/day). Methanogens prevent H₂ accumulation in the rumen, which otherwise may lead to adverse effects on fibre degradability and animal performance (Ellis *et al.* 2008). Given the importance of efficient fibre digestion, the use of CH₄ inhibitors must balance between reducing CH₄ production and avoiding negative impacts on animal performance and welfare. Inhibition of methanogenesis requires a redirection of reducing equivalents, H₂ in this case, to alternative sinks, instead of CO₂, unless the inhibitor's mode of action is a highly competitive electron acceptor. Malik *et al.* (2015) argued that H₂ clearance through pathways such as reductive acetogenesis and propionogenesis also has the advantage of energy conservation into end products such as meat and milk. Several of these alternative sinks will be reviewed herein and may also be implemented as independent feed additives or with an inhibitor. Studies have shown a decrease in CH₄ emissions paired with an increase in H₂ emissions without the addition of an

alternative sink (e.g. Roque *et al.* 2019b), indicating that elevated H₂ concentration in the rumen may not necessarily result in decreased fermentation, and hence, productivity.

3-nitroxypropanol (3NOP, marketed as Bovaer in the European Union)

Methyl-coenzyme M reductase (MCR) is the enzyme that catalyses the final step of the methanogenesis pathway from intermediate methyl-CoM to CH₄ as illustrated in Fig. 1. As a nickel enzyme, MCR can catalyse this step only when its Ni ion is in the +1-oxidation state and can be inactivated due to the existing redox potential (Duin *et al.* 2016). The position of 3NOP binding to an active site of MCR places the reducing nitrate group in close proximity to Ni(I), a distance in which electrons could be transferred. Although 3NOP inhibits methanogenesis and reduces methanogen growth, it does not negatively affect other microbial groups in the rumen (Duin *et al.* 2016).

More than 15 studies have been conducted using 3NOP, showing a marked reduction of enteric CH₄ emissions with a range of effectiveness. 3NOP added to ruminant diets in small quantities has been shown to persistently reduce enteric CH₄ emissions by inhibiting an important step in the methanogenesis metabolic pathway, without apparent negative side effects (Hristov *et al.* 2015). Figure 2 shows a forest plot illustrating the effect sizes as a mean difference between the control and treatment-group mean CH₄ production. For example, Vyas *et al.* (2016) reported that with 0.2 g 3NOP/kg dry-matter (DM) supplementation, CH₄ production in backgrounding and finishing beef cattle reduced 37.6% and 84.3% compared with the control group, whereas Vyas *et al.* (2018), using the same amount of supplementation in

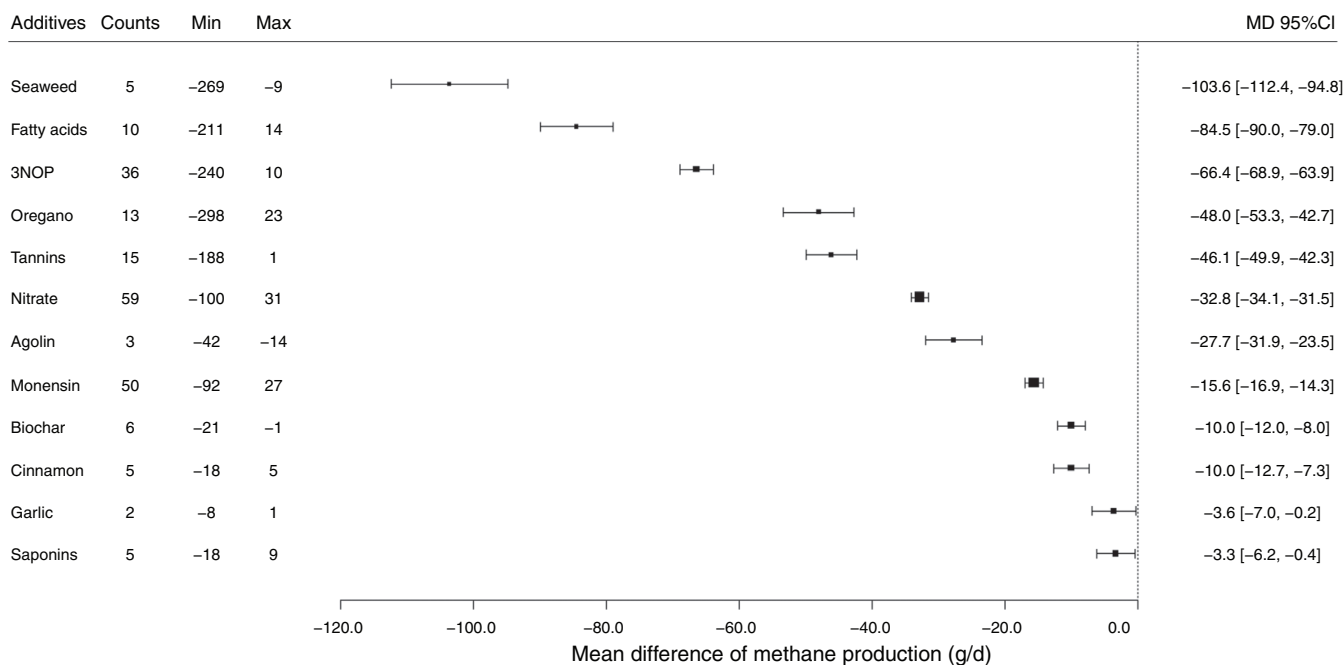


Fig. 2. Forest plot of mean difference (MD) of methane production for different feed additives, counts of studies, minimum and maximum of MD. Only studies conducted *in vivo* were included in the analysis.

