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# A meta-analysis of methane-mitigation potential of feed additives evaluated in vitro

**L. F. Martins,[1] S. F. Cueva,[1] C. F. A. Lage,[1,2] M. Ramin,[3] T. Silvestre,[1,4] J. Tricarico,[5] and A. N. Hristov[1]***
[1]Department of Animal Science, The Pennsylvania State University, University Park, PA 16802
[2]Cornell Cooperative Extension, Bath, NY 14810
[3]Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Umeå, Sweden SE-901 83
[4]Kemin Industries, Singapore, 758200
[5]Innovation Center for U.S. Dairy, Rosemont, IL 60018

## ABSTRACT

A systematic literature review of in vitro studies was performed to identify methane ($CH_4$) mitigation interventions with a potential to reduce $CH_4$ emission in vivo. Data from 277 peer-reviewed studies published between 1979 and 2018 were reviewed. Individual $CH_4$ mitigation interventions were classified into 14 categories of feed additives based on their type, chemical composition, and mode of action. Response variables evaluated were absolute $CH_4$ emission (number of treatment means comparisons = 1,325); total volatile fatty acids (n = 1,007), acetate (n = 783), propionate (n = 792), and butyrate (n = 776) concentrations; acetate to propionate ratio (n = 675); digestibility of dry matter (n = 489), organic matter (n = 277), and neutral detergent fiber (n = 177). Total gas production was used as an explanatory variable in the model for $CH_4$ production. Relative mean difference between treatment and control means reported in the studies was calculated and used for statistical analysis. The robust variance estimation method was used to analyze the effects of $CH_4$ mitigation interventions. In vitro $CH_4$ production was decreased by antibodies (−38.9%), chemical inhibitors (−29.2%), electron sinks (−18.9%), essential oils (−18.2%), plant extracts (−14.5%), plant inclusion (−11.7%), saponins (−14.8%), and tannins (−14.5%). Overall effects of direct-fed microbials, enzymes, macroalgae, and organic acids supplementation did not affect $CH_4$ production in the current meta-analysis. When considering the effects of individual mitigation interventions containing a minimum number of 4 degrees of freedom within feed additives categories, *Enterococcus* spp. (i.e., direct-fed microbial), nitrophenol (i.e., electron sink), and *Leucaena* spp. (i.e., tannins) decreased $CH_4$ production by 20.3%, 27.1%, and 23.5%, respectively, without extensively, or only slightly, affecting ruminal fermentation and digestibility of nutrients. It should be noted, however, that although the total number of publications (n = 277) and treatment means comparisons (n = 1,325 for $CH_4$ production) in the current analysis were high, data for most mitigation interventions were obtained from less than 5 observations (e.g., maximum number of observations was 4, 7, and 22 for nitrophenol, *Enterococcus* spp., and *Leucaena* spp., respectively), because of limited data available in the literature. These should be further evaluated in vitro and in vivo to determine their true potential to decrease enteric $CH_4$ production, yield, and intensity. Some mitigation interventions (e.g., magnesium, *Heracleum* spp., nitroglycerin, β-cyclodextrin, *Leptospermum pattersoni*, *Fructulus Ligustri*, *Salix caprea*, and *Sesbania grandiflora*) decreased in vitro $CH_4$ production by over 50% but did not have enough observations in the database. These should be more extensively investigated in vitro, and the dose effect must be considered before adoption of mitigation interventions in vivo.

**Key words:** enteric methane, ruminal fermentation, in vitro

## INTRODUCTION

Decreasing enteric $CH_4$ emission to improve animal efficiency has been a research focus since early studies reported up to 12% of gross energy intake losses by $CH_4$ production (Czerkawski, 1969; Moe, 1981; Johnson and Johnson, 1995). More recently, the rising interest of governments and the society in climate change has directed researchers to better understand rumen methanogenesis and develop strategies to decrease greenhouse gas (**GHG**) emissions by livestock, especially enteric $CH_4$ in ruminants (Hristov et al., 2013; Congio et al., 2021; Arndt et al., 2022). The effectiveness of mitigation strategies, however, is inconsistent and data are, in some cases, controversial. For example, Hegarty et

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*Corresponding author: anh13@psu.edu

al. (2021) classified a low to medium agreement for efficacy of most enteric $CH_4$ mitigation strategies tested in vivo.

In vitro systems are considered as a preliminary step in the investigation of novel nutrition and rumen manipulation interventions, and they can be used to screen large number of treatments and doses in a short period of time, compared with in vivo experiments (Flachowsky and Lebzien, 2012; Hristov et al., 2012; Vinyard and Faciola, 2022). Ruminal fermentation and digestibility data generated by in vitro techniques, however, do not usually accurately represent in vivo responses, as demonstrated by studies evaluating grass and corn silages differing in plant maturity (Macome et al., 2017a, 2018) and N fertilization (Macome et al., 2017b), as well as dietary starch sources and levels (Hatew et al., 2015). On the contrary, a strong relationship (adjusted $R^2 = 0.94$) for $CH_4$ production data between in vitro and in vivo systems was reported by Danielsson et al. (2017), and accurate predictions of in vivo $CH_4$ production using an in vitro gas production system have been reported by Ramin and Huhtanen (2012). Differences in fluid and particle dilution rates, feed substrate to rumen volume ratio, and the lack of absorption of fermentation end products (Hristov et al., 2012) are some of the reasons why in vitro data are more variable than, and in some cases not representative of, in vivo experimental data. Nevertheless, when $CH_4$ mitigation strategies are subjected to an in vitro evaluation, it is reasonable to assume that differences from control, although not representative in absolute terms, would be unidirectional with in vivo effects. This has been described, for example, in a meta-analysis by Brandao et al. (2020), in which the relationship between independent and dependent variables used in their models were similar for data collected from an omasal sampling technique and a dual-flow continuous culture system, even though the magnitude of the measured responses was different.

Considering the feasibility of in vitro systems to preliminarily investigate nutritional interventions that may decrease GHG emissions in ruminants, the objective of the current study was to perform a systematic meta-analysis of in vitro studies to identify feed additives with a potential to reduce enteric $CH_4$ emission. Our goal was to reveal mitigation strategies that effectively decreased in vitro $CH_4$ production, had no negative effect on ruminal fermentation and nutrient digestibility, and have not been extensively studied in vivo.

## MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

### Literature Search

A comprehensive search of the literature was conducted to identify experiments evaluating $CH_4$ mitigation interventions in vitro. Databases of the Commonwealth Agricultural Bureau International, the EBSCO Discovery Service, and the Web of Science were searched, and data were compiled for this meta-analysis. The search was conducted in February 2019 using the terms in vitro in combination with "methane," "fermentation," or "gas production." The abstract content of the publications retrieved by the searched criteria (n = 1,199) was reviewed, and publications were selected for further consideration if they included in vitro measurements of $CH_4$ production, a clearly defined treatment and control, and multiple experimental replications (at least 2 or more replicates for each treatment within a study). Additional publications were searched whenever a citation in a manuscript identified a reference not listed in the searched database. Only peer-reviewed manuscripts published in English were selected for this meta-analysis.

