Can electron acceptor supplementation improve ruminant production when methanogenesis is inhibited?

Rongcai Huang

Université Clermont Auvergne

UMRH-INRAE

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HUANG Rongcai

Microbiology

Supervisors: Dr. MORGAVI Diego and Dr. POPOVA Milka

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Defence committee:

Dr. Christiane FORESTIER, Professor, Université Clermont Auvergne, president (examiner, France)

Dr. Christophe CHASSARD, Directeur de recherche, INRAE (examiner, France)

Dr. Eva RAMOS-MORALES, Senior researcher, CSIC (reporter and examiner, Spain)

Dr. Gabriel DE LA FUENTE OLIVER, Assistant professor, Universitat de Lleida (reporter and examiner, Spain)

RÉSUMÉ

Le secteur de l'élevage est une source majeure de méthane d'origine anthropique, un facteur du réchauffement climatique. Tandis que les stratégies disponibles peuvent réduire les émissions de méthane d'environ 25 %, la diminution de la méthanogenèse ne profite pas facilement à l'animal hôte car l'énergie de l'aliment correspondant au méthane perdu n'est pas récupérée à des fins productives. Dans le rumen, les micro-organismes fermentent les glucides, produisant des acides gras volatils (AGV) ainsi que des équivalents réducteurs, principalement sous forme de dihydrogène. Les méthanogènes du rumen utilisent le CO2 comme principal accepteur d'électrons, qui réagit avec le dihydrogène pour générer du méthane. D'autres voies métaboliques hydrogénotrophes existent également. Nous avons postulé que si la méthanogenèse est inhibée, la supplémentation en un accepteur d'électrons externe pourrait encourager des voies hydrogénotrophes générant des nutriments tels que les AGV, améliorant ainsi la production animale.

Cette thèse visait à explorer le potentiel des composés phénoliques en tant qu'accepteurs d'électrons et leur aptitude à optimiser la production animale lorsque la méthanogenèse est inhibée. Nous avons évalué divers composés phénoliques comme accepteurs d'hydrogène in vitro et avons examiné les effets d'un composé phénolique sélectionné chez les vaches laitières. Nos résultats indiquent que l’acide gallique et le phloroglucinol étaient les plus efficaces en tant qu’accepteurs d'hydrogène in vitro, augmentant la production d'acétate et de gaz total lorsque la méthanogenèse était

inhibée par du 2-bromoéthanesulfonate de sodium (BES) ou par l’algue rouge Asparagopsis taxiformis. De plus, le phloroglucinol a réduit l'accumulation de dihydrogène, la production de méthane et la présence de méthanogènes tout en augmentant celle des bactéries dégradant potentiellement le phénolique, quand la méthanogenèse était inhibée par le BES. Pour des raisons pratiques et réglementaires, dans l'expérience in vivo réalisée sur 28 vaches laitières, l'acide gallique et l'inhibiteur de méthanogenèse Asparagopsis armata ont été utilisés. Contrairement aux résultats in vitro, l'acide gallique n'a pas réduit les émissions de dihydrogène et de méthane ni augmenté les proportions d'acétate et de propionate. Toutefois, la supplémentation en acide gallique a mitigé l'effet négatif de l'Asparagopsis armata sur la production laitière et l’association acide gallique et Asparagopsis armata a eu un effet d'interaction positive sur la production laitière dans cette étude.

Nos découvertes suggèrent que l’acide gallique et le phloroglucinol ont le potentiel pour agir comme accepteurs d'hydrogène dans l'écosystème du rumen. Toutefois, dans l'expérimentation in vivo, l'acide gallique combiné avec Asparagopsis armata n'a pas eu d'effet d'interaction sur l'émission de dihydrogène. Des études supplémentaires sur les animaux sont nécessaires pour validation car le manque d'effet observé pourrait être attribuable aux niveaux d'inclusion de l'acide gallique. Les recherches futures devraient aussi examiner l'impact de l'acide gallique, seul ou en combinaison avec un inhibiteur de méthanogenèse, sur la production laitière.

Mots clés : composé phénolique, accepteur d'électrons, dihydrogène, méthane, production animale, microbiote du rumen

ABSTRACT

The livestock sector is one of the major anthropogenic sources of methane, which contributes to global warming. While available strategies can reduce methane emissions by around 25%, decreasing methanogenesis does not readily benefit the host animal as the feed energy corresponding to methane lost is not recovered for productive purposes. In the rumen, microbes ferment carbohydrates, to produce volatile fatty acids (VFAs), and reducing equivalents mainly dihydrogen. Rumen methanogens use carbon dioxide as the main electron acceptor to react with dihydrogen to produce methane, but other hydrogenotrophic pathways exist. We hypothesized that when methanogenesis is inhibited, the supplementation of an external electron acceptor could favour hydrogenotrophic pathways that produce nutrients such as VFAs, thus improving animal production.

The objectives of this thesis were to investigate the potential of phenolic compounds as electron acceptors and their ability to improve animal production when methanogenesis is inhibited. We tested a range of phenolic compounds as hydrogen acceptors *in vitro* and investigated the effect of the selected phenolic compound in dairy cows. Our results showed that gallic acid and phloroglucinol were the best hydrogen acceptor candidates *in vitro* as they improved acetate and total gas production when methanogenesis was inhibited by 2-bromoethanesulfonate sodium (BES) or by the red seaweed *Asparagopsis taxiformis*. Moreover, phloroglucinol decreased dihydrogen accumulation, methane production, methanogen abundance while increasing the abundance of potential phenolic-degrading bacteria when methanogenesis was inhibited

by BES. For practical and regulatory reasons, in the *in vivo* trial with 28 lactating cows, gallic acid and the methanogenesis inhibitor *Asparagopsis armata* were used. Contrary to *in vitro* results, gallic acid did not decrease dihydrogen and methane emissions or increase acetate and propionate proportions. However, gallic acid supplementation alleviated the negative effect of *Asparagopsis armata* effect on milk production and gallic acid combined with *Asparagopsis armata* had a positive interaction effect on milk production in this study.

Our findings suggest that gallic acid and phloroglucinol have the potential to act as hydrogen acceptors in the rumen ecosystem. Notwithstanding, no interaction effect on dihydrogen emission was observed *in vivo* when gallic was combined with *Asparagopsis armata*. Further animal studies are needed for validation as the lack of effect observed might be attributable to the inclusion levels of gallic acid. Subsequent research should also explore the impact of gallic acid, both independently and in combination with a methanogenesis inhibitor, on milk production.

Keywords: phenolic compound, electron acceptor, dihydrogen, methane, animal production, rumen microbiota

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ABBREVIATIONS

Alkaline phosphatase	ALP
Acetate:propionate ratio	A:P
Acid detergent fiber	ADF
Acid detergent lignin	ADL
Adenosine triphosphate	ATP
Alanine transaminase	ALT
Asparagopsis armata	A. armata
Asparagopsis taxiformis	A. taxiformis
Aspartate aminotransferase	AST
Beta-hydroxybutyrate	BHB
Crude protein	CP
Dihydrogen	H2
Dry matter	DM
Dry matter intake	DMI
Electron transferring flavoprotein/butyryl-CoA dehydrogenase	EtfAB-Bcd
Energy corrected milk production	ECM
Energy-conserving methyltransferase	MtrA-H
Energy-independent soluble transhydrogenase	STH
Erredoxin-dependent and membrane-associated Hydrogenase	Ech
Ferredoxin: NADP+ Oxidoreductase	FNRs
Gamma-glutamyl transferase	GGT
Gross energy	GE
Hydrogenase/heterodisulfide reductase complex	HdrABC
Membrane-bound pyridine nucleotide transhydrogenase	PntAB
Methane	CH_4

Milk urea nitrogen	MUN
NADH-dependent reduced ferredoxin: NADP+ oxidoreductase	NfnAB
Neutral detergent fiber	NDF
Non-esterified fatty acids	NEFA
Organic matter	OM
Oxidized nicotinamide adenine dinucleotide	NAD^+
Oxidized nicotinamide adenine dinucleotide	$NADP^+$
Oxidized nicotinamide adenine dinucleotide phosphate	Fd_{ox}
Pyruvate dehydrogenase	PDH
Pyruvate formate-lyase	PFL
Pyruvate-ferredoxin oxidoreductase	PFOR
Quantitative polymerase chain reaction	qPCR
Reduced ferredoxin	Fd_{red}
Reduced flavin adenine dinucleotide	FADH2
Reduced nicotinamide adenine dinucleotide	NADH
Reduced nicotinamide adenine dinucleotide phosphate	NADPH
Rhodobacter nitrogen fixation	Rnf
Somatic cell count	SCC
Sulfur hexafluoride	SF6
Total gas production	TGP
Volatile fatty acids	VFA
2-bromoethanesulfonate sodium	BES
3-nitrooxypropanol	3-NOP

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CHAPTER 1

Literature review

The generation and transformation of reducing equivalents in the rumen metabolisms: part Ⅰ

1 Introduction

Ruminants differ from other animals in that they have four stomachs: the rumen, reticulum, omasum, and abomasum before the small intestine. This unique digestive tract gives ruminants having the ability to digest structural carbohydrates. In the rumen, the interactions between microbes, and between microbes and the host animal allow ruminants to digest plant material that is non-utilizable to monogastric animal including humans. In the highly anaerobic environment of the rumen, bacteria, protozoa, and fungi ferment structural and non-structural carbohydrates to volatile fatty acids (VFAs), carbon dioxide, and reducing equivalents. Methanogens use the reducing equivalents (e.g., dihydrogen, formate) and carbon dioxide as precursors to synthesize methane. However, enteric methane emissions contribute to climate change, and waste feed energy. Methane production from the livestock sector accounts for 6% of anthropogenic greenhouse gas emissions (Gerber et al., 2013), and represents 2 to 12% of the gross energy intake in cattle (Johnson and Johnson, 1995).

Over last fifty years, researchers have developed many strategies to decrease methane emissions from ruminant (Beauchemin et al., 2020). Among them, feed

additives such as 3-nitrooxypropanol and red seaweed show the greatest potential to decrease methane emissions. However, the energy saved by decreasing methane emissions is not correspondingly used to increase animal production (Ungerfeld, 2018, Melgar et al., 2020). Researchers believe that electron flow in the rumen is the key link to improving animal production when using methanogenesis inhibitors (Ungerfeld, 2018, Leahy et al., 2022). A few papers have reviewed rumen electron flow (Hegarty and Gerdes, 1999, Ungerfeld, 2020, Leahy et al., 2022), however the information is not detailed enough. Therefore, this literature review detailed 1) the nutrients fermentation couple with reducing equivalents generation; 2) the electron transfer between different reducing equivalents; 3) dihydrogen and formate generation. All this information can give us with an insight into the electron flow in the rumen and possible methods to redirect the electron flow.

2 Reducing equivalents generation in the rumen

Reducing equivalents are electron donors in redox reactions. Rumen microbes ferment carbohydrates, amino acids, and lipids by several pathways that couple reducing equivalents, including the reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced ferredoxin (Fd_{red}), dihydrogen, formate, and small amounts of reduced flavin adenine dinucleotide (FADH2), generation. These reducing equivalents have different redox potentials and can provide different numbers of electrons in the redox reaction. For example, per mol of NADH, NADPH, FADH2, dihydrogen, and formate can provide 2 moles of electrons, while per

mole of Fd_{red} can only provide 1 mole of electron.

2.1 Carbohydrate metabolism couples with reducing equivalents generation

Carbohydrates account for 60 - 70% of total diet, which are the major energy source for ruminants (National Research Council, 2001). Approximately 75% of the digestible carbohydrate energy is converted to VFA, which provides up to 75% of the metabolizable energy for the host animal (Bergman, 1990). Dietary carbohydrates include cellulose, hemicellulose, starch, pectin, oligosaccharides, disaccharides (e.g., sucrose, and fructans), monosaccharides (e.g., glucose, and fructose), and organic acid (e.g., lactate). Cellulose, hemicellulose, and pectin are the major components of the plant cell wall. Starch is mainly found in the concentrate feed. Sucrose and fructans are the major nonstructural carbohydrates of forages and lactate is abundant in silage (National Research Council, 2001). Glycosidases hydrolyze cellulose, hemicellulose, starch, sucrose, and fructans to different monomers: cellulose to glucose; hemicellulose to fucose, galactose, arabinose, xylose, and rhamnose (Solden et al., 2017); starch to glucose; sucrose to glucose and fructose; fructans to fructose; and pectin mainly to galacturonate (Marounek and Dušková, 1999). Except glucose, other monosaccharides participate in glucose metabolism pathways by converting themselves into the intermediate products of the glucose metabolism pathways.

2.1.1 Glycolysis pathway

Glycolysis pathway is the core pathway of the carbohydrate metabolic, linking

carbohydrate, amino acid, and lipid metabolism. Figure 1 shows the major intermediates and the end products of this pathway. In this pathway, glyceraldehyde-3-phosphate oxidation is the only reaction coupled to NADH generation. One mole of glucose can be converted into 2 moles of glyceraldehyde-3-phosphates; thus 1 mole of glucose can generate 2 moles of NADH via this pathway. It should be noted that oxidized nicotinamide adenine dinucleotide (NAD^+) is not the only cofactor for glyceraldehyde-3-phosphate oxidation. Some microbes also use oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) or oxidized ferredoxin (Fd_{ox}) as the cofactor (Spaans et al., 2015), although these microbes unlikely to dominate VFA production in the rumen.

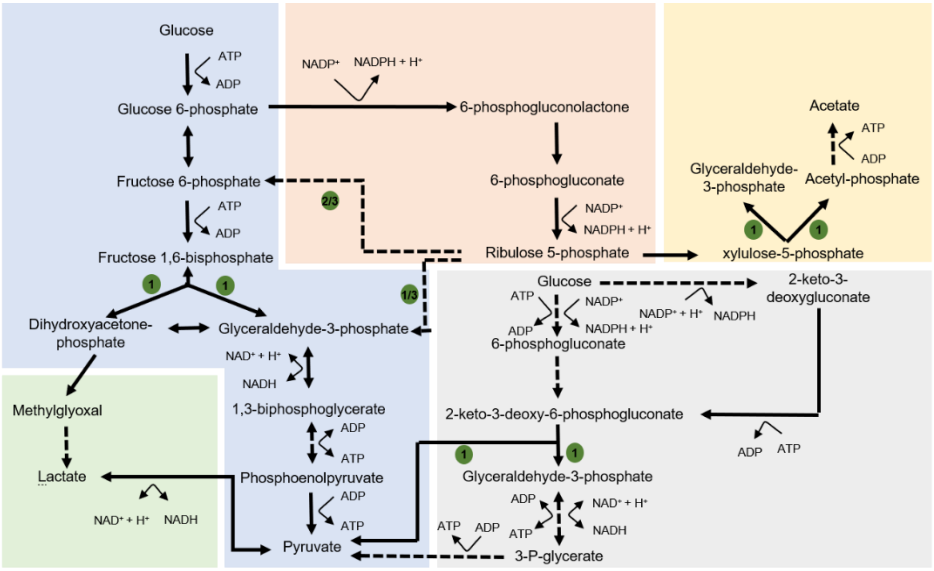

Figure 1 Carbohydrate metabolism pathways.

Blue area represents the glycolysis pathway; pink area represents the oxidation reactions of pentose phosphate pathway; gray area represents the Entner-Doudoroff pathway; green area represents the methylglyoxal pathway; and the yellow area represents the phosphoketolase pathway. Solid and dashed arrows represent single and lumped enzymatic reaction, respectively.

2.1.2 Pentose phosphate pathway

The pentose phosphate pathway includes both oxidative and non-oxidative reactions. Figure 1 shows only the oxidative reaction because the reducing equivalents are generated only in the oxidative reaction. The oxidation of glucose-6-phosphate and 6-phosphogluconate produce NADPH. The end products of the pentose phosphate pathway are glyceraldehyde-3-phosphate and fructose 6-phosphate which both are the intermediates of the glycolysis pathway. One moles of glucose can be converted to 1 moles of ribulose 5-phosphates and 2 moles of NADPH in the oxidative reaction, then 1 moles of ribulose 5-phosphates can be converted to 1/3 mole of glyceraldehyde-3-phosphate and 2/3 moles of fructose 6-phosphate in the non-oxidative reaction.

The pentose phosphate pathway primarily produces NADPH rather than NADH,

which is the main source of NADPH used for biosynthesis (Spaans et al., 2015). The intermediate metabolite ribulose-5-phosphate is the precursor of nucleotides. Therefore, this pathway is very important for rumen microbes because rumen microbes, especially bacteria, have a short generation time.

2.1.3 Entner-Doudoroff pathway

The Entner-Doudoroff pathway is another carbohydrate metabolic pathway (Figure 1). There are two types of Entner-Doudoroff pathways, the classical Entner-Doudoroff pathway and the modified Entner-Doudoroff pathway. The modified Entner-Doudoroff pathway comprises of a semi-phosphorylated Entner-Doudoroff pathway and a non-phosphorylated Entner-Doudoroff pathway (Ahmed et al., 2005). Both the classical and the modified Entner-Doudoroff pathways use 1 mole of glucose to produce 2 moles of pyruvate, 1 mole of NADPH, and 1 mole of NADH (Ettema et al., 2008, Spaans et al., 2015). The classical Entner-Doudoroff pathway is mainly found in prokaryotes (Fabris et al., 2012), while the modified Entner-Doudoroff pathway is mainly found in several *Clostridium* species such as *Halobacterium saccharocorum*, and extreme thermoacidophiles (Conway, 1992). Similar to the pentose phosphate pathway, $NADP^+$ is the preferred electron acceptor in the Entner-Doudoroff pathway (Spaans et al., 2015).

2.1.4 Methylglyoxal pathway

Some rumen bacteria lack the genes encoding for enolase; therefore, the glycolysis pathway is incomplete for these bacteria as enolase catalyzes the dehydration of 2-phospho-D-glycerate to produce phosphoenolpyruvate. Seshadri et. al. (2018) reported

that ruminal isolates belonging to the genera *Butyrivibrio*, *Prevotella*, and uncharacterized members of the family *Lachnospiraceae* have a high proportion of enolase-negative strains (approximately 50% for *Butyrivibrio*). Therefore, the methylglyoxal pathway has been proposed for these microbes (Kelly et al., 2010) (Figure 1). In the methylglyoxal pathway, glyceraldehyde-3-phosphate is converted to dihydroxyacetone-phosphate, and then dihydroxyacetone-phosphate is converted to pyruvate through intermediate metabolites including methylglyoxal and lactate. However, this metabolic pathway has not been confirmed. Also, some enolase-negative *Butyrivibrio* strains do not produce lactate and they lack the gene for L-lactate dehydrogenase (Seshadri et al., 2018). Furthermore, Hackmann et. al., (2017) did not find the genes encoding the methylglyoxal pathway in enolase-negative rumen bacteria through genome analysis. Therefore, the methylglyoxal pathway is probably not widespread in rumen bacteria. Further studies are needed to investigate carbohydrate metabolism in these enolase-negative microbes.

2.1.5 Phosphoketolase pathway

Phosphoketolase is a promiscuous enzyme that catalyzes three different reactions (Valk et. al. 2020). Here we focus on only one of the three reactions, xylulose 5-phosphate cleavage to acetyl-phosphate and glyceraldehyde-3-phosphate (Figure 1), as there is little information on the other two reactions for rumen microbes. Some rumen microbes such as *Prevotella ruminicola* B14 (this microbe has now been assigned to the genus *Xylanibacter* (Hitch et al., 2022)) and *Fibrobacter succinogenes* S85 showed significant phosphoketolase activity (Matte et al., 1992). Valk et. al. (2020) identified the gene of phosphoketolase by whole-genome analysis and found the phosphoketolase by

proteomic analysis of the rumen bacterium *Lactobacillus suebicus*. However, the incubation pH (pH = 4) and temperature (30 ℃) used in this study were very different from the rumen environment. For some rumen microbes, such as *Butyrivibrio fibrisolvens* 787, *Prevotella ruminicola* AR29, and *Butyrivibrio fibrisolvens* ATCC 19171, researchers did not find the phosphoketolase activity in pure culture (Marounek and Petr, 1995, Marounek and Dušková, 1999). More research is needed to investigate the importance of the phosphoketolase pathway in rumen carbohydrate metabolism.

