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Feed additives for methane mitigation: A guideline to uncover the mode of action of antimethanogenic feed additives for ruminants

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ABSTRACT

This publication aims to provide guidelines of the knowledge required and the potential research to be conducted in order to understand the mode of action of antimethanogenic feed additives (AMFA). In the first part of the paper, we classify AMFA into 4 categories according to their mode of action: (1) lowering dihydrogen (H_2) production; (2) inhibiting methanogens; (3) promoting alternative H_2-incorporating pathways; and (4) oxidizing methane (CH_4). The second part of the paper presents questions that guide the research to identify the mode of action of an AMFA on the rumen CH_4 production from 5 different perspectives: (1) microbiology; (2) cell and molecular biochemistry; (3) microbial ecology; (4) animal metabolism; and (5) cross-cutting aspects. Recommendations are provided to address various research questions within each perspective, along with examples of how aspects of the mode of action of AMFA have been elucidated before. In summary, this paper offers timely and comprehensive guidelines to better understand and reveal the mode of action of current and emerging AMFA.
Key words: methanogens, mitigation, rumen methanogenesis, rumen microbiota

INTRODUCTION

Enteric methane (CH_4) from ruminant livestock accounts for 30% of anthropogenic CH_4 emissions and 5% of anthropogenic GHG emissions worldwide (Jackson et al., 2020). Given that 88% of the livestock CH_4 emissions are contributed by enteric fermentation, interest is increasing in mitigating CH_4 emissions from ruminants as a way to achieve the goals of the Paris Agreement (limit global warming to 2.0°C, and preferably 1.5°C, above preindustrial levels; Arndt et al., 2022). Meta-analyses have consistently shown that antimethanogenic feed additives (**AMFA**) are the most potent abatement strategy to mitigate enteric CH_4 emissions from ruminants (Almeida et al., 2021; Arndt et al., 2022).

The adoption of AMFA as a mitigation strategy in ruminant production requires a consistent and sustained reduction of rumen CH_4 production with no negative effect on animal health, well-being, or productivity. Moreover, AMFA should also be safe for workers handling the additives, as well as consumers, and the environment. A thorough understanding of how AMFA inhibit CH_4 production in the rumen is necessary, as is an understanding of the metabolic fate of AMFA in the animal, the environment, and humans consuming animal products. Without this understanding, it may be more difficult to approve or register AMFA (Tricarico et al., 2025), or to recommend or account for its use (del Prado et al., 2025; Dijkstra et al., 2025). Defining specific targets and understanding

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the modes of action can also enable the search for and discovery of new AMFA and decrease social resistance to the adoption of AMFA in livestock production.

Although inhibiting the formation of CH_4 in the rumen and its release to the atmosphere would theoretically save energy for the ruminant host, this intervention has not resulted in consistent improvements in animal performance; instead, it increases feeding costs (Ungerfeld, 2018; Morgavi et al., 2023). Understanding how AMFA affect the rumen microbial ecosystem and the host metabolism, could also allow developing more tailored and cost-effective mitigation strategies. For example, selecting the appropriate type of AMFA, adjusting the dose or delivery mechanism according to diet, animal type and production system (Ungerfeld et al., 2022). Diets could also be reformulated considering changes in feed intake and nutrient digestion and absorption caused by the inhibition of CH_4 production, if those changes are understood.

This paper provides guidelines and recommendations about the knowledge required to define the mode of action of commercial or investigational AMFA and the research needed to fill existing knowledge gaps. The first part of this paper provides a brief overview of rumen metabolism and methanogenesis to enable the classification of the AMFA into 4 categories according to their broad mode of action. The second part of the paper focuses on research strategies recommended to establish the mode of action of an AMFA from various perspectives. We use the term "mode of action" in a broad sense to describe the overall effects of AMFA on specific microorganisms, the microbial community, and the collective metabolism of the rumen microbial ecosystem, rumen hydrogen dynamics, and methanogenesis, as well as any effects on the host. The term "mechanism of action" refers to the understanding of how a compound interacts with a microbial cell at a molecular level.

Investigating the mode of action of an AMFA involves considerable financial resources, specialized equipment, and the dedication of expert human resources. Therefore, the present guidelines are intended for AMFA that have demonstrated consistent efficacy in vitro or in animal trials without showing negative effects on animal production and health. In this regard, the present guidelines should be viewed within the context of the collection of 6 papers of Technical Guidelines to Develop Feed Additives to Reduce Enteric Methane. The research discussed in the first 2 papers of this series present guidelines for in vitro discovery and identification of bioactive compounds (Durmic et al., 2025) and testing at animal level (Hristov et al., 2025), which will usually precede the research herein for elucidating the modes of action of AMFA. Elucidating the modes of action of AMFA will aid in the interpretation and modelization of the enteric CH_4 mitigating effects (Dijkstra et al., 2025), in the registration and regulation process (Tricarico et al., 2025), and in carbon accounting (del Prado et al., 2025).

POTENTIAL TARGETS FOR AMFA

The AMFA can be broadly classified into 4 categories according to the targeted microbes and metabolic pathways or processes affected (Figure 1): (1) modulation of the rumen microbial fermentation to decrease H_2 production, (2) direct inhibition of methanogens, (3) H_2 redirection toward alternative electron (e^-)-incorporating pathways, and (4) CH_4 oxidation. However, many AMFA may have multiple modes of action to decrease CH_4 emissions.

H_2 Production and Its Inhibition

In the rumen, a variety of polysaccharides, including cellulose, hemicellulose, pectin, and starch, undergo hydrolysis, ultimately yielding glucose and other hexoses and pentoses (Figure 1). Monosaccharides experience subsequent catabolism, leading to the production of VFA (mainly acetate, propionate, and butyrate), and carbon dioxide (CO_2), releasing e^- that are accepted by microbial intracellular cofactors NAD^+ and oxidized ferredoxins (Ungerfeld, 2020). Cofactors, once reduced, must be re-oxidized through transferring e^- for rumen fermentation to continue (Wolin, 1979). Re-oxidation of reduced cofactors is largely (but not solely) facilitated by hydrogenases, enzymes catalyzing the generation or uptake of dihydrogen (molecular hydrogen or H_2; Ungerfeld, 2020). Genes encoding hydrogenases are present in about two-thirds of the sequenced genomes from cultivated rumen bacteria (Greening et al., 2019). Dihydrogen-evolving hydrogenases catalyze the transfer of e^- from reduced cofactors to protons (H^+) to form H_2. Most rumen H_2-evolving hydrogenases genes and transcripts correspond to the bifurcative type [FeFe] A3 and prototypical hydrogenases [FeFe] A1 (Greening et al., 2019; Pitta et al., 2022b). In the rumen, H_2 exists in 2 phases: dissolved H_2 in the liquid phase and H_2 in the gaseous phase, with only dissolved H_2 being available for microorganisms (Beauchemin et al., 2020). Under typical conditions, dissolved H_2 is at low concentration in the rumen (0.1–50 μ*M*; Janssen, 2010) and does not accumulate as it is used by methanogenic archaea to produce CH_4. Removal of H_2 helps the re-oxidation of NADH to NAD^+, with NAD^+ being required to accept e^- from the oxidation of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate in glycolysis (Voet and Voet, 1995), the central catabolic pathway of hexoses in the rumen (Russell and Wallace, 1997). Thus, ultimately, removal of H_2 is important for the continuity of fer-