***Exclusion Criteria and Studies Included in the Database.*** A Preferred Reporting Items for Systematic Reviews and Meta-Analysis diagram (Moher et al., 2009) of the flow of data collection for the meta-analysis is presented in Figure 1. After the initial search and screening, 1,059 publications including multiple experiments were assessed for eligibility. From those, 513 studies were excluded because of the following reasons: abstract in English but full article in other language (n = 98); lack of control (n = 155); data published as abstract only (n = 121); data reported as figures (n = 37); incomplete methodology (n = 13); publication was not peer-reviewed (n = 9); error terms were not reported (n = 16); number of observations was not clear (n = 26); treatments could not be defined (n = 38). The complete database consisted of 546 publications from 1979 through 2018, containing $CH_4$ mitigation interventions related to dietary formulation (n = 269 publications) and feed additives supplementation (n = 277 publications). The database is available at The Pennsylvania State University's ScholarSphere repository (https://scholarsphere.psu.edu/resources/fdfe07ea-d631-459c-80c1-ddd9efb3dfc0; Martins et al., 2023a). Given the extent of the database, $CH_4$ mitigation interventions related to dietary formulation were removed, and the current meta-analysis was focused on the in vitro mitigation effects of feed additives only. A list of the 277 publications is provided in Supplemental Table S1

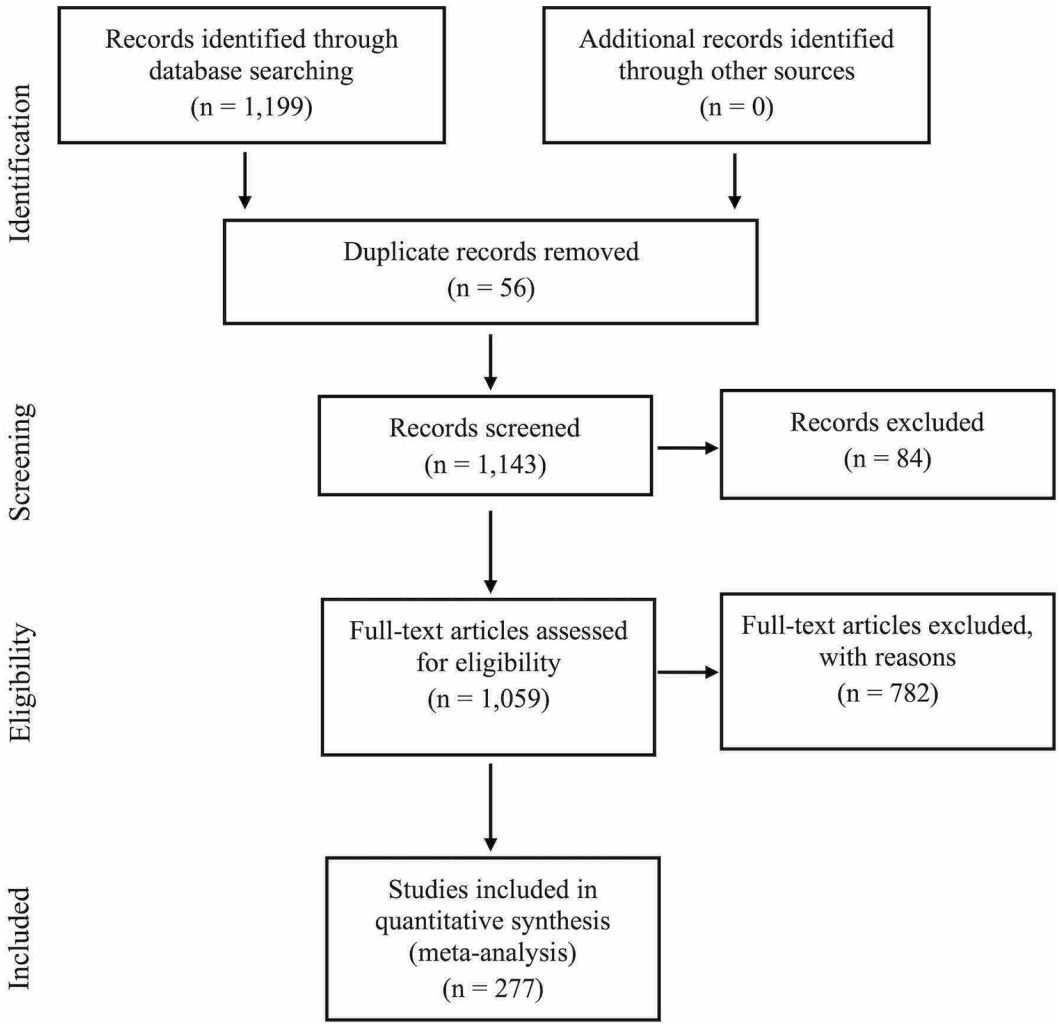


**Figure 1.** Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram adapted from Moher et al. (2009). The flow describes the systematic review from initial search and screening to final selection of publications to be included in the meta-analysis. The 277 articles selected for inclusion in the meta-analysis contained one or multiple experiments.

(https://scholarsphere.psu.edu/resources/89508fb6-e603-41a3-8daa-41aa579449e5; Martins et al., 2023b).

***Data Extraction and Classification of Mitigation Interventions.*** Individual $CH_4$ mitigation interventions were identified and classified into 14 categories of feed additives based on their type, chemical composition, and mode of action, as follows: antibodies, chemical elements, chemical inhibitors, direct-fed microbials, electron sinks, enzymes, essential oils, flavonoids, macroalgae, organic acids, plant extracts, plant inclusion, saponin, and tannins. Plant inclusion, although not necessarily being used as feed additives in the original publications, was included in the current meta-analysis because they were mostly nonconventional forage sources containing different bioactive compounds (e.g., tannins, saponins, and polyphenols), which, could have a potential to modify ruminal fermentation. Response variables considered in this meta-analysis were $CH_4$ production (number of treatment means comparisons = 1,325); total VFA (n = 1,007), acetate (n = 783), propionate (n = 792), and butyrate (n = 776) concentrations; acetate to propionate ratio (**A:P**; n = 675); digestibility of DM (n = 489), OM (n = 277), and NDF (n = 177). Each study was examined individually to assign treatments as control (i.e., baseline condition) and treatment (i.e., an intervention aimed at reducing enteric $CH_4$ emission). In studies where more than one treatment dose was used, estimates (i.e., treatment means) and error terms across doses were averaged and compared with the control mean. Treatment means and error terms in studies with a factorial arrangement (i.e., inclusion of multiple and different treatments in the same study) were analyzed separately (not averaged) and compared with the control.

