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# Exploring the combination of *Asparagopsis taxiformis* and phloroglucinol to decrease rumen methanogenesis and redirect hydrogen production in goats

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ABSTRACT

Many strategies for mitigating enteric methane ($CH_4$) emissions in ruminants have focused on suppressing the activity of rumen methanogens, but this often leads to excess dihydrogen ($H_2$) accumulation in the rumen, which is subsequently expelled and represents a potential energy loss. We hypothesized that phloroglucinol could act as a $H_2$ acceptor when rumen methanogenesis is inhibited and be potentially transformed into beneficial compounds for the animal. Eight adult goats were randomly assigned to a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: two levels of *Asparagopsis taxiformis* as $CH_4$ inhibitor [0 vs. 5 g/kg on a dry matter (DM) basis; AT- and AT+, respectively] and two levels of phloroglucinol as alternative $H_2$ acceptor (0 vs. 20 g/kg DM, PG- and PG+, respectively). Therefore, four dietary treatments were considered: i) basal diet (AT-PG-); ii) *A. taxiformis* alone (AT+PG-); iii) phloroglucinol alone (AT-PG+); and iv) the combination of *A. taxiformis* and phloroglucinol (AT+PG+). Animals were fed a maintenance diet with a 70:30 forage-to-concentrate ratio. After 10 d of adaptation to the diet, enteric gas emissions were measured in respiration chambers during 3 d prior to rumen content sampling on d 14. Dietary supplementation with *A. taxiformis* decreased $CH_4$ production (-33.9 %) and increased $H_2$ emissions (+3465 %), along with greater rumen propionate concentration. In contrast, phloroglucinol supplementation alone did not impact $CH_4$ emissions or the rumen concentration of the main microbial groups but substantially increased acetate molar proportion (+10.2 %) which could act as an alternative $H_2$ acceptor. Moreover, when *A. taxiformis* was combined with phloroglucinol, it resulted in a decrease in $H_2$ emissions (-68.1 %). However, this decrease in $H_2$ emissions was not fully explained by the increase in the acetate as phloroglucinol led to an increase in acetate both when methanogenesis was inhibited and when it was not. These findings suggest that the rumen fermentation of phloroglucinol may capture some of the additional $H_2$ arising from the inhibition of

*Abbreviations:* 3NOP, 3-nitrooxypropanol; A:P, Acetate:propionate ratio; ADF, Acid detergent fiber; ADL, Acid detergent lignin; BW, Body weight; CH4, Methane; CO2, Carbon dioxide; CP, Crude protein CP; DM, Dry matter; DMI, Dry matter intake; EE, Ether extract; H2, Dihydrogen; NDF, Neutral detergent fiber; OM, Organic matter; SEM, Standard error of the mean; VFA, Volatile fatty acids.

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methanogenesis by *A. taxiformis* through pathways other than acetate formation. Moreover, $H_2$ emissions were not eliminated and most of the decrease occurred during the post-prandial stage, suggesting that the efficiency of $H_2$ redirection could be further improved.

## 1. Introduction

Methane ($CH_4$) is a greenhouse gas with a global warming potential 28–34 times greater than carbon dioxide ($CO_2$) over a 100-year time horizon and 84–86 times more potent over a 20-year time horizon (Gerber et al., 2013). Methane production from ruminant livestock is responsible for approximately 40 % of total greenhouse gas emissions from livestock and 26 % of global anthropogenic $CH_4$ emissions (Gill et al., 2010; Tseten et al., 2022). The main source of enteric $CH_4$ emissions in ruminants is the rumen methanogenesis which is a biological process in which methanogens primarily utilize dihydrogen ($H_2$) generated from feed fermentation to reduce $CO_2$, thereby lowering the partial pressure of $H_2$ in the rumen (Ellis et al., 2008). Enteric $CH_4$ is then exhaled or eructated by the ruminant into the atmosphere, having a notable short-term impact on global warming but also representing a loss of 2–12 % of the gross energy intake to the animal (Johnson and Johnson, 1995). In recent years, different additive-based nutritional technologies to mitigate $CH_4$ emissions from ruminants have been developed (Honan et al., 2021). Novel synthesized nitrooxycompounds, such as 3-nitrooxypropanol (3NOP) (Kebreab et al., 2023) and plant-based additives rich in halogenated compounds, such as the red macroalgae *Asparagopsis* spp. (Lean et al., 2021; O'Hara et al., 2023) have been found to decrease $CH_4$ production under *in vitro* and *in vivo* conditions. In particular, *Asparagopsis taxiformis* has shown high efficacy with $CH_4$ reductions of up to 80 % in cattle (Kinley et al., 2020; Roque et al., 2021) due to its high bromoform content (Glasson et al., 2022). Bromoform is a halogenated $CH_4$ analogue that specifically targets the nickel tetrapyrrole (cofactor $F_{430}$) of methyl-coenzyme M reductase, thereby inhibiting the last step of methanogenesis (Ermler, 2005; Yang et al., 2007; Glasson et al., 2022). Additionally, bromoform inhibits the cobamide-dependent methyl-transferase step of methanogenesis by cross-reacting with reduced vitamin $B_{12}$ of methyl-tetrahydromethanopterin:coenzyme M methyltransferase (Wood et al., 1968; Honan et al., 2021).

The inhibition of rumen methanogenesis often leads to $H_2$ accumulation in the rumen, making $H_2$ available for being redirected towards alternative metabolic pathways that consume $H_2$ and yield fermentation products such as volatile fatty acids (VFA) (Ungerfeld, 2015). This has been evidenced by the shift in the fermentation profile towards propionate production (a $H_2$-incorporating pathway) that has been consistently reported when $CH_4$ inhibitors were included in the diet of ruminants (Hristov et al., 2015; Martinez-Fernandez et al., 2016; Kinley et al., 2020). However, methanogenesis inhibition is yet accompanied by a substantial increase in $H_2$ expelled across all these studies, indicating that the rumen system is unable to capture all the $H_2$ in excess into nutritionally valuable metabolic products for the ruminant. This suggests that this process can still be optimized in order to minimize the energy lost by the host (Ungerfeld et al., 2022). Novel nutritional strategies based on feeding substrates that can modify the rumen microbiota and act as $H_2$ acceptors could facilitate the redirection of energy towards beneficial fermentation products, resulting in potential increases in productivity.

Reductive acetogenesis (Gagen et al., 2015), propionogenesis (Newbold et al., 2005), nitrate and sulphate reduction (van Zijderveld et al., 2010), increases of microbial biomass production (Ungerfeld, 2015) and the metabolism of organic acids (Ungerfeld et al., 2003; Newbold et al., 2005) have been identified as alternative pathways in the rumen for allocating the excess $H_2$ when $CH_4$ is inhibited (Newbold et al., 2005). However, not all of them promote the synthesis of metabolites that are nutritionally usable by the animal. A proposed nutritional strategy consists of dietary supplementation with phenolic compounds such as flavonoids that can be degraded by rumen bacteria into energy yielding products (Krumholz and Bryant, 1986; McSweeney et al., 2001). The microbial degradation of flavonoids in the rumen generally results in the formation of phloroglucinol as an intermediate metabolite (Krumholz et al., 1987). This metabolite can be reduced by specific rumen bacteria into acetate, consuming $H_2$ (Tsai and Jones, 1975; Tsai et al., 1976; Patel et al., 1981). Martinez-Fernandez et al. (2017) showed that, under the methanogenesis inhibition scenario induced by chloroform, phloroglucinol supplementation decreased $H_2$ expelled by redirecting $H_2$ towards acetate production in beef cattle. In previous *in vitro* work, we evaluated the potential of different phenolic compounds as $H_2$ acceptors, including phloroglucinol using rumen fluid from cattle (Huang et al., 2023) and goats (Romero et al., 2023b). We found that rumen microorganisms can utilize the excess $H_2$ resulting from methanogenesis inhibition by *A. taxiformis* in the catabolism of phloroglucinol to acetate, thereby shifting the fermentation profile and decreasing the amount of $H_2$ released. The present study hypothesized that a similar redirection of $H_2$ could also occur *in vivo*. Thus, the aim of this study was to analyze the effect of combining *A. taxiformis* and phloroglucinol to decrease $CH_4$ emissions while improving rumen fermentation through a more efficient $H_2$ utilization in goats.

