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P. Romero [a], R. Huang [b], E. Jiménez [a], J.M. Palma-Hidalgo [a], E.M. Ungerfeld [c], M. Popova [b], D.P. Morgavi [b], A. Belanche [a,d], D.R. Yáñez-Ruiz [a,*]

[a] *Estación Experimental del Zaidín (CSIC), Profesor Albareda, 1, 18008 Granada, Spain*
[b] *Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genès Champanelle, France*
[c] *Centro Regional de Investigación Carillanca, Instituto de Investigaciones Agropecuarias INIA, Temuco 4880000, Chile*
[d] *Departamento de Producción Animal y Ciencia de los Alimentos, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain*

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ABSTRACT

Most mitigation strategies to reduce enteric methane (**CH_4**) production in the rumen induce an excess of rumen dihydrogen (**H_2**) that is expelled and consequently not redirected to the synthesis of metabolites that can be utilised by the ruminant. We hypothesised that phenolic compounds can be potential H_2 acceptors when added to the diet, as they can be degraded to compounds that may be beneficial for the animal, using part of the H_2 available when ruminal methanogenesis is inhibited. We performed four *in vitro* incubation experiments using rumen inoculum from Murciano-Granadina adult goats: Experiment 1 examined the inhibitory potential of *Asparagopsis taxiformis* (**AT**) at different concentrations (0, 1, 2, 3, 4 and 5% of the substrate on a DM basis) in 24 h incubations; Experiment 2 investigated the effect of a wide range of phenolic compounds (phenol, catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, gallic acid and formic acid) at different doses (0, 2, 4, and 6 mM) on rumen fermentation for 24 h; Experiment 3 evaluated the combined effect of each phenolic compound at 6 mM with AT at 2% DM in sequential batch cultures for 5 days; and Experiment 4 examined the dose–response effect of phloroglucinol at different concentrations (0, 6, 16, 26 and 36 mM) combined with AT in sequential batch cultures for 5 days. Results from Experiment 1 confirmed that AT at 2% DM substantially inhibited CH_4 production while significantly increasing H_2 accumulation and decreasing the acetate:propionate ratio. Results from Experiment 2 showed that phenolic compounds did not negatively affect rumen fermentation at any dose. In Experiment 3, each phenolic compound at 6 mM combined with AT at 2% DM inhibited CH_4 production. Phloroglucinol numerically decreased H_2 accumulation and significantly increased total gas production (**TGP**), volatile fatty acid (**VFA**) production and the acetate:propionate ratio. In Experiment 4, phloroglucinol at increasing doses supplemented with AT at 2% DM significantly decreased H_2 accumulation and the abundances of archaea, protozoa and fungi abundances, and increased TGP, total VFA production and the acetate:propionate ratio in a dose-dependent way. In conclusion, combined treatment with AT and phloroglucinol was successful to mitigate CH_4 production while preventing the accumulation of H_2, leading to an increase in acetate and total VFA production and therefore an improvement in rumen fermentation in goats.

Implications

The strategies developed to reduce methane emissions in ruminants by using inhibitors have not managed to translate methane decrease into improved animal productivity, which could be partly due to the inability to convert the excess dihydrogen in the rumen into compounds that the animal can use. In this study, we evaluated the use of seven phenolic compounds combined with a methanogenesis inhibitor to capture excess rumen dihydrogen and potentially improve fermentation efficiency. Phloroglucinol decreased dihydrogen accumulation and increased total volatile fatty acid, through acetate production. The results show that dihydrogen acceptors like phloroglucinol have the potential to improve fermentation patterns when methane inhibitors are used in the diet.

* Corresponding author.
E-mail address: david.yanez@eez.csic.es (D.R. Yáñez-Ruiz).

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Introduction

The contribution of enteric methane (**CH_4**) to greenhouse gas emissions (Gill et al., 2010) and its associated loss of gross energy intake to the animal (2–12%; Johnson and Johnson, 1995) have evidenced the need for strategies to reduce CH_4 production by ruminants (Ungerfeld, 2018). Enteric CH_4 is generated to maintain a low dihydrogen partial pressure in the rumen (Janssen, 2010). Dihydrogen (**H_2**) is produced through microbial degradation of carbohydrates in the rumen and used by methanogenic archaea, among other substrates such as formate, to reduce one- or two-carbon substrates. If H_2 partial pressure increases above a certain threshold, hydrogenases involved in electron transfer reactions during feed fermentation can be inhibited by negative feedback mechanisms, compromising some rumen fermentation pathways (Wolin et al., 1997).

Numerous additive-based strategies to reduce CH_4 emissions have been investigated (Hegarty et al., 2021). Among others, the halogenated compounds bromochloromethane, 2-bromoethanesulfonate (**BES**) and chloroform have demonstrated their specific inhibitory effect on rumen methanogenic archaea under *in vitro* and *in vivo* conditions (Patra et al., 2017). However, these compounds are not accepted for practical use due to the short-lived persistence of their effects on the rumen microbiota and their potential carcinogenic or contaminating properties.

Other alternatives have arisen recently. Several *in vitro* (Kinley et al., 2016a and 2016b; Machado et al., 2014 and 2016; Roque et al., 2019) and *in vivo* studies (Kinley et al., 2020; Li et al., 2016; Roque et al., 2021; Stefenoni et al., 2021) have demonstrated the inhibitory potential of certain brown and red macroalgae when used as feed supplements, particularly the red macroalgae from the genus *Asparagopsis*. Two species of *Asparagopsis* distributed across tropical and temperate marine ecosystems, *A. taxiformis* and *A. armata,* naturally produce a range of halogenated methane analogue metabolites, such as bromoform, encapsulated into specialised gland cells until its release as a natural defence mechanism (Paul et al., 2006). *A. taxiformis* (**AT**) is currently considered as one of the most promising species due to its high bromoform content (1–15.8 mg/g DM; Glasson et al., 2022) and its capability for decreasing CH_4 production when added to both grass- and grain-based diets (Min et al., 2021). It has been proven that AT at low inclusion rates can reduce CH_4 emissions from *in vitro* fermentations by up to 99% (Machado et al., 2016), without negative impact on microbial fermentation (Kinley et al., 2016a).

However, the above-mentioned studies also reported an increase in H_2 expelled, suggesting the inefficiency of the rumen microbial community under methanogenesis inhibition conditions to capture excess H_2 and redirect it into other fermentation end products to be used by the animal, resulting in a moderate energy loss to the host (Ungerfeld et al., 2022). This can clearly discourage farmers to adopt additive-base mitigation strategies, if there are no benefits to production.

Reductive acetogenesis, propionogenesis, reduction of nitrate and sulphate, formate accumulation and an increase of microbial biomass production are the main alternative metabolic routes in the rumen for allocating the excess H_2 when methanogenesis is inhibited (Gagen et al., 2015; Leng, 2014; Newbold et al., 2005; Ungerfeld, 2015; van Zijderveld et al., 2010). However, not all of them promote the synthesis of metabolites that are usable by the animal. The reduction of phenolic compounds by rumen microorganisms could represent a pathway for H_2 incorporation into beneficial products that the animal host can potentially use to improve production efficiency. Phenolic compounds (Barba et al., 2014; Minatel et al., 2017) form a large group of secondary metabolites in plants characterised by containing benzene rings with one or more hydroxyl groups including functional derivatives (esters, methyl esters, glycosides, etc.). Specific rumen bacteria can reduce phenolic compounds such as gallate, pyrogallol, and phloroglucinol with H_2 or formate consumption and transform them into acetate as an energy-yielding product for the host (Tsai and Jones, 1975; Patel et al., 1981; Krumholz and Bryant, 1986; McSweeney et al., 2001; de Paula et al., 2016).

