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Recent Advances in Enteric Methane Mitigation and the Long Road to Sustainable Ruminant Production

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Abstract

Mitigation of methane emission, a potent greenhouse gas, is a worldwide priority to limit global warming. A substantial part of anthropogenic methane is emitted by the livestock sector, as methane is a normal product of ruminant digestion. We present the latest developments and challenges ahead of the main efficient mitigation strategies of enteric methane production in ruminants. Numerous mitigation strategies have been developed in the last decades, from dietary manipulation and breeding to targeting of methanogens, the microbes that produce methane. The most recent advances focus on specific inhibition of key enzymes involved in methanogenesis. But these inhibitors, although efficient, are not affordable and not adapted to the extensive farming systems prevalent in low- and middle-income countries. Effective global mitigation of methane emissions from

livestock should be based not only on scientific progress but also on the feasibility and accessibility of mitigation strategies.

1. INTRODUCTION

The production of food for human consumption challenges the sustainability of resources at a planetary level (1). Livestock production, particularly ruminant production, is at risk of exceeding some planetary boundaries beyond natural recovery (2). One boundary at risk associated with the ruminant sector is climate change, where enteric methane emissions are particularly relevant for global warming (3).

Methane is a greenhouse gas (GHG) that is second only to carbon dioxide in its importance for global warming. Methane concentrations in the atmosphere have accelerated over the past 15 years to the point where this increase could jeopardize the ongoing efforts to reduce carbon dioxide emissions (4). Besides, the radiative forcing of methane, the greenhouse effect, is now estimated to be 25% higher (5) than the values used in international agreements, including the UN Paris Agreement on climate change. Nevertheless, methane has a short perturbation lifetime in the atmosphere of approximately 12 years. This advocates methane as a preferred target for mitigating global warming in the near future, as proposed by the Global Methane Pledge initiative (**https://www.globalmethanepledge.org**).

The share of livestock emissions in anthropogenic GHG emissions (11%), and especially the share of methane in these emissions (45%) (6), highlights the need to consider the impact of livestock on the environment when considering global warming and the GHG mitigation targets set by the UN Framework Convention on Climate Change. Whether the recent increase in atmospheric methane concentration is due to increased emissions, reduced sinks, or both is unclear (4). This increase is accompanied by a shift in the $^{13}C/^{12}C$ isotopic ratio ($\delta^{13}C_{CH4}$), with an increase in negative $\delta^{13}C_{CH4}$ values that are characteristic of methane from biogenic sources, including ruminants but also wetlands and waste (4). Notwithstanding the uncertainties mentioned above, recent adjusted estimates suggest that enteric methane from ruminants is a major contributor to the drop in atmospheric $\delta^{13}C_{CH4}$ and to the increase in overall methane emissions observed since the beginning of the twenty-first century (7).

Although ruminants contribute substantially to GHG emissions, ruminant-derived food plays a pillar role in food security and human nutrition, and that should not be underestimated (8, 9). Meat and milk from ruminants are major sources of high-quality protein and essential minerals and vitamins for many people, particularly in developing countries (10). The carbon footprint of meat and milk protein from ruminants is higher than that of plant proteins, but when quality aspects such as the digestible essential amino acids score are taken into account, the carbon footprint decreases by up to 40% compared to unadjusted values (11). Another comparative advantage of ruminant production systems is that up to 90% of feeds consumed are not human edible, such as forages and co-/by-products of the feed industry (12). Recent research shows that proteins from ruminants have on average a smaller GHG footprint than proteins from monogastric animals, which is particularly evident when feed production from crops is considered and the use of human-nonedible feeds is maximized for ruminants (13). If ruminants are "walking wetlands" (4) from a global warming perspective, they are also walking biological converters of cellulosic biomass into food and other useful products. Therefore, the urgent need to reduce the environmental impact of ruminants is often considered from the perspective of, and possible trade-offs with, providing people with an adequate supply of nutritious food.

Strategies to decrease enteric methane emissions have been explored for several decades already, with a steady increase since the mid-2000s following the publication of the UN Food and Agriculture Organization (14) report *Livestock's Long Shadow*. Enteric methane production is a complex trait driven by the rumen microbiome (all microbes present in the rumen, the microbiota, and their genes) and modulated by multiple factors, including the diet, the ruminant animal, and their interactions (15, 16). This review considers these animal-related approaches to mitigate methane emissions from farmed ruminants, focusing on the most recent and promising developments in the area. We critically assess present and future challenges the sector faces in adopting these solutions.

2. METHANOGENESIS AND EMISSIONS METRICS

2.1. How Enteric Methane Is Produced

Methanogenesis, the formation of methane, is a normal process that occurs during feed digestion in the ruminant gastrointestinal tract. Enteric methanogenesis takes place mostly in the rumen due to its size and anterior position in the ruminant gastrointestinal tract. It is a process of microbial origin that occurs in the absence of oxygen, when carbohydrates from the feeds are fermented by a collection of microbes to produce energetic substrates used by the animal (17). In normal conditions, fermentation produces hydrogen, which does not accumulate as it is assumed it would inhibit fermentation (18, 19), although animal studies in which methane inhibition increased hydrogen concentration without affecting fermentation of feeds challenge this assumption (20–22). Instead, most of the hydrogen is used by methanogenic microbes to produce methane (19), whereas a small part is emitted through belching.