All of the above carbohydrate metabolic pathways are related to VFA production as the end products of these pathways are either pyruvate or can be converted to pyruvate, which is the precursor of acetate, propionate, and butyrate. However, their importance in VFA production varies. Glucose metabolism via the pentose phosphate pathway or the phosphoketolase pathway produces more reducing equivalents but less pyruvate and ATP. For example, 1 mole of glucose can produce 2 moles of NADH, 2 moles of pyruvate, and 2 moles of ATP through the glycolysis pathway; whereas 1 mole of glucose can produce 2 moles of NADPH, 5/3 moles of NADH, 5/3 moles of pyruvate, and 5/3 moles of ATP through the pentose phosphate pathway; 1 mole of glucose can produce 2 moles of NADPH, 1 mole of NADH, 1 mole of pyruvate, 1 mole of acetate, and 2 moles of ATP via the phosphoketolase pathway (Figure 1). Therefore, the pentose phosphate and phosphoketolase pathways are probably not preferred for VFA production as these two pathways produce less VFA precursor pyruvate, and less ATP. Another reason why these two pathways are not preferred is that they produce more reducing equivalents, however the rumen environment is in a very high reducing potential state. The glycolysis and

methylglyoxal pathways produce the same amount of pyruvate and reducing equivalents; however, the methylglyoxal pathway consumes ATP. The glycolysis and Entner-Doudoroff pathways also produce the same amount of reducing equivalents and pyruvate (Figure 1). The glycolysis pathway uses NAD^+ as its electron acceptor, whereas the Entner-Doudoroff pathway uses $NADP^+$ and NAD^+ as its electron acceptor. In addition, the glycolysis pathway produces more ATP than the Entner-Doudoroff pathway. For example, 1 mole of glucose is metabolized to 2 moles of pyruvate and produces 2 moles of ATP by the glycolysis pathway, whereas 1 mole of glucose is metabolized to 2 moles of pyruvate and produces only 1 mole of ATP by the Entner-Doudoroff pathway (Figure 1). Considering the above factors mentioned, the glycolysis pathway is likely to be the core pathway for VFA production in the rumen.

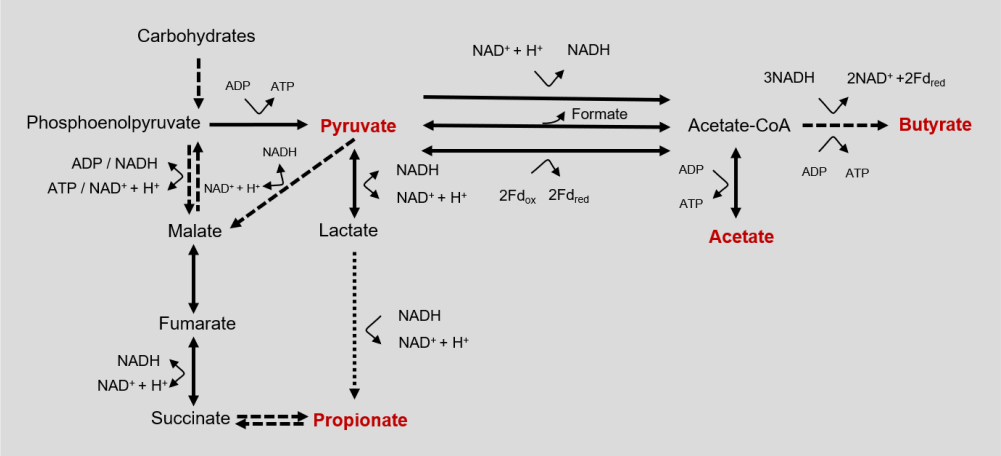

Figure 2 Acetate, propionate, and butyrate generation in the rumen. Solid and dashed arrows represent single and lumped Enzymatic reactions, respectively.

2.2 Volatile fatty acid production couples with reducing equivalents generation

Pyruvate is the major end product of different monosaccharides via different carbohydrate metabolic pathways (Figure 1). It is also the main precursor for VFA production (Figure 2). Pyruvate can be converted to acetyl-CoA via three different pathways: 1) pyruvate dehydrogenase (PDH) catalyzes pyruvate to acetyl-CoA using NAD^+ as electron acceptor; 2) pyruvate-ferredoxin oxidoreductase (PFOR) catalyzes pyruvate to acetyl-CoA using Fd_{ox} as the electron acceptor; 3) and pyruvate formate-lyase (PFL) catalyzes pyruvate to acetyl-CoA and produces formate (detailed in the following section). Pyruvate-ferredoxin oxidoreductase and PFL catalyze reversible reactions, whereas PDH catalyzes irreversible reaction. Therefore, acetyl-CoA production by PFOR and PFL is likely to be inhibited when dihydrogen accumulates in the rumen caused by the methanogenesis inhibitor. Certainly, the use of a methanogenesis inhibitor generally decrease acetate proportion.

Pyruvate-ferredoxin oxidoreductase has been found in methanogens such as *Methanosarcina barkeri* (Bock et al., 1997), most anaerobic bacteria (Chabrière et al., 1999), and rumen protozoa (Yarlett et al., 1981). This enzyme is important because it catalyzes Fd_{red} generation instead of NADH. The negative redox potential of Fd_{red} is higher than that of NADH, allowing Fd_{red} to be used in reactions that require stronger reductants than NADH such as dihydrogen formation or formate formation (Ragsdale, 2003). Under anaerobic conditions, PFOR was thought to be one of the main pathways for acetyl-CoA generation (Kerscher and Oesterhelt, 1982). Acetyl-CoA is the precursor for the acetate production. Acetyl-CoA synthetase catalyzes acetyl-CoA to generate acetate and ATP. Acetyl-CoA is also the precursor for the generation of butyrate. Figure 2 shows that 2 moles of acetyl-CoA consume 3 moles of NADH to generate 1 mole of butyrate and 2 moles of Fd_{red} (Hackmann and Firkins, 2015). There are three different pathways for propionate formation: 1) phosphoenolpyruvate conversion via intermediates such as malate, fumarate, and succinate; 2) pyruvate conversion via the same intermediates such as malate, fumarate, and succinate; 3) pyruvate conversion via lactate (Hackmann et al., 2017). Propionate generation via phosphoenolpyruvate or pyruvate metabolism consumes reducing equivalents (Figure 2). Valerate, caproate, isobutyrate, and isovalerate generation are not discussed here because their concentrations in the rumen fluid are low.

Table 1 The quantitative relationship between the production of 1 mole of acetate, butyrate, and propionate and the availability of reducing equivalents, ATP, and dihydrogen[1].

VFA	NADH	Fd_{red}^{-1}	ATP	H_2
Acetate	1	2	2	2
Butyrate	-1	6	3	2
Propionate	-1	0	1	-1

[1]We assume that pyruvate is the precursor for acetate, butyrate, and propionate production, and the pyruvate is generated by glucose via glycolysis pathway.

From Figure 1 and Figure 2, we can calculate the number of moles of dihydrogen generated when 1 mole of acetate, or propionate, or butyrate is produced via glycolysis pathway (Table 1). It is noteworthy that acetate and butyrate production generates the same amount of dihydrogen, while propionate production consumes dihydrogen. Although converting more carbohydrates to propionate may theoretically allow the animal to retain more feed energy, rumen microbes do not appear to adapt to this strategy because dihydrogen is mainly used to produce methane instead of more propionate. The reasons might because different VFA have different physiological functions. For example, acetate and butyrate mainly used to synthesis milk fat in dairy cow (Bauman and Griinari, 2003), and propionate is mainly used to produce glucose in the liver (Bergman, 1990). Additionally, acetate and butyrate production generate more ATP than propionate production (Table 1), which can support microbial growth to maintain rumen functions. Further research is required to understand why propionate production is not the primary pathway for dihydrogen consumption by ruminal microbes.

2.3 Protein metabolism couples with reducing equivalents generation

The rumen digestibility of proteins from different feedstuff varies, with plant protein degradation rates ranging from 28 to 56% after 12-h of rumen incubation (Taghizadeh et al., 2005). Bacteria, protozoa, and anaerobic fungi in the rumen generate various proteases, peptidases, and deaminases to digest feed protein. Among the rumen microbes, bacteria are considered the principal proteolytic microorganisms because of their high abundance and up to 40% of cultured bacteria have proteolytic activity (National Research Council , 2001). In the rumen, the digestible feed proteins are hydrolyzed to peptides and amino acids, which are mainly used to synthesize microbial protein (Wu, 2013). Amino acid is also undergoing deamination to ammonia and α-ketoacids, which are involved in metabolic pathways including amino acid, fatty acid, and VFA synthesis (Bach et al., 2005). The deamination of amino acids can generate NAD(P)H via dehydrogenases or FADH2 via oxidases (**Figure 3**) (Wu, 2013). Additionally, the synthesis of amino acids using α-ketoacids and ammonia, as well as the synthesis of glutamate from glutamine, consume NAD(P)H. The metabolism of α-ketoacid via carbohydrate metabolism pathway also linked to NAD(P)H generation or consumption.

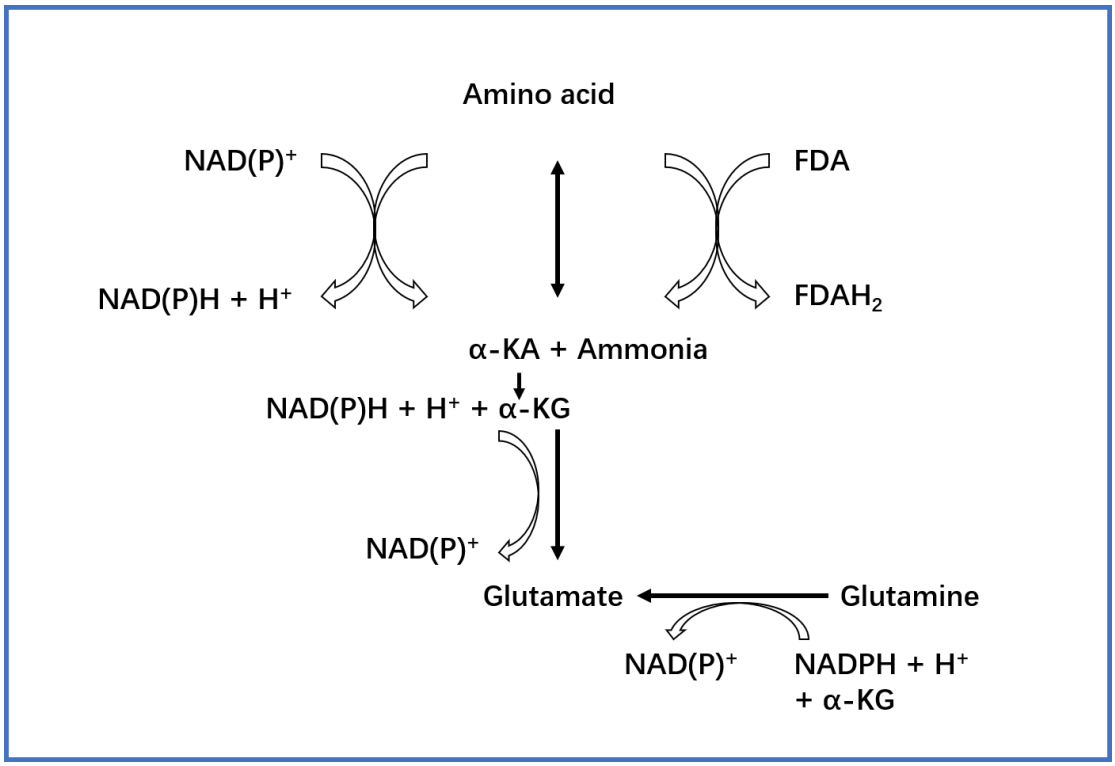

Figure 3 Reducing equivalents generation and consumption by amino acids metabolism: example of glutamate and glutamine which is in the central status in amino acids synthesis in the rumen. (Modified from Wu, 2013)

2.4 Lipid metabolism couples with reducing equivalents generation

Microbial lipases extensively hydrolyze esterified lipids from the diet in the rumen, yielding free fatty acids and glycerol. Glycerol can be absorbed by the rumen epithelium (Werner Omazic et al., 2015) or metabolized by rumen microbes to produce mainly propionate and butyrate via the intermediate glyceraldehyde-3-phosphate (Rémond et al., 1993, Wang et al., 2009). The conversion of 1 mole of glycerol to 1 mole of propionate consumes 1 mole of NADH, while the conversion of 2 moles of glycerol to 1 mole of butyrate generates 6 moles of Fd_{red} and consumes 1 mole of NADH. Meanwhile, free fatty acid can undergo degradation via the β-oxidation pathway by rumen microbes,

using FAD and NAD^+ as electron acceptors. However, the rumen's capacity to degrade long-chain fatty acids is limited, with less than 1% being degraded (Jenkins, 1993). As a result, the generation of FADH2 and NADH from β-oxidation of long-chain free fatty acids is negligible.

3 Electron transfer occurs among NAD(H), NADP(H), and ferredoxin

NAD^+, $NADP^+$, and Fd_{ox} are the electron acceptors for anaerobic dehydrogenase reactions. Different anaerobic dehydrogenases exhibit varying preferences for these electron acceptors, likely due to the difference in the structure and the difference in reduction potential of these electron acceptors. For example, PFOR and flavoprotein/butyryl-CoA dehydrogenases (EtfAB-Bcd) more commonly utilize Fd_{ox} as the electron acceptor. In physiological conditions, the reduction potential of these electron acceptors follows the order of $NAD^+ > NADP^+ > Fd_{ox}$. Although the reduction potential of these electron acceptors is different, the electrons can transfer between each other under certain conditions.

3.1 Mutual transformation between NAD(H) and NADP(H)

NADH and NADPH are commonly used as reducing equivalents in microbes. As mentioned above, NADH is primarily generated via the glycolysis pathway, while NADPH is primarily generated via the pentose phosphate and Entner-Doudoroff pathways. Under standard physiological conditions, $NADP^+$/NADPH and NAD^+/NADH have identical redox potential (E′0: −320mV). However, in bacterial cells, the $NADP^+$/NADPH ratio is lower

than the NAD^+/NADH ratio (Bennett et al., 2009, Amador-Noguez et al., 2011), which makes $NADP^+$/NADPH having a higher reducing capacity than NAD^+/NADH. The functions of NADPH and NADH are also different: NADPH is used for anabolic redox reactions including lipid, cholesterol, nucleic acid, and amino acid synthesis, whereas NADH is used for oxidation reactions (Spaans et al., 2015). The varying metabolic functions of NADPH and NDAH may arise from the specificity of enzymes that utilize these reducing equivalents (Cracan et al., 2017).

There are two pathways for NAD(H) biosynthesis: the de novo pathway and the salvage pathway (Begley et al., 2001). However, NAD(H) kinase is the only enzyme responsible for NADP(H) de novo biosynthesis, which phosphorylates NAD^+ to $NADP^+$ and NADH to NADPH (Spaans et al., 2015) (Figure 4). While, NADP(H) phosphatase hydrolyzes $NADP^+$ to NAD^+ and NADPH to NADH (Figure 4). NAD(H) kinase together with NADP(H) phosphatase regulate the intracellular balance of NAD(H) and NADP(H) (Kawai and Murata, 2008). Energy-independent soluble transhydrogenase (STH) is the enzyme that catalyzes electron transfer between NAD(H) and NADP(H) (Figure 4). Energy-independent soluble transhydrogenase is found in Gammaproteobacteria, such as *Pseudomonas fluorescens*, and catalyzes a reversible reaction (French et al., 1997). Membrane-bound pyridine nucleotide transhydrogenase (PntAB) is an energy-dependent or proton-translocating dependent transhydrogenase that facilitates electron transfer between NAD(H) and NADP(H) (Figure 4). NADPH formation is coupled with H^+ flow from extracellular to intracellular, which provides energy for this reaction. Membrane-bound pyridine nucleotide transhydrogenase is widely distributed in the mitochondria of

eukaryotes and certain bacteria but is rare in archaea (Jackson, 2012). Although STH and PntAB catalyze reversible reactions, STH favors NADH generation (Reddy et al., 2015), while PntAB favors NADPH generation (Pedersen et al., 2008) under physiological conditions.

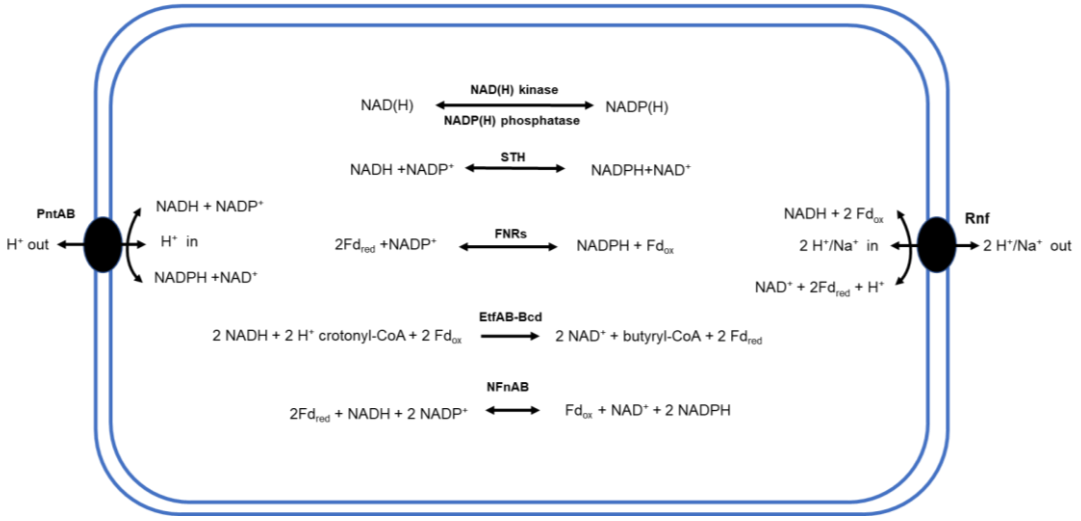

Figure 4 Electron transfer occurs among NAD(H), NADP(H), and ferredoxin.

STH, energy-independent soluble transhydrogenase; PntAB, membrane-bound pyridine nucleotide transhydrogenase; EtfAB-Bcd, electron transferring flavoprotein/butyryl-CoA dehydrogenase; FNRs, ferredoxin: $NADP^+$ oxidoreductases; NfnAB, NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase; Rnf, rhodobacter nitrogen fixation.

3.2 Electron transfer between NAD(H) and ferredoxin

NADH transfer electrons to Fd_{ox} occurs in the butyrate generation pathway. Specifically, EtfAB-Bcd complex reduces crotonyl-CoA to butyryl-CoA with NADH and generates Fd_{red} (Buckel and Thauer, 2018a) (Figure 4). This reaction is called electron bifurcation which couples exergonic and endergonic redox reactions to simultaneously generate low- and high-potential electron carriers (Garcia Costas et al., 2017). In this reaction, 1 mole of NADH transfers 2 moles of electrons to 1 mole of crotonyl-Co A to generate 1 mole of butyryl-CoA, and 1 mole of NADH transfers 2 moles of electrons to 2 moles of Fd_{ox} to generate 2 moles of Fd_{red}. Reduced ferredoxin generation requires energy which is provided by butyryl-CoA generation (Garcia Costas et al., 2017). Therefore, chemical energy is saved in this reaction. Electron bifurcation is recognized as

the third energy conservation reaction in biology (the others are substrate-level phosphorylation and oxidative phosphorylation). Additionally, EtfAB-Bcd catalyzes irreversible reaction in microorganisms (Buckel and Thauer, 2018b).

The Rhodobacter nitrogen fixation (Rnf) complex is a membrane-associated electron-transport protein that also couples electron transfer between ferredoxin and NAD(H) with proton or sodium ion translocation (Figure 4) (Buckel and Thauer, 2018b). The Rnf complex is found in strict anaerobes, including most of *butyrivibrios* (Hackmann and Firkins, 2015), acetogenic bacteria (Tremblay et al., 2012), acetoclactic methanogens (Schlegel et al., 2012), methanotrophic archaea (Wang et al., 2014), and sulfate reducers (Pereira et al., 2011). This reaction couples with 2 moles of H^+/Na^+ translocation out the cell when NADH is generated by oxidation of Fd_{red}. Oxidation of Fd_{red} is an exergonic process because Fd_{red} has a higher reducing potential than NADH, and the energy is saved by the translocation of H^+/Na^+ out the cell. When H^+/Na^+ is transported from the extracellular to intracellular the through ATP synthase, ATP is generated.

3.3 Electron transfer between NADP(H) and ferredoxin

Ferredoxin: $NADP^+$ oxidoreductases (FNRs) are flavoenzymes that typically feature a non-covalently bound flavin. They catalyze a reversible reaction that involves electron transfer between the one-electron carrier reduced ferredoxin and the two-electron carrier NADPH (Figure 3). These enzymes play a crucial role in photosynthesis. However, in contrast to photosynthetic cells, FNRs in microbes tend to favor the generation of Fd_{red}

(Spaans et al., 2015). Generating Fd_{red} via the reduction of NADPH requires energy because Fd_{red} has a lower reduction potential. Thus, it is unlikely that this reaction is prevalent among rumen microorganisms. Additionally, there is another type of FNR NADH-dependent reduced ferredoxin: NADP+ oxidoreductase (NfnAB), which couples the exergonic reduction of $NADP^+$ with Fd_{red} and the endergonic reduction of $NADP^+$ with NADH (Figure 4) (Spaans et al., 2015). This reaction is known as electron confurcation, which is the reverse reaction of electron bifurcation. Genome analysis has shown that NfnAB gene is present in many anaerobic bacteria and archaea (Huang et al., 2012, Buckel and Thauer, 2018b).

4 Interspecies electron transfer

Interspecies electron transfer occurs in the rumen. Rumen bacteria, protozoa, and fungi break down carbohydrates to produce VFA and reducing equivalents such as dihydrogen and formate. These reducing equivalents then diffuse to methanogens, which utilize them to synthesize methane (Leng, 2014). This process has a dual effect: on one hand, the consumption of dihydrogen and formate by methanogens accelerates VFA production, while on the other hand, methanogens obtain energy via methane production.