backgrounding phase (0.2 g/kg DM) of beef cattle, found a 54.1% reduction in CH₄ production. These authors reduced the level of supplementation of 3NOP to 0.125 g/kg DM during the finishing phase and reported 53.8% reduction in CH₄ production. There was also an improvement in gain-to-feed ratio during treatment, with a 7% drop in DM intake (DMI). Similarly, Martinez-Fernandez *et al.* (2018) reported a decrease in CH₄ production of 38% and daily weight gain of 0.571 kg/day compared with the control in steers supplemented with 0.30 g 3NOP/kg DM. Hristov *et al.* (2015) demonstrated that CH₄ production in lactating cows was reduced by 30% by feeding 3NOP at 0.04–0.08 g/kg DM without affecting feed intake and milk production. Lopes *et al.* (2016) reported a 31% decrease in CH₄ production in lactating dairy cattle fed diets supplemented with 0.06 g/kg DM. In a meta-analysis of the anti-methanogenic effects of 3NOP, Dijkstra *et al.* (2018) reported that enteric CH₄ production was reduced 39% in dairy and 22% in beef cattle at a mean dose of 0.123 g/kg DM. Additive dose and the neutral detergent fibre (NDF) content of diet had a significant impact on the effectiveness of 3NOP in reducing enteric CH₄ emissions. Furthermore, an increase in 3NOP dose of 0.010 g/kg DM from the mean dose further reduced CH₄ production by 2.56 ± 0.55%. Similarly, Jayanegara *et al.* (2018) reported that the methanogenic archaea population was reduced through 3NOP supplementation and the magnitude of reduction was positively correlated with 3NOP dose in small and large ruminants. Addition of 3NOP is also associated with shifting H₂ production in the rumen and results in an increase in molar proportion for propionate and decreases acetate production (Haisan *et al.* 2014; Kim *et al.* 2019; Lopes *et al.* 2016).

There are no known adverse effects of supplementing 3NOP on the animal or the subsequent product. The feed additive 3NOP continues to be studied and, after approval by regulatory bodies, it is expected to be on the market in the near future.

Halogens

Plant species that accumulate halogenic compounds in their tissues have been investigated for their potential to reduce enteric CH₄ emissions. Halogens are elements that hold a large, negative electron affinity and seek to combine with other compounds to reach stability through satisfaction of the valence shell in the rumen environment (Gribble 2004). Bromoform and chloroform are halogens that have been found to interfere directly with the methanogenesis pathway by serving as competitive inhibitors (or analogues) of the MCR, preventing the final catalysis step (Goel *et al.* 2009). The mode of action is through reacting with reduced vitamin B12 and inhibiting the cobamide-dependent methyl-transferase step of methanogenesis (Wood *et al.* 1968; Chalupa 1977). The B12-dependent methyl-transferases also play an important role in one carbon metabolism in acetogenic bacteria (Banerjee and Ragsdale 2003), and, therefore, halogenated compounds may have an effect on reductive acetogenesis.

At supplementation level of 1.50–1.59 g/kg DM (2.6 g/100 kg liveweight; mean liveweight = 288 kg) of chloroform–

cyclodextrin, steers have demonstrated a 30–35% reduction in enteric CH₄ production, with no detectable differences in rumen fermentability (Martinez-Fernandez *et al.* 2016). Steers dosed daily with 0.267 g/kg DM of chloroform were shown to decrease 94–95% of CH₄ production within 4–5 days of treatment. However, CH₄ production has been shown to slowly recover to 62% of the pre-treatment levels by Day 42 of treatment (Knight *et al.* 2011). The macroalgae species *Asparagopsis taxiformis* and *A. armata* have been evaluated for their mitigation potential both *in vitro* and *in vivo* (Roque *et al.* 2019a, 2019b). *Asparagopsis* spp. contain relatively high concentrations of bromoform and other halogenated compounds such as bromochloromethane (Paul *et al.* 2006; Machado *et al.* 2016). An *in vitro* trial analysing effectiveness across seaweed species found *A. taxiformis* to be the most effective species among 20 freshwater and marine macroalgae in reducing CH₄ output (98.9%), but also reduced total gas production (62%), likely indicating inhibition of digestion (Machado *et al.* 2016). Increasing the dose to 5% *in vitro*, Roque *et al.* (2019a) reported a 95% reduction in the level of CH₄ production. Three papers have been published so far, reporting the effect of *Asparagopsis* spp. in sheep, dairy and beef cattle *in vivo*. Li *et al.* (2018) supplemented *A. taxiformis* at 67.5 g/kg DM (30 g/kg of organic matter, OM) in sheep diets and reported a reduction of up to 80% in enteric CH₄ production. However, rumen volatile fatty acid (VFA) concentrations in the 0%, 0.5%, 1.0%, 2.0% and 3.0% macroalgae inclusion groups declined from 92.0, to 86.5, 74.9, 69.1 and 65.4 mM respectively. Reductions in VFA concentrations are not desirable as they provide energy to the ruminant. In lactating dairy cattle, Roque *et al.* (2019b) observed up to 67.2% reduction in CH₄ intensity (g/kg milk produced) using *A. armata* at an inclusion rate of 18.3 g/kg DM (10 g/kg of OM). In Brangus beef cattle, Kinley *et al.* (2020) reported a reduction of enteric CH₄ production of up to 98% by supplementing a feedlot diet with *A. taxiformis* at 3.26 g/kg DM (2 g/kg of OM). In addition, there was an improvement of 42% in average daily gain with a supplementation level of 1.63 g/kg DM (1.0 g/kg of OM) and it went up to 53% at an inclusion rate of 3.26 g/kg DM (2.0 g/kg of OM). The study by Kinley *et al.* (2020) reported a greater effectiveness at a lower dose than did that of Roque *et al.* (2019b), which was likely due to the large differences in the bromoform concentration in *A. taxiformis* and *A. armata*, while also acknowledging the inclusion of monensin in the Kinley *et al.* (2020) experimental diets. The bromoform concentration in Roque *et al.* (2019b) study was 1.32 mg/g compared with 6.55 mg/g in the Kinley *et al.* (2020) study.