Martinez-Fernandez et al. (2017) showed that, when rumen methanogenesis was inhibited by chloroform, H_2 gas was redirected towards alternative sinks through the supplementation with phloroglucinol that favoured specific microbial groups in beef cattle. However, this observation has not been proven under practical CH_4 mitigation strategies such as AT and 3-nitrooxypropanol. We hypothesised that phenolic compounds might be reduced to beneficial metabolites for the host animal when added to the diet, capturing in that conversion part of the excess H_2 produced in a CH_4 inhibition scenario.

The aims of the present study were (1) to confirm the potential of AT as a methanogenesis inhibitor with rumen inoculum from goats (Exp. 1); (2) to investigate the effect of a wide range of phenolic compounds at different doses on rumen fermentation (Exp. 2); (3) to evaluate the most promising combinations of AT and phenolic compounds as H_2 acceptors (Exp. 3); and (4) to determine the optimal dose of the most promising phenolic compound that combined with AT allowed maximising CH_4 mitigation, H_2 capture and volatile fatty acid (**VFA**) production (Exp. 4). Different *in vitro* models consisting of batch cultures and sequential batch cultures were used to evaluate these effects specifically with goats' rumen inoculum. This latter approach was chosen to allow the rumen microbiota to be adapted to the presence of different phenolic compounds in the diet.

This work is part of a broader study in which the same experimental hypothesis was applied *in vitro* using rumen fluid from dairy cows (Huang et al., companion paper). Goats and cows have distinct rumen microbiota composition (Henderson et al., 2015; Corral-Jara et al., 2022) and are fed with different types of diets. Moreover, small and large ruminants have shown different responses to the presence of phenolic compounds in the diet (Robbins et al., 1987), which all together could result in differing effects of the experimental treatments evaluated in this work. The four consecutive experiments conducted in this study including batch cultures and semi-continuous incubations allowed to have a greater insight into the mode of action of the proposed CH_4 mitigation strategy.

Material and methods

Animal procedures 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 EEZ-CSIC (A/18/03/2019/042). Four consecutive *in vitro* experiments were performed, and the outcomes from each experiment were used to tailor subsequent experimental designs.

Substrate, methanogenesis inhibitors and phenolic compounds preparation

Alfalfa hay and barley grain were used as a substrate for the incubations in a 70:30 ratio in DM. They were ground with a hammer mill with a 1 mm^2 pore size. *Asparagopsis taxiformis* (Exp. 1, 3 and 4) and 2-bromoethanesulfonate (Exp. 1) were chosen as methanogenesis inhibitors. Phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid and formic acid were selected for Exp. 2 and 3 due to their potential activity as H_2 acceptors based on their catabolic pathways (Tsai et al., 1976;

Patel et al., 1981; Krumholz and Bryant, 1986; McSweeney et al., 2001). *Asparagopsis taxiformis* was obtained from SeaExpert (Faial, Portugal), and its bromoform concentration was 6 mg/g DM. It was freeze-dried and ground to 1 mm^2 particles using a laboratory mill (IKA All analytical mill, Staufen, Germany). The mill equipment was previously cooled in liquid nitrogen to avoid overheating and potential damage of AT chemical integrity. Then, AT was ground 2 × 30 s with a 30 s interval between cycles to cool the mill. Milled AT was stored at 4 °C in a desiccator.

BES and organic compounds were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Stock solutions of BES (10 mM) and each phenolic compound (3 M) were prepared and stored at 4 °C. The nutrient composition of the substrate was analysed as described in Arco-Pérez et al. (2017). Chemical composition (in g/kg DM) of the alfalfa hay was 901 OM, 27.9 N, 428 NDF, 303 ADF, 63 ADL and 13.7 EE, while for the barley grain was 975 OM, 21.5 N, 285 NDF, 67.8 ADF, 8.7 ADL and 20.1 EE.

Experiments 1 and 2: Selecting inclusion levels of methanogenesis inhibitor and phenolic compounds

Experiment 1 consisted of an *in vitro* batch culture incubation for 24 h to evaluate the dose–response effect of AT at different concentrations (0, 1, 2, 3, 4 and 5% of the substrate on a DM basis) on rumen fermentation. The methanogenesis inhibitor BES was also evaluated at six doses (0, 4, 8, 12, 16 and 20 μM). A total of 48 Wheaton bottles were used according to the following design: two inhibitors (AT and BES) × six doses for each animal inocula (n = 4). The 0% dose of AT was considered the control treatment.

Experiment 2 consisted of a dose–response *in vitro* batch culture incubation for 24 h to investigate the effect of various preselected organic compounds on rumen fermentation. Phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid as electron acceptors, and formic acid as an electron donor, were evaluated at different doses (0, 2, 4 and 6 mM), to examine their effects on rumen fermentation, and identify concentrations that could negatively affect VFA production or feed degradation. The phenolic compounds were selected based on the degradation pathways described by Tsai et al. (1976), Patel et al. (1981), Krumholz and Bryant (1986), and McSweeney et al. (2001). The incubation used a total of 128 Wheaton bottles based on the following design: eight phenolic compounds × four doses for each animal inocula (n = 4). The 0 mM dose for each phenolic compound was considered the control treatment.

Experiments 1 and 2 followed the protocol described in Yáñez-Ruiz et al., (2016). Rumen fluid was obtained from four rumen-fistulised Murciano-Granadina adult goats (n = 4) adapted (>3 wks) to a diet of 70% alfalfa hay and 30% barley grain on a DM basis (same feeds and proportions of substrates used in the incubations). Rumen fluid was sampled before the morning feeding, filtrated through a double layer of cheese cloth, and mixed with prewarmed incubation buffer (0.35 g/L $NaHCO_3$, 0.04 g/L $(NH_4)HCO_3$; Mould et al., 2005) in a 1:2 ratio. At that point, 50 mL of inoculated medium was anaerobically dispensed to 120-mL Wheaton bottles containing 0.5 g DM substrate.

Immediately after inoculations, bottles were sealed, gently mixed, and kept in an incubator at 39 °C for 24 h. Gas pressure in the headspace of the bottles was measured at 6 and 24 h using a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ, USA) and sampled in standard PVDF gas-tight bags (Cole-Parmer Kynar, Vernon Hills, IL, USA) for CH_4 and H_2 analysis. Culture content was also sampled at 24 h and divided into two subsamples: the first sample (0.8 mL) was diluted with 0.8 mL of an acid solution (0.5 N HCl, 20 g/L metaphosphoric acid containing 0.8 g/L of crotonic acid as internal standard) and stored at −20 °C until VFA determination. The second sample (1.6 mL) was diluted with 0.4 mL of trichloroacetate solution (250 g/L) and stored at −20 °C for lactate and ammonia analysis. Incubation pH was measured at the beginning and end of the incubation.