## 2. Material and methods

All management and experimental procedures involving animals were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013). Protocols were approved by the Ethical Committee for Animal Research at the Estación Experimental del Zaidín (EEZ-CSIC) (A/18/03/2019/042).

### 2.1. Feed additives

The anti-methanogenic additive used was *A. taxiformis* (Blue Ocean Barns Inc., Redwood City, CA, US) containing bromoform at a

concentration of 5 g/kg on a dry matter (DM) basis. Bromoform concentration was measured by headspace solid-phase micro extraction (CTC Analytics PAL Combi-xt Autosampler, Zwingen, Switzerland) according to Colomb et al. (2008) at the Centre for Scientific Instrumentation of the University of Granada (CIC-UGR), Spain. The macroalgae was freeze-dried and ground to pass through a 1-mm screen. The mill equipment was previously cooled in liquid to avoid overheating and altering the active compound. Afterwards, *A. taxiformis* was ground for 2 × 30 s with a 30 s interval between cycles to cool the mill. Milled seaweed material was stored in an airtight recipient contained in a desiccator at 4 °C. The phenolic compound used as substrate was phloroglucinol anhydrous (MBL LAB CÓRDOBA SL, Córdoba, Spain; 99 % purity).

### 2.2. Experimental design

Eight Murciano-Granadina adult female goats with an average body weight (BW) of 40.8±1.8 kg were used as experimental animals at EEZ-CSIC, Granada, Spain. They were randomly assigned to 1 of 4 experimental treatments and the study was conducted over 8 experimental periods according to a replicated 4 × 4 Latin square design (alternating the animals from the first and second Latin Square in consecutive periods) with a 2 × 2 factorial arrangement of treatments: two levels of *A. taxiformis* (0 vs. 5 g/kg DM; AT- and AT+, respectively) and two levels of phloroglucinol (0 vs. 20 g/kg DM; PG- and PG+, respectively). Therefore, four treatments were considered: i) a basal diet without supplementation (AT-PG-); ii) a basal diet supplemented with *A. taxiformis* (AT+PG-); iii) a basal diet supplemented with phloroglucinol (AT-PG+); and iv) a basal diet supplemented with *A. taxiformis* and phloroglucinol (AT+PG+). The doses of *A. taxiformis* and phloroglucinol were determined based on a previous *in vitro* study (Romero et al., 2023b). Throughout the experiment, all goats had free access to drinking water and were fed the same basal diet (Table 1) consisting of alfalfa hay chopped at 15–20 cm and barley grain in a 70:30 ratio in DM. Feed intake was restricted to 1 kg DM/d representing approximately 90 % of the *ad libitum* DMI based on measurements conducted during one week before the experiment began. Diet was divided into two equal daily meals at 0900 and 1400 h (representing 0 h and 5 h in Fig. 1, respectively). This feed distribution ensured enough time for the animals to eat the second meal before the artificial illumination was turned off (from 1900 to 0700 h), as they had negligible feed consumption during the overnight period. Additives were mixed with ground barley grain (20 g DM) and administered directly into the animal's mouth via a top-cut syringe at feeding times. The animals from AT-PG- group were dosed similar amount of ground barley without additives.

Each 14-d experimental period consisted of 10 d for adaptation to the treatments in individual pens, followed by 3 d for $CH_4$ and $H_2$ measurements, and the last day for rumen fluid sampling. As rumen microbiota needs to be adapted to the presence of phenolic compounds to facilitate their degradation (Theodorou et al., 1987), phloroglucinol was administered in increasing doses over the first 3 d (5, 10, and 15 g/kg DM, respectively). From d 4 onwards, animals were supplemented with the complete doses of phloroglucinol and *A. taxiformis* (20 g/kg DM). During d 11, 12 and 13, animals were confined in open-circuit respiration chambers to quantify enteric $CH_4$ and $H_2$ emissions. Each goat was always allocated to the same chamber across all periods to minimize intra-animal variability. For each animal, feed intake was daily recorded, as well as the temperature, humidity, $CO_2$ concentration and air turnover rate within each respiration chamber.

On d 14, animals returned into the individual pens and were fed at 0900 h, along with the administration of the treatments. At 3 h post-feeding (1200 h), rumen content was withdrawn by orogastric intubation (100 mL approx.) as described in Belanche et al. (2020). Rumen samples were filtered through sterile cheesecloth (1 mm pore size) and fluid subsamples were stored at −20 °C for lactate and VFA analysis, and at −80 °C for quantification of the main microbial groups in the liquid fraction, as described in Romero et al. (2023b). Goats were weighed twice within each period (d 1 and 14) before feeding (0900 h) using a digital scale (Bosche GmbH & Co. KG, Damme, Germany) corresponding to the entrance and exit of the chamber, respectively. Furthermore, feed samples were collected once a week and pooled to conduct compositional analysis.

### 2.3. Methane and dihydrogen measurements

Enteric $CH_4$ and $H_2$ production by each goat was measured using 4 open-circuit respiration chambers as described by Abecia et al. (2012). Chamber air temperature was maintained between 15 and 20°C. Measurements were interrupted twice a day, when the chamber floor was cleaned and the goats received the morning feed (0900 h), and when animals received the afternoon feed (1400 h). These 10-min interruptions had minimal effect on the daily emissions as they coincided when the corresponding exhaust duct was not being sampled. Airflow (L/min) was measured twice a day for the exhaust duct of each chamber using an anemometer (PCE Ibérica SL, Albacete, Spain). The air stream in each duct was subsampled and directed into a gas analyzer. Methane concentration was measured using an infrared gas analyzer (ULTRAMAT 23, Siemens AG, Munich, Germany), whereas $H_2$ concentration was measured using a

**Table 1**
Chemical composition (g/kg DM) of the diet ingredients.

| | DM | OM | CP | EE | NDF | ADF | ADL |
|---|---|---|---|---|---|---|---|
| Alfalfa hay | 904 | 895 | 148 | 9.01 | 566 | 423 | 314 |
| Barley | 909 | 907 | 106 | 19.5 | 296 | 60.8 | 8.26 |
| *A. taxiformis* | 924 | 788 | 393 | 3.59 | 424 | 112 | 79.4 |

DM: Dry matter; OM: Organic matter; CP: Crude protein; EE: Ether extract; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin.