METABOLIC PATHWAYS AND POSSIBLE MODES OF ACTION

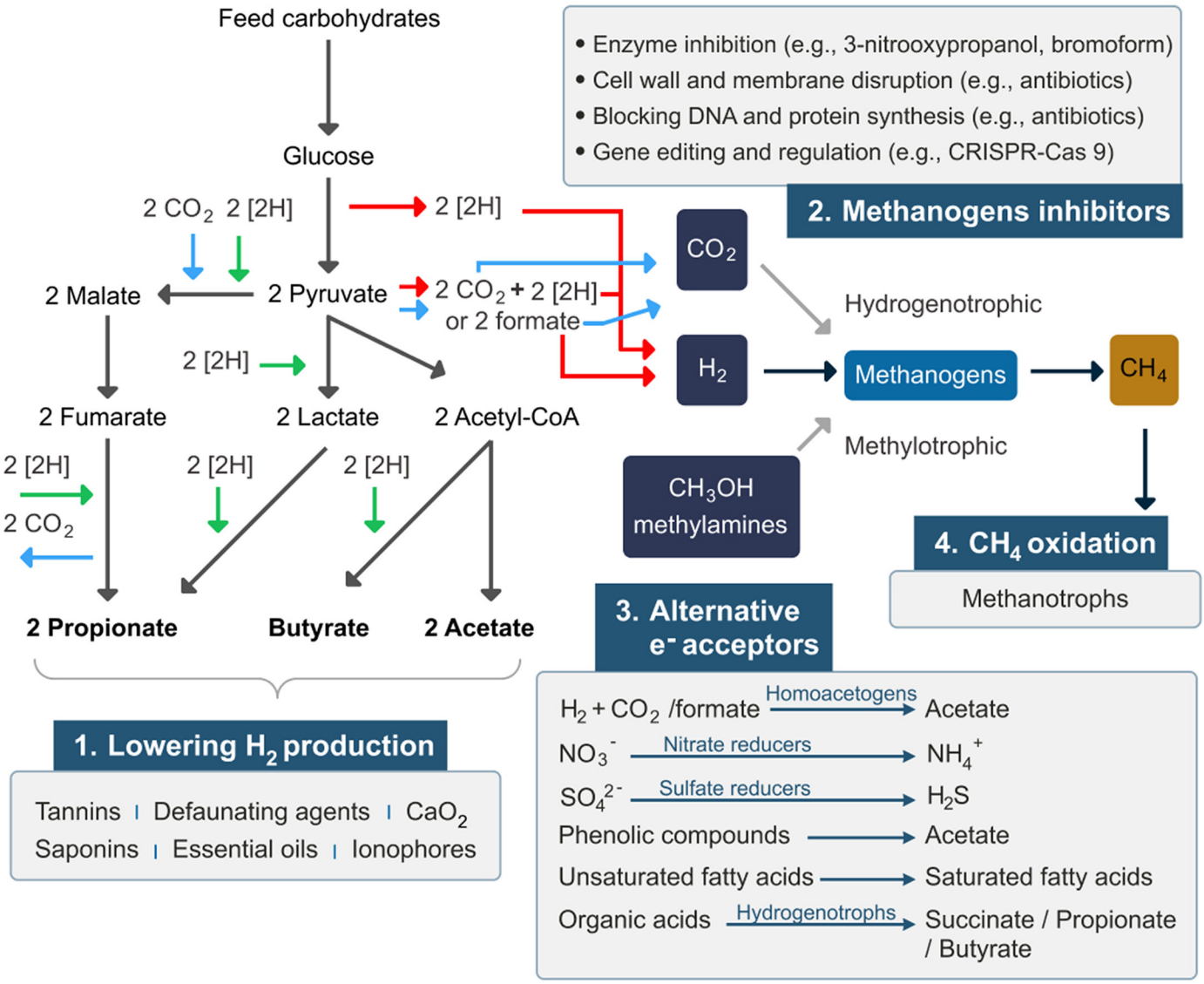

Figure 1. Illustration of the rumen methanogenesis and the main categories of antimethanogenic feed additives (AMFA) according to their intended mode of action. Blue arrows represent flows of CO_2 release and incorporation. Red and green arrows indicate release and incorporation of reducing equivalent pairs, respectively. Unintended effects on nontargeted microbes/processes are not included. Not all AMFA in the figure show consistent effects and variation exists. Only catabolic pathways with glucose as a substrate are presented for simplicity: Glucose + 2 H_2O → 2 $Acetate^-$ + 2 H^+ + 2 CO_2 + 4 [2H]; Glucose → $Butyrate^-$ + H^+ + 2 CO_2 + 2 [2H]; Glucose + 2 [2H] → 2 $Propionate^-$ + 2 H^+ + 2 H_2O; CO_2 + 4 [2H] → CH_4 + 2 H_2O. Created by A. Belanche and Sabrina Garay; used with permission.

mentation (van Lingen et al., 2016). Protons in solution do not count for electron flows and balances, as H^+ in solution are devoid of e^- and instead participate in acid-base reactions. It is redox reactions that generate the Gibbs energy changes that drives microbial ATP generation and transmembrane electrochemical gradients that fuel nonspontaneous anabolic processes. The pool of hydrogen atoms composed of H^+ and an e^-, which can be exchanged in fermentation, is collectively termed metabolic hydrogen ([H]). A portion of [H] released in fermentation will become H_2. When conducting e^- balances for pathways and overall fermentation, it can be convenient to quantify [H] sinks as pairs of reducing equivalents ([2H]; Ungerfeld, 2020).

Methane production can be decreased using AMFA that inhibit the rumen microbiota involved in the production of acetate (e.g., *Ruminococcus albus* and *R. flavefaciens*), to lower the availability of H_2 for methanogens (Figure 1; Pereira et al., 2022). Because [H] release is associated with the production of acetate and, to a lesser extent, of butyrate, and conversely, propionate production incorporates [H], inhibiting microorganisms producing H_2 (or modifying their metabolism) generally shifts fermentation from acetate toward propionate (Janssen, 2010). These AMFA inhibiting the H_2-producing microbiota can be divided into several subcategories and described according to the main targeted microbes and the mechanism of inhibition at the cell level (see "The Cell and Molecular Biochemistry Perspective" section).

The modulation of the type and amount of substrate available for the rumen microbes represents another alternative to decrease H_2 production. Tannins are a heterogeneous group of high molecular weight phenolic compounds with the capacity to form reversible and irreversible complexes with feed proteins, and to a lesser extent, with polysaccharides, alkaloids, nucleic acids, minerals, as well as metabolically active microbial compounds such as enzymes and structural membrane proteins, ultimately limiting microbial digestion and feed fermentation (Frutos et al., 2004). Calcium peroxide (CaO_2) can indirectly affect H_2-producing microorganisms through increasing the oxidation-reduction potential by slowly decomposing into $Ca(OH)_2$ and H_2O_2, with H_2O_2 decomposing to H_2O and O_2 (Demeyer, 1982; Morgavi et al., 2010).

Methanogens and Their Inhibition

There are about 20 species of methanogens isolated from the rumen and characterized, whereas other isolates remain uncharacterized (Khairunisa et al., 2023). Three methanogenic pathways exist, depending on the substrate used as a carbon source (Liu and Whitman, 2008): hydrogenotrophic, methylotrophic (in turn, divided into methyl dismutation and methyl-reducing pathways), and acetoclastic pathways. In the hydrogenotrophic pathway, CH_4 is produced through the reduction of CO_2, primarily using electrons donated by H_2, and to a lesser extent by formate (Ungerfeld, 2020). In this pathway, CO_2 is successively reduced to CH_4 through a series of 8 steps known as the Wolfe cycle (Figure 2). The majority of hydrogenotrophic rumen methanogens belong to the genus *Methanobrevibacter*, including *Methanobrevibacter gottschalkii*, *Methanobrevibacter ruminantium*, and *Methanobrevibacter smithii* (Janssen and Kirs, 2008). In the methylotrophic pathway the primary substrates contain methyl groups, such as methanol, methylamines, and methylsulfides. Methyl groups are transferred to a cognate corrinoid protein and subsequently enter

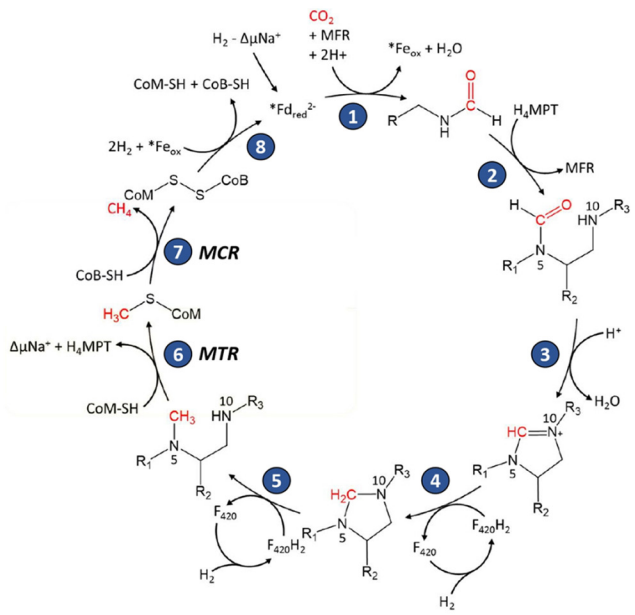

Figure 2. The Wolfe cycle for the reduction of CO_2 to CH_4 in hydrogenotrophic methanogens. Steps: (1) CO_2 reacts with methanofuran (MFR) to produce formyl-MFR; (2) The formyl group is transferred to tetrahydromethanopterin (H_4MPT); (3–5) Intramolecular imine formation and successive reductions; (6) Methyl transfer from methyl-H_4MPT to CoM-SH catalyzed by coenzyme M (CoM) methyl-transferase (cobalamin); (7) Methyl group reduced to CH_4 catalyzed by methyl-CoM reductase (cofactor F_{430}); (8) Ferredoxin mediated regeneration of CoM. Adapted from Glasson et al. (2022) by Sabrina Garay, with permission from Elsevier.

methanogenesis via methyl coenzyme M (**CoM**), where they are further reduced to CH_4 (Ferguson et al., 2000). Methylotrophic methanogens are primarily represented by Methanomassiliicoccaceae-affiliated groups and by *Methanosphaera*, belonging to the order *Methanobacteriales*, and to a lesser extent by *Methanosarcinales*. Only *Methanosaeta* and *Methanosarcina* are known to utilize the acetoclastic pathway to oxidize the carboxyl group of acetate to CO_2 and reduce the methyl group to CH_4.