4.1 Dihydrogen generation

Hydrogenases are a diverse group of metalloenzymes that reversibly transfer electrons to protons to generate dihydrogen. Depending on the metal ion composition in the active site, hydrogenases can be classified into [NiFe], [FeFe], and [Fe] groups (Lubitz et al., 2014). Both [NiFe] and [FeFe] hydrogenases have proton channel,

hydrophobic gas channels for dihydrogen, and an electron transfer chain consisting of three [FeS] clusters, while [Fe] hydrogenase uses methenyltetrahydromethanopterin as an electron transfer chain component (Lubitz et al., 2014). Although hydrogenases catalyze a reversible reaction, [NiFe] hydrogenases favor dihydrogen oxidation, and [FeFe] hydrogenases favor dihydrogen generation. In the rumen microbes, hydrogenases present widespread traits. Greening et al. (2019) reported that 65%, 42%, and 2.4% rumen bacteria and archaea encode [FeFe], [NiFe], and [Fe] hydrogenase genes, respectively (Greening et al., 2019). To further classify these hydrogenases into functional groups based on their primary sequence, Søndergaard et al. (2016) developed an online tool that identified 6 different groups. [NiFe] hydrogenases were classified into 4 groups: group 1 (1a to 1k) and group 4 (4a to 4i) have the function of dihydrogen uptake, group 2 has the function of dihydrogen uptake and sensory, and group 3 (3a to 3d) is bidirectional. [FeFe] hydrogenases (A1 to A4, B, C1 to C3) and [Fe] hydrogenases were classified into 1 group, respectively.

4.1.1 Ferredoxin- and NAD- dependent electron-bifurcating [FeFe]-hydrogenase

Genome analysis of rumen microbes showed that the dominant hydrogenase reads are from A1, A2, A3, B, 3a, 3c, 4e, 4g, 4h, 4i, and [Fe] subgroup, which are mainly found in *Clostridiales*, *Methanobacteriales*, and *Selenomonadales* (Greening et al., 2019). Moreover, the authors found that A3, 1d, 3a, 3c, and 4g genes were highly expressed (RNA/DNA expression ratio > 4), with A3 accounted for 54% of hydrogenase transcripts (Greening et al., 2019). Hydrogenase A3 belongs to the [FeFe] group, which utilizes Fd_{red}

and NADH as stoichiometric electron donors to produce dihydrogen (Figure 5). The oxidation of reduced ferredoxin is an exergonic reaction, coupled with endergonic reaction of NADH oxidation, thereby conserving energy through electron confurcation. The hydrogenase A3 complex comprises three components: HydA, HydB, and HydC. HydA possesses the proton and dihydrogen gas channel, HydB contains an FMN subunit that transfers electrons from NADH, and HydC is an iron-sulfur protein responsible for electron transfer from Fd_{red} (Buckel and Thauer, 2018b). As a result, the hydrogenase A3 complex is also referred to as the HydABC complex (or MvhAGD complex). Reduced Fd, primarily produced by the EtfAB-Bcd and PFOR enzymes, serves as the electron donor for hydrogenase A3. This efficient utilization of Fd_{red} likely explains why hydrogenase A3 is the predominant hydrogenase in anaerobic fermenting microbes. Furthermore, it should be noted that this reaction is reversible and the reverse reaction has been observed in certain hydrogenotrophic acetogens (Schuchmann and Müller, 2012).

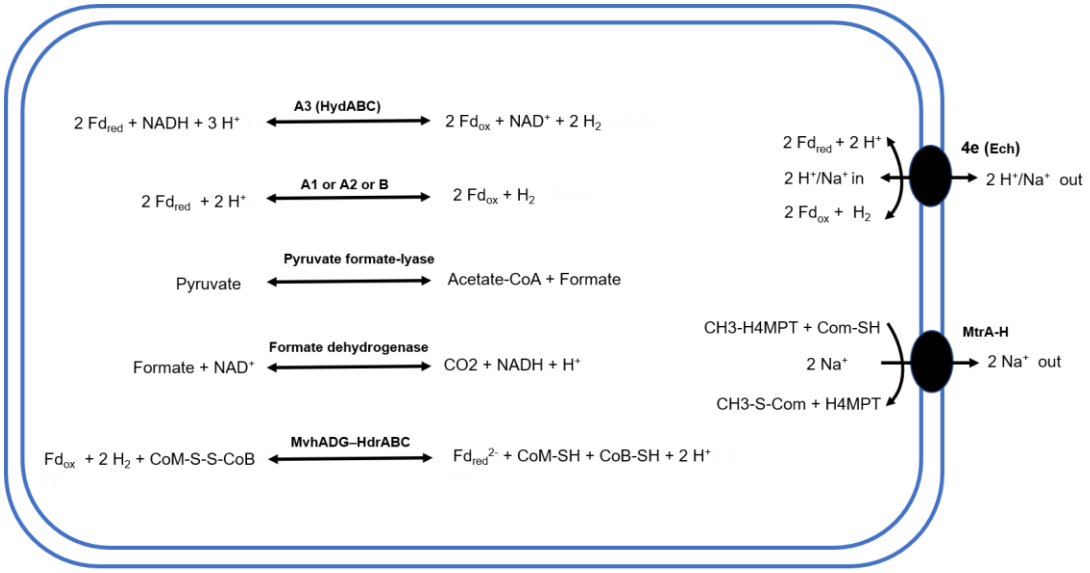

Figure 5 Common hydrogenases and the bifurcation reaction in Wolfe cycle.

HydABC, electron-bifurcating Fd- and NAD- dependent [FeFe]-hydrogenase; MtrA-H, energy-conserving methyltransferase; Ech, ferredoxin-dependent and membrane-associated hydrogenase.

4.1.2 Ferredoxin-dependent hydrogenase

Hydrogenases A1, A2, B, and C are the most abundant ferredoxin-dependent [FeFe]-hydrogenases, and both metagenomic and metatranscriptomic analyses showed that these hydrogenase genes are abundant and highly expressed in the rumen microorganisms of sheep (Greening et al., 2019). Hydrogenases A1, A2, and B catalyze the dihydrogen generation by using Fd_{red} as the electron donor (Figure 5), while hydrogenase C works as dihydrogen concentration sensor (Søndergaard et al., 2016). Hydrogenases A1, A2, and B are mainly found in fermentative bacteria such as *Clostridia*, *Negativicutes*, and *Bacteroidia*, while hydrogenase C is mainly found in *Clostridia* (Greening et al., 2019). When dihydrogen accumulated in the rumen, *Ruminococcus albus* downregulated the genes expression of hydrogenases C and A1 both by 111 folds, thus total dihydrogen production by A1 likely decreased (Greening et

al., 2019). A similar phenomenon was observed in another study by Zheng et al. (2014).

Hydrogenase 4e, also known as the Ech complex, is a ferredoxin-dependent and membrane-associated [NiFe]-hydrogenase that catalyzes dihydrogen formation through Fd_{red} oxidation and H^+/Na^+ translocation from intracellular to extracellular (Figure 5) (Buckel and Thauer, 2018b), thereby conserving energy. This enzyme is abundant and highly expressed in sheep rumen microorganisms, and is dominant in *Clostridia* (Greening et al., 2019). Meanwhile, the functions of highly expressed hydrogenase 4g in *Clostridia* remains unclear (Greening et al., 2019).

4.2 Formate generation

Rumen microbes have different pathways to generate formate. Pyruvate formate-lyase catalyzes pyruvate to acetyl-CoA and formate, which is thought to be the main source of formate production in the rumen fluid (Figure 5) (Asanuma et al., 1998). The genes of hydrogenases 4f, formate hydrogenlyase 4a, and formate dehydrogenase A4 are dominant in *Clostridia*, *Gammaproteobacteria*, and *Negativicutes*, respectively (Greening et al., 2019). Transportation of formate out of the cell is inefficient (Stams and Plugge, 2009), therefore 4f, 4a, and A4 likely catalyze formate oxidation to H_2 evolution (Greening et al., 2016, Søndergaard et al., 2016). Moreover, dihydrogen production via hydrogenase A3 is more efficient than pyruvate formatelyase. Considering the factors mentioned above, formate is likely not as important as dihydrogen in interspecies electron transfer under physiologic rumen conditions. According to Hungate et al. (1970), utilizing formate as electron donor produces 18% methane, while using dihydrogen

produces 82% methane.

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Rumen methanogenesis, methanogenesis inhibition methods, knowledge gaps and objectives of this study: part Ⅱ

1 Rumen microbiota

The rumen microbiota comprises of a diverse range of microbes, including bacteria, fungi, archaea, protozoa, and phage, which interact with each other and the host animal to facilitate the feed digestion. The host animal provides a habitat with an anaerobic environment, steady temperature, and abundant nutrients to the microbes, and in return, the microbes provide nutrients such as VFA and microbial protein to the host animal. The rumen bacteria, fungi, and protozoa digest feed nutrients (mainly carbohydrates) to VFA and dihydrogen. Rumen wall absorbs VFA as the primarily energy source of the ruminant, and archaea use the diffused dihydrogen with carbon dioxide to produce methane. On one hand, archaea obtain energy (ATP) through methanogenesis. On the other hand, dihydrogen consumption promotes VFA production. Here we give a detailed description of the rumen archaea because of their role in methane production.

1.1 Rumen archaea

Archaea are present in the rumen at low abundance (10^4 / mL rumen fluid), with 16s rRNA gene sequencing indicating that they account for only 0.3%~3.3% of total rumen prokaryotes (Janssen and Kirs, 2008). Methanogenic archaea (methanogens) are the dominant component, which use carbon dioxide and dihydrogen to produce methane as

the end product (Patra et al., 2017). Analysis of 8623 archaeal 16s rRNA gene sequences from the Ribosomal Database Project database Release 11, showed that 63.2% of the sequences were classified as *Methanobrevibacter*, followed by 9.8% as *Methanosphaera*, 7.7% as *Methanomicrobium*, and 1.2% as *Methanobacterium* (Patra et al., 2017). More recently, Xing et al. (2020) identified *Methanomassiliicoccus*, *Methanobrevibacter*, *Methanosphaera*, *Methanoculleus*, and *Methanothrix* as the most abundant archaea in the rumen fluid. Most of the rumen methanogens have not yet been isolated. It was reported that cultured methanogens only accounted for approximately 0.7% of the total rumen original archaeal sequences, and most of the isolates were classified as Methanobacteriaceae (Patra et al., 2017). Meanwhile, methanogens inhabit rumen fluid, attach to the feed and rumen epithelium, and even have an endosymbiotic relationship with rumen protozoa (Patra et al., 2017). The abundance and diversity of methanogens differ between rumen fluid, solid, and epithelium (Pei et al., 2010).

Methanogens can be classified into three categories based on the substrates used to produce methane: hydrogenotrophic methanogens, methylotrophic methanogens, and acetotrophic methanogens (Morgavi et al., 2010). Most rumen methanogens are hydrogenotrophic methanogens that use dihydrogen and carbon dioxide as substrates to produce methane. Members of the genus *Methanobrevibacter* belong to this category; Methylotrophic methanogens use methyl compounds as carbon sources and are classified as *Methanosarcinales*, *Methanosphaera*, and *Methanomassiliicoccaceae*; Acetotrophic methanogens including *Methanosarcinales* using acetate to produce methane and carbon dioxide (Huws et al., 2018).

2 Implications of enteric methane emissions

According to the United Nations, the world population is expected to reach 9.7 billion by 2050 and 11.1 billion by 2100 (Abel et al, 2016). As a results, there will be a greater need for animal products, especially milk and meat, to meet the growing demand for nutrition. Moreover, the requirement for animal protein is significantly increasing with the economic improvement in low- and medium-developed countries. However, the animal industry is one of the main sources of greenhouse gas (e.g., carbon dioxide, methane, and nitrous oxide). In fact, livestock sector produces 7.1 gigatonnes CO2-eq per annum, representing 14.5% of human-induced greenhouse gas emissions (Gerber et al., 2013).

In addition, methane emissions from ruminants also results in feed energy waste. About 2 ~ 12% of feed energy is lost because of methane production (Johnson and Johnson, 1995). Thus, decreasing methane production could potentially save feed energy and produce more animal products. Decreasing enteric methane emissions, while simultaneously improving animal productivity is one of the most effective ways to meet the increasing demand for animal protein and the challenge of climate change. Also, if decreasing enteric methane emissions leads to increase animal productivity, farmers will have the motivation to use methanogenesis inhibitors in practice.

3 Methane metrics and methane measurement techniques

To evaluate the effectiveness of mitigation strategies, reliable tools to measure methane and express methane emissions are mandatory. The methane metrics used to

describe methane production by ruminants have been standardized to methane production, methane yield, and methane intensity. The term “methane production” refers to the amount of methane produced per day (g of CH_4 /d); “methane yield” refers to the amount of methane produced per kg dry matter intake (g of CH_4/kg DMI), and “methane intensity” refers to the amount of methane produced per kg animal products (e.g., g of CH_4/kg animal products) (Melgar et al, 2020). Residual methane emissions, primarily used in breeding, refers to the discrepancy between an animal's actual methane production and its expected production based on its feed intake and body weight (Herd et al., 2014).

Measuring animal methane emissions is essential for the fundamental understanding of methanogenesis and evaluating any mitigation strategies. The accuracy, precision, repeatability, and cost-effectiveness are the key factors for the methane measurement methods (Patra, 2016). With around 50 years of work in this area, many enteric methane measurement methods have been evaluated. In general, those methods can be classified into two categories: direct methods that directly measure methane emissions from animal, and indirect methods that estimate methane emissions based on other parameters such as feed intake and digestibility.

3.1 Direct methods

3.1.1 Respiration Chamber

The respiration chamber, which can be classified as either open-circuit chamber or closed-circuit chamber, is considered the “gold standard” for animal enteric methane

measurement, because it provides highly accurate and repeatable results (Tedeschi et al., 2022). Animals are confined in the chamber for several successive days (usually 3-5 days), and gas samples are collected at a given frequency (for instance, 15 min) and analyzed for composition, while the outlet airflow is recorded. To prevent potential internal gas leaks, the respiration chamber maintains a slightly negative atmospheric pressure (Zhao et al., 2020).

Compared to other methane measurement methods, the respiration chamber can measure enteric methane produced from the rumen and the hindgut, and it can be used to study feed digestibility and energy metabolism, which are directly related to methane production. Additionally, the respiration chamber can continuously monitor methane and dihydrogen production in a 24-h cycle, which is important because ruminant enteric gas emissions, especially methane and dihydrogen emissions, are not evenly distributed throughout the day (van Gastelen et al., 2020).

However, the respiration chamber is costly and requires intensive labor, which limits the number of animals that can be included in each trial. Furthermore, animal behavior may be disturbed while they are confined in the chamber, potentially affecting their performances, even if the animals are acclimated to the chamber prior to the experiment. Milking, cleaning, sampling, and feeding operations may also interrupt methane and dihydrogen measurement, however a recent study had suggested that these impacts can be negligible (van Gastelen et al., 2020).

3.1.2 Greenfeed

Greenfeed is designed by C-Lock incorporation (Rapid City, SD, USA) to measure

methane, dihydrogen, carbon dioxide emissions, and oxygen consumption by ruminants. It is an automated head-chamber system equipped with a radio-frequency identification system, a bait feed delivery system, a head position sensor, an airflow meter, and gas sensors. The radio-frequency identification system can recognize the animal, and the bait feed delivery system can allure animal to visit this device and control the interval time of the adjacent visiting. For each measurement, it takes 7 mins on average. We can use Greenfeed in grazing system because it is portable and automatic. Although the bait feed and environmental conditions such as wind speed may introduce variances to the measurement, the Greenfeed is a reliable method to measure methane and dihydrogen emissions when the number of animals and visiting frequency are appropriate (Coppa et al., 2021).

3.1.3 Other methods

The sulfur hexafluoride (SF6) tracer technique is a minimally invasive and specific method for measuring methane emission in both stall feeding and grazing systems. A small brass permeation tube filled with liquid SF6 is orally dosed into the rumen, after which the liquid SF6 gasifies at a constant rate. Gas samples are collected from the ruminant’s mouth and nose using tubes (Arbre et al., 2016). Methane emission per day can be calculated according to the SF6 release rate and the concentration ratio of methane to SF6 in the collected gas sample. The key assumption of this method is that the rumen's methane production rate is constant throughout the day. However, it is well established that rumen methane production rate fluctuates mainly depending on feeding (Van Gastelen et al., 2018). Also, this method requires a well-trained technician to

operate to minimize error. There are other direct measurement methods, including the sniffer technique, ventilated hood, and facial mask, however these methods are not widely used due to issues related to accuracy, cost, and/or difficulty of operation. Further information about these methods can be found in review papers (Hammond et al., 2016, Zhao et al., 2020, Tedeschi et al., 2022).

3.2 Indirect methods

While the methods of respiration chamber, Greenfeed, and SF6 tracer technique for measuring ruminant methane emissions are well-established, their intensive labor and high expenditure requirements limit their application. To address this issues, indirect methane emission measurement methods have been developed. For example, the in vitro technique is used to predict the methane production of ruminants. However, this method only reflects relative methane emission among different treatment groups under *in vitro* conditions. Recently, researchers have sought to identify biomarkers to predict rumen methane emission. Chilliard et. al. (2009) found that milk-saturated fatty acids of C6:0 to 16:0 and C10:1 have correlation coefficients of 0.87 and 0.91, respectively, with methane emission; and some unsaturated fatty acids have a negative correlation with methane emission. A meta-analysis also found that milk fatty acid is a good indicator to predict dairy methane emission (Bougouin et al., 2019).

Van Gastelen et. al. (2018) further compared the different milk fatty acid analysis methods, gas chromatography and Fourier-transform infrared spectroscopy, to calculate the correlation coefficients of milk fatty acid and methane emission. They found that the

correlation coefficient is 0.77 for milk fatty acid analyzed by gas chromatography and 0.72 for milk fatty acid analyzed by Fourier-transform infrared spectroscopy. And the cross-validation results indicate that both fatty acid measurement methods were robust to predict methane emission. However, the authors thought that these methods are not yet ready to use in practice. Additionally, researchers have identified some blood discriminant metabolites (Yanibada et al., 2020) and milk discriminant metabolites (Yanibada et al., 2021) that have the potential to predict methane emission.

4 Methanogenesis mitigation strategies

Methane is a potent greenhouse gas that has a global warming potential equivalent to 28 times than that of carbon dioxide over a period of 100 years (Tian et al., 2016), however, methane has a shorter lifespan than carbon dioxide. Therefore, decreasing methane emissions could have a rapid and significant effect on decreasing global warming (Tian et al., 2016).

To date, researchers have developed various methods to mitigate ruminant methane emissions, including the use of feed additives (e.g., 3-nitrooxypropanol, seaweed, lipid, secondary plant compounds, nitrate, and phenolic compounds) (Li et al., 2016, Martinez-Fernandez et al., 2017, Doreau et al., 2018), diet formulation (e.g., high-quality forage, increase the ratio of concentrate) (Eugène et al., 2021), management intervention (e.g., improve feed efficiency), breeding, immunization, and defaunation (Beauchemin et al., 2020).

Recently, studies have shown that algae have a high ability to mitigate ruminal

methane emissions *in vitro* and *in vivo* (Machado et al., 2014, Li et al., 2016). Algae is a natural animal feed source in many countries (Tiwari and Troy, 2015), making it a potential methanogenesis inhibitor in ruminants. Additionally, electron acceptors such as nitrate and phenolic compounds are effective methanogenesis inhibitors by completing available dihydrogen with methanogens (Doreau et al., 2018, Rongcai et al., 2021). In this review, we discuss the use of algae and electron acceptors as methanogenesis inhibitors in the ruminant sector.

4.1 Algae

Algae can be categorized as either macroalgae (seaweed) or microalgae. Seaweed can be further categorized into three main groups based on their pigmentation: green seaweeds (*Chlorophyta*), red seaweeds (*Rhodophyta*), and brown seaweeds (*Ochrophyta Phaeophyceae*) (Hashim and Chu, 2004). The nutrient composition of algae is highly variable and depends on the factors such as species, habitat, and collection time. Algae are known to contain various bioactive compounds, including steroids and unsaturated fatty acids (Harwood et al., 2009). Additionally, red and brown seaweeds are known to contain halogenated compounds, and some brown seaweeds (such as *Sargassum Fucus,* and *Ascophyllum nodosum*) possess antioxidant activity due to the presence of phenolic compounds (Holdt and Kraan, 2011).

4.1.1 The history of using algae as methanogenesis inhibitor and the related bioactive compounds

The use of algae as potential methanogenesis inhibitor started in the mid-2000

based on the amount and composition of their lipids because the role of lipids in decreasing methane production in ruminants was already known (Swift et al., 1948). Ungerfeld et. al. (2005) found that hexadecatrienoic acid extracted from the Hawaiian *Chaetoceros* (microalgae) inhibited methane production *in vitro* batch fermentation. Later, Wang et. al. (2008) reported that phlorotannins extracted from *Ascophyllum nodosum* (brown seaweed) inhibited methane production in 24-h fermentation. Subsequently, Bozic et al. (2009) investigated the use of whole *Chaetoceros* (microalgae) powder as a feed additive to inhibit rumen methane production. They found that adding *Chaetoceros* greatly reduced methane production, hypothesizing that the bioactive compound was hexadecatrienoic acid.

