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Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited *in vitro* – Part 1. Dairy cows

R. Huang [a], P. Romero [b], A. Belanche [b,c], E.M. Ungerfeld [d], D. Yanez-Ruiz [b], D.P. Morgavi [a,*], M. Popova [a]

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

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ABSTRACT

Some antimethanogenic feed additives for ruminants promote rumen dihydrogen (**H_2**) accumulation potentially affecting the optimal fermentation of diets. We hypothesised that combining an H_2 acceptor with a methanogenesis inhibitor can decrease rumen H_2 build-up and improve the production of metabolites that can be useful for the host ruminant. We performed three *in vitro* incubation experiments using rumen fluid from lactating Holstein cows: Experiment 1 examined the effect of phenolic compounds (phenol, catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, and gallic acid) at 0, 2, 4, and 6 mM on ruminal fermentation for 24 h; Experiment 2 examined the combined effect of each phenolic compound from Experiment 1 at 6 mM with two different methanogenesis inhibitors (*Asparagopsis taxiformis* or 2-bromoethanesulfonate (**BES**)) for 24 h incubation; Experiment 3 examined the effect of a selected phenolic compound, phloroglucinol, with or without BES over a longer term using sequential incubations for seven days. Results from Experiment 1 showed that phenolic compounds, independently of the dose, did not negatively affect rumen fermentation, whereas results from Experiment 2 showed that phenolic compounds did not decrease H_2 accumulation or modify CH_4 production when methanogenesis was decreased by up to 75% by inhibitors. In Experiment 3, after three sequential incubations, phloroglucinol combined with BES decreased H_2 accumulation by 72% and further inhibited CH_4 production, compared to BES alone. Interestingly, supplementation with phloroglucinol (alone or in combination with the CH_4 inhibitor) decreased CH_4 production by 99% and the abundance of methanogenic archaea, with just a nominal increase in H_2 accumulation. Supplementation of phloroglucinol also increased total volatile fatty acid (**VFA**), acetate, butyrate, and total gas production, and decreased ammonia concentration. This study indicates that some phenolic compounds, particularly phloroglucinol, which are naturally found in plants, could improve VFA production, decrease H_2 accumulation and synergistically decrease CH_4 production in the presence of antimethanogenic compounds.

Implications

Antimethanogenic additives can decrease the environmental hoofprint of ruminants. However, inhibition of methane production increases H_2 accumulation in the rumen and does not result in the production of useful end products for the host ruminant. This work evaluated the capacity of seven phenolic compounds to decrease rumen H_2 build-up and improve fermentation when methane production was inhibited in an *in vitro* dairy cow model. Phloroglucinol and also gallic acid decreased H_2 accumulation, archaeal abundance, and increased total volatile fatty acids, notably through acetate production. This study shows that H_2 acceptors like phloroglucinol have the potential to improve fermentation when methane inhibitors are used in the diet.

Introduction

Ruminants produce enteric methane (**CH_4**), accounting for 6% of anthropogenic greenhouse gas emissions and ~40% of greenhouse gas emissions from livestock (Gerber et al., 2013). Reducing enteric CH_4 emissions is important for the sustainability of the ruminant sector, and several approaches are being investigated (Beauchemin et al., 2020). One of the most effective strategies is

* Corresponding author.
E-mail address: diego.morgavi@inrae.fr (D.P. Morgavi).

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the inhibition of methanogenesis using feed additives. The macroalgae *Asparagopsis taxiformis* (**AT**) and the synthetic compound 3-nitrooxypropanol are effective feed additives showing a consistent reduction in enteric CH_4 emissions (Li et al., 2016, Dijkstra et al., 2018, Roque et al., 2021). Methane production also results in dietary energy losses of between 2 and 12% for ruminants (Johnson and Johnson, 1995). Theoretically, it may be expected that the feed energy saved by decreasing CH_4 production would improve the energy balance of the host animal. However, animal productivity does not increase correspondingly (Ungerfeld, 2018). For example, no differences in milk production were observed in a dairy cow study where CH_4 production was decreased 26% by 3-nitrooxypropanol (Melgar et al., 2020), although the authors estimated an additional 0.4 kg/d of milk could have potentially been produced. In another study on sheep, a decrease of up to 80% in CH_4 yield induced by AT inclusion did not improve live weight gain (Li et al., 2016).

This lack of concordance between energy saved by decreasing enteric CH_4 production and theoretical increases in animal performance remains largely unexplained (Ungerfeld, 2018). Dihydrogen (**H_2**) is the main substrate for rumen methanogens to produce CH_4 (Morgavi et al., 2010), and it accumulates in the rumen when methanogenesis is inhibited (Janssen, 2010, Ungerfeld, 2020). In theory, H_2 accumulation could limit the regeneration of reduced cofactors (NADH, Fd_{red}), decreasing nutrient catabolism (Wolin et al., 1997). However, *in situ* (Nolan et al., 2010, Martínez-Fernández et al., 2014, Zhang et al., 2020) and *in vivo* total tract (Jayanegara et al., 2017, Ungerfeld, 2018, Kim et al., 2020) apparent digestibility was not negatively affected by increased H_2 concentrations. Also, volatile fatty acid (**VFA**) concentrations were unaffected when adjusted by changes in DM intake, although effects on VFA production have not been fully characterised. Feed energy lost via H_2 eructation resulting from the inhibition of methanogenesis is relatively minor, although variable and dependent on the extent of methanogenesis inhibition (Ungerfeld et al., 2022). It is of interest to investigate if, when methanogenesis is inhibited, the rumen microbiota could use H_2 (otherwise expelled) to retain its energy in useful end products for the host animal, and if this could result in improved feed efficiency and productivity.