Among these 3 known pathways, the hydrogenotrophic predominates in the rumen, followed by the methylotrophic pathway (Poulsen et al., 2013). The acetoclastic pathway is virtually absent in the rumen (Morgavi et al., 2010), as evidenced by the fact that methanogens that can utilize acetate, such as *Methanosarcina* and *Methanosaeta*, are consistently in very low abundances across ruminant species and diets (Henderson et al., 2015; Malik et al., 2022), and the acetoclastic pathway was shown to be of negligible importance in rumen batch culture (He et al., 2018). Likely, acetoclastic methanogens are not found in the rumen because the rumen passage rate of rumen contents is greater than their rate (Janssen and Kirs, 2008). Importantly, all 3 pathways share the final steps of the Wolfe cycle (Figure 2), which is catalyzed by

methyl coenzyme M reductase (**MCR**), an enzyme that is a common target of inhibitors (Rouvière and Wolfe, 1988). Methyl coenzyme M reductase utilizes coenzyme B as an electron donor for the reduction of the methyl group in methyl-CoM to CH_4, and it contains a Ni^{+1} ion, forming part of a tetrapyrrole derivative named cofactor F_{430}. The enzyme methyl-tetrahydromethanopterin: coenzyme M methyltransferase (**MTR**) catalyzes the transfer of a methyl group to CoM, generating methyl-CoM, in a reaction dependent on cobamides. This is the antepenultimate step in rumen methanogenesis and is also shared across most methanogens except certain methylotrophs that have similar enzymes (methanol- or methylamine corrinoid methyltransferases). Similarly, heterodisulfide reductase, which regenerates the CoM and coenzyme B, is always present (Hedderich et al., 1990). Therefore, these enzymes also represent relevant targets for the development of AMFA.

Direct inhibition of methanogens to inhibit CH_4 production offers several advantages over attempts to alter the upstream H_2 flow. As archaea lack the taxonomical and biochemical diversity of bacteria, protozoa, and fungi (Ferry, 1992; Wright et al., 2004; Henderson et al., 2015), their ability to inactivate AMFA or adapt is theoretically more limited, so targeting methanogens could reduce the likelihood of adaptation to AMFA. Furthermore, direct inhibition of methanogens may reduce the likelihood of undesirable consequences on fermentative microorganisms. Although there are a variety of potential approaches to inhibit methanogens directly (e.g., enzyme inhibition, cell wall and cell membrane disruption, blocking DNA and protein synthesis, and gene editing and regulation; Figure 1), few of them have been evaluated in vivo. Those AMFA that inhibit enzymes involved in CH_4 production (i.e., 3-nitrooxypropanol and bromoform forming part of red algae *Asparagopsis*, which inhibit MCR and MTR, respectively), are closer to practical application.

Alternative Electron Acceptors

Redirection of [H] toward desirable sinks is one strategy for decreasing rumen methanogenesis (Figure 1). Organic acids such as malate, fumarate, or acrylate have been investigated as a potential strategy to direct [H] toward propionate formation (Newbold et al., 2005). The reduction of fumarate to succinate is thermodynamically more favorable than methanogenesis (Ungerfeld and Kohn, 2006). However, much of the added fumarate and malate seems to be converted to acetate, releasing [H] rather than incorporating it; as a result, decreases in CH_4 are much lower than theoretically predicted (Ungerfeld et al., 2007; Ungerfeld and Forster, 2011). Phenols such as phloroglucinol incorporate e^- in its reduction to acetate. Phloroglucinol was effective to incorporate part of excess H_2 resulting from the inhibition of methanogenesis in in vitro rumen cultures but did not affect (Huang et al., 2023) or decreased numerically (Romero et al., 2023b) CH_4 production in the absence of an inhibitor of methanogenesis. Polyunsaturated fatty acids inhibit CH_4 production in the rumen, but they mostly act by inhibiting methanogens and H_2-producing microorganisms rather than as e^- acceptors (Nagaraja et al., 1997). Nitrate is a strong mineral e^- acceptor in the rumen when reduced to nitrite, and subsequently to ammonia, and can also directly inhibit methanogens and some fibrolytic bacteria through its reduction intermediate—nitrite—in nonadapted animals (Zhou et al., 2011; Lee and Beauchemin, 2014). Rumen microbes efficiently reduce nitrate to nitrite but the subsequent conversion of nitrite to ammonium occurs at a slower rate, leading to the potential risk of accumulation and absorption of this toxic intermediate (Iwamoto et al., 1999). Moreover, nitrite can also be metabolized to other undesirable end products, such as small amounts of N_2O, a GHG even more potent than CH_4 (Petersen et al., 2015; Yang et al., 2016). Sulfate is another mineral e^- acceptor in which reduction is slightly energetically more favorable than methanogenesis, but the toxic e^- sink hydrogen sulfide can be absorbed through the rumen wall and pose a health risk to the host animal (Gibson et al., 1993).

There is also interest in the use of homoacetogenic bacteria as direct-fed microbials. Homoacetogens conduct reductive acetogenesis, the production of acetate from H_2/CO_2, formate, or CO (autotrophic reductive acetogenesis) or NADH and reduced ferredoxin (heterotrophic reductive acetogenesis; Ljungdahl, 1986). Note that in this respect, reductive acetogenesis is an e^--incorporating pathway, rather than an e^- acceptor. Reductive acetogenesis competes with methanogenesis for [H] in gut environments, such as the hindgut of some termites and cockroaches (Liu and Whitman, 2008). It is thought that very little reductive acetogenesis occurs in the rumen with functional methanogenesis, because the partial pressure of H_2 is lower than the threshold required for homoacetogens (Ungerfeld and Kohn, 2006). Raju (2016) showed in vitro and ex vivo that reductive acetogenesis was a minor e^--incorporating pathway in the rumen with functional methanogenesis, which increased when CH_4 production was inhibited by chemical inhibitors of methanogens acetylene or 2-bromoethanesulfonate.

Methane Oxidation

Aerobic methanotrophs belong to phyla *Proteobacteria* and *Verrucomicrobia*. Anaerobic oxidation of CH_4 involves the coupling of CH_4 oxidation with various e^- acceptors, including nitrate, nitrite, iron, manganese, and sulfate, and occurs in conjunction with a syntrophic

bacterial or archaeal partner (Guerrero-Cruz et al., 2021). Methanotrophy in the rumen was found to be very low (Kajikawa et al., 2003), but the abundance of methanotrophs seems to increase with nitrate supplementation in the diet (Liu et al., 2017), and methanotrophic genus *Methylomonas* was more abundant in low- than in high-CH_4-emitting cattle (Auffret et al., 2018). A central aspect to the success or potential of this strategy is whether methanotrophy is an obligate catabolic pathway for rumen methanotrophs or for externally dosed methanotrophs to obtain energy and carbon.

A PROPOSED PIPELINE TO UNDERSTAND THE MODE OF ACTION OF AMFA

The questions in this section address the research needed to establish the mode of action of AMFA. These questions are proposed from 5 levels of aggregation or perspectives: microbiology, cell and molecular biochemistry, microbial ecology, animal metabolism, and cross-cutting. For each perspective, we recommend research strategies to gather further understanding of the mode of action of AMFA.

The Microbiology Perspective

Which Microbes Are Targeted by the AMFA? Identifying the target microbial group is the first step in classifying any AMFA into one or more of the different modes of action described in the previous section. Initial insights on whether an AMFA is inhibiting methanogens directly or inhibiting H_2-producing microbes can be provided by cultivating mixed rumen microorganisms with and without the AMFA, and with both the control and methanogenesis-inhibition treatment with and without added H_2. If the inhibition of CH_4 production by an AMFA dissipates when H_2 is added, it is likely that the AMFA is acting on H_2-producing microorganisms rather than on methanogens. This preliminary approach, however, can yield incorrect results with AMFA such as nitrate with a dual mode of action (Lee and Beauchemin, 2014). Nitrate will inhibit methanogenesis through its reduction intermediate nitrite, but its mode of action as an alternative e^- acceptor would not be captured using this approach, as nitrate will continue to inhibit CH_4 production even under nonlimiting H_2.