### Statistical Analysis

Treatment and control means, error terms, and number of observations extracted from each study were compiled in a database using Microsoft Excel (version 16.64; Microsoft Corporation). The error terms coefficients of variation, least significant difference, relative standard deviation, relative standard error (SE), SE, and standard error of the mean were converted to standard deviations using SAS (SAS, v9.4; SAS Institute). Effect-size estimates and corresponding-sampling variances were obtained using the "metafor" (version 4.2–0) and "robumeta" (version 2.1) packages in RStudio (version v2023.03, R Foundation for Statistical Computing), following the methodology detailed in the meta-analysis by Dijkstra et al. (2018). Briefly, the mean difference (**MD**) of the response variables was calculated as the difference between treatment mean and its respective control mean using the "escalc" function in the "metafor" package. The magnitude of response variables and the units of $CH_4$ production reported varied greatly from study to study; therefore, relative MD (MD expressed as a fraction [in %] of observed control mean) was the effect size in further analysis. Relative MD was checked for normality using the "boxplot" function, and extreme values were excluded. Considering that studies included in the current meta-analysis contained multiple treatment groups sharing a common control group, the robust variance estimation (**RVE**) method (Tanner-Smith et al., 2016) was used to analyze statistically dependent effect sizes. Random-effect models were fitted using the "robu" function in the "robumeta" package (Fisher et al., 2023) to estimate between-study variance and heterogeneity statistics. The RVE random-effect model included the effect of mitigation interventions (i.e., categories of feed additives and individual $CH_4$ mitigation interventions within categories) for all response variables, except for $CH_4$ production. An RVE mixed-effect meta-regression model was constructed by including relative total gas production (i.e., treatment total gas production ÷ control total gas production) as an explanatory variable for the effects of mitigation interventions on $CH_4$ production. Estimated effect size for $CH_4$ production obtained from interventions with less than 4 degrees of freedom (df) were omitted from the present study, following the package recommendation (Fisher et al., 2023). Statistical differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

## RESULTS

To facilitate the interpretation and discussion of the data in the current manuscript, only tendencies and statistically significant results (i.e., $P \leq 0.10$) from feed additives decreasing $CH_4$ production by more than 20% were presented. A complete list of individual $CH_4$ mitigation interventions with more than 4 df (n = 170) is available in Supplemental Dataset S1 (https://scholarsphere.psu.edu/resources/89508fb6-e603-41a3-8daa-41aa579449e5; Martins et al., 2023b). A summary of the main statistical parameters for random and mixed-effect models evaluating feed additives categories is described in Table 1. It should be noticed that total gas production was a significant ($P = 0.02$) explanatory variable, and its inclusion clearly changed the estimates for relative MD of $CH_4$ production across mitigation categories, as it can be observed by the discrepancy between estimates presented in Table 1 and Figure 2. Additionally, due to diversity of the data, we observed high heterogeneity (i.e., $I^2$ statistic) and between-study variance (i.e., $\tau^2$) across the models evaluating feed additives in the current study. Forest plots with the relative MD ± SE, number of treatment and control mean comparisons, and *P*-values are presented in Figures 2, 3, 4, and 5 summarizing the effects of the feed additives categories on $CH_4$ production, total VFA concentration, A:P, and DM digestibility. Estimated effect size or 95% confidence intervals (CI) are described in text to help readers better interpret the results, where appropriate.

### Overall Mitigation Effects of Feed Additives Categories

In vitro $CH_4$ production was decreased ($P < 0.02$) by antibodies (average mitigation effect = −39.0%; 95% CI = −71.7 to −6.2%), chemical inhibitors (−29.2%; 95% CI = −48.1 to −10.3%), electron sinks (−19.0%; 95% CI = −25.8 to −12.1%), essential oils (−18.2%; 95% CI = −25.1 to −11.3%), plant extracts (−14.5%; 95% CI = −22.4 to −6.6%), plant inclusion (−11.7%; 95% CI = −16.4 to −7.2%), saponins (−14.8%; 95% CI = −22.5 to −7.0%), and tannins (−14.5%; 95% CI = −19.6 to −9.3%; Figure 2). Chemical elements, direct-fed microbials, enzymes, macroalgae, and organic acid supplementation did not affect $CH_4$ production in the current meta-analysis. Total VFA concentration was increased ($P \leq 0.09$) or not affected by the mitigation categories. Acetate concentration was decreased ($P \leq 0.08$) by most mitigation categories evaluated, except for chemical elements, enzymes, and macroalgae supplementation. It should be noted, however, that reduction in acetate concentration ranged from −1.8% (by direct-fed microbials) to −9.9% (by flavonoids), and that antibodies and chemical inhibitors (i.e., the most efficient $CH_4$ mitigation categories) increased and decreased acetate concentrations by 2.2% and −7.8%, respectively. Propionate concentration was increased

**Table 1.** Number of publications (publ.), number of mean comparisons (n), estimated effect size (mean), SE, heterogeneity ($I^2$), and between-study variance ($\tau^2$) obtained from the models evaluating the effects of mitigation interventions on in vitro $CH_4$ production

| | $CH_4$ production | | | | | | |
|---|---|---|---|---|---|---|---|
| Mitigation intervention | Publ. | n | Mean[1] | SE | *P*-value | $I^2$ | $\tau^2$ |
| Random-effect model, intercept only | 277 | 1,219 | −13.7 | 1.13 | <0.001 | 99.9 | 176 |
| Random-effect model | | | | | | 99.9 | 352 |
| Antibodies | 1 | 8 | 0.5 | 0.01 | <0.001 | | |
| Chemical elements | 8 | 20 | −14.5 | 7.29 | 0.09 | | |
| Chemical inhibitors | 16 | 56 | −30.7 | 9.35 | 0.01 | | |
| Direct-fed microbials | 26 | 151 | −1.2 | 1.99 | 0.56 | | |
| Electron sinks | 37 | 98 | −18.9 | 3.46 | <0.001 | | |
| Enzymes | 12 | 57 | 8.1 | 4.79 | 0.12 | | |
| Essential oils | 37 | 49 | −21.1 | 3.31 | <0.001 | | |
| Flavonoids | 7 | 21 | −9.7 | 5.74 | 0.14 | | |
| Macroalgae | 7 | 102 | −17.8 | 10.46 | 0.14 | | |
| Organic acids | 17 | 39 | −4.4 | 3.92 | 0.28 | | |
| Plant extracts | 27 | 159 | −13.1 | 3.78 | 0.002 | | |
| Plant inclusion | 51 | 187 | −12.6 | 2.19 | <0.001 | | |
| Saponins | 14 | 50 | −14.7 | 3.11 | 0.001 | | |
| Tannins | 51 | 328 | −17.9 | 2.55 | <0.001 | | |
| Final mixed-effect model, 1 explanatory variable[2,3] | | | | | | 99.9 | 409 |
| Total gas production | 277 | 1,325 | 39.6 | 14.97 | 0.02 | | |

[1]Mean represents the estimate effect size of the relative mean difference (%) between control (i.e., baseline condition) and treatment (i.e., a strategy aimed at reducing enteric $CH_4$ emission). Relative mean difference was calculated as follows: relative mean difference, % = [(treatment mean − control mean) ÷ control mean].

