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Feed additives for methane mitigation: Recommendations for testing enteric methane-mitigating feed additives in ruminant studies

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ABSTRACT

There is a need for rigorous and scientifically-based testing standards for existing and new enteric methane mitigation technologies, including antimethanogenic feed additives (AMFA). The current review provides guidelines for conducting and analyzing data from experiments with ruminants intended to test the antimethanogenic and production effects of feed additives. Recommendations include study design and statistical analysis of the data, dietary effects, associative effect of AMFA with other mitigation strategies, appropriate methods for measuring methane emissions, production and physiological responses to AMFA, and their effects on animal health and product quality. Animal experiments should be planned based on clear hypotheses, and experimental designs must be chosen to best answer the scientific questions asked, with pre-experimental power analysis and robust post-experimental statistical analyses being important requisites. Long-term studies for evaluating AMFA are currently lacking and are highly needed. Experimental conditions should be representative of the production system of interest, so results and conclusions are applicable and practical. Methane-mitigating effects of AMFA may be combined with other mitigation strategies to explore additivity and synergism, as well as trade-offs, including relevant manure emissions, and these need to be studied in appropriately designed experiments. Methane emissions can be successfully measured, and efficacy of AMFA determined, using respiration chambers, the sulfur hexafluoride method, and the GreenFeed system. Other techniques, such as hood and face masks, can also be used in short-term studies, ensuring they do not significantly affect feed intake, feeding behavior, and animal production. For the success of an AMFA, it is critically important that representative animal production data are collected, analyzed, and reported. In addition, evaluating the effects of AMFA on

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nutrient digestibility, animal physiology, animal health and reproduction, product quality, and how AMFA interact with nutrient composition of the diet is necessary and should be conducted at various stages of the evaluation process. The authors emphasize that enteric methane mitigation claims should not be made until the efficacy of AMFA is confirmed in animal studies designed and conducted considering the guidelines provided herein.
Key words: feed additive, enteric methane mitigation, guideline, ruminant animal

INTRODUCTION

Increasing public interest in reducing GHG emissions in general, and specifically methane (CH_4) emissions from livestock enteric fermentation, has substantially increased funding opportunities for government and private research to develop CH_4 mitigation strategies. These opportunities, however, have highlighted the need for rigorous, repeatable, and scientifically-based testing systems for existing and new CH_4 mitigation technologies, including strategies based on use of antimethanogenic feed additives (**AMFA**). A critical part of this evaluation process is testing AMFA in live animals in an environment that is as close as possible to region-specific farming practices and diets. Although all segments of the development process of AMFA (see research topics included in the current special *Journal of Dairy Science* issue) are needed, the importance of animal testing cannot be overemphasized. Indeed, there are numerous examples of AMFA that performed well in in vitro laboratory tests but failed to produce desirable effects in vivo or resulted in unwanted trade-offs. Therefore, the objective this paper is to provide recommendations for conducting and analyzing data from animal experiments intended to test AMFA. Discussion and recommendations include experimental aspects such as study design and statistical analysis of the data, dietary interactions, additivity of AMFA effects, appropriate methods for measuring enteric CH_4 emissions, production, and physiological responses to AMFA, and their effects on animal health and product quality. Although there is not always sufficient information about all of these aforementioned aspects, this paper provides recommendations for experimental testing based on the existing literature and the expertise and experience of the authors. The authors emphasize the point that enteric CH_4 mitigation claims should not be made until efficacy of AMFA is confirmed in animal studies designed and conducted considering the guidelines provided in this document. This work is part of the collection of papers of the Technical Guidelines to Develop and Implement Antimethanogenic Feed Additives, which includes: (1) identification of bioactive compounds (Durmic et al., 2025); (2) testing at the animal level (this paper); (3) model development (animal, farm; Dijkstra et al., 2025); (4) uncovering the mode of action (Belanche et al., 2025); (5) registration and regulation (Tricarico et al., 2025); and (6) carbon accounting (del Prado et al., 2025).

EXPERIMENTAL DESIGN

When designing an experiment to measure efficacy of AMFA it is necessary to consider several factors, such as ruminant species (e.g., cattle, sheep, goats, buffaloes), type (e.g., beef, dairy cattle), age, and stage of production cycle (e.g., parity, DIM) for which efficacy of the AMFA is to be assessed. Also, the choices on how to design experiments and perform statistical analysis, including power analysis, depend on the focus of the experiment (e.g., a study for determining the variability in efficacy of an AMFA or a study to generate label claims or inventory values).

Animal Age and Production Stage

Age and production stage of the animal are critical factors when designing an experiment to assess the mitigation potential of AMFA because the development and characteristics of the rumen microbiome are fundamentally linked to both. During the first 3 mo of age, a young ruminant goes through a nutritional transition from a "pseudo-monogastric animal" to a functional ruminant. From 6 mo of age onwards, the rumen microbial community is rather similar to that of adult ruminants. Hence, when priming the rumen microbiome composition during its development toward a low-CH_4 emission composition in later life, this should be done between 0 to 6 mo of age (Furman et al., 2020; Morgavi et al., 2020). However, only a limited number of studies investigated the effect of early-life antimethanogenic interventions on CH_4 emission later in life (Beauchemin et al., 2020), and some showed that CH_4 emission was reduced during the use of AMFA but without persistency of the effect after the intervention was stopped (e.g., Cristobal-Carballo et al., 2021). Based on the few studies available, it is recommended that early-life AMFA supplementation should be carried out as soon as possible after birth (Yáñez-Ruiz et al., 2015), and CH_4 measurements should continue for at least 3 mo after the intervention, and preferably longer (6–9 mo) or even into adulthood.

Contrary to youngstock, adult ruminants have a more stable rumen microbiome composition, due to the general anatomy of the rumen (e.g., size and flow rate) as well as an active effect of the immune system by means of antibody secretion in saliva (Yáñez-Ruiz et al., 2015). It is

therefore generally concluded that, apart from the effects of nutrition or AMFA supplementation, changing the microbiome in adult ruminant is difficult to achieve (e.g., Weimer, 2015). Despite this rather stable rumen microbiome composition, adulthood represents a long period and different production stages, affecting the level of CH_4 emission, mostly caused by changes in DMI, metabolic processes, and dietary composition. Primiparous cows, for example, have a lower daily CH_4 production, but higher CH_4 intensity (i.e., CH_4 expressed as g/kg milk or ECM) compared with older lactating cows, because they have generally a lower DMI and are growing animals that use part of the energy intake for BW gain at the expense of milk production. Additionally, over the first 10 wk of lactation, an increase in CH_4 production is consistent with the increase in DMI as milk yield increases. However, early lactating cows are often in negative energy balance and thus part of the milk production is driven by energy coming from body reserves resulting in a lower CH_4 intensity. Furthermore, in late lactation animals, GE intake can be above the requirements for milk production and maintenance because of pregnancy, affecting the daily amount and intensity of CH_4 emission. Next to these metabolic changes, it is generally accepted that animals in early lactation receive a relatively large proportion of concentrate in the diet, whereas the proportion of forage in the diet increases with advanced lactation stage. These dietary differences lead to different CH_4 emission metrics. Therefore, it is recommended that the efficacy of an AMFA should be tested in the physiological state, feeding level, and production system in which it will later be used. Variation in responses to AMFA can be considerably reduced by selecting similar animals in terms of production stage and by blocking, as described in the following section.

Statistical Analysis—Before the Experiment

Blocking and Baseline Period. Randomization through blocking animals by breed, parity, DIM, milk production, and body mass is recommended to decrease experimental error. It is recommended to measure blocking variables such as milk production during a 2 to 3-wk pre-experimental period, which will serve as a baseline period to adjust the experimental results for initial variation among treatment groups, and it is recommended to report the baseline data of each treatment group (Winder et al., 2019). These variables can be included in experimental models as covariables after the experiment has been conducted to adjust for their initial variation among experimental groups (see the "Including Baseline Measurements as Covariables" section). In studies with lactating animals, unless there is a specific experimental objective related to interactions between an AMFA and lactations stage, it is recommended, wherever possible, that all animals begin and finish the experiment at the same lactation stage.

Power Analysis. Power analysis is conducted to determine the minimum number of animals per treatment needed to decrease the probability of making a Type II error (i.e., incorrectly not rejecting a false null hypothesis), to a predetermined acceptable level. Power analysis requires establishing a difference of magnitude in response variables that researchers consider to be of biological or productive importance and intend to be detected as significant. A power analysis can also be conducted to calculate the effect size that would be detected as significant with a certain power, for a given number of animals, which may be restricted by the resources available, to determine whether conducting the experiment is justified under those restrictions (Festing and Altman, 2002).

Response Variable of Choice for Power Analysis. With AMFA eliciting moderate effects on CH_4 or AMFA not previously tested in vivo, the power analysis should be based on the minimal decrease (e.g., 10%) in CH_4 production intended to be detected as statistically significant. Because CH_4 measurement techniques differ in their precision, that precision also needs to be considered when deciding on the number of animals to be used. If the investigators are testing a hypothesis related to animal production, digestion, or metabolism response variables of an AMFA already proved effective in reducing CH_4 (e.g., 3-nitrooxypropanol [**3-NOP**] or bromoform) it is recommended to conduct the power calculation based on those response variables deemed central to the experimental hypothesis and for which the expected effect size may be relatively small or unknown. If a large number of animals is required to evaluate other response variables, performing CH_4 measurements in an unbiased subset of those animals may be sufficient.

Experimental power should also consider response variables of importance even if they are not part of the central hypothesis of a study. For example, numerical decreases in digestibility caused by an AMFA should be a warning signal that supplementing with the AMFA could have resulted in significant decreases in digestibility if more animals had been used, that is, a type II error.

Power Calculation. For power calculations, estimates of variation are ideally based on previous experiments conducted under similar conditions. When this is not possible, the investigators could resort to statistical parameters from studies published in the literature. More difficult situations are pioneering studies where the response of a variable has not been reported or experiments where AMFA is to be evaluated under conditions very different from those reported in the literature. Pilot

studies may then be conducted to obtain an estimate of standard deviation, although this involves additional resources and effort.

Most experimental designs in this field often include random factors, repeated measures, or dose–response designs. Furthermore, Latin square and crossover designs in which all animals are subjected to all treatments and fixed or random period effects are present in the model are generally used in digestion and metabolism studies. Thus, the classical power calculations presented in most or many general statistical textbooks are not applicable, because they are based on a mathematical derivation from a *t*-test comparison between 2 treatments under a fixed-effects model. Given the variety and complexity of experimental designs and models and corresponding calculations of power, it is recommended to seek advice from a professional statistician for conducting power calculations for each specific statistical model.

Statistical Analysis—After the Experiment

Including Baseline Measurements as Covariables. The statistical model for data analysis can include, in addition to treatment and random effects, other variables that are measured and recorded before the experiment and which may be included as covariables to adjust the results for initial variation of those variables among experimental groups (Gaines Das, 2002; see "Baseline Period and Blocking"). In this regard, covariables include pre-experimental baseline levels of response variables in individual animals. A covariable phase may be included to also determine baseline levels of other variables that otherwise would not be measured. Variation in pre-experimental covariables might be partially controlled through restricting randomization (e.g., with randomized block designs). In addition, we recommend adjusting responses through baseline levels in the statistical model of each particular response variable. Even if the effect of the baseline level covariable is found to be nonsignificant ($P > 0.05$), we still recommend its inclusion in the statistical model considering the possibility of a Type II error in the assessment of the covariable effect, unless this effect is found to be well above significance (e.g., $P > 0.50$). That said, it is still recommended to include covariables in the model if they have been a criterion for blocking experimental subjects, for example, different breeds or genotypes of animals, or parity.

Outlier Analysis—How and When? Outliers are extreme observations that differ appreciably from the rest of the data and in a fitted model have high residuals in absolute value that can have a disproportionate influence on the analysis (Gaines Das, 2002). As a first measure to detect outliers, it is recommended to examine plots of the original data visually, as well as of residuals for large deviations (i.e., biological outliers). Formal statistical methods can be also used to identify influential statistical outliers, such as the Cook's D statistic, calculated by fitting the model with and without each observation.

Unless obvious mistakes or technical problems are identified, automatically eliminating outliers is not recommended. If obvious technical problems occur (e.g., sick animals), those observations should be removed from the dataset before running statistical analysis and their removal reported in the Materials and Methods section of the manuscript. Investigators should try identifying whether the reason for an observation having a high absolute value residual is technical or truly biological. Some aspects to examine are the following: If an observation is nonphysiological, is it nonphysiological for only one response variable or for more than one response? If observations are an outliers for more than one response variable, do all those observations belong to the same animal? Do more than one outlier belonging to the same treatment cluster follow any pattern? If the answer to any of those questions is "yes," the outlier is probably a true biological result rather than a technical problem. With regards to nonphysiological values in some response variables, one should consider that inhibiting methanogenesis in itself is a nonphysiological intervention, and it is possible that some experimental outcomes might be beyond normally expected physiological ranges (e.g., H_2 emission). If after carefully examining an anomalous observation, the investigators are still unable to discern whether its cause obeys to a technical problem or is truly biological, the analysis may be run with and without the outlier, and if the conclusions differ substantially when the outlier is excluded, both results may be presented.

Exploring Associations Between Different Response Variables. Authors sometimes present multiple correlation analyses between response variables reported in a controlled experiment. Depending on the dataset structure (i.e., number of observations relative to response variables), this analysis may also be conducted using multivariate methods such as principal components analysis. Although these exploratory analyses can lead to new scientific insights, we caution authors against implicitly inferring cause–effect relationships from these types of associations. Also, if multiple correlation analyses are conducted with many variables, it is recommended to adjust *P*-values considering an adjusted experiment-wise Type I error rate (e.g., false discovery rate) or declaring significance at *P*-values more stringent than 0.05. Variance inflation factor analysis is useful for understanding whether the importance of a covariable in a model may be explained by it being associated with other independent variables, that is, multicollinearity.

Pros and Cons for Different Experimental Designs. The design of an in vivo experiment depends on several

factors, such as experimental infrastructure and number of animals available, but primarily, the choice of experimental design must be dictated by the objectives of the experiment, which will largely determine the experiment duration (see following section) and type of treatments.

