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In vitro comparison of naturally bioactive plant extracts, essential oils, and marine algae targeting different modes of action for mitigation of enteric methane emissions in ruminants

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Feed additives to reduce enteric methane (CH_4) emissions from ruminants are gaining attention to help curb agriculture's 24% share of global CH_4 emissions. Several mechanisms of action of feed additives for mitigating rumen methanogenesis have been identified from ongoing research, however, there is still a need to determine the most effective method and explore potential synergies between these different approaches. This study evaluates the CH_4 mitigation potential of nine natural feed additives, focusing on their mode of action in reducing CH_4 emissions during *in vitro* fermentation. The natural feed additives assessed include garlic oil (GO), garlic powder (GP), allicin (ALL), *yucca schidigera* plant extract (Yucca), and an essential oil blend (EO), all functioning as rumen microbiome modifiers. Calcareous marine algae rumen buffer (CMA) and its magnesium oxide-fortified form (CMA.MgO) acted as hydrogen sinks, while *Asparagopsis taxiformis* (cultured and ocean forms) inhibited the central enzyme involved in methane metabolism. Total gas, CH_4, and volatile fatty acid (VFA) outputs were recorded after *in vitro* batch fermentations simulating rumen 24 h metabolic events. The CMA.MgO rumen buffer displayed significant reductions ($P=0.02$) in both CH_4 emissions relative to the control (Rel % CH_4; 40.1%) and total gas production relative to the control (Rel % Total gas; 22.9%). The greatest synergistic effect on gas emissions was achieved by combining GP with the CMA buffer treatment, leading to significant reductions ($P<0.05$) in Rel % Total gas by 37% and Rel % CH_4 by 64.5%. Additionally, sole supplementation of CMA.MgO, GO, and Yucca demonstrated improved rumen productivity by increasing total VFAs by 39.8%, 24.4%, and 22.6% compared to the control result, respectively. Feed additives altering rumen microbial populations by reducing methanogens and promoting VFA production increase readily available energy for the animal

while reducing CH_4 generation significantly. Semi-continuous rumen culture fermentations or *in vivo* studies can confirm the long-term stability of synergistic antimethanogenic mechanisms, potentially optimizing CH_4 inhibitors like *A. taxi* and EO for commercial use.

KEYWORDS

CMA, essential oils, asparagopsis, methane reduction, enzyme inhibitor, microbiome modifying, buffering, Ankom

1 Introduction

There is considerable interest in identifying natural bioactive compounds that have the potential to manipulate the rumen microbiome and fermentation processes. Manipulation of the rumen environment has the potential to protect animals against prevalent diseases, improve energy by increasing volatile fatty acids (VFAs) availability (Huang et al., 2021), increase product yield by elevating organic matter digestibility (Campanile et al., 2008), and minimize the production of enteric methane (CH_4) (Tong et al., 2020; Wasson et al., 2023). Mitigation strategies implemented to reduce the increase in enteric gases, especially CH_4, include dietary supplementation, animal management, and rumen manipulation. This study was aimed at investigating dietary supplementation approaches to support ruminant productivity and mitigate enteric CH_4 in line with emission reduction targets (Andersen et al., 2005; Masson-Delmotte et al., 2018).

Commercially available feed additive products claiming to mitigate enteric CH_4 emissions include red seaweed *Asparagopsis taxiformis* (Lome^{TM}, Solna, Sweden) and 3-Nitroooxypropanol (3-NOP), marketed as *Bovaer* (DSM, Kaiseraugst, Switzerland). Both additives work by inhibiting the central methyl-coenzyme M reductase (MCR) enzyme in the methanogenesis pathway, effectively inhibiting CH_4 production. Methanogenesis inhibitors provided as feed additives to ruminants have been the best performers thus far, with the highly bioactive seaweeds from the *Asparagopsis* genus demonstrating the greatest antimethanogenic potential (Roque et al., 2019, 2021). Recent reviews by Hristov (2024) and Arndt et al. (2022) summarize the CH_4 mitigation potential of enzyme inhibitors exceeds 28% in daily CH_4 emission yield and intensity. Despite the significant reduction in enteric CH_4 achieved with both enzyme inhibiting additives, questions remain regarding their consistency and the potential for rumen ecosystem adaptation (Hristov, 2024). This underscores the need for continued research into alternative feed additive CH_4 mitigating strategies and the exploration of ruminal adaptation to commercial feed additives during extended supplementation periods.

An essential oil blend of coriander (*Coriandrum sativum*) seed oil (up to 10%), eugenol (up to 7%), geranyl acetate (up to 7%), and geraniol (up to 6%) (Agolin SA, Bière, Switzerland), as well as a mix of garlic and citrus extract (Mootral SA, Rolle, Switzerland) are both labeled as antimicrobial agents in the rumen against methanogens. Essential oils demonstrate initial antibacterial activity by inhibiting the growth of gram-positive ruminal microorganisms and antiprotozoal activity (Patra and Yu, 2012), thus, reducing CH_4 emissions from dairy cattle (McIntosh et al., 2003). In recent reviews investigating effective strategies for mitigating enteric CH_4 emissions, oil and lipid supplementation displayed an average decrease of 12.9% in CH_4 intensity for dairy and beef cattle (Arndt et al., 2022; Hristov, 2024). Furthermore, in a literature review by Honan et al. (2022), the garlic and citrus extract mix demonstrated greater average CH_4 reduction potential in beef and dairy cattle studies compared to the commercial essential oil blend at an increased average dose rate of 1.14 g/kg DM compared to 0.07 g/kg DM, respectively. However, evidence of rumen adaptation to the supplementation of the garlic and citrus extracts is evident in trials by Klop et al. (2017) and Hart et al. (2019) that lasted 10 and 23 weeks, respectively. Rotation of rumen microbial modifying essential oils (EO) and lauric acid supplementation to lactating dairy cows on a weekly basis showed no improvement in CH_4 mitigation in contrast to sole supplementation of EO over a 10-week period (Klop et al., 2017). Alternatively, rotation of rumen feed additives with different modes of action for CH_4 mitigation may reduce the occurrence of rumen adaptation and possibly induce greater CH_4 mitigation compared to individual feed additive supplementation. Rotational supplementation of feed additives targeting different CH_4 production pathways may allow for the overlap in their antimethanogenic effects, preventing microbial adaption and maintaining the treatments effectiveness. Therefore, implementing a similar 2-week or weekly supplementation rotation structure, as described by Guan et al. (2006) and Klop et al. (2017) respectively, may benefit from rotating feed additives with different modes of antimethanogenic action. Antimicrobial rumen modifiers supplemented as mixtures to the rumen appear to induce enteric CH_4 mitigation without impacting ruminant feeding and productivity behavior (Khurana et al., 2023; Miller et al., 2023), suggesting the opportunity to apply further feed supplementation with an alternative CH_4 mitigating approach to explore possible synergistic effects.

Additionally, feed grade calcium nitrate (Bolifor, York, United Kingdom) is implemented as an additional hydrogen sink in the rumen to divert free hydrogen away from methanogenesis. Calcium

nitrate has previously displayed a linear decrease in CH_4 emissions with increasing inclusion rates (Olijhoek et al., 2016). Hydrogen sinks that act on rumen conditions to demote methanogenesis display an average decrease of 15% in CH_4 yield in beef and dairy animals as seen in the meta-analysis of CH_4 mitigation strategies by Arndt et al. (2022). Nitrate appears to be the most effective electron sink treatment for mitigating enteric methane without transient effect over long-term studies (Feng et al., 2020; Van Zijderveld et al., 2011). However, adaptation periods and gradual build-up of nitrate dietary inclusion are required to reduce the risk of nitrite build-up in the rumen and absorption into the bloodstream resulting in the development of methemoglobinemia (Nolan et al., 2016). Alternative rumen hydrogen-sequestering approaches, such as calcareous marine algae (CMA), have demonstrated hydrogen ion neutralization and stability within the rumen physiology, as reported in previous studies (Neville et al., 2019; Rossi et al., 2019). Therefore, CMA may serve as an effective alternative to nitrate-based feed additives for methanogenesis inhibition. This study follows on from previous *in vitro* research by Durmic et al. (2014), where a moderate reduction in methane production was found with calcareous marine algae supplementation.