[2]A robust variance estimate mixed-effect meta-regression model was constructed by including relative total gas production (i.e., treatment total gas production ÷ control total gas production) as an explanatory variable in the final mixed-effect model for $CH_4$ production.

[3]Final mixed-effect model contained all categories included in the random-effect model. Estimate effect size of the relative mean differences (%) ± SE, number of observations, and *P*-values are described in Figure 2.

by electron sinks (95% CI = 0.1 to 10.8%, $P = 0.05$), organic acids (95% CI = 2.5 to 18.8%, $P = 0.02$), plant inclusion (95% CI = −0.25 to 7.1%, $P = 0.07$), and saponins (95% CI = 1.2 to 13.2%, $P = 0.02$). Tannins, in contrast, decreased propionate concentration by −3.4% (95% CI = −6.8 to 0.03%, $P = 0.05$). Additionally, when considering the most effective $CH_4$ mitigation categories, propionate concentration was increased ($P = 0.02$) by 3.6% by antibodies but was not affected by chemical inhibitor supplementation. Butyrate concentration was increased ($P \leq 0.04$) or not affected by most mitigation categories, except it was decreased by tannins supplementation (95% CI = −8.4 to −0.4%, $P = 0.03$). Chemical inhibitors increased ($P < 0.001$) butyrate concentration by 10.2% (95% CI = 5.9 to 14.5%). Acetate to propionate ratio was decreased by chemical inhibitors (95% CI = −15.5 to −4.5%, $P < 0.001$), direct-fed microbials (95% CI = −13.8 to −0.7%, $P = 0.03$), electron sinks (95% CI = −13.9 to −3.9%, $P < 0.001$), organic acids (95% CI = −29.2 to −8.2%, $P < 0.001$), plant inclusion (95% CI = −9.4 to −2.1%, $P < 0.001$), and saponins (95% CI = −16.2 to −4.5%, $P < 0.001$; Figure 4). Digestibility of DM and OM was not extensively affected, but NDF digestibility was decreased ($P \leq 0.10$; up to −24.3%) by most feed additives included in the current meta-analysis. It should be noted, however, that the number of treatment means comparisons for NDF digestibility was substantially lower (n = 177) compared with other response variables (e.g., n = 489 for DM digestibility; Figure 5), and data should be interpreted with caution.

### *Mitigation Effects of Individual Interventions Within Feed Additives Categories*

Five hundred four individual $CH_4$ mitigation interventions within feed additives categories were identified in the current study, but only 70 decreased in vitro $CH_4$ production by more than 20%. Direct-fed microbials, electron sinks, essential oils, plant extracts, plant inclusion, and tannins were the only categories that contained individual $CH_4$ mitigation interventions producing estimates with 4 or more df in the statistical model. A summary of these variables will be presented in the current section of the manuscript. A complete description of statistically significant $CH_4$ interventions (n = 170) can be accessed in Supplemental Dataset S1.

*Enterococcus* spp. decreased $CH_4$ production by 20.3% and was the only effective individual mitigation intervention identified in the direct-fed microbials category. Total VFA and acetate concentrations were not affected, but propionate was decreased (−30.0%, $P <$

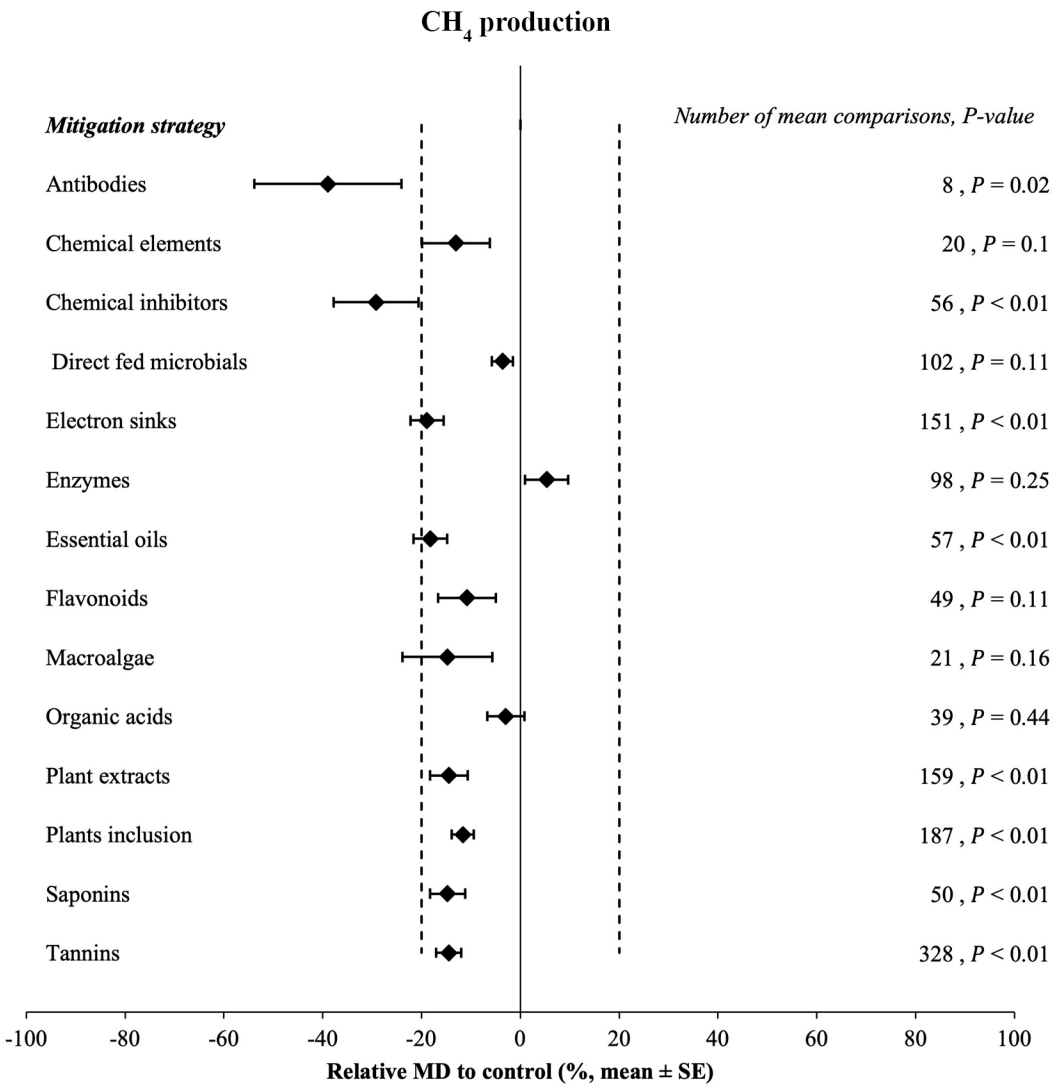


**Figure 2.** Forest plot of the relative mean difference (MD) to control (%, mean ± SE) of methane (CH4) mitigation intervention effects on in vitro CH4 production. The solid vertical line represents a mean difference of zero or no effect. Points to the left of the solid line represent a reduction in CH4 production, whereas points on the right of the line indicate an increase. Dashed vertical lines on the left and right represent, respectively, −20% and +20% effect.