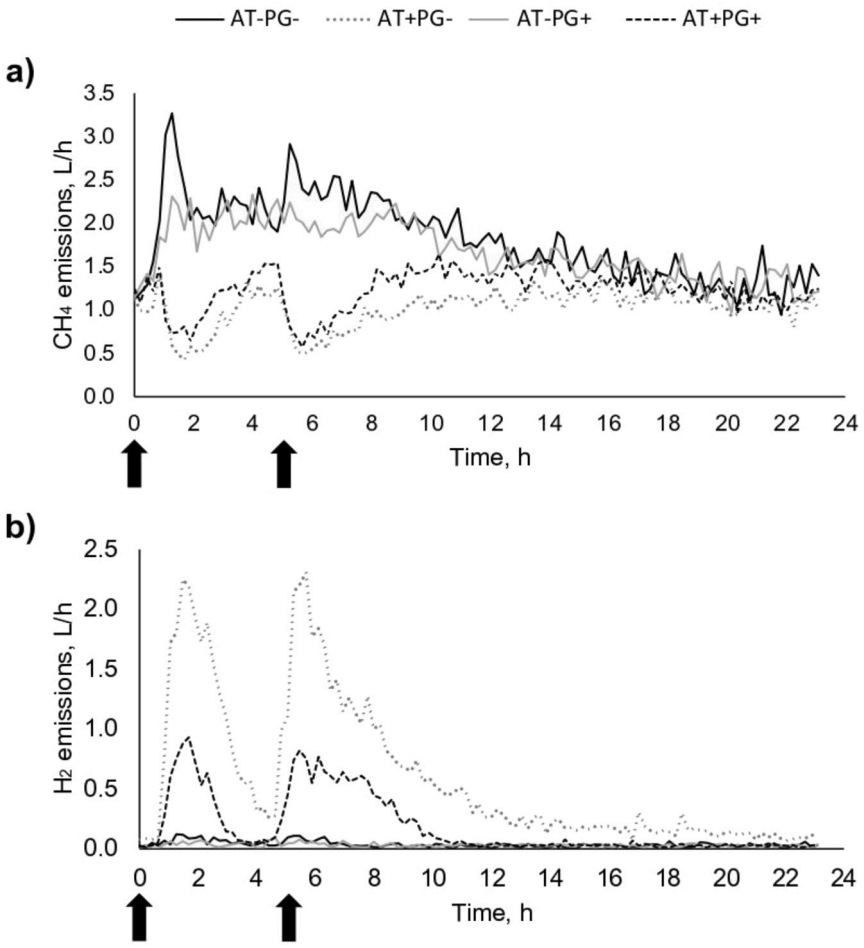


**Fig. 1.** Average diurnal pattern of $CH_4$ (**a**) and $H_2$ emissions (**b**) in goats (n=8 for 3 d) fed a basal diet (AT-PG-); supplemented with *A. taxiformis* at 5 g/kg DM (AT+PG-); supplemented with phloroglucinol at 20 g/kg DM (AT-PG+); or supplemented with *A. taxiformis* and phloroglucinol (AT+PG+). Arrows represents the feeding/dosing times at 0900 h and 1400 h, respectively.

tin-dioxide semiconductor sensor (Q-S121, Qubit Systems Inc., Kingston, Ontario, Canada). Additionally, room air was also sub-sampled and analyzed to determine the background $CH_4$ and $H_2$ concentrations. Gases in each chamber were measured for 90 s every 12.5 min (equivalent to 115 measurements/d). The daily $CH_4$ and $H_2$ emissions from each chamber were calculated as the air flow multiplied by the gas concentration in the chamber exhaust after background subtraction.

### *2.4. Chemical analyses*

Feed samples were passed through a 1-mm sieve for analyses, and DM, organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined as described in Arco-Pérez et al. (2017). Dry matter (method 934.01) and ash (OM) (method 942.05) were determined according to AOAC (2005). Nitrogen content (AOAC method 990.03) was determined using the Dumas method (Leco TruSpec CN, St. Joseph, MI, USA) and converted to CP by multiplying by 6.25. Ether extract was measured by extraction with petroleum ether (AOAC method 920.39). The analyses of NDF and ADF were carried out according to Van Soest et al. (1991) using an Ankom 220 fiber analyzer unit (Ankom Technology Corp., Macedon, NY, USA), with a heat stable α-amylase, while ADL was determined by solubilization of cellulose with 72 % sulfuric acid, being all expressed exclusive of residual ash (aNDFom, aNDFom and aADLom, respectively). Concentrations of individual VFA were analyzed with a GC system coupled with a flame ionization detector (Auto-System PerkinElmer, Norwalk, CT, USA) using a crosslinked 100 % polyethylene glycol column (TRB-FFAP, 30 m × 0.53 mm i.d. × 1 μm film thickness, Teknokroma, Spain), as described in Arco-Pérez et al. (2017). Lactate concentration was measured using the colorimetric method described by Barker and Summerson (1941) consisting in the oxidation of lactic acid to acetaldehyde in hot concentrated sulfuric acid. The color reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid (cold) is then quantified by colorimetry.

### 2.5. Real-time PCR analysis

Samples of rumen fluid were freeze-dried, and microbial DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Germany). Eluted DNA (2 μl) was used to assess the abundance of the main microbial groups by quantitative PCR (qPCR) using an iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA). Specific primers and standards for the 16 S rRNA gene for bacteria, mcrA gene for archaea, 18 S rRNA gene for protozoa, and the region between 18 S rRNA and ITS1 gene for anaerobic fungi were used as previously described in Palma-Hidalgo et al. (2021).

### 2.6. Statistical analyses

Prior to conducting the ANOVA, the assumptions of normality and homogeneity of variance were checked using the Shapiro-Wilk and Bartlett's tests, respectively. Results were statistically analyzed by a 2 × 2 factorial ANOVA as follows:

$$Y_{ijk} = \mu + AT_i + PG_j + AT{\times}PG_{ij} + G_k + PR_l + e_{ijkl}$$

Where $Y_{ijk}$ represents a dependent, continuous variable, $\mu$ is the overall mean, $AT_i$ is the fixed effect of *A. taxiformis* inclusion ($i$ = 0 vs. 5 g/kg DM), $PG_j$ is the fixed effect of phloroglucinol supplementation ($j$ = 0 vs. 20 g/kg DM), $AT{\times}PG_{ij}$ represents the interaction term, $G_k$ represents the random effect of the goat ($k$ = 1–8), $PR_l$ represents the random effect of the period ($l$ = 1–8), and $e_{ijkl}$ is the residual error. Means were compared by Fisher's protected LSD test when significant interactions were found ($P < 0.05$), using the StatGraphics Centurion 19 software (StatPoint Technologies, Inc. USA, 2020). Significant effects were declared at $P < 0.05$ and tendencies to differences at $0.05 \leq P < 0.10$.

## 3. Results

### 3.1. Animal performance, and methane and dihydrogen emissions

No significant differences were observed for DMI and BW across treatments (Table 2). The supplementation with *A. taxiformis* decreased daily $CH_4$ emissions ($CH_4$ production), per unit of DMI ($CH_4$ yield) and per kg of BW by 33.9, 31.4 and 34.7 %, respectively, compared to treatments without *A. taxiformis* ($P < 0.001$). Phloroglucinol supplementation did not affect $CH_4$ emissions.

The supplementation with *A. taxiformis* also promoted a substantial increase in $H_2$ emission per day, per DMI and per BW being 3465, 3711 and 3888 % higher than treatments without *A. taxiformis* supplementation, respectively ($P < 0.001$). There was an interaction between supplementation of *A. taxiformis* and phloroglucinol on $H_2$ emissions ($P = 0.01$), whereby combining both supplements decreased total expelled $H_2$ (-68.1 %), $H_2$ per unit of DMI (-71.4 %), and $H_2$ per kg BW (-72.3 %) compared with the supplementation of *A. taxiformis* alone. Likewise, the $H_2$ expelled per mol of $CH_4$ decreased was greater with *A. taxiformis* supplementation ($P < 0.001$), although the significant interaction (AT×PG, $P = 0.02$) revealed that this increase was 56.1 % lower when *A. taxiformis* was combined with phloroglucinol, in comparison with *A. taxiformis* alone.

### 3.2. Diurnal pattern of methane and dihydrogen emissions

Methane and $H_2$ emissions (L/h) followed a diurnal variation which was determined by the feeding pattern as shown in Fig. 1. Methane production in treatments without *A. taxiformis* supplementation peaked at hours 1 and 6, corresponding to post-prandial

**Table 2**
Effect of dietary supplementation with *A. taxiformis* (AT) and phloroglucinol (PG) on body weight, dry matter intake, $CH_4$ and $H_2$ production in goats after 14 d of treatment.

| | AT- | | AT+ | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | PG- | PG+ | PG- | PG+ | | AT | PG | AT×PG |
| BW, kg | 40.4 | 39.3 | 39.8 | 39.7 | 0.89 | 0.97 | 0.76 | 0.78 |
| DMI, kg/d | 0.90 | 0.89 | 0.85 | 0.86 | 0.020 | 0.29 | 0.96 | 0.81 |
| $CH_4$ emissions | | | | | | | | |
| $CH_4$, L/d | 45.4 | 42.2 | 27.1 | 30.8 | 1.78 | <0.001 | 0.91 | 0.13 |
| $CH_4$, L/kg DMI | 50.8 | 47.5 | 31.6 | 35.9 | 1.86 | <0.001 | 0.82 | 0.10 |
| $CH_4$, L/kg BW | 1.18 | 1.13 | 0.68 | 0.83 | 0.056 | <0.001 | 0.55 | 0.23 |
| $H_2$ emissions | | | | | | | | |
| $H_2$, L/d | 0.29$^{b}$ | 0.19$^{b}$ | 13.0$^{a}$ | 4.15$^{b}$ | 1.254 | <0.001 | 0.01 | 0.01 |
| $H_2$, L/kg DMI | 0.31$^{b}$ | 0.22$^{b}$ | 15.6$^{a}$ | 4.47$^{b}$ | 1.519 | <0.001 | 0.01 | 0.01 |
| $H_2$, L/kg BW | 0.01$^{b}$ | 0.005$^{b}$ | 0.37$^{a}$ | 0.10$^{b}$ | 0.0359 | <0.001 | 0.01 | 0.01 |
| $H_2/CH_4$ decr.[1] | 0$^{c}$ | 0$^{c}$ | 0.71$^{a}$ | 0.31$^{b}$ | 0.070 | <0.001 | 0.08 | 0.02 |

AT-: A. taxiformis at 0 g/kg DM; AT+: A. taxiformis at 5 g/kg DM; PG-: Phloroglucinol at 0 g/kg DM; PG+: Phloroglucinol at 20 g/kg DM; SEM: Standard error of the mean; BW: Body weight; DMI: Dry matter intake.