The effects of an AMFA on specific microbes or microbial groups can be investigated by studying changes in the absolute abundance of microorganisms in vitro or in vivo using classical cultivation techniques (Harrison and Vickers, 1990; Gruninger et al., 2022), quantitative (**q**) PCR (Pitta et al., 2021), or high throughput sequencing of the 16S rRNA (prokaryotes), 18S rRNA (protozoa), and large subunit rRNA (fungi) genes (Abecia et al., 2014; Pitta et al., 2021). However, while studying the effects of AMFA on microbial abundance in mixed rumen cultures or in vivo can help identify microbes affected by an AMFA, these methods do not demonstrate direct effects of an AMFA on a particular microbial group, but rather show associations. The inhibition of CH_4 production by an AMFA does not only affect the target microorganisms directly, but also affects many other microbial groups indirectly through changes in e^- flow (see "The Microbial Fermentation and Ecosystem Perspective" section).

Direct effects of an AMFA on a particular microbial group or species eventually need to be demonstrated via cultivation in pure culture, with and without the additive being studied, under ideal cultivation conditions (Table 1; e.g., Abecia et al., 2014; Romero et al., 2023a). A limitation of this approach is that not all rumen microorganisms are culturable in the laboratory. Additionally, it is important to determine whether the specific AMFA exhibits a microbe-ostatic or microbe-cidal effect. When the effects are microbe-ostatic rather than microbe-cidal, discontinuation of the AMFA enables the population or activity of the targeted microorganisms to quickly recover (Banik et al., 2016). Nevertheless, also if the microbe-cidal effects of the AMFA are incomplete, surviving cells may re-establish the original population size.

Can AMFA Act Through Inhibiting More than One Microbial Group Simultaneously? Antimethanogenic feed additives designed primarily for a specific mode of action can exert other modes of action simultaneously, also leading to the inhibition of methanogenesis (Table 1). For example, tannins can mitigate CH_4 by decreasing substrate degradation, which decreases the amount of H_2 available for methanogens (Tavendale et al., 2005), but they also have antiprotozoal activity (Morgavi et al., 2010). Similarly, some essential oils derived from garlic, cinnamon, rhubarb, and frangula, apart from decreasing H_2 production, may also directly inhibit methanogens (Benchaar and Greathead, 2011). Nitrate has a dual mode of action as e^- acceptor and as a toxin to methanogens through its intermediate nitrite (see the "Alternative Electron Acceptors" section). Various nitrocompounds can act as [H] acceptors and also inhibit formate dehydrogenase or formate hydrogen lyase in methanogens and rumen bacteria, as shown by inhibited CH_4 production after external addition of H_2 and formate (Anderson et al., 2008). Thus, it is relevant to identify all potential modes of action of a given AMFA to better interpret their efficacy (or lack of it) under different conditions (del Prado et al., 2025; Dijkstra et al., 2025; Hristov et al., 2025).

Can Targeted Microbes Develop Resistance to the AMFA? It is important to understand the extent of variation in sensitivity to AMFA among targeted microbes, so that more resistant microbes might occupy niches left

Table 1. Summary of recommended research to understand the mode of action of antimethanogenic feed additives (AMFA) at various knowledge levels

Knowledge level	Recommendations
The microbiology perspective	
Minimal knowledge	Conduct incubations with mixed rumen microbes with and without added H_2 to gain a preliminary understanding if the AMFA is inhibiting methanogens or H_2-producing microorganisms; explore changes in the target rumen microbes using quantitative PCR and high throughput sequencing; demonstrate direct effects with pure cultures grown with the AMFA
Deep understanding	Dose-responses with pure cultures of different species of the targeted microbes; investigate differences among target microbes in their sensitivity to the AMFA; long-term (≥84 d) in vivo monitoring of target and other microbes with high throughput sequencing; investigate direct unwanted effects in nontargeted microbes
Future research	Research on culturomics to assess the effects of AMFA on currently nonculturable microbes
The cell and molecular perspective	
Minimal knowledge	Identify the active compound or compounds
Deep understanding	Identify the type of mechanism involved: enzyme inhibition, membrane disruption, blocking DNA and protein synthesis; demonstrate target enzyme with pure enzyme: substrate systems; demonstrate the specific mechanism of inhibition, including binding sites and reaction intermediates and mechanism; establish the type (competitive or noncompetitive) and kinetics (i.e., the concentration of compound inhibiting 50% of the enzyme activity)
Future research	Study the mechanisms of action explaining variations in sensitivity and acquisition of resistance by targeted microbes
The microbial fermentation and ecosystem perspective	
Minimal knowledge	Investigate changes in the total VFA concentration and individual VFA molar percentages; determine H_2 accumulation in vitro and H_2 expelled in vivo; when the active compounds or metabolites are not naturally present in the rumen, or when degradation pathways are understood and research focuses on parameterizing rates, analytical chemistry might suffice to study degradation rates; conduct in vitro radiolabeling assays to understand the rumen metabolism of active compounds if the active compound or its metabolites may be present in the rumen
Deep understanding	Determine concentration of dissolved H_2, formate, succinate, lactate, methanol, and ethanol; conduct metagenomics and metatranscriptomics with a particular focus on hydrogenases
Future research	Metabolomics analysis to fully understand effects of the AMFA on electron sinks and metabolic intermediates; metaproteomic analyses for a closer understanding of changes in rates of metabolic pathways; determine the effects of inhibiting methanogenesis on VFA production; isolation and identification of microorganisms that metabolize the active compounds
The animal perspective	
Minimal knowledge	Understand the absorption, metabolism, excretion, and accumulation in animal products (milk or meat) and tissues (rumen, liver, mammary gland, blood, and other organs) of the active compounds and its rumen metabolites; toxicological and mutagenic examination of active compounds as well as intermediary and end product metabolites; if feed intake is decreased, examine the possibility of palatability or inflammation problems
Deep understanding	Oral administration of isotopic-labeled compound to ruminants and nonruminants followed by its quantification in tissues, feces, urine, milk, and exhaled air; conduct metabolomics analyses of rumen fluid and plasma screening for potentially toxic compounds, or compounds potentially affecting feed intake
Future research	Determine effects on VFA absorption, gluconeogenesis, and other postabsorptive processes; investigate mechanisms through which the inhibition of CH_4 production in the rumen can decrease feed intake
The cross-cutting perspective	
Minimal knowledge	Thoroughly report information of animal species, breed, production type, age, stage and level of production, diet, feeding management and behavior, intake pattern, and other details of the methods used in scientific papers
Deep understanding	Characterize the daily evolution of the concentration of the active compound in the rumen; optimize the design of combinations of various AMFA with synergistic modes of action
Future research	Investigate how the efficacy of different additives is affected by physiological and microbiological variables both at the in vivo level and in controlled experiments with mixed and defined cultures

empty by more sensitive microbes, and the extent that targeted microbes can develop resistance to the AMFA. This may also help explain if variation in the efficacy of AMFA across different diets may reflect differences in the composition of the communities of targeted microbes. For example, species of rumen methanogens grown in pure cultures differ in their tolerance to increasing doses of chemical inhibitors (Abecia et al., 2014; Duin et al., 2016). Co-occurrence analysis in vivo or in mixed cultures can provide insights into the potential replacement (or competition) of one AMFA targeted microbe by another that possesses similar metabolic and ecological activity (Ghanbari Maman et al., 2020).

Development of tolerance to ionophores, including cross-resistance to ionophores to which organisms had not been exposed and to the non-ionophore antibiotic avoparcin, was reported for *Fibrobacter succinogenes*, *Prevotella ruminicola*, and *Veillonela parvula* (Chen and Wolin, 1979; Newbold et al., 1993). Approaches to investigate the possibility of development of resistance to AMFA include detection of resistant microorganisms in sequential cultures of pure strains exposed to suboptimal doses of the AMFA, in vitro assessment of horizontal transfer of antimicrobial resistance genes, and isolation of resistant microorganisms from the rumens of animals supplemented the AMFA (Toomey et al., 2009).