In the rumen, as in other bioenvironments, methanogenesis is ensured by the methanogenic archaeal community. These microbes represent 0.3–3.3% of the rumen microbiota and are found in the rumen fluid, attached to feed particles, or associated to protozoa (23). As a reaction, methanogenesis is a reduction (the transfer of an electron) of carbon dioxide by dihydrogen to methane and water. Methane produced in this way, referred as the hydrogenotrophic pathway, represents a large proportion of the methane formed in normal conditions (24) and is performed mostly by archaea belonging to the *Methanobrevibacter* genus. However, other methanogenic archaea can synthetize methane using different pathways depending on available substrates. Two other pathways have been described, the aceticlastic pathway, which produces methane from acetate, and methyl-based pathways (including methyl dismutase and methyl-reducing pathways) that use methylated compounds as substrates (24, 25). Other unusual pathways have been described in the literature (26) but are not yet demonstrated as active in ruminants. As mentioned above, the hydrogenotrophic pathway is dominant in the rumen, with the characteristic that methane produced from formate, once inside methanogens and transformed to carbon dioxide and dihydrogen, can represent up to 18% of total ruminal methane (27). The acetate pathway is not important in the rumen (28, 29), but the methyl-based pathway, once considered minor due to the low number of rumen methanogens using the methyl dismutation (methylotrophic) pathway (29), can contribute a significant proportion of the methane emitted by ruminants (30). The main methylotrophic pathway in the rumen, however, uses hydrogen as the electron donor to reduce methyl compounds. *Methanosphaera* spp. and methanogens from the *Methanomassiliicoccales* order are the main representatives carrying out this process and account for approximately 30% of the total number of methanogens (29) when adjusted by 16S ribosomal RNA copy numbers.

2.2. A Note on Expression Metrics

Several metrics are commonly used to account for methane emissions and to assess the efficacy of mitigation strategies. These are absolute emissions (g/day), yield [g/kg dry matter intake (DMI)],

and intensity (g/kg of milk, meat). We focus on absolute emissions. Methane intensity, convenient for calculating the carbon footprint of animal products, is linked to production efficiency. Improving efficiency, particularly in low-performing systems, will improve resource use and food security in line with the UN Sustainable Development Goals but not necessarily reduce overall enteric methane emissions. Another metric used is the methane conversion factor (Ym), expressed as the proportion of the gross energy in the ingested feed that is lost as methane. It is often referred as the energy that could be used for productive purposes if it were saved by decreasing methane production in the rumen. Methane inhibition seems not to improve the energy balance of ruminants (31). Methane yield is, however, useful to decouple methane emissions from feed intake, which is the first dietary factor to modulate methane production.

3. DIETARY STRATEGIES

Enteric methanogenesis cannot be dissociated from feeds and the digestive processes that occur in the gastrointestinal tract (32). It is thus not surprising that mitigation strategies targeting diet composition and feed quality have been reported widely in the literature and continue to be explored. Extensive overviews of available dietary options have been published, some recently, covering the most successful strategies (16, 33, 34). The mitigation potential of these strategies has been quantitatively assessed in a recent meta-analysis (35). Of note, some successful dietary strategies decrease methane intensity but not absolute emissions (35). Here we present the main concepts by which dietary strategies, excluding additives that specifically affect methanogenesis (presented in Section 5), modulate methanogenesis, and we discuss knowledge gaps and shortcomings of current claims.

Dietary strategies modulate methanogenesis in two ways: by influencing the production of substrates used by methanogens or by affecting the methanogens themselves (**Figure 1*b*,*c***). These two ways can, and often do, coexist. The main factors involved in the effect of diet on the rumen ecosystem can be summarized as those affecting feed digestibility, fermentation products, and pH. These factors are interrelated and interact mutually. Diet digestibility is positively correlated with methane emissions (36). It follows that rumen digestibility is related to intake and passage rate as determinants of methane production. In short, intake increases when animals are fed more digestible diets with a concomitant increase in emissions, up to the point where the residence time of feed in the rumen is shortened, i.e., less fermentation, causing a decrease in methane produced per kilogram of DMI. Similarly, including in the diet feeds that are not fermented in the rumen—those that are naturally inert, such as lipids, or technologically treated to bypass the rumen—decreases emissions. The use of lipids in ruminant diets as a carbohydrate substitute effectively reduces absolute methane emissions in a dose-dependent manner (35, 37). No to minor effects on productive traits have been reported when dietary lipids are not overfed, although lipid efficacy depends on the type of fatty acids. Medium-chain 12:0 and 14:0 and unsaturated long-chain (C18 and higher) fatty acids are the most effective (38). Another lipid-induced mechanism contributing to methanogenesis reduction, which is related to fatty acid composition, is toxicity to sensitive microbial groups, including microbes contributing to dihydrogen production, such as cellulolytic bacteria and protozoa (39, 40). Maia et al. (39) showed that microbial growth is particularly affected in cellulolytic, hydrogen-producing *Ruminococcus albus*, *Ruminococcus flavefaciens*, and some *Butyrivibrio* spp. This negative effect on microbial growth depends on carbon chain length and degree of saturation, i.e., from more to less toxic eicosapentaenoic acid (20:5), docosahexaenoic acid (22:6), α-linolenic acid (18:3), and linoleic acid (39). Hydrogenation of unsaturated fatty acids also acts as an electron sink, decreasing the amount of substrate available for methanogenesis, but the effect is minor (41).