To identify natural bioactive compounds with the most effective mode of action for enhancing CH_4 mitigation and ruminant productivity, a high throughput *in vitro* batch culture method for simulating rumen enteric fermentation products was assembled. This method was adapted from existing methodologies in the literature to screen CH_4 mitigating compounds, categorized by their mechanisms of action, for use in ruminant feed supplementation (Brooke et al., 2020; Sarker et al., 2018). The feed supplements were selected for further *in vitro* investigation based on a meta-analysis originating from literature-based candidates with CH_4-mitigating potential by Hodge et al. (2024) and existing literature. The selected feed additives underwent *in vitro* screening to collect fermentation gas levels and profiles of the primary VFAs, acetate (or acetic acid), propionate (or propanoic acid), and butyrate (or butyric acid), through corresponding fermentation runs. This method was employed to facilitate direct comparisons and evaluate the effectiveness of each CH_4 reduction strategy across various representative treatments.

2 Materials and methods

2.1 Donor animals and rumen fluid collection

All procedures described in this experiment were approved by the Animal Welfare Body (AWB) at Munster Technological University (MTU), Kerry, Ireland. It was concluded that the animals in this project were slaughtered for another purpose before sampling. As the animals were not alive at the point of scientific sampling, there was no requirement for a Health Products Regulatory Authority (HPRA) licence, nor does it come under the remit of the AWB. A certificate of registration was obtained from the Department of Agriculture, Food and the Marine (DAFM) which authorized the transport and use of cattle rumen fluid obtained from ABP in accordance with Regulation (EC) No. 1069 of 2009 and Regulation (EU) No. 142 of 2011, Registration No. HAC2426. Procedures related to the care and handling of the experimental animals were conducted under protocols approved by the ABP food group quality team. Prior to every *in vitro* run, all glassware was autoclaved to achieve steam sterilization. Rumen fluid samples were collected from different areas of the rumen within Friesian and Friesian cross animals. The donor animals with a mean age of 4.3 ± 0.5 years and mean hot weight of 318.1 ± 21.0 kg were managed in ABP Food Group, Bandon and the rumen fluid was obtained within 2 minutes of each donor animal's time of death. The rumen digesta was strained over two stainless steel sieves with a pore aperture of 1.18 mm and 600 µm and the fluid was directly funneled into a sterilized 1.2 L thermos flask for insulated transportation. The thermos flask was filled 1 inch from the top of the flask and purged with CO_2 for 20 seconds before being tightly sealed in an upright position for transportation to the fermentation lab set-up, preventing spills and maintaining the CO_2 headspace. A second sterilized thermos flask was used to collect filtered rumen fluid through two layers of cheesecloth, removing larger particles of rumen digesta. The pH and temperature of the rumen fluid were recorded upon arrival at the laboratory after each collection. The headspace of the thermos flask was again purged with CO_2 for 20 seconds and sealed for relocation to a vertical laminar flow hood to prevent contamination of fermentation vessel contents during filling.

2.2 Experimental design

Following collection, rumen fluid was diluted (1:2 v/v) with pre-heated (39°C) artificial saliva buffer (pH 8 ± 0.015) (Cone, 1998) to closely resemble realistic ruminal pH conditions in the preheated septa port glass bottles over the 24 h fermentation period (see Supplementary Figure S1A). Fresh forage was added to each 250 mL bottle contents at 1.15 g dry matter (DM) dosage simulating the Bord Bia grass-fed standard conformance by ABP food group, Bandon (Bord Bia, 2020) and the average dry matter intake of 16.7± 0.25 kg of DM/d investigated for grazing cattle (Coleman et al., 2010). The fresh material was 3-leaf stage perennial ryegrass collected fresh on the morning of each *in vitro* incubation. Composition analysis of the grass diet was carried out by a forage analysis laboratory (FBA Laboratories, Waterford, Ireland) for wet chemistry-based analysis (Table 1). The procedures used in the wet-chemistry analysis of the forage portion used in this experiment is detailed in the next section (section 2.3).

Fresh forage was sealed in Ankom 5cm x 5cm 50-micron porosity feed bags (ANKOM Technology Ltd., New York, United States) and submerged in the buffered rumen fluid vessel contents to allow for gradual digestion within the fermentation units (see Supplementary Figure S1A). Treatments assigned to the 250 mL vessels included the following: rumen microbiome modifying *Allium sativum* derived garlic oil (GO), garlic powder (GP) and the plants bioactive compound allicin (ALL) (Axenic health

TABLE 1 Composition of perennial ryegrass fed to *in vitro* fermentation vessels at the beginning of each 24 h fermentation run.

Composition (% DM)	Perennial ryegrass
DM%	18.6
CP% *	28.4
NDF% *	47.8
ADF% *	25.2
OMD% *	82.5
Ash% *	10.8
WSC% *	5.9
Ether Extract (Oil)% *	4.0
Crude Fibre% *	20.0
DMD%	73.6%

DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; OMD, organic matter digestibility; WSC, water soluble carbohydrates; DMD, dry matter digestibility *–Results on dry matter basis.

solutions, Texas, US), *Yucca schidigera* plant extract (Yucca) (Agroin, Baja California, México), and an essential oil blend (EO) (Agolin SA, Bière, Switzerland), hydrogen-sequestering rumen buffers CMA (*Lithothamnion glaciale)* and the algae's magnesium oxide-fortified form (CMA.MgO) (Celtic Sea Minerals, Cork, Ireland), and the MCR inhibitor *Asparagopsis taxiformis*, in both its cultured form (*A. taxi* cultured) and its ocean form (*A. taxi* ocean) (Lome^{TM}, Solna, Sweden).The screening period included a total of 15 treatment variations and combinations that were investigated in at least 3 biological replicates. All individual biological replicates are the average of duplicate technical replicates for 24 h *in vitro* incubation. Optimum doses for both the marine based rumen buffer (CMA) and the essential oil (GO) were determined for combination treatment investigations based on dose response results and suitable doses for on-farm applications. Once the vessel filling was completed an airtight seal was established with the numbered ANKOM RF modules (ANKOM Technology Ltd., New York, United States). The sealed 250 ml bottles were incubated in a temperature-controlled orbital shaking water bath with pre-set parameters of 39°C and 85 rpm (see Supplementary Figure S1B). The current *in vitro* setup was adapted for optimum fermentation conditions based on previous *in vitro* methodologies (Brooke et al., 2020; Sarker et al., 2018), and to simulate rumen temperature conditions, rumination and, animal activity (Abeni and Galli, 2017). Initially, 99.9% CO_2 was flushed through the headspace of each fermentation bottle and evacuated through the septa port for 10 seconds. The septa ports were then sealed before being purged with 99.9% CO_2 to allow for a build-up of 5 pounds per square inch of CO_2 within the headspace of each sealed bottle. The CO_2 gas was held within the headspace for 30 minutes while incubated and in orbital motion to allow for absorption of the CO_2 gas within the rumen fluid inoculum. Venting valves for each of the modules were opened in unison to allow for a controlled release of the CO_2 purge gas. The valves were then sealed after purge gas evacuation to allow for the collection and recording of enteric gas. Headspace gas readings are detected via radio frequency technology which is transmitted to a base coordinator and recorded on ANKOM software as raw data in an Excel file. Total gas production was measured continuously for a maximum of 24 h with pressure readings for each module being recorded in 10-minute intervals. Enteric gas values expressed in mL/g DM were determined by application of the natural gas law to the final gas pressures while accounting for individual vessel headspace volumes.