Is the AMFA Toxic to Nontargeted Microbiota? Determining the effects of a given AMFA on nontargeted microbiota is important when designing a strategy to inhibit CH_4 production and concomitantly redirect [H] toward desirable sinks. For example, chloroform has been shown to be toxic to homoacetogens (Raju, 2016) and thus chloroform and other CH_4 halogenated analogs may not be ideal for inhibiting methanogenesis if the objective is to simultaneously promote reductive acetogenesis as a [H] sink alternative to CH_4. Direct effects, or lack of them, of an AMFA on nontargeted microbes need to be proven in pure culture studies in which the microbial species of interest is grown with the AMFA, and functional measurements can be conducted (Duin et al., 2016; Raju, 2016). Rumen protozoa favor methanogenesis by transferring H_2 to their endo- and epi-symbiotic methanogens (Belanche et al., 2014). Therefore, an indirect inhibition of symbiotic methanogens may occur as a result of inhibition of protozoa by the AMFA. This problem could be partially circumvented by using fluorescence staining to monitor changes in protozoal-associated endo- and epi-symbiotics methanogens (Belanche et al., 2014).

Recommendations for Research at the Microbiology Perspective

- Initially, explore changes in the rumen microbial community using qPCR to identify changes in the absolute abundance of the main microbial groups (bacteria, methanogens, protozoa, and fungi) and high throughput amplicon sequencing of rumen contents from animals fed different diets to identify those microbes that might be targeted by AMFA.
- Final confirmation of direct effects on microbes are to be obtained through cultivation in pure cultures with increasing concentrations of the AMFA without nutrient limitation.
- Evaluate if some target microbes are inherently more resistant or can develop resistance to the AMFA. Both questions can be addressed through assays with pure cultures and isolation of resistant microorganisms from the rumens of animals that have been supplemented with AMFA.

The Cell and Molecular Biochemistry Perspective

Which Are the Active Compounds? Identifying the active compound or compounds is straightforward in AMFA that are composed of a single chemical. If the chemical is mixed with a carrier, the control treatments should include the carrier alone in the same dietary proportion as when it is mixed with the active compound, to identify any carrier effects (e.g., Melgar et al., 2020). Identifying the active compound or compounds in AMFA that are natural products derived from plant extracts or algae is more complex and involves relating in vitro results of antimethanogenic activity to the primary components and secondary metabolites within the mixture (Table 1). Machado et al. (2016) noted that the highly effective AMFA red algae *Asparagopsis* is rich in bromoform and other halogenated CH_4 analogs, which have previously been shown to inhibit CH_4 production in vitro (Bauchop, 1967) and in vivo (Lanigan, 1972), and whose mechanism of action has been elucidated already (Wood et al., 1968). Following, Machado et al. (2016) confirmed in in vitro cultures the antimethanogenic activity of extracts from *Asparagopsis*, and that bromoform was both the most abundant secondary metabolite and the most potent inhibitor as a pure compound. A subsequent study reported similar effects of *Asparagopsis* and pure bromoform on the relative abundance of methanogens orders in mixed rumen cultures, confirming bromoform as the main active compound in *Asparagopsis* (Machado et al., 2018). If an AMFA contains tannins, for example, the importance of tannins at inhibiting CH_4 production can be verified by incubations with and without the tannin inactivating agent, polyethylene glycol.

Which Cellular and Intracellular Processes and Molecules Are Targeted by AMFA? There are a variety of mechanisms of action at the cell and molecular level through which AMFA can inhibit CH_4 formation. The greatest historical research effort and recent years' success has taken place with AMFA that specifically block the action of enzymes that are unique to methanogens (Figure 1). The enzyme MCR, which catalyzes the last step of methanogenesis, is shared by all methanogenic pathways and has thus been specifically targeted to inhibit all different methanogens. Two inhibitors of MCR that have been synthesized as analogs of its cofactor CoM and its methylated form methyl-CoM are 2-bromoethanesulfonate (**BES**; Gunsalus et al., 1978) and 3-nitrooxypropanol (**3-NOP**; Duin et al., 2016), respectively. First, it was shown that BES was a potent inhibitor of CH_4 production with cell extracts of *Methanobacterium thermoautotrophicum* (Gunsalus et al., 1978). It was later demonstrated that BES specifically inhibits MCR in experiments with pure (Ellermann et al., 1989) or partially purified MCR enzyme (Gräwert et al., 2014). 2-Bromoethanesulfonate binds MCR catalytic site and alkylates the Ni^{+1} in cofactor F_{430} to a transient Ni^{+3} intermediate, eventually yielding ethylene, sulfite, and Ni^{2+} (Goenrich et al., 2004; Li et al., 2010; Thauer, 2019).

The enzymology work for uncovering the mechanism of action of 3-NOP was conducted with purified MCR (Duin et al., 2016). The specific mechanism of inactivation of MCR by 3-NOP is through oxidation of the Ni atom in F_{430} at the catalytic site from Ni^{1+} to Ni^{2+} as demonstrated using electron paramagnetic resonance

spectroscopy (Duin et al., 2016). The presence of 3-NOP reduction products, nitrite and 1,3-propanediol was revealed using mass spectrometry and colorimetric measurements. Nitrite itself was found to inactivate MCR through oxidizing Ni^{1+}, implying a dual mechanism of action through which 3-NOP inhibits methanogenesis (Duin et al., 2016).

Methane halogenated analogs such as chloroform, bromoform, bromochloromethane, and others, inhibit CH_4 formation by impeding the transfer of a methyl group attached to a cobamide in MTR to CoM to yield methyl-CoM. Competitive inhibition of extracts containing MTR by dichloromethane, chloroform, or carbon tetrachloride with formation of halomethylcobalamins and the corresponding halogen anions, was shown by Wood et al. (1968).

There are other mechanisms through which AMFA can inhibit methanogens (Figure 1). Acetylene disrupts transmembrane electrochemical gradients in methanogens (Sprott et al., 1982). Methanogens inhibitors can also target enzymes involved in other anabolic processes, such as the synthesis of membrane lipids (Miller and Wolin, 2001), the cofactor tetrahydromethanopterin (Dumitru et al., 2003), and DNA gyrases (Bergerat et al., 1994). For example, the antibiotic squalamine causes disruption of methanogens pseudomurein cell wall and cytoplasm leakage, as shown by electron microscopy (Khelaifia and Drancourt, 2012). Antibiotics such as imidazoles can inhibit methanogens and other microbes through blocking DNA and protein synthesis (Khelaifia and Drancourt, 2012). Conceptual approaches include the control of regulatory noncoding RNA genes (**ncRNA**) in methanogens (Wurtzel et al., 2010; Qi et al., 2017), as ncRNA are expressed in rumen methanogens and are present in the rumen metatranscriptome (Zhou et al., 2023). Editing methanogens genomes using clustered regularly interspaced palindromic repeats and the associated cas genes is another approach that may be investigated in the future (Nayak and Metcalf, 2017; Bao et al., 2022).

There is also ongoing research on archaeal viruses as a potential tool to control rumen methanogens through cell lysis (Gilbert et al., 2020). The lytic enzyme PeiR from the φmru prophage was linked to nanoparticles and shown to inhibit pure cultures of methanogens and decrease CH_4 production in rumen continuous cultures (Altermann et al., 2022). Historically, the presence of viruses in cells was studied using transmission electron microscopy, whereas molecular methods are now used to identify viral sequences (Gilbert and Ouwerkerk, 2020).

Research has also been conducted to understand how some H_2-producing microorganisms are affected by AMFA at the cellular level. For example, gram-positive H_2-producing bacteria (e.g., *R. albus* and *R. flavefaciens*) are inhibited by ionophores through increased membrane porosity and disruption of transmembrane electrochemical gradients (Russell and Strobel, 1989; Duffield et al., 2008). The hydrophobicity of essential oils enables these molecules to disrupt the bacterial cell membrane, resulting in the leakage of ions (Knobloch et al., 1986). Saponins and several defaunating agents, such as capric, lauric or myristic acids can reduce CH_4 production by forming irreversible complexes with cholesterol in protozoal cell membranes, causing cell lysis, and thus lowering protozoal concentrations (Wallace et al., 2002).