[a-c] Values within a row with different superscripts differ significantly at $P < 0.05$ ($n$=8) due to AT × PG interaction.

[1] Mol of $H_2$ per mol of $CH_4$ decreased in relation to the AT-PG- diet.

situations, and was lower with the macroalgae supplementation at the corresponding times. Similarly, two large peaks were observed for $H_2$ emissions at 1 and 6 h in animals supplemented with *A. taxiformis* alone, which correspond with the post-prandial $CH_4$ inhibition. However, these peaks were less pronounced in animals supplemented with *A. taxiformis* combined with phloroglucinol and absent in those supplemented with phloroglucinol alone or fed the basal diet.

Considering the diurnal fluctuations of $CH_4$ production and $H_2$ emissions within each treatment, three-time intervals were identified which correspond with i) the post-prandial effects after the first feeding/dosing (0–5 h interval), ii) the second feeding/dosing (5–10 h interval) and iii) the overnight period (10–24 h interval) (Table 3). Supplementation with *A. taxiformis* promoted a decrease in the $CH_4$ production in all the three-time intervals, this decrease being much larger during the post-prandial periods (-60 %, $P < 0.001$) than overnight (-23 %, $P = 0.02$). During the period between 5 and 10 h, the decrease in $CH_4$ production induced by *A. taxiformis* supplementation tended to be higher when the macroalgae was not combined with phloroglucinol (interaction, $P = 0.07$). As described before, $H_2$ emissions were increased by *A. taxiformis* ($P < 0.001$) and decreased by phloroglucinol supplementation ($P = 0.003$ and $P = 0.05$), but these effects only occurred during post-prandial periods (i and ii) and not overnight (iii). Moreover, the increase in $H_2$ emissions induced by *A. taxiformis* supplementation during the post-prandial periods was significantly lower when *A. taxiformis* was combined with phloroglucinol (interaction, $P \leq 0.06$). This interaction was not detected during the 10–24 h period which corresponded with a non-feeding period.

### 3.3. Rumen fermentation and microbial abundances

There were no differences in lactate and total VFA concentrations across experimental groups (Table 4). However, a shift in the VFA profile was observed when *A. taxiformis* and phloroglucinol were administered. The supplementation of *A. taxiformis* significantly decreased the acetate ($P < 0.001$) and increased propionate ($P < 0.001$), butyrate ($P = 0.02$) and valerate molar proportions ($P < 0.001$), resulting in decreased acetate:propionate (A:P) ratio ($P < 0.001$). Conversely, phloroglucinol promoted a shift in the fermentation pattern towards a higher acetate ($P < 0.001$) and A:P ratio ($P < 0.001$), and lower propionate ($P < 0.001$), isobutyrate ($P = 0.001$), butyrate ($P = 0.01$), isovalerate ($P = 0.03$) and valerate molar proportions ($P = 0.001$).

Quantitative PCR analysis showed a tendency (interaction, $P = 0.10$) to decrease the concentration of total bacteria when *A. taxiformis* and phloroglucinol were combined, compared to separate supplementations (Table 4). The rumen concentrations of archaea, protozoa and anaerobic fungi were unaffected across experimental treatments.

## 4. Discussion

### 4.1. Methane and dihydrogen emissions

The rumen anti-methanogenic effects of *A. taxiformis* have been previously reported *in vitro* (Kinley et al., 2016; Machado et al., 2016, 2018; Roque et al., 2019a; Brooke et al., 2020), in sheep (Li et al., 2018), beef cattle (Kinley et al., 2020; Roque et al., 2021), and dairy cows (Stefenoni et al., 2021; Krizsan et al., 2023). The effectiveness of *A. taxiformis* has shown a large variation across these studies due to differences in inclusion rate, bromoform concentration, animal species, and diet formulation (Hegarty et al., 2021). Li et al. (2018) reported a 15 % decrease in $CH_4$ production in Merino sheep fed *A. taxiformis* at 5 g/kg OM, whereas Stefenoni et al. (2021) indicated reductions up to 80 % using a similar concentration in dairy cows. The 33.9 % reduction in $CH_4$ production observed in the present study when supplementing *A. taxiformis* at 5 g/kg DM agrees with a meta-analysis of the effects of supplementing *A. taxiformis* to cattle, which found a 37 % mean reduction in $CH_4$ yield (Lean et al., 2021).

In terms of $H_2$ fluxes, the literature shows that inhibiting methanogenesis through the supplementation of *A. taxiformis* (Kinley et al., 2020; Roque et al., 2021; Stefenoni et al., 2021; Krizsan et al., 2023) and other anti-methanogenic feed additives such as

**Table 3**
Effect of the dietary supplementation with *A. taxiformis* (AT) and phloroglucinol (PG) on averaged $CH_4$ and $H_2$ production (L/h) at different time intervals during 24 h: after the first feeding/dosing (0–5 h interval), ii) the second feeding/dosing (5–10 h interval) and iii) the overnight period (10–24 h interval).

| | AT- | | AT+ | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | PG- | PG+ | PG- | PG+ | | AT | PG | AT×PG |
| $CH_4$ emissions, L/h | | | | | | | | |
| 0–5 h | 2.32 | 2.04 | 0.86 | 1.08 | 0.138 | <0.001 | 0.84 | 0.15 |
| 5–10 h | 2.19 | 1.95 | 0.92 | 1.25 | 0.119 | <0.001 | 0.75 | 0.07 |
| 10–24 h | 1.46 | 1.42 | 1.12 | 1.28 | 0.056 | 0.02 | 0.55 | 0.34 |
| $H_2$ emissions, L/h | | | | | | | | |
| 0–5 h | 0.07[b] | 0.05[b] | 1.30[a] | 0.41[b] | 0.113 | <0.001 | 0.003 | 0.004 |
| 5–10 h | 0.03 | 0.03 | 0.92 | 0.36 | 0.098 | <0.001 | 0.05 | 0.06 |
| 10–24 h | 0.03 | 0.03 | 0.17 | 0.03 | 0.028 | 0.17 | 0.20 | 0.18 |

AT-: A. taxiformis at 0 g/kg DM; AT+: A. taxiformis at 5 g/kg DM; PG-: Phloroglucinol at 0 g/kg DM; PG+: Phloroglucinol at 20 g/kg DM; SEM: Standard error of the mean. The 0–5 h interval represents the period following the first feeding/dosing (0900 h), the 5–10 h interval represents the period following the second feeding/dosing (1400 h), and the 10–24 h interval represents the overnight period.
[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$ ($n$=8) due to AT × PG interaction.