Rumen microbes can use H_2 or formate to catabolise phenolic compounds such as gallate, pyrogallol, and phloroglucinol to generate VFA (Evans, 1977, Krumholz and Bryant, 1986). It was reported that phloroglucinol decreased the ratio of mol H_2/mol CH_4, increased acetate concentration, and improved weight gain in beef cattle when methanogenesis was inhibited by chloroform (Martinez-Fernandez et al., 2017). In this study, we screened a range of phenolic compounds for their potential as H_2 acceptors in rumen fluid from dairy cows. We hypothesised that when methanogenesis is inhibited in the rumen, phenolic compounds can act as H_2 acceptors to capture excess H_2 and produce useful end products. This study used rumen fluid from lactating dairy cows to investigate in *in vitro* incubations: (1) the dose responses of various phenolic compounds on fermentation; (2) the effects of combining phenolic compound with a methanogenesis inhibitor; (3) the effects of a selected phenolic compound combined with a methanogenesis inhibitor in a longer incubation using sequential batch incubations. This work is part of a larger study in which these experiments were replicated using goats as rumen fluid donors (Romero *et al.*, companion paper). These two ruminant species develop different rumen microbial communities as a result of their production system and host control (Henderson et al., 2015, Corral-Jara et al., 2022). In addition, goats and cows have shown different responses to the presence of phenolic compounds in the diet (Robbins et al., 1987), which could result in distinct responses to the treatments evaluated in this study.

Material and methods

Holstein dairy cows used as rumen fluid donors were housed at the INRAE UE1414 Herbipôle Unit (Saint-Genès Champanelle, France; https://doi.org/10.15454/1.5572318050509348E12). The study consisted of three *in vitro* experiments: dose responses of preselected phenolic compounds (**Exp.1**), individual phenolic compounds at 6 mM combined with a methanogenesis inhibitor (AT at 1.5 or 2.5% of substrate on a DM basis or 3 μM 2-bromoethanesulfonate (**BES**)) (**Exp.2**), and longer-term effects of phloroglucinol combined with a methanogenesis inhibitor (**Exp.3**).

Substrates and methanogenesis inhibitors preparation

Alfalfa hay and barley grain ground through a 1-mm sieve were used as substrates for incubation. *Asparagopsis taxiformis*, a red macroalgae, and BES were chosen as methanogenesis inhibitors in this study. *Asparagopsis taxiformis* was obtained from SeaExpert (Faial, Portugal), and its bromoform concentration was 6 mg/g DM. It was freeze-dried and ground using a laboratory mill (IKA All analytical mill, Staufen, Germany). Two milling cycles (30 s) were performed, cooling down the mill with liquid nitrogen between cycles to preserve AT chemical integrity. The milled AT was filtered through a polyester monofilament fabric (1 mm aperture) and stored at 4 °C in a glass bottle sealed with a rubber stopper. 2-Bromoethanesulfonate was obtained from Sigma-Aldrich (Darmstadt, Germany), and a 10 mM stock solution was prepared and stored at 4 °C.

Experiment 1: Dose-responses of phenolic compounds

This experiment focused on selecting the highest inclusion concentration of phenolic compounds without negatively affecting fermentation. Seven phenolic compounds were preselected based on their theoretical capacity to incorporate H_2 during their degradation process. The phenolic compounds used in this study were phenol, catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, and gallic acid. Also, formic acid, a fermentation intermediate, which releases H_2 and thus acts as an electron donor in the rumen (Leng, 2014), was used as a positive control. All phenolic compounds were obtained from Sigma-Aldrich (Darmstadt, Germany). Phenolic compounds were dissolved in ethanol to prepare stock solutions at a concentration of 1 mol/L for phenol, catechol, resorcinol, hydroquinone and pyrogallol, and 0.5 mol/L for phloroglucinol and gallic acid. The stock solutions were stored in amber glass bottles at 4 °C. We tested four concentrations (0, 2, 4, and 6 mM in the fermentation fluid) of each phenolic compound based on published work (Murray et al., 1996, Getachew et al., 2008, Sarwono et al., 2019). The 0 mM concentration contained only the mixed alfalfa hay and barley grain substrate as a control. The required amount of each stock solution was added into a 125-mL serum bottle used for the incubation, and the ethanol was evaporated under a stream of O_2-free CO_2 before adding the substrates. Formic acid was directly added to the bottle after inoculation.

Four rumen-cannulated lactating Holstein cows were used as rumen inoculum donors. The cows were fed *ad libitum* a ration containing 67% forage (corn silage and grass silage) and 33% of concentrate (corn and soybean meal) on a DM basis, twice per day. Cows had free access to water and mineral salt blocks. Rumen contents were collected through the rumen cannula before the morning feeding, placed into preheated 1-L thermal flasks and immediately transported to the laboratory. The rumen content from each animal was processed separately by straining through a polyester monofilament fabric (250 μm aperture) to obtain individual rumen fluids. The rumen fluid from each cow was

subsequently mixed with warm (39 °C) anaerobic buffer solution at a 1:2 (volume to volume) ratio under a stream of O_2-free CO_2 (Mould et al., 2005, Yáñez-Ruiz et al., 2016). A 50 mL rumen fluid-buffer mixture was anaerobically dispensed into 125-mL serum bottles containing 500 mg of substrates composed of alfalfa hay and barley grain (70 and 30% in DM, respectively). Each batch incubation included a blank consisting of rumen fluid-buffer mixture from each cow with no substrate. The bottles were incubated in a water bath at 39 °C for 24 h. At 6 and 24 h incubation, gas pressure was measured using a pressure transducer (GE Sensing, Druck), and a 5-mL gas sample was collected with a syringe followed by the release of excess gas until the pressure in the headspace equalised to the atmospheric pressure. Following gas sampling at 24 h incubation, fermentation liquid was collected for VFA and ammonia analysis as described below. The total number of experimental units was [eight treatments (seven phenolic compounds and formic acid) × 3 concentrations (2, 4, and 6 mM) + 1 control (substrate alone without treatment)] × 4 (cows) = 100 that were used in the statistical analysis (see below). Rumen inocula from individual cows were considered biological replicates (n = 4).