2.3 Feed sample analysis

Subsamples of the fresh perennial ryegrass were dried and ground for wet chemistry analysis. The gravimetric method with sample drying in a Genlab fan oven at 103°C for 4 hours was used to determine DM by difference in initial weight vs final weight. Crude protein was determined using a LECO ®928 series carbon and nitrogen determinator instrument (LECO Instruments UK Ltd., Stockport, United Kingdom). Ether extract (oil) was extracted using a Gerhardt Soxtherm Variostat extraction system (Gerhardt UK Ltd., Northamptonshire, United Kingdom) by weighing sample into cellulose thimbles and subjected to an automated extraction in petroleum ether. Petroleum ether was selected over alternative solvents such as diethyl ether for lipid extraction due to its greater selectivity for more nonpolar lipids. The percentage crude fibre was determined by digesting the silage sample in 0.26N H_2SO_4 and 0.31N NaOH solutions under specific conditions. Subsequently, the digested sample was dried in a Genlab fan oven. The crude fibre content was then determined by measuring the loss of ignition of the remaining dried residue in a Carbolite muffle furnace (Carbolite Gero Ltd., Hope, United Kingdom) kept at 500°C for at least 4 hours. Acid detergent fibre (ADF) was reported using the filter bag technique in an Ankom 220 Fibre Analyser digestion instrument using acid detergent solution (20 g cetyltrimethylammonium bromide (CTAB) to 1 L of 1N H_2SO_4 previously standardized) (Ankom Technology, New York, United States). The neutral detergent fibre (NDF) procedure was carried out using the filter bag technique in an Ankom 220 Fibre Analyser digestion instrument treated with neutral detergent solution with an average pH value of 7.0 ± 0.1. The neutral detergent solution contained 30 g of Sodium dodecyl sulfate (USP), 18.61 g of Ethylenediaminetetraacetic disodium salt (dehydrate), 6.81 g of Sodium borate, 4.56 g of Sodium phosphate dibasic (anhydrous), and 10.0 ml of Triethylene glycol in 1 L of distilled H_2O. To determine the digestible organic matter percentage (OMD%), samples were placed into an Ankom Daisy Incubator digestion jar along with cellulase and acetate buffer solution. The digestion was run for 48 hours at 39°C, followed by removing the sample bags, washing with water, and subject to NDF digestion as discussed above. Ash was determined with the loss on ignition method using a Carbolite muffle furnace at 500°C. Water soluble carbohydrates (WSC) were determined by phenol-sulfuric acid sample preparation followed by UV spectrophotometry using a Genesys 150 UV-Visible Spectrophotometer (Genesys, California, United States) and 10 mm cuvettes. Absorbance was measured at 485

nm as it corresponds to the distinct absorption peak of the phenol-sulfuric acid complex.

2.4 Determination of methane and VFA production

In vitro end products (enteric gas and volatile fatty acids) collected post-24-hour incubations were analzed via gas chromatography. Headspace gas was collected using 12 mL disposable syringes with removable needles via the septa port of each 250 mL bottle after 24 h incubation (see Supplementary Figure S1A). A total of 12 mL was extracted from each headspace and dispensed into separate 12 mL pre-evacuated glass vials with double-wadded PTFE/silicone septa (Labco Ltd., Lampeter, United Kingdom). The vials were labeled with the date, headspace pressure in psi, treatment added, and module head number for identification. Gas chromatography (GC) (Shimadzu GC-2010, Shimadzu Corporation, Kyoto, Japan) equipped with a 30 m x 0.53 mm Carboxen 1010 capillary column with a flame ionization detector (FID) was used to determine the amount of CH_4 in each of the samples. 150 µL samples were manually injected into the column. The oven temperature was held at a 75°C iso thermic temperature. The FID was held at 270°C and the injector was set to 200°C. Nitrogen was used as a carrier gas with a total flow rate of 21.0 mL/min and a column flow of 3 mL/min. The average DM supplemented (1.15 g) to fermentation vessels throughout the *in vitro* treatment screening period was used to standardize gas concentrations. Accumulated control total gas and CH_4 values (mL) achieved were set to 100% and the other treatment values were presented as a percentage of total gas and CH_4 relative to the untreated control in each 24-hour run (Tables 2, 3). The control in each run was set as the benchmark 24 h gas accumulation to ensure comparability between novel batches of rumen fluid each week. Standard calibration curves were generated using standards prepared manually using Nitrogen and CH_4 certified by Air Products (Air Products Plc, Cheshire, England) (see Supplementary Figure 2).

Seven treatments that exhibited the greatest CH_4 mitigation capabilities which were inoculated at doses feasible for on-farm supplementation were analyzed for VFA profiles. Volatile fatty acid sample deproteinization was performed by adding 200 µL of 25% metaphosphoric acid and formic acid (3:1) mixture to 1 mL of rumen fluid from each fermentation vessel post-24-hour incubation, based on a method adapted from Poblete et al. (2020). The mentioned ratio and solutions were selected based on the ability for 25% metaphosphoric acid to precipitate the proteins and the prevention of ghost peaks appearing on the chromatogram by using formic acid (Cottyn and Boucque, 1968). Samples were allowed to sit for 30 minutes before being centrifuged twice for 30 minutes at 3900 rpm in a benchtop centrifuge (Centrifuge 5810, Eppendorf) to separate the precipitated protein in the clear supernatant from the particulate debris pellet. Each supernatant (1 mL) was stored in 1.5 mL Eppendorf tubes at 4°C until GC analysis. The samples were diluted 10-fold with 3:1 methanol to deionized water in 1.5 mL chromatography vials to prevent the smearing phenomenon in chromatographs described by Luo et al. (2015). After dilution, the samples were injected from 1.5 mL chromatography vials (1.0 µL quantities, in split mode with a ratio of 5.0) via an AOC-20i autosampler into a Shimadzu GC-2010 gas chromatography unit equipped with a capillary column (SH-Stabilwax-DA, 30 m x 0.53 mmID x 0.25 µm, Shimadzu) with nitrogen as the carrier gas. The GC set-up was adapted from Luo et al. (2015), the flow rate was 37.4 mL/min, column temperature was held at 100°C for 2 minutes, increased at a rate of 8°C/min to 164°C and held for 1 minute, followed by an increase in temperature by 20°C/min to 200°C and held for 1 minute. The temperatures for the injector and the FID were held at 280°C and the cycle time for each analysis was 21 minutes. Total VFA concentrations are measured in mmol VFA/L of rumen fluid incubated and the individual acid concentrations are recorded in percentage mmol/mmol total VFA. An external standard column test mixture of free fatty acids (Thames Restek Ltd., Wycombe, United Kingdom) was prepared before the beginning of the sample analysis. The standard mix underwent a ten-fold dilution in a 3:1 solution of methanol and deionized water and was analyzed to achieve a good correlation between the measured response and concentrations of each acid ($R^2 \geq 0.99$) (see Supplementary Figures 3–8). The acids included acetic, propanoic, isobutyric, butyric, isovaleric, and valeric acid which are commonly found in the rumen (Lee et al., 2021; Luo et al., 2015).