Experiment 3: Effect of combined treatment of phenolic compounds and methanogenesis inhibitor

Considering the previous results, experiment 3 was carried out to evaluate the effect of combining AT with the eight previously selected phenolic compounds at a fixed dose of 6 mM, compared to AT alone. The *in vitro* experiment consisted of a 5-day sequential batch incubation using 68 Wheaton bottles according to the following design: eight phenolic compounds combined with AT at 2% DM × 2 doses of the phenolic compound (0 and 6 mM), plus one bottle with substrate alone for each animal inocula (n = 4). The 0 mM dose for each phenolic compound was equivalent to the treatment including only AT, which was considered the control treatment. A 5-day sequential batch incubation was used instead of a 24 h incubation period to promote adaptation of the rumen microbiota to the presence of phenolic compounds and facilitate their degradation (Theodorou et al., 1987). The same inocula, basal substrate and incubation set up (Wheaton bottles) described in Exp. 1 and 2 were used. To maintain the microbial fermentation for 5 days, one-third of the incubation volume was daily transferred using a 20 mL syringe from one *in vitro* batch culture to a new one with 33.3 mL fresh buffer and 0.5 g DM substrate, and a new dose of each phenolic compound was added to the corresponding bottle. AT at 2% DM was not added to the substrate of each bottle until the last transfer. Gas pressure in the headspace of the bottles was measured and released daily at 6 h and 24 h after each transfer. On the fifth day of incubation, headspace gas and culture content were sampled for CH_4 and H_2 determination as described above.

Experiment 4: The optimum concentration of phloroglucinol combined with methanogenesis inhibitor

Based on the results obtained in Exp. 3, another 5-day *in vitro* sequential batch incubation was performed to identify the optimal dose of the phenolic compound showing the greatest potential as H_2 acceptor when the microbiota was adapted to metabolise such substances. Phloroglucinol, the selected phenolic compound, was evaluated at five doses: 0, 6, 16, 26 and 36 mM. A total of 24 Wheaton bottles were used based on the following design: phloroglucinol combined with AT at 2% DM × five doses of phloroglucinol, plus one bottle with substrate alone for each animal inocula (n = 4). The 0 mM dose of phloroglucinol was equivalent to the AT-only treatment, which was considered the control treatment. The procedure followed during this incubation was the same as described in Exp. 3. Additionally, 1 mL of culture content was collected and centrifuged at 16 000 *g* for 15 min at 4 °C. After the centrifugation, the supernatant was discarded, and the pellet was stored at −20 °C until gDNA extraction.

Sample analyses

Methane and dihydrogen concentrations in the headspace samples were determined using a micro gas chromatography system (Agilent 490, Santa Clara, CA, USA) and a certified standard gas mix (Messer Gases for Life, Tarragona, Spain) with the following composition: 1% H_2, 3% O_2, 20% CH_4, 26% N_2, 50% CO_2. The GC analyses were carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (SIC-EEZ), CSIC, Granada, Spain. Total gas production (**TGP**) was calculated by transforming pressure measurements into volume units using the Ideal Gas Law under 1 atm and 39 °C. Concentrations of individual VFA (acetate,

propionate, isobutyrate, butyrate, isovalerate and valerate) were determined with a GC system (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). Lactate and ammonia concentrations were measured using the colorimetric methods described by Barker and Summerson (1941) and Weatherburn (1967), respectively. In the Barker and Summerson method, lactic acid is oxidised to acetaldehyde in hot concentrated sulfuric acid. Then, the colour reaction between acetaldehyde and p-hydroxydiphenyl in concentrated sulfuric acid (cold) is utilised for colorimetric measurement. In the Weatherburn method, phenol plus nitroprusside and alkaline hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonia concentration present. Microbial gDNA was extracted from frozen samples using DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative PCR was used to quantify the copies of the 16S rRNA gene for bacteria, mcrA gene for archaea, 18S rRNA genes for protozoa, and the region between 18S rRNA gene and ITS1 for anaerobic fungi. Primers and qPCR conditions used have been previously described in Palma-Hidalgo et al. (2021).

Statistical analyses

Data from *in vitro* experiments were statistically analysed by one-way ANOVA as follows:

Experiment 1:

$Y_{ij} = \mu + I_i + G_j + e_{ij}$ (one for each inhibitor).

Experiment 2:

$Y_{ij} = \mu + D_i + G_j + e_{ij}$ (one for each phenolic compound).
$Y_{ij} = \mu + C_i + G_j + e_{ij}$ (to compare phenolic compounds).

Experiment 3:

$Y_{ij} = \mu + W_i + G_j + e_{ij}$.

Experiment 4:

$Y_{ij} = \mu + X_i + G_j + e_{ij}$.

where Y_{ij} represents a dependent, continuous variable, μ is the overall population mean, I_i is the fixed effect of the dose of the methanogenesis inhibitor (AT or BES), D_i is the fixed effect of the dose of the phenolic compound (phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid or formic acid), C_i is the fixed effect of the type of phenolic compound at 6 mM, W_i is the fixed effect of the type of phenolic compound at 6 mM when combined with AT at 2% DM, X_i is the fixed effect of the dose of phloroglucinol when combined with AT at 2% DM, G_j represents the random effect of the goat used as donor, and e_{ij} is the residual error. When significant effects were detected, polynomial contrasts were used to determine linear and/or quadratic responses in Exp. 1, 2 and 4, and means were compared by Fisher's protected LSD test using the StatGraphics Centurion 19 software (StatPoint Technologies, Inc. USA, 2020). Significant effects were declared at $P < 0.05$ and tendencies at $0.05 \leq P < 0.10$.

Results

Selecting the inclusion level of methanogenesis inhibitor (Exp. 1)

Table 1 shows the dose–response effect of AT at the inclusion rates of 0, 1, 2, 3, 4, and 5% DM, on *in vitro* rumen fermentation. Increasing the concentration of AT promoted a decrease ($P < 0.001$) in rumen CH_4 production by up to 99%. The inhibition of methanogenesis was accompanied by an increase ($P < 0.01$) in rumen H_2 accumulation, reaching a plateau above 2% DM. Moreover, the VFA profile changed by the inclusion of AT, with acetate molar proportion decreased ($P < 0.001$) and propionate increased ($P < 0.001$) in a dose-dependent way. As a result, the acetate:propionate ratio decreased ($P < 0.001$). No significant effects were observed on other fermentation variables, such as TGP, pH, lactate, and ammonia concentration.

Experiment 1 also revealed the dose–response effect of BES at 4, 8, 12, 16, or 20 μM on *in vitro* rumen fermentation (Supplementary Table S1). Methane emissions decreased ($P < 0.01$) with the dose, reaching a 74% reduction at the highest concentration tested (BES at 20 μM). Methane inhibition resulted in a tendency to increase ($P = 0.092$) H_2 accumulation and a shift in the fermentation pattern towards less acetate ($P < 0.01$) and more propionate formation ($P < 0.001$), decreasing the acetate:propionate ratio ($P < 0.001$).