Experiment 2: Effect of phenolic compounds when methanogenesis was inhibited

This experiment was designed to assess the effects of phenolic compounds under methanogenesis inhibition in a 24-h incubation period. Based on Exp. 1 results, phenolic compounds were used at a concentration of 6 mM. A preliminary dose–response study with both AT and BES (performed under the same conditions and using the same donor cows) was used to obtain three distinct methanogenesis inhibition rates. These two different anti-methanogenic additives were chosen to verify that the effect of phenolic compounds, as H_2 acceptors, was not limited to a specific inhibitor. A low inhibition rate (~20%) was achieved with 1.5% AT, a medium inhibition rate (~50%) with 3 μM BES, and a high inhibition rate (~75%) with 2.5% AT. An independent run was performed with 10 treatments for each methanogenesis inhibitor: substrate alone, substrate + methanogenesis inhibitor (control), and substrate + m ethanogenesis inhibitor + individual phenolic compounds or formic acid. Incubation procedures and sample collection were as Exp.1. The total number of experimental units was 10 treatments × 3 (inhibitors) × 4 cows = 120 observations that were used in the statistical analysis (see below). Rumen inocula from individual cows were considered biological replicates (n = 4).

Experiment 3: Sequential batch incubation with phloroglucinol combined with 2-bromoethanesulfonate

This experiment used sequential batch incubations to evaluate the effect of phloroglucinol on fermentation in the presence or absence of BES over a longer incubation period (Fig. 1). Phloroglucinol was chosen because it was the most promising compound in the previous experiment among all seven tested phenolic compounds, whereas BES was chosen for practical reasons as it was easier to dose in the sequential batch incubations. We conducted three sequential 24-h incubations for stabilising and adapting the rumen microbes to phloroglucinol, followed by a fourth 24-h incubation and a fifth 72-h incubation with or without BES, in addition to the phloroglucinol treatment. There were two treatments in the first, second, and third incubations: control and 36 mM phloroglucinol. The concentration of phloroglucinol was based on the absence of negative effects on fermentations that was tested in preliminary experiments using the same conditions (not shown), and it was similar to the estimated concentration used in steers by Martinez-Fernandez et al. (2017). At the end of the third incubation (day 4), control flasks were split into control (as in previous batches) and 3 μM BES treatments, whereas phloroglucinol flasks were split into phloroglucinol (as in previous batches) and 3 μM BES + 36 mM phloroglucinol treatments. We used eight lactating Holstein cows as rumen fluid donors; cows were fed the same diets as described for Exp. 1 and 2. The first inoculation was performed

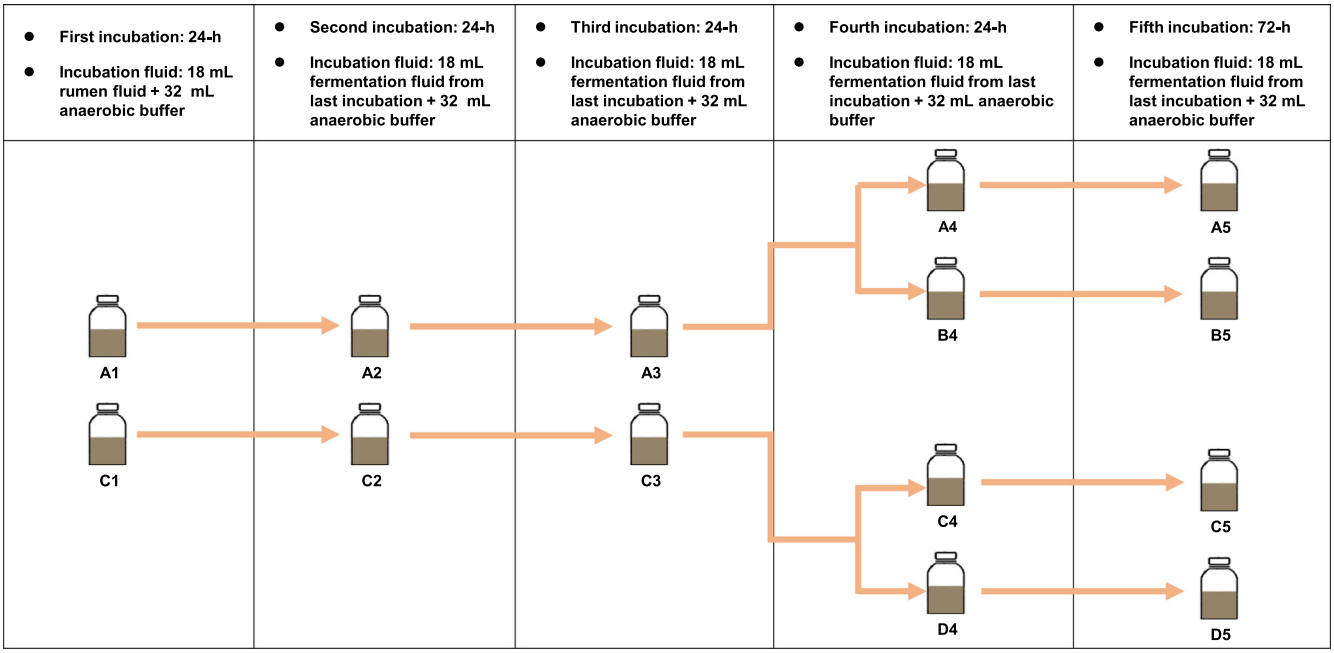

Fig. 1. Experimental design of the sequential batch incubation (Exp. 3) that used rumen fluid inocula from dairy cows (n = 8). Abbreviations: A = Substrate only, B = Substrate + 2-bromoethanesulfonic sodium, C = Substrate + phloroglucinol, D = Substrate + phloroglucinol + 2-bromoethanesulfonic sodium. The number next to the capital letter indicates the sequential of incubation. The inclusion levels of phloroglucinol and 2-bromoethanesulfonic sodium are 36 mM and 3 μM, respectively.

as in Exp. 1 and for the subsequent batches, and one-third of incubation fluid from the previous batch bottle was mixed with two-thirds of anaerobic buffer and used to inoculate the next corresponding serum bottle containing fresh substrate. After 6 and 24 h incubation of the first and second incubations, gas pressure was measured, followed by the release of excessive gas. For the third to fifth incubations, gas samples for gas composition analysis and liquid samples for VFA and ammonia analysis were collected as in Exp. 1. Additionally, 1 mL of incubation fluid 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. For phloroglucinol analysis, 5 mL of fermentation fluid was collected and stored at −20 °C.