**Table 4**
Effect of dietary supplementation with *A. taxiformis* (AT) and phloroglucinol (PG) on rumen fermentation parameters and the abundance of the main rumen microbial groups in goats after 14 d of treatment.

| | AT- | | AT+ | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | PG- | PG+ | PG- | PG+ | | AT | PG | AT×PG |
| Lactate, μg/mL | 47.4 | 53.6 | 52.9 | 51.5 | 3.16 | 0.82 | 0.75 | 0.60 |
| Total VFA, mM | 81.5 | 78.5 | 77.8 | 82.7 | 1.87 | 0.94 | 0.79 | 0.29 |
| Proportions, % | | | | | | | | |
| Acetate | 69.1 | 75.0 | 61.7 | 69.0 | 1.03 | <0.001 | <0.001 | 0.57 |
| Propionate | 15.7 | 13.2 | 20.9 | 16.2 | 0.64 | <0.001 | <0.001 | 0.19 |
| Isobutyrate | 1.12 | 0.88 | 1.16 | 0.95 | 0.037 | 0.39 | 0.001 | 0.78 |
| Butyrate | 11.3 | 8.80 | 13.0 | 11.1 | 0.476 | 0.02 | 0.01 | 0.70 |
| Isovalerate | 1.18 | 0.88 | 1.15 | 1.05 | 0.049 | 0.46 | 0.03 | 0.25 |
| Valerate | 1.65 | 1.27 | 2.13 | 1.69 | 0.077 | <0.001 | 0.001 | 0.79 |
| A:P ratio | 4.44 | 5.79 | 3.09 | 4.28 | 0.207 | <0.001 | <0.001 | 0.75 |
| Microbes[1] | | | | | | | | |
| Bacteria | 10.5 | 10.8 | 10.8 | 10.7 | 0.06 | 0.40 | 0.64 | 0.10 |
| Protozoa | 7.74 | 7.71 | 7.89 | 7.79 | 0.040 | 0.17 | 0.40 | 0.69 |
| Archaea | 4.88 | 4.93 | 5.31 | 4.91 | 0.221 | 0.67 | 0.71 | 0.64 |
| Fungi | 6.15 | 6.30 | 6.65 | 6.45 | 0.140 | 0.24 | 0.91 | 0.52 |

AT-: A. taxiformis at 0 g/kg DM; AT+: A. taxiformis at 5 g/kg DM; PG-: Phloroglucinol at 0 g/kg DM; PG+: Phloroglucinol at 20 g/kg DM; SEM: Standard error of the mean; VFA: Volatile fatty acid; A:P: Acetate:propionate ratio.
[1]Microbial concentration based in qPCR in log10 DNA copies / mg DM.

synthetic halogenated compounds (Mitsumori et al., 2012; Martinez-Fernandez et al., 2016) and 3NOP (Hristov et al., 2015; Lopes et al., 2016; Vyas et al., 2016) leads to an excess of rumen $H_2$ which can be redirected toward alternative sinks (Janssen, 2010). Our study confirmed that the supplementation of *A. taxiformis* inhibited $H_2$ consumption by methanogens, resulting in $H_2$ accumulation in the rumen. However, an important proportion of the $H_2$ accumulated in the rumen was exhaled, representing a moderate energy loss to the animal (Ungerfeld et al., 2022). In contrast to *A. taxiformis*, the supplementation with phloroglucinol showed no effect on $CH_4$ emissions *in vivo*.

Several $H_2$-incorporating metabolic pathways have been identified to potentially capture the excess rumen $H_2$ when methanogenesis is inhibited (Ungerfeld, 2015). However, there is limited data on the evaluation of combining $CH_4$ inhibitors and $H_2$ acceptors *in vivo*. Martinez-Fernandez et al. (2017) showed that phloroglucinol supplementation decreased $H_2$ emissions (-51 %) in cattle when methanogenesis was inhibited by chloroform. A similar decrease in $H_2$ emission (-68.1 %) were observed in the present study when *A. taxiformis* supplementation was combined with phloroglucinol as compared with supplementation of *A. taxiformis* alone. These findings are in line with our dose-response *in vitro* experiment (Romero et al., 2023b) which showed a progressive decline in expelled $H_2$ (up to −63 %) with increasing phloroglucinol doses (from 0 to 36 mM) under a $CH_4$ inhibition scenario (*A. taxiformis* at 2 %). Moreover, previous *in vitro* (Huang et al., 2023; Romero et al., 2023b) and *in vivo* studies (Martinez-Fernandez et al., 2017) reported a small but additional decrease in $CH_4$ production when phloroglucinol was combined with a methanogenesis inhibitor (-14 %). This aspect was not observed in our study, likely due to the differences between *in vitro* and *in vivo* conditions, as well as the type of inhibitor used (*A. taxiformis vs*. chloroform). Animals supplemented with *A. taxiformis* and phloroglucinol still expelled more $H_2$ than those supplemented with phloroglucinol alone or fed the basal diet, suggesting that a further optimization of this nutritional strategy could still be possible. In this sense, alternative combinations of $CH_4$ inhibitors and $H_2$ acceptors have been recently explored, such as *A. taxiformis* combined with fumaric acid *in vitro* (Thorsteinsson et al., 2023) and 3NOP combined with nitrate fumarate or acrylate in dairy cows (Maigaard et al., 2024a; 2024b). However, they did not result in further decreased in expelled $H_2$. Therefore, phloroglucinol remains as one of the most promising $H_2$ acceptor to date to capture excess $H_2$ resulting from methanogenesis inhibition.

The pattern of $CH_4$ and $H_2$ emissions throughout the day revealed larger post-prandial differences across treatments that gradually came closer towards the end of the day. This distinct pattern agrees with that observed in goats treated with bromochloromethane (Martínez-Fernández et al., 2013). The lower inhibitory activity towards the end of the day could indicate a rapid degradation of bromoform in the rumen. Romero et al. (2023a) reported using batch culture that 90 % of the bromoform content in *A. taxiformis* was degraded within the first 3 h of fermentation. Thus, the efficacy of this mitigation strategy could be improved by using novel delivery formats that allow sustained slow release of bromoform into the rumen. This would prolong the anti-methanogenic effect of bromoform in the rumen and ameliorate $H_2$ peaks, possibly improving the incorporation of $H_2$ into phloroglucinol reduction.

### *4.2. Feed intake and body weight*

Animals were fed a restricted amount of feed, therefore no differences in DMI were observed across treatments. However, *A. taxiformis* supplementation could be an issue when animals are fed *ad libitum* as DMI typically decreases in experiments with $CH_4$ reductions above 50 %. This potential decrease in DMI has been linked to the accumulation of excess $H_2$ and total gas in the rumen (Ungerfeld, 2018; Kjeldsen et al., 2022). It could be expected that phloroglucinol supplementation could mitigate this effect as it decreased $H_2$ accumulation. However, no differences in BW were observed between treatments in the present study, possibly as a result of the experimental design (Latin square) and the use of non-growing and non-lactating animals. Thus, the potential effects of

combining *A. taxiformis* and phloroglucinol on production parameters would need further investigation including experiments with a larger number of growing or lactating ruminants over longer experimental periods.

### 4.3. Rumen microbial fermentation

A shift in rumen fermentation toward propionate was observed in this study with *A. taxiformis* supplementation, leading to a decrease in the A:P ratio (-30.4 %). This agrees with previous studies (Li et al., 2018; Kinley et al., 2020; Stefenoni et al., 2021) that observed a 12–45 % decrease in the A:P ratio when methanogenesis was inhibited by *A. taxiformis* supplementation. Moreover, phloroglucinol supplementation resulted in an increase in the acetate molar proportion and the A:P ratio in our work, as observed in previous *in vitro* studies (Huang et al., 2023; Romero et al., 2023b). Sarwono et al. (2019) observed that *in vitro* incubation with phloroglucinol redirected rumen fermentation towards acetate production in high-forage diets (as noted in the present study), but not in high-concentrate diets, possibly as a result of lower $H_2$ availability. The increase in acetate and A:P ratio resulting from phloroglucinol supplementation can be explained by the degradation pathway of phloroglucinol in the rumen. Among other phenolic compounds, phloroglucinol can be reduced by specific rumen bacteria classified as *Eubacterium oxidoreducens*, *Streptococcus bovis*, and *Coprococcus* spp. to dihydrophloroglucinol, using NADPH or formate as the electron donors, thereby alleviating the partial pressure of $H_2$ in the rumen (Tsai and Jones, 1975; Tsai et al., 1976; Patel et al., 1981; Krumholz et al., 1987). Specifically, three phloroglucinol reductases belonging to the family of NADPH dehydrogenases/reductases have been identified as responsible for the anaerobic degradation of phloroglucinol (Conradt et al., 2016). Then, the hydrolytic ring cleavage of the dihydrophloroglucinol molecule predominantly produces $CO_2$ and acetate (and butyrate to a lesser extent) as end products (Conradt et al., 2016).