Table 1
Dose-response effect of *A. taxiformis* (0, 1, 2, 3, 4, 5% DM) on 24 h *in vitro* fermentation in rumen fluid from goats (Exp. 1).

Item	CTL	AT1	AT2	AT3	AT4	AT5	SEM	*P*-value	Contrasts
pH	6.45	6.35	6.36	6.38	6.37	6.40	0.02	0.612	NS
TGP (mL)	125	117	121	122	124	122	3.11	0.992	NS
CH_4 (mL)	38.0^{a}	2.13^{b}	0.34^{b}	0.10^{b}	0.12^{b}	0.10^{b}	3.43	<0.001	L**, Q***
H_2 (mL)	0.28^{c}	2.46^{c}	10.5^{b}	16.5^{ab}	29.0^{a}	19.9^{ab}	2.62	0.004	L***
NH_3-N (mg/100 mL)	53.0	42.9	50.0	47.8	43.4	42.2	3.08	0.912	NS
Lactate (μg/mL)	11.7	11.2	10.9	12.5	12.2	12.5	0.62	0.974	NS
Total VFA (mM)	113	106	109	105	105	103	3.79	0.987	NS
VFA (mol/100 mol)									
Acetate	61.7^{a}	53.9^{b}	53.1^{b}	52.3^{b}	52.5^{b}	52.4^{b}	0.76	<0.001	L***, Q***
Propionate	19.3^{b}	25.6^{a}	25.7^{a}	26.3^{a}	26.1^{a}	26.0^{a}	0.54	<0.001	L***, Q***
Isobutyrate	1.38	1.10	1.15	1.03	0.93	0.98	0.06	0.352	NS
Butyrate	14.0	15.9	16.4	16.9	17.0	17.2	0.38	0.142	NS
Isovalerate	1.98	1.53	1.55	1.48	1.38	1.45	0.09	0.446	NS
Valerate	1.69	2.05	2.10	2.05	2.08	2.13	0.06	0.283	NS
A:P	3.20^{a}	2.11^{b}	2.06^{b}	1.99^{b}	2.01^{b}	2.01^{b}	0.09	<0.001	L***, Q***

Abbreviations: CTL = control (substrate alone); AT = *A. taxiformis* at different concentrations: 0 (AT0), 1 (AT1), 2 (AT2), 3 (AT3) 4 (AT4) and 5% DM (AT5); VFA = volatile fatty acid; TGP = total gas production; A:P = acetate:propionate ratio.
$^{a-c}$Values within a row with different superscripts differ significantly at $P < 0.05$ (n = 4).
Contrast: NS, not significant; L, linear response; Q, quadratic response; $^{***}P < 0.001$.
$^{**}P < 0.01$.

The addition of AT at 2% DM was selected for the subsequent experiments, as it promoted a strong inhibition of rumen methanogenesis without impairing rumen fermentation.

Selecting the inclusion level of phenolic compounds (Exp. 2)

The inclusion of different phenolic compounds at concentrations of 2, 4 and 6 mM did not negatively alter rumen fermentation. The dose–response of each individual phenolic compound is provided in Supplementary Tables S2–S9. Table 2 summarises the effects of the eight phenolic compounds at their highest dose (6 mM) on rumen fermentation variables. Phloroglucinol and pyrogallol numerically decreased CH_4 production by 47% and 42%, respectively. No significant differences were observed in H_2 and TGP for any of the compounds. Phloroglucinol numerically increased total VFA production and significantly increased ($P < 0.01$) acetate molar proportion and acetate:propionate ratio ($P < 0.001$). In view of the above, the inclusion level of 6 mM was selected for the following experiment.

Effect of combined treatment of phenolic compounds and methanogenesis inhibitor (Exp. 3)

Table 3 shows the effect of combining the supplementation with a methanogenesis inhibitor (AT at 2% DM) and one of the eight phenolic compounds previously evaluated at a concentration of 6 mM compared with the AT-alone treatment. Methane production was significantly decreased ($P < 0.01$) when AT was supplemented in combination with every phenolic compound. Pyrogallol and phloroglucinol were the compounds that achieved the greatest nominal decrease in H_2 accumulation. Total gas production was increased with phloroglucinol or formic acid supplementation ($P < 0.001$). Phloroglucinol also increased ($P < 0.001$) total VFA production reaching a higher value than the AT treatment alone. Phloroglucinol supplementation also changed the VFA profile by increasing ($P < 0.001$) the acetate molar proportion and decreasing the molar proportion of propionate ($P = 0.028$) and isovalerate ($P = 0.025$). Although phloroglucinol significantly decreased the pH of the rumen culture, ammonia and lactate concentrations were not affected by the addition of the compound. Thus, phloroglucinol was chosen to be further evaluated at increasing concentrations in a CH_4 inhibition scenario in the subsequent experiment.

The optimum concentration of phloroglucinol combined with methanogenesis inhibitor (Exp. 4)

Table 4 shows the effect of increasing doses of phloroglucinol (6, 16, 26 and 36 mM) when added in combination with AT at 2% DM compared with the inhibitor-only treatment (the 0 mM dose). Methane production was further inhibited ($P < 0.001$) by the addition of phloroglucinol at the lowest concentration used (6 mM) compared with the addition of the AT alone, whereas H_2 accumulation decreased ($P < 0.001$) as dosage increased. Phloroglucinol at a dose of 16 mM and above promoted a decrease ($P < 0.01$) in H_2 released per mol of CH_4 decreased, as well as an increase ($P < 0.001$) in TGP. An increase in total VFA production was also observed in a dose-dependent way, reaching the maximum level with the 36 mM dose. Phloroglucinol at the maximum effective dose increased acetate ($P < 0.001$) and butyrate ($P = 0.031$) molar proportions, whereas propionate, isobutyrate, isovalerate and valerate molar proportions were decreased ($P < 0.001$). Consequently, the acetate:propionate ratio was increased ($P < 0.001$). In addition, phloroglucinol at 36 mM caused a reduction ($P < 0.001$) of the pH and the ammonia concentration, and an increment ($P < 0.001$) of the lactate concentration. The minimum and maximum doses of phloroglucinol (6 and 36 mM) were considered to evaluate the effect of phloroglucinol on the microbial communities. Phloroglucinol at 6 mM did not affect the abundance of the microbial groups. Phloroglucinol at 36 mM had no significant effect on the abundance of total bacteria, whereas it significantly decreased the abundances of protozoa ($P < 0.001$), archaea ($P < 0.001$) and fungi ($P = 0.013$) compared to the AT treatment.

Discussion

Effects of Asparagopsis taxiformis

In agreement with previous studies (Kinley et al., 2016a; Machado et al., 2016), our results evidenced that AT is a potent inhibitor of rumen methanogenesis, being as effective as the model inhibitor BES. In Exp. 1, the lowest dose of AT used (1% DM) was sufficient to decrease CH_4 production by 95%, while higher doses (2–5% DM) inhibited CH_4 production to undetectable levels. The effect of AT observed in this study agreed with Machado et al. (2016), which reported an 85% reduction of CH_4 production with 1% (OM basis) and a nearly total reduction at doses above 2%. Kinley et al. (2016a) also reported that CH_4 production was virtually eliminated at an inclusion rate of 2% of the OM intake. At 5%

Table 2
Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid and formic acid at 6 mM on 24 h *in vitro* fermentation in rumen fluid from goats (Exp. 2).