Sample analysis

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 organic matter, 27.9 nitrogen, 428 NDF, 303 ADF, 63 ADL and 13.7 ether extract, while barley grain contained 975 organic matter, 21.5 nitrogen, 285 NDF, 67.8 ADF, 8.7 ADL and 20.1 ether extract.

Gas composition (CH_4, H_2, and CO_2) was analysed within 12 h after sample collection using gas chromatography (Micro GC 3000A, Agilent Technologies, Les Ulis, France). The GC was calibrated using a certified gas standard mixture (Messer, France) containing CH_4, O_2, H_2, CO_2, and N_2 (Muñoz-Tamayo et al., 2019). For VFA analysis, 0.8 mL of filtrate was mixed with 0.5 mL of 4 mg/mL crotonic acid and 20 mg/mL metaphosphoric acid in 0.5 M HCl and analysed by gas chromatography (PerkinElmer Clarus 580, Waltham, USA) as described (Rira et al., 2015). For ammonia, 1 mL fermentation fluid was mixed with 5% orthophosphate solution (0.1 mL) and measured according to the phenol-hypochlorite reaction (Weatherburn, 1967). Total gas production (**TGP**) in mL was calculated using the Ideal Gas Law under standard atmospheric pressure and 39 °C. Microbial gDNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's instruction. Quantitative PCR (qPCR) 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 were previously reported (Palma-Hidalgo et al., 2021). Ethyl acetate was used to extract residual phloroglucinol (Kim et al., 2003), and HPLC (LC1260, Agilent, Les Ulis, France) was used to determine phloroglucinol concentration as described (Maxin et al., 2020). The stochiometric metabolic hydrogen recovery was calculated from fermentation products VFA, CH_4 and H_2 production (adapted from Demeyer, 1991) and was used as an indirect indicator of the reduction of phenolic compounds.

Statistical analysis

All data were checked for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) before statistical analysis. Non-normally distributed data (gene copy counts) were $\log_{10}$-transformed before statistical analysis. For all experiments, the following model was run using the PROC MIXED procedure of SAS:

$$Y_{ij} = \mu + P_i + A_j + e_{ij}$$

where Y_{ij} represents a dependent, continuous variable, μ is the overall population mean, P_i represents the fixed effect of treatment, A_j represents the random effect of the cow donor of rumen fluid, and e_{ij} is the residual error. The degree of freedom was calculated using the Satterth statement. The PDIFF statement was used to make multiple comparisons, *P* values were adjusted using the Dunnett statement for comparisons against the control in Exp.1 and Exp.2, and by the Tukey statement to account for multiple pair-wise comparisons for Exp. 3. Differences were considered significant at $P < 0.05$; trends were discussed at $P \leq 0.10$.

Results

Dose-response effects of phenolic compounds (Exp. 1)

There was no negative effect of phenolic compounds or formic acid on ruminal fermentation and VFA concentration for any dose used (2, 4 and 6 mM). Table 1 shows the effect of phenolic compounds or formic acid at a concentration of 6 mM on fermentation parameters compared to the control (substrate alone) treatment. After 24 h incubation, 6 mM phloroglucinol and gallic acid increased ($P = 0.017$ and $P < 0.001$ respectively) TGP by 4 and 7%, respectively. Phloroglucinol increased ($P < 0.001$) TGP in the 6–24 h incubation period, while gallic acid increased TGP in the 0–6 h and 6–24 h periods ($P = 0.001$ and $P = 0.007$, respectively). Phloroglucinol also increased ($P = 0.027$) total VFA concentration by 20%, with a 10% increase ($P < 0.001$) in acetate proportion; additionally, propionate proportion decreased by 22% resulting in an increase ($P < 0.001$) in the acetate:propionate ratio. The electron donor formic acid was the only compound that influenced CH_4 production, which increased ($P < 0.001$) by 14% between 0 and 6 h of incubation. None of the compounds affected H_2 accumulation. Metabolic hydrogen recovery rate was ~76% under the assay conditions and was not affected by the phenolic compounds except for phloroglucinol and pyrogallol that showed lower values ($P < 0.001$ and $P = 0.020$, respectively). Supplementary Tables S1 and S2 show gas production values for 2- and 4-mM doses across treatments, respectively. These lower concentrations were not used in subsequent experiments.

Effect of phenolic compounds when methanogenesis was inhibited (Exp. 2)

Methane production was decreased by 22, 51 and 75% by 1.5% AT, 3 μM BES, and 2.5% AT, respectively, compared to controls (Table 2 and Supplementary Tables S3 and S4). Table 2 shows the effects of phenolic compounds combined with 2.5% AT. The effects of phenolic compounds combined with 1.5% AT, which induced a low-medium inhibition rate (22%), and with 3 μM BES, which induced a medium inhibition rate (51%), were similar to that of 2.5% AT and are shown in Supplementary Tables S3 and S4, respectively. Dihydrogen accumulation in the gas headspace after 6-h incubation was negligible in the substrate-only group, whereas it was readily detected when CH_4 production was inhibited by 2.5% AT. Table 2 shows that none of the phenolic compounds decreased H_2 accumulation or CH_4 production. In contrast, formic acid increased ($P = 0.016$) H_2 accumulation. Phloroglucinol ($P = 0.003$), gallic acid ($P = 0.004$), and formic acid ($P = 0.010$) increased TGP by ~8% after 24 h incubation. The addition of phloroglucinol decreased ($P = 0.005$) metabolic hydrogen recovery, whereas it increased ($P = 0.006$) total VFA concentration by 18%, mainly due to a 41% increase ($P < 0.007$) in acetate proportion. Similarly to phloroglucinol, gallic acid increased ($P = 0.050$) acetate proportion by 23%. Most phenolic compounds had no effect on ammonia concentration except hydroquinone and phloroglucinol, which decreased ($P = 0.018$ and $P = 0.047$, respectively) ammonia concentration by 32% and 26%, respectively.