2.5 Statistical analysis

Enteric gas expressed as a percentage of the control vials, and VFA level changes were analyzed using the repeated measures analysis of variance using mixed models via Addinsoft XLSTAT-Basic+ software (version 2021.2.2). The model included the fixed effect of treatment, and the random effect of the rumen fluid inoculum used in each fermentation run to determine the presence of significant influence induced by both fixed and random effects. Significance of fixed effects was determined using Fisher's F-test with consideration for the random effect of rumen fluid variation (via orthogonal decomposition) to enable the study of the unbalanced dataset. The Tukey honestly significant difference (HSD) test was used to test differences between sample means for significance. For non-parametric enteric gas production data (milliliters of gas per 1.15 g DM incubated), the Kruskal-Wallis test was used for comparison of k samples. Dunn's *post-hoc* test for multiple comparisons was used between each independent treatment to identify which treatments were significantly different. Differences were declared significant at $P < 0.05$.

3 Results

3.1 *In vitro* gas production

Initially the treatments were categorized based on their mechanism of action and analyzed for their corresponding gas production response, as shown in Table 2. The results indicate

significant reduction potential in CH_4 emissions for hydrogen sequestering (−23.6%; P=0.01), enzyme inhibiting (−20.6%; P=0.03), and rumen microbiome modifying additives (−29.6%; P<0.01) compared to the control values (Rel % CH_4). Additionally, hydrogen sequestering (−12%) and enzyme inhibiting (−11%) categories showed significant reductions (P<0.03) in total gas production relative to the control values (Rel % Total gas), while a trend towards significance (−8.5%) was observed for rumen microbiome modifying additives. Subsequently, the investigation into each treatment's optimal dose–response on *in vitro* gas emissions are presented in Table 3 and discussed under the related subheadings of this section. Dose-response curves for the hydrogen-sequestering CMA treatment and rumen microbiome-modifying GO treatment are shown in Figures 1, 2, respectively.

3.1.1 Rumen microbiome modifying bioactives

Maintenance of Rel % Total gas production and significant reductions in Rel % CH_4 with GO at its optimum dose depicted from literature (42.2 g/kg DM), facilitated the dose response investigation seen in Figure 2. The lower dose of GO (14.3 g/kg DM) was identified as a more practical level of GO for on-farm supplementation based on gas production results (Table 3, Figure 2) and was therefore the selected dose for VFA analysis, as discussed in later sections. Supplementation with GO resulted in reduction in total gas compared to the control equating to 9.2, 15.1, and 13.7% when the *in vitro* administration dosage was increased in increments of 14.3, 42.2, and 128 g/kg DM (Figure 2), respectively. There was a trend between GO dosage and significant reduction (P < 0.04) in Rel % CH_4 of 36.9, 56.7, and 59.3% as the concentration of GO was increased to 14.3, 42.2, and 128 g/kg DM, respectively (Figure 2). The plant active component ALL was investigated at a concentration of 7.2 g/kg DM, to simulate the natural amount found in the high dose of garlic oil (128 g/kg DM) extracted from *Allium sativum* (Busquet et al., 2005; Rahman, 2007). The plant component induced a reduction of 17.8 in Rel % total gas and 30.8 in Rel % CH_4 production compared to control values (Table 3). The essential oil blend (EO) when supplemented at 0.14 g/kg DM, demonstrated a decrease of 1.4% accumulated total gas from the control value and induced an increase of 1% in Rel % CH_4 to the control across the four biological replicate *in vitro* runs (Table 3). Yucca plus powder derived from the *Yucca schidigera* plant was supplemented at a singular dose of 1.14 g/kg DM. The yucca plant supplementation was the only investigated treatment that resulted in increased Rel % Total gas production (0.8%). However, the associated percentage CH_4 amount (Rel % CH_4) was reduced by 28.2% when Yucca was supplemented over 24 hours.

3.1.2 Hydrogen sequestering bioactives

The selected optimum dose of CMA at 10 g/kg DM maintained Rel % Total gas (89.5%) and induced a 24.7% reduction in Rel % CH_4 (Table 3) leading to the dose response graphed in Figure 1. The CMA induced a decrease of 2.1% in Rel % Total gas production when supplemented at the lower dose of 5 g/kg DM. Total gas production declined with respect to the control value as CMA dosage was increased from 5 to 10 g/kg DM, producing an 8.4% reduction (Figure 1). Conversely, Inclusion of 15 g/kg DM CMA elicited a total gas decline of 4.3%. The CMA middle dose (10 g/kg DM) caused the greatest drop of 24.7% CH_4 compared to the control (Figure 1). A 13% decrease in CH_4 in relation to the control value was identified after 15 g/kg DM supplementation of CMA, surpassing the recommended dose for rumen buffering (5 g/kg DM) with five times its CH_4 reduction compared to the control (2.5%).

The supplemented CMA.MgO is a magnesium ion-fortified calcareous marine algae that was investigated for rumen productivity and CH_4 mitigation in five separate rumen fluid runs. Average total gas volumes after supplementation of 10 g/kg DM CMA.MgO were decreased significantly (P = 0.02) by 22.9% relative to the control (Table 3). A significant drop (P = 0.02) in CH_4 production (40.1%) was observed across *in vitro* runs after 10 g/kg DM of CMA.MgO was included. The CMA.MgO replicated

TABLE 2 Effect of additive's mechanisms of action on gas production post 24 h incubation in buffered rumen fluid from Friesian cow donors.

	Total gas production (mL) ± SD*[1]	Rel % Total gas ± SEM[2]	CH_4 (mL) ± SD*[3]	Rel % CH_4 ± SEM[4]	N[5]
Factor					
Mechanism of action	<0.0001	0.001	<0.0001	<0.0001	
Mechanism of action					
None (Control)	75.79 ± 0.76[a]	100.05 ± 2.37[a]	5.34 ± 0.10[a]	99.77 ± 4.27[a]	44
Hydrogen Sequestration	67.18± 0.43[ab]	88.01 ± 3.17[b]	4.19 ± 0.07[ab]	76.21 ± 5.76[b]	35
Enzyme Inhibitor	66.41 ± 0.1.36[b]	89.09 ± 5.11[b]	4.25 ± 0.22[ab]	79.19 ± 9.25[b]	12
Rumen Microbiome Modifying	66.05 ± 0.43[b]	91.58 ± 3.18[ab]	3.15 ± 0.07[b]	70.12 ± 5.77[b]	37
SEM	2.01	2.72	0.45	6.43	

[1]*–milliliters of gas per 1.15 g dry matter incubated; SD–standard deviation; [a,b]–means in a column with different superscripts are significantly different (p < 0.05); [2]Rel% Total gas–relative percentage total gas of the mean; SEM, standard error of the mean; [3]CH_4–methane; [4]Rel% CH_4–relative percentage methane of the control, [5]N–number of biological replicates.

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TABLE 3 Effect of additives on volatile fatty acid concentrations and gas production post 24 h incubation in buffered rumen fluid from Friesian cow donors.