Item	CTL	Phe	Cat	Res	Hyd	Phl	Pyr	GA	FA	SEM	*P*-value
pH	6.46^{a}	6.46^{a}	6.48^{a}	6.47^{a}	6.47^{a}	6.35^{b}	6.46^{a}	6.47^{a}	6.44^{a}	0.01	0.009
TGP (mL)	132	132	131	131	130	133	132	132	136	1.32	0.994
CH_4 (mL)	18.3	20.2	23.5	19.1	19.3	9.78	10.7	21.6	13.0	1.65	0.562
H_2 (mL)	0.27	0	0	0	0	0.01	0.01	0	0	0.05	0.884
NH_3-N (mg/100 mL)	157	141	141	161	133	151	157	139	157	6.72	0.987
Lactate (μg/mL)	10.4	11.1	11.3	7.72	11.6	8.02	12.0	13.0	11.2	0.87	0.936
Total VFA (mM)	110	108	111	112	114	126	108	111	113	1.50	0.206
VFA (mol/100 mol)											
Acetate	61.2^{b}	61.2^{b}	60.6^{b}	61.2^{b}	60.7^{b}	64.3^{a}	61.0^{b}	60.8^{b}	60.4^{b}	0.23	0.002
Propionate	18.5	18.9	19.0	18.7	19.0	17.0	18.7	18.7	18.7	0.17	0.214
Isobutyrate	1.21	1.10	1.20	1.20	1.20	1.13	1.10	1.08	1.13	0.03	0.899
Butyrate	15.3	14.8	15.3	15.0	15.2	14.2	15.3	15.5	15.6	0.21	0.942
Isovalerate	2.30	2.25	2.38	2.45	2.48	2.15	2.60	2.53	2.75	0.06	0.444
Valerate	1.46^{bc}	1.80^{a}	1.48^{bc}	1.43^{bc}	1.43^{bc}	1.28^{c}	1.35^{bc}	1.40^{bc}	1.60^{ab}	0.04	0.049
A:P	3.31^{b}	3.24^{b}	3.19^{b}	3.28^{b}	3.21^{b}	3.81^{a}	3.27^{a}	3.25^{a}	3.25^{a}	0.04	<0.001

Abbreviations: CTL = control (substrate alone); Phe = phenol; Cat = catechol; Res = resorcinol; Hyd = hydroquinone; Phl = phloroglucinol; Pyr = pyrogallol; GA = gallic acid; FA = formic acid; TGP = total gas production; VFA = volatile fatty acid; A:P = acetate:propionate ratio.
$^{a–c}$Values within a row with different superscripts differ significantly at $P < 0.05$ (n = 4).

Table 3
Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid and formic acid at 6 mM when combined with *A. taxiformis* at 2% DM on *in vitro* fermentation after 5 days of incubation in rumen fluid from goats (Exp. 3).

Item	S	Treatments									SEM	*P*-value
		AT	AT + Phe	AT + Cat	AT + Res	AT + Hyd	AT + Phl	AT + Pyr	AT + GA	AT + FA		
pH	6.53 ± 0.01	6.50^{ab}	6.51^{a}	6.51^{a}	6.50^{ab}	6.50^{ab}	6.35^{c}	6.52^{a}	6.51^{a}	6.47^{b}	0.01	<0.001
TGP (mL)	95.9 ± 2.46	77.6 ^{cd}	77.8 ^{cd}	77.1 ^{cd}	79.2^{bcd}	75.0^{d}	87.0^{a}	76.9 ^{cd}	80.1^{bc}	82.6^{ab}	0.68	<0.001
CH_4 (mL)	8.91 ± 0.93	0.11^{a}	0.00^{b}	0.01^{b}	0.01^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.01	0.007
mL CH_4/mmol VFA	1.81 ± 0.20	0.03^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.002	0.007
H_2 (mL)	0.14 ± 0.02	2.17	1.93	2.52	2.12	1.52	1.36	1.02	1.70	2.30	0.13	0.155
mL H_2/mmol VFA	0.03 ± 0.00	0.54^{a}	0.51^{abc}	0.66^{a}	0.55^{a}	0.39^{abc}	0.28^{bc}	0.25^{c}	0.41^{abc}	0.58^{a}	0.04	0.081
mol H_2/mol CH_4 decreased	–	0.25	0.23	0.29	0.25	0.20	0.16	0.12	0.19	0.27	0.02	0.211
NH_3-N (mg/100 mL)	15.5 ± 2.86	13.0	13.6	13.4	14.8	14.3	14.7	12.0	11.6	15.2	0.68	0.954
Lactate (μg/mL)	5.09 ± 0.31	4.23^{a}	2.22^{bc}	3.77^{ab}	1.64^{c}	3.55^{abc}	3.06^{abc}	1.62^{c}	2.25^{bc}	2.68^{abc}	0.24	0.025
Total VFA (mM)	98.9 ± 2.99	81.3^{bc}	74.4^{c}	77.3^{bc}	79.1^{bc}	77.9^{bc}	95.7^{a}	81.5^{bc}	80.5^{bc}	79.2^{bc}	1.16	<0.001
VFA (mol/100 mol)												
Acetate	60.2 ± 0.36	53.1^{b}	50.5 ^{cd}	47.1^{e}	49.2^{d}	50.8 ^{cd}	57.3^{a}	51.2^{c}	50.8 ^{cd}	52.0^{bc}	0.46	<0.001
Propionate	24.4 ± 0.36	28.0^{ab}	24.9^{bc}	25.0^{bc}	27.8^{ab}	29.8^{a}	23.1^{c}	26.6^{abc}	27.4^{ab}	28.4^{ab}	0.48	0.028
Isobutyrate	1.40 ± 0.16	1.19	1.25	1.13	1.18	1.03	1.08	1.10	1.28	1.18	0.04	0.895
Butyrate	10.1 ± 0.53	13.5^{c}	18.3^{a}	16.6^{ab}	15.2^{bc}	13.9^{bc}	14.9^{bc}	16.0^{abc}	15.5^{abc}	14.0^{bc}	0.38	0.042
Isovalerate	1.38 ± 0.14	1.04^{ab}	1.13^{a}	1.05^{ab}	1.00^{abc}	0.90^{d}	0.88^{d}	0.98 ^{cd}	1.05^{ab}	0.98 ^{cd}	0.02	0.025
Valerate	2.63 ± 0.18	3.30^{c}	4.00^{bc}	9.23^{a}	5.75^{b}	3.50^{bc}	2.75^{c}	4.20^{bc}	4.03^{bc}	3.48^{bc}	0.38	<0.001
A:P	2.47 ± 0.02	1.90^{bc}	2.05^{b}	1.91^{bc}	1.79^{c}	1.71^{c}	2.49^{a}	1.96^{bc}	1.87^{bc}	1.83^{bc}	0.04	<0.001

Abbreviations: S = substrate alone; AT = *A. taxiformis* at 2% DM tested alone or in combination with a phenolic compound at 6 mM; Phe = phenol; Cat = catechol; Res = resorcinol; Hyd = hydroquinone; Phl = phloroglucinol; Pyr = pyrogallol; GA = gallic acid; FA = formic acid; TGP = total gas production; VFA = volatile fatty acid; A:P = acetate:propionate ratio.
In the first column, mean ± SE. For the treatments, SEM.
The treatments were included in the statistical analysis (substrate alone was not).
[a–e]Values within a row with different superscripts differ significantly at $P < 0.05$ (n = 4).