A few years later, Machado et. al. (2014) evaluated the effect of 20 different seaweeds (including green, brown, and red seaweeds) on rumen methane production *in vitro*. They found that all seaweeds decreased methane production, with *Dictyota bartayresii* (brown seaweed) and *A. taxiformis* (red seaweed) exhibiting the greatest inhibitory effects, decreasing methane production by 92% and 99%, respectively. The authors suggested that the secondary metabolites, in particular isoprenoids for Dictyota bartayresii and volatile halogen compounds for *A. taxiformis*, were responsible for the decrease in methane production (Machado et al., 2014). It was reported that bromoform was the most abundant volatile halogenated compound, accounting for 80% of the essential oil extracted from *A. taxiformis* (Burreson et al., 1976). Moreover, researchers found that bromoform concentration was 109-fold higher than the second abundant halogenated compound (dibromochloromethane) in *A. taxiformis* (Machado et al., 2016).

Paul et al. (2006) reported that bromoform extracted from *A. armata* had anti-bacterial properties. More recently, it has been confirmed that *A. taxiformis* effectively inhibits methane production primarily due to its high concentration of bromoform (Machado et al., 2016). And Glasson et al. (2022) reported that bromoform acts both on the coenzyme M methyltransferase and the methyl-coenzyme M reductase to decrease methane production.

4.1.2 Seaweed inhibits methanogenesis *in vitro*

In vitro work showed that red seaweed, especially the red seaweed from the genus *Asparagopsis,* has been shown to be more effective than green and brown seaweeds in decreasing methane production (Machado et al., 2014, Maia et al., 2016, Brooke et al., 2020, Choi et al., 2021a, Choi et al., 2021b). This is likely because the high concentration of bromoform in *Asparagopsis* species (Brooke et al., 2020, Nørskov et al., 2021).

Machado et al., (2014) reported that including 16.7% of *A. taxiformis* (based on OM) decreased methane production by 99%, but also decreased total gas production by 62%, and total VFA concentration by 47%. To determine the optimal inclusion dose, a lower dose-response experiment was conducted with ten levels of inclusion (0, 0.07, 0.125, 0.25, 0.5, 1, 2, 5, 10, and 16.7 %, based on OM) (Machado et al., 2016). They found that 1% inclusion level of *A. taxiformis* decreased methane production by 85% with a 17% decrease in total VFA and a 32% decrease in total gas production. Later, Kinley et. al. (2016) found that 2% inclusion level of *A. taxiformis* (based on OM) eliminated methane production without negatively effecting total VFA concentration or feed digestion. However, these three *in vitro* studies did not measure the bromoform concentration in the

A. taxiformis. Thus, we still do not know the optimal inclusion level of *A. taxiformis* in *in vitro* fermentation. Later, Machado et al. (2016) measured the bromoform concentration (1.7 mg/g bromoform based on DM) in *A. taxiformis* and found that 2% of *A. taxiformis* (based on OM) decreased methane production by 95%, with TGP decreased by 25% without negatively affecting total VFA production and organic matter digestion. Therefore, 2% inclusion level of *A. taxiformis* was recommended as the highest inclusion level when the bromoform concentration is 1.7 mg/g as it has minimal negative effects on *in vitro* fermentation (Machado et al., 2016).

In addition, 2% inclusion of *A. taxiformis* (based on OM) altered the community structure of methanogens by decreasing the abundance of *Methanobacteriales*, *Methanomassiliicoccales*, and *Methanomicrobiales* (Machado et al., 2018). Roque et. al. (2019a) also reported that 5% inclusion level of *A. taxiformis* altered the relative abundance of methanogens and changed the bacterial community structure by beta-diversity analysis.

4.1.3 Seaweed inhibits methanogenesis *in vivo*

Li et. al. (2016) first reported that *A. taxiformis* decreased methane production in sheep in a dose-dependent manner, with inhibition rate from 53% to 81% when the *A. taxiformis* (based on OM, lower than 0.4 g/kg halogenated compounds on DM basis) was included at levels between 0.5% and 3%. Total VFA concentration, dry matter intake (DMI), and live weight were not affected when inclusion of 0.5% *A. taxiformis* (Li et al., 2016). In another study, Roque et al. (2019b) found that inclusion of 0.5% and 1% *A. armata* (based on OM, bromoform concentration was 1.3 mg/g based on DM) decreased

methane yield by 20.3% and 42.7%, respectively, but also decreased DMI by 10.8% and 38%, respectively. Moreover, milk yield decreased by 11.6% at the 1% inclusion level (Roque et al., 2019b). The effects of *Asparagopsis* on methane inhibition rate and DMI differ in these two studies despite the similar *Asparagopsis* inclusion level. These discrepancies could be explained by the factors such as bromoform concentration in *Asparagopsis*, animal species, diet, and the inclusion method of *Asparagopsis*. Kinley et al. (2020) investigated the effects of *A. taxiformis* with a high concentration of bromoform (6.6 g/kg DM) at inclusion levels of 0.1% and 0.2% (based on OM). They found that these low inclusion levels decreased methane yield by 38% and 98%, respectively, while increasing weight gain by 53% and 42%, respectively. Importantly, these inclusion levels did not negatively impact DMI or total VFA concentration. In another study, which used *A. taxiformis* with a high concentration of bromoform (based on OM, bromoform concentration was 7.8 g/kg based on the DM), the authors found that inclusion of 0.25% *A. taxiformis* decreased steer methane production by 36%, 51%, and 72% when fed high (60%), medium (40%), and low (11%) forage diets, respectively. Moreover, this inclusion level in different diets did not have negative effects on DMI, average daily gain, and feed conversion efficiency (Roque et al., 2021).

Overall, these studies suggest that using high-bromoform-containing *Asparagopsis* allows for decreased inclusion levels, which may minimize negative effects on DMI and animal performance. However, more research is needed to fully understand the effects of *Asparagopsis* on methane production and DMI, particularly when using low inclusion levels and high-bromoform-containing seaweed.

4.1.4 Factors affecting the antimethanogenesis ability of seaweed

The efficacy of *Asparagopsis* in inhibiting methanogenesis is directly related to the concentration of the bioactive compound bromoform. Any factors that decrease the concentration of bromoform in *Asparagopsis* will decrease its inhibitory efficacy. Stefenoni et. al. (2021) observed that the methane-inhibition power of *A. taxiformis* was not stable in a four-month duration study, with high efficiency in the first two months that decreased thereafter. They have confirmed that bromoform concentration in the *A. taxiformis* linearly decreases during storage, while exposure to light accelerates the decrease process but storage temperature does not have a significant effect on bromoform concentration (Stefenoni et al., 2021). Additionally, the processing method of *A. taxiformis* is another key factor in preserving bromoform concentration. Vucko et. al. (2017) examined the effect of processing factors including rinsing (unrinsed/dip rinsed/submerged), freezing (frozen/not frozen), and drying (freeze-dried/ kiln-dried/ dehydrated) on bromoform concentration in the *A. taxiformis,* and found that unrinsed, frozen, and subsequently freeze-dried were the most effective processing method to maintain bromoform concentration. Other factors such as harvest time, growth phase, habitat, and bromide concentration in the sea may also influence bromoform concentration in *A. taxiformis* (Paul et al., 2006, Félix et al., 2021).

4.1.5 Animal health and animal products safety concerns when using seaweed as a methanogenesis inhibitor

The use of seaweed as a feed additive may raise concerns about animal health and

safety of animal products. Li et. al. (2016) first reported that inclusion of *A. taxiformis* led to ruminal mucosa granulomatosis and keratosis. These findings were confirmed by Muizelaar et. al. (2021), who observed rumen wall inflammation and the absence of rumen papillae. However, these observations were based on a small number of animals, thus, further experiments with a larger sample size are necessary to verify these results. In addition, both Li et. al. (2016) and Stefenoni et. al. (2021) reported that *A. taxiformis* decreased alanine transaminase (ALT) concentration in plasma, which is an indicator of liver cell damage.

A. taxiformis has a high concentration of bromide (mainly bromoform) and iodine, which may transfer into milk and meat when used it as feed additive. Stefenoni et. al. (2021) reported that inclusion of 0.5% *A. taxiformis* (based on the DM) for 112 d did not increase bromoform concentration in the milk or alter the milk sensory properties, while it significantly increased milk bromide and iodine concentrations. In contrast, Roque et. al. (2019b) reported that the inclusion of 1% *A. armata* (based on OM) for 63 d numerically increased milk bromoform concentration, although this concentration is much lower than the allowed bromoform concentration in drinking water. For meet, bromoform was not detected in the muscle and adipose fat of sheep when inclusion of 3% *A. taxiformis* (based on OM) in the diet for 72 d (Li et al., 2016). Similarly, Kinley et. al. (2020) observed that the meat quality of steers was not affected by inclusion of 0.2% *A. taxiformis* (based on OM) for 90 d and bromoform was not detected in the fat, meat, and kidney tissues. Roque et. al. (2021) found that bromoform was not detected in meat and liver tissues, and the quality of the meat was not affected by inclusion of *A. taxiformis* for

147 d, however, the iodine concentration in strip loin was significantly increased by *A. taxiformis* supplementation, although this concentration is below the tolerable upper intake level for human consumption of foods specified by the US Food and Nutrition Board of the National Academy of Sciences (Roque et al., 2021).

4.2 Electron acceptors

An electron acceptor is a chemical compound that can accept electrons in redox reactions. In the rumen, fumarate and carbon dioxide are the primarily electron acceptors (Ungerfeld, 2020). The mechanism by which electron acceptors decrease methane production is by competing with hydrogenotrophic methanogens for available dihydrogen. Meanwhile, some electron acceptors, such as nitrate, could also directly inhibit methanogens (Guyader et al., 2015b).

Several compounds have been tested as electron acceptors to reduce ruminal methane production, including nitrate (Van Zijderveld et al., 2010, Doreau et al., 2018), sulfate (Judy et al., 2019), fumarate (Asanuma et al., 1999), and phenolic compound such as phloroglucinol (Martinez-Fernandez et al., 2017). Calcium nitrate has been shown to decrease methane production in nonlactating cows (Guyader et al., 2015a, Guyader et al., 2015b). However, high nitrate inclusion levels may result in side effects such as a decrease in milk protein yield, an increase in blood methemoglobin level, and a decrease in hemoglobin level (Van Zijderveld et al., 2011). Therefore, Beauchemin et al. (2020) recommended that the inclusion level of nitrate should not be exceed 2% (based on DM).

Inclusion of 0.93% calcium sulfate (based on DM) has been shown to decrease methane yield by 11.2%, and to decrease apparent organic matter digestibility, but did not have adverse effects on DMI or milk yield (Judy et al., 2019). However, sulfate is less effective than nitrate and can be toxic to animals when included at high level (Hegarty, 1999).

4.2.1 phenolic compounds

Phenolic compounds, including flavonoids, phenolic acids, and tannins, share a common chemical structure that includes at least one aromatic ring with one or more hydroxyl substituents (Ayad and Akkal, 2019). This literature review focuses on gallic acid, which is the subunit of hydrolysable tannins. Tannins are plant secondary metabolites present in legumes, cereals, grains, and other feedstuffs (Roca-Fernández et al., 2020). There are two classes of tannins in plants: hydrolysable tannins and condensed tannins. Hydrolysable tannins can be broken down into phenolic constituents, mainly gallic acid, by the ruminal microbiota. The ruminal microbiota then converts gallic acid into pyrogallol, which is further converted into phloroglucinol or resorcinol (McSweeney et al., 2001). Phloroglucinol degradation requires NADPH, this is the mechanism by which phenolic compounds have the potential to work as electron acceptors.

Several researchers have isolated rumen bacteria that can degrade phloroglucinol. Tsai and Jones (Tsai and Jones, 1975) isolated 8 strains of phloroglucinol-degradation bacteria from rumen, 5 of them belong to *Streptococcus bovis* and the other belong to *Coprococcus.* Later, Patel et al. (1981) purified phloroglucinol reductase from

Coprococcus sp. Pe15 and found that it was NADPH-dependent reductase. Meanwhile, other researchers isolated another phloroglucinol-degradation bacterium *Eubacterium oxidoreducens* sp. nov. from rumen (Krumholz and Bryant, 1986).

Tsai et. al. (1976) speculated that *Coprococcus* sp. Pe15 decomposes 1 molecule of phloroglucinol to produce 2 molecules of carbon dioxide, and 2 molecules of acetate according to the end products and their ratios in pure culture. Evans (1977) speculated that *Rhodopseudomonas gelatinosa* decomposes 1 molecule of phloroglucinol to produce 3 molecules of acetate and consumes 1 molecule NAD(P)H, while Krumholz et. al. (1987) speculated that *Eubacterium oxidoreducens* decomposes 1 molecule of phloroglucinol to produce 3 molecules of acetate and consumes 0 molecules of NAD(P)H or to produce 1 molecule of acetate, 1 molecule of butyrate, and consumes 2 molecules of NAD(P)H based on the enzymes isolated from the pure culture. The pathways of phloroglucinol degradation mentioned above only represent the specific bacteria and they have not been confirmed. However, an *in vivo* study has been observed that phloroglucinol decreased dihydrogen expelled (g/kg DMI) and decreased formate concentration in the rumen fluid when methanogenesis was inhibited (Martinez-Fernandez et al., 2017).

5 Knowledge gap

In the rumen, methanogens use dihydrogen and carbon dioxide to produce methane. To date, we can achieve around a 25% methanogenesis inhibition rate without negatively affecting ruminants' health and productivity. When methane production is inhibited,

dihydrogen accumulates in the rumen; though alternative hydrogenotrophic pathways are present, all the dihydrogen is not consumed, and just a small proportion erupts. Our current knowledge of the dihydrogen economy in rumen is incomplete, and an improved understanding of microbial pathways involved in dihydrogen metabolism is required.

6 Hypotheses and objectives

Evidence suggests that alternative hydrogenotrophs can prosper in the rumen and uptake dihydrogen when methanogenesis is inhibited. We hypothesized that adding alternative electron acceptors to the rumen could enhance these pathways and redirect the excessive dihydrogen toward nutritionally beneficial sinks. The objective of the work presented in this manuscript was to evaluate the potential of phenolic compounds to serve as alternative hydrogen acceptors; as phenolic compounds are metabolized to VFA, we aimed at assessing the effects on ruminant production. To achieve this aim, we performed an *in vitro* experiment to test a range of phenolic compounds as hydrogen acceptors and investigate their effects on rumen microbiota when methanogenesis was inhibited. We also performed an *in vivo* experiment to build a methanogenesis-inhibited model in cows and test the impact of the selected phenolic compound on animal performance, gas emissions, rumen microbiota, and blood metabolites.

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CHAPTER 2

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

Introduction

In this *in vitro* experiment, we screened a range of phenolic compounds as dihydrogen acceptors and selected the best candidate for the *in vivo* experiment. We hypothesized that when methanogenesis is inhibited, the addition of phenolic compounds may enhance the rumen phenolic-degrading bacteria to capture the excessive dihydrogen and generate nutrients. The objective of this chapter is to find the best phenolic compounds as dihydrogen acceptor by screening a range of phenolic compounds *in vitro*.

Experiment design

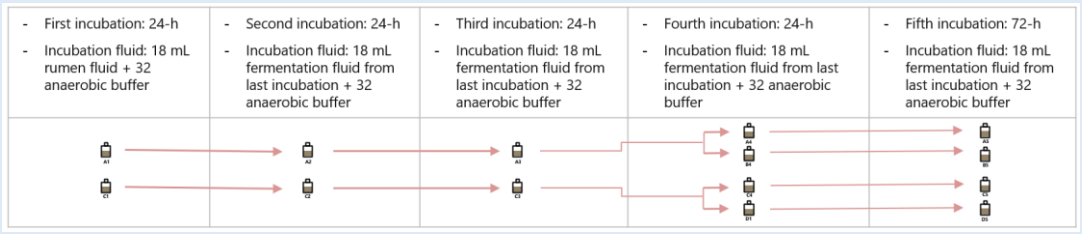
Exp.1

- Treatment: 2, 4, and 6 mM of each phenolic compounds, control
- Incubation time: 24 h

Exp.2

- Treatment: 6 mM of each phenolic compounds + 3 μM BES
- Incubation time: 24 h

Exp.3 (sequential incubation)



Sample collection: gas and fermentation fluid were collected at end of the fourth and fifth incubations.

Main finding

- 6 mM phenolic compound alone didn't have negative effect on fermentation
- 6 mM phloroglucinol and gallic acid combined with BES increased acetate proportion
- After adaptation, 36 mM phloroglucinol combined with BES decreased dihydrogen accumulation
- Supplementation with 36 mM phloroglucinol decreased methanogens abundance

Conclusion

Phloroglucinol and the gallic acid have the potential to serve as dihydrogen acceptors *in vitro*. In addition, phloroglucinol supplementation likely have the negative effect on methanogens abundance.

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*

Corresponding author: Diego Morgavi. E-mail address: diego.morgavi@inrae.fr

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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 hypothesized that combining a 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 (*A. 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 cow's VFA production, decrease H_2 accumulation and synergistically decrease CH_4 production in the presence of antimethanogenic compounds.

Key words: phloroglucinol; methane inhibitor; dihydrogen accumulation; rumen fermentation; volatile fatty acid

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 vitro*. 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.

1 Introduction

Methane (**CH_4**) production in livestock sector accounting for 6% of anthropogenic

greenhouse gas emissions and ~40% of total greenhouse gas emissions in livestock sector (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 the inhibition of methanogenesis using feed additives. The macroalgae *A. taxiformis* 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, 1995b). 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 liveweight 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., 2010b), 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 dry matter 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 catabolize phenolic compounds such as gallate, pyrogallol, and phloroglucinol to generate VFA (Evans, 1977, Krumholz and Bryant, 1986b). It was reported that phloroglucinol decreased the ratio 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 hypothesized 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., 2015a, Corral-Jara et al., 2022b). In addition, goats and cows have shown different response 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.

2 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 pre-selected 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-bromoethanesulfonic sodium (BES)) (Exp.2), and longer-term effects of phloroglucinol combined with a methanogenesis inhibitor (Exp.3).

2.1 Substrates and methanogenesis inhibitors preparation

Alfalfa hay and barley grain ground through a 1-mm sieve were used as substrates for incubation. *A. taxiformis,* a red macroalgae, and BES were chosen as

methanogenesis inhibitors in this study. *A. 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 (30s) 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.

2.2 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 pre-selected 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, 2014b), 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 pre-heated 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 equalized 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 [8 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).

2.3 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 + methanogenesis inhibitor + individual

phenolic compounds or formic acid. Incubation procedures and sample collection were as Exp.1. The total number of experimental units were 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).

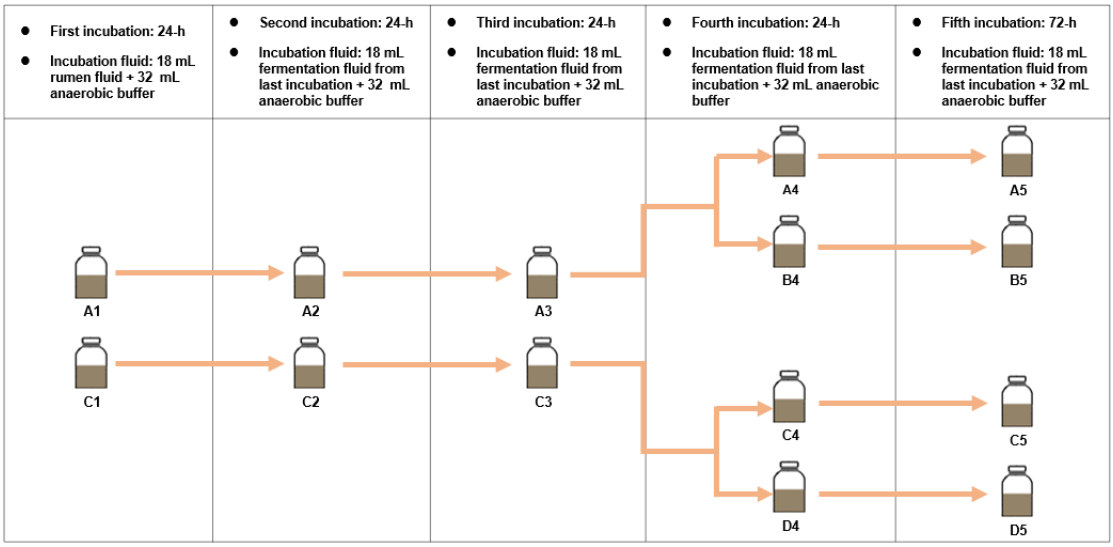

Figure 6 Experimental design of the sequential batch incubation that used rumen fluid inocula from dairy cows (n=8). Abbreviations: A = Substrate only; B = Substrate + 2-bromoethanesulfonic sodium; C = Substrate + phloroglucinol; D = Substrate + phloroglucinol + 2-bromoethanesulfonic sodium. The number 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.