Treatment[1]	Total VFAs (mmol/L) ± SD[2]	C2:C3[3]	Total gas production (mL) ± SD*[4]	Rel % Total gas ± SEM[5]	CH_4 (mL) ± SD*[6]	Rel % CH_4 ± SEM[7]	N[8]
No Additive							
Control	80.49 ± 35.88	3.49	76.09^{a} ± 25.48	100.00^{a} ± 6.30	5.43^{ab} ± 3.88	100.00^{a} ± 10.46	24
Hydrogen Sequestration							
CMA (10 g/kg DM)	80.28 ± 28.00	3.63	55.16^{a} ± 32.64	89.52^{ab} ± 8.58	2.97^{ab} ± 2.92	75.31^{abcde} ± 14.18	3
CMA.MgO (10 g/kg DM)	112.55 ± 30.42	3.65	60.86^{a} ± 31.45	77.06^{bc} ± 7.39	2.47^{ab} ± 2.92	59.86^{cdef} ± 12.17	5
Enzyme Inhibitor							
A. taxi (cultured) (2.5 g/kg DM)	–	–	66.41^{a} ± 11.62	86.90^{ab} ± 7.42	2.45^{ab} ± 0.27	78.41^{abcd} ± 12.68	3
A. taxi (ocean) (2.5 g/kg DM)	–	–	68.47^{a} ± 11.56	91.33^{ab} ± 6.16	2.78^{ab} ± 0.70	80.20^{abc} ± 10.11	3
Rumen Microbiome Modifying							
EO (0.14 g/kg DM)	72.74 ± 30.59	3.39	81.05^{a} ± 22.89	98.59^{ab} ± 7.82	7.87^{a} ± 4.77	101.01^{a} ± 12.87	4
ALL (7.2 g/kg DM)	–	–	67.30^{a} ± 22.85	82.22^{abc} ± 8.51	3.72^{ab} ± 2.56	69.20^{abcdef} ± 14.10	3
GO (14.3 g/kg DM)	100.07 ± 29.48	3.37	52.59^{a} ± 11.77	90.78^{ab} ± 6.29	3.42^{ab} ± 2.38	63.07^{bcdef} ± 10.46	3
Yucca (1.14 g/kg DM)	98.70 ± 30.59	3.39	80.22^{a} ± 24.76	100.75^{a} ± 8.06	6.13^{ab} ± 3.63	71.78^{abcdef} ± 13.292.87	4
Combinations							
CMA (10 g/kg DM) + GP (0.5 g/kg DM)	92.37 ± 28.00	3.80	55.98^{a} ± 23.42	63.01^{c} ± 8.00	1.69^{b} ± 1.59	35.55^{f} ± 13.18	4
CMA (15 g/kg DM) + GO (0.5 g/kg DM)	86.60 ± 25.73	3.52	53.77^{a} ± 10.26	77.70^{bc} ± 8.11	1.80^{ab} ± 1.05	59.16^{cdef} ± 13.40	3
SEM	4.60	0.05	2.72	2.67	0.45	5.41	
p-value	0.257	0.232	0.044	< 0.0001	< 0.0001	< 0.0001	

[1]CMA, calcareous marine algae; CMA.MgO, calcareous marine algae fortified with magnesium oxide; *A. taxi* cultured, *Asparagopsis taxiformis* in cultured form; *A. taxi* ocean, *Asparagopsis taxiformis* in ocean form; EO, Essential oil blend; ALL, allicin powder; GO, garlic oil; GP, garlic powder; Yucca, *Yucca schidigera*; g/kg DM–grams per kilogram dry matter; SEM–standard error of the mean; [2]Total VFAs (mmol/L)–total volatile fatty acids in millimoles per litre; SD, standard deviation [3]C2:C3–acetic acid-to-propanoic acid ratio; [4]*–milliliters of gas per 1.15 g dry matter incubated; [a,b,c,d,e,f]–means in a column with different superscripts are significantly different ($p < 0.05$); [5] Rel % Total gas–relative percentage total gas of the mean; [6]CH_4–methane; [7]Rel % CH_4 –relative percentage methane of the control, [8]N–number of biological replicates.

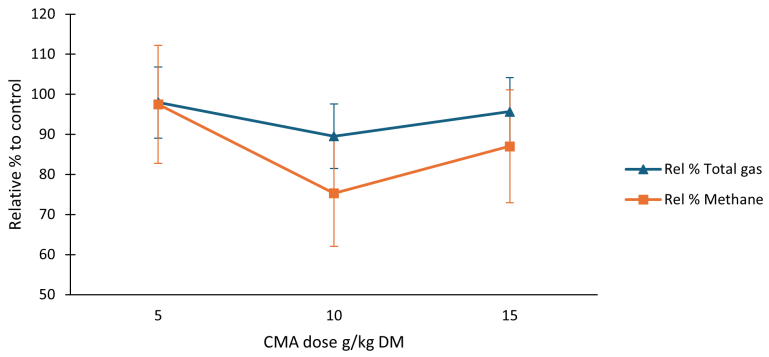

FIGURE 1
Dose response of CMA for total gas and methane post 24 h rumen simulation. n ≥ 3. CMA, calcareous marine algae; Rel % Total gas, relative percentage total gas to control; Rel % CH_4, relative % methane to control.

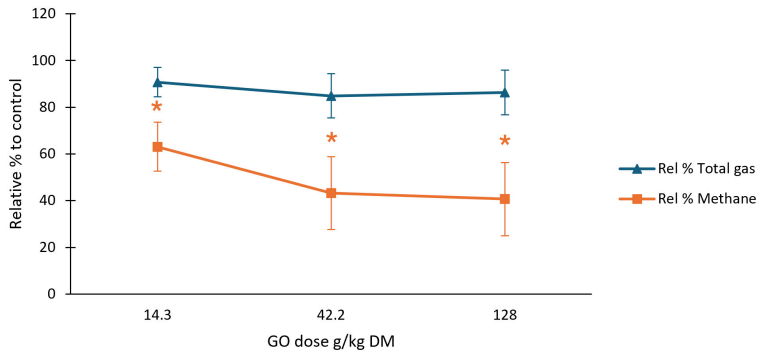

FIGURE 2
Dose response of GO for total gas and methane post 24 h rumen simulation. n=3. GO, garlic oil; colour coordinated four-point shapes (*) signify significant differences ($p < 0.05$); Rel % Total gas, relative percentage total gas to control; Rel % CH_4, relative % methane to control.

the middle dose of the CMA but induced a greater as well as significant total gas and CH_4 reduction compared to the reference control values.

3.1.3 Enzyme inhibiting bioactives

The red seaweed (*A. taxi*) was supplemented in both its freeze-dried oceanic and cultured forms, with each form included at the same dose (2.5 g/kg DM). *Asparagopsis taxiformis* from cultured origin caused a 13.1% decrease in total gas compared to its oceanic form which decreased Rel % total gas by 8.7%. Similarly, the cultured *A. taxi* induced a greater reduction in Rel % CH_4 production (21.6%) in contrast to the capabilities of CH_4 reduction with supplementation of the *A. taxi* in its ocean form (19.8%).

3.1.4 Combination bioactives

The combination of GP (0.5 g/kg DM) with the middle dose of CMA (10 g/kg DM) significantly decreased ($P < 0.001$) total gas production by 37% in comparison to the control, correspondingly, the CMA (15 g/kg DM) and GO (0.5 g/kg DM) combination induced a significant 22.3% reduction in Rel % Total gas ($P = 0.04$; Table 3). The combination supplementation of CMA and *Allium sativum* derivatives induced significant CH_4 reductions ($P < 0.01$; Rel % CH_4) of 64.5% and 40.8% with GP and GO supplementation, respectively, suggesting a synergistic effect (Table 3).

3.2 *In vitro* VFA profiles

The total VFA concentrations and acetic acid-to-propanoic acid ratios (C2:C3) collected from the treatment vessels are presented in Table 3 and discussed under the corresponding mode of action subheadings. Individual VFA proportions in percentage millimole per millimole of total VFA corresponding to each treatment VFA profile are presented in Table 4. The levels of acetic acid, propanoic acid, and butyric acid can collectively account for 95% of the total VFAs found in rumen fluid (Luo et al., 2015). Among the six types of VFA analzed in this study, acetic acid, propanoic acid, and butyric acid accounted for 87.1% of the VFAs analzed. No significant differences were observed between the control mean values and the treatment values concerning total VFA concentrations, whereas significance was identified with individual VFA component propanoic acid and pairwise comparison significance for n-valeric acid.

3.2.1 Rumen microbiome modifying bioactives

Volatile fatty acid profiling of the lower GO dosage (14.3 g/kg DM) generated the second highest concentration of total VFAs (100.1 mmol/L RF; Table 3), 24.4% greater than the control concentration. There were no significant differences in individual VFA concentrations between GO (14.3g/kg DM) and the control after 24 h fermentation. Total VFA's were decreased by 9.6%, with no instances of significant changes between control and EO treated individual VFA concentrations. Volatile fatty acid profiling of Yucca-treated rumen fluid revealed a 22.6% rise in the total concentration of VFAs. Individual VFA proportions were not significantly influenced by Yucca supplementation.