This study demonstrated that phloroglucinol supplementation significantly increased acetate molar proportion (+10.2 %) across treatments. Our experimental hypothesis was that acetate production derived from phloroglucinol degradation performs as an alternative $H_2$ acceptor, therefore a higher increase in acetate would be expected assuming that all $H_2$ not used in the methanogenesis pathway was diverted to acetate production. This hypothesis was only partially demonstrated as the increase in the acetate molar proportion associated to phloroglucinol degradation was only slightly higher in presence than in absence of *A. taxiformis* (+11.8 vs +8.1 %), without reaching statistical differences. Part of the discrepancy can be explained by the fact that while $CH_4$ and $H_2$ productions were measured continuously during 3 days, VFA concentrations derived from a single time sample, as further discussed. Interestingly, the similar A:P ratio observed between the AT-PG- and AT+PG+ diets is possibly because the increase in the acetate production induced by phloroglucinol degradation was counterbalanced by the increase in the propionate production induced by *A. taxiformis* supplementation. In agreement with Martinez-Fernandez et al. (2017), no differences were observed in total VFA concentration when phloroglucinol was supplemented in a $CH_4$ inhibition scenario in the present study. Conversely, the previous *in vitro* studies reported an increase in total VFA concentration when *A. taxiformis* and phloroglucinol were combined (Huang et al., 2023; Romero et al., 2023b). Differences in $CH_4$ inhibition rates and rumen fermentation pattern between *in vitro* (Kinley et al., 2016; Machado et al., 2016, 2018; Roque et al., 2019a) and *in vivo* studies using *Asparagopsis* spp. have been previously reported (Li et al., 2018; Roque et al., 2019b; Krizsan et al., 2023). In concordance, our previous *in vitro* studies showed a more substantial reduction of $CH_4$ production (> 99 %), resulting in greater availability of $H_2$ to be utilized by phloroglucinol-reducing bacteria (Huang et al., 2023; Romero et al., 2023b). Thus, the degradation rate of phloroglucinol to acetate *in vivo* could be expected to be lower than observed under *in vitro* conditions. This lower phloroglucinol degradation could be partially explained by its lower availability for the rumen microbes as a result of the higher liquid passage rate than under *in vitro* conditions. Other possible explanations could be associated to the different sampling time (24 h *vs* 3 h after feeding) and units (VFA production *vs* VFA concentration) considered *in vitro* or *in vivo*, respectively (Romero et al., 2023b). In relation to the sampling method, Ramos-Morales et al., (2014) demonstrated that stomach tubing is a feasible alternative for sampling rumen digesta allowing to detect most of the effects observed when sampling through cannula in terms of rumen fermentation and microbiome. However, our experimental protocol consisting in rumen sampling 3 h after the morning feeding did not allow to capture the diurnal variation in the rumen fermentation pattern nor the total VFA production which could be linked with the $CH_4$ and $H_2$ emissions.

### 4.4. Abundance of the main microbial groups

Rumen concentrations of total bacteria, methanogens, protozoa, and anaerobic fungi were not affected by the dietary supplementation with *A. taxiformis*, phloroglucinol or the combination of both. A reduction in the number of methanogens by *A. taxiformis* supplementation was expected, as observed in one of the previous *in vitro* experiments (Romero et al., 2023b). However, *A. taxiformis* supplementation had no effect on the abundance of archaeal communities under *in vivo* conditions. Krizsan et al. (2023) did not find any significant differences in the abundance of archaeal communities but showed a shift in the archaeal community composition upon *A. taxiformis* supplementation. This observation supports previous findings suggesting that rumen methanogenesis depends to a large extent on the relative abundance of different archaeal species and their activity rather than their absolute numbers (Zhou et al., 2010). Phloroglucinol did not affect the abundance of total bacteria, but a shift in bacterial community structure with increased levels of bacterial taxa able to catabolize phloroglucinol to acetate and butyrate would be expected. As mentioned above, several rumen bacteria have been identified as phloroglucinol utilizers and their relative abundance was affected when phloroglucinol was supplemented as an available substrate (Martinez-Fernandez et al., 2017). In addition, de Paula et al. (2016) observed a decrease of *Entodinium* protozoa with supplementation of different phenolic compounds, whereas Zuhainis et al. (2007) found that certain phenolic monomers decreased rumen fungal population. Thus, further and more detailed analyses of the rumen microbiota are needed to clarify the mechanisms of action of phloroglucinol in the rumen microbial ecosystem.

## 5. Conclusions

This *in vivo* study demonstrated that dietary phloroglucinol supplementation attenuated the postprandial peak of $H_2$ emissions resulting from the inhibition of methanogenesis by *A. taxiformis*. Supplementation with phloroglucinol promoted an increase in acetate molar proportion in the rumen both when methanogenesis was inhibited and when it was not and therefore, the $H_2$ redirection cannot be fully explained by the acetate acting as the key $H_2$ acceptor. Moreover, most of this $H_2$ redirection occurred during the post-prandial stage and $H_2$ production was still detected across treatments, suggesting that $H_2$ redirection efficiency could be further improved. These findings warrant additional research to optimize this nutritional strategy and to assess its long-term effects on animal productivity. Given the high phloroglucinol cost, alternative $H_2$ acceptors should be investigated in order to implement similar strategies on farm conditions.

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## CRediT authorship contribution statement

**Emilio M. Ungerfeld:** Writing – review & editing, Conceptualization. **Pedro Romero:** Writing – original draft, Methodology, Investigation, Formal analysis. **David R. Yáñez-Ruiz:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Diego P. Morgavi:** Writing – review & editing, Conceptualization. **Milka Popova:** Writing – review & editing. **Alejandro Belanche:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare no competing interests.

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## References

Abecia, L., Toral, P.G., Martín-García, A.I., Martínez, G., Tomkins, N.W., Molina-Alcaide, E., Newbold, C.J., Yáñez-Ruiz, D.R., 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95, 2027–2036. https://doi.org/10.3168/jds.2011-4831.

Arco-Pérez, A., Ramos-Morales, E., Yáñez-Ruiz, D.R., Abecia, L., Martín-García, A.I., 2017. Nutritive evaluation and milk quality of including of tomato or olive by-products silages with sunflower oil in the diet of dairy goats. Anim. Feed Sci. Technol. 232, 57–70. https://doi.org/10.1016/j.anifeedsci.2017.08.008.

Barker, S.B., Summerson, W.H., 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138, 535–554. https://doi.org/10.1016/s0021-9258(18)51379-x.

Belanche, A., Palma-Hidalgo, J.M., Nejjam, I., Jiménez, E., Martín-García, A.I., Yáñez-Ruiz, D.R., 2020. Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. J. Dairy Sci. 103, 5047–5060. https://doi.org/10.3168/jds.2019-18002.

Brooke, C.G., Roque, B.M., Shaw, C., Najafi, N., Gonzalez, M., Pfefferlen, A., De Anda, V., Ginsburg, D.W., Harden, M.C., Nuzhdin, S.V., Salwen, J.K., Kebreab, E., Hess, M., 2020. Methane reduction potential of two pacific coast macroalgae during in vitro ruminant fermentation. Front. Mar. Sci. 7, 561. https://doi.org/10.3389/fmars.2020.00561.

Colomb, A., Yassaa, N., Williams, J., Peeken, I., Lochte, K., 2008. Screening volatile organic compounds (VOCs) emissions from five marine phytoplankton species by head space gas chromatography/mass spectrometry (HS-GC/MS). J. Environ. Monit. 10, 325–330. https://doi.org/10.1039/b715312k.

Conradt, D., Hermann, B., Gerhardt, S., Einsle, O., Michael, M., 2016. Biocatalytic properties and structural analysis of phloroglucinol reductases. Angew. Chem. Int. Ed. 55, 15531–15534. https://doi.org/10.1002/anie.201607494.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J., 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J. Agric. Sci. 146, 213–233. https://doi.org/10.1017/S0021859608007752.

Ermler, U., 2005. On the mechanism of methyl-coenzyme M reductase. Dalton Trans. 3451–3458. https://doi.org/10.1039/b506697b.

Gagen, E.J., Denman, S.E., McSweeney, C.S., 2015. Acetogenesis as an alternative to methanogenesis in the rumen. In: Malik, P.K., Bhatta, R., Takahashi, J., Kohn, R. A., Prasad, C.S. (Eds.), Livestock production and climate change. CABI, Wallingford, pp. 292–303. https://doi.org/10.1079/9781780644325.0292.