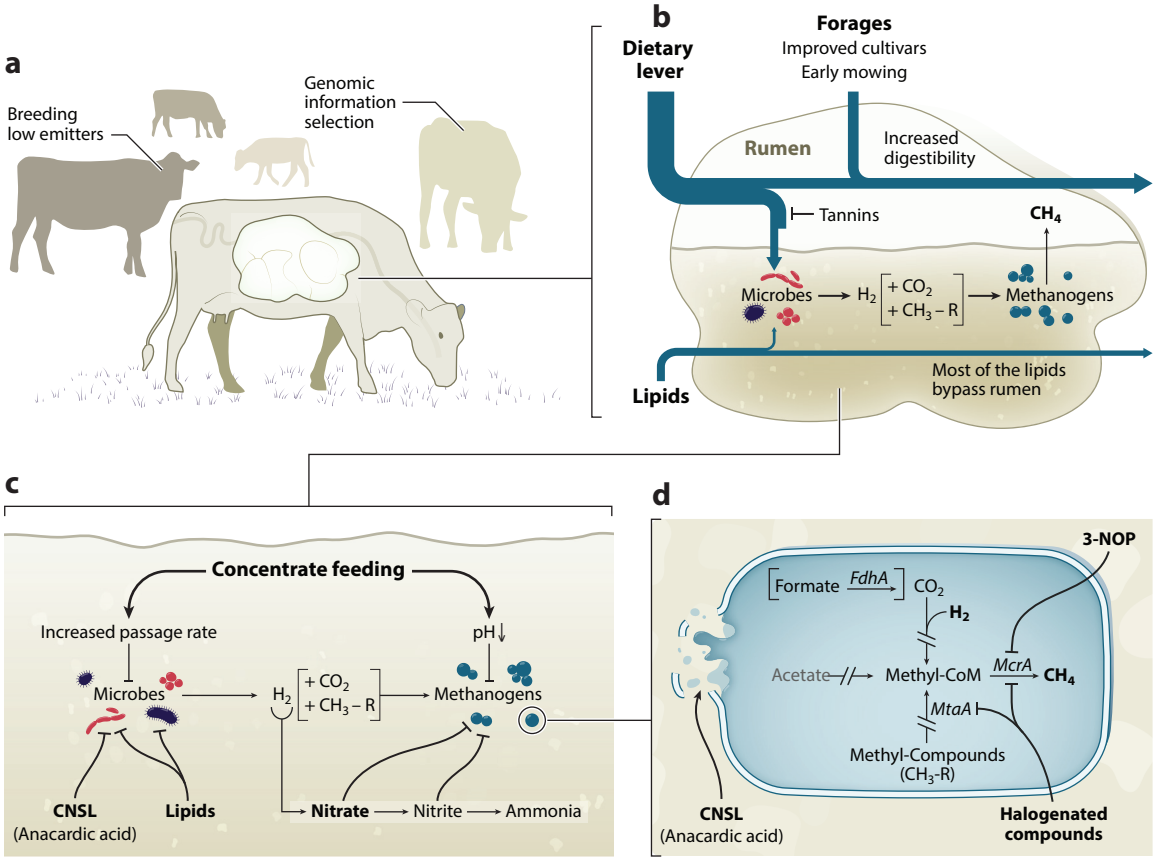

Figure 1

Main strategies and their mechanisms for reducing methane emissions in ruminants. (*a*) At the herd level, methane emissions are reduced by breeding for low emitters, either by selecting for low emitters or by selecting individuals based on their genomic information related to methane emission. (*b*) Methane emissions are reduced by several dietary strategies, by increasing digestibility and passage rates or even bypassing the rumen, to limit nutrient availability to microbes. (*c*) In the rumen, methane emissions are mitigated by strategies that inhibit hydrogen-producing microbes; shift the substrate of methanogenesis; or inhibit methanogens, the microbes that produce methane. (*d*) Targeted strategies reduce methane emissions by inhibiting key enzymes in the methanogenesis pathway or by directly affecting methanogens' viability. Abbreviations: CNSL, cashew nut shell liquid; 3-NOP, 3-nitrooxypropanol.

Reducing the forage-to-concentrate ratio is another way to modulate the production of substrates used by methanogens. Concentrates are rapidly fermentable carbohydrates that shift fermentation pathways toward the production of propionate, a major electron sink under normal conditions (25, 42). In addition, these rapidly fermentable carbohydrates lower the pH of the rumen fluid and therefore inhibit methanogen growth. Methanogens found in the rumen have an optimum pH of approximately 7 and are sensitive to moderate changes, especially lower pH values (43–45). In mixed rumen cultures, methanogenesis was minimal at pH 6 and completely inhibited at pH 5.5 (46). Although excessive concentrate feeding leads to subacute acidosis impairing animal production, rumen pH values <6 are usually observed in lactating dairy cows after feeding (47). Low pH has a direct effect on methanogenesis that is independent of other factors, such as passage rate or substrate type (48), that usually co-occur in the rumen. Janssen (18) reviewed the effects of pH and other co-occurring effects on methanogens. Chemical composition

of the feed and specifically of the carbohydrate fraction also modulates methane emission, which has led to numerous dietary strategies to mitigate methane emissions. For instance, the hydrogenotrophic CO_2-reducing pathway is associated with structural plant carbohydrates cellulose and hemicellulose, whereas the hydrogenotrophic methyl-reducing pathway is associated with other dietary components, such as pectin, carnitine, and choline (49). This duality in substrate dependence influences the effect of dietary strategies on emissions and, more generally, on methanogenesis persistence.

Plants used to feed ruminants also contain secondary metabolites useful for methane mitigation. Particularly, tannins, and essential oils extracted from plants, have been shown to reduce absolute methane production (50–54), but not in all situations, as there are contrasting results. Meta-analyses show modest methane reduction effects of ~10% or less (53, 54), whereas single studies can show significant effects; e.g., a ~30% decrease in emissions was observed in sheep fed tannin-containing forages leucaena and glyricidia (51). These differences may arise because both tannins and essential oils are plant secondary metabolites comprising a diverse group of compounds with different chemical structures and, in many cases, specific biological activity. Contributing to this is the variation found in plants and extracts in the concentration and composition of the mixture of bioactive compounds. Plant secondary metabolites can affect overall feed digestibility and fermentation via mixed mechanisms involving the ones cited above and can also inhibit the microbiota. Tannins reduce the availability of substrates for fermentation in the rumen; affect the colonization of forages by microbes; and have a direct effect on microbes, particularly the fibrolytic microbial community and methanogens (55, 56). The mode of action of essential oils is attributed to their antimicrobial activities (57), and methanogens are more sensitive to some essential oils than fermentative microbes (57, 58). A recent meta-analysis shows that some, but not all, specific mixtures of essential oils can decrease methane production and increase milk yield, but reported changes were small, and more research is needed (53).

All these dietary strategies may, however, prove insufficient or irrelevant for the worldwide majority of farmed ruminants that are raised on extensive and mixed grazing systems (12). In addition, producers, particularly in low- and middle-income countries (LMICs), may not have the economic and technical means to implement these strategies. In this case, grassland management could be an easier solution to implement. Options include use of improved cultivars (e.g., brown midrib maize, high-sugar grasses), grazing at earlier stages of grass maturity for improved digestibility, and legumes containing tannins and other secondary plant metabolites. However, the efficacy of forage and fresh legumes on methane mitigation in ruminants is debated due to the multiple cofactors that might affect their efficacy. These include factors such as the temperature differences in regions of cultivation, where certain climates tend to favor the growth of plants with a lower methane mitigation potential, or animal-related traits such as increased feed intake (59, 60).