What Are the Molecular Mechanisms of Resistance or Adaptation to AMFA? As outlined in the first publication of this series (Durmic et al., 2025), the selection and progress of an AMFA requires persistent effects not subject to the development of resistance. It is therefore necessary to understand the mechanism by which these resistances may develop. Several mechanisms of resistance to AMFA have been described: for example, some rumen bacteria can respond to tannins by changing their morphology by developing a thick glycocalyx (Chiquette et al., 1988). Similarly, the antiprotozoal effect of saponins is often transitory after an adaptation period in which rumen bacteria develop the ability to inactivate saponins through deglycosylation of the sugar moiety (Patra and Saxena, 2009), although modifying saponins' chemical structure may enhance their resistance to degradation in the rumen (Ramos-Morales et al., 2017). So far, no mechanisms of microbial adaptation to 3-NOP have been described even after 1 yr of continuous treatment (van Gastelen et al., 2024). However, the efficacy of 3-NOP to inhibit different methanogens is variable among ruminant species (Duin et al., 2016), suggesting structural differences in MCR are responsible for differences in tolerance to 3-NOP (Pitta et al., 2022a). The existence of differences among methanogens in their capacity to synthesize coenzymes involved in methanogenesis has also been suggested to affect the efficacy of methanogenesis inhibitors (Ungerfeld, 2022). Methanogens may also avoid the effects of AMFA through metabolism of the active molecules to a less toxic form, such as the reduction of halogenated CH_4 analogs (Glasson et al., 2022). Long et al. (2021) identified mutations in the genome of *Methanococcus maripaludis* that increased resistance to chloroform, neomycin, and echinomycin, with not all mutations being related to the genes encoding targets of the inhibitors. This study suggested that mutations in the gene encoding geranyl-geranyl-glyceryl-phosphate synthase, for example, may have enhanced the resistance of this methanogen by reducing their uptake of chloroform and neomycin.

Recommendations for Research at the Cell and Molecular Biochemistry Perspective

- The mechanism of action of active compounds in AMFA needs to be hypothesized from their chemical composition, and molecular structure.
- When the AMFA is a plant extract consisting of a blend of compounds of natural origin, its molecular composition needs to be thoroughly characterized to formulate hypotheses about possible mechanisms of action.
- A sole suite of biochemical methods cannot elucidate all possible intracellular mechanisms of action of AMFA, and complementary techniques such as enzymology, isotope labeling, metagenomics, or microbial pure cultures are generally required.
- For chemical inhibitors hypothesized to inhibit enzymes, enzymology assays with purified or partially purified enzymes, substrates, cofactors, and inhibitors, are required to define a mechanism of action. It is recommended to understand the sequence, structure, and function of the enzyme in question and the binding site for AMFA to fully elucidate the mechanism of action of that AMFA.
- For groups of organisms such as methanogens, it is recommended that mechanisms of action are studied with a model organism relevant to the rumen ecosystem (e.g., *Methanobrevibacter ruminantium*).
- Mechanisms of methanogens resistance to inhibitors may exist and need to be investigated.

The Microbial Fermentation and Ecosystem Perspective

Which Are the Consequences of Inhibiting CH_4 Production on Rumen Fermentation Pathways? The inhibition of methanogenesis is not an isolated intervention and has indirect consequences on metabolism of the rumen microbial ecosystem. A comprehensive understanding of the effect of AMFA on e^- flow and fermentation pathways is important. Antimethanogenic feed additives affect fermentation pathways directly (i.e., affecting microbes responsible for these pathways), or indirectly through altering the concentration of intermediate products. This requires a system-wide perspective of the flows of production and utilization of H_2 (Greening et al., 2019) and other e^- carriers.

Typically, inhibiting rumen methanogenesis results in a marked accumulation of H_2 in vitro (Ungerfeld, 2015) and an increase in exhaled H_2 in vivo (Ungerfeld et al., 2022), along with increases in dissolved H_2 in rumen fluid (Melgar et al., 2020). Other intermediate e^- carriers, such as formate, ethanol (Martinez-Fernandez et al., 2016; Melgar et al., 2020), and lactate (Amgarten et al., 1981) can also accumulate. Succinate has been reported to accumulate in methanogenesis-inhibited in vitro cultures (Ungerfeld et al., 2003). Methanol and methylamines, which are substrates for methylotrophic methanogenesis, have also been reported to accumulate when rumen methanogenesis is inhibited (Martinez-Fernandez et al., 2018). It is recommended to routinely measure the concentrations of dissolved H_2 and the metabolic intermediates succinate, ethanol, methanol, formate and lactate when investigating the inhibition of rumen CH_4 production by AMFA (Martinez-Fernandez et al., 2016; Melgar et al., 2020) to understand which metabolic pathways are constrained and at which steps. Metabolomics can be used to explore changes in a larger suite of compounds with biological and production implications (Martinez-Fernandez et al., 2018). Examination of reductive acetogenesis can be conducted with ^{13}C-labeled bicarbonate (Nollet et al., 1997; Le Van et al., 1998). Demonstration of rumen CH_4 oxidation requires quantifying the fate of ^{13}C in CH_4 (Kajikawa et al., 2003).

As different methanogens possess different methanogenesis pathways, they also have different thermodynamic thresholds for H_2 (Feldewert et al., 2020). Consequently, AMFA that differentially inhibit methanogens with different H_2 thresholds may also lead to differences in H_2 concentration in the rumen, which in turn will differentially favor nonmethanogenic H_2-incorporating pathways depending on their H_2 threshold, affinity *Km*, and maximum rate at substrate saturation. This is relevant because the possibility of redirecting e^- toward nutritionally beneficial pathways may make the adoption of some AMFA more appealing than others (Beauchemin et al., 2022).

In vitro mixed rumen culture systems have been extensively used to examine effects on balances of [2H] produced, incorporated, and recovered (Durmic et al., 2025). As in vitro systems lack absorption of metabolites, and dilution rates are fixed and known in case of continuous and semi-continuous incubations, the production, incorporation, and recovery of e^- can be estimated from the net production of metabolites and known biochemical pathways (Ungerfeld, 2015). Importantly, the genetic capacity of some microbial species to conduct alternative fermentation pathways indicated by the examination of genomes of cultivated rumen bacteria (Hackmann et al., 2017) is not considered in classical [2H] balances, although their quantitative importance for redirection of e^- flow in the rumen remains unknown. Furthermore, accounting for aspects such as the possibility of acetate being formed in the rumen via reductive acetogenesis, the balance between hydrogenotrophic and methyl-reducing methanogenesis, as well as utilization of H_2 for microbial growth affects the estimation of [2H] balances (Ungerfeld, 2015).

In vitro systems are extremely useful for testing basic hypotheses and mechanisms about the effects of AMFA on e^- flow. Ultimately, it is important to verify changes elicited by AMFA on rumen metabolism in experiments in vivo. Studying e^- flows in vivo requires estimating VFA production in as close to steady state conditions as possible (e.g., Markantonatos and Varga, 2017; Hristov et al., 2025) (see How does the AMFA Affect the Absorption of Metabolites from the Rumen and Postabsorptive Metabolism?).

How Do AMFA Affect the Composition of the Rumen Microbial Community? Apart from direct inhibition of microorganisms (see Which Microbes are Targeted by the AMFA?), AMFA affect the composition of the rumen microbiota indirectly through changes in rumen conditions affecting microbial populations. Examples of important indirect effects are AMFA inhibiting methanogens and the resulting H_2 accumulation affecting hydrogenogenic and hydrogenotrophic microorganisms negatively and positively, respectively (Ungerfeld, 2020). Although research of the effects of AMFA on the composition of the rumen microbial community can be conducted in batch, continuous or semicontinuous cultures (Romero-Pérez et al., 2016; Roque et al., 2019), in vitro methods may not fully reflect the composition of microbial communities in vivo (Johnson et al., 2009; Weimer et al., 2011). Hence, in vivo experiments are preferred to study the effects of the AMFA on microbial α diversity, community composition, and the correlation among microbial taxa (e.g., co-occurrence analysis and microbial networks). Preliminary insights about changes in metabolic pathways resulting from changes in the composition of the microbial communities can be predicted using the Kyoto Encyclopedia of Genes and Genomes (Horinaka et al., 2021). When interpreting the amplicon sequencing results it is recommended to pay special attention to changes in known hydrogenogenic and hydrogenotrophic rumen organisms. Whether an organism produces or takes up H_2 (or formate) can be characterized from previous isolates, or through metagenome-assembled genomes, to predict its capacity for H_2 metabolism. The use of shotgun metagenomics in conjunction with metatranscriptomics provides information not only about the diversity and abundance of microbial taxa, but also about the abundance of functional genes and transcripts involved in different metabolic pathways and changes in gene expression (Pitta et al., 2022b). As a step further, metaproteomics encompasses mRNA translation through generating information about microbial enzymes and other proteins present in the microbial ecosystem. Information on proteins is theoretically expected to be closer to metabolism, as the half-life of proteins is longer than their mRNA transcripts (Huws et al., 2018). However, in preliminary studies, metaproteomics failed to discriminate between high- and low-emitting animals (Wallace et al., 2017).