3.2.2 Hydrogen sequestering bioactives

The middle dose of CMA significantly decreased ($P = 0.05$) propanoic acid concentrations by 10.2% in contrast to the control (Table 4). The total VFAs and remaining individual VFA concentrations remained largely unchanged by CMA (10 g/kg DM) supplementation. The overall VFA concentration experienced a 39.8% increase compared to the control when fermentation vessels were supplemented with 10 g/kg DM of CMA.MgO. However, the increase in total VFAs associated with CMA.MgO was not significant, with similar trends observed in the individual VFA percentages.

3.2.3 Bioactive combinations

Combining CMA (10 g/kg DM) and GP (0.5 g/kg DM) in the simulated rumen led to an increase in total VFA concentration (14.8%) with a significant reduction ($p = 0.02$) in propanoic acid (10.7%) compared to the control. Additionally, the second combination treatment of CMA (15 g/kg DM) and GO (0.5 g/kg

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TABLE 4 Effect of additives on individual VFA millimolar proportions (Percentage mmol/mmol total VFA) of acetic acid, propanoic acid, isobutyric acid, n-butyric acid, isovaleric acid, and n-valeric acid after 24 h incubation.

Treatment[1]	Acetic acid ± SD[2]	Propanoic acid ± SD[3]	Isobutyric ± SD	n-Butyric ± SD	Isovaleric ± SD	n-Valeric ± SD	N[4]
No Additive							
Control (1.15 g DM)	57.75 ± 5.06	17.75^{a} ± 1.39	4.67 ± 2.57	13.52 ± 2.56	2.96 ± 0.29	3.35^{ab} ± 2.22	6
Hydrogen Sequestration							
CMA (10 g/kg DM)	53.82 ± 3.89	15.94^{b} ± 1.06	7.80 ± 1.98	14.47 ± 1.97	2.96 ± 0.23	5.07^{a} ± 1.72	3
CMA.MgO (10 g/kg DM)	55.01 ± 4.33	16.99^{ab} ± 1.19	6.22 ± 2.20	13.92 ± 2.19	2.77 ± 0.25	5.08^{a} ± 1.90	3
Rumen Microbiome Modifying							
EO (0.14 g/kg DM)	55.71 ± 4.36	17.64^{ab} ± 1.20	5.98 ± 2.22	13.88 ± 2.21	3.11 ± 0.25	3.69^{ab} ± 1.91	3
GO (14.3 g/kg DM)	54.66 ± 4.18	17.91^{a} ± 1.15	5.39 ± 2.12	15.67 ± 2.11	2.77 ± 0.24	3.61^{ab} ± 1.83	3
Yucca (1.14 g/kg DM)	55.27 ± 4.36	17.46^{ab} ± 1.20	6.99 ± 2.22	13.75 ± 2.21	2.90 ± 0.25	3.64^{ab} ± 1.91	3
Combinations							
CMA (10 g/kg DM) + GP (0.5 g/kg DM)	58.62 ± 1.86	15.85^{b} ± 1.06	7.71 ± 1.98	12.77 ± 1.97	3.08 ± 0.23	2.02^{b} ± 1.72	3
CMA (15 g/kg DM) + GO (0.5 g/kg DM)	57.55 ± 6.74	17.05^{ab} ± 3.21	6.09 ± 3.82	13.53 ± 3.74	3.07 ± 0.65	2.77^{ab} ± 2.15	3
SEM	0.60	0.28	0.39	0.30	0.05	0.37	
p-value	0.297	0.015	0.065	0.422	0.108	0.066	

[1]CMA, calcareous marine algae; CMA.MgO, calcareous marine algae fortified with magnesium oxide; EO, essential oil blend; GO, garlic oil; GP, garlic powder; Yucca, *Yucca schidigera*; g/kg DM–grams per kilogram dry matter. SEM-standard error of the mean. [2]S–standard deviation [3a,b]–means in a column with different superscripts are significantly different ($p < 0.05$); [4]N–number of biological replicates.

DM) tended to increase total VFA concentration by 7.6% and inflicted no significant influence on individual VFAs compared to the control value.

4 Discussion

This study investigates the potential of natural bioactive compounds as feed additives, examining their diverse mechanisms of action to enhance ruminal productivity and reduce enteric CH_4 emissions, supporting further *in vivo* trials and potential on-farm applications. The effectiveness of the mentioned feed additives and combinations on rumen productivity was evaluated based on total gas volumes, enteric CH_4 levels, and VFA profiles collected and analyzed at the end of each fermentation run. A similar *in vitro* study achieved similar rumen fermentation gas levels and included a comparable scale fermentation system fed a high forage diet (Ahmed et al., 2021). This study also used the 2:1 ratio of buffer to rumen fluid with a grass-dominant diet for control vessels which was also closely reproduced in the current *in vitro* study. The control vessels set the benchmark for enteric gas levels and VFA concentrations when investigating treatments for effects on the simulated rumen fermentation and outputs. Total gas production in control vessels was comparable to the headspace enteric gas volumes obtained by Ahmed et al. (2021) using the same forage dominant diet composition, reinforcing the reproducibility and robustness of the experimental setup. This consistency enhances confidence in the methodology and strengthens the credibility of novel treatments and treatment combinations for their observed effects in the current study.

The results in Table 2 support the hypothesis that the bioactive mechanisms of action play a significant role in CH_4 abatement, while significant reductions in Rel % Total gas were observed with hydrogen sequestering and enzyme inhibiting mechanisms (Table 2). The promising reductions in Rel % CH_4 observed across the three investigated mechanisms, along with the need to identify treatments that enhance fermentation productivity, prompted further analysis of treatments at optimal doses to evaluate their individual and combined impact on fermentation parameters (Tables 3, 4). The inclusion of GO resulted in comparable overall gas percentages, amounting to 90.8%, 84.9%, and 86.3% in relation to the control, as the concentrations of 14.3, 42.2, and 128 g/kg DM GO increased. A corresponding trend with GO inclusion was observed by Patra and Yu (2012), and they reported that as GO was increased in dosage increments there was a linear decrease in CH_4 emissions. There were numerical increases in total VFAs, propanoic acid and butyric acid with drops in acetic acid in fermentations supplemented with GO (14.3 g/kg DM). The detected reduction in acetic acid (3.1%) may be credited to the antimicrobial properties of GO, directly inhibiting methanogenic *Archaea* (Busquet et al., 2006; Kim et al., 2012). As a result, the increased intensity of dissolved hydrogen in the buffered rumen fluid due to methanogenesis suppression, may have suppressed acetic acid production based on the expected stoichiometry of acetate, as reported by Wang et al. (2017, 2018). Hence, hydrogen

ions that would have been utilized in the formation of CH_4, could have been redirected towards hydrogen sinks, such as propanoic acid. A meta-analysis by Ungerfeld (Ungerfeld, 2015) supporting this concept found that inhibiting CH_4 production in ruminal fermentation systems increased hydrogen availability, which was subsequently utilized in the formation of VFAs, particularly propionate. The strength of the rumen buffer used across the *in vitro* fermentations may have suppressed the potential production of propanoic acid with treatment supplementations. Evidence of VFA shifts to higher levels of propanoic acid in lower ruminal pH conditions supports the theory (Morgante et al., 2007). Similarly, ruminal VFA concentration has been shown to be related negatively to ruminal pH (Dijkstra et al., 2012), suggesting the opportunity to increase total VFAs and decrease acetic acid to propanoic acid concentrations in higher fermentable diets with supplemented treatments such as GO. Intensifying sulfhydryl-modifying activity linked with GO's bioactive compounds and the GO dosage increase in this study support the possible inhibition of SH-containing enzymes involved in the synthesis of isoprenoid chains found on the cell membrane of methanogenic archaea (Busquet et al., 2006; Patra and Yu, 2012). Steady decreases in CH_4 volumes with increasing GO doses may signify the specific targeting of GO's active components on the rumen archaea domain. Stability in total gas production, as the concentration is increased, may be explained by the predominance of gram-negative bacteria in animals being fed high forage diets (Seshadri et al., 2018; Ternouth, 1968) and GO's antibacterial activity against susceptible gram-positive bacteria is suspected to be due to the lack of a protectant outer membrane of the cell wall (Burt, 2004; Yasin et al., 2022). Microbial profiling and inclusion of the current GO dosages to rumen fluid fed a high grain diet *in vitro* could uncover the versatility of GO as a CH_4 mitigating feed additive in livestock provided with diverse diets.