All of these dietary strategies, including promoting intake, could decrease methane intensity by up to 18% (35). However, they generally do not decrease absolute methane emissions unless the number of ruminants is reduced. For instance, DMI is closely correlated with absolute methane emissions and production; thus, when methane is expressed per unit of animal product, such as energy-corrected milk, intensity is reduced (34). As intake increases and energy requirements for maintenance are met, more nutrients are available for production (61). Reducing the carbon footprint intensity of animal products is particularly relevant in LMICs, where production efficiency is still low and consumption of ruminant-based foods is expected to grow in the coming years (62). This is acceptable only if increased production efficiency contributes to food security and nutrition for vulnerable populations, but clearly it does not contribute to the long-term sustainability perspective of the global livestock sector. Considering the looming prospect of GHGs leading to rising temperatures, we stress the negative impact of increasing productivity without reducing the

number of ruminants. Importantly, concerted efforts from all stakeholders and the right policies are needed to avoid negative impacts on vulnerable populations in LMICs.

4. ANIMAL BREEDING AND GENETICS

The individual variation in enteric methane emissions that is observed in animals under the same feeding and management conditions suggests the possibility of breeding for low-emissions cattle and sheep (63–66). To breed low-emitting ruminants, animals with the genetic potential for lower enteric methane emissions must be identified and selected (**Figure 1*a***). The heritability (h^2) of methane production, which quantifies the impact of genetic background on methane variations (0: no impact, 1: extreme impact), ranges from 0.13 to 0.29 in sheep (65, 67) and 0.11 to 0.45 in dairy cattle (68–71). However, because direct and accurate individual measurements of methane in a large number of animals are not always possible, the use of proxies for indirect selection or prediction of methane has been recommended. These proxies include traits associated with methane production, such as rumen morphology, feed efficiency, milk mid-infrared spectra, milk volatile fatty acids, signatures of host-associated gastrointestinal microbes, or feeding behavior traits (72–75). Among these, milk mid-infrared spectra, which are readily available from milk recording agencies, and milk volatile fatty acids have been studied extensively. Diet influences milk composition, and these proxies show a wide range of accuracy depending on diet composition (76) or statistical model (77). Predicting methane levels from efficiency-related traits, such as intake or residual feed intake, has also been proposed, but considerable variation has been reported with conflicting results, including a lack of association with methane emissions (63, 78–80). Therefore, further studies are needed that consider the variability due to external factors such as diet, production system, recording period, and season. Alternatives based on the use of residual methane metrics estimated from variables such as DMI, body weight, and milk yield have also been recommended. Residual methane emission is the difference between the actual and expected methane output (81). For example, Manzanilla-Pech et al. (64) showed in dairy cattle that residual methane adjusted by metabolic body weight and energy-corrected milk appears to be a more suitable metric for inclusion in breeding selection indices than methane production, methane yield, or methane intensity.

Another critical limitation to the implementation of breeding programs to reduce methane is the economic value associated with methane emissions. To the best of our knowledge, there are currently no benefits to farmers associated with reducing methane emissions. In fact, several authors agreed that setting the price of methane production in the breeding target too low may make it impractical to implement (66, 82, 83). Therefore, government support, policies, and regulations should be put in place to support the adoption of breeding as a mitigation strategy to reduce methane emissions.

In addition to traditional breeding programs, the use of genomic information offers alternatives for selecting low-emitting ruminants. Today, hundreds of thousands of genetic markers covering the entire genome can be genotyped at reasonable cost. Having established the genetic basis of a trait of interest, a logical next step is to explore its genetic architecture using genome-wide association studies. In dairy cattle, the polygenic nature of individual variability in methane production has been demonstrated, and quantitative trait loci, single-nucleotide polymorphisms, and candidate genes associated with methane traits have been reported (84–87). However, current studies have ignored genotype × environment interactions, and some have used relatively small numbers of animals (<300). Furthermore, independent replication between studies is lacking; interestingly, however, most of the studies report overlaps between genomic intervals associated with methane emissions and quantitative trait loci associated with feed efficiency, milk production, and conformation traits. Therefore, before considering the inclusion of associated single-nucleotide

polymorphisms in breeding programs, additional research must be conducted to investigate the potential pleiotropic effects of the observed associations, because they may have unintended negative effects on productivity or farm profitability.

The genomic information can also be used to improve the accuracy of breeding value prediction through genomic selection. However, implementing genomic selection requires a reference population of individuals with genotypes and accurate phenotypic records. For low-heritability traits such as methane emission, many genotyped animals with low levels of relatedness and accurate measurements of methane are essential to ensure accurate predictions (66, 88). However, accurate measurement of methane based on the gold standard of respiration chambers in thousands of samples is prohibitively expensive and impractical in extensive conditions. Although alternative technologies are available to measure methane emissions, many have been tested only in intensive dairy systems and are not suitable for pastoral production systems. Two options for quantifying methane under grazing conditions are the sulfur hexafluoride tracer technique and the GreenFeed system. However, these systems can be expensive to install and maintain and require Internet access for data collection, which can be an issue for LMICs with limited resources and infrastructure. In addition, performing genomic analyses requires access to computational infrastructure and expertise in data analysis, quantitative genetics, and modeling (89).

Importantly, breeding for low methane emissions has several advantages over other methane mitigation strategies, including a permanent and cumulative effect over generations. It is also a practical choice for extensive pastoral ruminant production systems. These advantages should be considered when assessing the suitability of genetic selection as a methane mitigation strategy. As a proof of concept, a pioneering initiative in New Zealand has demonstrated the potential of genetic selection to reduce methane emissions. After 10 years of divergent selection in ewes, Rowe et al. (65) reported a 12% difference in methane yield between selected lines. This demonstrates an effective response to selection and host genetic control. Integrative multi-omics approaches also offer many novel opportunities. Recent studies have confirmed that methane production results from a joint contribution of the host genome and the ruminal microbiome (70, 75, 84, 90, 91), opening up the possibility of developing innovative hologenomic methods for mitigation. Although still in its early stages, promising research results indicate that microbial data can significantly improve phenotype predictions, regardless of whether some microbes are under direct genetic control by the host (92). A recent study in sheep confirmed that holobiont models, incorporating host genome and metagenome information, provide better predictive accuracy compared to genome-centric approaches (91). The authors also showed that a reference-free metagenome profile performs better than a metagenome profile restricted to a reference database, explaining in combination with the host genotype more than 70% of the variation in methane emissions and residual feed intake. However, the reliability of these estimates is not yet well established, microbial profiling remains costly, and which statistical approach is most effective for implementing hologenomic predictions is unclear. Further research is therefore needed with larger sample sizes, during key life events, and covering different environments.