Table 1
Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, and gallic acid or formic acid at 6 mM on 24-h *in vitro* ruminal fermentation from dairy cows (n = 4).

Item	Treatment									SEM	*P*-value[2]
	Control[1]	Phenol	Catechol	Resorcinol	Hydroquinone	Phloroglucinol	Pyrogallol	Gallic acid	Formic acid		
Gas production (mL)											
TGP/0–6 h	58.7	58.0	58.2	57.9	60.7	58.1	58.5	62.6*	62.2*	10.08	<0.0001
TGP/6–24 h	65.9	64.5	65.9	65.6	67.2	71.9*	66.6	70.4*	66.4	8.59	<0.0001
TGP/0–24 h	124.6	122.5	124.1	123.4	127.9	129.9*	125.2	133.0*	128.7	18.64	<0.0001
CH_4/0–6 h	13.3	13.3	13.0	13.3	14.1	13.4	13.6	14.1	15.2*	1.79	<0.0001
CH_4/6–24 h	17.2	16.7	17.2	16.9	17.8	16.0	16.7	18.0	16.6	2.29	0.028
CH_4/0–24 h	30.4	30.0	30.3	30.2	31.9	29.4	30.3	32.1	31.7	4.06	0.004
H_2/0–6 h	0.06	0.03	0.13	0.12	0.13	0.04	0.10	0.07	0.00	0.038	0.034
Metabolic hydrogen recovery (%)	75.8	74.6	76.6	75.7	76.7	56.7*	66.3*	72.8	78.2	2.25	<0.0001
NH_3-N (mg/100 mL)	31.9	35.3	28.4	27.6	38.3	23.6	32.3	31.9	35.3	4.63	0.061
pH	6.72	6.26*	6.18*	6.25*	6.19*	6.98	7.02	6.66	6.72	0.120	<0.001
Total VFA (mM)	105.8	105.3	104.6	105.1	109.2	126.6*	123.6	112.5	106.7	15.56	0.013
VFA, mol/100 mol											
Acetate	59.8	59.7	58.9	59.5	59.6	65.5*	61.4	61.6	59.3	2.28	<0.0001
Propionate	16.5	16.3	16.3	16.6	15.4*	12.8*	15.3*	14.8*	16.8	0.55	<0.0001
Isobutyrate	2.0	2.0	2.2	2.1	2.0	1.7*	1.9	1.9	2.0	0.07	<0.0001
Butyrate	13.0	13.2	13.2	13.0	12.7	12.0	12.5	12.4	13.2	1.23	0.024
Isovalerate	4.2	4.1	4.6	4.3	4.8*	3.8	4.2	4.3	4.2	0.39	0.005
Valerate	3.7	3.7	3.8	3.6	3.9	3.0*	3.5	3.5	3.7	0.12	0.002
A:P	3.6	3.7	3.6	3.6	3.8	5.1*	4.0	4.2*	3.6	0.23	0.001

Abbreviations: TGP = total gas production; VFA = volatile fatty acid; A:P = acetate:propionate ratio.
[1] Control: substrate alone with no phenolic compound or formic acid added.
[2] Dunnett-Hsu was used to adjust *P*-value.
* Indicates $P < 0.05$, compared to control containing substrate alone and no phenolic compound added.

Table 2
Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid, and formic acid at 6 mM when combined with *Asparagopsis taxiformis* at 2.5% DM on *in vitro* ruminal fermentation from dairy cows (n = 4).

Item		Treatment									SEM	*P*-value[2]
	Substrate alone[1]	AT	AT + Phe	AT + Cat	AT + Res	AT + Hyd	AT + Phl	AT + Pyr	AT + GA	AT + FA		
Gas production (mL)												
TGP/0–6 h	56.0 ± 6.4	46.6	46.9	47.4	48.8	49.1	50.0	47.9	50.9*	53.0*	2.12	0.002
TGP/6–24 h	55.1 ± 3.8	49.4	50.7	48.8	49.3	49.3	53.7	49.9	52.7	49.8	3.56	0.206
TGP/0–24 h	111.1 ± 4.5	96.0	97.6	96.2	98.1	98.4	103.6*	97.8	103.6*	102.8*	4.19	0.001
CH_4/0–6 h	12.8 ± 1.0	2.3	4.1	3.6	3.1	2.1	1.0	2.5	2.6	1.2	1.15	0.034
CH_4/6–24 h	14.3 ± 2.1	4.3	8.4	6.5	5.3	3.5	0.7	3.2	3.7	0.5	2.91	0.164
CH_4/0–24 h	27.0 ± 2.4	6.6	12.5	10.1	8.4	5.6	1.7	5.6	6.2	1.7	4.02	0.107
H_2/0–6 h	0.0	5.41	3.42	3.97	5.10	6.26	5.95	5.28	5.58	8.94*	1.220	0.002
Metabolic hydrogen recovery (%)	60.3 ± 5.9	48.2	56.4	49.2	48.3	46.5	26.8*	42.0	39.8	42.4	5.42	0.002
NH_3-N, (mg/100 mL)	45.0 ± 6.0	39.9	37.6	37.7	30.4	26.9*	29.6	34.6	37.7	32.7	3.77	0.031
Total VFA (mM)	120.0 ± 5.5	93.4	93.1	98.7	96.4	92.3	110.2*	95.3	99.2	93.4	3.18	0.014
VFA, mol/100 mol												
Acetate	64.0 ± 4.1	43.7	46.3	51.2	49.3	45.4	61.6*	48.2	53.7	45.0	3.08	0.035
Propionate	18.3 ± 2.5	24.7	23.4	23.6	24.2	24.7	18.7*	23.7	22.5	26.1	1.84	<0.0001
Isobutyrate	2.1 ± 0.9	1.8	1.8	1.6	1.6	1.5	1.3	1.7	1.5	1.2	0.185	0.416
Butyrate	9.2 ± 1.2	17.5	16.2	15.1	15.3	16.5	17.4	16.1	14.9	17.0	1.58	0.158
Isovalerate	3.3 ± 1.3	3.9	3.9	3.1	3.2	3.1	2.5*	3.2	2.9	2.6	0.26	0.008
Valerate	2.7 ± 0.7	4.7	4.4	4.2	4.0	4.4	3.8	4.2	4.0	4.4	0.30	0.200
A:P	3.6 ± 0.6	1.9	2.2	2.3	2.2	2.1	3.2*	2.2	2.5	1.9	0.30	0.002