The integration of garlic-derived treatments with CMA as an additional antimethanogenic agent broadens the potential of garlic beyond its use as a standalone strategy for enteric methane mitigation as discussed in previous research (Patra and Yu, 2012). Co-dosage of GO with CMA evoked a synergistic effect in terms of CH_4 mitigation. GO was supplemented at a much lower dose (0.5 g/kg DM) with 15 g/kg DM of CMA and caused a significant decrease ($P = 0.01$) in Rel % CH_4 (40.8%) (Table 3). In correspondence with this result, at a lower dose of CMA (10 g/kg DM) co-dosed with GP (0.5 g/kg DM) also induced a significant reduction ($P < 0.001$) in CH_4 with respect to the control (64.5%). The significant drops in CH_4 levels appear to correlate with significant drops in total gas with reference to the control values, as a greater drop in total gas was observed with CMA and GP (37%) compared to the CMA and GO combination (22.3%) (Table 3). Increases in total VFA concentrations in both combination treatments compared to the control suggest the redirection of metabolic hydrogen in the rumen away from enteric CH_4 production and toward VFAs (Ungerfeld, 2015), this in turn would increase the efficiency of ruminant production and decrease environmental impact from rumen fermentation. Additionally, the direct inhibition of methanogens as a hydrogen sink by garlic-derived compounds can increase hydrogen availability (Sari et al., 2022), potentially enhancing CMA's buffering capacity. This shift in hydrogen utilization may favour pH stabilization over its role in branched-chain fatty acid synthesis, such as valeric acid. This is supported by the significant reduction in valeric acid observed in the CMA with GP treatment compared to CMA and CMA.MgO treatments ($P = 0.02$). Further research on dosage rates for both CMA and GO is necessary to find optimum levels of both but a potential explanation for this synergistic effect with CMA addition may be the rumen buffer's ability to neutralize the acidic rumen environment (Cruywagen et al., 2015; Rossi et al., 2019) caused by the GO supplement (pH 4.91). The rumen buffers' ability to reduce pH fluctuation and improve overall digestion described by Rossi et al. (2019) might explain the maintenance of total gas production as the concentration of CMA was increased. The introduction of CMA with *Allium sativum* derivatives *in vitro* may maintain the rumen fluid at a more stable range of pH 6 to 7 when supplemented with more acidic diets, preventing acidosis-like conditions, and providing an optimum pH range for microorganisms (Krause and Oetzel, 2006), including those that are affected by GO's inhibitory effects. Further *in vitro* studies with a rapidly fermentable acidic diet and less potent buffer solution are necessary to unveil the potential of CMA in mitigating enteric CH_4 and influencing fermentation outcomes in rumen inoculum susceptible to lower ruminal pH conditions.

The supplementation of three CMA doses (5, 10, and 15 g/kg DM) induced non-significant reductions in total gas percentages relative to the mean, with the greatest reduction in 10 g/kg DM CMA supplemented vessels (10.5%). The middle CMA dose (10 g/kg DM) also caused the greatest CH_4 reductions relative to the control mean (24.7%) out of the three dosages administered (Figure 1). The middle CMA dose appears to be the optimum amount for enteric CH_4 mitigation as improvements in mitigation were not observed as CMA introduction was increased to 15 g/kg DM. Based on CH_4 percentages relative to control values in Figure 1, the middle and higher CMA quantities appear to provide adequate buffering capacity to act as sufficient proton acceptors to sequester H_2 from methanogenesis over the 24-hour *in vitro* period. Increasing the dosage of CMA has previously shown improvements in rumen pH ranges when the dose was increased from 0.125% to 1.2% dietary DM (Cruywagen et al., 2004). Similarly, the CMA dose increase from 5 to 10 g/kg DM enhanced rumen fermentation parameters in this *in vitro* study by mitigating methanogenesis without significantly reducing total gas volume and total VFA concentrations after 24 h incubation. The significant reduction in propanoic acid proportions influenced by CMA at the middle dose may be due to its buffering properties, which stabilize ruminal pH in conjunction with the artificial saliva buffer. This stabilising effect likely inhibits the proliferation of propionate-producing bacteria, as lower ruminal pH has been shown to favour these bacteria (Russell, 1998). Subsequent research may involve conducting additional tests with diets that challenge rumen pH, aiming to investigate the CH_4 mitigating capabilities of rumen buffers under conditions resembling rumen fluid susceptibility to acidosis.

The CMA with additional magnesium oxide (CMA.MgO) was supplemented at 10 g/kg DM. CMA.MgO reduced Rel % total gas production by 22.9%, 12.5% more than that observed by CMA alone,

and caused a 15.5% greater reduction in Rel % CH_4 levels when compared to CMA at the same concentration (10 g/kg DM). Magnesium (Mg) has a higher cofactor capacity than Ca due to its higher coordination number as a central atom for free ions, and greater surface area may stimulate additional enzymes within the rumen. Considering Mg's cofactor abilities, Mg is linked with promoting approximately 300 cellular enzymes which are responsible for oxidative phosphorylation and metabolism of carbohydrates, lipids, and proteins in the rumen (Ebel, 1980). Promotion of the fermenting rumen species through increased enzymatic action could increase interaction with H_2 – utilizing bacteria through "interspecies hydrogen transfer" (Iannotti et al., 1973), thus, maintaining rumen fermentation pathways, pH, and synthesis of VFA's. A shift towards a 40% increase in total VFAs produced compared to CMA at the same concentration and the control total VFA result (Table 2), strongly suggests enhanced enzymatic catalysis and an increased supply of the primary source of metabolizable energy in ruminants (Bergman, 1990). This, in turn, promotes higher levels of productivity. Furthermore, the carbonate ion (CO_3^-) found in the CMA and CMA.MgO calcium carbonate structure can utilize hydrogen byproducts, such as those of pyruvate metabolism, in the reduction reaction to bicarbonate (HCO_3^-) and ultimately carbonic acid (H_2CO_3). Therefore scavenging H_2 from the largest H_2 sink being the formation of CH_4 where 4 moles of H_2 are consumed through the following reaction:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The *in vitro* results in this study point toward the optimum effect of the CMA on rumen productivity and H_2 redirection from CH_4 production using the middle dose of 10 g/kg DM.

The principal bioactive component ALL, obtained from *Allium sativum*, induced a 17.8% decrease in Rel % total gas production when supplemented at a concentration of 7.2 g/kg DM. An average decrease in Rel % CH_4 of 30.8% was observed when ALL was supplemented at concentrations resembling the higher GO treatment allicin content (1.4%) typically found in GO (Ahmed et al., 2021; Busquet et al., 2005). In terms of enteric CH_4 reduction relative to the control, the GO higher dose with expected equivalent allicin content induced a 28.5% greater reduction compared to the ALL 25% allicin concentrated powder suggesting the importance of the synergistic CH_4 mitigating capabilities of GO's compounds diallyl sulfide, diallyl disulfide, and allyl mercaptan. Busquet et al. (2005) identified significant CH_4 mitigation by diallyl disulfide and allyl mercaptan in a separate *in vitro* microbial fermentation, suggesting the requirement of the primary GO components for optimal anti-methanogenic activity. Our study results display greater anti-methanogenic activity and total gas maintenance across all three GO doses with suspected lower allicin levels in the lower and middle GO dose than the 25% allicin powder supplementation.