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

This experiment used sequential batch incubation to evaluate the effect of phloroglucinol on fermentation in the presence or absence of BES over a longer incubation period (Figure 6). 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 stabilizing 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 8 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 as in Exp. 1 and for the subsequent batches, 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 were collected and stored at -20 °C.

2.5 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 micro gas chromatography (Micro GC Fusion, INFICON). The micro-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, 1967b). 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 polymerase chain reaction (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 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.

2.6 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 $P \leq 0.10$.

3 Results

3.1 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 2 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.

Table 2 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.

3.2 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 3 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, with 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 3 Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid, and formic acid at 6 mM when combined with *A. taxiformis* at 2.5% DM on *in vitro* rumen fermentation from dairy cows (n= 4).

Item	Substrate alone[1]	Treatment									SEM	*P*-value[2]
		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 = *A. taxiformis*; 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 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.

3.3 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 4 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 proportion (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 (P = 0.002), and had no effect on protozoal (P = 0.80) abundance, compared to the control.

Table 4 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				SEM	*P*-value
	Control[1]	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^{c}	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] Control: Substrate alone, no chemical compound added.

[a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$.

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 ($P = 0.001$) 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 archaeal ($P < 0.001$), and higher bacterial abundance ($P = 0.013$) than BES alone. In contrast, 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).

4 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 pyrogallol, phloroglucinol or resorcinol by the ruminal microbiota (McSweeney et al., 2001b). 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., 1987b). *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 total gas and

VFA concentration, and in particular acetate proportion. The latter is in accordance with the expected end product of phloroglucinol degradation (Krumholz and Bryant, 1986b). 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., 1970b).

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 donor, 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, 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 catabolize phloroglucinol (Tsai and Jones, 1975b), but this bacterium is a minor member of the normal rumen microbiota (Petri et al., 2013). Other more specialized bacteria known to utilize 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 hypothesized that populations of rumen microbes that can metabolize phloroglucinol needed longer incubation times to grow to metabolize 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 utilize 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 4). 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., 2001b). 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 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.

5 Conclusion

We evaluated the effect of seven phenolic compounds on 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.

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: 0000-0002-3094-7700

Pedro Romero: 0000-0001-5746-8286

Alejandro Belanche: 0000-0001-5880-6021

Emilio M. Ungerfeld: 0000-0002-5422-5462

David Yanez-Ruiz: 0000-0003-4397-3905

Milka Popova: 0000-0001-6695-5502

Diego P. Morgavi: 0000-0002-3883-0937

Author contributions

Rongcai Huang: Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Writing - Review & Editing; **Pedro Romero:** Visualization, Writing - Review & Editing; **Alejandro Belanche:** Conceptualization, Resources, Writing - Review & Editing, Visualization; **Emilio M. Ungerfeld:** Writing - Review & Editing, Visualization; **David Yanez-Ruiz:** Conceptualization, Resources, Writing - Review & Editing,

Visualization; **Milka Popova:** Conceptualization, Validation, Formal analysis, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition; **Diego P. Morgavi:** Conceptualization, Validation, Formal analysis, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of interest

None.

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Supplemental material

Table S1 Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, and gallic acid or formic acid at 2 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	58.68	56.58	57.71	57.30	58.42	60.27	58.92	59.94	59.19	10.43	0.21
TGP / 6-24 h	65.90	61.79	65.10	65.87	66.98	68.84	65.88	66.91	65.85	8.44	0.13
TGP / 0-24 h	124.58	118.79	122.81	123.17	125.41	129.11	124.81	126.85	125.04	18.89	0.13
CH_4 / 0-6 h	13.30	12.47	12.25	13.14	13.48	13.50	13.48	13.74	14.00	1.87	0.35
CH_4 / 6-24 h	17.15	16.15	16.68	16.90	17.36	16.94	16.61	16.49	16.35	2.35	0.50
CH_4 / 0-24 h	30.45	28.65	28.93	30.04	30.83	30.44	30.09	30.22	30.34	4.21	0.64
H_2 / 0-6 h	0.06	0.07	0.34	0.14	0.10	0.03	0.07	0.06	0.02	0.09	0.29

Abbreviations: TGP = total gas production.

[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 S2 Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, and gallic acid or formic acid at 4 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	58.68	58.98	58.83	58.64	58.31	59.07	60.34	60.94	60.47	9.86	0.25
TGP / 6-24 h	65.90	65.48	65.84	65.84	65.45	69.95*	67.86	66.78	66.74	9.07	<0.01
TGP / 0-24 h	124.58	124.46	124.66	124.28	123.76	129.03*	128.20	127.71	127.20	18.91	<0.01
CH_4 / 0-6 h	13.30	13.47	13.18	13.82	13.62	13.57	14.30	13.80	14.12	1.81	0.19
CH_4 / 6-24 h	17.15	16.81	17.04	16.68	17.15	16.17*	16.70	16.56	17.15	2.48	0.05
CH_4 / 0-24 h	30.45	30.28	30.22	30.50	30.77	29.74	31.01	30.36	31.27	4.27	0.20
H_2 / 0-6 h	0.06	0.03	0.08	0.02	0.06	0.06	0.06	0.07	0.00	0.03	0.76

Abbreviations: TGP = total gas production.

[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 S3 Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid, and formic acid at 6 mM when combined with *A. taxiformis* at 1.5% DM on *in vitro* ruminal fermentation from dairy cows (n=4).

Item	Substrate alone[1]	Treatment									SEM	*P*-value[2]
		AT	AT+Phe	AT+Cat	AT+Res	AT+Hyd	AT+Phl	AT+Pyr	AT+GA	AT+FA		
Gas production (mL)												
TGP / 0-6 h	56.04±6.40	49.39	48.41	50.49	49.64	48.54	54.41	48.88	51.92	51.70	3.06	0.39
TGP / 6-24 h	55.07±3.75	55.69	55.62	56.23	58.38	55.92	64.00*	56.98	56.88	56.17	2.23	0.01
TGP / 0-24 h	111.11±4.48	105.08	104.04	106.73	108.02	104.50	118.41*	105.86	108.80	107.87	4.41	0.09
CH_4 / 0-6 h	12.76±0.99	7.49	8.87	8.94	8.70	7.82	6.64	8.12	7.35	7.71	1.66	0.02
CH_4 / 6-24 h	14.26±2.09	13.68	14.08	14.44	14.85	14.28	10.07	12.92	12.16	13.31	1.86	0.09
CH_4 / 0-24 h	27.02±2.40	21.17	22.94	23.39	23.55	22.10	16.70	21.04	19.50	21.02	3.42	0.06
H_2 / 0-6 h	0.00±0.00	1.04	0.24	0.41	0.34	0.69	2.60	0.89	1.24	1.54	1.04	0.49
Metabolic H recovery (%)	60.27±5.85	62.08	72.02	69.99	67.45	63.52	45.04*	59.15	56.30	64.86	4.32	<0.01
NH_3-N (mg/100 mL)	45.00±6.02	42.10	43.20	40.98	38.76	36.61	34.33	33.97*	38.24	40.84	3.53	0.02
Total VFA (mM)	120.0±5.50	109.0	95.6	101.0	104.7	106.6	127.3*	110.5	109.7	105.2	3.69	<0.01
VFA, mol/100 mol												
Acetate	64.0±4.14	55.7	56.4	56.3	57.2	57.4	59.8	57.6	57.0	54.5	3.20	0.74
Propionate	18.3±2.46	20.5	19.9	19.8	19.9	20.2	18.2	19.1	19.2	21.5	1.90	0.25
Isobutyrate	2.09±0.861	1.90	1.92	1.94	1.88	1.80	1.32	1.81	1.85	1.89	0.174	0.06
Butyrate	9.17±1.238	13.2	13.0	13.0	12.6	12.5	14.2	13.0	13.3	13.7	1.70	0.92
Isovalerate	3.33±1.335	3.96	4.00	4.43	3.96	3.95	2.85	4.19	4.16	3.92	0.336	0.09
Valerate	2.72±0.690	3.91	3.89	3.79	3.68	3.52	3.13	3.60	3.77	3.96	0.373	0.17
A:P	3.55±0.60	2.80	2.87	2.88	2.90	2.91	3.77	3.10	3.11	2.69	0.46	0.24

Abbreviations: AT = *A. taxiformis*; 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 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 but no phenolic compound added.

Table S4 Effect of phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, gallic acid and formic acid at 6 mM when combined with BES at 3 μM on *in vitro* ruminal fermentation from dairy cows (n=4).

Item	Substrate alone[1]	Treatment									SEM	*P*-value[2]
		BES	BES+Phe	BES+Cat	BES+Res	BES+Hyd	BES+Phl	BES+Pyr	BES+GA	BES+FA		
Gas production (mL)												
TGP / 0-6 h	52.99±6.22	49.59	47.67	47.83	49.02	46.98	47.73	47.90	51.47	51.24	1.99	0.06
TGP / 6-24 h	56.75±5.83	51.25	50.70	50.90	50.02	50.20	59.05*	53.78	55.95*	52.10	2.63	<0.01
TGP / 0-24 h	109.74±7.48	100.84	99.04	98.73	99.05	97.19	106.88*	101.67	107.42*	103.33	3.42	<0.01
CH_4 / 0-6 h	11.82±0.88	2.93	4.36	4.39	5.05	4.81	5.88*	4.72	4.96	5.24	0.60	0.12
CH_4 / 6-24 h	14.75±2.47	10.14	9.00	9.50	9.39	9.94	9.06	9.46	9.43	10.80	1.05	0.46
CH_4 / 0-24 h	26.57±3.24	13.07	13.54	13.89	14.44	14.74	14.35	14.18	14.39	16.04*	1.23	0.10
H_2 / 0-6 h	0.09±0.08	5.25	4.50	4.24	5.36	3.60	3.37	4.02	3.71	4.27	0.89	0.56
Metabolic H recovery / %	79.36±2.46	65.06	54.11	60.02	58.97	60.01	44.06*	49.95*	48.92*	65.47	3.03	<0.01
NH_3-N (mg/100 mL)	35.46±10.20	36.35	29.15	24.99	29.13	24.25	23.98	23.30	26.10	31.73	4.37	0.47
Total VFA (mM)	93.0±12.14	95.0	88.3	86.3	91.7	88.5	105.0	99.0	105.4	88.7	6.03	<0.01
VFA, mol/100 mol												
Acetate	59.8±8.16	44.2	56.7*	54.0*	55.6*	53.5*	59.5*	58.8*	55.8*	52.0	3.94	<0.01
Propionate	18.7±4.91	24.8	20.6	21.6	20.9	20.8	16.5*	18.9*	18.7*	21.2	2.12	<0.01
Isobutyrate	1.99±0.315	2.20	1.64	1.83	1.79	1.92	1.55*	1.57*	1.78	1.94	0.164	0.03
Butyrate	11.6±1.09	15.8	13.4	14.5	13.4	14.5	15.0	13.4	15.5	15.0	0.917	0.48
Isovalerate	3.71±0.990	6.12	3.53*	3.73*	3.79*	4.38*	3.39*	3.24*	3.43*	4.62*	0.766	<0.01
Valerate	3.68±1.310	5.69	3.44*	3.80*	3.94*	4.16*	3.44*	3.58*	4.10*	4.44	0.486	<0.01
A:P	3.43±1.19	1.87	2.88*	2.56	2.88*	2.70	3.77*	3.27*	3.04*	2.63	0.45	<0.01

Abbreviations: BES = 2-bromoethanesulfonic sodium; 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 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.

Table S5 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, fifth sequential batch).

Item	Treatment				SEM	*P*-value
	Control	BES	PHL	PHL + BES		
TGP (mL)	103.37[a]	92.06[b]	106.30[a]	104.75[a]	1.37	<0.01
CH_4 (mL)	12.14[a]	5.69[b]	0.03[c]	0.00[c]	0.45	<0.01
H_2 (mL)	0.30[c]	1.39[b]	1.95[ab]	1.72[ab]	0.19	<0.01
NH_3-N (mg/100 mL)	50.09[a]	47.04[a]	24.56[b]	23.59[b]	1.35	<0.01
Total VFA (mM)	121.3[b]	122.2[b]	207.5[a]	208.7[a]	3.12	<0.01
VFA, mol/100 mol						
Acetate	57.8[b]	54.0[c]	76.0[a]	76.4[a]	0.83	<0.01
Propionate	20.6[b]	22.0[a]	3.9[c]	3.7[c]	0.29	<0.01
Isobutyrate	1.93[a]	1.94[a]	0.76[b]	0.73[b]	0.035	<0.01
Butyrate	11.5[b]	12.9[b]	15.8[a]	15.8[a]	0.67	<0.01
Isovalerate	2.92[a]	2.92[a]	1.22[b]	1.18[b]	0.088	<0.01
Valerate	4.58[b]	5.37[a]	0.74[c]	0.67[c]	0.188	<0.01
A:P	2.81[b]	2.42[b]	18.68[a]	19.33[a]	1.07	<0.01
Microbe (log copies mL^{-1})						
Bacteria	10.11	10.23	10.36	10.32	0.08	0.16
Protozoa	1.05	0.70	1.68	1.44	0.30	0.09
Archaea	5.98[a]	5.63[a]	2.45[b]	2.40[b]	0.17	<0.01
Fungi	2.34	2.16	1.22	1.82	0.38	0.05

Abbreviations: BES = 2-bromoethanesulfonic sodium; PHL = phloroglucinol; TGP = total gas production; VFA = volatile fatty acid; A:P = acetate: propionate ratio.

[1] Control: Substrate alone, no chemical compound added.

[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

CHAPTER 3

Phenolic compounds increased phenolic utilization bacteria abundance when methanogenesis was inhibited in vitro

Introduction

The samples used in this trial were from the longer-term incubation described in chapter 2 and the longer-term incubation from our Spanish co-operators (detailed in M&M). In the longer-term incubation, we observed that phloroglucinol and pyrogallol increased acetate production and decreased dihydrogen accumulation. We hypothesized that changes in fermentation parameters were due to changes in rumen microbiota. The objectives of this study were to identify the microbes responsible for the phloroglucinol and pyrogallol degradation and to compare the responses of cow and goat's rumen microbiota to phenolic compounds when using methanogenesis inhibitor.

Experiment design

Cow inocula experiment: using rumen fluid from cows

- After three 24-h adaptation, there were four treatments: CTL, (control), BES (3 µM 2-bromoethanesulfonate), PHL (36 mM phloroglucinol), and BES+PHL (3 µM BES + 36 mM PHL)

Goat inocula experiment: using rumen fluid from goats

- After three 24-h adaptation, there were six treatments: CTL (control), AT (2% *A.taxiformis*), AT+PHL1 (2% AT + 6 mM phloroglucinol), AT+PHL2 (2% AT + 36 mM PHL); AT+PYR1 (2% AT + 6 mM pyrogallol), and AT+PYR2 (2% AT+ 36 mM PYR)

Main finding

- In both experiments, PHL and PYR at 36 mM decreased bacterial α-diversity
- Most bacteria taxa in cow and goat inocula experiments with increased abundances (including *Lactobacillaceae*, *Bifidobacteriaceae*, and *Lachnospiraceae*) were known with the phenolic compounds utilization, while the abundance of cellulolytic bacteria (including *Fibrobacteraceae*, *Ruminococcaceae*, and *Succinivibrionaceae*) decreased with this supplementation

Conclusion

The same microbial taxa were prompted in both experiments, attesting that 1) phenolic-degradating bacteria are naturally present in the two ruminant species and 2) their growth can be enhanced when methanogens are inhibited and phenolic compounds supplemented.

Phenolic compounds increased phenolic utilization bacteria abundance when methanogenesis was inhibited *in vitro*

Abstract

Two previous *in vitro* studies found that phloroglucinol and pyrogallol decreased ruminal dihydrogen accumulation and increased volatile fatty acid production when methanogenesis was inhibited. The present work builds upon the previous study describing the effects of these phenolic compounds on the bacterial community when methanogenesis was inhibited with rumen inoculum from cows and goats. There were four treatments in the cow inocula experiment: CTL, (control), BES (3 μM 2-bromoethanesulfonate), PHL (36 mM phloroglucinol), and BES+PHL (3 μM BES + 36 mM PHL). There were six treatments in the goat inocula experiment: CTL (control), AT (2% *A. taxiformis*), AT+PHL1 (2% AT + 6 mM phloroglucinol), AT+PHL2 (2% AT + 36 mM PHL); AT+PYR1 (2% AT + 6 mM pyrogallol), and AT+PYR2 (2% AT+ 36 mM PYR). In both experiments, PHL and PYR at 36 mM decreased bacterial alpha-diversity. Most bacteria taxa with increased abundances (including *Lactobacillaceae*, *Bifidobacteriaceae*, and *Lachnospiraceae*) were known with the phenolic compound utilization, while the abundance of cellulolytic bacteria (including *Fibrobacteraceae*, *Ruminococcaceae*, and *Succinivibrionaceae*) decreased with this supplementation. Although the structure of rumen bacterial community differed between cows and goats, about half of the bacterial

clades whose abundance was affected by 36 mM PHL in the cow innocula experiment were equally affected by 36 mM PHL and PYR in the goat innocula experiment. These findings suggest that nutritional supplementation with phenolic compounds could modify the rumen bacterial community towards a more efficient rumen dihydrogen metabolism and ultimately as a methane mitigation strategy.

1 Introduction

The livestock sector plays an important role in climate change because it is one of the main anthropogenic methane sources (Gerber et al., 2013). Also, methane production in cattle wastes 2 to 12% of gross energy intake (Johnson and Johnson, 1995). In the rumen, bacteria, fungi, and protozoa ferment carbohydrates to volatile fatty acid (VFA) and reducing equivalents. The VFA is the primary energy source, while the reducing equivalents are used by hydrogenase to synthesize dihydrogen. Methanogens then use the dihydrogen and carbon dioxide to synthesize methane (Morgavi et al., 2010).

While current methane mitigation strategies can decrease methane production by approximately 25%, the saved feed energy resulting from decreased methane emissions is often not utilized to increase animal production (Ungerfeld, 2018). The key factor in improving animal production lies in the metabolism of dihydrogen when a methanogenesis inhibitor is used (Ungerfeld, 2020, Leahy et al., 2022). Therefore, different chemical compounds are used to redirect dihydrogen metabolism when methanogenesis is inhibited. For example, our previous works used nitrate as hydrogen acceptor (Guyader et al., 2015, Popova et al., 2017).

Additionally, in our previous *in vitro* works (Romero et at., 2023; Chapter 2), we observed that combing phloroglucinol with 2-bromoethanesulfonate (BES), and combing phloroglucinol or pyrogallol with *A. taxiformis*, decreased dihydrogen accumulation and further decreased methane production. Moreover, combing either phloroglucinol or pyrogallol with a methanogenesis inhibitor increased total VFA production. As both methane and VFA are produced by rumen microbes, we hypothesized that the changes in rumen fermentation were due to changes in the rumen microbes. Indeed, previous research has shown that bulls receiving a diet supplemented with phloroglucinol and the methanogenesis inhibitor chloroform had an increased abundance of *Prevotella*, *Coprococcus*, *Ruminococcus*, *Fibrobacter*, CF231, and YRC22 (Martinez-Fernandez et al., 2017). The objectives of the present study were to identify the microbes in cows and goats involved in phloroglucinol and pyrogallol degradation and to compare the responses of rumen microbiota from small and big ruminants (goat and cow) to hydrogen acceptor when using methanogenesis inhibitor.

2 Materials and Methods

This study consists of two experiments: one conducted in France at INRAE using cow's rumen fluid as innocula, and the other conducted in Spain at CSIC using goat's rumen fluid as inocula. The experimental procedures involving cows were approved by the local animal ethics committee (APAFIS #8218-20161151782412) and were conducted in compliance with relevant regulations in France and the EU. Similarly, the procedures involving goats were approved by Ethical Committee for Animal Research at EEZ-CSIC

(A/18/03/2019/042) and were carried out in accordance with relevant regulations in Spain and the EU.

2.1 Cow inocula experiment: sequential batch incubation using rumen fluid from cows.

The inocula media preparation were described in Chapter 2 Exp.3. Briefly, rumen fluid were collected from 8 lactating Holstein cows what were fed ad libitum a diet consisting of 67% forage (corn silage and grass silage) and 33% of concentrate (corn and soybean meal) on a DM basis, twice per day. The cows had free access to water and mineral salt blocks. Rumen fluid was collected via esophageal tubing before morning feeding and transferred to preheated 1-L thermal flasks, which was transported to the laboratory without delay. Each animal's rumen fluid was processed separately, mixed with a warmed (39 °C) anaerobic buffer solution at 1:2 (volume to volume) ratio under a stream of O_2-free CO_2 (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).