Sourcing naturally occurring halogens circumvents the need to use synthetic halogens. Historically, these synthetics have had detrimental effects on the environment (Gribble 2004). Kinley *et al.* (2020) and Roque *et al.* (2019b) tested for residual bromoform content in meat (or edible offal) and milk respectively. In both cases, concentrations of bromoform were either undetectable or not significantly different from the control, suggesting no safety issues arising from the active ingredient. At present, *A. taxiformis* is not produced commercially; so, accessibility is an issue. The use of

macroalgae also needs to be approved by regulatory agencies before widespread use by producers.

Nitrate

Adding nitrate to ruminant diets can be an effective CH₄ mitigation strategy because nitrate competes with methanogens for H₂ in the rumen. Nitrate (NO₃⁻) is reduced to nitrite (NO₂⁻; NO₃⁻ + H₂ → NO₂⁻ + H₂O) and further to ammonia (NH₄⁺; NO₂⁻ + 3H₂ + 2H⁺ → NH₄⁺ + 2H₂O) by rumen microbes. However, small quantities of nitrous oxide may also be produced (Latham *et al.* 2016). This pathway is highly competitive with methanogens for H₂ utilisation in the rumen due to greater changes in Gibbs energy than with methanogenesis (CO₂ + 4H₂ → CH₄ + 2H₂O) pathway (Villar *et al.* 2020). The result is a redirection of H⁺ flow from CO₂ to nitrate reduction, thereby reducing the generation of CH₄ (Olijhoek *et al.* 2016).

About 24 *in vivo* studies showed that the efficacy of nitrate additives varied widely, ranging from +1.25% to -29.8%, and may be affected by several factors. A meta-analysis conducted by Feng *et al.* (2020) investigated the potential explanatory variables for anti-methanogenic effects of *in vivo* nitrate supplementation in cattle. These included DMI, roughage proportion, NDF content, crude protein (CP) content, bodyweight, nitrate dose, cattle type, and CH₄ measurement methods. The authors reported that nitrate significantly reduced CH₄ emissions in a dose-response manner and the mitigating effect increased with the level of nitrate inclusion. Methane production reduced 14.6% in cattle supplemented with nitrate at 17.7 g/kg DM (Feng *et al.* 2020). Hulshof *et al.* (2012) reported that nitrate supplementation increased ammonia-nitrogen concentrations in the rumen by 34%, decreased propionate concentrations by 16%, but did not affect the total VFA concentrations. Persistency of nitrate was tested by van Zijderveld *et al.* (2011a), by including 21 g/kg DM during four successive 24-day periods and a consistent 16% reduction in daily CH₄ production (g/day) and yield (g CH₄/kg DMI) was demonstrated. An additive effect of nitrate and linseed oil was reported by Guyader *et al.* (2015a) in multiparous, non-lactating dairy cattle. These authors reported that adding 4% linseed oil to 3% calcium nitrate further reduced CH₄ production from 22.8% (nitrate only) to 33.0%.

Concerns about the toxicity of the intermediate product of nitrate, namely nitrite, to ruminants necessitate management, as animal poisoning may occur via methaemoglobinemia (Latham *et al.* 2016). Nitrite is toxic in blood because it converts haemoglobin to methaemoglobin, which is incapable of carrying oxygen. Blood methaemoglobin concentrations in ruminants increase with a greater nitrate consumption and could cause nitrate poisoning (Lee and Beauchemin 2014). Apparent nitrate-poisoning symptoms such as depressed feed intake, slow or no weight gain, reproduction failure, respiratory distress, coma and death have been reported in previous studies with methaemoglobin concentrations of 30–40% of total haemoglobin (Bruning-Fann and Kaneene 1993). Lee and Beauchemin (2014) discussed several critical factors related to nitrate toxicity, including the

dietary nitrate concentrations, nitrate consumption rate, incomplete reduction of nitrate and nitrite to ammonia, and rumen outflow rates. Toxic effects of nitrite on the populations of main cellulolytic bacteria, which may be caused by the negative effects of nitrate/nitrite on cellulolytic and xylanolytic activity, have also been observed (Iwamoto *et al.* 2002; Asanuma *et al.* 2015; Granja-Salcedo *et al.* 2019). However, the risk of nitrate toxicity can be reduced by gradual acclimation of ruminants to dietary nitrate or utilisation of encapsulated nitrate (Lee and Beauchemin 2014). Currently, nitrate inclusion may not be advisable in commercial operations due to its potential toxicity. However, a denitrifying probiotic, *Paenibacillus fortis*, that can enhance nitrite detoxification in nitrate treated ruminants, has been identified (Latham *et al.* 2019). If successful, nitrate and the probiotic might be a practical mitigation strategy to reduce CH₄ production from ruminants.