Table 4
Effect of phloroglucinol at different doses (6, 16, 26, 36 mM) when combined with *A. taxiformis* at 2% DM on *in vitro* fermentation after 5 days of incubation in rumen fluid from goats (Exp. 4).

Item	S	Treatments					SEM	*P*-value	Contrasts
		AT	AT + Phl6	AT + Phl16	AT + Phl26	AT + Phl36			
pH	6.73 ± 0.02	6.68^{a}	6.50^{b}	6.18^{c}	5.86^{d}	5.78^{d}	0.07	<0.001	L***, Q**
TGP (mL)	94.7 ± 1.95	78.6^{c}	88.3^{b}	97.3^{a}	99.2^{a}	99.2^{a}	1.93	<0.001	L***, Q***
CH_4 (mL)	10.4 ± 1.42	0.13^{a}	0.03^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.02	0.014	L**, Q*
mL CH_4/mmol VFA	2.98 ± 0.42	0.05^{a}	0.01^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.01	0.015	L**, Q*
H_2 (mL)	0.13 ± 0.03	3.15^{a}	2.30^{ab}	1.61^{bc}	1.15^{c}	1.71^{bc}	0.20	0.002	L***, Q**
mL H_2/mmol VFA	0.04 ± 0.01	1.05^{a}	0.60^{b}	0.31^{bc}	0.21^{c}	0.28^{c}	0.08	<0.001	L***, Q***
mol H_2/mol CH_4 decreased	–	0.33^{a}	0.23^{ab}	0.16^{b}	0.13^{b}	0.18^{b}	0.02	0.009	L**, Q*
NH_3-N (mg/100 mL)	15.3 ± 1.98	18.0^{a}	13.9^{ab}	10.6^{b}	5.13^{c}	4.31^{c}	1.33	<0.001	L***
Lactate (μg/mL)	3.18 ± 0.47	2.20^{b}	1.97^{b}	3.46^{b}	7.89^{a}	8.42^{a}	0.60	<0.001	L***
Total VFA (mM)	70.0 ± 0.89	60.2^{e}	76.2^{d}	103^{c}	113^{b}	120^{a}	5.11	<0.001	L***, Q***
VFA (mol/100 mol)									
Acetate	61.6 ± 0.60	53.4^{d}	58.8^{c}	65.4^{b}	70.5^{a}	71.8^{a}	1.58	<0.001	L***, Q***
Propionate	19.2 ± 0.97	21.8^{a}	18.0^{b}	12.1^{c}	7.40^{d}	6.45^{d}	1.34	<0.001	L***, Q***
Isobutyrate	1.05 ± 0.06	1.04^{a}	0.90^{ab}	0.78^{b}	0.50^{c}	0.45^{c}	0.05	<0.001	L***
Butyrate	12.9 ± 0.92	17.3^{c}	17.7^{bc}	18.4^{abc}	19.7^{ab}	19.8^{a}	0.35	0.031	L***
Isovalerate	1.35 ± 0.06	1.16^{a}	0.95^{b}	0.65^{c}	0.48^{d}	0.38^{d}	0.07	<0.001	L***, Q**
Valerate	4.03 ± 0.46	5.31^{a}	3.72^{b}	2.58^{c}	1.35^{d}	1.10^{d}	0.37	<0.001	L***, Q**
A:P	3.24 ± 0.15	2.47^{d}	3.28^{d}	5.45^{c}	9.68^{b}	11.3^{a}	0.75	<0.001	L***
Microbe (log copies/mL)									
Bacteria	11.6 ± 0.07	11.6	11.6			11.5	0.03	0.593	NS
Protozoa	8.32 ± 0.33	8.22^{a}	8.31^{a}			5.82^{b}	0.38	<0.001	L***
Archaea	8.14 ± 0.01	6.65^{a}	6.81^{a}			5.23^{b}	0.22	<0.001	L***, Q*
Fungi	6.44 ± 1.10	6.44^{a}	5.54^{a}			2.75^{b}	0.60	0.013	L**

Abbreviations: S = substrate alone; AT = *A. taxiformis* at 2% DM tested alone or in combination with phloroglucinol at different concentrations: 6 (AT + Phe6), 16 (AT + Phe16), 26 (AT + Phe26) and 36 mM (AT + Phe36). TGP = total gas production; VFA = volatile fatty acid; A:P = acetate:propionate ratio.
In the first column, mean ± SE. For the treatments, SEM.
The treatments were included in the statistical analysis (substrate alone was not).
[a–e]Values within a row with different superscripts differ significantly at $P < 0.05$ (n = 4).
Contrast: NS, not significant; L, linear response; Q, quadratic response; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

AT in OM, Roque et al. (2019) reported a 95% reduction in CH_4 production.

The CH_4 reduction reached at the lowest dose of AT (−95%) was greater than that provided by the highest dose of BES (−74%). Other *in vitro* studies have reported that BES at a dose range of 0.03 to 0.04 mM is able to reduce CH_4 by more than 95% (Nollet et al., 1997; Ungerfeld et al., 2004; Zhang and Yang, 2012). Based on this, AT and BES would have similar inhibitory activity than other halogenated compounds (bromochloromethane, 89–94%; Goel et al., 2009) or substances of different nature, such as synthetic mononitrate compounds (3-nitrooxypropanol, 75%; Guyader et al., 2017). Moreover, the addition of AT also increased

dihydrogen accumulation in an inverse manner to CH_4 production, as observed with expelled H_2 in previous *in vivo* studies with 3-nitrooxypropanol (Hristov et al., 2015; Vyas et al., 2018). Elevation of H_2 was expected as methanogenesis is the main pathway of H_2 incorporation in the rumen. This increase of H_2 partial pressure might lead to mechanisms of H_2 regulation, mainly a displacement from H_2-producing acetate to H_2-incorporating propionate production (Janssen, 2010). This could explain the decrease in the acetate: propionate ratio observed in this study, as reported by Roque et al. (2019) and Kinley et al. (2016a). Acetate production decreases as it generates H_2, while propionate production increases as it consumes H_2. This change in VFA profile did not result in negative effects on meat quality in beef cattle (Kinley et al., 2020), although it is hypothesised that AT could have a negative impact on the milk fat content of lactating ruminants (Roque et al., 2019). In contrast to Machado et al. (2016), total VFA production did not decrease. The rest of the fermentation variables studied were not affected by the inclusion of AT. Based on these results and previous studies, AT at a dose of 2% DM was decided to be optimal to provide a strong inhibition of methanogenesis without compromising microbial fermentation in subsequent experiments.