Latin square or crossover designs have the advantage of having all animals subjected to all experimental treatments, which allows considerable statistical power with relatively few animals, because all animals serve as their own controls. For example, Latin square designs are useful in digestion and metabolism experiments in which availability of rumen-cannulated animals or the number of animals with permanent catheters may be limited. Latin square designs are sometimes used for testing dose-effects of a given AMFA and they can also be useful in experiments with factorial arrangements of treatments (e.g., testing the effects of an AMFA with 2 basal diets). A full 4 × 4 Latin square would require a minimum of 4 animals, but because replicating the square decreases the experimental error and increases the statistical power of the experiment, it is generally recommended that 8 or 12 animals be used (i.e., 2 or 3 animals per treatment and per experimental period). Moreover, including treatment by square interactions in the experimental model helps assess whether responses to treatments may vary among groups of animals.

The advantage of crossover designs is that they allow a relatively low number of animals and other resources to be used. However, their key limitation is that the short period of testing precludes evaluating any long-term effects or changes, such as adaptation of rumen microbes to the AMFA. Even when allowing washout periods, if there is no rigorous knowledge available on the minimum length of adaptation or washout periods specific to the particular AMFA being evaluated, longer-term carryover effects cannot be discarded. For instance, it has been observed that after diet changes the ruminal fermentation patterns and microbiome stabilize after a few days, ranging from 4 to 16 d in beef cattle (Petri et al., 2013; Machado et al., 2016; Rabaza et al., 2020), dairy cattle (de Menezes et al., 2011; Dieho et al., 2017; Weimer et al., 2017; Ricci et al., 2022), sheep (Xie et al., 2018), or yaks (Zhang et al., 2020), and up to 21 d in buffalo (Dixit et al., 2022). Other studies have found the evolution of the bacterial community composition of dairy cows to be variable among cows after exchange of rumen contents (Weimer et al., 2010). Clemmons et al. (2019) suggested, however, that adaptation and washout periods must be re-evaluated as the rumen microbiome did not stabilize until 9 to 10 wk following a change from a forage-based to concentrate-based diet. To our knowledge, similar studies have not been conducted with AMFA. Considering that the length of experimental periods in most crossover design experiments is limited, this type of design must be complemented with longer longitudinal studies, and the possible lack of complete microbial adaptation should be considered when interpreting the results. Another limitation of crossover designs is that it is not feasible to take samples (e.g., muscle biopsy) to examine the presence of residues of the additive or metabolites, or it can only be done on a smaller number of animals (i.e., at the end of the last experimental period).

Another alternative is continuous designs in which all animals first undergo a baseline, control (or covariate) period, followed by a period in which the AMFA is administered. In this design, each animal act as its own control (i.e., treatment period vs. baseline/covariate period). To avoid confounding treatment and period effects, this type of experimental design is appropriate when animal physiology (except for the treatment effects), diet, and the environment are not expected to change appreciably during the experimental period; for example, animals fed at, or close to, maintenance (Mitsumori et al., 2012; Martinez-Fernandez et al., 2016). This design can be also used with animals in mid lactation. Continuous design is not appropriate for grazing experiments, and with early lactation animals, unless the control and methanogenesis-inhibition periods can be brought close in time by shortening the treatment adaptation period in animals already adapted to their basal diets (Garcia et al., 2022).

Continuous-design experiments testing one or more AMFA or doses against a control treatment are appropriate for assessing long-term effects of AMFA on production traits, including BW change, and CH_4 emission. For example, CH_4 production partially recovered during a 42-d period of chloroform supplementation (Knight et al., 2011). Similar concerns have been reported with 3-NOP (Hristov et al., 2022) and the red macroalga *Asparagopsis taxiformis* (Wasson et al., 2023), but length of treatment, lactation stage, and diet often confound the mitigation effect of AMFA. For example, in a year-long study, van Gastelen et al. (2024) reported that the efficacy of 3-NOP appeared to decline, but not continuously, over time and the authors suggested that the mitigation potential of 3-NOP may have been influenced by diet type, diet composition, and its nutritive value. In long-term continuous-design experiments (i.e., 1 year or covering more than 1 lactation), in addition to antimethanogenic effects, evaluating the effects on animal health and welfare, production (milk production, BW change), and residues in products (milk, and if a slaughtering fattening study, meat) should be included, wherever possible. Even progeny effects of the treatments in the offspring could be tested in long-term studies. Any production effects may become clearer the longer an experiment is. In long-term studies, the measurement frequency and sampling schedules may vary for the different variables. Treatment

(including AMFA) effects on animal reproduction must be tested with a larger number of animals (i.e., ≥200/treatment) and, in the case of dairy cows, over a full lactation (or multiple lactations). If the experimental AMFA causes any health- or welfare-related issues to the animals, the experiment must be terminated immediately.

More animals per treatment are needed in continuous rather than in crossover designs (see discussion on statistical power analysis for number of animals per treatment). Additionally, in long-term experiments, special attention needs to be paid to the consistency in the formulation of the diet and nutritional quality of the feed ingredients, particularly forages, throughout the duration of the trial. This is important to be able to differentiate between changes in CH_4-mitigating efficacy over time associated exclusively with the AMFA (i.e., rumen microbial adaptation) or with the dietary conditions (Kebreab et al., 2023; van Gastelen et al., 2024).

Continuous designs have to use completely randomized block allocation, meaning that as identical as possible animals are grouped within block to be randomly allocated to one of the treatments (including controls). Outcome of the blocking procedure during the baseline period should be evaluated in all cases and demonstrate absence of or minimal differences between groups allocated to treatments. The arrangement of treatments can also be important for the power of an experiment. Structured designs such as dose–response trials have greater power for the same number of experimental units compared with treatments that are only qualitatively different.

The disadvantage of the continuous experiments is that they are generally costly and laborious. Changes in the production cycle (lactation stage, growing stage, age, and so on) must be considered when interpreting the data. The advantage being, however, that this design represents how AMFA would be applied in practice, and there are no changeovers between treatments and thereby risks of carry-over effects. Other types of study designs, such as group/pen and field experiments, may also be used in AMFA efficacy studies, particularly in the later stages of the evaluation process, and these have been extensively discussed elsewhere (Tempelman, 2004; St-Pierre, 2007; Bello et al., 2016; Hristov et al., 2019).

Experimental Length

The duration of a study depends, among other aspects, on the research objective. If the study is purely meant to provide evidence about the CH_4 mitigating properties of AMFA, a short-term efficacy study may suffice. The results of these short-term studies cannot be extrapolated to life stages or diets that were not tested, and persistence of the effect or effects that may start to appear after a certain lag period, cannot be properly evaluated. Long-term studies are essential to confirm the persistence of the antimethanogenic effect; they will also provide information on whether the rumen microbiome or metabolic processes adapt or adjust, resulting in a loss of efficacy over time. Long-term studies are also essential to evaluate feed efficiency, product quality, and animal health and reproduction, and, particularly, to make sure that supplementation of the AMFA does not result in negative effects in terms of animal safety, both of the animals to which the AMFA is given and their offspring.

In the past, AMFA efficacy studies typically lasted between 3 and 8 wk (e.g., Bhatta et al., 2013; Castro-Montoya et al., 2015; Guyader et al., 2015b; Olijhoek et al., 2016). During the past decade, the duration of efficacy studies has increased, partially as a result of the possibility to use spot-sampling measurement techniques in a more practical setting rather than respiration chambers to measure the CH_4 reduction potential of AMFA.

Following the requests from the regulatory bodies for registration of AMFA to be marketed some regulatory agencies dictate the minimal experimental length when the efficacy of AMFA is tested in ruminants (Tricarico et al., 2025). As an example, the European Food Safety Authority (**EFSA**) specifies the experimental length according to the animal species and category (Supplemental Table S1; see Notes). In accordance, nowadays experiments last for about 12 to 15 wk (e.g., Hristov et al., 2015b; van Gastelen et al., 2020; Melgar et al., 2021; Miller et al., 2023b). However, considering the complete lifetime of a ruminant (depending on production systems, dairy or beef), these 12 to 15 wk still only represent a relatively short period of their life. This will become more relevant in relation to accounting for reduction of GHG emissions in the livestock sector with carbon trading (del Prado et al., 2025). Long-term studies should preferably aim for a full production cycle, with the duration of actual measurement depending on stop/go decisions. For example, every 2 mo, where the study will continue (go) when the AMFA is still effectively reducing CH_4 emission, where the study will be terminated (stop) or adjusted (dose increased) when the level of CH_4 emission of supplemented animals is not different from nonsupplemented animals or the efficacy of AMFA has dramatically decreased.

Frequency of Delivery and Residual Effect of AMFA Supplementation

The frequency of supplementation required will depend on the release rate and mode of action of the active ingredient within the rumen (Belanche et al., 2025). Frequency of supplementation can vary from AMFA

with transient effects (3-NOP), to biochemicals that suppress or inhibit microbial growth or functionality either directly or indirectly (i.e., fatty acids, nitrate/nitrite, tannins, and halomethanes such as bromoform), to those that provide alternative pathways resulting in diversion of H_2 away from methanogenesis (nitrate). The experimental design can aid in identifying the frequency with which the AMFA has to be supplemented (e.g., intermittently, once or twice a day), or if it requires continuous feeding of a TMR diet.

Another important consideration is the residual effect, that is, if the antimethanogenic effect persists after cessation of AMFA supplementation. It has been demonstrated that after switching cows from 3-NOP to control treatment at the end of a continuous-design experiment the mitigation effect of 3-NOP disappears rapidly (Hristov et al., 2022), and intraruminal 3-NOP pulse doses indicated that this occurs within hours (Reynolds et al., 2014). Despite the importance of the continuation of measurement of enteric CH_4 emissions for days or weeks following cessation of the supplementation period, studies analyzing residual effects of AMFA are scarce. This information is key for future application. For example, once-a-day feeding or once in several days would particularly facilitate delivery in grazing or extensive systems. Furthermore, the development of rumen slow-release formulations or AMFA delivered in drinking water would be valuable to regulate the delivery frequency, and it may require further considerations in the future.

Recommendations

In this section, the key considerations for experimental design when assessing AMFA have been outlined. An appropriate pre-experiment power analysis and detection and impact of outliers on conclusions should focus on biologically relevant outcomes, with careful interpretation of collinearity, ensuring results are not over-extrapolated. Detailed information on health status, AMFA delivery methods, and studies with different ruminant species and production systems are needed. Similarly, more focus on long-term studies over multiple production cycles, and the effect of AMFA withdrawal on CH_4 emissions post-supplementation would determine any residual effects, which would help formulate application protocols and efficacy assessments.

BASAL AND EXPERIMENTAL DIETS

Diet Type and Feeding Practices

Studies have demonstrated a stark contrast in not only CH_4 yield (CH_4 expressed as g/kg of DMI) but also the ruminal microbiota controlling CH_4 production between animals offered a high concentrate compared with an exclusively forage-based diet (Miller et al., 2023a). Thus, it may be expected that the efficacy of AMFA is modulated by the chemical composition of the overall diet (mainly sources of carbohydrates and nitrogen). For example, van Gastelen et al. (2022) reported greater reductions in CH_4 yield when the AMFA 3-NOP was added to starch-rich diets compared with fibrous diets. Similarly, Roque et al. (2021) reported an interaction between dosing of the seaweed *Asparagopsis* spp. and diet, with greater methanogenic inhibitory effects detected when diets low in forage were offered to beef steers. In addition to defining the extent of feed degradation in the rumen, which is a driver of methanogenesis, conditions in the rumen environment could be expected to interact with the AMFA itself by influencing the rate of release from their excipients, degradation and inactivation by the rumen microbes, the rate for distribution in the rumen pool, formation of microbial biofilms, and the washout of the AMFA in the rumen where it has its effect (e.g., passage rate).

In general, ruminant feeding systems are classified as confined, grazing, or a combination of both. In confinement systems, the ruminant receives rations that are usually formulated to meet the animal's nutrient requirements for a given level of production, while in grazing systems the animals harvest their diet from forage swards, where selectivity in the process depends on grazing management, pasture abundance, and social interactions. Ad libitum feed intake in confined systems can also be affected by palatability, feeding behavior, and social interactions. Because CH_4 production patterns are closely linked to feed intake patterns and diet composition, any influence on what the animal eats, the amount consumed, and size and frequency of meals needs to be well-defined and reported when assessing AMFA. In confined systems, the feeding strategies (e.g., top-dressing of concentrates vs. concentrate feed stations, TMR vs. partial mixed ration, and precision feeding) will determine the diurnal pattern of rumen fermentation and CH_4 production. Similarly, grazing management practices, such as the time of allocation of new pasture, influence grazing bouts (Vibart et al., 2017) and rumen fermentation patterns (Vibart et al., 2019; Santana et al., 2023). Testing AMFA under grazing conditions can be challenging, due to the logistic difficulties of both delivery of the additive in the diet of the animal as well as measuring key response outcomes such as intake of pasture. Beyond the influence of diurnal patterns on methanogenesis, the different feeding strategies will influence the ease with which an AMFA can be dosed to animals (see section on supplementation methods).

Feeding Level and Passage Rate

Typically, although not necessarily, ad libitum feeding results in greater feed intake and passage rate compared with restricted feeding. In the absence of AMFA, feeding level influences CH_4 production and CH_4 yield through its effect on passage rate (Hammond et al., 2014), which in turn defines the extent of degradation of fermentable OM in the rumen. Fermentation patterns characterized by lower acetate-to-propionate ratio, have been proposed to interact with AMFA to increase their efficacy (e.g., 3-NOP; van Gastelen et al., 2022). However, the rumen passage rate and fermentation patterns appear to interact differently with AMFA depending on their mode of action. For example, Feng et al. (2020) reported in their meta-analysis that the efficacy of nitrate, an alternative hydrogen acceptor, as a mitigation option for CH_4 is reduced by increased feed intake, which the authors explained by a concurrent change to fermentation profiles toward more reduced fermentation products.

As mentioned previously, increases in passage rate associated with increased feeding level will also directly affect AMFA efficacy via increased washout of digesta and the AMFA itself from the rumen. In this regard, it is important that solubility, particle size, and density of AMFA formulations are well characterized, both during development and in efficacy studies, given the role that these variables have on the mean retention time in the rumen (Dufreneix et al., 2019).