In summary, the genetic basis of methane emissions in ruminants is still not understood fully, and further research is needed, particularly regarding the use of proxies to predict methane emissions. Similarly, a comprehensive understanding of host–microbiome interactions is critical to the successful implementation of novel hologenomic strategies to reduce enteric methane emissions. Although we acknowledge the promise of breeding to reduce methane emissions, we believe it is insufficient as a stand-alone approach. Therefore, this challenging journey from classical breeding to hologenomic-based methane mitigation strategies requires additional multidisciplinary research and collaborative efforts to effectively reduce the environmental impact of ruminant production systems.

5. MODULATION OF THE RUMEN MICROBIOTA AND FERMENTATION

Dietary and management strategies described in previous sections have an average mitigation effect at the herd level estimated at 18% (35), but globally this figure is much lower (<10%) given the adoption rates and economics associated with the changes (93). In addition, most of the strategies would reduce the intensity of ruminant-based products, but emissions would be reduced only if ruminant numbers were reduced. In contrast, strategies that specifically target methanogens and the methanogenic pathway (**Figure 1*d***) are effective in reducing emissions, often with limited negative side effects on the rumen microbial ecosystem. These strategies take the form of feed additives, some of which are now commercially available in some countries. In this section, we present the recent development of such targeted strategies and discuss the technical knowledge gaps for expanding their use.

5.1. Inhibition of Methanogenesis by 3-Nitrooxypropanol

The ability of 3-nitrooxypropanol (3-NOP) to inhibit methane production in ruminants results from its highly specific microbial target, the active Ni(I) site of the methyl-coenzyme M reductase that catalyzes the last step of methanogenesis (94). As a result, the relationship between 3-NOP dose and methane inhibition appears to be linear (95), with an average reduction in daily methane emissions of 32.5% (96). Despite its known mode of action, its effects on animal performance, rumen fermentation, and microbiota vary with dose, animal type, and basal diet (97, 98). Interestingly, inhibition of the methanogen population in the rumen is not always realized, even when reductions in methane emissions are reported (99, 100). When feeding beef cattle a 90% forage diet with 3-NOP at 200 mg/kg dry matter, Gruninger et al. (101) reported minimal impact on the relative abundance of predominant bacteria and archaeal communities but observed a reduction in the *Bacillota:Bacteroidota* (homotypic synonym *Firmicutes*:*Bacteroidetes*) ratio. Conversely, a reduction in the total methanogen population was observed when 3-NOP was included at doses of 2–2.5 g/animal in beef cattle fed a tropical forage diet (102), indicating that further research on the effects of 3-NOP on the microbiome is needed.

The anti-methanogenic effect of 3-NOP is linked directly to its presence in the rumen, yet as a result of its highly soluble nature, its ruminal residence time is short-lived (94). Consequently, current use of 3-NOP is limited to intensive feeding systems with the capability to offer at least one and preferably two or more feeding bouts per day. In attempts to overcome this, slow-releasing formulations of 3-NOP are being investigated to prolong the time that 3-NOP is active in the rumen (103). Further, a stacking of additives, such as that seen by Gruninger et al. (101) when 3-NOP was combined with canola oil, suggests an additive effect on methane inhibition with a reduction in methane observed for 21 h after feeding. A stacking of additives could provide productivity gains not commonly seen with 3-NOP supplementation alone. Despite an increase in propionate concentration in the rumen with 3-NOP supplementation (96), the expected increase in animal performance associated with an increase in energy-dense volatile fatty acids has not yet been realized. The ultimate fate of excess H_2 remains to be understood, although it does not appear to be fully used by the main fermentation pathways and is instead expelled as gaseous H_2 (104).

Early-life administration of 3-NOP as a mechanism to program the rumen for lower methane emissions has shown considerable promise. Meale et al. (100) successfully intervened with 3-NOP supplementation from birth to 11 weeks of age to imprint a methane reduction still observed at one year of life. An intervention targeting crucial periods of development, including microbial establishment in the newborn and the weaning transition, is necessary to overcome the highly resilient nature of the mature ruminal microbiota and the strong host–microbiota interaction, which often forces the microbial population to revert back to its preintervention state (105). However,

such an intervention is labor intensive, as the method of administration must not induce the reflexive closure of the esophageal grove in neonates, as would normally occur if the animal is suckling milk.

5.2. Inhibition of Methanogenesis by Halogenated Compounds

Halogenated compounds, synthetic (e.g., bromocloromethane, chloroform, and bromoform) and naturally occurring (e.g., bromoform produced by marine algae species), are very effective in inhibiting methane production in ruminants. Halogenated methane analogs have a direct inhibitory effect on methanogenesis, mainly by binding with coenzyme M methyltransferase, thus inhibiting methyl transfer in methanogenesis (106). Recently, Glasson et al. (106) suggested that halogenated compounds can inhibit both coenzyme M methyltransferase and methyl coenzyme M reductase in vivo, although the exact extent of the inhibition of both pathways requires confirmation. Bromochloromethane supplementation to cattle, sheep, and goats decreased methane production between 30% and 91%, with an increase in expelled H_2 levels (when measured), showing no detrimental effect on DMI or ruminal fermentation (21, 107, 108). Chloroform supplementation also decreases methane production in ruminants (38–89%), while increasing the H_2 expelled by the animal (20, 102, 109). These studies showed no apparent detrimental effects on rumen fermentation or feed intakes in cattle fed forage alone or supplemented with concentrate. Regarding naturally occurring halogens, marine algae are important producers of halogenated, low–molecular weight compounds (such as brominated and chlorinated haloforms) as a defense mechanism against predators or environmental stressors (110). Red seaweed species (*Asparagopsis taxiformis* and *Asparagopsis armata*) contain high concentrations of bromoform and inhibit methanogenesis in vivo. In recent years, *Asparagopsis* has been tested in sheep (111), dairy cattle (112, 113), and beef cattle (22, 114), showing a linear decrease in methane production (9–98% reduction) with increases in H_2 expelled. Although most studies reported nonapparent detrimental effects on DMI or ruminal fermentation, some studies found a decrease in DMI and milk yield with high doses above 1% of *Asparagopsis* per kilogram of DMI (112, 113, 115), which could be also due to a greater concentration of iodine or other minerals. In addition, some studies reported an increase in productivity and feed conversion efficiency (22, 114); however, these findings must be considered with caution due to the experimental designs used and the small number of animals.