Effects of phenolic compounds

Some CH_4 mitigation strategies promote metabolic pathways that can compete with archaea for H_2 (McAllister and Newbold, 2008). Organic acids, such as propionate precursors, can be reduced by rumen bacterial species using H_2 as electron donor. Aspartate, malate, fumarate and acrylate have been tested as direct H_2 acceptors, or compounds metabolised to electron acceptors (Newbold et al., 2005; Ungerfeld et al., 2003). However, the use of these organic acids as feed additives presents some drawbacks that limit their practical application, such as concerns over palatability (Bayaru et al., 2001), rumen pH decrease (Asanuma et al., 1999), inconsistency of the effects produced, or their high cost (Newbold and Rode, 2006).

Thus, there is an interest in searching for compounds with commercial potential that can be metabolically reduced by rumen microbes and, at the same time, yield end products that can be used by the host animal as energy sources. There is a group of tannins that can be hydrolysed into carbohydrates and phenolic constituents, mainly gallic acid, which is further converted to pyrogallol, phloroglucinol or resorcinol by the rumen microbiota (McSweeney et al., 2001). These can finally be reduced to acetate while incorporating H_2 (Tsai et al., 1976).

Results from Exp. 2 demonstrated that the inclusion of phenolic compounds at 2, 4 or 6 mM in the rumen did not have adverse effects on rumen fermentation. Phloroglucinol at 6 mM decreased CH_4 production by 47%, increased total VFA production by 15% and changed the VFA profile by increasing acetate production. In agreement with our results, Sarwono et al. (2019) reported an 18–32% reduction in CH_4 production when phloroglucinol was supplemented at 6 mM. They also reported that phloroglucinol could redirect rumen fermentation towards acetate production in high forage:concentrate diets (as noted in our study), but inhibited rumen fermentation without increasing acetate in low forage:concentrate diet.

Effects of phenolic compounds in a methane inhibition scenario

Fermentation of carbohydrates in the rumen, particularly the formation of the three main VFAs (acetate, propionate and butyrate; Russell and Wallace, 1997), involves an important flow of metabolic hydrogen that living organisms can use for their anabolic functions (Ungerfeld, 2020). Under normal rumen metabolic conditions, the majority of the H_2 produced is transferred to rumen methanogens to produce CH_4 (Morgavi et al., 2010). When methanogenesis is inhibited, a proportion of H_2 can be redirected into energy-yielding metabolites such as propionate while an important proportion of the unutilised H_2 accumulates in the rumen, limiting the reoxidation of reduced cofactors (NADH, Fd_{red}), and thus decreasing nutrient catabolism (Wolin et al., 1997). Furthermore, H_2 eructation is considered as a moderate energy loss to the animal (Guyader et al., 2017; Martinez-Fernandez et al., 2016). To overcome this problem, several reports have identified substrates that can capture rumen H_2 and promote alternative metabolic pathways: acetogenesis (Gagen et al., 2015), propionogenesis (Newbold et al., 2005) or increases in microbial biomass production (Ungerfeld, 2015). Theoretically, phenolic compounds are considered H_2 acceptors as they can be degraded by several rumen bacteria classified as *Eubacterium oxidoreducens*, *Streptococcus bovis* and *Coprococcus* spp., with metabolic hydrogen consumption (Tsai et al, 1976; Patel et al., 1981; Krumholz and Bryant, 1986). *Eubacterium oxidoreducens* degrade phloroglucinol to acetate and butyrate, whereas *Coprococcus* spp. degrade phloroglucinol to acetate (Conradt et al., 2016). Martinez-Fernandez et al. (2017) demonstrated that the potential of the organic compound phloroglucinol as H_2 acceptor was maximised when supplemented in combination with chloroform as a methanogenesis inhibitor in the rumen of steers. The present research described for the first time *in vitro* in goat rumen content the effect of a range of phenolic compounds supplemented in combination with AT, an algae-based methanogenesis inhibitor with promising *in vivo* applications.

In Exp. 3, CH_4 production was further reduced by all phenolic compounds when combined with AT compared with AT alone. Phloroglucinol and pyrogallol at 6 mM reduced H_2 accumulation, but only phloroglucinol increased total VFA production and acetate molar proportion. These results agree with studies by Tsai et al. (1976), Patel et al. (1981), and Conradt et al. (2016), who reported that *Coprococcus* spp. could reduce phloroglucinol to acetate using NADPH as the electron donor, thereby alleviating the partial pressure of H_2 in the rumen. Specifically, three phloroglucinol reductases belonging to the family of NADPH dehydrogenases/reductases have been identified as responsible for the anaerobic degradation of phloroglucinol, which involves hydrolytic ring cleavage to 3-hydroxy-5-oxohexanoic followed by acetate formation (Conradt et al., 2016). In agreement with Martinez-Fernandez et al. (2017), the present study showed that phloroglucinol addition in the rumen when methanogenesis had been inhibited resulted in greater acetate formation with less gas H_2 produced per mole of CH_4 decreased. Therefore, the negative impact of H_2 accumulation in the rumen caused by AT supplementation can be alleviated by the addition of phloroglucinol, stimulating microbial groups which utilise H_2 as reductant in the metabolism of phloroglucinol to acetate and thus decrease the partial pressure of H_2.

Given these positive outcomes, it was important to evaluate a wider range of phloroglucinol concentrations in order to achieve the maximum potential efficiency on rumen fermentation. The results showed that increasing doses of phloroglucinol led to a response on the fermentation variables studied. Tsai et al. (1976) reported that one molecule of H_2 is used to degrade one molecule of phloroglucinol to two molecules of acetate and two molecules of CO_2. In agreement, our study found an inverse relationship between H_2 accumulation and acetate. As H_2 accumulation (expressed in relation to VFA production) decreased with the dose of phloroglucinol, acetate concentration increased in a dose-dependent manner (Fig. 1). Moreover, the progressive increase in TGP observed in this study could be related to the increase in VFA production but also to the CO_2 production caused by the degradation of phloroglucinol (Tsai et al., 1976).

The increasing doses of phloroglucinol also resulted in a decrease in pH that could be explained by the increase in total

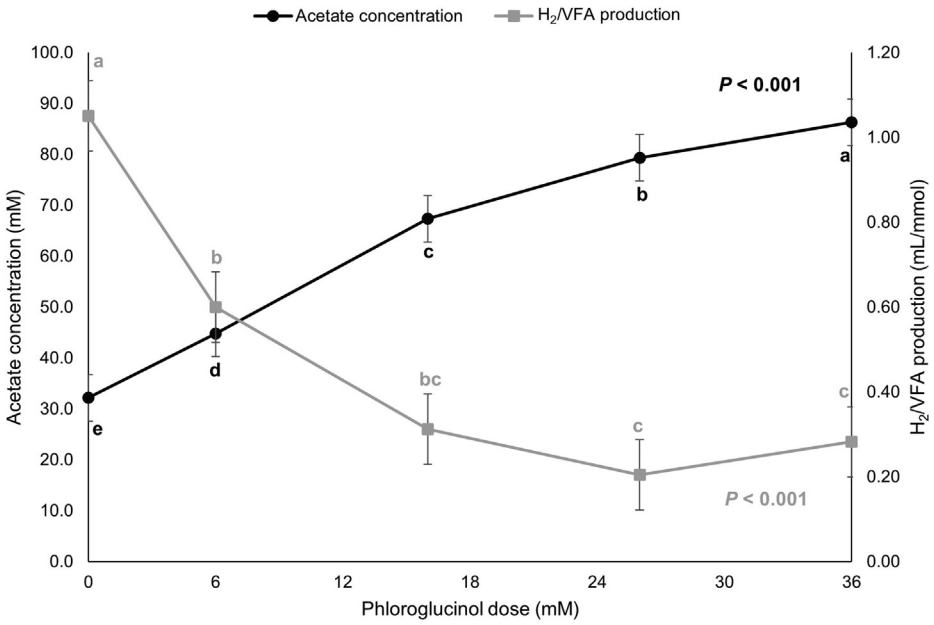

Fig. 1. Effect of phloroglucinol at different initial concentrations (0, 6, 16, 26, 36 mM) when combined with *A. taxiformis* at 2% DM on dihydrogen production per total volatile fatty acid production (mL/mmol) and acetate concentration (mM) after 5 days of incubation using rumen fluid from goats (Exp. 4). VFA = volatile fatty acid. [a–e]Within a row means with different superscripts differ (n = 4).