Abbreviations: AT = *Asparagopsis taxiformis*; Phe = phenol; Cat = catechol; Res = resorcinol; Hyd = hydroquinone; Phl = phloroglucinol; Pyr = pyrogallol; GA = gallic acid; FA = formic acid; TGP = total gas production; VFAs = volatile fatty acids; A:P = acetate:propionate ratio.
[1] Substrate alone in the first column (mean ± SE) is provided for information.
[2] Dunnett-Hsu was used to adjust *P*-value.
* Indicates $P < 0.05$, compared to AT containing substrate and AT but no phenolic compound added.

Longer-term effect of phloroglucinol on in vitro incubation (Exp. 3)

The sequential batch incubation technique evaluated the effect of phloroglucinol supplementation on fermentation parameters and microbial abundance over several incubation days. Table 3 shows the results with and without addition of the methanogenesis inhibitor BES after three 24-h sequential incubations used to adapt rumen cultures to phloroglucinol. Phloroglucinol treatment nominally increased ($P = 0.074$) TGP and inhibited ($P < 0.001$) CH_4 production in comparison with the control. Interestingly, although CH_4 production was inhibited, phloroglucinol increased H_2 accumulation only nominally ($P = 0.37$) compared to the control. Phloroglucinol increased ($P < 0.001$) total VFA concentration by 59%, with increments in acetate ($P < 0.001$) and butyrate ($P < 0.001$) proportion of 29% and 58%, respectively, compared to the control. In contrast, phloroglucinol decreased propionate pro-

Table 3
Effect of phloroglucinol with or without BES as methanogenesis inhibitor using a sequential batch culture incubation method and rumen fluid from dairy cows (n = 8).

Item	Treatment[1]				SEM	*P*-value
	Control[2]	BES	PHL	PHL + BES		
TGP (mL)	92.4[c]	84.1[d]	95.3[abc]	97.9[a]	1.03	<0.0001
CH_4 (mL)	9.71[a]	2.90[b]	0.03	0.00[c]	0.290	<0.0001
H_2 (mL)	0.29[b]	2.99[a]	1.13[b]	0.84[b]	0.363	<0.0001
Metabolic hydrogen recovery (%)	29.7[a]	33.1[a]	5.6[b]	4.3[b]	4.10	<0.0001
NH_3-N (mg/100 mL)	40.6[a]	40.4[a]	17.4[b]	16.8[b]	0.60	<0.0001
Total VFA (mM)	113.2[b]	106.9[b]	180.5[a]	189.6[a]	5.52	<0.0001
VFA, mol/100 mol						
Acetate	60.0[b]	56.0[b]	77.3[a]	79.4[a]	1.34	<0.0001
Propionate	21.2[a]	22.7[a]	3.4[b]	3.1[b]	0.48	<0.0001
Isobutyrate	1.6[a]	1.6[a]	0.7[b]	0.6[b]	0.05	<0.0001
Butyrate	10.4[b]	12.3[bc]	16.4[a]	15.0[ac]	0.99	<0.0001
Isovalerate	2.3[a]	2.3[a]	1.0[b]	1.0[b]	0.09	<0.0001
Valerate	4.0[a]	4.7[b]	0.5[c]	0.4[c]	0.15	<0.0001
A:P	2.9[b]	2.3[b]	20.5[a]	19.6[a]	1.16	<0.0001
Microbe (log10 copies/mL)						
Bacteria	10.03[b]	10.06[b]	10.34[a]	10.31[a]	0.054	0.001
Protozoa	1.37	1.42	1.70	1.68	0.304	0.732
Archaea	6.04[a]	5.42[b]	3.65[c]	3.50[c]	0.162	<0.0001
Fungi	2.61	1.95	1.11	1.61	0.550	0.092

Abbreviations: BES = 2-bromoethanesulfonic sodium; PHL = phloroglucinol; TGP = total gas production; VFA = volatile fatty acid; A:P = acetate:propionate ratio.
[1] Data shown are from the fourth 24-h sequential incubation.
[2] Control: Substrate alone, no chemical compound added.
[a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$.

portion ($P < 0.001$) and ammonia concentration ($P < 0.001$). Thus, phloroglucinol sharply increased ($P < 0.001$) the acetate:propionate ratio. Also, phloroglucinol decreased archaeal ($P < 0.001$) and fungal ($P = 0.079$) abundances, whereas it increased bacterial abundance ($P = 0.002$), and had no effect on protozoal ($P = 0.80$) abundance, compared to the control.