The use of synthetic or naturally occurring halogenated compounds could also have collateral effects, as they might inhibit the methyl transferases in other microorganisms (116). For instance, these compounds may affect reductive acetogenesis, as B12-dependent methyl transferases play a key role in one-carbon metabolism in this process (117). Other considerations for the use of these compounds in livestock are the environmental impact (ozone depletion), effects on animal health, and potential residues in products for human consumption. Some studies and reviews (22, 106, 112) suggested that animal health and product residues are not compromised with the *Asparagopsis* levels used for inhibiting methanogenesis in vivo, and that contribution to ozone depletion is minimal relative to the total sources of anthropogenic bromine (106). However, further research (e.g., longer-term studies using greater numbers of animals) must be carried out to confirm these claims.

5.3. Inhibition of Methanogenesis by Surfactant Anacardic Acid

Cashew nut shell liquid (CNSL) is an abundant coproduct of cashew production. It has been used for broad applications ranging from the chemical industry to biological applications such as antimicrobials (118). Indeed, CNSL contains phenolic compounds with antimicrobial activities, namely, anacardic acid, cardanol, and cardol, with anacardic acid being the main one. The antimicrobial activity of anacardic acid was already advocated a few decades ago to reduce methane

produced in the rumen (119). Watanabe et al. (120) later confirmed the anti-methanogenic potential of raw CNSL. They demonstrated a reduction of methane production along a shift of rumen microbes toward propionate producers in three independent in vitro experiments, namely, a batch culture and a semicontinuous culture of rumen fluid experiment and a pure culture of selected bacteria. Anacardic acid exhibits a surfactant activity that disrupts the cell wall of some microbes. Methanogens are particularly sensitive to anacardic acid, requiring lower inhibitory concentrations than most bacteria (121). Methanogens lacking an external proteinaceous surface layer in their cell envelope, such as the genus *Methanobrevibacter*, are susceptible, whereas other methanogens, such as *Methanosarcina barkeri* and *Methanomicrobium mobile*, do present such a surface and are not disrupted by CNSL. However, there are exceptions in both cases (121), and other membrane components seem to play a role in the resistance to CNSL that needs to be elucidated. Note that the presence of pseudomurein in the sensitive genera *Methanobrevibacter* and *Methanobacterium*, which are positive to Gram staining, is replaced by protein subunits and heteropolysaccharides, respectively, in the resistant genera *Methanomicrobium* and *Methanosarcina* (122). The surfactant property of anacardic acid also disrupts Gram-positive bacteria (118). For instance, CNSL disrupted the cell walls of *R. flavefaciens* and *Butyrivibrio fibrisolvens*, which are fibrolytic bacteria producers of H_2, in an in vitro assay (123).

CNSL's effects on methane emission were also observed in Holstein cows fed CNSL, with up to a 38% decrease in emissions (CH_4/kg of DMI) (124). Further in vivo trials confirmed the reduction of methane emission by CNSL (125–127). Together, these studies revealed the mode of action of CNSL. The reduction in the number of hydrogen producers is compensated, at least in relative abundance, by succinate and propionate producers, including *Succinivibrionaceae* and *Prevotellaceae*. Moreover, these studies highlighted a decrease in the relative abundance of *Methanobrevibacter*, the main methanogen genus in the rumen, characterized by both 16S rRNA and *mcrA* gene sequencing.

Although promising, CNSL use is limited by the stability of its main antimicrobial compound, anacardic acid. Indeed, heating of CNSL, a common process to enrich CNSL in cardanol for industrial uses, decarboxylates anacardic acid, thus converting it to cardanol (128). Cardanol itself has much lower antimicrobial activity, and heated CNSL does not reduce methane emission in vitro or in vivo (120, 129). Thus, the chemical industry's demand for cardanol reduces the availability of raw CNSL for the livestock sector. A second minor limitation is the nonadditive effect of CNSL with another inhibitor of methanogenesis, although only one study assessed a combined effect with encapsulated nitrate (130).

5.4. Inhibition of Methanogenesis by Nitrate

Nitrate has been evaluated in several methane mitigation studies and has shown consistent and persistent reductions in enteric methane emissions (53, 131). Nitrate supplementation is assumed to lower methane emissions by decreasing hydrogen availability in the rumen. Screening of the 501 reference genomes of cultured rumen bacteria showed that the *Selenomonadales* and *Campylobacterales* clades encode enzymes involved in the reduction of nitrate (NO_3^-) to ammonia (NH_3) (132). Furthermore, in vitro studies with pure cultures of *Selenomonas ruminantium* ssp. *lactilytica*, *Wolinella succinogenes*, and *Veillonella parvula* have shown these species to be active for nitrate and nitrite reduction (133). In sheep with a low methane-emitting phenotype, the expression level of ammonia-forming nitrite reductases increased along with hydrogenotrophic acetogenesis and fumarate reduction, demonstrating that non-methanogenic pathways can be effective hydrogen sinks (132). New evidence suggests nitrate could reduce methane emissions via direct inhibition of methanogens. Dietary nitrate supplementation in dairy cows increases the concentration of rumen dissolved hydrogen and expelled hydrogen (134). These results suggest nitrate's

toxic effect on rumen methanogens as hydrogen consumption is reduced. Importantly, a significant decrease in the number of methanogens was reported in sheep receiving nitrate supplementation (135). In steers, changes in absolute numbers were not significant (136), but methanogen diversity was affected. Similarly, *mcrA* gene expression was reduced after nitrate feeding to dairy cows, but no change in the number of methanogens was observed (137). Lastly, a recent dynamic mechanistic modeling approach supported the claim that the methane mitigation effect of nitrate supplementation was due to methanogen inhibition by nitrites rather than reduced hydrogen availability (138).