Cultured *A. taxi* provided by a Swedish Greentech company (Volta Greentech, Stockholm, Sweden), showed comparable results with ocean-sourced *A. taxi* in terms of enteric gas production post-24-hour incubation when supplemented at the same dose rate (2.5 g/kg DM). *A. taxiformis* halogenated CH_4 analogue, bromoform (Merck group Ltd., Darmstadt, Germany), was initially investigated in this *in vitro* system for its ability to inhibit the MCR enzyme required for methanogenesis in rumen archaea (Duin et al., 2016) and proved very effective with almost complete CH_4 inhibition but significantly reduced total gas yield (data not shown). The bromoform concentration of *A. taxi* is linked to the CH_4-mitigating efficacy of this additive (Kinley et al., 2020; Roque et al., 2019, 2021), and the active compound has been shown to deliver the greatest suppression of MCR synthesis in the final step of methanogenesis (Alvarez-Hess et al., 2023; Machado et al., 2016). Similarly, 3-NOP inhibits enteric CH_4 emissions by binding to the nickel cofactor active site of MCR in a position that allows for electron transfer with 3-NOPs nitrate group, resulting in MCR inactivation (Duin et al., 2016). Providing *A. taxi* and 3-NOP containing halogenated methane analogue (HMA) components at 1% organic matter inclusion increases ruminal H_2 production (Kirwan et al., 2024; Roque et al., 2019), probably due to the suppression of methanogenesis as the largest H_2 sink in the rumen. Based on the current results, bromoform may be at a higher concentration in the cultured seaweed (unspecified) compared to the ocean-sourced seaweed (6–10 mg/g DM) due to the greater reduction induced by the seaweed cultured form on gas production (3%) and CH_4 emissions (11.9%) in this study.

The essential oil blend (EO) formulated for direct ruminant consumption caused a 1% increase on CH_4 emissions and resulted in a 1.4% decrease in overall gas production, suggesting negligible influence with the recommended dose of EO on *in vitro* fermentation over 24 hours. The inclusion of the essential oil blend over 6 and 22-week-long studies *in vivo* appear to allow the essential oils anti-methanogenic properties to unfold by inducing a 14.7% and 6% decrease in CH_4 g/day with its recommended dose, respectively (Castro-Montoya et al., 2015; Hart et al., 2019). An *in vitro* system with continuous culture capabilities such as the rumen simulation technique (RUSITEC) used in a recent study by Foggi et al. (2024), may be more suited for EO treatment investigation to reveal the ruminal changes in metabolite concentrations and abundance of bacteria that occur when the essential oil blend is applied for extended periods.

Supplementation with Yucca slightly (0.8%) increased total gas production compared to the control benchmark value – the only feed additive to do so in this study. The adequacy of Yucca extracts in maintaining overall gas levels and increasing total VFA concentration compared to the control (22.6%) in this study reinforce the proposed ability of saponins to enhance the growth of specific rumen microbes associated with aiding digestion (Goel et al., 2008; Wang et al., 2000). Furthermore, investigated cultures in the study by Wang et al. (2000) showed a sustained higher optical density at 14 and 24 hours during *in vitro* incubation when yucca extract was included. This implies a flourishing population of rumen microbes through increased metabolism of complex carbohydrates which is supported by improved total gas and VFA production with the current *in vitro* Yucca supplementation. The Yucca extract caused a decrease of CH_4 emissions by 28.2% which may be explained by linkages to protozoa and methanogen reduction with saponin supplementation (Wina et al., 2005; Goel et al., 2008). While the preservation of total gas production suggests the absence of an impact on *in vitro* ruminal fermentation, the present dosage of Yucca necessitates additional *in vivo* validation for beneficial microbial community proliferation and the described

CH_4 mitigating effect that was not observed when the plant extract was fed at a similar dose *in vivo* (Holtshausen et al., 2009). In alignment with our findings, numerous authors have consistently reported reductions ranging from 22% to 27% in CH_4 concentration *in vitro* through the supplementation of saponins (Anele et al., 2022; Hess et al., 2003; Hu et al., 2005). These findings reinforce the effectiveness of the current *in vitro* rumen simulation system to accurately replicate rumen fermentation and supplement effects. Simultaneously, it emphasizes the need for additional research on Yucca supplements for CH_4 mitigation in live animals.

Trends towards significant CH_4 reduction with ALL further support garlic's antimicrobial activity. Results from this 24-hour batch fermentation study indicate that rumen microbiome modifiers and hydrogen-sequestering bioactive compounds are the most effective CH_4 mitigators. However, it is worth noting that the enzyme-inhibiting compound bromoform, found in *A. taxi* and the rumen modifying saponins in Yucca as well as the essential oil mixture (EO) may demonstrate greater efficiency in extended fermentation cycles. This study aligns with the imperative to achieve greenhouse gas reduction targets, as outlined in the Global Methane Pledge launched at COP 26 in November 2021 in Glasgow to reduce global methane emissions by at least 30% from 2020 levels by 2030 (UN Climate Change Conference UK, 2021). In accordance with these objectives, this high throughput *in vitro* system has demonstrated its capability to accurately replicate rumen conditions allowing for the swift identification of treatments with CH_4 mitigating potential that enhance rumen productivity. The development of *in vitro* screening systems offers a cost-effective and ethically responsible approach to evaluating CH_4 reduction strategies before *in vivo* testing, providing a practical pathway for the livestock industry to achieve its methane reduction targets. Limitations associated with this set-up include the absence of host palatability influence for higher GO dosages, the short duration of *in vitro* screening fermentations in comparison to RUSITEC fermentations, and the accompanying costs associated with routine feeding of higher dose supplements to animals. Nevertheless, a potential effective strategy for mitigating enteric CH_4 could involve combining lower GO concentrations with rumen buffers or sole supplementation of rumen buffers fortified with magnesium oxide.

5 Conclusion

The *in vitro* fermentation analysis of potential rumen feed additives with CH_4 mitigating capabilities revealed mechanisms that either enhanced or inhibited rumen metabolism. These variations were supported by noticeable fluctuations in gas emissions as well as distinct comparative patterns in concentrations of total VFAs. Supplementation of rumen modifying garlic derivatives GO and GP, as well as hydrogen sequestering rumen buffers CMA and CMA.MgO induced CH_4 reductions that were backed up by ruminal VFA fluctuations indicative of rumen functionality and trends towards increased total VFA concentrations. Conducting additional *in vitro* research with CMA and CMA.MgO rumen buffers added to rumen fluid at lower pH ranges may elucidate the acetic acid to propanoic acid ratios linked to the inhibited methanogenesis observed in this study. Combining the antimicrobial garlic derivatives at lower concentrations with the hydrogen sink rumen buffers implied synergistic effects with significant CH_4 mitigation and improved VFA profiles at concentrations that are suitable for *in vivo* investigations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Welfare Body (AWB) at Munster Technological University (MTU), Kerry, Ireland. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

IH: Writing – original draft, Formal analysis, Investigation. PQ: Writing – review & editing, Conceptualization, Methodology, Supervision. MA: Writing – review & editing, Methodology. SO: Writing – review & editing, Conceptualization, Methodology, Supervision.

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Conflict of interest

Authors MA and SC were employed by the company Marigot Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Generative AI statement

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2025.1546486/full#supplementary-material

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