How Are AMFA Metabolized in the Rumen, at Which Rates, and by Which Microbes? Investigating how the active compounds inhibiting CH_4 production are metabolized in the rumen is important to understand the effective rumen AMFA concentrations (Dijkstra et al., 2025) and daily patterns of CH_4 inhibition and whether the AMFA or its metabolites can be transferred to animal products, the environment, or accumulate in animal organs. The degradation of newly developed AMFA with unknown pathways of metabolism can be difficult to assess. One challenge for studying the degradation pathways of small molecular CH_4 inhibitors is their low molecular weight (i.e., 121.09 g/mol for 3-NOP), which lacks strong UV absorbing structural elements, making them difficult to measure and identify at relatively low concentrations in complex biological matrices. The high volatility of some molecules (i.e., halogenated compounds) represents another challenge that complicates sampling and analyses (Glasson et al., 2022). To overcome these challenges, rumen culture studies with isotopic or radiolabeled active compounds can be conducted to understand the metabolic fate of active compounds in the rumen. For example, it was found that the carbon skeleton of ^{14}C-labeled 3-NOP was rapidly metabolized to 1,3-propanediol by rumen mixed cultures (Duin et al., 2016; EFSA Panel on FEEDAP, 2021).

Studying the metabolic fate of compounds which are typically not present in the rumen may preclude the need for radiolabeling making it more affordable and technically easier, although this will ultimately depend on the requirements of regulatory agencies (Tricarico et al., 2025). Analytical chemistry may suffice if pathways of metabolism are reasonably understood and rates of conversion to intermediates are determined under different ruminal conditions. For example, the metabolic fate and pathways of e^- acceptors such as nitrate and sulfate in the rumen are well known (Drewnoski et al., 2014; Yang et al., 2016), so the extent of their metabolism can be assessed by quantifying the concentration and emissions of their intermediates and end products (Zhao et al., 2015). Similarly, pathways that methanogens use to reduce CH_4 halogenated analogs are understood (Glasson et al., 2022). Romero et al. (2023a) found that bromoform was rapidly degraded by rumen cultures and that the concentration of the end product of the first dehalogenation, dibromomethane, increased during the first 6 h of incubation and gradually declined thereafter. It is recommended to fully characterize the fate of not only the active compounds in the AMFA, but of also all possible intermediates as discussed in the further section.

Determining which microbes metabolize each compound requires the use of pure cultures (Krone et al.,

1989). Microorganisms are, in principle, able to utilize a particular compound that may be identified through sequential dilutions and enrichment by providing the AMFA as the sole energy and carbon substrate. Subsequently, in vivo changes in the rumen abundance of AMFA-degraders so identified can be monitored using qPCR or high-throughput sequencing.

Recommendations for Research from the Microbial Fermentation and Ecosystem Perspective

- A minimal understanding of the effects of an AMFA on rumen fermentation involves characterizing changes in the concentration of fermentation products (mainly, VFA and ammonium), accumulation of fermentation intermediates (primarily H_2; reporting formate, lactate, succinate, and alcohols is also recommended).
- Initial studies to understand the above aspects may be conducted in vitro but in vivo results are required to confirm the applicability of findings.
- A more advanced mechanistic understanding can be provided through the study of abundance of genes and transcripts, and gene expression, through metagenomics, metatranscriptomics, metaproteomics and metabolomics.
- Research to understand how an AMFA is metabolized in the rumen will have to vary depending on whether its components and their metabolites are generally present in the rumen.

The Animal Metabolism Perspective

How Are AMFA Effects on Feed Intake and Digestibility Mediated? Meta-analyses revealed that inhibition of rumen methanogenesis generally causes a decrease in DMI (Ungerfeld et al., 2022), although this may also depend on the design of trials (Hristov et al., 2025). In some studies, the dose-dependent, negative effects of the bromoform-containing algae *Asparagopsis* on DMI might have been explained by palatability issues or inflammation of the rumen epithelium (Li et al., 2018; Muizelaar et al., 2021; Stefenoni et al., 2021).

Likewise, tannins can reduce palatability by reacting with taste receptors and by the complexation and precipitation of salivary proline-rich proteins with subsequent loss of the lubricating effect of saliva (Horne et al., 2002), although some ruminants can reverse this effect by modifying the quantity and type of salivary proteins (Frutos et al., 2004). Whether an AMFA reduces DMI because of diminished palatability can be evaluated through comparing responses in feed intake and feeding behavior to the AMFA when it is administered in a mixed diet or dosed into the rumen through a rumen cannula (Kim et al., 2019). Cafeteria-type studies in which animals can voluntarily choose different feeds are another option to study the effect of AMFA on palatability (Lee et al., 2020). The possibility of an AMFA causing epithelial inflammation can be evaluated in intestinal cell culture models (Ponce de León-Rodríguez et al., 2019), and through the analysis of inflammatory acute phase proteins such as haptoglobin, serum amyloid, and α1 acid glycoprotein (Eckersall and Bell, 2010), or via postmortem examination of rumen mucosa (Li et al., 2018; Muizelaar et al., 2021).

One physiological explanation for the decrease in DMI when rumen methanogenesis is inhibited is the increased production and absorption of propionate acting as a satiety signal (Ungerfeld et al., 2022). Other proposed hypotheses through which inhibiting CH_4 production decreases DMI include mechanisms related to increased gut fill (Allen, 1996), which is proposed to be caused by the accumulation of H_2 hindering NADH oxidation, fermentation, and eventually digestion, and increased total gas pressure in the rumen as 1 mol of CH_4 is replaced by 4 moles of H_2 plus 1 mol of CO_2 (Kjeldsen et al., 2022). The possibility of toxic compounds being formed in the rumen and absorbed has also been speculated upon (Kjeldsen et al., 2022).

How Do AMFA Affect the Absorption of Metabolites from the Rumen and Postabsorptive Metabolism? Individual VFA have different effects on postabsorptive metabolism because they are metabolized through different pathways in ruminant tissues (van Houtert, 1993). Therefore, it is important to understand the effects of AMFA on VFA production in the rumen and their absorption (Table 1). Theoretical considerations (Janssen, 2010) and meta-analyses from in vitro batch cultures (Ungerfeld, 2015) suggest that inhibiting CH_4 production in the rumen may increase propionate production and absorption, although this has not been verified with in vivo measurements of VFA production. Measuring VFA in vivo would eventually require estimating rumen propionate production beyond merely measuring propionate concentration or molar percentage (for a review of tracer and nontracer methods to determine VFA production in the rumen, see France and Dijkstra, 2005). Inhibition of rumen methanogenesis can also result in accumulation of methanol and methylamines (Martinez-Fernandez et al., 2018). Ruminants are typically limited in their supply of absorbed methyl donors (McFadden et al., 2020), and therefore it is of interest to determine if AMFA increase the absorption of methanol or methylamines, although the possibility that excess absorption of methanol and methylamines from the rumen leading to toxicity should also be considered. Metabolomics of rumen fluid (Martinez-Fernandez et al., 2018) and plasma (Yanibada et al., 2020) can reveal changes in the concentration of methyl donors.

How Are AMFA or Their Metabolites Absorbed, Metabolized, and Excreted? Studies determining the metabolism of an AMFA in the rumen would be followed by studies investigating the absorption, distribution, metabolism, and excretion of the AMFA and its metabolites by the ruminant. Most AMFA and their metabolic products are small molecules that can be absorbed through the rumen wall or in the distal digestive tract. Tracing AMFA or their degradation products in animal tissues, milk, feces, and urine, can be challenging because concentrations are generally low due to dosage and metabolites are diluted by digesta and may not be absorbed or accumulated in body tissues. Labeling the active compounds in the AMFA with isotopes would enable to trace them and their nongaseous rumen-derived metabolites in animal tissues. The isotopic metabolites of the active compound could be identified using nuclear magnetic resonance or gas or liquid chromatography coupled to mass spectrometry (Thiel et al., 2019a; EFSA Panel on FEEDAP, 2021). Studies conducted with rodents or other nonruminants may be useful to assess the postabsorptive fate and potential toxicity of active compounds that might be absorbed without undergoing rumen metabolism, acknowledging that postabsorptive metabolism in these animal models may differ from ruminants (e.g., metabolism of 3-NOP to 3-hydroxypropionic acid in rats; Thiel et al., 2019a,b; Tricarico et al., 2025). Once degradation products are identified, they should be assessed in toxicology and mutagenic tests in ruminants and nonruminants. An indirect assessment of possible toxicity of AMFA can also be provided by a detailed analysis of blood parameters, such as plasma minerals, metabolites, and enzyme concentrations (Kung et al., 2003).

Recommendations for Research from the Animal Metabolism Perspective

- If the AMFA being investigated decreases DMI, evaluate if it reduces palatability, produces inflammation within the gastrointestinal tract, or if it affects passage rate.
- If productive performance is consistently improved by supplementation of an AMFA, it should be investigated if this is linked to an improved nutrient absorption or utilization resulted from rumen methanogenesis inhibition.
- Investigating safety for animals, consumers, and the environment of new AMFA without a history of use in animal production requires characterizing the absorption and postabsorptive metabolism of AMFA and their rumen metabolites as well as their possible accumulation in animal products and organs, excretion in feces and urine, and their possible toxicity and mutagenic effects.