Gerber, P.J., Hristov, A.N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A.T., Yang, W.Z., Tricarico, J.M., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: A review. Animal 7, 220–234. https://doi.org/10.1017/S1751731113000876.

Gill, M., Smith, P., Wilkinson, J.M., 2010. Mitigating climate change: The role of domestic livestock. Animal 4, 323–333. https://doi.org/10.1017/S1751731109004662.

Glasson, C.R.K., Kinley, R.D., de Nys, R., King, N., Adams, S.L., Packer, M.A., Svenson, J., Eason, C.T., Magnusson, M., 2022. Benefits and risks of including the bromoform containing seaweed Asparagopsis in feed for the reduction of methane production from ruminants. Algal Res 64, 102673. https://doi.org/10.1016/j.algal.2022.102673.

Hegarty, R.S., Passetti, R.A.C., Dittmer, K.M., Wang, Y., Shelton, S., Emmet-Booth, J., Wollenberg, E., McAllister, T., Leahy, S., Beauchemin, K., Gurwick, N., 2021. An evaluation of evidence for efficacy and applicability of methane inhibiting feed additives for livestock. Edition 1. Global Research Alliance. ⟨https://hdl.handle.net/10568/116489⟩.

Honan, M., Feng, X., Tricarico, J.M., Kebreab, E., 2021. Feed additives as a strategic approach to reduce enteric methane production in cattle: Modes of action, effectiveness and safety. Anim. Prod. Sci. 62, 1303–1317. https://doi.org/10.1071/AN20295.

Hristov, A.N., Oh, J., Frederick, T.W., Harper, M.T., Weeks, H.L., Branco, A.F., Moate, P.J., Deighton, M.H., Williams, S.R.O., Kindermann, M., Duval, S., Hristov, A.N., Oh, J., Giallongo, F., Frederick, T.W., Harper, M.T., Weeks, H.L., 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. U. S. A. 112, 10663–10668. https://doi.org/10.1073/pnas.1515515112.

Huang, R., Romero, P., Belanche, A., Ungerfeld, E.M., Yanez-Ruiz, D., Morgavi, D.P., Popova, M., 2023. Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited in vitro – Part 1. Dairy cows. Animal 17, 100788. https://doi.org/10.1016/j.animal.2023.100788.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1–22. https://doi.org/10.1016/j.anifeedsci.2010.07.002.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492. https://doi.org/10.2527/1995.7382483x.

Kebreab, E., Bannink, A., Pressman, E.M., Walker, N., Karagiannis, A., van Gastelen, S., Dijkstra, J., 2023. A meta-analysis of effects of 3-nitrooxypropanol on methane production, yield, and intensity in dairy cattle. J. Dairy Sci. 106, 927–936. https://doi.org/10.3168/jds.2022-22211.

Kinley, R.D., de Nys, R., Vucko, M.J., Machado, L., Tomkins, N.W., 2016. The red macroalgae Asparagopsis taxiformis is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. Anim. Prod. Sci. 56, 282–289. https://doi.org/10.1071/AN15576.

Kinley, R.D., Martinez-Fernandez, G., Matthews, M.K., de Nys, R., Magnusson, M., Tomkins, N.W., 2020. Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. J. Clean. Prod. 259, 120836 https://doi.org/10.1016/j.jclepro.2020.120836.

Kjeldsen, M.H., Jensen, M.B., Lund, P., 2022. Potent methane reducing feed additives in a Danish context, and their reduction potential, additive effects, risks related to animal welfare and carry-over to milk, and potential trade-offs. 88 pp. Advisory report from DCA – Danish Centre for Food and Agriculture, Aarhus University.

Krizsan, S.J., Ramin, M., Chagas, J.C.C., Halmemies-Beauchet-Filleau, A., Singh, A., Schnürer, A., Danielsson, R., 2023. Effects on rumen microbiome and milk quality of dairy cows fed a grass silage-based diet supplemented with the macroalga Asparagopsis taxiformis. Front. Anim. Sci. 4, 1112969 https://doi.org/10.3389/fanim.2023.1112969.

Krumholz, L.R., Bryant, M., 1986. Eubacterium oxidoreducens sp. nov. requiring H2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. 144, 8–14. https://doi.org/10.1007/BF00454948.

Krumholz, L.R., Crawford, R.L., Hemling, M.E., Bryant, M.P., 1987. Metabolism of gallate and phloroglucinol in Eubacterium oxidoreducens via 3-hydroxy-5-oxohexanoate. J. Bacteriol. 169, 1886–1890. https://doi.org/10.1128/jb.169.5.1886-1890.1987.

Lean, I.J., Golder, H.M., Grant, T.M.D., Moate, P.J., 2021. A meta-analysis of effects of dietary seaweed on beef and dairy cattle performance and methane yield. PLoS One 16, e0249053. https://doi.org/10.1371/journal.pone.0249053.

Li, X., Norman, H.C., Kinley, R.D., Laurence, M., Wilmot, M., Bender, H., de Nys, R., Tomkins, N., 2018. Asparagopsis taxiformis decreases enteric methane production from sheep. Anim. Prod. Sci. 58, 681–688. https://doi.org/10.1071/AN15883.

Lopes, J.C., de Matos, L.F., Harper, M.T., Giallongo, F., Oh, J., Gruen, D., Ono, S., Kindermann, M., Duval, S., Hristov, A.N., 2016. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. J. Dairy Sci. 99, 5335–5344. https://doi.org/10.3168/jds.2015-10832.

Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016. Dose-response effects of Asparagopsis taxiformis and Oedogonium sp. on in vitro fermentation and methane production. J. Appl. Phycol. 28, 1443–1452. https://doi.org/10.1007/s10811-015-0639-9.

Machado, L., Tomkins, N., Magnusson, M., Midgley, D.J., de Nys, R., Rosewarne, C.P., 2018. In vitro response of rumen microbiota to the antimethanogenic red macroalga Asparagopsis taxiformis. Microb. Ecol. 75, 811–818. https://doi.org/10.1007/s00248-017-1086-8.

Maigaard, M., Weisbjerg, M.R., Hellwing, A.L.F., Larsen, M., Andersen, F.B., Lund, P., 2024a. The acute effects of rumen pulse-dosing of hydrogen acceptors during methane inhibition with nitrate or 3-nitrooxypropanol in dairy cows, 00728-00728 J. Dairy Sci. S0022-0302 (24). https://doi.org/10.3168/jds.2023-24343.

Maigaard, M., Weisbjerg, M.R., Johansen, M., Walker, N., Ohlsson, C., Lund, P., 2024b. Effects of dietary fat, nitrate, and 3-nitrooxypropanol and their combinations on methane emission, feed intake, and milk production in dairy cows. J. Dairy Sci. 107, 220–241. https://doi.org/10.3168/jds.2023-23420.

Martinez-Fernandez, G., Denman, S.E., Yang, C., Cheung, J., Mitsumori, M., McSweeney, C.S., 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. Front. Microbiol. 7, 1122. https://doi.org/10.3389/fmicb.2016.01122.

Martinez-Fernandez, G., Denman, S.E., Cheung, J., McSweeney, C.S., Min, W., 2017. Phloroglucinol degradation in the rumen promotes the capture of excess hydrogen generated from methanogenesis inhibition. Front. Microbiol. 8, 1871. https://doi.org/10.3389/fmicb.2017.01871.

Martínez-Fernández, G., Abecia, L., Martín-García, A.I., Ramos-Morales, E., Hervás, G., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2013. In vitro-in vivo study on the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats. Animal 7, 1925–1934. https://doi.org/10.1017/S1751731113001699.

McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O., 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91, 83–93. https://doi.org/10.1016/S0377-8401(01)00232-2.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S.E., McSweeney, C.S., 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108, 482–491. https://doi.org/10.1017/S0007114511005794.

Newbold, C.J., López, S., Nelson, N., Ouda, J.O., Wallace, R.J., Moss, A.R., 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation. Br. J. Nutr. 94, 27–35. https://doi.org/10.1079/BJN20051445.