Adding BES decreased ($P < 0.001$) CH_4 production by 70% and increased ($P < 0.001$) H_2 accumulation tenfold, compared to control. Phloroglucinol + BES increased ($P < 0.001$) TGP and suppressed ($P < 0.001$) CH_4 production, compared to BES treatment. Interestingly, the combination of phloroglucinol + BES decreased ($P = 0.001$) H_2 accumulation by 72% compared to BES alone. Phloroglucinol + BES increased ($P < 0.001$) total VFA concentration by 77%, with increments in acetate ($P < 0.001$) and butyrate ($P < 0.074$) proportions of 42% and 22%, respectively, compared to the BES treatment. In contrast, phloroglucinol + BES decreased ($P < 0.001$) propionate proportion, and consequently, markedly increased ($P < 0.001$) the acetate:propionate ratio. Phloroglucinol + BES treated cultures had lower ($P < 0.001$) ammonia concentration, lower ($P < 0.001$) archaeal, and higher ($P = 0.013$) bacterial abundance than phloroglucinol alone. In contrast, the abundance of protozoa ($P = 0.90$) and anaerobic fungi ($P = 0.93$) were similar across treatments. Data from HPLC analysis at the third 24-h incubation show a reduction of phloroglucinol of more than 80% from the initial concentration. Similar results on fermentation parameters and on microbial abundance were found in the fifth sequential incubation (supplementary Table S5).

Discussion

Inhibiting methanogenesis in the rumen is accompanied by an increase in accumulated H_2 *in vitro* and expelled H_2 *in vivo* (Janssen, 2010). We speculated that the extra H_2 released could be used by rumen microbes to produce useful compounds for the host ruminant provided the presence of enough concentration of phenolic compounds that could incorporate H_2 in their reductive pathways. Phenolic compounds were considered suitable H_2 acceptor alternatives because they are naturally present in plants containing hydrolysable tannins, mainly gallic acid, which is further converted to phloroglucinol or resorcinol by the ruminal microbiota (McSweeney et al., 2001). In addition, it was reported that *Eubacterium oxidoreducens* sp. nov. and *Coprococcus* sp. Pe15 isolated from the rumen reduce some phenolic compounds using H_2 or formate as electron donors to produce VFAs and/or CO_2 (Tsai et al., 1976, Krumholz et al., 1987). *Eubacterium oxidoreducens* degrades phloroglucinol to acetate and butyrate, whereas *Coprococcus* sp. degrade phloroglucinol to acetate (Conradt et al., 2016).

The seven phenolic compounds examined did not negatively affect *in vitro* fermentation at the doses used. Instead, 6 mM phloroglucinol increased TGP and VFA concentration, and in particular acetate proportion. The latter is in accordance with the expected end product of phloroglucinol degradation (Krumholz and Bryant, 1986). In contrast, Sarwono et al. (2019) reported that adding 6 mM phloroglucinol decreased gas production, ammonia concentration, and CH_4 production. Our results agree with Wei et al. (2019), who found that up to 4.8 mM gallic acid did not affect total VFA, ammonia concentration, or CH_4 production but increased TGP. We also observed that formic acid increased ($P < 0.001$) CH_4 production after 6-h incubation. This was expected as formic acid is a substrate for rumen methanogens (Hungate et al., 1970).

When CH_4 production was inhibited, we observed, as anticipated, H_2 accumulation in the headspace at 6 h of incubation. However, none of the phenolic compounds modified the concentration of H_2. Despite this result, phloroglucinol in particular increased the proportion of acetate by up to 41% with a concomitant decrease in the extent of H_2 recovery. The low hydrogen recovery suggests the reduction of these phenolic compounds to acetate. Likewise, gallic acid increased acetate proportion by 23%. In the rumen, gallic acid can be transformed by decarboxylation to pyrogallol that can be further converted to phloroglucinol or resorcinol. Phloroglucinol can then be reduced to dihydrophloroglucinol using H_2 or formate as electron donors, and the ring cleavage of the dihydrophloroglucinol molecule can produce acetate and CO_2 (Tsai et al., 1976, Lotfi, 2020). In contrast, the other phenolic compounds examined in this study did not increase acetate proportion, likely because they were not used by the rumen microbiota in the conditions of the study.

A possible reason to explain why we did not observe any decrease in H_2 could be due to the incorporation by phenolic compounds of other electron donors such as formate (Krumholz et al., 1987), although we did not determine formate concentration so as

to understand the extent of formate utilisation in the reduction of phenolic compounds. However, it is likely that the abundance of the rumen microbes capable of degrading phenolic compounds is low in the non-adapted rumen. In the rumen. *Streptococcus bovis* have the ability to catabolise phloroglucinol (Tsai and Jones, 1975), but this bacterium is a minor member of the normal rumen microbiota (Petri et al., 2013). Other more specialised bacteria known to utilise phloroglucinol as substrate, such as *Coprococcus* spp and *Eubacterium oxidoreducens,* are also not predominant in the rumen.

Because of the expected low abundance of the bacteria able to degrade phenolic compounds and the low phloroglucinol concentration (lower than 5 μg/mL) in the donor cows' rumen fluid, we hypothesised that populations of rumen microbes that can metabolise phloroglucinol needed longer incubation times to grow to metabolise phenolic compounds. In this regard, it is known that diets containing tannins favour the overgrowth of resistant microbes able to degrade hydrolysable tannins (Nelson et al., 1995, Krause et al., 2005). In order to examine this hypothesis, we performed a sequential batch incubation experiment using phloroglucinol as H_2 acceptor and BES as the CH_4 inhibitor. Prolonged incubation with phloroglucinol increased total gas, total VFA, and acetate production, and decreased metabolic hydrogen recovery corroborating the results obtained with shorter 24-h incubations (Exp.2). Interestingly, we found that prolonged phloroglucinol treatment decreased CH_4 production to undetectable concentrations, while only minimally increased H_2 accumulation compared to the control. The phloroglucinol + BES treatment decreased H_2 accumulation by 72% and almost totally inhibited CH_4 production compared to BES alone. These results indicate that prolonged incubation could favour microbial communities able to utilise phloroglucinol as a H_2 acceptor reducing H_2 accumulation. Additionally, phloroglucinol without BES also increased butyrate proportion, which may be explained by the increase in acetate that can be converted to butyrate (Hackmann and Firkins, 2015). Butyrate production from acetate consumes metabolic hydrogen, which may also contribute to explain why phloroglucinol decreased H_2 accumulation. Moreover, butyrate may also be a product of phloroglucinol degradation (Conradt et al., 2016).