Rumen modifiers

The rumen environment can be modified with feed additives to limit the growth of methanogens and to suppress CH₄ production, without targeting the specific methanogenesis pathway. The factors influencing CH₄ production include those involved in H₂ and carbohydrate metabolism (Morgavi *et al.* 2010). Understanding rumen metabolic processes that affect CH₄ formation is still advancing; however, feed additives were used to modify the rumen environment to reduce CH₄ production without compromising animal health or productivity. This section discusses feed additives that can potentially reduce CH₄ production by modifying the rumen environment.

Dietary lipids

Dietary lipids modify the rumen environment in several ways, including (1) toxic characteristics on methanogens and protozoa, (2) hydrogenation of unsaturated fatty acids (alternative H₂ sink) and (3) shifts to propionic production, leading to reduction of enteric CH₄ production (Johnson and Johnson 1995; Beauchemin *et al.* 2008, 2009b). Efficacy of lipids to reduce CH₄ emissions are dependent on the form and level of supplementation, as well as the source and fatty acid profile (Beauchemin *et al.* 2008; Eugène *et al.* 2008). Several meta-analyses were conducted to estimate the impact of dietary lipids on CH₄ production (e.g. Beauchemin *et al.* 2008; Eugène *et al.* 2008; Martin *et al.* 2010). For example, Beauchemin *et al.* (2008) evaluated 17 studies in sheep, beef and dairy cattle and reported a 5.6% reduction in CH₄ production for every 1% additional inclusion of supplemental fat. In dairy cattle, Eugène *et al.* (2008) reported a decrease of 9% through lipid-supplementation (average 6.4%) compared with control diets (average 2.5%), mostly as a consequence of reduced DMI. Similarly, Patra (2013) reported 3.77% decline in CH₄ emissions for each percentage inclusion of lipid in dairy cattle diets. Prediction inconsistencies by the inclusion of supplemental lipid are likely to be due to differences in lipid source and diet composition. In a review, Rasmussen and Harrison (2011) reported that the most effective fatty acid profiles that reduce CH₄ production were medium-chain (8–16 carbon chains);

MCFA) and polyunsaturated (PUFA) fatty acids. However, reductions in DMI due to high levels of dietary lipids are well characterised and ration formulation programs often are set not to exceed 6–7% of total DMI (NRC 2001).

Medium-chain fatty acids

These include lauric, myristic, capric and caprylic acids (Hollmann *et al.* 2012). *In vitro* studies have reported coconut oil, which contains 75% of MCFA, to reduce CH₄ production by 43–85% (Dong *et al.* 1997; Machmüller *et al.* 1998). Application of coconut oil in *in vivo* trials also showed similar patterns in CH₄ reduction (Hollmann *et al.* 2012). Ruminants fed diets containing 13, 27 and 33 g coconut oil/kg DM had 3%, 37% and 45% reduction in CH₄ output compared with the control respectively. DMI, solids-corrected milk yield, and milk fat yield (no difference between the two greatest levels of inclusion on milk fat yield) decreased linearly with an increase in coconut oil application. Inclusion of myristic acid at a rate of 50.0 g/kg DM in dairy cattle diets reduced CH₄ production by 36%, but also reduced milk fat by 2.4%, with a tendency to reduce DMI (Odongo *et al.* 2007). Lauric acid had no negating effects on methanogenesis in dairy cattle when they received it at 10.0 g/kg DM (Hristov *et al.* 2009). Within the same trial, the treatment group receiving 21.6 g/kg DM of coconut oil reduced their CH₄ production by 61% compared with the control.

Polyunsaturated fatty acids

Polyunsaturated fatty acids have also been shown to reduce CH₄ production. For example, Bayat *et al.* (2015) found that enteric CH₄ production reduced by 29.5% with supplementation of 60 g/kg DM of camelina oil, but other parameters such as milk yield and milk components were compromised. In contrast, Duthie *et al.* (2018) did not find significant differences in enteric CH₄ production in steers fed increasing amounts of dietary lipid sourced from maize distillers dark grains, which increased diet ether extract from 24 to 37 g/kg DM for 17 weeks. Supplementation of diets with cottonseed oil has been shown to decrease enteric CH₄ production by ~42% (Nogueira *et al.* 2020). These authors suggested that bio-hydrogenation of lipids served as an alternative H₂ sink, and with each percentage point of lipid added to the diet, CH₄ production was reduced by 8%. Further characterisation and understanding of the impact and longevity of dietary lipid inclusion on methanogenesis would be valuable in selecting plant sources and estimating their impact. Dietary lipid additives (both MCFA and PUFA) show substantial decreases in CH₄ production with a wider range of effectiveness compared with other feed additives (Fig. 2).

Probiotics

Microorganisms included in diets are often referred to as probiotics, cultures, or direct-fed microbials. Introducing microorganisms to a digestive microbiome is practiced on farms to influence the rumen flora for improved digestion. Results of feeding fungi, yeast or bacteria to reduce CH₄

production have not been consistent in studies conducted *in vitro* or *in vivo*. Application of live yeast cultures (various strains of *Saccharomyces cerevisiae*) have not been shown to significantly change CH₄ production, rumen fermentation or apparent total tract nutrient digestibility in dairy cattle (Bayat *et al.* 2015). Additionally, inclusion of either a dead or live form of *S. cerevisiae* has little to no impact on nutrient digestibility or rumen fermentation patterns in beef heifers (Vyas *et al.* 2014). A meta-analysis by Darabighane *et al.* (2019) using data from 1990–2016 observed no significant reduction in CH₄ production through the use of probiotics.