Precision Feeding—Automated Feeders, Robotic Milking Systems

The advent of technology and the desire to improve efficiency have enabled precision feeding, in which some feed components are fed separately to better match the nutrient requirements of individual animals (Morey et al., 2023; Martins et al., 2024a). Precision feeding relies on the ability to feed different amounts of concentrate mixes or even individual ingredients depending on the performance of the animals, meaning that allocation of separate feeds can occur at different frequencies during the day. As a consequence, DMI, forage-to-concentrate ratio, and the size and frequency of meals will be different for each individual in a herd. Hence, precision feeding may induce individual differences in rumen digesta passage, substrate fermentation, and fermentation patterns, and, in turn, lead to potential differences in the inhibitory effect of AMFA. These individualized or precision feeding systems may be beneficial in terms of efficiency of utilization of nutrients such as N (Morey et al., 2023); however, they increase the complexity of assessing the efficacy of AMFA because of the large number of possible permutations of concentrate mixes or ingredients, which will determine both the rumen fermentation conditions that AMFA would encounter and the opportunity to consistently and homogeneously deliver AMFA for all animals in the herd, throughout the day. Moreover, because animals may regulate their total DMI depending on the amount of concentrate offered (specifically through voluntary intake of the partial-TMR), accurate DMI measurements are critical, as in any experiment involving measurements of enteric CH_4.

Methods of Supplementing the AMFA and the Placebo to the Diet

The optimum strategy for delivering AMFA will depend on the bioactive agents within the AMFA itself and will also be governed by whether the animals are fed indoors or are grazing. In confined feeding, AMFA can be supplied in the ration so that the animal is constantly receiving the AMFA throughout the day. In contrast, grazing systems pose a significant challenge in terms of consistent diurnal delivery of AMFA, with current options limited to the inclusion of AMFA within supplements offered to grazing animals. Furthermore, the mode of action and latency of effect of AMFA varies (Belanche et al., 2025), and this is an important consideration when determining methods of delivery to grazing animals. For example, 3-NOP has an immediate effect through direct targeting the enzyme methyl-coenzyme M reductase in rumen archaea that catalyzes the last step of formation of CH_4 (Duin et al., 2016), and for optimum effect it must be continuously available within the rumen digesta (Hristov and Melgar, 2020). Additives such as 3-NOP have demonstrated high efficacy in confined feeding because the animals receive the inhibitor in every mouthful eaten, but that will not apply in grazing systems.

In the absence of a continuous (or regular with milking) supply of concentrate feed supplement during grazing, different technologies are under development, such as controlled-release intraruminal boluses to provide long-term delivery of AMFA. Such a formulation should facilitate ongoing exposure to the active ingredient, circumventing the requirement for dietary supplementation. Notwithstanding this, the possibility of significant variability in compound release from devices deposited within the reticulo-rumen has been reported (Cardinal, 1997). In New Zealand, there are current research efforts to create a prolonged-release bolus, containing bromoform, specifically designed for graze-based cattle. Preliminary reports indicate up to a 70% reduction in daily CH_4 emissions over a 6-mo period (AgriZero, 2024). Other options for more extensive pastoral systems may include the incorporation of AMFA through the water

supply or within molasses feed blocks, though variable and likely inadequate consumption has been reported (Callaghan et al., 2021).

In situations where the diet is amended to include AMFA, the additive can be delivered as part of an individual feed supplement. Recent research has also focused on the development of AMFA that can evoke modifications to the ruminal environment to specifically target archaea (Pitta et al., 2018). For example, data from Ireland, indicate that an oxidizing AMFA can be incorporated into a pelleted format and offered in discrete feeds to beef cattle with some evidence of residual persistency (Roskam et al., 2023). The specific mode of action is an increase in rumen redox potential. Where animals are managed under a more intensive grazing production system, or with partial grazing, such AMFA can be delivered within a concentrate supplement offered when cows enter the milking parlor or with supplemental feeding in the barn.

Diet Nutrient Composition and Analyses

Forage-to-concentrate ratio has been identified as a key dietary attribute regulating the efficacy of some AMFA (Roque et al., 2021; van Gastelen et al., 2022) but not others (Feng et al., 2020). Key compositional variables that have shown to influence the effect of inhibitors include NDF for *Asparagopsis* spp. (Roque et al., 2021) and 3-NOP (Kebreab et al., 2023) as well as starch and crude fat for 3-NOP (van Gastelen et al., 2022; Kebreab et al., 2023). A recent meta-analysis of an expanded version of the Arndt et al. (2022) database concluded that the efficacy of 3-NOP, nitrate, and lipids can be partially explained by differences in dietary nutrient composition and other factors such as dose and length of supplementation period (Martins et al., 2024b). Neutral-detergent fiber has been reported as a key compositional variable, for example, explaining differences in methanogenesis for dairy (Niu et al., 2018), and beef (van Lingen et al., 2019) diets and for sheep fed a variety of forage crops (van Lingen et al., 2021). Neutral-detergent fiber and NFC intake have been reported as crucial for CH_4 emissions in buffalo (Prusty et al., 2014). Both starch and NDF as key dietary variables are consistent with the conceptual model of the effect of variables such as pH and rate of passage on rumen methanogenesis (Janssen, 2010). Using a dataset of studies conducted in New Zealand with sheep fed fresh forages, Pacheco et al. (2014) reported that the moisture content of the forage had a similar correlation with CH_4 yield as NDF concentration ($\sim r = 0.75$), but moisture content had a stronger correlation with CH_4 yield when expressed per unit of digestible OM ($r = -0.80$ vs. 0.54). These authors stated that the stronger correlation of moisture with CH_4 yield could be due to differences in passage rate of liquids from the rumen. Solutes present in the intrinsic moisture of forages (e.g., minerals and cell contents) affect rumen osmolarity, leading to differences in liquid passage rate and the concomitant shifts toward more reduced products of fermentation (e.g., Adams et al., 1987). Moisture and individual mineral content of diets are rarely reported in the literature, with the emphasis typically placed on the composition of the DM. Given their possible involvement in passage rate, moisture and individual mineral content are important variables to measure and report when evaluating AMFA, and especially in animals fed fresh forages.

Because of the influence of diet on CH_4 production and on the efficacy of AMFA, studies that evaluate AMFA must include a detailed description of the diet used, both in terms of ingredients and their chemical composition. Reporting physical attributes of the diet (e.g., particle size of forages and processing of grains) is to be considered, because of their influence on feed intake behavior, salivation, rates of passage, and, consequently, the rumen degradation of dietary components mediated by the rumen residence time. Such information is not only useful for interpretation of the results of a study, but also critical to expand databases, which can be used to further improve our understanding of the interactions between diet composition and CH_4 production. Analysis of chemical composition should be conducted using published methods, such as those from the Association of Official Analytical Chemists (**AOAC**). If near-infrared spectroscopy (**NIRS**) is used to determine the chemical composition of the diet, the suitability of NIRS calibration for the feeds under consideration needs to be ascertained.

Both in experimental and practical conditions, variability in diet composition is just as important for formulated rations (i.e., confined systems) as for fresh forage diets. Studies examining the efficacy of AMFA in which grazed or cut-and-carry forages form the basal diet, require a different approach for diet sampling. Although weekly sampling may be considered sufficient in confined feeding systems under well-controlled conditions, daily sampling with subsequent pooling is needed when fresh forages are used. Nevertheless, it is recommended that AMFA are tested with diets differing in feed ingredients and nutrient composition and roughage qualities to get a better understanding of the relationship between AMFA efficacy and diet composition and quality (i.e., crude protein, NDF, starch, ether extract/fatty acids, ash/minerals, digestibility).

Recommendations

In this section, we have outlined how feed characteristics and feeding practices determine key rumen environmental variables that influence the efficacy of AMFA,

mainly through changes in intake pattern, rumen degradation and passage rates, and type of fermentation, which affect the diurnal pattern of CH_4 production. Because of these interactions, it is not possible to generalize that a reduction determined for one AMFA employed under a particular set of dietary and feeding circumstances will equally translate to other situations. The evaluation of AMFA should use diets and feeding management that are representative of and applicable to common feeding practices in a region of interest. The optimum strategy for delivering AMFA is also affected by feeding systems; there is need for developing strategies (e.g., slow-release intra-ruminal bolus, water supply, or feed block) to provide long-term delivery. Last, some emerging feeding practices that pursue improvements in the efficiency of nutrient utilization, such as precision feeding, are likely to generate greater variability in CH_4 production between individual animals compared with group feeding, increasing the complexity of assessing the effectiveness of AMFA.

COMBINING AMFA WITH OTHER METHANE MITIGATION STRATEGIES

Arndt et al. (2022) stated that "...combinations of multiple mitigation strategies are likely needed to sufficiently mitigate CH_4 to limit global warming to 1.5°C," but the net antimethanogenic effect of combining 2 or more strategies may differ from the sum of their individual mitigation effects. This potential lack of additive effects poses major problems in the simultaneous implementation of different CH_4 mitigation strategies and in setting up reliable accounting systems (del Prado et al., 2025). Most reports on AMFA are short-term studies focused on testing strategies separately and do not evaluate combined strategies. Based on the available literature, recommendations are formulated and discussed herein for testing a given enteric CH_4 mitigating AMFA in combination with (1) other nutritional mitigating strategies, (2) a second AMFA, and (3) with non-nutritional interventions. Recommendations for testing the effect of feed management (feeding level, concentrate level, forage quality, and so on) on AMFA efficacy have been addressed in previous sections and will not be repeated here, but must be considered when testing combinations of mitigating strategies.

An effect that is additive is defined as the sum of the reduction of CH_4 yield/intensity by the individual AMFA, for example 10% reduction by AMFA "A" and 10% by AMFA "B" adds up to an ~20% reduction when AMFA "A + B" are fed in combination (along with a nonsignificant *P*-value of the interaction,that is, no interaction; Figure 1). In some reports no interactions became apparent (e.g., Guyader et al., 2015b) but in other reports, additivity was not achieved and the combined AMFA interacted resulting in a mitigation effect smaller than the sum (Liu et al., 2011; Maigaard et al., 2024). This type of interaction is defined as an antagonistic effect; in contrast, a combined effect greater than the sum of the individual effects is defined as a synergistic effect (Figure 1).

The existence of additive or associative CH_4 mitigation effects should only be tested for AMFA with proven mitigating efficacy. Experiments should include a minimum of 4 treatments, control (no AMFA), the AMFA being tested (AMFA "A"), the concomitant CH_4 mitigation strategy (AMFA "B"/strategy "B"), and the combination of AMFA "A" + AMFA "B"/strategy "B." There are several options for the design of the combined treatment. Most commonly, a 100% dose (with dose being expressed on an active-ingredient basis) of each AMFA as in studies where individual AMFA treatments are used, 100:100 (Kolling et al., 2018), but also 50% of each AMFA, 50:50 (Aboagye et al., 2018), or 60:40 (Poornachandra et al., 2019) have been used. The latter designs are not ideal for evaluating additive effects per se, but still allow for detecting associative effects. Design of the combined treatment therefore should depend on the specific hypotheses of the study, the CH_4 mitigating efficacy, and the mode of action of the AMFA and strategy in question as outlined in the following discussion. Prior in vitro testing of the range of permutations, including different compounds and dosages (Durmic et al., 2025), can provide initial useful insights to better design the in vivo experiment.

Additive and Associative Effects of AMFA with Other Nutritional Mitigation Strategies

This section provides recommendations for testing in vivo combinations of AMFA with other nutritional mitigation strategies (e.g., lipids), including AMFA such as plant secondary compounds (e.g., tannins) and synthetic AMFA such as 3-NOP and nitrate (Supplemental Table S2). Lipids and tannins are often used in combination, which can only slightly improve production and could potentially reduce CH_4 emission, but because high doses have negative side effects on feed intake, digestibility, and production, there is a limit to the amounts that can be added to the diet. Identification of additivity of effects and associative effects when combining AMFA (e.g., tannin, 3-NOP, nitrate) with a nutritional strategy (e.g., lipid supplementation) facilitates a more widespread implementation than the use of single strategies, because greater CH_4 reduction could be achieved while maintaining intake, digestibility, and production.

An additive or associative mitigation effect on CH_4 yield was observed when lipids were combined with hy-

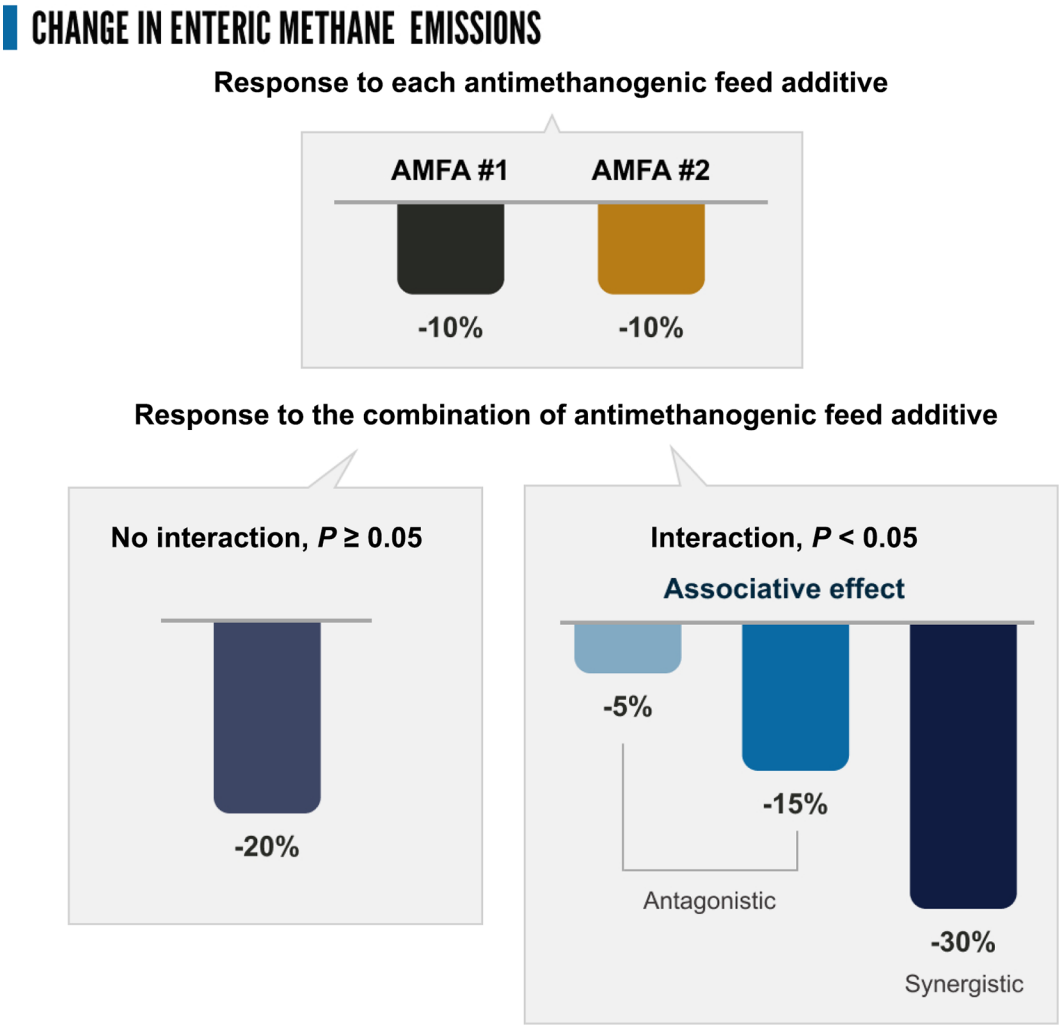

Figure 1. Terminology of possible effects on methane reduction of the combination of one feed additive with another feed additive or with a concomitant methane mitigation strategy. Interaction means that the mitigation effect of the additive is affected by the presence of the other additive, either antagonistically or synergistically. In case of an additive effect, the combined effect is the sum of the effects of the individual additives; there is no interaction between the 2 feed additives. Created by A. Schwarm and Sabrina Garay; used with permission.

drolyzable (**HT**) or condensed tannins (**CT**; Liu et al., 2011: 14% + 25% → 33%; Williams et al., 2020: 14% + 11% → 20%), with 3-NOP (Zhang et al., 2021: 24% + 28% → 51%), and with nitrate (Guyader et al., 2015b: 17% + 22% → 32%). The mode of action for lipids and tannins is both direct and indirect, thus multifactorial, and additive and associative effects were observed in the above-mentioned studies by Liu et al. (2011) and Williams et al. (2020), but not in the study by Lima et al. (2019): 39% + 14% → 35%. The discrepancy may be explained by the relatively high addition of refined soybean oil (50 g/kg of DM) by Lima et al. (2019), resulting in a mitigation threshold being reached. Thus, it is recommended to carefully consider the inclusion rate of an AMFA when used in combination (e.g., based on the aforementioned example, the concentration of oil used in the combination should be lower than 50 g/kg of DM).