0.001) and butyrate was increased (25.7%, $P = 0.01$) by *Enterococcus* spp. supplementation. Digestibility of DM was slightly decreased (−5.1%, $P = 0.04$), and OM and NDF digestibility were not affected by *Enterococcus* spp. Within the electron sinks category, nitrate, nitroglycerin, and nitrophenol decreased $CH_4$ production by 20.2%, 53.1%, and 27.1%, respectively. Total VFA concentration was decreased ($P < 0.001$) by up to 7.9% with nitrate and nitroglycerin, and A:P was decreased ($P < 0.001$) by 6.9% and 18.8% by nitroglycerin and nitrophenol, respectively. Nitrate decreased ($P < 0.001$) DM digestibility by 8.4%. *Agronis fragrans*, β-cyclodextrin, cinnamaldehyde and garlic, *Leptospermum petersonii*, *Santalum spicatum*, and *Tagetes minuta* essential oils decreased ($P < 0.001$) $CH_4$ production by 24.6%, 75.7%, 54.5%, 50.7%, 26.8%, and 33.1%, respectively. The essential oil β-cyclodextrin increased ($P < 0.001$) total VFA by 11.8% and decreased ($P < 0.001$) A:P by 23.2%, but its effects on digestibility of nutrients were not reported in the studies included in the current meta-analysis. Plant extracts from *Rheum emodi* (i.e., emodin) and *Terminalia chebula* decreased ($P = 0.02$) and tended to decrease ($P = 0.08$) $CH_4$ production by 27.1% and 21.2%, respectively. Emodin also decreased ($P < 0.001$) total VFA concentration by 14.3%. Within the plant inclusion category, 20 individual interventions decreased $CH_4$ production by more than 20% (Supplemental Dataset S1) *Fructus ligustri*

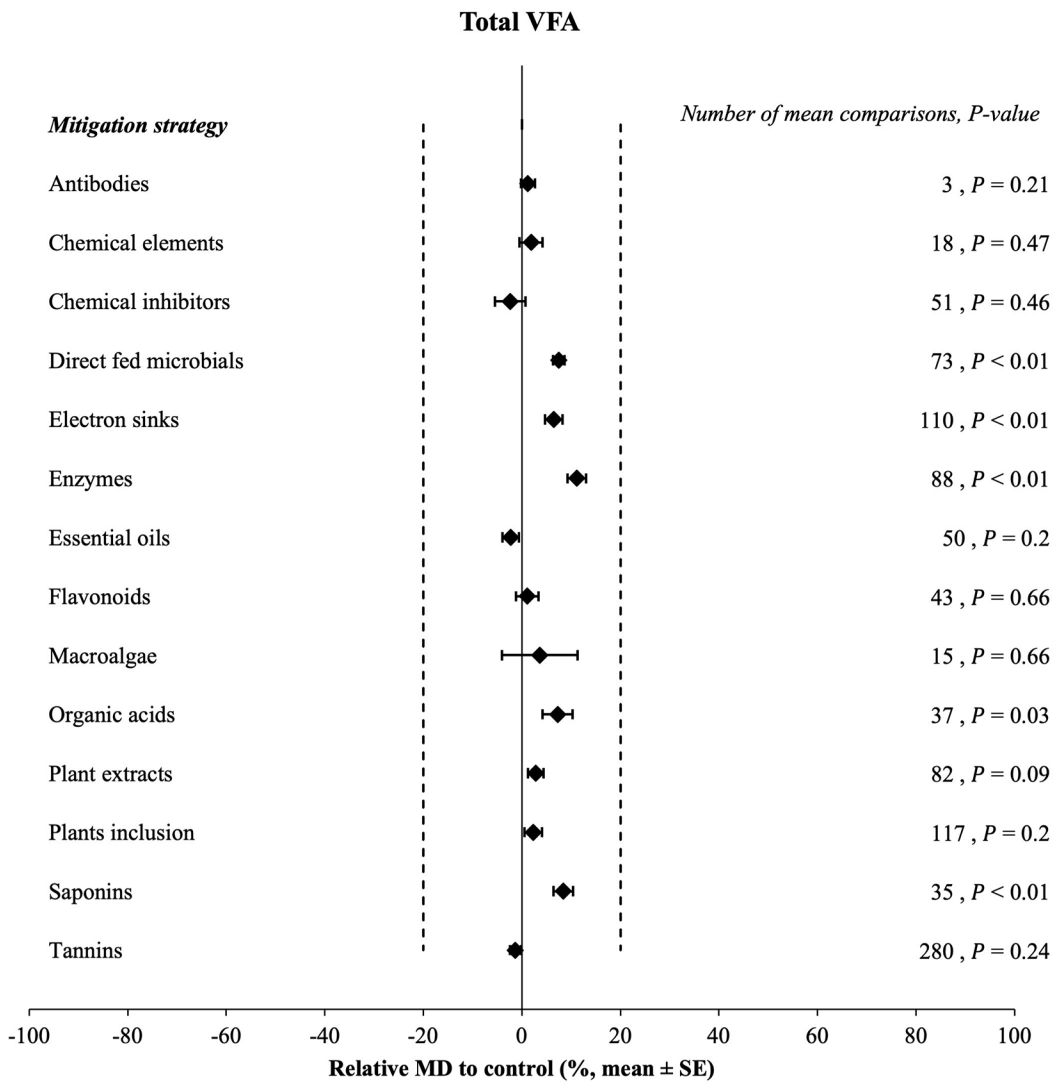


**Figure 3.** Forest plot of the relative mean difference (MD) to control (%, mean ± SE) of methane mitigation–intervention effects on in vitro total VFA concentration. The solid vertical line represents a mean difference of zero or no effect. Points to the left of the solid line represent a reduction in VFA concentration, whereas points on the right of the line indicate an increase. Dashed vertical lines on the left and right represent, respectively, −20% and +20% effect.