Introducing propionate-producing bacteria has been evaluated as a possible solution because propionate production consumes H₂ as a reducing equivalent and, thereby, competes with methanogenesis (Ungerfeld 2013). This has not been effective with all strains of bacteria but *Propionibacterium thoenii* T159 reduced CH₄ production by 20% and increased VFA production by 21% in a study that screened 31 different strains within *in vitro* models (Chen *et al.* 2020). However, in lactating primiparous cows, *P. freudenreichii* 53-W was shown to increase CH₄ production by 27% (Jeyanathan *et al.* 2019). The mechanisms of reduction in CH₄ production (if any) are still unknown and could be either directly by microbes or indirectly through metabolites that affect the rumen microbiome (Doyle *et al.* 2019). Jeyanathan *et al.* (2019) found no effect on CH₄ output when feeding *Lactobacillus pentosus* D31, and *L. bulgaricus* D1 *in vivo*. Currently, there is no concrete evidence that probiotics are an effective method of CH₄ mitigation.

Acetogenesis, or reductive acetogenesis, is another H₂-utilising metabolic pathway in which acetogens utilise CO₂ and H₂ as substrates to produce acetate. While more prevalent in other mammalian guts, acetogens cohabit with methanogens in the rumen, but are either lacking a substantial population density, preferred environment conditions, or the competitiveness to be the favourable pathway of H₂ 'disposal' (Joblin 1999). Redirection of H₂ into the acetogenesis pathway to yield acetate would allow the recapture of energy compared with the loss due to methanogenesis. Enhancing this pathway in the rumen may be approached by sourcing acetogens from other ecosystems and transplanting them into the rumen (Gagen *et al.* 2014) or uncovering a method to enhance the existing rumen acetogen population if they can outcompete native methanogens.

Biochar

Organic matter that has undergone pyrolysis, commonly known as biochar, has a wide range of impacts on livestock systems due to its unique characteristics. Biochar has been utilised for generations as a remedy for digestive disorders and is sourced by the livestock industry to address issues surrounding animal husbandry, metabolism and waste management (Kalus *et al.* 2019; Schmidt *et al.* 2019). Abatement of CH₄ production through the application of biochar has been shown in soil (Yu *et al.* 2013) and compost (Sonoki *et al.* 2013). Considering that there is already an existing market for biochar as a beneficial feed additive, *in vivo* evidence for GHG mitigation will be

significant (Schmidt *et al.* 2019). Possible mechanisms have been elucidated through a study that observed that application of biochar to paddy soils stimulated methanotrophic proteobacteria and reduced CH₄, despite methanogens also being stimulated (Feng *et al.* 2012). Additionally, biochar may provide a habitat for methanogens or possibly absorb gases when consumed due to its porous nature, but the mechanisms of action for CH₄ mitigation in cattle are not well understood (Terry *et al.* 2019; Man *et al.* 2020).

Rice husks sourced for biochar and fed at an inclusion rate of 6 g/kg DM reduced CH₄ production by 22%, increased liveweight gain by 25%, and had no impact on DMI over a 98-day period (Leng *et al.* 2012). Biochar supplemented at 8 g/kg DM reduced CH₄ production by 9.5% in growing steers and 18.4% in finishing steers (Winders *et al.* 2019). Contrary to these findings, inclusion levels of 'pine-enhanced biochar' at 5, 10 and 20 g/kg DM in the diets of Angus × Hereford heifers did not reduce CH₄ emissions (Terry *et al.* 2019). However, it altered the microbiota, notably selecting against *Fibrobacter* species, which is one of the dominating phyla of the rumen responsible for cellulose degradation (Béra-Maillet *et al.* 2004). The wide variation in effectiveness precludes biochar as proven feed additive to reduce CH₄ production at present. More research, particularly *in vivo*, is required to understand the conditions under which biochar can mitigate CH₄ production.

Ionophores

Ionophores, such as monensin, alter rumen microbial populations to improve digestive efficiency by depriving methanogens of substrates that are typically provided by Gram-positive bacterial and ciliate protozoal populations (Russell and Strobel 1989; Hook *et al.* 2010). This fermentation shift favours the production of propionate over acetate, which reduces the amount of H₂ available for methanogens.

A meta-analysis by Appuhamy *et al.* (2013) quantitatively determined the impact of monensin in cattle. In beef cattle supplemented with monensin at an average monensin dose of 0.032 g/kg DM, CH₄ production was reduced by 19 g/day, which was further reduced as the NDF content of the diet increased. In dairy cattle, CH₄ production was reduced by 6 g/day at the same average dose and was further reduced as the dietary lipid content increased. Appuhamy *et al.* (2013) concluded that although there were reductions in CH₄ production through supplementation with monensin, the effect was transient, lasting ~6 weeks. In contrast, Benchaar (2020) reported no suppression effect of monensin on CH₄ output when it was administered to dairy cattle (0.024 g/kg DM), but there was an increase in the proportion of a biohydrogenation intermediate, thus altering rumen metabolism patterns.

The antimicrobial nature of ionophores has caused a concern to human health (Guan *et al.* 2006; Hook *et al.* 2010). Long-term use of ionophores is limited due to a low efficacy, transient nature and safety concerns.