We conducted three sequential 24-h incubations to stabilize and adapt the rumen microbes to phloroglucinol, followed by a fourth 24-h incubation combing phloroglucinol with BES as methanogenesis inhibitor. The first, second, and third incubations had two treatments: control and 36 mM phloroglucinol, the concentration of which was determined based on the absence of negative effects on fermentations from our preliminary trial. At the end of the third incubation, control flasks were split into control (CTL, as in previous

batches) and 3 μM BES (BES) treatments, whereas phloroglucinol flasks were split into phloroglucinol (PHL, as in previous batches) and 3 μM BES + 36 mM phloroglucinol (BES+PHL) treatments. In the second through fourth batches, one-third of the incubation fluid from the previous batch bottle was mixed with two-thirds of the anaerobic buffer and used to inoculate the next corresponding serum bottle with fresh substrates. At the end of the fourth incubation, 1 mL of incubation fluid was collected, centrifuged at 16 000 g for 15 min at 4 °C, and the supernatant was discarded. The pellet was stored at -20 °C until DNA extraction.

2.2 Goat inocula experiment: sequential batch incubation using rumen fluid from goats

Rumen fluid were obtained from four rumen-fistulized adult Murciano-Granadina goats (n=4) that had been adapted to the experimental diet for more than 3 weeks. The substrates and the inoculation media were prepared as Chapter 2. The *in vitro* experiment consisted of four sequential batch incubations using 125-mL serum bottles according to the following design: 6 mM or 36 mM phloroglucinol, 6 mM or 36 mM pyrogallol, 2% *A. taxiformis* (based on DM, *A. taxiformis* was added in the fourth incubation), and control treatments (n=4 for each treatment) for the first, second, and third incubations. In the fourth incubation, 2% *A. taxiformis* was added to each bottle except for the control treatment, resulting in six treatments: Control (CTL), 2% *A. taxiformis* (AT), 2% *A. taxiformis* + 6 mM phloroglucinol (AT+PHL1), 2% *A. taxiformis* + 36 mM phloroglucinol (AT+PHL2), 2% *A. taxiformis* + 6 mM pyrogallol (AT+PYR1), and

2% *A. taxiformis* + 36 mM pyrogallol (AT+PYR2). At the end of the fourth incubation, 1 mL of incubation fluid was collected, centrifuged at 16 000 g for 15 min at 4 °C, and the supernatant was discarded. The pellet was stored at -20 °C until DNA extraction.

2.3 Amplicon sequencing

Microbial DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's instructions. The rumen fluid DNA was submitted to Spanish National Research Council for DNA library preparation, which involved amplification of the V3-V4 hypervariable region of the 16S rRNA gene using Fluidigm primers pairs Miseq_F and Miseq_R targeting both archaea and bacteria, as previously described (Palma-Hidalgo et al., 2022). The resulting amplicons were sequenced on an Illumina MiSeq platform using a MiSeq 300-cycle sequencing kit. Downstream processing was performed using QIIME 2 (Bolyen et al., 2019). Specifically, demultiplexing was performed using Qiime demux emp-paired plugin; while denoising, quality control, and amplicon sequence variant (ASV) generation were performed using the DATA2 plugin. Finally, representative sequences from all ASVs were aligned against SILVA 138.1.

2.4 Statistical analysis

Alpha diversity and relative abundance of the rumen bacteria of ASVs were analyzed by the GLMMIX process considering treatment as a fixed effect, animal as a random effect, and *P*-value was adjusted by Bonferroni statement. Beta diversity was analyzed by R (Version R 4.2.2) Phyloseq package, and performed permanova multiple

comparisons using the PairwiseAdonis package (Martinez Arbizu, 2017), with *P*-value adjusted by Bonferroni statement. Microbial significant associations were analyzed by R package Masslin2 (Mallick et al., 2021). UpSetR plots were generated using the R package UpSetR (Conway et al., 2017), and modified according to our previous publication (Popova et al., 2022). We also performed distance-based redundancy analysis using R package ggvegan.

3 Results and discussion

In our previous work (Romero et al., 2023; Chapter 2), we found that phloroglucinol and pyrogallol decreased dihydrogen accumulation and increased total VFA production by promoting acetate production when methanogenesis was inhibited. In this study, we used a metataxonomic approach to gain insight into the potential changes in the rumen microbiota induced by phloroglucinol and pyrogallol alone or in combination with a methanogenesis inhibitor. We used rumen fluid as inocula from two ruminant species, dairy cows and goats, and amplified both the bacterial and archaeal communities with universal primers. However, we observed an extremely low number of amplicon sequence variants (ASVs) assigned as archaea in high-concentration phloroglucinol and pyrogallol groups, rendering further analysis infeasible. This low number of archaea ASVs was consistent with qPCR results, which showed a sharp reduction in archaea abundance in response to high phloroglucinol and pyrogallol supplementation, as compared to the control group (Romero et al., 2023; Chapter 2).

Table 5 Alpha diversity metrics of ruminal bacteria from cow inocula experiment.

Item	Treatment				SEM	P-value
	CTL	BES	PHL	BES+PHL		
Observed ASVs	246[a]	224[a]	71[b]	83[b]	9.5	<0.0001
Shannon index	4.41[a]	4.37[a]	3.60[b]	3.48[b]	0.080	<0.0001
Simpson index	0.96	0.97	0.96	0.95	0.004	0.0375
Faith's phylogenetic diversity	11.04[a]	10.48[a]	3.90[b]	3.85[b]	0.244	<0.0001

Abbreviations: CTL = control; BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol.

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$

Table 6 Alpha diversity metrics of ruminal bacteria from goat inocula experiment.

Item	Treatment						SEM	P-value
	CTL	AT	AT+PHL1	AT+PHL2	AT+PYR1	AT+PYR2		
Observed ASVs	317[a]	315[a]	301[a]	120[b]	310[a]	129[b]	14.7	<0.0001
Shannon index	4.46[a]	4.47[a]	4.56[a]	3.79[b]	4.58[a]	3.40[b]	0.090	<0.0001
Simpson index	0.96[a]	0.97[a]	0.98[a]	0.96[a]	0.97[a]	0.90[b]	0.009	0.0005
Faith's phylogenetic diversity	12.99[a]	11.95[a]	11.67[a]	6.18[b]	12.47[a]	6.28[b]	0.538	<0.0001

Abbreviations: CTL = control; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

3.1 Rumen bacterial diversity was affected by phenolic compounds but not by methanogenesis inhibitors

The methane inhibitors used in this study, BES and *A. taxiformis,* did not affect alpha diversity indicators (Shannon index, Simpson index, and Faith's phylogenetic, Tables 5 and 6). BES is the structural analogue of CoM that competes with methyl-CoM for binding to methyl-coenzyme M reductase, effectively inhibiting the reduction of methyl group into methane in the last step of the methanogenic pathway (Patra et al., 2017). Meanwhile, *A. taxiformis* contains bromoform, which acts on both coenzyme M methyltransferase and methyl-coenzyme M reductase to inhibit methanogenesis (Glasson et al., 2022). Both BES and bromoform are specific inhibitors that target the methanogenesis pathway, which is unique to methanogenic organisms. This likely explains why neither of these inhibitors affected bacterial alpha diversity.

In contrast, phenolic compounds phloroglucinol and pyrogallol, at a concentration of 36 mM significantly decreased alpha diversity indicators, except the Simpson index

(Tables 5 and 6). However, at a lower concentration of 6 mM, these phenolic compounds did not affect alpha-diversity indicators. Notably, the results from the 36 mM phloroglucinol treatment alone are inconsistent with previous *in vivo* work, which reported no effect of a similar concentration (around 40 mM) of phloroglucinol on alpha diversity (Martinez-Fernandez et al., 2017). The inconsistent results may be due to the differences between the *in vitro* and *in vivo* methods used in the studies.

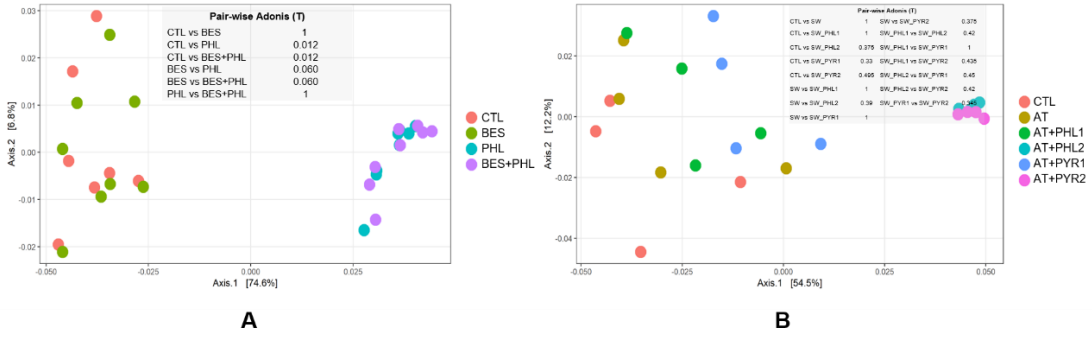

Figure 7 Beta diversity of ruminal bacteria from cow inocula (A) and goat inocula (B) experiment.

CTL = control; BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

Likewise, beta diversity analysis revealed differences in bacterial community structure in both fermentation fluid using inocula from cows and goats (Figure 7). In the cow inocula experiment, samples exposed to 36 mM phloroglucinol with and without BES clustered together and separated from the samples from control and BES treatments (Figure 7, A). Pairwise Adonis statistical analysis showed a significant difference between the control and PHL groups ($P = 0.012$), and a trend difference between BES and BES+PHL groups ($P = 0.060$), while there was no difference between the control and BES groups ($P = 1$) or between the PHL and BES+PHL groups ($P = 1$). In the goat inocula experiment, Pairwise Adonis statistical analysis showed no difference between groups, although samples from AT+PHL2 and AT+PYR2 groups were separated from other samples (Figure 7, B). The results of beta diversity of the cow inocula experiment are inconsistent with those of Martinez-Fernandez et al. (2017), who found that phloroglucinol (~40 mM) did not affect beta diversity. The differences between our study

and Martinez-Fernandez et al. (2017) could be due to the *in vitro* versus *in vivo* approach, the actual concentration and exposure time of the microbiota to phloroglucinol. The *in vivo* concentration was calculated based on the dose and steers' live weight (Martinez-Fernandez et al., 2017), with possible dilution due to passage rate out of the rumen and the assess pharmacokinetics.

We also investigated the effect of fermentation factors, including VFAs, NH3, and pH, on the microbiota structure of cow inocula and goat inocula experiments (Figure S1 and S2). In the cow inocula experiment, we found that acetate, butyrate, and caproate contributed to the samples from the PHL and BES+PHL groups, while isovalerate contributed to the samples from the control and BES groups. Previous studies have shown that phloroglucinol degradation can increase acetate and butyrate production (Conradt et al., 2016), and that inhibition of methane production can increase caproate production (Ungerfeld, 2015). Phloroglucinol supplementation can also decrease protein digestion in the rumen, thus indirectly decreasing isovalerate production (McSweeney et al., 2001). This could explain why isovalerate contributed to the samples from the control and BES groups. In goat inocula experiment, we found that samples from control, AT, AT+PHL1, and AT+PYR1 groups clustered based on pH value, isobutyrate, isovalerate, NH_3, and propionate. Interestingly, the AT+PHL2 group and the AT+PYR2 group were separated, with butyrate contributing to the AT+PHL2 grouping and lactate contributing to the AT+PYR2 grouping.

3.2 Bacterial community responses to phloroglucinol supplementation in cow inocula experiment

Hydrolysable tannins are secondary plant metabolites commonly found in leguminous forages (Reed, 1995). Previous studies reported that some rumen microbes have a tolerance to tannins by dihydrogen to break down tannins (Patra et al., 2012). Figure 8A shows the top 50 bacteria that showed significant associations (positive or negative) compared to the control group in the cow inocula experiment.

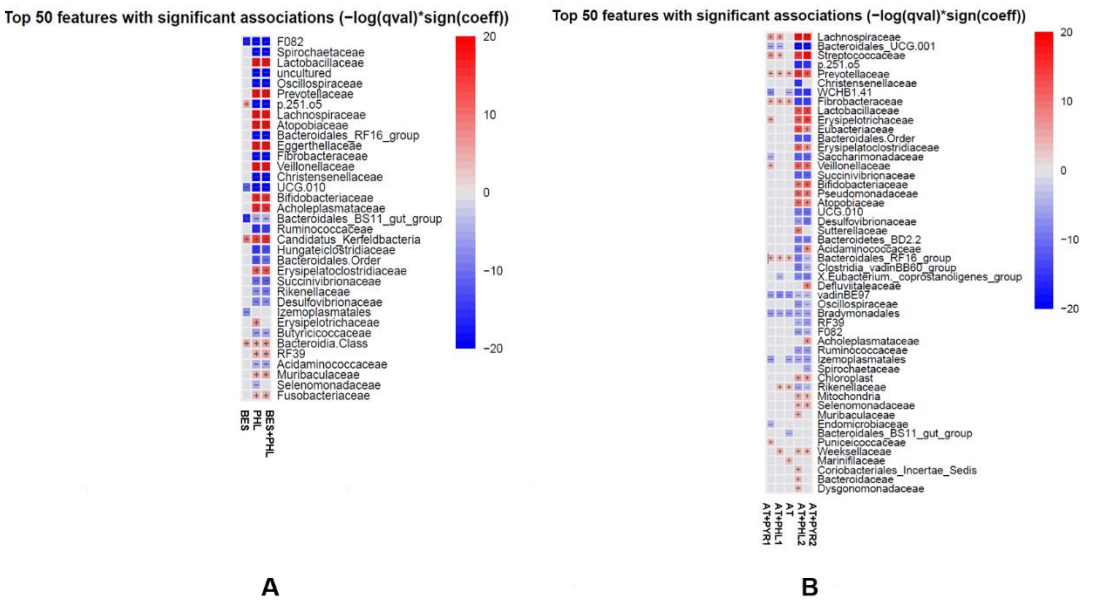

Figure 8 Top 50 features with significant associations of ruminal bacteria from cow inocula (A) and goat inocula (B) experiments compared to control, respectively.

BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

The effects of treatments PHL and BES+PHL on the top 50 bacteria (Figure 8A) were similar, indicating that phloroglucinol is the key driver for changes in bacterial structure. Phloroglucinol supplementation significantly increased the abundance of several bacterial families, including *Lactobacillaceae, Prevotellaceae*, *Lachnospiraceae*, *Atopobiaceae*, *Eggerthellaceae*, *Veillonellaceae*, *Bifidobacteriaceae*, *Acholeplasmataceae*, *Erysipelatoclostridiaceae, Bacterioidia Class,* RF39*, Muribaculaceae,* and *Fusobacteriaceae*. Some of these bacteria have been reported to utilize phloroglucinol or have their abundance increased by phloroglucinol supplementation. For example, *Coprococcus,* a genus from the family *Lachnospiraceae* that was isolated from rumen fluid, degraded 80% phloroglucinol after two days of pure

culture (Tsai and Jones, 1975). Martinez-Fernandez et al. (2017) reported that phloroglucinol increased the proportion and the absolute abundance of *Coprococcus* when methane production was inhibited by chloroform; Sarwono et al. (2019b) also found that 10 mM phloroglucinol increased the proportion of *Coprococcus* by twofold, compared to the control group. The abundance of *Prevotella,* the genus from the family *Prevotellaceae,* was also increased by phloroglucinol when methane production was inhibited by chloroform (Martinez-Fernandez et al., 2017). These results suggest that *Coprococcus* plays an important role in phloroglucinol degradation, and that *Prevotella* has the potential ability to utilize phloroglucinol.

Phloroglucinol supplementation increased the abundance of other bacteria that are also involved in phenolic compound metabolism. Regression analysis has shown that the enrichment of *Lactobacillaceae* in the gut of *Drosophila melanogaster* was associated with the content of phenolic compounds (Garcia-Lozano et al., 2020), and the abundance of *Lactobacillaceae* was fourfold higher in the group with high concentration of phenolic compound (Nissen et al., 2022). Another possible reason for the observed increase in *Lactobacillaceae* abundance is that they are known lactate producers. As phloroglucinol supplementation inhibited methane production and resulted reducing equivalents such as dihydrogen accumulation (Chapter 2), lactate production increased because it consumes reducing equivalents (Ungerfeld, 2020). This could also explain the increase in the abundance of *Veillonellaceae* because members of this family are lactate utilizers (Daly et al., 2012). *Fusobacteriaceae,* and *Atopobiaceae* are butyrate producers (Van den Abbeele et al., 2020, Kynkäänniemi et al., 2022). Butyrate production also consumes

reducing equivalents compared to acetate production. This agrees with our previous findings of phloroglucinol supplementation enhanced butyrate production (Chapter 2).

Supplementation with jabuticaba peel, which has a high concentration of total phenolic compounds (8220 mg/100g), significantly increased the abundance of *Eggerthellaceae* and *Muribaculaceae* in the gut (Loubet Filho et al., 2022). *Bifidobacteriaceae* abundance increased threefold in a high concentration of phenolic compound group (Nissen et al., 2022). *Flavobacterium,* from the class *Bacterioidia,* has been shown to efficiently degrade phenol (Wirth et al., 2015). However, little information is available about the functions of RF39, and *Acholeplasmataceae* as these bacteria they are either uncultured or their metabolic activities have not been well-established (Wang et al., 2020, Casar et al., 2021). Table S6 presents the results of the multiple comparisons of the bacterial abundance between the four groups.

Whereas phloroglucinol supplementation significantly decreased the abundance of F082, *Spirochaetaceae*, *Oscillospiraceae*, p-251-o5, *Bacteroidales* RF16 group, *Fibrobacteraceae*, *Christensenellaceae*, unclassified *Ruminococcaceae* UCG-010, *Ruminococcaceae*, and *Hungateiclostridiaceae*. Similar results were reported by Sarwono et al. (2019a), who found that 10 mM phloroglucinol decreased the abundance of *Ruminococcus flaveciens* and *Fibrobacter succinogenes* when using a high forage ratio as a substrate. There are two possible reasons for the observed decrease in abundance of these bacteria with high phloroglucinol concentration.

High concentration of phloroglucinol led to massive increase in total VFA production (Romero et al., 2023; Chapter 2), which likely resulted in a low pH value. However, some

bacteria, especially cellulolytic bacteria cannot grow at low pH values (Russell and Dombrowski, 1980; Russell and Wilson, 1996). The families *Spirochaetaceae* (Shivani et al., 2017), *Oscillospiraceae* (Fei et al., 2022), *Fibrobacteraceae* (Ozbayram et al., 2018)*, Ruminococcaceae,* and unclassified *Ruminococcaceae* UCG-010 (Wang et al., 2013, Guo et al., 2019) are known to be cellulose-degrading bacteria. *Christensenellaceae* and *Hungateiclostridiaceae* belong to the class *Clostridia,* which has a high cellulose-degrading ability (Fontes and Gilbert, 2010, Zhang et al., 2018). Although *Bacteroidales* RF16 *group* is unclassified, its abundance was significantly higher in the forage group than in the concentrate group in rumen of yak (Liu et al., 2019), suggesting that is might be a cellulose-degrading bacteria. The role of the *Bacteroidetes,* unclassified family F082 and p-251-o5 remain unclear (Chiariotti et al., 2020). Another possible reason for the decrease in bacterial abundance is that phenolic compounds may be toxic to some bacteria. Hierholtzer et al. (2013) reported that phloroglucinol (5.6 mM) can affect the membrane permeability of anaerobic bacteria.

Figure 8 also shows that the effects of BES (A) and *A. taxiformis* (B) on these 50 bacteria was small. These results are in line with the alpha and beta diversity results. The small impact of BES and *A. taxiformis* on these bacteria likely because BES and *A. taxiformis* are specific inhibitor to methanogens.

3.3 Bacterial community responses to phloroglucinol supplementation in goat inocula experiment and the comparison between cow and goat inocula experiments

Figure 8B shows the top 50 bacteria that showed significant associations (positive or negative), compared to the control group. When phloroglucinol and pyrogallol were combined with *A. taxiformis* at the same concentration, they displayed a highly similar impact on the bacteria structure (Figure 8B and Table S7). The similarity in effect could be attributed to the involvement of both phloroglucinol and pyrogallol in the metabolic pathway of gallic acid, as reported in various studies (Evans, 1977, Bhat et al., 1998; McSweeney et al., 2001; Conradt et al., 2016).

Interestingly, approximately 50% of the bacterial that displayed a positive association with phloroglucinol supplementation in the cow inocula experiment were also positively associated with 36 mM phloroglucinol and pyrogallol supplementation in the goat inocula experiment. This finding suggests that the metabolic effects of phloroglucinol and pyrogallol on these bacteria may be conserved across ruminant species. Additionally, in goat inocula experiment, we observed a significant increase in butyrate and lactate production following treatment with AT+PHL2 (Romero et al., 2023), which provides further support for the hypothesis that the increased abundance of *Lactobacillaceae, Veillonellaceae, Fusobacteriaceae, and Atopobiaceae* were responsible for the observed effects. Interestingly, in cow inocula experiment, approximately 60% of the bacteria that displayed a negative relationship with phloroglucinol supplementation were also negatively associated with 36 mM phloroglucinol and pyrogallol supplementation in the

goat inocula experiment. Consistent with these findings, we observed a significant decrease in pH value in the goat inocula experiment (Romero et al., 2023).