(i.e., *Ligustrum lucidum*) decreased ($P < 0.001$) $CH_4$ production by 65.8%, but it also tended to decrease ($P = 0.10$) DM digestibility by 12.8%. The tannins category contained 38 individual mitigation interventions that decreased $CH_4$ production by more than 20% in vitro. Tannins extracted from *Achras zapota*, *Dodonaea angustifolia*, *Mentha citrata*, and *Sesbania grandiflora* decreased $CH_4$ production by over 50%; however, total VFA, VFA profile, and digestibility of nutrients have not been reported for the former interventions in the studies included in the current meta-analysis.

The inclusion of *Caesalpinia sappan* (n = 8), and tannins from *Acacia* spp. extract (n = 30), chestnut (n = 7), ellagitannins (n = 7), *Ficus* spp. extract (n = 6), grape marc (n = 14), *Leucaena* spp. extract (n = 22), mimosin (n = 6), quebracho (n = 8), and sainfoin (n = 56) were the most extensively studied mitigation interventions relative to the number of treatment means comparisons included in the current meta-analysis. These strategies were responsible for decreasing or tended to decrease ($P \leq 0.10$) in vitro $CH_4$ production by 29.4%, 7.6%, 16.1%, 16.4%, 14.0%, 23.5%, 8.7%, 10.8%, and 8.5%, respectively; however, their effects on ruminal overall ruminal fermentation and digestibility of nutrients were not extensively reported by studies in the literature.

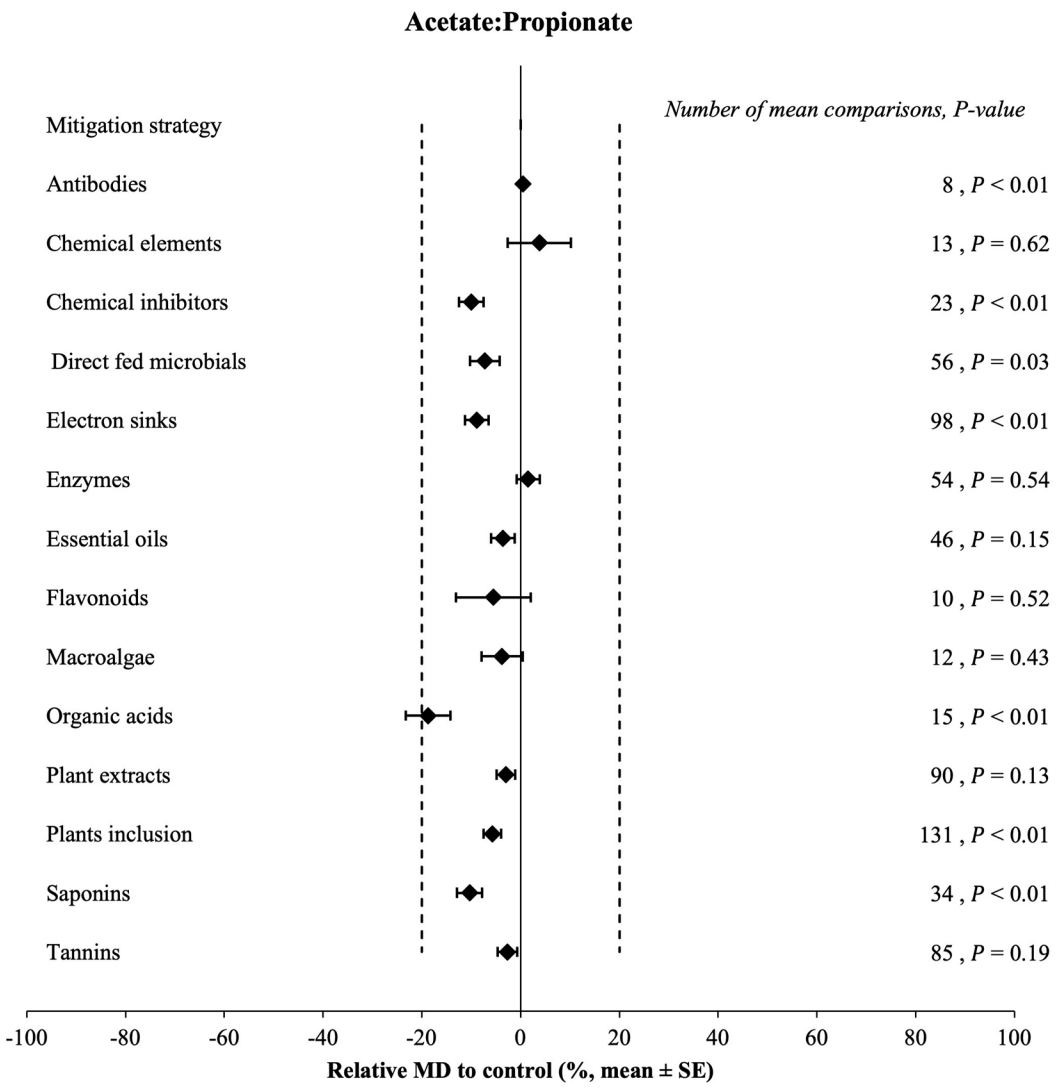


**Figure 4.** Forest plot of the relative mean difference (MD) to control (%, mean ± SE) of methane mitigation–intervention effects on in vitro total acetate-to-propionate ratio (A:P). The solid vertical line represents a mean difference of zero or no effect. Points to the left of the solid line represent a reduction in A:P, whereas points on the right of the line indicate an increase. Dashed vertical lines on the left and right represent, respectively, −20% and +20% effect.

### *Mitigation Effects of Individual Interventions with Limited Data*

As previously indicated, estimated effect size obtained from interventions with less than 4 df were omitted from the present study; however, we believe it is important to highlight the mitigation effect of individual interventions that resulted in a large decrease of $CH_4$ production in vitro, even though their efficacy remain to be confirmed in future studies. In vitro $CH_4$ production was decreased ($P \leq 0.03$) by 51.8% with the supplementation of chemical element magnesium and essential oil extracted from *Heracleum* spp. Similarly, the flavonoids Bioflavex (i.e., extracted from bitter orange and grapefruit), myricetin, and neohesperidin tended to decrease ($P \leq 0.10$) $CH_4$ production by 39.0%, 29.2%, and 34.9%, respectively. The saponins sarsaponin and *Tribulus terrestris* extract decreased $CH_4$ production by 38.1% and 26.5%, respectively, whereas tannins extracted from *Combretum* spp., *Salix caprea*, *Quercus* spp., and *Vitellaria paradoxa* (i.e., sheanut) decreased $CH_4$ production by 51.2%, 23.1%, 40.1%, and 34.9%, respectively. There were not enough data to describe the effects of the above-mentioned individual interventions on ruminal fermentation and nutrient digestibility.