The Cross-Cutting Perspective

How Is AMFA Effectiveness Modulated? The overall effectiveness of AMFA at mitigating enteric CH_4 emissions is influenced by numerous factors that modulate their mode of action and achieving a mechanistic understanding requires thorough research (Table 1). These factors are related to animal species, breed, age, as well as production stage, diet composition, the method of AMFA administration, and patterns of feed consumption (del Prado et al., 2025; Dijkstra et al., 2025; Hristov et al., 2025). Differences in rumen microbial communities are fundamental to variations in the magnitude of CH_4 emissions. A comprehensive global analysis across various foregut fermenter species revealed that the dominant archaeal groups were remarkably similar worldwide, with 5 dominant methanogen groups comprising 89% of archaeal communities (Henderson et al., 2015). Although in this analysis diet had a greater influence than animal species on rumen microbial communities, next to diet also differences between ruminant species and types, such as beef versus dairy cattle can impact CH_4 production and the efficacy of AMFA.

Differences among animals in their responses to AMFA may stem from variations in physiological traits and management practices, including feed intake level and pattern, rumen volume, and fractional passage rate affecting rumen conditions such as pH and microbiome composition (e.g., Colucci et al., 1990; Swainson et al., 2008). Reynolds et al. (2014) pulse-dosed 3-NOP twice daily and observed a transitory inhibition of CH_4 production lasting for about 2 h after dosing. Meta-analyses have demonstrated differences between beef and dairy cattle in the efficacy of monensin, nitrate, and 3-NOP, likely due to differences in diet, DMI, and passage rates causing variation in rumen microbial communities (Ranga Niroshan Appuhamy et al., 2013; Dijkstra et al., 2018, 2025; Feng et al., 2020). The effects of essential oils containing thymol are variable across different dietary conditions as their antimicrobial effects can increase with lower pH presumably because thymol becomes more hydrophobic and integrates better with microbial cell membranes when its hydroxyl group is protonated (Calsamiglia et al., 2007). Similarly, the supplementation of dairy cows with a blend of coriander seed oil, eugenol, geranyl acetate and geraniol caused greater CH_4 decrease after rumen microbiota adapted to the additives in the long term (>28 d; Belanche et al., 2020). The composition of plant extracts is affected by the type of plant, growth conditions, harvesting season, and extraction and storage methods, as highlighted in the companion publications Durmic et al. (2025) and Hristov et al. (2025). Hence it is not advised to draw conclusions about AMFA efficacy based on a single study or sample. It is recommended to

confirm findings of enteric CH_4 mitigation across different animal species, diet types, production systems (e.g., grazing vs. confinement), or treatment duration to prevent potentially erroneous extrapolations. For example, supplementation of 3-NOP to grazing dairy cows twice per day at milking generated a time gap between the delivery of the additive to the rumen and grass ingestion that resulted in considerably milder inhibition of CH_4 production (Costigan et al., 2024; Muñoz et al., 2024) compared with what has been reported in a meta-analysis of studies of dairy cows fed TMR (Kebreab et al., 2023).

The effect of AMFA is dose dependent. For example, greater levels of 3-NOP (van Gastelen et al., 2022) or *Asparagopsis* (Roque et al., 2021), resulted in greater reductions in CH_4 production in cattle. Key enzymes in the methanogenic pathway (i.e., MTR and MCR) are susceptible to competitive or oxidative inhibition (Glasson et al., 2022). Therefore, a greater supply of AMFA is likely to increase the inhibition of these enzymes. Alternative e^- acceptors such as nitrate and sulfate were also shown to decrease CH_4 in a dose-dependent manner (Olijhoek et al., 2016).

Storage time and conditions may also affect the stability of AMFA depending on AMFA characteristics and AMFA carrier used, thereby affecting their efficacy. For example, 75% to 84% of the bromoform in *Asparagopsis* was lost after 4 mo of storage at 4°C (Stefenoni et al., 2021). An understanding of the actual dose of the active compound and the dynamics of its concentration in the rumen are crucial for understanding the CH_4 inhibitory effect of AMFA.

There may be dietary effects on AMFA efficacy as demonstrated for the efficacy of *A. taxiformis* and 3-NOP which decreased with increasing dietary fiber content (Dijkstra et al., 2018; Roque et al., 2021; Kebreab et al., 2023), while the CH_4 decreasing effect of monensin in beef steers tended to increase as dietary fiber increased (Appuhamy et al., 2013). Variation in sensitivity of methanogen species to AMFA has been demonstrated for 3-NOP and other inhibitors of methanogen. In mature ruminants, hydrogenotrophs constitute the majority of methanogens, whereas the microbiomes of newborns have a higher proportion of methylotrophs (Furman et al., 2020). Thus, differences in methanogenic communities at different stages of life (Friedman et al., 2017) may also affect the efficacy of AMFA.

Understanding the mechanisms of action of active compounds may allow for the formulation of synergic combinations of AMFA. For example, addition of phloroglucinol to inhibitors of methanogenesis decreased H_2 accumulation in vitro (Huang et al., 2023; Romero et al., 2023b) and H_2 expelled in vivo (Martinez-Fernandez et al., 2017). Direct fed microbials such as homoacetogens acted synergistically with AMFA to incorporate H_2 into reductive acetogenesis rumen cultures (Nollet et al., 1997). Similarly, addition of nitrate- and nitrite-reducing bacteria to rumen cultures supplemented with nitrate was shown to inhibit CH_4 production and enhance the reduction of nitrate to ammonium (Jeyanathan et al., 2014), although this was not confirmed in vivo (de Raphélis-Soissan et al., 2014). When combining inhibitors of methanogens with feed additives promoting hydrogenotrophic pathways of interest, it is necessary to consider the possibility of unwanted effects of the inhibitor of methanogens on hydrogenotrophs of interest.

Recommendations for Research from the Cross-Cutting Perspective

- It is necessary to report the actual dose of the AMFA, storage conditions and duration, dosing system, feeding level, method of delivery, and the type of production system (type of animal and diet) for a better understanding of the mode of action and variation in effectiveness.
- Further research is needed to enhance our understanding of the mechanisms through which AMFA interact with diet composition, and with rumen conditions for various life and production stages of different ruminants.
- Monitoring rumen concentration of the active compounds in AMFA can help understand variation in their efficacy.
- Combining various AMFA to achieve synergistic effects of reduction in CH_4 emission requires a sound understanding of their mode of action and their interaction, as well as knowledge of their effects on nontargeted microbes.

SUMMARY OF RECOMMENDATIONS

Unveiling the mode of action of AMFA is a difficult and costly task that requires multidisciplinary approaches from different perspectives, involving specialized equipment, facilities, and human resources. Therefore, we recommend conducting extensive research on the mode of action of AMFA only after their effectiveness has been proven in vitro or in vivo, as well as their lack of detrimental effects on animal production and health. The proposed pipeline of research recommendations is summarized in Table 1.

At the microbiology level, it is necessary to demonstrate which microbes are targeted by AMFA, but also if there are unwanted effects on nontarget microbiota. It is also necessary to understand the mechanisms through which AMFA act at the cellular and molecular level to inhibit enzymes or disrupt other cellular processes in target microorganisms. Microbial ecology studies focus

on understanding the effects of AMFA on the microbial ecosystem and metabolic pathways, including the degradation of the active compounds. Safety for animals, consumers, and the environment requires understanding the kinetics of absorption, postabsorptive metabolism, excretion in urine and feces, and accumulation of active compounds from AMFA and their metabolites in animal products and tissues. Research is required to understand the mechanisms through which the type of animal, the diet, management, and other factors, affect the mode of action and effectiveness of AMFA.

To achieve these objectives this publication provides a guideline and recommendations for conducting experimental research we understand is required to elucidate the mode of action and efficacy of AMFA. The research proposed herein implies complementary approaches to claim a minimal knowledge, a comprehensive understanding of the mode of action of AMFA, and research considered as important to be carried out in the future to fill important knowledge gaps in this area.

NOTES

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Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; ADME = absorption, distribution, metabolism, and excretion; AMFA = antimethanogenic feed additives; BES = 2-bromoethanesulfonate; CoM = coenzyme M; H_4MPT = tetrahydromethanopterin; MCR = methyl coenzyme M reductase; MFR = methanofuran; MTR = methyl-tetrahydromethanopterin: coenzyme M methyltransferase; ncRNA = noncoding RNA genes; q = quantitative.

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