Plant secondary compounds

Plant secondary compounds are primarily synthesised in response to their environmental conditions and not for specific

physiological function (Morrissey 2009). Some plant secondary compounds that may possess antimethanogenic properties are variable in composition due to environmental condition in which they are grown. Seasonal variation, pollution, diseases, pests, storage, injuries and pollination activity influence secondary-compound production and composition (Figueiredo *et al.* 2008). These compounds are not commonly extracted or isolated before feeding to ruminants because of time and cost considerations, which may contribute to their concentrations being inconsistent. These obstacles present a challenge in determining or predicting efficacy.

Tannins

Tannins are soluble, phenolic compounds that accumulate within plant tissues likely due to ongoing metabolic processes and contribute to the plant defence system (Swanson 2003). The CH₄ mitigation mechanisms of tannins are not well understood but may be due to a combination of factors, including a reduction in fibre digestibility (decrease in H₂ production) or a direct inhibition of methanogens (Tavendale *et al.* 2005).

Jayanegara *et al.* (2012) conducted a meta-analysis describing the relationship between rumen CH₄ formation and the level of dietary tannin (hydrolysed or condensed) inclusion between *in vivo* and *in vitro* models. These authors reported that low levels of inclusions of tannins in animal experiments often yielded inconsistent results on CH₄ production, but that variability seemed to diminish at higher doses, leading to setting the threshold for detecting treatment differences in animals to be >20 g/kg DM of tanniferous inhibitors. Furthermore, reduction in CH₄ production was often followed by a suppression in OM and fibre digestibility. Methane measurements from goats fed *Kobe lespedeza*, a forage containing condensed tannins at 151, 101 and 49.9 g/kg of DM led to a 54%, 52% and 32% reduction compared with the control group respectively (Animut *et al.* 2008). Supplementing beef cattle diets with tannic acid at a 26 g/kg DM inclusion rate, CH₄ production decreased 33.6%, but the digestibility of DM and CP, and the concentration of VFA were negatively affected (Yang *et al.* 2017). Investigating different tannin-containing hays, Stewart *et al.* (2019) found small burnet (*Sanguisorba minor*) fed to Angus cows and heifers to reduce CH₄ production in comparison to a diet containing alfalfa hay (209 vs 289 g CH₄/day respectively). However, CP and DM digestibility was affected negatively.

Grape marc or pomace contains high concentrations of condensed tannins and it is a readily available biowaste from the viticulture industry. Moate *et al.* (2014) fed dried pelleted (274 g/kg DM) or ensiled grape marc (269 g/kg DM) to dairy cattle and found that the dried form was the most effective in reducing CH₄. The authors reported that the CH₄ production in dairy cattle fed the control, dried and ensiled grape marc was 470, 375 and 389 g CH₄/day. More recently, Caetano *et al.* (2019) fed ensiled grape marc at a rate of 31.2 g/kg DM, which equates to ~3–4 kg/day of ensiled grape marc (estimated on the basis of reported DMI). Treatment inclusion in the study of Caetano *et al.* (2019) study led to a

14% reduction in CH₄ production; however, it ultimately decreased the energy availability of the diet due to the greater contents of lignin and acid detergent fibre in the treatment diet. Cattle have exhibited intoxication sensitivities to tannins, particularly if diets do not meet nutrient requirements for growth or milk production. However, such issues can be avoided through appropriate dosages and adaptation periods paired with properly formulated diets (Doce *et al.* 2013).

Flavonoids

Flavonoids are not known to have extensive CH₄ reduction potential, but anti-microbial properties of the compounds have been reviewed (Patra and Saxena 2010). Several *in vitro* trials (Oskoueian *et al.* 2013; Kim *et al.* 2015) have been conducted to gain a better understanding of antimicrobial characteristics and its relation to methanogenesis, but studies utilising *in vivo* models are scarce. Kim *et al.* (2015) studied the mitigation potential of four plants containing flavonoids *in vitro*, by using rumen fluid sourced from a single cow. In all treatments, CH₄ production was reduced by 39–48%; however, results such as this have not yet been translated into animal models. Flavonoids derived from mulberry leaves (~1.3 g/kg DM) did not influence methanogenesis to a detectable level in sheep, but they increased digestibility (Chen *et al.* 2015). Rutin trihydrate, a flavonoid, was given to dairy cattle at a dose of 100 mg/kg bodyweight, which led to an elevated plasma glucose, β -hydroxybutyrate and albumin, but did not suppress CH₄ production (Stoldt *et al.* 2016).

Saponins

Saponins have been studied for their capacity to alter rumen fermentation by reducing protozoal communities, thus lowering H₂ availability and the production of CH₄ (Hess *et al.* 2003). Saponins are commonly found in low quantities in legume plants such as kidney beans, soya beans, chickpeas and green peas (Shi *et al.* 2004). Holtshausen *et al.* (2009) conducted a two-part study on saponins derived from *Yucca schidigera* and *Quillaja saponaria* and their effect on CH₄ production *in vitro* and *in vivo*. Inclusions of 15, 30, or 45 g/kg DM of *Y. schidigera* and *Q. saponaria* decreased CH₄ production ranging from 6 to 26% *in vitro*. However, *in vivo* study in dairy cattle using whole-plant *Y. schidigera* and *Q. saponaria* powders at 10 g/kg of DM did not show an impact on rumen fermentation. Cross-bred cattle supplemented with soapnut, a saponin-containing plant, did not have significant reductions in CH₄ production (Poornachandra *et al.* 2019). Tea saponins offered to ewes led to a decrease in CH₄ production if scaled to metabolic weight; otherwise, no differences were observed in absolute values (Liu *et al.* 2019). The same supplement was offered to steers at 2.44 and 3.85 g/kg DM, but no impact on gas output was observed (Ramírez-Restrepo *et al.* 2016). Lack of results in reducing CH₄ production may be linked to low concentrations of saponins within additives. However, in some circumstances, high concentrations of saponins have been linked to bloat through foaming properties, but no strong conclusions have been drawn (Lindahl *et al.* 1954;