The abundance of *Eubacteriaceae, Streptococcaceae, Chloroplast*, *Erysipelotrichaceae*, *Mitochondria*, *Pseudomonadaceae*, *Selenomonadaceae*, and *Weeksellaceae* increased only in the AT+PHL2 and AT+PYR2 groups in the goat inocula experiment, while no such increase in the cow inocula experiment for the PHL and BES+PHL groups. This difference is likely due to the variations in rumen microbiota between goats and cows, despite the presence of the same core microbes (Henderson et al., 2015, Corral-Jara et al., 2022).

Eubacterium oxidoreducens sp. nov. from the family *Eubacteriaceae* (Krumholz and Bryant, 1986; Krumholz et al., 1987) and *Streptococcus* from the family *Streptococcaceae* (Tsai and Jones, 1975) were isolated from steer rumen fluid, and studies have demonstrated that these two bacteria can utilize phloroglucinol and/or pyrogallol with reducing equivalents such as dihydrogen. However, it is unclear why *Eubacteriaceae* was not detected and why *Streptococcaceae* abundance was lower in PHL or BES+PHL group in the cow inocula experiment (Table S6).

Erysipelotrichaceae has been found to have a positive association with phenolic compounds in plant-based foods (Calderón-Pérez et al., 2021). *Pseudomonas* sp. from the family *Pseudomonadaceae* has been reported to be involved in phenol degradation (Lack and Fuchs, 1994). However, we did not find any publications that reported the involvement of *Selenomonadaceae* and *Weeksellaceae* in phenolic compound metabolism or reducing equivalent consumption.

3.4 Different treatments combination have different unique bacteria

We used UpSetR analysis to identify core ASVs that were present in all treatments and treatment-specific ASVs (Figure 9). In the cow inocula experiment (Figure 9 A), we identified 35 core ASVs, with *Prevotella* (16%), *Streptococcus* (11%), and *Megasphaera* (10%) being the dominant ASV. *Prevotella* was the dominant ASV, which is consistent with its prevalence in the rumen microbial ecosystem (Stevenson and Weimer, 2007). In the PHL group, we identified 2 unique ASVs, with the highest abundance ASV being *Erysipelotrichaceae* UCG 006. In the BES+PHL group, we identified 1 unique ASV, which was *Agathobacter*. Like *Coprococcus, Agathobacter* belongs to the family *Lachnospiraceae*. We found 8 unique ASVs that were present in both the PHL and BES+PHL groups, with *Ligilactobacillus* (68%), *Dialister* (18%), and *Limosilactobacillus* (5%) being the top three ASVs. Most of these ASVs belonged to the family *Lactobacillaceae* and *Veillonellaceae*. In the control and BES groups, we identified 75 unique ASVs, with *Succinivibrio* (24%), *Treponema* (15%), and F082 (8%) being the top three abundant ASVs. Additionally, we identified 18 unique ASVs in the control group and 4 unique ASVs in the BES group.

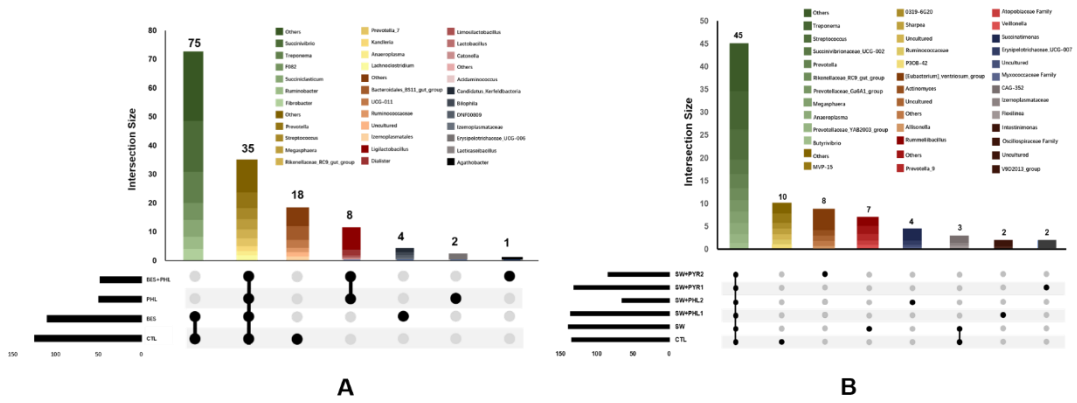

Figure 9 An UpSetR plot of unique ASVs across different treatments in cow inocula (A) and goat inocula (B) experiments. The number of ASV for each treatment is plotted on the left of the Upset plots of cows and goats, respectively. Dark circles indicate samples containing accessions and their counts are shown by the figures at the top of the charts. Connecting bars indicate overlap between multiple samples. CTL = control; BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

In the goat inocula experiment (Figure 9B), we identified 45 core ASVs, with top three most abundant ASVs affiliated with *Treponema* (19%), *Streptococcus* (15%), and *Succinivibrionaceae* UCG-002 (7%). *Streptococcus* was the second most abundant core ASV in cow inocula experiment. However, *Treponema* was not found in cow inocula experiment core ASVs, it was the second abundant ASV in control and BES groups. Also, the abundance of *Succinivibrionaceae* UCG-002 in cow inocula experiment of core ASVs was much lower (0.2%). The observed difference in core ASVs between cow inocula and goat inocula experiments could due to the varying bacteria composition in the original cow and goat's rumen fluid and the pyrogallol supplementation in the goat inocula experiment. Indeed, we found different ASVs in the control group from cow inocula experiment and the control group from goat inocula experiment.

In the AT+PHL2 group, we identified 4 unique ASVs including *Succinatimonas*

(61%), *Erysipelotrichaceae*_UCG-007 (22%), uncultured (12%), and Family *Myxococcaceae* (5%). Meanwhile in the BES+PHL group, there was only 1 unique ASV, which was *Agathobacter.* In the AT+PYR2 group, we identified 8 unique ASVs, with the top three most abundant ASVs affiliated with *Eubacterium ventriosum group* (53%), *Actinomyces* (14%), and uncultured (14%). We did not find any unique ASVs in both AT+PHL1 and AT+PHL2 groups, or in AT+PYR1 and AT+PYR2 groups.

4 Conclusion

This study aimed to explore the potential of rumen bacteria to degrade phloroglucinol and pyrogallol using dihydrogen. We observed that the responses of cow and goat rumen bacteria to phloroglucinol and pyrogallol were similar, with approximately 50% of the same bacterial showing increased or decreased abundance when supplemented with 36 mM phloroglucinol and/or pyrogallol in both experiments. Phloroglucinol and pyrogallol have a very similar and large effect on rumen bacteria structure, while methane inhibitors *A. taxiformis* and BES had a limited effects on rumen bacteria structure. Our findings also suggest that certain bacteria present in cow and goat rumen fluid possess the ability to utilize phloroglucinol and pyrogallol, although further studies, such as pure culture and genomic analysis, are necessary to confirm these findings.

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Supplemental materials

Table S6 Bacterial abundance from cow inocula experiment.

Bacteria family	Treatment (Relative abundance, %)				SEM	P-value
	CTL	BES	PHL	BES+PHL		
Bacteroidales_BS11_gut_group	0.15a	0.00b	0.00b	0.00b	0.014	<0.0001
p-251-o5	0.51a	0.77a	0.00b	0.00b	0.067	<0.0001
Eggerthellaceae	0.24b	0.22b	0.78a	0.74a	0.087	<0.0001
Christensenellaceae	0.76a	0.47a	0.00b	0.00b	0.093	<0.0001
Acidaminococcaceae	2.61a	2.38a	0.13b	0.10b	0.110	<0.0001
uncultured	0.47a	0.53a	0.00b	0.00b	0.035	<0.0001
Spirochaetaceae	4.33a	5.45a	0.00b	0.00b	0.703	<0.0001
Fibrobacteraceae	1.36a	1.88a	0.00b	0.00b	0.282	<0.0001
UCG-010	0.30a	0.11b	0.00c	0.00c	0.017	<0.0001
Bacteroidales_RF16_group	0.30a	0.29a	0.00b	0.00b	0.030	<0.0001
Bifidobacteriaceae	0.19b	0.26b	4.65a	4.16a	0.475	<0.0001
Succinivibrionaceae	10.90a	11.26a	0.16b	0.18b	1.244	<0.0001
Selenomonadaceae	1.72a	1.92a	0.14b	0.13b	0.141	<0.0001
Desulfovibrionaceae	0.18a	0.19a	0.00b	0.00b	0.020	<0.0001
Rikenellaceae	13.51a	13.54a	0.45b	0.23b	0.699	<0.0001
Bacteroidales Order	0.14a	0.14a	0.00b	0.00b	0.015	<0.0001
Oscillospiraceae	1.18a	0.95a	0.00b	0.00b	0.119	<0.0001
Acholeplasmataceae	1.12b	1.36b	5.65a	5.33a	0.762	<0.0001
Hungateiclostridiaceae	0.150a	0.111a	0.00b	0.00b	0.019	<0.0001
F082	3.63a	1.36b	0.00c	0.00c	0.146	<0.0001
Lachnospiraceae	12.56b	11.82b	18.51a	21.20a	0.998	<0.0001
Bacteroidaceae	0.12a	0.17a	0.00b	0.00b	0.029	<0.0001
Prevotellaceae	10.62b	12.43b	28.50a	29.54a	0.860	<0.0001
Eubacteriaceae	0.08a	0.05a	0.00b	0.00b	0.011	<0.0001
Ruminococcaceae	1.50a	1.19a	0.01b	0.02b	0.180	<0.0001
Lactobacillaceae	0.02b	0.00b	10.18a	8.82a	1.43	<0.0001
Atopobiaceae	1.30b	1.62b	3.17a	2.66a	0.368	0.0001
Bacteroidia Class	0.01b	0.05a	0.00b	0.00b	0.009	0.001
Izemoplasmatales	0.04a	0.00b	0.00b	0.00b	0.007	0.0013
Veillonellaceae	7.16b	7.41b	11.10a	9.51ab	0.736	0.0015
Butyricicoccaceae	0.45a	0.42a	0.00b	0.00b	0.116	0.0016
Enterobacteriaceae	0.04ab	0.04a	0.00b	0.00b	0.012	0.0032

Monoglobaceae	0.09a	0.07ab	0.00b	0.00b	0.021	0.0047
MVP-15	0.02a	0.02a	0.00b	0.00b	0.006	0.0051
Streptococcaceae	11.84a	10.96ab	5.46ab	4.87b	2.077	0.0056
Bacteroidales_UCG-001	0.07ab	0.08a	0.00b	0.00b	0.022	0.006
Enterococcaceae	0.05ab	0.06a	0.00b	0.00b	0.02	0.0067
Candidatus_Kerfeldbacteria	0.00b	0.02a	0.00b	0.00b	0.005	0.0075
Synergistaceae	0.20ab	0.22a	0.00b	0.00b	0.068	0.01
Erysipelotrichaceae	0.52ab	0.41b	0.95a	0.64ab	0.139	0.0228
Tannerellaceae	0.00	0.04	0.00	0.00	0.011	0.0239
Clostridiaceae	0.13	0.36	0.00	0.00	0.108	0.0341
Clostridia_UCG-014	0.25	0.26	0.00	0.00	0.100	0.0367
WCHB1-41	0.04	0.02	0.00	0.00	0.011	0.0544
Sutterellaceae	0.30	0.29	0.26	0.09	0.074	0.0614
Saccharimonadaceae	0.05	0.05	0.00	0.00	0.023	0.0664
UCG-011	0.06	0.00	0.00	0.00	0.020	0.0675
RF39	0.02	0.02	0.00	0.00	0.009	0.0678
Gastranaerophilales	0.02	0.00	0.00	0.00	0.007	0.0751
Pirellulaceae	0.02	0.00	0.00	0.00	0.007	0.0773
Anaerolineaceae	0.01	0.00	0.00	0.00	0.004	0.0835
uncultured	0.01	0.00	0.00	0.00	0.029	0.088
uncultured	0.13	0.08	0.07	0.05	0.031	0.1034
Izemoplasmataceae	0.00	0.01	0.00	0.00	0.004	0.1166
Fusobacteriaceae	0.03	0.02	0.00	0.00	0.013	0.1624
Muribaculaceae	0.01	0.02	0.00	0.00	0.010	0.2108
Erysipelatoclostridiaceae	7.71	7.94	8.94	10.71	1.898	0.4414
Clostridia_vadinBB60_group	0.28	0.28	0.40	0.17	0.141	0.4674
Anaerovoracaceae	0.42	0.33	0.49	0.86	0.309	0.6017

Abbreviations: CTL = control; BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table S7 Bacterial abundance from goat inocula experiment.

Bacteria family	Treatment (Relative abundance, %)						SEM	P-value
	CTL	SW	SW+PHL1	SW+PHL2	SW+PYR1	SW+PYR2		
Erysipelatoclostridiaceae	1.38b	0.74b	1.12b	3.98a	0.60b	1.70b	0.316	<0.0001
Rikenellaceae	5.58a	7.46a	7.52a	0.62b	6.18a	0.78b	0.542	<0.0001
Desulfovibrionaceae	0.22a	0.10bcd	0.14ab	0.02cd	0.13ab	0.00d	0.032	<0.0001
Prevotellaceae	10.60b	13.90b	14.88b	32.04a	15.64b	17.39b	1.579	<0.0001
Acidaminococcaceae	0.82a	0.73a	0.76a	0.03a	0.49a	2.08b	0.223	<0.0001
Ruminococcaceae	0.75a	0.32bc	0.33bc	0.02c	0.53ab	0.02c	0.087	<0.0001
Bradymonadales	0.39a	0.03b	0.02b	0.00b	0.02b	0.00b	0.036	<0.0001
Streptococcaceae	5.74b	6.95b	7.71b	14.04b	10.85b	27.70a	2.11	<0.0001
Atopobiaceae	0.24c	0.27c	0.27c	3.25a	0.64bc	0.86b	0.281	<0.0001
Spirochaetaceae	24.84a	22.35a	20.80a	4.99b	21.01a	1.29b	1.728	<0.0001
Succinivibrionaceae	14.20a	13.58a	10.23a	0.15b	7.80ab	0.19b	1.815	<0.0001
RF39	0.34a	0.28a	0.24a	0.03b	0.28a	0.01b	0.043	<0.0001
Bacteroidales_RF16_group	0.33bc	0.85ab	0.83ab	0.00c	1.24a	0.04c	0.154	<0.0001
Bacteroidales_UCG-001	0.71a	0.42b	0.28b	0.00c	0.22bc	0.00c	0.057	<0.0001
WCHB1-41	0.80a	0.21bc	0.38b	0.00c	0.08c	0.00c	0.07	<0.0001
Eubacteriaceae	0.00b	0.00b	0.02b	0.23a	0.01b	0.04b	0.025	<0.0001
Lachnospiraceae	6.42b	5.66b	7.58b	22.64a	7.93b	18.33a	1.384	<0.0001
Christensenellaceae	0.54ac	0.59a	0.64a	0.00b	0.67a	0.13bc	0.1045	0.0001
uncultured	0.33abc	0.44abc	0.66a	0.30bc	0.44ab	0.02c	0.079	0.0005
UCG-010	0.18a	0.08ab	0.07ab	0.00b	0.08ab	0.00b	0.026	0.0006
Lactobacillaceae	0.14b	0.13b	0.16b	1.56ab	0.18b	3.33a	0.454	0.0006
p-251-o5	3.92a	6.73a	6.91a	0.00b	4.74a	0.01b	1.202	0.0008
Oscillospiraceae	1.52a	0.94ab	0.74ab	0.02b	0.62ab	0.14b	0.242	0.0008
Izemoplasmatales	0.34a	0.02b	0.05b	0.00b	0.02b	0.00b	0.049	0.0009
Defluviitaleaceae	0.00b	0.01b	0.00b	0.00b	0.02b	0.27a	0.04	0.0011
Puniceicoccaceae	0.02ab	0.03ab	0.00b	0.00b	0.05a	0.00b	0.009	0.0016
Endomicrobiaceae	0.19a	0.04b	0.08ab	0.00b	0.01b	0.00b	0.033	0.0016
Fibrobacteraceae	1.64ab	5.50a	4.58ab	0.00b	4.23ab	0.00b	1.031	0.0019

Acholeplasmataceae	4.96ab	2.18b	2.03b	2.76b	3.44b	9.70a	1.16	0.0025
Veillonellaceae	2.80b	2.27c	3.63ab	7.53ab	5.07ab	7.98a	0.9766	0.0027
Bacteroidetes_BD2-2	0.27ab	0.53a	0.26ab	0.00b	0.28ab	0.00b	0.082	0.0032
Bifidobacteriaceae	0.28b	0.29b	0.28b	1.25b	0.28b	2.88a	0.552	0.0044
[Eubacterium]_coprostanoligenes_group	0.49a	0.22ab	0.10ab	0.00b	0.43ab	0.00b	0.111	0.0056
Sutterellaceae	0.04b	0.06b	0.03b	1.88a	0.13b	0.00b	0.339	0.0076
F082	2.94a	0.78ab	1.60ab	0.04b	1.71ab	0.11b	0.507	0.0083
vadinBE97	0.14a	0.00b	0.00b	0.00b	0.01b	0.00b	0.026	0.0096
Pseudomonadaceae	0.00	0.00	0.00	0.02	0.00	0.02	0.005	0.0103
Marinifilaceae	0.01ab	0.04a	0.01ab	0.00b	0.00b	0.00b	0.006	0.0121
Bacteroidales Order	1.09	1.78	1.54	0.00	0.30	0.00	0.4697	0.0146
uncultured	0.26a	0.23a	0.18ab	0.14ab	0.14ab	0.02b	0.055	0.0161
Saccharimonadaceae	0.91a	0.42ab	0.42ab	0.00b	0.10ab	0.00b	0.194	0.0178
Bacteroidales_BS11_gut_group	0.26	0.00	0.00	0.00	0.01	0.03	0.059	0.0352
Actinomycetaceae	0.00	0.00	0.00	0.00	0.00	0.04	0.01	0.046
Anaerovoracaceae	0.10	0.04	0.08	0.13	0.06	0.00	0.028	0.0558
Erysipelotrichaceae	0.06	0.12	0.17	1.08	0.30	2.57	0.207	0.0570
Clostridia_vadinBB60_group	1.55	1.20	1.24	0.00	1.61	0.12	0.256	0.0661
Muribaculaceae	0.14	0.10	0.13	0.17	0.12	0.09	0.059	0.0844
Bacteroidia Class	0.12	0.21	0.16	0.00	0.00	0.00	0.074	0.1027
Hungateiclostridiaceae	0.01	0.00	0.01	0.00	0.03	0.00	0.001	0.1066
Pedosphaeraceae	0.05	0.03	0.02	0.00	0.01	0.00	0.017	0.1119
uncultured	0.17	0.07	0.04	0.00	0.14	0.01	0.048	0.1131
Selenomonadaceae	0.82	0.73	0.84	0.94	0.90	1.60	0.284	0.1320
Weeksellaceae	0.00	0.01	0.02	0.00	0.00	0.00	0.005	0.1320
Elusimicrobiaceae	0.10	0.01	0.00	0.00	0.00	0.01	0.028	0.1336
Synergistaceae	0.09	0.06	0.06	0.00	0.06	0.00	0.031	0.1517
MVP-15	0.02	0.00	0.00	0.00	0.00	0.00	0.007	0.1625
0319-6G20	0.02	0.00	0.00	0.00	0.00	0.00	0.005	0.2232
Mitochondria	0.01	0.01	0.02	0.06	0.01	0.08	0.028	0.2350
Izemoplasmataceae	0.02	0.00	0.00	0.00	0.00	0.00	0.007	0.2799

Coriobacteriales_Incertae_Sedis	0.01	0.00	0.00	0.00	0.00	0.00	0.003	0.2858
Clostridia_UCG-014	0.04	0.02	0.02	0.00	0.02	0.03	0.015	0.3050
Chloroplast	0.00	0.02	0.00	0.07	0.00	0.35	0.125	0.3234
Enterobacteriaceae	0.03	0.10	0.02	0.00	0.10	0.00	0.052	0.3635
Bacteroidaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.005	0.4509
Anaerolineaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.004	0.4509
Pirellulaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.001	0.4509
Marinilabiliaceae	0.01	0	0.00	0.00	0.00	0.00	0.003	0.4509
PeH15	0.00	0.00	0.00	0.00	0.00	0.00	0.002	0.4509
Paludibacteraceae	0.00	0.01	0.00	0.00	0.00	0.00	0.004	0.4509
Planococcaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.005	0.4509
Butyricicoccaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.004	0.4509
Oscillospirales	0.00	0.00	0.00	0.00	0.00	0.00	0.002	0.4509
p-2534-18B5_gut_group	0.00	0.03	0.02	0.00	0.01	0.00	0.016	0.4509
Clostridia Class	0.00	0.00	0.00	0.00	0.00	0.00	0.001	0.4509
uncultured	0.00	0.00	0.00	0.02	0.00	0.00	0.006	0.4509
COB_P4-1_termite_group	0.00	0.00	0.00	0.00	0.00	0.00	0.0001	0.4509
Enterococcaceae	0.00	0.00	0.00	0.00	0.00	0.02	0.008	0.4509
Myxococcaceae	0.01	0.00	0.00	0.01	0.00	0.00	0.005	0.5617
Dysgonomonadaceae	0.00	0.01	0.00	0.00	0.00	0.00	0.004	0.6371
Eggerthellaceae	0.02	0.01	0.00	0.00	0.01	0.00	0.008	0.7412

Abbreviations: CTL = control; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

[a,b,c,d]Values within a row with different superscripts differ significantly at $P < 0.05$.