VFA, which in turn could be interpreted as a further indication of increased fermentative activity (Ungerfeld, 2020). Moreover, the lactate accumulation and the decrease in propionate molar proportion reported in this research could indicate an inhibition of the acrylate pathway (Prabhu et al., 2012), which would be competing for reducing equivalents with the phloroglucinol degradation pathway. The decrease in ammonia concentration observed in this study could be associated with the ability of phenolic compounds to bind dietary proteins via their hydroxyl groups, increasing the resistance of the proteins to microbial degradation (McSweeney et al., 2001). With some diets, a decrease in the concentration of ammonia in the rumen may lead to a decrease in urinary N excretion, which would result in reduced emissions of nitrous oxide and therefore of greenhouse gas (Carulla et al., 2005; Dijkstra et al., 2018). Additionally, the decrease in ammonia concentration is consistent with the decrease in the molar proportion of isobutyrate and isovalerate, which originate from deamination of branched-chain amino acids (Apajalahti et al., 2019).

The addition of phloroglucinol had no effect on the abundance of total bacteria. McSweeney et al. (2001) reported that polyphenolic compounds inhibit the population density of major cellulolytic bacteria in the rumen and lead to decrease on digestibility. Sarwono et al. (2019) showed that phloroglucinol supplementation lowered the relative abundance of *Ruminococcus albus*, but did not affect other cellulolytic species, such as *Ruminococcus flaveciens* or *Fibrobacter succinogenes*. Moreover, phloroglucinol would be expected to shift the composition of the bacterial population by stimulating specific microbial groups capable of using H_2 in the metabolism of phloroglucinol to acetate and butyrate. Martinez-Fernandez et al. (2017) reported that the combination of chloroform and phloroglucinol promoted an increase in the relative abundance of *Coprococcus* spp., which are relevant in phloroglucinol degradation. The present study also showed that the abundances of protozoa, archaea and fungi were decreased by phloroglucinol supplementation, which suggests that phloroglucinol could have a toxic effect on those communities. In agreement with that, de Paula et al. (2016) observed a reduction of *Entodinium* protozoa by different phenolic compounds. Kayembe et al. (2013) reported the toxicity of phenol, resorcinol, hydroquinone and pyrogallol to methanogens, and demonstrated the inverse correlation of the methanogenic toxicity with the number of hydroxyl groups on the aromatic compound. Sarwono et al. (2019) also observed a decrease in the relative abundance of methanogens with the addition of phloroglucinol. Zuhainis et al. (2007) found that certain phenolic monomers decreased rumen fungal population. Further analyses of the rumen microbiota are needed to clarify the mechanisms of action of phenolic compounds such as phloroglucinol in the rumen.

In Martinez-Fernandez et al. (2017) *in vivo* study, phloroglucinol was supplied in the rumen by progressively increasing its dose during the first 10 days up to 75 g/100 kg LW, which was estimated to be equivalent to about 40 mM in the rumen. In agreement, our study demonstrated that phloroglucinol could be added to the rumen up to similar concentrations to maximise H_2 capture and acetate production without compromising the rumen function. The present work with AT confirms the hypothesis previously demonstrated by Martinez-Fernandez et al. (2017) using chloroform as a methanogenesis inhibitor. The practical potential of this CH_4 mitigation strategy now needs to be verified by further *in vivo* studies.

The results presented here using rumen fluid from goats were similar to those obtained with rumen fluid from dairy cows (Huang et al., companion paper). However, the response of CH_4 production *in vitro* to the same batch of AT used in both studies was slightly different. AT provided at 2% DM substantially (>99%) inhibited CH_4 production when using goats' rumen fluid, whereas using cows' rumen fluid at slightly higher doses of AT (2.5% DM) promoted a somewhat smaller CH_4 inhibition (75%). The effects of the phenolic compounds were similar in both animal species, suggesting that the combination of AT and phloroglucinol has potential to be used across different ruminant species.

Conclusion

Methanogenesis inhibition in rumen fluid from goats caused by supplementation of *Asparagopsis taxiformis* resulted in the accumulation of excess H_2. After screening a wide range of phenolic com-

pounds, phloroglucinol was shown to be the most effective to decrease the H_2 partial pressure in the *in vitro* system. Increasing doses of phloroglucinol as a dietary supplement enhanced the efficiency of H_2 capture. Therefore, a combined treatment consisting of AT and phloroglucinol can prevent the accumulation of H_2 in the rumen, promote an increase in acetate production, and an improvement in rumen fermentation. The potential of such nutritional interventions would need a further confirmation using an *in vivo* approach.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100789.

Ethics approval

Animal procedures 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 EEZ-CSIC (A/18/03/2019/042).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

Author ORCIDs

P. Romero: https://orcid.org/0000-0001-5746-8286.
R. Huang: https://orcid.org/0000-0002-3094-7700.
E. Jiménez: https://orcid.org/0000-0001-5175-2251.
J.M. Palma-Hidalgo: https://orcid.org/0000-0002-6237-1822.
E.M. Ungerfeld: https://orcid.org/0000-0002-5422-5462.
M. Popova: https://orcid.org/0000-0001-6695-5502.
D.P. Morgavi: https://orcid.org/0000-0002-3883-0937.
A. Belanche: https://orcid.org/0000-0001-5880-6021.
D.R. Yáñez-Ruiz: https://orcid.org/0000-0003-4397-3905.

Author contributions

P. Romero: Methodology, investigation, resources, formal analysis, writing - original draft; **R. Huang:** Writing - review and editing; **E. Jiménez:** Methodology, investigation, resources; **J.M. Palma-Hidalgo:** Methodology, investigation, resources; **E.M. Ungerfeld:** Writing – review and editing; **M. Popova:** Conceptualisation, resources, writing - review and editing; **D.P. Morgavi:** Conceptualisation, resources, writing - review and editing; **A. Belanche:** Conceptualisation, validation, formal analysis, resources, writing - review and editing, supervision, project administration, funding acquisition; **D.R. Yáñez-Ruiz:** Conceptualisation, validation, formal analysis, resources, writing - review and editing, supervision, project administration, funding acquisition. All authors read and approved the final version.

Declaration of interest

The authors declare that they have no competing interests

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