The 100:100 combination of lipids with 3-NOP can result in an impressive 50% reduction in CH_4 yield compared with the control (Zhang et al., 2021). Still, the addition of lipids can result in a substantial reduction in the apparent digestibility of OM and fiber, both when added to the diet alone or in combination with 3-NOP (Zhang et al., 2021). Therefore, it is recommended to reduce the amount of lipids (e.g., 50% lipids + 100% 3-NOP) to maintain feed digestibility. It needs to be pointed out that nonsignificant, or "numerical" reductions in digestibility should also be considered and studied further because they may be indicative of a true negative effect of the AMFA being examined that was not detected due to underpowering of the experiment for secondary variables.

The combination of lipids and nitrate resulted in substantial mitigation effects (Guyader et al., 2015b), in a long-term study of 17 wk (Guyader et al., 2016). A synergistic effect was even observed when canola oil was combined with nitrate, meaning the combination of the 2 strategies had a greater mitigation effect than the numerical sum of the individual treatments (Villar et al., 2020: 6% + 9% → 25% CH_4 g/kg of DMI; Supplemental Table S2).

In the study by Maigaard et al. (2024) the combined use of lipids from cracked rapeseed with 2 AMFA, nitrate, and 3-NOP did not result in CH_4 yield reductions that were greater than what was obtained by separate supplementation of the most potent AMFA (18% to 23% 3-NOP >12% to 13% nitrate >6% to 7% lipid), therefore any combination of these 3 potent treatments resulted in effects lower than predicted based on the sum of their individual effects (i.e., negative interaction or antagonism).

Additive and Associative Effects of AMFA with Similar or Different Modes of Action

As mentioned previously, studies including AMFA with inconsistent efficacy, usually related to diverse composition of the active ingredients (e.g., tannins, saponins, essential oils), should be evaluated for mitigating efficacy before being used in combination to be able to draw conclusions on the additivity of CH_4 mitigation. In the studies by Guyader et al. (2015a), Klop et al. (2016), and Alemu et al. (2019), the additivity of CH_4 mitigation could not be tested, because one of the single AMFA did not reduce enteric CH_4. In principle, using 2 AMFA with similar efficacy is more promising for observing additive and associative effects than combining more and less effective AMFA, such as nitrate with essential oil or saponin, despite their different modes of action. The 100:100 combination in the mix is recommended in case of similar efficacy and diverse mode of action of the AMFA (e.g., 3-NOP and nitrate, 23% + 19% → 32% combined reduction; Maigaard et al., 2024). In some cases, however, the proportion of AMFA in the mix could be reduced to, for example, 50:50 of the individual doses (Supplemental Table S2). Examples of such combinations include the following: distinct efficacy and diverse mode of action of the 2 AMFA (e.g., 3-NOP and monensin; Romero-Perez et al., 2014), similar efficacy and similar mode of action (e.g., extracts of oregano and green tea; Kolling et al., 2018), or in case of expected adverse effects (e.g., tannins and saponins, Poornachandra et al., 2019). This dose reduction is justified because the combination of: (1) a strong (3-NOP) and a weak mitigant will likely cause only a marginal additivity if any, (2) 2 AMFA with similar modes of action are less likely to result in additive CH_4 mitigation, and (3) in case of secondary plant compounds (tannins, saponins) the negative side effects on production can be minimized when using a reduced amount in the mix. This scenario is different from combining AMFA with a nutritional strategy where 2 strong mitigants (e.g., 3-NOP and fat/oil) used in 100:100 combination can achieve an additive, substantial reduction in CH_4 yield (28% + 24% = 51%; Zhang et al., 2021). In the study by Maigaard et al. (2024), an associative effect of 3-NOP and nitrate was observed in primiparous cows, but was not conclusive for multiparous cows, which underpins that differences related to parity (e.g., feed intake level and rumen pool kinetics), may affect the individual and combined mitigation effects of AMFA.

It is also of utmost importance that studies testing AMFA combinations account for possible differences in lag time before the efficacy of the individual AMFA is at a maximum, and if the efficacy is reduced over time for one or both AMFA. For example, *Acacia* tannins are effective within 10 min of application (Denninger et al., 2020), whereas essential oils may only show an effect on CH_4 after several weeks (Belanche et al., 2020). These differences in lag times underline that experimental design always should reflect both the research questions raised and the mode of action, if known, of the potential AMFA under evaluation.

Rotational Use of AMFA. Rotations of more than one AMFA intend to amplify the spectrum of targeted microbial groups to enhance antimethanogenic effects and diminish potential adaptation over time. Most research in rotation of AMFA has examined antibacterial ionophores such as monensin and lasalocid, rather than more specific and potent AMFA, and focusing on animal performance, digestion, and rumen fermentation (e.g., Clary et al., 1993). Guan et al. (2006) did not find that a 2-wk rotation of monensin and lasalocid further decreased CH_4 yield or extended the persistency of the inhibition, compared with monensin alone. Lack of enhancement of antimethanogenic effects might have been due to similar mechanisms of action of monensin and lasalocid (Supplemental Table S2). Klop et al. (2017) did not find differences between supplementing a blend of essential oils alone or in rotation with lauric acid across 3 experimental periods, and only a decline in CH_4 yield during the first experimental period, indicating the AMFA effect was not persistent. The lack of a nonsupplemented control in those studies does not allow conclusions about effects on persistency and to assess whether there was an inhibition of CH_4 production with one AMFA that the rotation with a second AMFA did not enhance. We are unaware of in vivo studies comparing the rotational use of combined AMFA specifically inhibiting CH_4 production.

Different AMFA may target different microbial groups, or they may overlap in their effects on some microbes.

Affected microbes may have differential sensitivities to each AMFA. It may be speculated that when shifting from one AMFA to another with a different mode of action, a partial or total recovery of previously inhibited microbial groups would occur until the next AMFA change. The extent of this recovery would depend on the doses of the AMFA and periods of rotation. From a practical standpoint, rotation of AMFA could allow decreasing feed costs in comparison with the corresponding combination, provided that similar doses of each AMFA, whether combined or rotated, allowed achieving similar effects. On the other hand, rotation would make animal management more complex because 2 different rations would have to be mixed and fed. As research about the efficacy and other aspects of AMFA inhibiting CH_4 production progresses, it will be important to evaluate their combinations and rotations with different period lengths.

Combination of AMFA with Non-Nutritional Mitigation Strategies

There is scarce research about the interaction between AMFA and non-nutritional, CH_4 mitigating strategies. Various studies have compared productivity, rumen metabolites, microbiota composition, and metagenomics and metatranscriptomics of high- and low-CH_4 emitting animals (Shi et al., 2014; Wallace et al., 2015; Danielsson et al., 2017; Stepanchenko et al., 2023). However, the interaction between the individual animal's capacity to emit CH_4 and the response to nutritional manipulators of CH_4 production has only been minimally examined. An interesting analysis by Giagnoni et al. (2022) revealed a moderate negative association between individual CH_4 production and yield and the response to 3 nutritional strategies to mitigate enteric CH_4, including 3-NOP, nitrate, and fat. Low CH_4 yield cows seemed to respond better to the mitigating strategy than high CH_4 yield cows. Although heterogeneity of the phenotype is representative for today's herds, it is recommended to preselect individuals from a herd to minimize variation, and to be more representative for future herds consisting of high feed efficient animals or low CH_4 yielding/intensity animals. The combination of AMFA with non-nutritional mitigation strategies that are in early stages of investigation (e.g., vaccination, methanogens viruses, and lytic enzymes) has seldom been evaluated. Nguyen et al. (2016) found that nitrate decreased CH_4 yield in faunated lambs by 28% but increased it by 25% in defaunated lambs. In general, it is not expected that interactions between AMFA and non-nutritional mitigation strategies can be properly evaluated until the latter are fully developed.

Recommendations

It should be considered that CH_4 mitigating effects are not additive until evaluated in animal trials. Persistency of associative effects and additivity of AMFA combined with other nutritional strategies, with other AMFA, and with non-nutritional strategies needs to be evaluated in long-term studies. The design of the combined treatment should reflect the efficacy (similar/distinct) and mode of action (similar/diverse) as well as enable testing of adverse effects on production or animal health (Supplemental Table S2).

ENTERIC METHANE EMISSION MEASUREMENT TECHNIQUES AND PROTOCOLS

The main focus of this section is to describe and recommend CH_4 measurement methods and protocols for quantifying the potential effects of AMFA on CH_4 emissions in animal experiments. The initial phases of the AMFA screening program require CH_4 measurement methods that can provide an accurate and precise CH_4 production value in short (1–3 d) and medium (14–30 d) experimental periods with measurement of daily temporal emission profiles of CH_4, CO_2, and H_2 and measurement of DMI. To test the long-term efficacy of an AMFA, it is useful to use CH_4 measurement methods that are less laborious, do not interfere with the normal animal routine on-farm, and can be implemented in cycles (e.g., GreenFeed system; **GF**). For successful AMFA, larger area top-down methods could be used to verify CH_4 reductions on-farm or a group of farms in a region; however, these measurement methods are unlikely to distinguish CH_4 emission sources. Benefits and limitations of methods are best acknowledged when designing an experiment. In many cases, however, the equipment and expertise available in a given research unit, region, or country and the prevailing production system (confined or grazing) will determine which method to use.

Methane Measurement Methods

A recent analysis showed that the most commonly reported CH_4 measurement methods used in the past 25 years for individual animal measurements in descending order (% of studies) were respiration chamber (**RC**; whole animal chambers; 51%), the sulfur hexafluoride tracer technique (**SF_6**; 36%), the GF automated emissions monitoring system (7%), sniffer (2%), face masks (**FM**; 2%), portable accumulation chambers (**PAC**; 1%) and handheld laser CH_4 detectors (**LMD**; 1%; Della Rosa et al., 2023b). In addition, barn housing flux methods, open-air measurement methods, and unmanned aerial

vehicles (**UAV**) and satellites have been used to determine CH_4 emissions or hotspots from animals housed in groups, at the whole-farm level, or in a region (Vinković et al., 2022). There are important differences among these methods related to the following:

- Whether emissions can be determined from animals individually, in groups, on a whole farm, or in a region.
- Whether the CH_4 emissions captured include only those from the muzzle (exhaled + eructated), or also include flatus, manure/bedding emissions, total farm emissions, or all sources of CH_4 emissions in a larger area.
- Whether the methods determine emission fluxes directly or indirectly (e.g., via tracer, modeling based on airflow/direction measurement data) or measure CH_4 concentrations only.
- Whether emissions are measured continuously (24 h per day/feeding cycle) or via shorter sampling periods (e.g., spot samples of 2 min to ~60 min, at various frequencies).
- Whether emissions can be captured at short-, medium- or long-term scales (e.g., hours, days, weeks, or months/years).
- Whether the technique can correctly measure enteric CH_4 in rumen-fistulated animals.
- Accuracy, precision, repeatability, and uncertainties of the measurement.
- Cost of the equipment and of running the studies, as well as the measurement throughput.

Each CH_4 measurement technology has found a place in research because each method has its advantages and disadvantages, is suitable under particular conditions, and allows the user to address different research objectives (Table 1). For full detail of each CH_4 measurement method and technical aspects, the reader is referred to other reviews of CH_4 measurement methods (Goopy et al., 2016; Hammond et al., 2016; NASEM, 2018; Garnsworthy et al., 2019; Bekele et al., 2022; Tedeschi et al., 2022). The key to the accuracy of observed enteric CH_4 emission rates is the measurement of air flow (either directly or indirectly), greatly distinguishing alternative measurement methods in performance and expectations.

A few measurement methods will not be discussed in this text, including the polytunnel method (Lockyer and Jarvis, 1995), because it does not seem to have been sufficiently used in the past 2 decades, and LMD (Chagunda et al., 2009), because there are still large challenges to performing consistent measurements and generating repeatable results.

In choosing a measurement method, users should consider that enteric CH_4 is released from the rumen via eructation, produced in the hindgut and emitted in the flatus, or absorbed from the digestive tract into the bloodstream and exhaled from the lungs. The vast majority (97% to 98%) of the enteric CH_4 produced is emitted via the mouth and nostrils (i.e., muzzle; Murray et al., 1976; Muñoz et al., 2012). Therefore, emissions determined with a method measuring whole animal emissions (e.g., RC, PAC) or just emissions from the muzzle (e.g., SF_6, GF, hood, and FM) will be similar (Grainger et al., 2007; Hristov et al., 2018; Jonker et al., 2020; McGinn et al., 2021). If AMFA are expected to alter manure composition and, consequently, manure GHG emissions, the latter can be measured using methods that capture all farm emissions (e.g., UAV, larger scale open-air methods) and methods that capture all emissions in a larger area/region (e.g., large-scale open-air methods and satellites).