Sen *et al.* 1998). Low-level inclusions may have antiprotozoal and mild antibacterial characteristics and can be incorporated into livestock diets through a variety of plant options.

Essential oils

Essential oils (EO) are naturally occurring chemical compounds extracted from plants and used in fragrances and cosmetics and, to a lesser extent, pharmaceutical products for humans and animals. Volatile in nature, the EO contribute to the phenotypic expression of the plant including colour and scent (Edris 2007; Benchaar *et al.* 2008). Consumption of EO has been observed to affect rumen microbial communities and fermentation patterns in a varying manner, depending on the EO source (Benchaar and Greathead 2011). Many EO hold a high affinity for lipid and bacterial membranes, leading to disruption, but the broad antimicrobial effect is likely to be due to a combination of mechanisms (Helander *et al.* 1998). EO are non-specific in nature; therefore, there is a concern for their inclusion in diets because they may affect favourable microbe populations, leading to a decrease in feed efficiency. Numerous plants such as cinnamon, lemongrass, ginger, garlic, juniper berries, eucalyptus, thyme, citrus, oregano, mint, rosemary and coriander have been screened *in vitro* (Benchaar *et al.* 2008; Nanon *et al.* 2015). However, only few have been studied *in vivo*. Some studies include the whole plant (Olijhoek *et al.* 2019) into a diet, while other extract the EO before inclusion in a more concentrated treatment (Lejonklev *et al.* 2016), which introduces another level of variability.

Oregano contains EO compounds carvacrol and thymol that may stimulate general antimicrobial properties in the rumen (Kolling *et al.* 2018). Only two *in vivo* studies (Tekippe *et al.* 2011; Hristov *et al.* 2013) have shown reduction of CH₄ production of up to 40% in dairy cattle. Hristov *et al.* (2013) did not observe any adverse effects of supplementation (8.7, 18.9 and 28.2 g *Origanum vulgare* leaves/kg DM) on feed efficiency, rumen pH or VFA concentrations. In contrast, several other studies have shown no significant impact of supplementing oregano on CH₄ production. For example, lactating dairy cattle supplemented with oregano oil and carvacrol at 0.05 g/kg DM did not express any anti-methanogenic properties (Benchaar 2020). Kolling *et al.* (2018) reported a reduction in CH₄ yield (in g/kg digestible DMI), but no reductions surrounding other CH₄ emission parameters such as protozoal count, by using 0.56 g oregano extract/kg DM in lactating dairy cattle. Olijhoek *et al.* (2019) reported no significant reduction in dairy cows supplemented with either 18–53 g oregano plant/kg DM from *Origanum vulgare* ssp. *vulgare* containing 0.12% EO of oregano DM, or 7–21 g oregano DM/kg of DM from *Origanum vulgare* ssp. *hirtum* containing 4.21% EO of oregano DM. The authors speculated that the differences in reported effectiveness could be related to the duration of measurement (1–8 h post intake in those that reported reductions vs >24 h in studies with no effect). The observation by Hristov *et al.* (2013) who reported a linear decline in effectiveness after feed intake lends support to

measurement duration contributing to differences in reported effectiveness.

Garlic (*Allium sativum*) contains organosulfur compounds, specifically diallyl disulfide, as its main EO component. Organosulfur compounds are suspected of having a toxic effect on the enzyme system of the methanogenic archaea, inhibiting their activity, while also suppressing protozoal populations (Busquet *et al.* 2005a; Soliva *et al.* 2011). Soliva *et al.* (2011) reported a 91% reduction in CH₄ with 300 mg/L garlic oil *in vitro*, associated with an increase in bacterial counts and reduction in protozoa. Similarly, Busquet *et al.* (2005a) observed a 73.6% reduction in CH₄ production *in vitro* by using similar concentrations of garlic oil. However, most *in vivo* cattle studies have not found an impact of garlic oil on CH₄ production. For example, van Zijderveld *et al.* (2011b) used diallyl disulfide at 0.056 g/kg DM in dairy cattle and observed no reduction in CH₄ production. Staerfl *et al.* (2012) using dried garlic bulbs (treatment standardised for 15 g allicin/kg DM) in feedlot cattle reported no significant effect on CH₄ production measured at 5, 9 and 11 months of age. Similarly, Meale *et al.* (2014) reported no detectable differences in enteric CH₄ or CO₂ production in animals supplemented with garlic oil (15 g allicin/kg DM). Sheep models have reported similar results of no detectable difference in enteric CH₄ production (Patra *et al.* 2011; Klevenhusen *et al.* 2011); however, goat models supplemented with L propyl-propane-thiosulfinate, another organosulfur compound found in garlic, suppressed CH₄ production by roughly 33% (Martinez-Fernandez *et al.* 2013). Nevertheless, in their subsequent experiment, Martinez-Fernandez *et al.* (2014), using the same compound in goats *in vivo*, did not find a significant reduction in enteric CH₄ production.