O'Hara, E., Terry, S.A., Moote, P., Beauchemin, K.A., McAllister, T.A., Abbott, D.W., Gruninger, R.J., 2023. Comparative analysis of macroalgae supplementation on the rumen microbial community: Asparagopsis taxiformis inhibits major ruminal methanogenic, fibrolytic, and volatile fatty acid-producing microbes in vitro. Front. Microbiol. 14, 1104667 https://doi.org/10.3389/fmicb.2023.1104667.

Palma-Hidalgo, J.M., Jiménez, E., Popova, M., Morgavi, D.P., Martín-García, A.I., Yáñez-Ruiz, D.R., Belanche, A., 2021. Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. Anim. Micro 3, 11. https://doi.org/10.1186/s42523-021-00073-9.

Patel, T.R., Jure, K.G., Jones, G.A., 1981. Catabolism of phloroglucinol by the rumen anaerobe. Appl. Environ. Microbiol. 42, 1010–1017.

de Paula, E.M., Samensari, R.B., Machado, E., Pereira, L.M., Maia, F.J., Yoshimura, E.H., Franzolin, R., Faciola, A.P., Zeoula, L.M., 2016. Effects of phenolic compounds on ruminal protozoa population, ruminal fermentation, and digestion in water buffaloes. Livest. Sci. 185, 136–141. https://doi.org/10.1016/j.livsci.2016.01.021.

Ramos-Morales, E., Arco-Pérez, A., Martín-García, A.I., Yáñez-Ruiz, D.R., Frutos, P., Hervás, G., 2014. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. Anim. Feed Sci. Technol. 198, 57–66. https://doi.org/10.1016/j.anifeedsci.2014.09.016.

Romero, P., Huang, R., Jiménez, E., Palma-Hidalgo, J.M., Ungerfeld, E.M., Popova, M., Morgavi, D.P., Belanche, A., Yáñez-Ruiz, D.R., 2023b. Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited in vitro – Part 2. Dairy goats. Animal 17, 100789. https://doi.org/10.1016/j.animal.2023.100789.

Romero, P., Belanche, A., Jiménez, E., Hueso, R., Ramos-Morales, E., Salwen, J.K., Kebreab, E., Yáñez-Ruiz, D.R., 2023a. Rumen microbial degradation of bromoform from red seaweed (Asparagopsis taxiformis) and the impact on rumen fermentation and methanogenic archaea. J. Anim. Sci. Biotechnol. 14, 133. https://doi.org/10.1186/s40104-023-00935-z.

Roque, B.M., Salwen, J.K., Kinley, R., Kebreab, E., 2019b. Inclusion of Asparagopsis armata in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. J. Clean. Prod. 234, 132–138. https://doi.org/10.1016/j.jclepro.2019.06.193.

Roque, B.M., Brooke, C.G., Ladau, J., Polley, T., Marsh, L.J., Najafi, N., Pandey, P., Singh, L., Kinley, R., Salwen, J.K., Eloe-Fadrosh, E., Kebreab, E., Hess, M., 2019a. Effect of the macroalgae Asparagopsis taxiformis on methane production and rumen microbiome assemblage. Anim. Micro 1 (1), 14. https://doi.org/10.1186/s42523-019-0004-4.

Roque, B.M., Venegas, M., Kinley, R.D., de Nys, R., Duarte, T.L., Yang, X., Kebreab, E., 2021. Red seaweed (Asparagopsis taxiformis) supplementation reduces enteric methane by over 80 percent in beef steers. PLoS One 16, e0247820. https://doi.org/10.1371/journal.pone.0247820.

Sarwono, K.A., Kondo, M., Ban-Tokuda, T., Jayanegara, A., Matsui, H., 2019. Effects of phloroglucinol and the forage: Concentrate ratio on methanogenesis, in vitro rumen fermentation, and microbial population density. Adv. Anim. Vet. Sci. 7, 164–171. https://doi.org/10.17582/journal.aavs/2019/7.3.164.171.

Stefenoni, H.A., Räisänen, S.E., Cueva, S.F., Wasson, D.E., Lage, C.F.A., Melgar, A., Fetter, M.E., Smith, P., Hennessy, M., Vecchiarelli, B., Bender, J., Pitta, D., Cantrell, C.L., Yarish, C., Hristov, A.N., 2021. Effects of the macroalga Asparagopsis taxiformis and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. J. Dairy Sci. 104, 4157–4173. https://doi.org/10.3168/jds.2020-19686.

Theodorou, M.K., Gascoyne, D.J., Akin, D.E., Hartley, R.D., 1987. Effect of phenolic acids and phenolics from plant cell walls on rumenlike fermentation in consecutive batch culture. Appl. Environ. Microbiol. 53, 1046–1050. https://doi.org/10.1128/aem.53.5.1046-1050.1987.

Thorsteinsson, M., Maigaard, M., Lund, P., Weisbjerg, M.R., Nielsen, M.O., 2023. Effect of fumaric acid in combination with Asparagopsis taxiformis or nitrate on in vitro gas production, pH, and redox potential. JDS Commun. 4, 335–339. https://doi.org/10.3168/jdsc.2022-0259.

Tsai, C.-G., Jones, G., 1975. Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. Can. J. Microbiol. 21, 794–801. https://doi.org/10.1139/m75-117.

Tsai, C.-G., Gates, D.M., Ingledew, W.M., Jones, G.A., 1976. Products of anaerobic phloroglucinol degradation by Coprococcus sp. Pe151. Can. J. Microbiol. 22 (2), 159–164. https://doi.org/10.1139/m76-022.

Tseten, T., Sanjorjo, R.A., Kwon, M., Kim, S.W., 2022. Strategies to mitigate enteric methane emissions from ruminant animals. J. Microbiol. Biotechnol. 32, 269–277. https://doi.org/10.4014/jmb.2202.02019.

Ungerfeld, E.M., 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. Front. Microbiol. 6, 37. https://doi.org/10.3389/fmicb.2015.00037.

Ungerfeld, E.M., 2018. Inhibition of rumen methanogenesis and ruminant productivity: A meta-analysis. Front. Vet. Sci. 5, 113. https://doi.org/10.3389/fvets.2018.00113.

Ungerfeld, E.M., Rust, S.R., Burnett, R., 2003. Use of some novel alternative electron sinks to inhibit ruminal methanogenesis. Reprod. Nutr. Dev. 43, 189–202. https://doi.org/10.1051/rnd:2003016.

Ungerfeld, E.M., Beauchemin, K.A., Muñoz, C., 2022. Current perspectives on achieving pronounced enteric methane mitigation from ruminant production. Front. Anim. Sci. 2, 795200 https://doi.org/10.3389/fanim.2021.795200.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Vyas, D., McGinn, S.M., Duval, S.M., Kindermann, M.K., Beauchemin, K.A., 2016. Optimal dose of 3-nitrooxypropanol for decreasing enteric methane emissions from beef cattle fed high-forage and high-grain diets. Anim. Prod. Sci. 58, 1049–1055. https://doi.org/10.1071/AN15705.

Wood, J.M., Kennedy, F.S., Wolfe, R.S., 1968. Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. Biochemistry 7, 1707–1713. https://doi.org/10.1021/bi00845a013.

Yang, N., Reiher, M., Wang, M., Harmer, J., Duin, E.C., 2007. Formation of a nickel−methyl species in methyl-coenzyme M reductase, an enzyme catalyzing methane formation. J. Am. Chem. Soc. 129, 11028–11029. https://doi.org/10.1021/ja0734501.

Zhou, M., Hernandez-Sanabria, E., Guan, L.L., 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Environ. Microbiol. 76, 3776–3786. https://doi.org/10.1128/AEM.00010-10.

van Zijderveld, S.M., Gerrits, W.J.J., Apajalahti, J.A., Newbold, J.R., Dijkstra, J., Leng, R.A., 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93, 5856–5866. https://doi.org/10.3168/jds.2010-3281.

Zuhainis, S.W., Abdullah, N., Alimon, A.R., Ho, Y.W., 2007. Effects of phenolic monomers on the degradation of 14C-cellulose by rumen fungi. Res. J. Microbiol. 2, 918–925. ⟨https://scialert.net/abstract/?doi=jm.2007.918.925⟩.