Units to Express Emissions

The main factor affecting the amount of enteric CH_4 an animal produces when no AMFA is provided is DMI (Hristov et al., 2013; Ramin and Huhtanen, 2013; Jonker et al., 2017). Consequently, an observed reduction in CH_4 emission can be due to a treatment effect, a reduced DMI, or a combination of both. Thus, expressing efficacy as CH_4 yield allows evaluation of the potency of an AMFA, regardless of changes in DMI, but CH_4 yield itself can be more affected by changes in DMI than in CH_4 production, if the effects of the AMFA on CH_4 production are modest or moderate. However, in meta-analyses CH_4 yield generally appears to decline as absolute DMI (or DMI as a proportion of BW) increases (Niu et al., 2018; Swainson et al., 2018), as is the case with an identical diet (e.g., Warner et al., 2017), likely reflecting changes in passage rate due to changes in DMI. Herd et al. (2014) defined several residual CH_4 traits (measured CH_4 − predicted CH_4 production) that can rank treatments independently of variation in DMI. Dry matter intake is often not measured in studies with grazing animals, at a large scale or on commercial farms and, therefore, it has to be estimated using prediction equations, inert markers, or other proxies. However, it has to be acknowledged that the variance of the CH_4 trait usually increases greatly when DMI is estimated rather than measured (Appuhamy et al., 2016; Herd et al., 2016; Jonker et al., 2020).

Some AMFA might affect total-tract diet digestibility without affecting DMI, and therefore CH_4 emissions could also be expressed per kilogram of digested DM, OM, or NDF. Ultimately, CH_4 emissions per unit of animal production (i.e., emission intensity expressed per unit of ECM, fat and protein corrected milk [**FPCM**], or ADG) need to be determined to ensure an AMFA has no negative effects on animal production and product quality and to determine the carbon footprint of the animal

Table 1. Description and advantages and disadvantages of methods for measuring ruminant livestock enteric methane (CH_4) emissions

Method	Method description	Advantages and disadvantages
Respiration chamber (RC)	The animal is placed in an enclosed chamber with measured airflow (flux) and sampling for CH_4 and CO_2 concentration analysis every 0.16 to 30 min across 1 to 5 d. Up to 10 chambers connected to an analyzer and facilities with 2 to 24 chambers. Individual animals or groups of animals (e.g., up to 4 dairy cows or ~20 sheep). Sensors for other gases (e.g., H_2) can be added.	*Advantages:* accurate, precise, and repeatable CH_4 production and yield measurements in a 24 h period. Confident results (if properly calibrated and recovery determined to be 100% ± 2%). Lowest number of animals required of any CH_4 measurement method. Provides a daily pattern of CH_4 emissions. *Disadvantages:* Chamber does not reflect the animal production environment and cannot be used on the farm location. Animal movement is restricted, which may affect animal physiology. Feed intake can be affected, which can affect CH_4 emission. Animals need to be trained.
SF_6 tracer technique	Sampling gear worn by the animal continuously draws air from the muzzle of the animal into a canister for 3 to 6 d. A capsule placed in the rumen releases SF_6 at a known rate (tracer flux method). Up to 75 animals have been reported to be measured at once under grazing conditions or well-ventilated confinement conditions. Gas concentrations in samples collected are analyzed by GC (can be setup to also analyze H_2 and CO_2).	*Advantages:* Accurate CH_4 production measurements over 3 × 24 h periods. When implementing stringent protocols and using recommended equipment, a low number of animals per treatment is needed (similar to RC). Can be used with free housing and grazing animals. Measurements can be conducted simultaneously on animals housed in individual pens/stalls or across many groups. Any experimental design can be implemented. *Disadvantages:* Difficult to use in an environment with variable background air CH_4 and SF_6 concentrations. The signal-to-noise ratio (measure − background CH_4) becomes very small when CH_4 is greatly inhibited using an additive (signal-to-noise ratio is normally used as a data exclusion criterion). Animals need to be trained. Requires constant monitoring of the gear (blockages of the air sampling line with moisture or dust, or other breakages), which can lead to the need for additional measurement days. The method is tedious; labor intensive; and equipment, consumables (e.g., permeation tubes), and gas analysis can be expensive. Provides no information on temporal CH_4 emissions. SF_6 is a potent greenhouse gas.
GreenFeed (GF) voluntary visits	The automated concentrate feeder units have integrated airflow (flux) and continuous CH_4 and CO_2 concentration measurements in free housing or grazing animals. Sensors for other gases (e.g., H_2, H_2S and O_2) can be integrated. Up to 30 animals per unit.	*Advantages:* Accurate CH_4 emission measurements with 50 to 70 spot samples per animal across ≥14 d. Can be used in barns, confined or grazing environment. Commercial support for troubleshooting of equipment and data storage and calculation. Can be run by people with little knowledge of CH_4 measurement equipment. A standard calibration and recovery procedure is implemented by all users, leading to a small technique measurement variation among different institutes. Can also provide measurements over a long period of time. Provides daily pattern of CH_4 emissions. *Disadvantages*: Measurements rely on voluntary visitation of animals to the units. Visits per day can be low in some trials, and animals not visiting the unit can be high, particularly under grazing conditions, when the unit is used on commercial farms, or when animals are not trained. In these cases, animals can be replaced during the training period or the measurement period can be extended, and the number of animals at the beginning of the experiment should be high enough to ensure statistical power. New animals require training for 3 to 14 d. There is variation in bait feed/pellet intake among animals depending on the variation in GF visits. Under grazing conditions, the unit needs to be moved frequently to be close to the animals, making it more labor-intensive.
GreenFeed in tiestall barn	Independent or integrated into automated concentrate feeder (see previous entry). Unit is placed in front of the animal for 5 min at a time under tiestall housing conditions. Has been used to measure 8 to 20 animals/unit within a measurement period.	Similar to previous section, but in addition: *Advantages:* Accurate CH_4 production measurements if frequency of spot samples and measurement period is applied (8 or 12 spot samples of 5 min at 2- or 3-h intervals across 3 d). The operator decides the sampling schedule and sampling does not depend on voluntary visitation. Training the animals is still critically important. *Disadvantages*: Limited to animals housed in a tiestall facility. Limited number of animals can be measured simultaneously.
Portable accumulation chamber (PAC)	The animal is placed in an airtight chamber (with known volume) and CH_4 and CO_2 concentration is measured at the start, middle, and end of a measurement period. Currently, PAC are mainly used with sheep, although cattle versions are also available, for ranking CH_4 emissions, collecting 2 spot samples of 40 to 60 min at 14 d apart. A PAC system generally has 10 to 12 chambers and up to 7 groups of sheep are measured per day.	*Advantages:* Simple method. For sheep, 10 to 12 PAC units can be placed on a trailer to do measurements on-farm. Relatively large number of individual animals can be measured per day (70 to 84). Potential to screen a large number of treatments in a single trial. *Disadvantages:* Animals need to be removed from their production environment to perform the measurement and are usually not fed during the measurements. Usually, there are no intake measurements. Relatively labor intensive. Spot sampling that does not cover the full feeding cycle; an intensive spot-sampling schedule is needed to provide accurate CH_4 production measurements. Current setup and measurement protocols are mainly to rank animals for emissions (breeding programs).

Continued

Table 1 (Continued). Description and advantages and disadvantages of methods for measuring ruminant livestock enteric methane (CH_4) emissions

Method	Method description	Advantages and disadvantages
Face mask (FM)	Mask placed over the muzzle of an animal that is restrained in a crush/chute or similar or trained to lay or stand still. Air from the muzzle is either directly actively drawn through a flow meter and to a CH_4 analyzer or accumulated in a bag or similar, and then air volume and CH_4 concentration are measured. Two to 12 spot samples of 15 to 30 min each per animal across 1 to 3 d are collected.	*Advantages:* Relatively simple method that can provide accurate measurements. There are mobile versions of the method. *Disadvantages:* An intensive spot-sampling schedule is needed to provide accurate CH_4 production measurements. The animal cannot eat or drink during the measurements and needs to cooperate to enable measurements. Labor intensive. Limited number of animals can be measured simultaneously.
Sniffer	Installed AMS[1] in a freestall barn (also a few reports on use in automated concentrate feeders, over feed bins, over freestall cubicles, in the milking shed and on the halter of the animal) with up to ~65 cows per AMS. Sniffer only measures CH_4 and, in most cases, CO_2 concentrations; flux can only be estimated based on calculated CO_2 balance in combination with measured CH_4:CO_2 ratio.	*Advantages:* Can provide CH_4 and CO_2 concentration measurements over a long period of time (e.g., full lactation). No interference with normal animal routine. Animals typically visit the AMS 1.9 to 3.2 times per day (4- to 36-h interval) for 5 to 22 min. *Disadvantages:* Current setup is mainly to rank animals for emissions (breeding programs), not to accurately measure emissions. High between-animal variation within a treatment. There is no standard setup of the sniffer (e.g., analyzer used and shape of feed dish and surroundings) and no agreement across users on how to correct for background gas concentrations and how to aggregate the data of a visit (e.g., average of a visit, peak concentration). Aligning the gas sensor data and the EID[1] data from the AMS is tedious. Environmental conditions around each sniffer in a facility and animal head movement affect the dilution rate of the gas concentration measured. There is uncertainty around the predicted CO_2 balance and accurate animal data are needed for this calculation. Currently not recommended for testing AMFA.
House flux	The CH_4 concentration in outgoing air (and incoming air) in a whole or compartmented barn can be determined by an open-path laser or by subsampling air to a gas analyzer. The ventilation rate (flux) is measured, or estimated, based on mass balance or calculated CO_2 balance + measured CH_4:CO_2 ratio, or release of a known quantity of an external tracer such as SF_6. Usually, N_2O and NH_3 emissions are also measured.	*Advantages:* Enables group measurements in freestall systems. Enables measurements including the effect on manure emissions. Little or no interference with normal farm routine. *Disadvantages*: Large uncertainty around measured ventilation rate. Difficult to get accurate measurements in naturally ventilated barns. The quantity of manure in barns with slatted floors can have a large effect on emissions. The sensitivity of these methods to capture small (5% to 10%) difference in enteric CH_4 emissions is questionable. If used as a tracer, SF_6 is a potent greenhouse gas. Not recommended for testing AMFA.
Methods to determine fluxes combined with models	Groups of animals in the open air in confinement or grazing or indoors with concentrations of gases measured in the free atmosphere, using open-path FTIR[1] or open-path laser, and fluxes estimated using models such as perimeter line measurements, mass balance, flux-gradients technique, eddy covariance, inverse dispersion methods, or CH_4/CO_2 ratio method, direct concentration ratio of treatment versus control animal block measurements, or release of a known quantity of a tracer such as N_2O. Usually, N_2O emissions are also measured.	*Advantages*: Enables measurement of large groups of animals or the whole farm (or region) with no interference with normal animal routine. Long-term measurements can be performed in confinement. Enables measurements including the effect on manure emissions in confined and soil emissions under grazing conditions, including N_2O emissions. The large-scale setup (regional towers) can potentially be used as a top-down method for areas where the CH_4 mainly originates from ruminant livestock. *Disadvantages*: Not for indoor use. The location of measurements needs to have a relatively flat terrain, with minimal obstacles (e.g., buildings, trees) and other sources producing CH_4 (when measuring groups of animals). Measurements included in the analysis only occur during the right weather conditions (loss of data can be high). No statistical replication in most cases. The sensitivity of these methods to capture small (5% to 10%) difference in enteric CH_4 emissions is questionable. Not recommended for testing AMFA. N_2O is a potent greenhouse gas.

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product (del Prado et al., 2025). Measuring this metric, however, may be less important at the early stages of testing new AMFA, considering that DMI, the main driver of animal production, is typically measured in nutrition experiments and required for registration purposes (Tricarico et al., 2025).

We recommend CH_4 production be reported in grams or moles rather than as volume, because grams and moles are independent of temperature and pressure. Reporting CH_4 production in grams or moles allows genuine interstudy comparisons and analyses while disregarding different local conditions of temperature and pressure. In cases where CH_4 production is expressed as volume, air pressure, temperature, and humidity, must be measured and reported and CH_4 data must be reported at standard conditions.

Table 1 (Continued). Description and advantages and disadvantages of methods for measuring ruminant livestock enteric methane (CH_4) emissions

Method	Method description	Advantages and disadvantages
Unmanned aerial vehicle–mounted sensors	Airplane, or drone with CH_4 concentration analyzer and meteorological measurements on the ground (ideally near the emissions source) to estimate flux using mass balance equations (or other modeling methods, as described in the previous section). Releasing a known quantity of a tracer such as N_2O can also be used with these methods. Short-term measurement campaigns (e.g., 5 to 15 min of measurement time each).	*Advantages:* No interference with normal farm routine. Captures total farm CH_4 emissions. *Disadvantages:* Short and infrequent measurement campaigns do not provide representative sampling of livestock emissions. Difficult to representatively capture plume emissions of the farm. Extremely high variability within measurement campaigns, if reliant on regional weather station data and highly variable measurement with on-farm weather station data (error range sometimes includes zero emissions). Difficult to estimate airflow appropriately. Cannot distinguish between different sources of emissions on-farm (e.g., enteric, manure storage, young livestock, mature animals). Not possible to compare treatments under controlled conditions. Usually, farm data are needed (e.g., animal classes, animal numbers, quantity of manure stored, manure storage system, and so on) to make sense of the data. Not recommended for testing of AMFA.
Satellite	Absorption spectroscopy to measure CH_4 concentration. Inverse modeling to estimate the location of an emission source and emission rate. Measurements at every fly-by, usually daily, with successful reading only when the sky is largely cloud-free and during sunny conditions.	*Advantages:* Measurements of CH_4 concentration in a region over a long time series (seasonal and across years). Can potentially pinpoint hot spots with high CH_4 concentrations. Can potentially be used as a top-down method to track whether CH_4 concentration hotspots change over time. *Disadvantages:* Method not fully developed. Only CH_4 concentration measurements at a coarse resolution during sunlit and cloud-free conditions. At this stage, it is not clear if a satellite can point source and track livestock emissions. Cannot distinguish between different sources of CH_4 emissions in a region (e.g., livestock emissions, soil emissions, natural land emissions, landfill CH_4, fossil CH_4) nor between individual farms (maybe isolated megafarms). Only suitable for regions with mainly livestock farming activity. Only able to verify overall CH_4 abatement in a region due to all mitigation measures implemented, not for a specific mitigation option such as the use of additives on-farm. Not recommended for testing of AMFA.