At a low level, as it is in forage crops, nitrate is not a cause for concern for ruminants. However, the higher doses of nitrate required to decrease methane production in ruminants will result in absorption of nitrate and nitrite into the blood through the rumen wall due to incomplete reduction to ammonia. Nitrite in the blood binds to hemoglobin and converts it to methemoglobin, a metalloprotein incapable of carrying oxygen. High levels of methemoglobin are associated with a range of clinical symptoms, reviewed in detail by Lee & Beauchemin (131). Nitrate supplementation may contribute to increased N emissions from manure as it is further reduced to ammonia in the rumen and excreted as urea in the urine (139). However, as a methane mitigator, nitrate could also be a lever for dietary N reformulation, as nitrate increased microbial nitrogen synthesis in vitro (140), suggesting that it could enhance microbial protein flux from the rumen. Nitrate supplementation is a promising strategy for reducing methane production. However, animal health concerns may prevent its adoption in practice, and possible emissions trade-offs must be considered. Nitrate encapsulation is a commercially available technical solution that might circumvent potential health problems, as it induces a slow release of nitrate in the rumen.

Strategies that target the methanogens, either by inhibiting key enzymes in methanogenesis or by acting on the methanogens themselves, are highly effective, reducing enteric emissions by up to 80%. Nevertheless, questions remain about the fate of the breakdown products of some additives in the animal and the environment, as mentioned for halogenated compounds, or the possible adverse effects on animal health of nitrite and other compounds that are absorbed into the bloodstream. Although information on the long-term effects of these strategies appears promising, the results require confirmation with larger studies and under different conditions. Finally, the combination of strategies is promising but needs to be evaluated thoroughly both in terms of the real cumulative effect and in terms of market availability and economic constraints.

6. TECHNOLOGIES IN DEVELOPMENT

A much-hoped-for strategy in the field of enteric methane mitigation is the use of vaccines against rumen methanogens. The prospect of reducing emissions by stimulating the immunological response of the host ruminant is attractive because of its potential applicability under most farming conditions, including pastoral systems. The concept of modulating populations of specific rumen microbes by producing antibodies delivered via saliva after immunization was first demonstrated in the 1990s for ciliate protozoa and then for bacteria associated with rumen acidosis in cattle and sheep (141–143). Research into the approach's application to rumen methanogens soon followed (144, 145). However, the technology is challenging, and several hurdles to its application remain. Over the past 20 years, important advances have been made in several key fundamental areas needed to develop an effective vaccine. These include improved understanding and genomic information on rumen methanogens, which is essential for identifying potential antigens, as well as the demonstration of specific antibodies in blood, saliva, and rumen contents following immunization. Recently, the cross-reactivity of antibodies between various abundant species of the genus *Methanobrevibacter* has been shown (146). Cross-reactivity or the use of antigens

common to most rumen methanogens appears to be necessary to maintain the efficacy of produced antibodies in the long term and to avoid the replacement of targeted species by non-sensitive species (147). Studies on vaccines for methane mitigation were reviewed recently (148), summarizing advances in technology regarding antigen selection and adjuvant use, but also highlighting gaps in knowledge. Key findings of the systematic review (148) are the relatively limited number of publications and the lack of consistent evidence that enteric methane emissions can be reduced in vivo. Therefore, based on this publicly available information, the approach has not moved up the Technology Readiness Level ladder to be considered as a medium-term alternative for the ruminant sector. Potential emissions reductions from this technology have been assumed in some scenarios (149). However, based on published evidence, it is not yet possible to predict whether methane mitigation vaccines will be used in the field.

A novel approach being tested is the capture of exhaled methane from the animal. Different from other strategies that decrease enteric methane production by altering the ruminal environment or interfering with the methanogenic pathway, this proprietary technology uses a catalytic mechanism that converts exhaled methane into CO_2 and water. The portable catalyzer is positioned close to the nostrils via a halter. The developers' initial claims indicate a reduction potential of up to 50%, with no negative effect of the halter on animal behavior and welfare. Expected scientific publications in peer-reviewed journals should provide more information on the potential impact of this technology (150).

Novel slow-release technologies (such as rumen-bolus systems) are under development currently. These systems will allow release of anti-methanogenic compounds (e.g., bromoform, 3-NOP) at a constant rate in the rumen for prolonged periods of time. This is of particular interest in extensive grazing systems (such as the subtropical rangelands in Australia), where livestock consume less frequent and significantly lower amounts of supplements due to environmental conditions and availability of quality pastures during the wet season (3–5 months per year). Also, this technology could maximize the inhibition effect of the compounds, as they will be released continually in the rumen.

Various approaches at different stages of development are reported regularly in the scientific literature. It is beyond the scope of this review to list all these strategies (for comprehensive lists, see 33, 53, 54). However, some of these approaches have been studied for several years but have not progressed to higher Technology Readiness Levels and, if successful, may be available for field use only in the medium to long term. For instance, the use of homoacetogens as direct-fed microbials (DFMs) to decrease rumen methane emissions has been largely explored. The concept is attractive but has not been successful until now because the partial pressure of H_2 in the rumen is normally lower than the threshold for reductive acetogenesis, with homoacetogens having a lower H_2 affinity constant (Ks) compared to methanogens (15, 151). Other possible innovations in DFMs aimed at reducing methane production include the selection of lactic acid bacteria that produce methanogen-specific bacteriocins (152) and the use of sulfate-reducing bacteria that can oxidize hydrogen sulfide, thus avoiding the negative effects of this gas on animal performance and health (153). In the latter case, the DFMs must be combined with a sulfate-containing feed ingredient or additive. However, all these approaches have little chance of success in the field unless new DFMs are discovered or there is a breakthrough in the biology of these microbes, i.e., homoacetogens that compete with methanogens in the rumen. Expected emissions reductions from these innovations cannot be estimated at present.