The phloroglucinol concentration used in Exp.3 is similar to a previous study in steers (Martinez-Fernandez et al., 2017). Similar to our results, Martinez-Fernandez et al. (2017) reported that when CH_4 production was inhibited, phloroglucinol decreased the amount of H_2 expelled and increased rumen acetate proportion. In addition, we found that phloroglucinol alone decreased H_2 accumulation and CH_4 production. This latter disparity between the two studies may be due to differences between *in vitro* and *in vivo* methods and, eventually the types of diet and animals (dairy vs beef cattle).

The addition of phloroglucinol decreased archaeal abundance and increased bacterial abundance, but had no negative effect on protozoal and fungal abundance (Table 3). It is noted that protozoa were not expected to survive in the sequential batch incubations. Abundance of protozoal 18S rRNA was low and may be the remnants of dead cells transferred in successive inocula. Abundance of fungi was also low, whereas the results of methanogens suggest a toxic effect of phloroglucinol on this community. Similar effects were reported in anaerobic digestors of pig manure where the use of phenolic compounds such as pyrogallol decreased methanogenesis when incubated at comparable concentrations to our study (Kayembe et al., 2013). Further research is needed to clarify the mechanism of action of phenolic compounds on methanogens.

Phloroglucinol, as well as hydroquinone and pyrogallol, strongly decreased ammonia concentration *in vitro*. It is known that dietary proteins bind to the hydroxyl moieties of phenolic compounds and this complex is more resistant to microbial degradation (McSweeney et al., 2001). This phenomenon can decrease emissions of nitrous oxide, another potent greenhouse gas, from ruminant production systems. Low ammonia concentration in the rumen have been associated to decreases in urinary N excretion resulting in decreased emissions of nitrous oxide (Carulla et al., 2005, Dijkstra et al., 2013). Consistent with the lower ammonia concentration findings, we also observed that supplementation of phloroglucinol, with or without BES, decreased isobutyrate and isovalerate proportion, which originate from the deamination of branched-chain amino acids and serve as a carbon skeleton in the synthesis of branched-chain amino acids (Chen and Russell, 1988). Low branched-chain amino acid degradation or greater synthesis in the rumen may increase their availability for productive functions in the small intestine of the host ruminant.

The results presented here using the rumen fluid from lactating dairy cows as inocula are similar to those obtained with rumen fluid from goats (Romero et al., companion paper). However, the sensitivity to methane inhibitors was different as the same batch of AT that was used in both studies almost totally inhibited methane production from goat's rumen fluid at a concentration of 2%, whereas, a 2.5% AT concentration in cow's rumen fluid decreased methane production by 75%. As far as the effect of phenolic compounds, they were comparable in both animal species indicating that, at least for this parameter, the results can be extrapolated to different ruminant species.

Conclusion

We evaluated the effect of seven phenolic compounds on the rumen fermentation of dairy cows when methanogenesis was inhibited *in vitro* and found that phloroglucinol and gallic acid increased the proportion of acetate, a nutritionally important metabolite for the host animal. The decrease in H_2 accumulation indicates that these compounds were successfully used as H_2 acceptors. Phloroglucinol, probably because of its position at the end of the biotransformation of phenolic compounds into VFA, was more effective at incorporating accumulated H_2 and at generating acetate than gallic acid. Moreover, phloroglucinol alone also affected methanogenesis by decreasing methanogens' abundance. The dual effect of phloroglucinol as a H_2 acceptor and methanogenesis inhibitor in dairy cows needs to be verified by further studies.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100788.

Ethics approval

The use of experimental animals followed the French Ministry of Agriculture guidelines and other applicable guidelines and regulations for animal experimentation in the European Union. Procedures for collecting rumen fluid were approved by the French Ministry of Education and Research (APAFIS #8218-20161151782412).

Data and model availability statement

The raw data generated for this study are available at https://doi.org/10.57745/H3QJTU.

Author ORCIDs

Rongcai Huang: https://orcid.org/0000-0002-3094-7700.
Pedro Romero: https://orcid.org/0000-0001-5746-8286.

Alejandro Belanche: https://orcid.org/0000-0001-5880-6021.
Emilio M. Ungerfeld: https://orcid.org/0000-0002-5422-5462.
David Yanez-Ruiz: https://orcid.org/0000-0003-4397-3905.
Milka Popova: https://orcid.org/0000-0001-6695-5502.
Diego P. Morgavi: https://orcid.org/0000-0002-3883-0937.

Author contributions

Rongcai Huang: Validation, Formal analysis, Investigation, Writing - Original Draft, Visualisation, Writing - Review and Editing. **Pedro Romero:** Visualisation, Writing - Review and Editing. **Alejandro Belanche:** Conceptualisation, Resources, Writing - Review and Editing, Visualisation. **Emilio M. Ungerfeld:** Writing - Review and Editing, Visualisation. **David Yanez-Ruiz:** Conceptualisation, Resources, Writing - Review and Editing, Visualisation. **Milka Popova:** Conceptualisation, Validation, Formal analysis, Resources, Writing - Review and Editing, Visualisation, Supervision, Project administration, Funding acquisition. **Diego P. Morgavi:** Conceptualisation, Validation, Formal analysis, Resources, Writing - Review and Editing, Visualisation, Supervision, Project administration, Funding acquisition.

Declaration of interest

None.

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