[1]AMS = automatic milking system; EID = electronic identification system; FTIR = Fourier-transform infrared spectroscopy.

Measurements of Individual and Groups of Animals and Measurement Techniques According to the Length and Location of the Experiment

Next to methods that can only be used to measure groups of animals, methods that determine individual animal emissions can also be implemented with group-housed animals (e.g., SF_6, GF, sniffer) or with animals temporarily removed from the group just for the measurement (e.g., FM, PAC, RC). However, measurements with SF_6, FM, PAC, and RC require some interference with the normal animal husbandry routine. Use of GF requires dietary modification to include pellets as the attractant (if the diet is only forage, it is recommended to use pellets from forage, e.g., alfalfa pellets). The hood method is, by definition, on individual animals and RC can be with individual animals or small groups of animals in a chamber. These methods are generally used with a relatively small number (2–30) of animals being measured simultaneously, except for the SF_6 method, which has been reported to measure 75 animals at once under grazing conditions (McNaughton et al., 2005) or well-ventilated confinement conditions (with stable and low background air CH_4 and SF_6 concentrations). A large number of GF units can be used for measuring emissions from hundreds of animals simultaneously. With all techniques, a larger number of animals can be measured in consecutive measurement blocks or when experiments are repeated.

Respiration chambers, head boxes or hoods, the most updated version of the SF_6 technique (Deighton et al., 2014), GF used in a tiestall barn, and FM with an intensive sampling schedule (8 or 12 spot samples across 2–3 d, covering all segments of a 24-h feeding cycle) are suitable for use in short-term measurement periods to prove the immediate effect of AMFA, ensuring they do not significantly affect feed intake, production, and animal behavior. All of these methods can be used in repeated-measurement campaigns over time to test the medium and long-term effect of AMFA. It must be noted that the SF_6 release rate from the permeation tube declines over time and affects CH_4 estimates in long-term trials (e.g., beyond 30 d from time of calibration), leading to overestimating the CH_4 production by up to 10% when measurements are conducted more than 200 d from calibration (Moate et al., 2015). The GF system with voluntary visitation to the concentrate feeder can be implemented in medium- to longer-term studies (>14 d), enabling accurate measurements from up to 30 animals per GF unit and with 50 to 70 spot samples per animal collected across at least 14 d. The system can be set up under any farming condition, including for graz-

ing animals. When set up in a freestall barn or feedlot, it can be used continuously to determine the medium- to long-term effect of AMFA.

Sniffers, GF, and barn and open-air methods have been used to determine CH_4 emissions on commercial farms (Bell et al., 2014; Arndt et al., 2018; McGinn et al., 2019; van Breukelen et al., 2023) and mobile PAC can be used on-farm to phenotype sheep for CH_4 emissions (Rowe et al., 2020). The main methods used with grazing animals include SF_6, GF, PAC (animals off grazing) and field-based open-air methods. Instead of in-field grazing studies, zero-grazing cut-and-carry studies can be performed with RC, GF, and SF_6 to accurately measure DMI and CH_4 emissions, but one should be aware that grazing behavior cannot be accounted for.

The UAV methods have been used to estimate emissions from whole farms and were able to distinguish between farms using different manure storage. However, considering the distance of measurement, the dilution of the enteric CH_4 signal, and the size of the mitigation effect, together with need to determine air flow accurately, its usefulness for confirming the reduction in enteric CH_4 due to the use of AMFA is unclear and still has to be demonstrated. Furthermore, gaining representative measurements of AMFA effects from a livestock farm might be impractical, unless they are used for accounting purposes (i.e., regional or national inventories; del Prado et al., 2025). Large-scale regional open-air measurement towers and satellites are being developed to potentially determine long-term CH_4 flux, concentration, or hotspot changes in a region as top-down measurements, but usefulness for studying AMFA effect over time is currently not clear. What is important to note, and for how results should be analyzed, is that these methods only determine the change in total CH_4 due to joint changes in management across farms in a region (e.g., animal numbers, efficiency, and use of any mitigation option), next to some potential AMFA effects.

Flux Measurements

The simplest method for quantifying the total CH_4 produced during a short period (hours) is by placing the animal in a PAC with a known air volume and sealing the chamber (Turner and Thornton, 1966; Goopy et al., 2011). The increase in CH_4 concentration (relative to the 0 h reading, that is, the background level) in the chamber during the measurement in combination with the known air volume of the chamber minus the volume occupied by the animal (assumed 1 kg BW = 1.00 to 1.01 L) are used to calculate CH_4 produced (liters/time; please see text about units in the previous section) during the measurement period.

With RC, hood, GF, and FM, in many cases the air is drawn through the system via pipes with a pump and the volume of air flowing through the pipe is measured with a mass flow controller or other air flow meter (Gerrits and Labussière, 2015; Huhtanen et al., 2015) and a subsample of air (inlet and outlet with RC) for its gas concentrations. A similar approach can be used in barn systems with mechanical ventilation (van Gastelen et al., 2023), but this approach only allows group measurement, and conditions are generally less controlled (depending on airtightness of the barn and its design), resulting in more uncertainty in the flow measurements and including all CH_4 sources in the barn.

Another method of estimating CH_4 flux is by releasing a known quantity of a tracer gas and determining the ratio of CH_4 to tracer gas in collected air samples. This approach is employed in the SF_6 tracer technique (Johnson et al., 1994), where the animal is dosed with a calibrated SF_6 permeation tube into the rumen. A similar approach can be used with open-air, barn, and UAV methods releasing at a known rate N_2O, acetylene, or SF_6 as a tracer gas from the sources where the CH_4 emission occurs (Tedeschi et al., 2022). It should be noted, however, that some of the tracer gases used in these methods (i.e., SF_6, N_2O) are potent GHG themselves (US EPA, 2024) and their use goes against the overall goal of reducing total GHG emissions. Similar to the external tracer gas method, CO_2 balance has been used as an internal tracer method, originally to estimate CH_4 emissions from barns (Pedersen et al., 2008; Hassouna et al., 2016; Tedeschi et al., 2022) and now also used with the "sniffer" method (Madsen et al., 2010). However, Huhtanen et al. (2020) demonstrated that this method was not capable of ranking CH_4 emissions from dairy cows with different feed efficiencies. The necessity to estimate the CO_2 production is a weakness of the method and the estimate itself is not an independent measurement. Alternatively, the CH_4 and CO_2 concentrations ratio can be used directly to compare treatments as has been used with "sniffers" (Lassen et al., 2012) and for confined feeding measurements, eliminating the need to predict CO_2 production (McGinn et al., 2019).

For measurements in the open-air with confinement, grazing, or in a region, fluxes can be estimated with a range of models using locally measured data of wind speed and direction. Models and methods used to estimate the flux include perimeter line measurements, mass balance models, flux-gradients techniques, eddy covariance, and inverse dispersion methods (for details see Harper et al., 2011). Some of these methods are also used to estimate the flux when using UAV. The accuracy of these methods is to be demonstrated first for AMFA with a rather known efficacy, or by some recovery tests.

Technical Factors that Affect the Methane Emission Measurements

Important factors to consider with all CH_4 measurement methods include understanding the range of CH_4 concentrations one expects to measure and having a sensor that is suitable for measuring concentrations in this range with a constant small measurement error. Furthermore, the difference in CH_4 concentration between the background and source emission measurement needs to be sufficiently large (signal-to-noise ratio; **SNR**) to enable an accurate measurement of the CH_4 difference. The SNR can be improved by reducing the airflow through the system when using RC and hood (FM) and by keeping the animal in the chamber for longer when using PAC. With the SF_6 tracer technique, one should just consider looking at the SNR of SF_6 for data exclusion, rather than CH_4 because the SNR of CH_4 might be low due to the antimethanogenic effect of the AMFA. For the older version of the large ruminants GF unit, the manufacturer recommends measurements from animals that produce >150 g CH_4/d; the sensitivity has been improved in the new GF units (available since 2022), where sensitivity of the equipment is ≥4 g CH_4/d and 1,000 g CO_2/d. Hydrogen is a possible measurement in GF with additional sensors (sensitivity of 1 g H_2/d). For open-air and barn methods, data need to be excluded during periods of too low SNR (i.e., limit of detection) as described previously (McGinn, 2013; Laubach et al., 2024). For all methods, it is important to calibrate the CH_4 sensor properly with standard gas CH_4 concentrations in the range that can be expected to be measured in the animal or group of animals. Furthermore, CH_4 sensors drift over time and therefore regular standard gas checks need to be performed and the sensor recalibrated if needed (Aldhafeeri et al., 2020). Failing to do these can have consequences for the precision and accuracy (mean and slope bias) of the CH_4 measurements.

For systems that use measured flux such as RC, hood, GF, and FM, a whole-system gas recovery test needs to be performed at regular intervals to ensure that 100% ± 2% of gases is captured by the whole system (see the guideline of Mesgaran et al., 2021). Failing to confirm a near 100% recovery can have a serious consequence on the accuracy (i.e., mean bias) of a system, and (temporal) variation between units in a facility is even more problematic (Gardiner et al., 2015). For PAC, one needs to ensure and confirm each time a new animal enters the chamber that the chamber is fully sealed and no air is leaking out of the chamber (Jonker and Waghorn, 2020a).

The SF_6 tracer technique requires stringent data screening to identify equipment failure and outlier data to ensure data integrity (Jonker and Waghorn, 2020b). Usually less than 10%, and in most cases less than 5%, of the data are excluded. The open-air measurement methods (field/confined method, UAV, and satellite) also have a stringent screening of data to ensure weather conditions (e.g., wind speed, direction, precipitation, sunlight) were within the limits suitable to obtain accurate measurement from the source data. This data screening for limits of detection can result in more than 75% of the data being discarded for the final analysis (McGinn et al., 2019).

Another factor to consider is the number of measurement days required to get an accurate and precise CH_4 measurement. In general, accurate data are generated in 3 d (including the animal entry day) for RC, 2 to 3 d for FM and GF in tiestall barns with an intensive sampling schedule, 3 to 5 d for SF_6 (3 good sampling days needed), and ≥14 d for GF with voluntary visitation of the automated concentrate feeder. For other methods, the number of sampling days is less well-defined when performing treatment comparisons but is generally ≥14 d. For spot-sampling methods, the number of spot samples and the minimum duration of a spot sample is also important. For GF with voluntary visitations, only spot samples of >2 min are used for data analysis and a minimum of 50 to 70 spot samples per animal collected across at least 14 d are needed (Hammond et al., 2016; Melgar et al., 2021). This sampling intensity may allow detection of low mitigation effects of, for example, 5% to 10%. For GF used in the tiestall and for FM, 8 or 12 spot samples at 2- or 3-h intervals across 2 to 3 d are used to estimate CH_4 production (Washburn and Brody, 1937; Bhatta and Enishi, 2007; Hristov et al., 2015a). It must be emphasized that with all spot-sampling methods it is very important to collect enough samples that are well-dispersed across the whole feeding cycle of the animal to have representative and accurate estimates of daily enteric CH_4 emissions. For other spot-sampling methods when used to compare treatments, these criteria are less well-defined.

Unmanned aerial vehicles and satellites (and similar) are not yet fully developed into robust methods to measure emissions from livestock. Usually, with these methods there is no direct comparison (mainly to track change over time within a source area) and it first needs to be demonstrated whether these methods can provide representative sampling because emissions of large groups of livestock can be very variable within a day and across seasons, considering practical and less-controllable farming conditions. Data from trials with CH_4 measured using RC, GF, and SF_6 are currently accepted to be accurate and are extensively used to develop bottom-up prediction equations for use in national GHG inventories and farm models (Hristov et al., 2018; Vibart et al., 2021; del Prado et al., 2025).

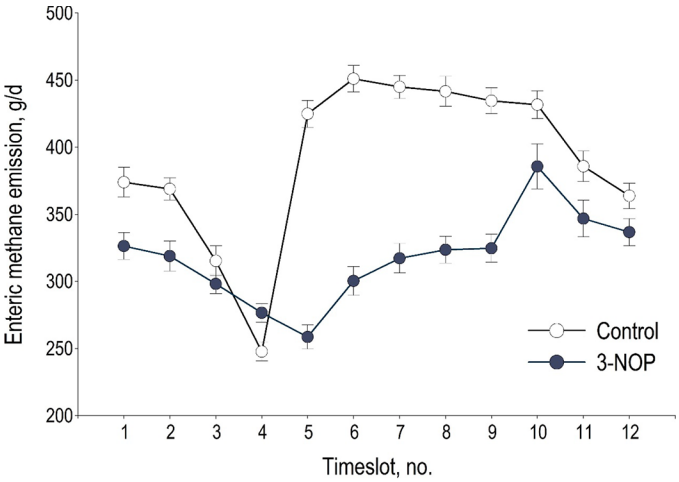

Figure 2. Diurnal variation in enteric methane emission in lactating dairy cows with normal or inhibited rumen methanogenesis. Data are arithmetic means (± SE) extrapolated to a 24-h period from the measurement taken during individual timeslot visits (from Hristov and Melgar, 2020). Timeslots 1 through 12 represent 2-h periods (0000 to 0200, 0200 to 0400, 0400 to 0600, 0600 to 0800, 0800 to 1000, 1000 to 1200, 1200 to 1400, 1400 to 1600, 1600 to 1800, 1800 to 2000, 2000 to 2200 and 2200 to 0000 h, respectively). The difference in methane emission between control and 3-nitrooxypropanol (3-NOP) was statistically significant ($P < 0.05$) for all timeslots, except timeslot 3 (i.e., 0400 to 0600 h). Feeding, including 3-NOP provision (mixed with the TMR), was once daily, during timeslot 5 (i.e., 0800 to 0900 h). Cows were milked twice daily: morning milking was from around 0600 to 0800 h; evening milking was from around 1745 to 1930 h. Enteric methane emission was measured using the GreenFeed (C-Lock, Inc., Rapid City, SD) systems.