A decade ago, Leng (154) reported that biochar, a charcoal product from organic matter pyrolysis, decreased enteric methane emissions and hypothesized that biochar's increased surface area would favor biofilm formation and methane oxidation. This has led to numerous research projects, but overall, the effects reported in vivo are variable, limited, and often contradictory,

although this might also be due to inaccurate ways to measure methane emission (155, 156). A recent report found that not all biochars can decrease enteric methane emissions in ruminants, and the effectiveness might depend on several factors, such as biomass type, pre- and post-pyrolysis manipulation, and compounds in the biochar (157). The in vivo methane reduction of the biochars and doses tested was less than 12% under controlled feeding conditions, and no significant effect was observed under grazing conditions (157). Further research is required to identify a biochar with much greater anti-methanogenic properties to be viable as feed supplement.

7. CHALLENGES AHEAD

The UN goal of achieving net zero emissions by 2050 to limit the rise in global temperatures will require a contribution from all sectors. Agriculture is no exception, and ruminant production, with its recognized high environmental impact, is under pressure to be sustainable. However, sustainability also rests on social and economic pillars that must be fulfilled for long-term solutions. Ruminant livestock also contribute substantially to UN Sustainable Development Goal 2, Zero Hunger, through their contribution to food security and improved nutrition and livelihoods, particularly for vulnerable populations in LMICs. Reducing emissions to net zero is a major challenge for the sector, compounded by global warming's increasing impact on animals and resources. Droughts and rising temperatures are already affecting forage and crop production, with tropical and subtropical regions with rain-fed pastoral and mixed systems most at risk (158). The estimated reduction in livestock production by the end of the century ranges from 4% to 10%, depending on the GHG emission pathway chosen (159). This figure, calculated from the reduction in DMI due to heat stress, is rather conservative, as the effects of heat stress on reproduction and health (158) were not considered (159). Breeding for heat-tolerant animals is one of the adaptation strategies the sector is exploring. These breeding programs should consider incorporating methane emission traits into selection indices to avoid unintended selection for high methane emitters.

Several aspects link enteric methane emissions and global warming. Higher ambient temperature decreases DMI and, consequently, enteric methane emissions. Concurrently, under heat stress, there is a decrease in the acetate-to-propionate ratio, along with an increase in lactate and a lower pH in the rumen (160). As described in previous sections of this review, all these factors contribute to lower methane emissions per animal, but for these poorly performing animals, the methane intensity of the meat and milk produced is much higher. However, temperature's effect on methane does not appear to be linear. In crossbred cattle adapted to tropical conditions, increases in temperature from 25°C to 35°C decreased methane emissions, but at 40°C, methane output was the same as at 25°C, whereas DMI continued to be affected (161). Why this happens is unclear, but if confirmed, it will have implications for overall emissions in hot regions and temperate regions now becoming warmer. A related aspect is that underfed ruminants, a common situation during the dry season in tropical areas, have higher emission yields (162).

Most farmed ruminants, 75% of large ruminants and 85% of small ruminants, come from grazing and mixed crop-livestock systems (163), with the highest concentration in tropical and subtropical regions (**Supplemental Figure 1**). The trend of increasing numbers of ruminants from LMICs in these regions has not abated since global data became available in the early 1960s (**https://www.fao.org/faostat/**). In contrast, ruminant numbers in high-income countries, mostly in temperate regions, have not increased or have even decreased (**Supplemental Figure 1**). Improvements in production efficiency have led to reductions in ruminant numbers and methane emissions, whereas milk and meat production has increased [e.g., for Germany and the United States (61, 164)]. Mitigation options (available and in development) can be applied to the intensified production system prevalent in temperate regions but are more difficult to apply in grazing

Supplemental Material >

and extensive systems prevalent in Africa, Australia, and South America. In addition, in some regions, such as dry tropics rangelands in northern Australia, animals graze in very extensive areas with little human interaction, in an environment dominated by a short wet season and a long dry season, making it difficult to deploy feed supplements or other methane abatement strategies consistently through the whole year. Other critical challenges, particularly for grazing systems, are that anti-methanogenic compounds must be continuously available in the rumen to be effective; thus, the timing, frequency, and quantity consumed are critical to maximize methane inhibition. Therefore, novel ways of delivering these strategies to cattle under these conditions must be developed and evaluated.

For global impact, novel strategies, including delivery methods, applicable to grazing and less-intensive mixed systems are needed. This is because the trajectory of intensification observed in high-income countries cannot be followed in LMICs due to the current and future constraints of global warming on production outlined above, as well as economic and infrastructure limitations. Intensification based on concentrate feeds also would not be recommended, given food competition with human feed production. Globally, increased energy costs render the use of cereals from arable land to feed ruminants less competitive, which naturally leads to more grazing and fewer animals (165).

8. CONCLUDING REMARKS

Recent years have seen significant progress in understanding and reducing enteric methane emissions in ruminants. Whereas dietary and breeding strategies have been shown to be effective, their impact on global emissions is limited. Research on and expansion of these strategies should continue, as no single solution can be applied to all production systems. The improved production efficiency achieved by these strategies decreases methane intensity and has allowed some regions of the world to reduce herd size and total methane emissions while increasing milk and meat production. However, the global increase in ruminant livestock numbers offsets these regional improvements.

Inhibition of enteric methane by targeting the microbiota is the most successful strategy with potential for high impact. The most promising and novel approaches are in this area, but technical hurdles for long-term delivery and efficacy must be overcome to have a global impact. Importantly, the cost of treatment is not offset by increased production and, if not offset by other mechanisms, will effectively prohibit its global adoption. Ruminant methane emission is a global problem. Part of the solution is to reduce enteric methane production, as highlighted in this review. Progress in this area is encouraging, but a massive research effort is needed to find solutions that can be applied globally and have a lasting positive effect. For this to happen, the right funding, policies, and government interventions must be in place to ensure access and improve equity.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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