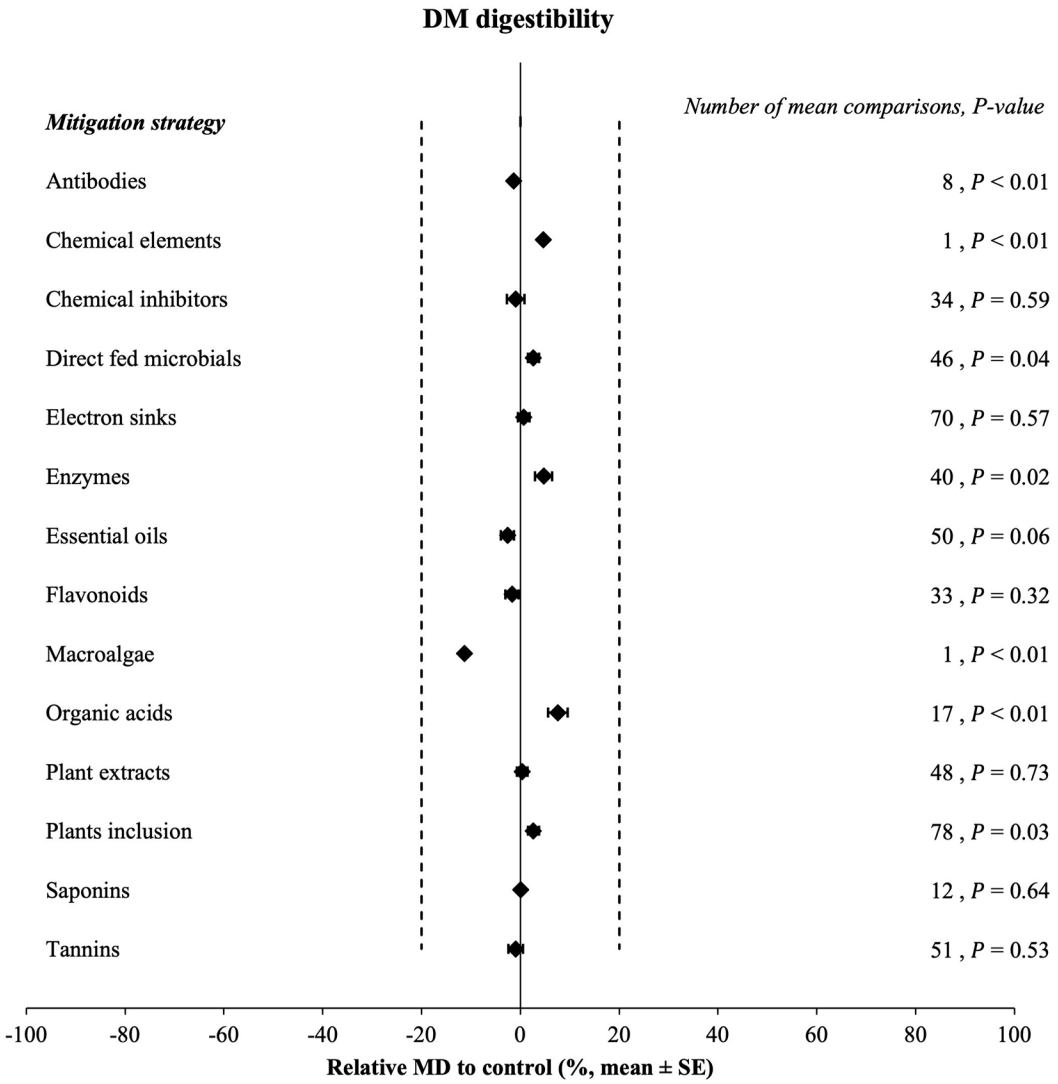


**Figure 5.** Forest plot of the relative mean difference (MD) to control (%, mean ± SE) of methane mitigation–intervention effects on in vitro DM digestibility. The solid vertical line represents a mean difference of zero or no effect. Points to the left of the solid line represent a reduction in DM digestibility, whereas points on the right of the line indicate an increase. Dashed vertical lines on the left and right represent, respectively, −20% and +20% effect.

## DISCUSSION

The data included in the present meta-analysis was generated from all types of in vitro systems (e.g., batch culture and non-rumen simulation technique and rumen simulation technique continuous culture), performed with different types of rumen inoculums (identified by authors as filtered, liquid, liquid and solid, and strained ruminal fluid) collected from different donor species (e.g., sheep, goats, dairy and beef cattle, and buffalos). Donors were also from different breeds, raised in diverse environmental conditions, fed with different diets, and were at different physiological states. The results from this meta-analysis can be considered representative of a broad spectrum of ruminant production systems, and mitigation interventions identified as effective are likely applicable to ruminants fed different diets and managed at different environmental conditions.

Although the above-mentioned variables could interact with $CH_4$ mitigation interventions, the main objective of the current study was to identify potential strategies that have not been extensively evaluated in vivo. Therefore, the interactions between $CH_4$ mitigation interventions and diet composition, animal species, and animal physiological state were not tested and should be addressed in future analyses. The antimethanogenic effect of some feed additives determined in the current analysis, such as the essential oils β-cyclodextrin, cinna-

maldehyde and garlic, *Leptospermum petersonii*, *Santalum spicatum*, and *Tagetes minuta*, are not comparable to effects of essential oils reported in vivo (Hegarty et al., 2021). It is known that in vitro studies tend to use higher doses than what would be practical or safe to the animal (Calsamiglia et al., 2007; Benchaar and Greathead, 2011), and toxic levels for some feed additives have not yet been well stablished in vivo. As an example, the 75.7% decrease in $CH_4$ production by β-cyclodextrin supplementation was obtained from an average of effects ranging from −25% to −97% in a study evaluating 3 doses of supplementation (0.1, 0.2, and 0.4 m*M*; Mohammed et al., 2004). Data reported in Mohammed et al. (2004) supported a high potential of β-cyclodextrin to mitigate $CH_4$ production, but there are no toxicological data for its dietary supplementation in ruminants. A 52-wk toxicity study indicated that the nontoxic effect level for β-cyclodextrin in rats and dogs was 11 and 44 m*M* (i.e., equivalent to 654 to 1967 mg/kg per day), respectively (Bellringer et al., 1995), indicating that 0.4 m*M* (i.e., equivalent to 4.5 g/cow per day for a cow with a rumen volume of 100 L) could be safe to be further evaluated in vivo. In this sense, for strategies where $CH_4$ production was decreased by more than 20% and further evaluations have not yet been conducted, estimates should not be considered as representative of in vivo responses until more studies are conducted and nontoxic levels are well stablished.