Continuous or Short-Term Sampling and Temporal Variation in Methane Emissions

It is well documented that CH_4 emissions throughout the day are not constant at a temporal time scale (Jonker et al., 2014; Biswas and Jonker, 2019). Furthermore, some AMFA have been found to only have a temporal effect on CH_4 and H_2 emissions depending on AMFA presence in the rumen (Figure 2; Hristov and Melgar, 2020). For example, Reynolds et al. (2014) using RC observed a temporal short-term effect on CH_4 emissions (2–3 h) when dairy cows received 3-NOP as a pulse dose around the main feeding, and a similar profile was observed in sheep (but lasting ~10 h) receiving the electron acceptor nitrate (van Zijderveld et al., 2010). In a long-term study with 3-NOP, Hristov and Melgar (2020) reported a 45% reduction in CH_4 yield 2 h after the morning feeding of dairy cows, but no effects before feeding (Figure 2). Clearly, in these cases, if a short-term CH_4 measurement (2–60 min) was performed before feeding, no effect on CH_4 emissions would be observed (i.e., underestimate the daily effect of AMFA), while measuring for a short-term in the first 2 to 4 h after feeding would result in a very large overestimation of the CH_4 reduction potential of AMFA. Therefore, CH_4 measurement distribution across the day, particularly with spot-sampling techniques, is critically important to enable capturing the temporal variation in CH_4 emissions due to feeding events and the potential temporal effect of AMFA. With GF in tiestalls, FM, and PAC, the temporal variation can be captured by implementing an evenly distributed sampling schedule across 24 h in a 2- to 3-d period as described before (Hristov et al., 2015a). For H_2 emissions the sampling window of time is even narrower than for CH_4 (e.g., van Lingen et al., 2023).

Continuous measurement methods, such as RC and SF_6, by definition, determine daily CH_4 emissions and therefore can directly determine reduction due to supplementing AMFA. The SF_6 method does not provide information on diurnal variation in CH_4 emissions and therefore might not be able to identify AMFA that result only in a minor and very short-term CH_4 reduction (<3 h).

In grazing systems where supplementation is employed, discrete supplementation with concentrates causes variation in the dynamics of rumen fermentation and the microbial microenvironment throughout the day (Cajarville et al., 2006; Aguerre et al., 2013). Thus, it is expected that there will also be variation in the kinetics of ruminal methanogenesis. This variation is particularly pertinent to the scheduling of CH_4 measurements, especially when using methods that estimate daily emissions from specific sampling points such as GF or spot sampling through PAC (Hammond et al., 2016b).

For RC, a CH_4 reading for an individual chamber will in most cases occur every 0.16 to 30 min for systems used across different institutes, and in one case occurring once every 120 min (Della Rosa et al., 2021). Wang et al. (2019b) found that the CH_4 reading interval within a chamber (up to 3 h) had little effect on daily CH_4 production, but the max/min CH_4 ratio decreased linearly with increasing CH_4 reading interval. Therefore, the interpretation of temporal CH_4 profiles is affected by CH_4 recording frequency (Lee et al., 2022; Della Rosa et al., 2023a). A further consideration for the interpretation of RC data is the air exchange rate (times per hour) in the chamber, which ranged from 1 to 50 times/h across RC systems reviewed by Della Rosa et al. (2021), as well as the accuracy with which the air exchange rate is controlled and measured.

For methods integrated into an automatic milking system (**AMS**), automated concentrate feeder, or feed bin, such as GF and sniffer, spot sampling is voluntary when the animal is visiting the device to be milked or to eat. The main difference for these spot-sampling locations is the frequency of visits, with animals typically visiting the AMS 2 or 3 times a day, the automated concentrate feeder 1 to 5 times a day (Della Rosa et al., 2021), and the feed bin 9 to 27 times per day (Troy et al., 2016; Flay et al., 2019; Melgar et al., 2021; Biswas et al., 2022). For

any method of spot sampling, it is important to evaluate whether the entire daily pattern is well captured.

Recommendations

Each CH_4 measurement method has its strengths, weaknesses, and uncertainties, and different methods may be suited for determining emissions in different environments and experimental designs. Currently, data from trials with CH_4 measured using RC, GF, and SF_6 are accepted to be accurate. The user needs to be aware of the limitations of a method and ensure the proper equipment setup, calibration, monitoring, alignment of multiple sensor information, and data screening and exclusion, as well as implementing appropriate animal measurement protocols, including animal adaptation and training and measurement duration and timing (see Table 1 for requirements for each method). Failing to do so can result in faulty, highly variable, or biased CH_4 measurements and hence wrong conclusions on the effectiveness of AMFA on reducing enteric CH_4.

OTHER PROCESSES TO BE MONITORED AND MEASUREMENTS TO BE TAKEN

In addition to the technical measurement of the efficacy of AMFA in reducing enteric CH_4 emissions, several other aspects of animal performance are also key elements of experimental evaluation. General information on experimental conditions helps to better contextualize the productive situation and environment (type of animal, feed intake, diet composition, animal behavior, animal productivity, and farming conditions) in which AMFA are evaluated. This information is furthermore highly relevant if prediction equations are to be developed to predict efficacy of AMFA (Dijkstra et al., 2025), which preferably include information that allows evaluation of synergies and trade-offs as well. At the least, feed intake and animal productivity must be included in any study; also, monitoring of feed intake behavior may deliver important additional information. Animal activity and feed intake behavior influenced by AMFA, or a certain dosing of AMFA, may affect palatability or feeding behavior (Melgar et al., 2020b). The method of recording gaseous emissions may be sensitive to changes in activity and feeding behavior, which influences the recorded CH_4 mitigating effect of AMFA. Furthermore, such information is relevant when attempting to explain observed synergies or trade-offs. Health status and health treatments carried out before and during testing the AMFA should be reported as well. The occurrence of trade-offs as well as synergies of an AMFA with any or all of these aspects, as well as with N and OM, and especially NDF, digestibility is of particular relevance. As discussed in the previous section, feed intake is the main driver of CH_4 production and as such is single most important variable, together with feed composition, for monitoring AMFA effects.

Besides animal performance, there must be a continuous monitoring of animal's BW change, and in long periods of measurement, BCS. Traits related to animal welfare may also be recorded. Although seldom reported, it is recommendable to gather information on water intake and drinking behavior because water intake level may influence productive efficiency (Pires et al., 2022), and thus indirectly CH_4 emission intensity. Measurements of the target animal performance may go beyond the productive performance and health if the long-term effects of AMFA are to be tested. Only long-term studies can shed light on the absence of negative effects on growth, fertility, longevity, resilience, colostrum quality, or offspring development. Reports of long-term studies are rather scarce but are highly recommended and can be performed as field studies (see previous sections) following up experimental testing of AMFA under well-controlled study conditions (Tricarico et al., 2025).

Although feed intake, BW, and animal performance themselves deliver information on feed utilization, measurement of total-tract digestibility would be an important extra verification, particularly in the case of a short-term study design, which is less suitable for testing for performance effects. This information is highly valuable, as discussed previously, because increasing feeding level potentially increases the passage rate and shortens rumen retention time, leading to lower feed degradation and CH_4 yield (Molano and Clark, 2008; Hammond et al., 2013). Moreover, these measurements should be combined with measurements of intake of individual nutrients because it determines the level of CH_4 emission for the control treatment (Niu et al., 2018) and is a representative sampling of the diet offered as well as refusals for cases where sorting of feed ingredients occurred.

Analysis of H_2 emissions is relevant when the aim is to better understand the mode of action of AMFA (Belanche et al., 2025; Dijkstra et al., 2025). For example, increased H_2 production is observed with the single use of direct methanogen inhibitors, but this may, however, be reversed when an inhibitor is used in combination with alternative electron acceptors such as nitrate (Olijhoek et al., 2016) or phloroglucinol (Martinez-Fernandez et al., 2017). Although nitrate as an electron acceptor increases H_2 emission (Olijhoek et al., 2016), with 3-NOP as an inhibitor, the recent study of Maigaard et al. (2024) demonstrated a significant interaction effect of nitrate and 3-NOP on H_2 emission and a lower numerical increase in H_2 emissions was shown for the combination of nitrate and 3-NOP compared with nitrate or 3-NOP alone. Nevertheless, more information is needed to identify vari-

ability in rumen dissolved H_2 dynamics and to what extent observed H_2 emission reflects rumen H_2 production. Although energy losses as H_2 as a proportion of energy saved in CH_4 not formed have been reported to be moderate (Hristov et al., 2015b; Ungerfeld et al., 2022), H_2 concentration has a pivotal role in the thermodynamics of rumen fermentation and VFA profile (Janssen, 2010). Hence, understanding the variation among experiments in expelled and dissolved H_2 is considered important.

Another parameter to measure and compare when testing AMFA is CO_2 emission (Reynolds et al., 2014; Melgar et al., 2020a; van Gastelen et al., 2020). In the absence of differences in digestibility between treatments and animal productivity, CO_2 emission would directly reflect the level of feed intake, and hence serve as an extra verification of the accuracy of reported feed intake and whether a study was executed as planned. It is important to note that feed intake is always vulnerable to experimental bias and error and is particularly difficult to monitor in pastoral systems and under grazing conditions.

In addition to measurement of total-tract digestibility, measurement of rumen fermentation parameters and rumen digestibility may deliver important supplementary information on efficacy and mode of action of AMFA, with the downside that mostly invasive methods and rumen-fistulated animals are needed instead of using fully intact animals, unless the ororuminal tubing technique is used in which case care should be taken to avoid saliva contamination (de Assis Lage et al., 2020; Muizelaar et al., 2020). Caution is needed to ensure that the adopted rumen sampling (or content evacuation) schemes capture the entire diurnal pattern of rumen fermentation dynamics, and researchers should realize that the best sampling scheme depends on feeding management and feed intake behavior. Highly important parameters to measure are concentrations and profile of VFA (because rate of production of individual VFA is a too difficult to measure determinant of rate of fermentation and H_2 and CH_4 production). Together with the observed changes in H_2 and CH_4 emission rate at the animal level, changes in rumen VFA may shed light on the mode of action of AMFA and its consequences within the rumen environment. In this regard, it is highly recommended to monitor other electron carriers intermediate in rumen fermentation such as formate, methanol, ethanol, and methylamines, which have been shown to accumulate when methanogenesis was inhibited (Olijhoek et al., 2016; Martinez-Fernandez et al., 2018; Melgar et al., 2020a), as well as the electron carriers lactate and succinate. For example, different mechanisms and consequences are involved with 3-NOP (Reynolds et al., 2014; Melgar et al., 2020a) and bromoform (Stefenoni et al., 2021) compared with nitrate as an electron acceptor (van Zijderveld et al., 2011; Olijhoek et al., 2016). With both options, a higher emission of H_2 is reported but the fermentation profile shifts toward less acetate and increased propionate and other longer chain and more reduced VFA with the specific inhibitors 3-NOP and bromoform, and instead toward more acetate (a shorter chain and less reduced VFA) and less propionate and longer chain and more reduced VFA with the electron acceptor nitrate. Alternatively, combining these modes of action, for example, with degradation of phloroglucinol as an alternative electron acceptor, in addition to it being an inhibitor of methanogenesis, was reported to revert the increase of H_2 production and decrease of acetate with inhibitor activity into a decrease of H_2 and increase of acetate concentrations in steers (Martinez-Fernandez et al., 2017). As mentioned in the section on basal and experimental diets, rumen passage rate and pH are important determinants of rumen microbial activity and methanogenesis and may modulate the effect of AMFA on CH_4 emission. Rumen pH can be measured in rumen fluid samples together with VFA, but some measurements (i.e., pH) can also be made from intact animals using intraruminal dwelling sensors, and their use should be encouraged where available (and where their use is not limited by the length of the experiment). Rumen passage rate measurements are technically more demanding, requiring intensive handling of rumen-fistulated animals, but should be considered if data are to be generated to quantify rumen function in-depth and improve modeling of rumen fermentation and methanogenesis, including efficacy of AMFA (Dijkstra et al., 2025). Using rumen-cannulated animals in AMFA experiments can be problematic, depending on the CH_4 emission technique used. It has been reported that gas leakage through the cannula can increase variability with the SF_6 technique (Beauchemin et al., 2012) and GF data from rumen-cannulated cows were discarded in the study of Melgar et al. (2020a) because of unrealistic daily CH_4 emission values, even though a cannula extension device designed to capture gases leaking through cannula (Lopes et al., 2016) was used. Furthermore, Wang et al. (2019a) reported that rumen cannulation may alter headspace gaseous composition and rumen methanogen community. Thus, unless enteric gas emissions are measured in enclosures such as RC, the use of rumen-cannulated animals in experiments designed to evaluate AMFA is not recommended.

Recommendations

In this section, measurements that are highly recommended for AMFA evaluation trials, such as digestibility, animal performance, and monitoring feed intake behavior (and, if possible, drinking behavior as well), and animal behavior are outlined. Highly useful additional information on gaseous emissions includes the measure-

ment of H_2 and CO_2. In short-term studies, general information on experimental conditions and total-tract nutrient digestibility delivers important additional insight in to how animal performance may be affected. These measurements are pivotal for estimating synergies or trade-offs between the effect on enteric CH_4 mitigation and effects on N and OM excretion as sources of GHG and N emissions from manure. Measurements of rumen function may deliver important insight on AMFA mode of action and how the effect was achieved. Likewise, measurements of post-absorptive metabolic changes may help to explain effects on animal performance and well-being. Effects of AMFA on animal performance are best verified in well-designed and controlled long-term trials under practical conditions instead of short-term AMFA efficacy experiments.

ANIMAL HEALTH

Feed additives, including AMFA, can have a positive, a negative, or no effect on animal health, and it is important to monitor and evaluate the health status of animals during the trials and beyond. In case of disease during the trial, clinical evaluation is required and documentation on the cause of death and performing postmortem examinations are part of the animal ethics approval process. For example, it has been shown that the bromoform-containing algae *Asparagopsis* spp. can cause inflammation and damage of the rumen epithelium, which is detectable in living animals by inflammation markers in the blood and by feed refusal, and in slaughtered animals by histological and visual examination (Muizelaar et al., 2021). Nitrate is naturally present in forage at low levels. As a feed additive, nitrate must be introduced gradually to reduce the risk of accumulation of the toxic intermediate nitrite in the rumen and absorption into the blood where it disables the ability of red blood cells to carry oxygen (methemoglobinaemia) measured as blood methemoglobin level (percent of total hemoglobin; Lee and Beauchemin, 2014).