Lemongrass (*Cymbopogon* spp.) has been assessed *in vitro* for potential antimicrobial effects due to citral, an aldehyde sourced from the EO fraction contributing to aromatic characteristic of the plant (Pawar *et al.* 2014; Joch *et al.* 2016; Singh *et al.* 2018). While CH₄ was not measured, Wanapat *et al.* (2008) detected an improvement in microbial protein supply, DM digestibility and microbial populations when Brahman-native beef cattle consumed 18.5 g lemongrass powder/kg DM. In lactating Barki goats, 4 g/kg DM elicited a slight increase in protozoal counts and CH₄ production (Khatab *et al.* 2017).

Supplementing cinnamaldehyde and cinnamon oil (containing 78% cinnamaldehyde) to dairy cattle diet at inclusion rates ranging from 0.003 to 0.16 g/kg did not reduce CH₄ production (Benchaar 2016). Methanogen numbers decreased in a study adding 0.5 g/kg DM of cinnamon oil, but the study did not measure gases directly, so any CH₄ reduction was speculative (Khorrami *et al.* 2015). Eugenol, an active EO component of cinnamon, was added to diets at 0.025, 0.050 or 0.075 g/kg DM, but no treatment group demonstrated a difference in enteric CH₄ compared with the control (Benchaar *et al.* 2015). Shifts away from acetate production and towards propionogenesis have been observed in artificial conditions when cinnamon-sourced additives were introduced (Busquet *et al.* 2005b). Inclusion of EO in livestock diets has not rendered any safety concerns for animal husbandry or consumption of subsequent products.

Essential oil blends

Taking advantage of the unique composition among plants, some studies have used an EO 'blend' or 'complex' containing extracts from multiple plants. The antimicrobial nature of a variety of the EO may imply a capacity to modify rumen fermentation. EO blends have demonstrated a greater feed efficiency and a higher production of energy-corrected milk in dairy cattle through modification of rumen fermentation (Elcoso *et al.* 2019; Silva *et al.* 2020). Blends have become commercially available, typically containing at least two different EO. For example, Agolin Ruminant (Agolin, Bière, Switzerland; AR) contains a blend of eugenol, geranyl acetate and coriander EO. Agolin Ruminant is an antimicrobial EO product and has shown 20% reduction in CH₄ intensity in dairy cattle (Hart *et al.* 2019). Klop *et al.* (2017) alternated AR (0.17 g/kg DM) with lauric acid (0.65 g/kg DM) for 2-week periods over 10 weeks, but CH₄ production was not altered. Elcoso *et al.* (2019) estimated 15% lower CH₄ production in lactating dairy cattle consuming AR. Castro-Montoya *et al.* (2015) fed 0.0128 and 0.0240 g AR/kg DM to dairy and beef cattle respectively, but detected only tendencies towards CH₄ reduction in both groups, with no significant differences occurring.

Mootral[®] is synthesised from natural products including garlic- and flavonoid-containing citrus extract and has demonstrated anti-methanogenic properties (Eger *et al.* 2018; Roque *et al.* 2019c; Vrancken *et al.* 2019). The garlic component in Mootral[®] targets methanogenic archaea populations and protozoal communities in the rumen and has led to nearly complete inhibition of CH₄ production *in vitro* at a dosage of 2 g experimental mixture/day, without compromising bacterial population (Eger *et al.* 2018). The experimental mix contained 1.5% (w/w) allicin and 45% (w/w) polyphenolics (Eger *et al.* 2018). A 23.2% decrease in CH₄ yield (26.8% expressed in CH₄ production) was observed in Angus × Hereford crosses after 12 weeks of treatment by supplementing Mootral[®] at 1.58 g/kg DM (Roque *et al.* 2019c). Adverse effects on DMI, ADG and feed efficiency were not detected over the 12-week trial. Lactating cattle offered Mootral incorporated in pellets at a rate of 0.640 g/kg DM for Holstein-Friesian and 1.21 g/kg DM for Jersey herd experienced suppression of CH₄ of 20.7% and 38.3% respectively (Vrancken *et al.* 2019). Additionally, 3–5% increase in milk yield across breeds was observed with increased feed efficiency in the Jersey cattle. Further research is required to determine the effective dose and magnitude of reduction from ruminants supplemented with Mootral[®].

Conclusions

Several feed additives provide a promising option that could increase the sustainability of animal-sourced foods by substantially reducing enteric CH₄ emissions. Rumen inhibitors have shown potential of up to 98% reduction in enteric CH₄ production, although they differ in accessibility and risk to animal welfare. Although none of the inhibitors are currently on the market, on the basis of the volume of available literature, 3NOP may be offered to producers in the

near future, with nitrate and microalgae to follow after further research. Rumen modifiers including EO, tannins, saponins, biochar and lipids can be sourced globally but vary in composition and are not always effective. Consistency is a factor to consider with plant-based feed additives, but it can be addressed, as demonstrated, in commercial applications such as Mootral[®] and Agolin Ruminant. Direct-fed microbials or probiotics have not demonstrated strong evidence to be considered a rumen modifier to suppress CH₄ production. Due to increased interest in this area, research is expected to accelerate in production of feed additives that reduce enteric CH₄ production.

Conflicts of interest

The authors declare no conflicts of interest.

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