From all mitigation interventions evaluated, chemical inhibitors category was the most effective to decrease $CH_4$ production without largely affecting ruminal fermentation and digestibility of nutrients, and with a relatively large number of studies (n = 16). As reviewed by Hristov et al. (2013), studies investigating supplementation of chemical inhibitors suggested a reduction of approximately 50% of enteric $CH_4$ production, with a possible adaptation by the rumen microbes to this class of compounds over time. A successful example of a chemical inhibitor used in vivo is 3-nitrooxypropanol (**3-NOP**; Dijkstra et al., 2018; Melgar et al., 2021; Kebreab et al., 2023), which has a potential to decrease enteric $CH_4$ emissions by up to 30% in dairy cows without negatively affecting, and even improving (i.e., milk fat concentration), animal performance (Hristov et al., 2022). In a recent meta-analysis, Arndt et al. (2022) reported 3-NOP and bromochloromethane to have the largest $CH_4$ mitigation effect in sheep and cattle, with no effect on DMI, fiber digestibility, MY, or weight gain (bromochloromethane data only). Despite the high mitigation effect of the category, individual interventions within chemical inhibitors category (e.g., 3-nitro-1-propionate, 3-NOP, bromochloroacetic acid, and bromoethanesulfonate) did not significantly affect $CH_4$ production. This result could be likely explained by the limited number of studies evaluating chemical inhibitors included in the current database (e.g., maximum of 4 observations for bromoethanesulfonate).

There is a gap in the scientific knowledge regarding long-term efficacy of most $CH_4$ mitigation strategies (Hristov et al., 2022), especially for those related to rumen manipulation. Hristov et al. (2013) reviewed the literature and described up to 50% decrease of enteric $CH_4$ production by electron sinks supplementation, which was comparable to the reduction observed for chemical inhibitors in the same report. Overall, electron sinks supplementation effectively reduced $CH_4$ production in the current analysis, and nitrate, nitroglycerin, and nitrophenol were the most effective individual interventions within the category; however, their negative effects on total VFA concentration and DM digestibility could be detrimental to the in vivo application of these electron sinks. Fumaric acid and nitrate were reported as effective enteric $CH_4$ mitigation strategies in the meta-analysis by Arndt et al. (2022), and nitrate decreased $CH_4$ production by 20% across different species in the meta-analysis by Congio et al. (2021). This result aligns with the 20.2% decreased $CH_4$ production by nitrate supplementation in the current analysis. As reviewed by Hristov et al. (2013), the adaptability of the rumen environment, the potential increase in ammonia production, and the potential toxicity caused from intermediate products of nitrate metabolism are some of the concerns regarding the use of electron sinks in ruminant nutrition. The use of different combinations of electron sinks may increase their mitigation potential, but additive effects of these compounds have not been evaluated in the present study.

Supplementation of direct-fed microbials and enzymes, which are some of the most traditional ruminal fermentation modifiers evaluated in the present study, did not affect in vitro $CH_4$ production when considering the overall category effect. Nevertheless, *Enterococcus* spp., as an individual $CH_4$ mitigation intervention, was one of the most prominent strategies identified in the current analysis. The supplementation *Enterococcus* spp. decreased $CH_4$ production without affecting total VFA concentration and digestibility of nutrients and should be further investigated in vivo. The negative effect of *Enterococcus* spp. supplementation on propionate concentration, however, should be addressed in future studies. It is important to note that the supplementation of direct-fed microbials and exogenous enzymes, although not effective in reducing $CH_4$ production in the current study, could contribute to decreasing the intensity of $CH_4$ emissions by increasing feed efficiency, animal productivity, and nutrient digestibility. For example, enhanced animal performance with yeast products supplementation has been reported across ru-

minant species (Desnoyers et al., 2009), including dairy cows (Poppy et al., 2012).

Plant secondary compounds are generally classified into saponins, tannins, and essential oils categories (Calsamiglia et al., 2007), and they have been extensively investigated as rumen modifiers in vitro and in vivo (Benchaar and Greathead, 2011; Cobellis et al., 2016; Honan et al., 2021). Tannins and saponins are known to have antinutritional effects, which can be especially problematic when dietary protein is limiting animal production. The supplementation of tanniferous forages to diets containing adequate levels of nutrients, in contrast, decreased enteric $CH_4$ production by 12% without affecting milk yield or weight gain, even though DM digestibility was decreased by 12% in the meta-analysis by Arndt et al. (2022). Supplementation of different sources of tannins decreased enteric $CH_4$ yield by up to 27%, but also dramatically decreased (i.e., 51% reduction by *Leucaena* spp.) DMI in the meta-analysis by Congio et al. (2021). Tannins can also decrease nutrient digestibility, which also corroborated with the slightly decreased OM digestibility (−5.7%) data presented in the current study.

The relatively low mitigation efficacy of most plant secondary compounds described in the current study corroborates with in vivo and other in vitro analyses (Hristov et al., 2013; Hegarty et al., 2021). It should be noted, however, that many of these compounds have not yet been extensively studied in vivo, and they might have additional physiological effects other than modifying ruminal fermentation. Most essential oils have a broad spectrum of activities, in some cases negatively affecting overall ruminal fermentation, and may also interact with dietary composition and animal metabolism. For example, Silvestre et al. (2022) reported up to 7.5% reduction in enteric $CH_4$ production in dairy cows supplemented with increasing doses of a combination of *Capsicum* oleoresin and clove oil, and a quadratic effect of the same blend on blood β-hydroxybutyrate concentrations. Overall, plant secondary metabolites are expected to produce less than 10% reduction of enteric $CH_4$ emission (Hegarty et al., 2021). Future research should attempt to evaluate different combinations of compounds and their interactions with diet and animal metabolism, as well as the additive effect of combining plant secondary compounds with other rumen manipulation strategies.

## CONCLUSIONS

The meta-analysis characterized the in vitro effects of 170 $CH_4$ mitigation interventions with different efficacies. However, many of these interventions had estimated effect sizes based on less than 5 observations due to limited available data. The most effective category was chemical inhibitors, decreasing in vitro $CH_4$ production by 29.2% without negatively affecting ruminal fermentation and digestibility of nutrients. Among individual interventions within categories, Enterococcus spp. (i.e., direct-fed microbials) and nitrophenol (i.e., electron sinks) decreased $CH_4$ production by 20% and 27.1%, respectively, with minimal impact on ruminal fermentation. Plant secondary compound categories (e.g., essential oils, plant extracts, plant inclusion, saponins, and tannins) decreased $CH_4$ production by up to 18.2%. Notably, tannin extracted from *Leucaena* spp. decreased in ivtro CH4 production by , 23.5% without extensively affecting ruminal fermentation and nutrient digestibility. Number of observations used for statistical analysis, lack of data regarding ruminal fermentation, and nutrient digestibility reported in the current analysis (see Supplemental Dataset S1), and overestimation of effect of treatments due to high doses should be considered before selecting individual mitigation interventions to be tested in vivo.

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