The majority of polyphenol-rich plants contain more CT than HT (Jayanegara et al., 2012). In contrast to CT, HT are (partially) degradable by ruminal microbes (Bhat et al., 1998; Makkar, 2003). The absorption of metabolites from polyphenol degradation, and maybe HT, may improve the systemic antioxidant status of ruminants (Zhou et al., 2019). However, hepatotoxicity and even toxicity in cattle and sheep have been reported after consumption of plants rich in HT, for example, hepatotoxic punicalagin from pomegranate fruit (cattle; Hawes and Gill, 2018; Niu et al., 2023) and from yellow wood, *Terminalia oblongata* (sheep; McSweeney et al., 1988). Signs of toxicity were observed at 0.9 g HT/kg of BW (McSweeney et al., 1988), and at 0.67 g/kg of BW (Niu et al., 2023); therefore it is recommended to formulate rations below these levels. The liver stress, measured as serum alanine aminotransferase activity, was shown to be reversed after discontinuation of dietary HT supplementation (Niu et al., 2023). Bhat et al. (1998) suggested stepwise adaptation to dietary HT to reduce liver stress, potentially enabling the metabolism to (partially) detoxify HT metabolites. Hence, when using tannin-rich plants, care must be taken to include as much CT as needed to reduce enteric CH_4 production, but as little unfavorable HT (e.g., punicalagin) as possible, to reduce negative health effects. Other AMFA are not reported to have negative effects on animal health (Hegarty et al., 2021).

Additionally, compromised animal health and differences in the health status across animals can substantially increase the variability in the CH_4 emissions and production data. When animals experience health issues, DMI and metabolic processes change, and the immune response is activated. Treatment may also include antibiotics, which affect the general microbiome composition of the animal. This does not allow for reliable AMFA testing, because compromised animal health or differences in the health status across animals can substantially increase the variability in the CH_4 emissions and production data. It is therefore recommended that animals used for AMFA testing follow standard farming practices such as colostrum feeding, vaccination, and deworming programs as well as drug treatments to guarantee the highest possible health standards, and the availability of this information has to be ensured. When selecting animals for AMFA testing, it is recommended to select only animals that fulfill health criteria using, for example, a clinical scoring procedure (e.g., van Dixhoorn et al., 2018).

Recommendations

In this section, it was stressed how animal health can be affected by AMFA and that it is important to have healthy animals when testing AMFA. For this reason, recording and reporting animal health status before and during the experiment (particularly important in long-term studies) should be done as a standard when investigating AMFA efficacy. This is of particular importance when the AMFA mode of action is unknown.

ANIMAL PRODUCT QUALITY

The nutritional composition of milk, meat, and the associated products is strongly influenced by livestock farming practices (management, feeding, breeding), with the animal's diet being the most influential driver for the concentration of nutrients, essential for human nutrition,

in milk (Qin et al., 2021; Ormston et al., 2023) and meat (Clinquart et al., 2022). Beyond the nutritional aspect, animal nutrition highly affects the organoleptic characteristics of the final product (Kilcawley et al., 2018). Methane mitigation via nutritional intervention typically aims to, or inevitably does, drastically affect the rumen microbiome. Therefore, using AMFA that modify the rumen microbiome and its metabolic pathways, the ruminal synthesis of nutrients for their transport into food, and the generation of precursors in the rumen for synthesis of nutrients elsewhere in the body can alter the nutritional composition of the milk and meat that ruminants are producing (see Belanche et al., 2025 for discussions about the effects of AMFA on rumen microbiome composition, metabolism, and the consequences on post-absorptive metabolism).

Nutritional Quality

Fatty acids are synthesized in milk and meat via different metabolic pathways associated with the diet and rumen microbiota and its function (Palmquist, 2006; Dinh et al., 2021). The rumen microbiome provides an abundance of acetate and butyrate, which are absorbed in the blood and diffused in the mammary gland, where they are used as carbon sources for the de novo synthesis of milk fat (Tian et al., 2022). Altering the composition of the rumen microbial community via use of AMFA can affect the transfer rate of fatty acids from feed to food, the extent of their hydrogenation in the rumen and the production of mid- and long-chain fatty acids in the mammary gland; and consequently, the fatty acids profile of milk and meat (Palmquist, 2006; Buccioni et al., 2012; Dinh et al., 2021; Wu et al., 2021).

Marine-based AMFA (e.g., macroalgae) may be particularly high in iodine and increase the iodine concentrations in milk and meat; an effect that has been previously observed in studies feeding red seaweed to dairy cows (Stefenoni et al., 2021; Newton et al., 2023; Qin et al., 2023) and finishing lamb (Grabež et al., 2022). This effect can be advantageous in populations with documented iodine deficiency or in consumers with higher iodine requirements (children, pregnant and nursing women, or women of childbearing age), but also as a means for increasing the iodine concentrations in milk from production systems (pasture-based) and months (grazing season) where iodine concentrations are expected to be lower (Brito, 2020; Newton et al., 2023). However, in any case, care should be taken to maintain the iodine supply via the animal diet within the legislative limits, for example, the EFSA Panel on Additives Products or Substances used in Animal Feed (FEEDAP, 2013).

The B vitamins are synthesized by rumen microbiota and there is large variation in which microbiota affect the different pathways for vitamins synthesis (Jiang et al., 2022). The site of synthesis may also vary for the different vitamins, with studies supporting that B vitamins are mainly synthesized in the rumen (Girard and Graulet, 2021). There is also increased knowledge on the specificity on genome function because out of the 2,366 genomes that were identified to synthesize vitamins, most were able to synthesize only one vitamin, and no genome was capable of synthesizing more than 5 (Jiang et al., 2022). Given the extensive role of the rumen microbiome in vitamin synthesis and the fact that AMFA target manipulation of the rumen microbiota to reduce CH_4 formation, potential effects of AMFA on vitamin synthesis in animal gastrointestinal tract, presence in the circulatory system, and concentrations in the final product (milk or meat) should be considered, alongside any potential effects on animal health and productivity.

Organoleptic Parameters

Feeding AMFA in the form of garlic and citrus extracts to dairy cows may affect the flavor and taste of dairy products, as well as potentially some rheological properties, as a result of their high content of organosulfur compounds (Rossi et al., 2018). Seaweeds rich in glutamic acid, an amino acid delivering umami taste (Yamaguchi and Ninomiya, 2000; Makkar et al., 2016; Morais et al., 2020), may potentially cause its increase in milk and dairy products, thereby affecting their taste, flavor, and consumer acceptance. A sensory panel indicated that milk from cows fed *A. taxiformis* was not organoleptically different from milk from control cows, but 43 out of 109 participants (i.e., 39%) correctly identified milk from treatment cows as being different from control milk (with the *P*-value approaching a trend at $P = 0.11$; Stefenoni et al., 2021). Organoleptic characteristics of dairy products can be affected by compounds of rumen origin at relatively low concentrations in the animal product (e.g., skatole and indole, Bendall, 2001; Young et al., 2003). Our understanding of the fermentation products of a CH_4-inhibited rumen is limited primarily to VFA, but the fate of other rumen metabolites needs to be investigated for early identification of trade-offs (or co-benefits) between AMFA and organoleptic characteristics of products.

Safety Characteristics

In addition to proving the CH_4-mitigation efficacy, in vivo trials are required by authorization authorities to provide information on the safety of the AMFA in terms of animal and human health as well as the environment (Tricarico et al., 2025). The in vivo experiments can be combined with in vitro tests to describe how the active

component is metabolized in the rumen (Romero et al., 2023; Belanche et al., 2025); however, quantification of the potential absorption, deposition in tissues, or excretion requires in vivo experimentation.

Bromoform is the bioactive compound in *Asparagopsis* spp. that is considered responsible for the reduction in CH_4 emissions after dietary supplementation in dairy cows and beef cattle (Wasson et al., 2022). The US Environmental Protection Agency classifies bromoform as "potential human carcinogenic" compound, setting a limit of 0.7 mg/kg in water (Agency for Toxic Substances and Disease Registry, 2005), and the World Health Organization recommends ≤0.4 mg bromide intake per kilogram of BW, which would be an approximate maximum concentration of bromide in water of 6 mg/kg in adults and 2 mg/kg in children (International Agency for Research on Cancer, 2003). Previous work has shown that bromoform and bromide concentrations in milk may be increased when cows are fed bromoform-containing seaweed (*A. taxiformis*; Stefenoni et al., 2021; Wasson et al., 2022), particularly when feeding amounts in excess of recommended levels for reduction in CH_4 emissions (Muizelaar et al., 2021). When fed at near minimum inclusion rates as part of the basal diet, bromoform was not detectable at higher than background levels in milk and meat (Glasson et al., 2022).

As mentioned previously, marine-based AMFA (e.g., macroalgae) may be particularly high in iodine. The high iodine levels pose a strong limitation on the amounts that can be included in the animal diets, given that EFSA recommends a maximum iodine inclusion in dairy cows and small dairy ruminants at 2 mg I/kg and does not permit an inclusion rate of more than 5 mg I/kg of diet (FEEDAP, 2013). Based on these recommendations, assuming a dairy cow consuming 25 kg of DM per day, the total iodine intake must not exceed 125 mg per day. At the concentration of 2.27 mg/g of DM of iodine previously reported for *A. taxiformis* (Roque et al., 2021), and assuming the basal diet provides only the minimum iodine supply for meeting dairy cows' requirements at 0.5 mg I/kg of DM (National Research Council, 2001; 12.5 mg/cow per day), the seaweed cannot be included at more than 50 g/cow per day (0.20% DM inclusion rate) or 16.5 g/cow per day (0.07% DM inclusion rate) before the diet exceeds the maximum permitted or recommended iodine concentration, respectively. Notably, these maximum inclusion rates are 2.5 to 14.3 times lower to those previously found to reduce CH_4 emissions in dairy cows (0.5%–1.0% of DM; Roque et al., 2021; Stefenoni et al., 2021). If the dietary iodine supplementation is controlled and remains within the permitted limits, there is no potential health risk for consumers. However, in practice it may be expensive and impractical to constantly monitor iodine concentrations of all feed ingredients alongside marine-based AMFA. Studies in which cows' diets exceeded iodine permitted inclusion rates resulted in milk iodine concentrations that would pose significant nutritional risk for consumers, and in particular children (Newton et al., 2023). Certain marine-based AMFA may also be rich in heavy metals, but previous studies have shown that increased intake of seaweeds in cows' diet does not affect heavy metals concentrations in milk (Newton et al., 2021; Newton et al., 2023; Qin et al., 2023). Seaweeds (91% *Ascophyllum nodosum*: 9% *Laminaria digitata*, DM basis) in dairy diets have increased milk arsenic concentrations in other studies; however, milk arsenic concentrations were negligible and milk consumption appears to pose no apparent arsenic-related risks to human health even when cows' diets are high in arsenic (Newton et al., 2021).

Recommendations

In this section, key considerations to assess animal product quality, essential to guaranteeing that AMFA are safe to use and do not impair ruminant food products are presented. It is recommended to screen AMFA for potential antinutritional and toxic compounds before embarking on animal trials. If it is expected or known that the AMFA contain antinutritional, harmful, or toxic compounds to human health, it is imperative to assess the final product for potential contamination, to ensure that there has been no transport of the harmful compounds or its residues from feed to food. Along the same line, it is necessary to ensure that the animal diet is designed in a way that is commercially applicable and does not exceed the upper tolerable limits for certain nutrients and compounds toxic to animals and humans. Nutritional quality of milk and meat needs to be assessed, and flavor and sensory tests are to be conducted to ensure that AMFA do not affect the organoleptic properties of the foods (e.g., pasteurized milk, cheese, yogurt, fresh cuts of meat, processed meats).

CONCLUSIONS

The 2 basic questions that need to be considered in designing ruminant experiments involving AMFA are "What is the research question that needs to be addressed?" and "What are the results going to be used for?" A study for determining efficacy may have to maximize statistical power of the test by including few or only one dietary treatment. A study to generate label claims or inventory values may need to consider the farming practices in which the AMFA will be used in a broader term. The experimental design should therefore mirror the research question, and proper experimental design and thorough post-experimental statistical analyses are pre-

requisites. Long-term studies are often absent in evaluating AMFA but are highly warranted and encouraged. The methane-mitigating effects of combinations of AMFA with nutritional or non-nutritional practices should not be considered additive before being evaluated in animal trials. The selection of treatments when evaluating combinations of mitigation strategies should account for the individual efficacy and mode of action of each strategy. Respiration chambers, the SF_6 method, GreenFeed, and hood and FM techniques can be used to measure enteric CH_4 emission and determine the efficacy of AMFA in short-term studies, but it is necessary to ensure application of these techniques does not significantly affect feed intake, production, and normal animal behavior. These methods can also be used in repeated-measurement campaigns over time to test the medium- and long-term effects of AMFA. Pivotal for the evaluation of AMFA efficacy are representative measurements of feed intake, feed composition, and possibly feed intake behavior, milk production and composition, BW and BCS changes (continuous-design experiments only), and enteric gaseous emission measurements. Supporting information could include measurement of H_2 and CO_2 emissions, rumen VFA, and other fermentation variables, as well as total-tract digestibility to determine potential synergies or trade-offs in GHG emissions exerted via excreta. Determining the effects of AMFA on the animal's health status and product quality is essential, and relevant analysis, including nutrient composition; antinutritional, harmful, or toxic compounds; and organoleptic evaluation of animal products should be conducted as part of the AMFA assessment process. The content of any such compound in the potential AMFA should therefore be quantified before embarking on animal trials, and tolerable limits of potentially harmful compounds to animals and humans, respectively, should be consulted. In conclusion, enteric CH_4 mitigation claims should not be made until efficacy of AMFA is confirmed in animal studies designed and conducted according to the guidelines provided herein.

NOTES

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Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; AMFA = antimethanogenic feed additives; AMS = automatic milking system; AOAC = Association of Official Analytical Chemists; CT = condensed tannins; EFSA = European Food Safety Authority; EID = electronic identification system; FM = face masks; FPCM = fat and protein corrected milk; FTIR = Fourier-transform infrared spectroscopy; GF = GreenFeed; HT = hydrolysable tannins; LMD = laser methane detectors; NIRS = near-infrared spectroscopy; PAC = portable accumulation chambers; RC = respiration chamber; SNR = signal-to-noise ratio; SF_6 = sulfur hexafluoride tracer technique; UAV = unmanned aerial vehicle.

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