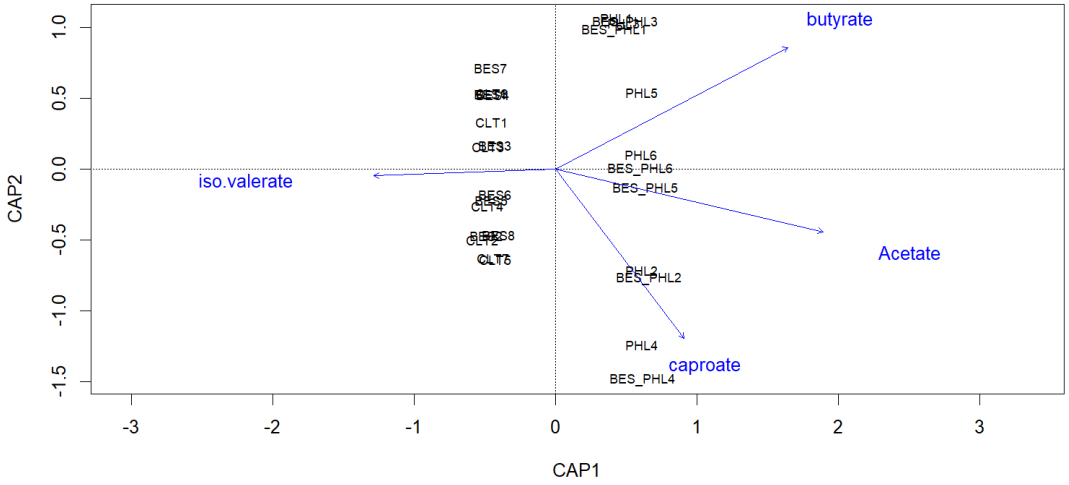


Figure S1 Distance-based redundancy analysis for cow inocula experiment.

CTL = control; BES = 3 μM 2-bromoethanesulfonic sodium; PHL = 36 mM phloroglucinol; BES+PHL = 3 μM 2-bromoethanesulfonic sodium + 36 mM phloroglucinol.

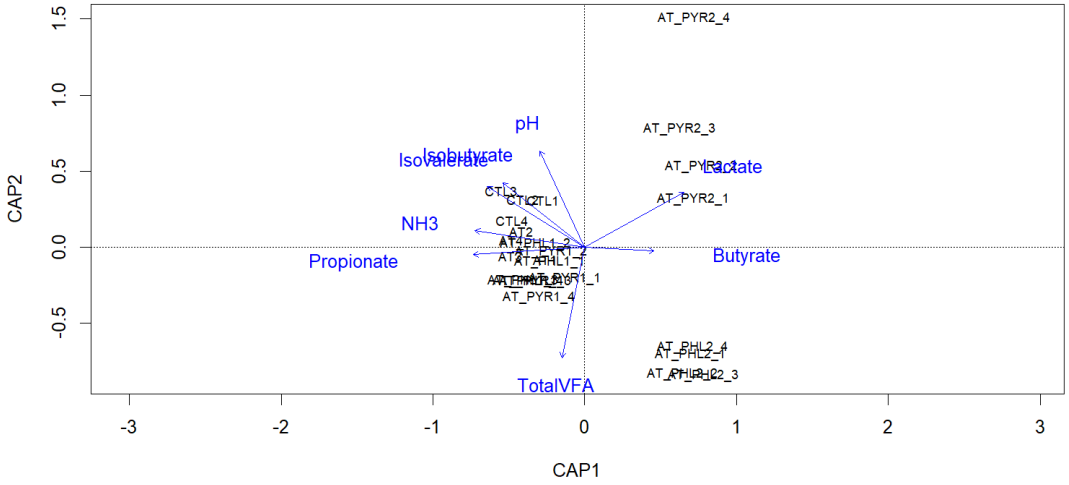


Figure S2 Distance-based redundancy analysis for goat inocula experiment.

CTL = control; AT = 2% *A. taxiformis*; AT+PHL1 = 2% *A. taxiformis* + 6 mM phloroglucinol; PHL2 = 2% *A. taxiformis* + 36 mM phloroglucinol; AT+PYR1 = 2% *A. taxiformis* + 6 mM pyrogallol; AT+PYR2 = 2% *A. taxiformis* + 36 mM pyrogallol.

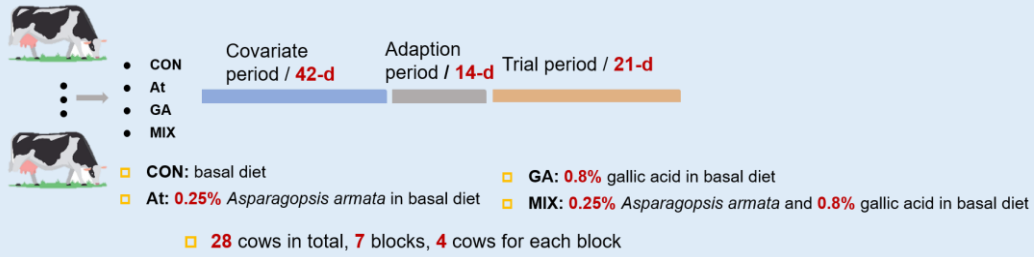
CHAPTER 4

Gallic Acid alleviates the negative effect of A. armata on milk yield without affecting methanogenesis inhibition in dairy cows

Introduction

In this trial, we used gallic acid for an *in vivo* supplementation, as this compound is the subunit of hydrolysable tannins and its ability to act as dihydrogen acceptor was demonstrated in vitro in Chapter 2. We hypothesized that combining methanogenesis inhibitor with gallic acid would stimulate phenolic-degrading rumen bacteria to uptake dihydrogen and produce more VFA, thus improving animal production. The objective of this study was to investigate the effects of gallic acid, acting as a dihydrogen acceptor, on milk production, gas emissions, and rumen fermentation when using methanogenesis inhibitor.

Experimental design



Main finding

- 0.25% AT alone decreased methane yield, increased dihydrogen emissions
- 0.8% GA alone did not affect methane or dihydrogen emissions, nor acetate or propionate molar proportions
- Feed intake decreased by 10% in the AT group with a consequent decrease in milk yield by 18%
- In the MIX group, feed intake was also decreased (8%), but the drop in milk yield was less severe (11%), compared to AT group

Conclusion

This study confirmed the antimethanogenic effect of *A. armata* but also shows a negative effect on feed intake and milk yield. Although there was no interaction effect of gallic acid and *A. armata* on dihydrogen and methane emission or acetate and butyrate proportion, they have a positive interaction on milk yield.

Gallic Acid alleviates the negative effect of *A. armata* on milk yield without affecting methanogenesis inhibition in dairy cows

Abstract

We have shown that when ruminal methanogenesis was inhibited *in vitro*, gallic acted as a hydrogen acceptor improved ruminal fermentation. In this work, we tested the effect of gallic acid and the methanogenesis inhibitor *A. armata* on dihydrogen emissions and milk production in dairy cows. Lactating Holstein cows (n=28) were separated into four treatment groups as follow: CON, basal diet; AT, basal diet with 0.25% *A. armata*; GA, basal diet with 0.8% gallic acid; and MIX, basal diet with 0.25% *A. armata* and 0.8% gallic acid. This study used a randomized complete block design with 6 weeks of covariate period, 2 weeks of adaptation period, and 3 weeks of experimental period. The AT treatment decreased methane yield by 25%, increased dihydrogen emissions threefold, and reduced the acetate to propionate ratio in the rumen. In contrast, gallic acid did not affect methane or dihydrogen emissions, nor acetate or propionate molar proportions. Feed intake decreased by 10% in the AT group with a consequent decrease in milk yield of 18%. In the MIX group, feed intake was also decreased (8%), but the drop in milk yield was less severe (11%). This study confirmed the antimethanogenic effect of *A. armata* but also shows a negative effect on feed intake and milk yield. While gallic acid alleviated the negative effect of *A. armata* on milk production and gallic acid combined

with *A. armata* had positive effects on milk yield, given the conditions in this study.

1 Introduction

The livestock sector contributes 14.5% of anthropogenic greenhouse gas emissions, and enteric methane emissions accounting for almost half of total enteric greenhouse gas emissions (Gerber et al., 2013). Effective strategies for decreasing methane emissions in the rumen exist, but they are not readily adopted by farmers. Recently, research focused on decreasing methane emissions without altering the fermentation process; the actual challenge is simultaneously reducing methane emissions and improving fermentation efficiency.

This challenge could be met by considering the pivotal place of dihydrogen in the rumen metabolism. Thermodynamic conditions in the rumen give an advantage to methanogenic archaea, makes them the main consumers (Morgavi et al., 2010). However, alternative dihydrogen consumers are still present and could redirect dihydrogen toward metabolic pathways providing the host animal with extra energy. For instance, some phenolic compounds such as gallic acid and phloroglucinol can capture dihydrogen and generate acetate and butyrate (Tsai et al., 1976, Krumholz et al., 1987). To develop alternative hydrogenotrophic pathways, two conditions should be met: methanogenic archaea need to be inhibited and an alternative hydrogen acceptor should be available. One effective methanogen-specific inhibitor is the red seaweed *A. taxiformis* (Li et al., 2016), which bioactive compound bromoform can inhibit methanogenesis (Machado et al., 2016). Glasson et al. (2022) reported that bromoform acts both on the

coenzyme M methyltransferase and the methyl-coenzyme M reductase to decrease methane production.

Several compounds have been evaluated as hydrogen acceptors in previous studies, including nitrate (Popova et al., 2017), and phloroglucinol when methanogenesis was inhibited (Martinez-Fernandez et al., 2017). Our *in vitro* studies also showed the potential of phenolic compounds gallic acid, phloroglucinol, and pyrogallol to capture excessive dihydrogen, and to increase VFA production when methane production was inhibited (Chapter 2). These results from our *in vitro* study are positive as consuming excessive dihydrogen would improve rumen fermentation and more VFA production would provide more energy for the host animal.

While our *in vitro* studies have provided promising results, it remains unclear how these phenolic compounds might impact animal productivity and the rumen microbiota with a methanogenesis inhibitor *in vivo*. To address this gap in knowledge, we conducted an animal study, in which we tested the interaction effects of *A. armata*[1] as a methanogenesis inhibitor with gallic acid as a hydrogen acceptor on lactating dairy cows. We hypothesized that gallic acid can improve animal productivity by redirecting excessive dihydrogen metabolism and modify rumen microbiota when methanogenesis is inhibited by *A. armata.* In this study, we investigated the effects of gallic acid alone, *A. armata* alone, and the combination of gallic acid with *A. armata* on animal production, rumen fermentation, gas emissions, rumen microbial structure and gene expression, blood metabolism, and cow's health.

[1] *Asparagopsis armata*, red seaweed, is similar to *A.taxiformis.* In this trial, we used *Asparagopsis armata* as it was the only available variety in our provider.

2 Material and Methods

This experiment was performed at Herbipôle, which is INRAE facility for low mountain ruminant experiments located in Marcenat (doi:10.15454/1.5572318050509348E12). This study was carried out from February 2022 to May 2022 in accordance with the French Ministry of Education and Research and applicable European Union directive 2010/63/EU guidelines for animal experimentation. The local ethics committee on animal experimentation in the Auvergne-region committee approved the experiment (approval number APAFIS#32276-2021070514093708 v1), and the study was registered on the Animal Study Registry (DOI: 10.17590/asr.0000282).

2.1 Animals, Experimental Design, and Diets

The study involved 28 multiparous Holstein cows with an average body weight of 642 ± 59 kg and daily milk production of 19 ± 2.5 kg. The experiment used a randomized complete block design with three periods: a covariate period of 6 weeks, an adaptation period of 2 weeks, and an experimental period of 3 weeks. During the covariate period, dry matter intake (DMI), milk yield, and methane production were recorded and these data were used for the blocking. The cows were blocked into 7 blocks with 4 animals each, based on parity, calving date, DMI, milk yield, and methane production during the covariate period. Within each block, cows were randomly assigned to one of the four treatments: CON (Control, basal diet), AT (0.25% *A. armata* on a DM basis), GA (0.8% gallic acid on a DM basis), and MIX (0.25% *A. armata* + 0.8% gallic acid on a DM basis).

A partial mixed ration feeding strategy was used, as it is common in European

counties. *A. armata* (Volta Greentech, Solna, Sweden) and gallic acid (Acros organics, China) were added to the pellets. The pelleting duration and temperature were controlled to minimize the loss of bioactive compound in the *A. armata*, the highest temperature was 54 °C during the pelleting. Three different pellets were made: the pellet without *A. armata* and gallic acid, the pellet containing 3% *A. armata,* and the pellet containing 6% gallic acid. The ingredients and nutrient composition of mixed forage and pellets are shown in Table 7. In the AT group, the pellet containing 3% *A. armata* was mixed with the pellet without *A. armata* and gallic acid to create a mixed pellet with 1% *A. armata.* In the GA group, the pellet containing 6% gallic acid was mixed with the pellet without *A. armata* and gallic acid to create a mixed pellet with 3% gallic acid. In the MIX group, the pellet containing 3% *A. armata,* the pellet containing 6% gallic acid, and the pellet without *A. armata* and gallic acid were mixed to create a mixed pellet with 1% *A. armata* and 3% gallic acid.

During the covariate period, the supply of concentrate was adjusted so that the average forage intake to concentrate intake ratio was 75:25. Based on this ratio, the *A. armata* inclusion level based on DMI was 0.25%, and the gallic acid inclusion level based on DMI was 0.8%. The cows were housed in a free-stall barn and fed ad libitum with free access to drinking water. The mixed forage was prepared with a double screw mixer (Solomix 2 VLH-B, Trioliet, Oldenzaal, Netherlands) and provided once daily in the morning at 08h30. The mixed forage and the mixed pellet were provided by different automatic feeding systems that could record the feed intake of individual cows.

In the first week of the adaption period (week 7), the inclusion level of *A. armata*

gradually increased to 0.5% and the gallic acid inclusion level gradually increased to 0.8%. However, the concentrate intake of 3 cows in the AT group and 4 cows in the MIX group sharply decreased in the second week of the adaption period (week 8). Therefore, the *A. armata* inclusion level was adjusted to 0.25% in the AT and MIX groups for the trial period. The cows were milked twice daily at 07h30 and 16h30.

Table 7 Ingredients and chemical composition of the experimental diets.

Mixed forage				
Ingredient of forage, % of DM				
Silage	46	46	46	46
Hay	43	43	43	43
Beet pulp	10	10	10	10
Premix[1]	1	1	1	1
Chemical composition of the Forage, % of DM				
OM	89.5	89.5	89.5	89.5
CP	11.1	11.1	11.1	11.1
NDF	59.6	59.6	59.6	59.6
ADF	32.1	32.1	32.1	32.1
GE, MJ/kg of DM	17.8	17.8	17.8	17.8
Pellet	CON pellet	3% AT pellet	6 GA pellet	
Ingredient of concentrate, % of DM				
Corn	20	22	21	-
Wheat	19	19	19	-
Barley	15	15	15	-
Wheat bran	11	10	9	-
Sunflower cake	18	14	14	-
Rapeseed	12	12	11	-
Calcium carbonate	1	1	1	-
Premixer	1.5	1.5	1.5	-
Salt	0.5	0.5	0.5	-
A. armata	0	3	0	-
Gallic acid	0	0	6	-
Molasses	2	2	2	-
Chemical composition of the Concentrate, % of DM				
OM	92.8	92.4	93.2	-
CP	19.1	19.0	16.7	-
NDF	21.6	21.6	20.0	-
ADF	10.1	9.3	8.7	-
Starch	31.5	31.9	34.4	-
GE, MJ/kg of DM	17.8	17.6	17.6	-

Abbreviations: CON = pellet without *A. armata* and gallic acid; AT = pellet with 3% *A. armata*; GA = pellet with 6% gallic acid; OM = organic matter; CP =crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; GE = gross energy.

[1]1% commercial mineral-vitamin premix (Galaphos, CCPA, France).

2.2 Sampling and measurement

During the experiment, we recorded the feed intake of each cow using automatic feeding systems to measure daily feed intake. To calculate dry matter intake (DMI), we collected pellets and mixed forage twice weekly and dried them at 60 °C for 72 h in a forced-air oven to determine their dry matter contents. Additionally, we collected the

mixed forage, the refusal of mixed forage, and the three different pellets weekly and stored them at -20 °C. After the experiment, we composited the sample of the mixed forage, refusal of mixed forage, and the different three pellets into one sample, respectively, for each period. The composited sample was then dried at 60 °C for 72 h in a forced-air oven. The mixed forage and refusal of mixed forage were ground through a 1-mm sieve using a mill (BJL8500-2, Boisson Jean-Luc, Lusignan, France), while the mixed pellet was ground through a 1-mm sieve using a Foss mill (Cyclotec CT193, Hilleroed, Denmark).

We used the ground samples for chemical composition analysis, following the methods previously described by Guyader et al. (2015b). Briefly, we determined the dry matter (DM) content by drying at 104 °C for 4 h. We determined organic matter (OM) and ash content by ignition at 550 °C for 6 h (Method 942.05; AOAC, 2005). We determined total N content by combustion using the Dumas method (Method 968.06; AOAC, 2005), and calculated the crude protein (CP) content as N × 6.25. We determined neutral detergent fiber (NDF) and acid detergent fiber (ADF) following the method described by Van Soest (1991). We determined starch content using an enzymatic method (Faisant et al., 1995), and determined gross energy (GE) by isoperibolic calorimetry (model C200, IKA, Staufen, Germany).

Before the experiment, we collected *A. armata* powder for bromoform analysis. During the adaption and experimental period, we collected the 3 % *A. armata* containing pellet once in week 7 (the beginning of the adaption period), week 9 (the beginning of the experimental period), and week 11 (the end of the experimental period) and stored them

at -20 °C for bromoform analysis. We sent these samples to Bigelow Laboratory for Ocean Sciences (East Boothbay, USA) for bromoform analysis by GC/MS (QP2010 Ultra, Shimadzu), following the method described by Paul et al. (2006).

2.3 Gas measurement

We used two Greenfeed systems (C-Lock Inc., Rapid City, SD) to measure methane, dihydrogen, and carbon dioxide production of cows. The Greenfeed systems were installed in the stall, and cows had free access to the system during the experiment. The Greenfeed system dispensed 4 drops of pellets to each visiting cow, with 40 g of pellet in each drop and 45 s intervals between adjacent drops. Each cow was allowed up to 6 visits per day, with at least 4 h between adjacent visits. Pellet consumption from the Greenfeed system was included in the calculation of DMI.

Before the experiment, cows were trained to use the Greenfeed system for two weeks. The Greenfeed system was calibrated once a day by injecting a calibration gas mixture with certificated concentrations of methane and carbon dioxide. Additionally, the carbon dioxide recovery rate of the system was measured once a month.

The raw gas emission data was processed by C-Lock, Inc. (Rapid City, USA) following their established protocols. Methane, dihydrogen, and carbon dioxide emissions (g/day) were determined based on the concentrations of these gases, the airflow in the collection pipe, the concentration of these gases in the ambient air, the rate of air capture, and the air temperature. C-Lock was not informed of the treatment of each animal. We calculated methane production (g/d), methane yield (g/kg DMI), and methane

intensity (g/kg milk yield or g/kg energy corrected milk yield) during the covariate and experimental periods.

2.4 Milk production, composition, body weight， and body condition score

The milk production data were recorded for each cow at every milking event. Milk samples were collected for 2 consecutive days weekly both in the morning and afternoon, to determine milk fat, milk true protein, milk lactate, milk urea concentration, and somatic cell count (SCC) using a Foss MilkoScan FT6000 (Foss Electric, Hillerød, Denmark). To obtain each milk sample, 30 mL of milk was collected and preserved with 0.02% bronopol at 4°C. Milk composition data were weighted according to the corresponding milk yield during each sampling event.

On Tuesday during week 11 in the experimental period, 10 mL milk from each cow in CON, AT, and MIX groups was collected twice in the morning and evening, and stored at -20 °C. After the experiment, equal amounts of these milk samples from the same group were pooled to obtain one sample for bromoform analysis, as previously described. The weight of each animal was manually recorded every other week, and their body condition score was assessed every other week by parallel-trained staff.

2.5 Rumen fluid and blood

Rumen fluid samples were collected on Thursday in week 3 and on Tuesday in week 4 during the covariate period, and on Tuesday and Thursday in week11 during the experimental period, using oesophageal tubing 5 h after the morning feeding (Popova et

al., 2022). The collected rumen fluid was filtered through a polyester monofilament fabric (250 μm pore size), and the resulting filtrate was subsampled for VFA, ammonia, pH, protozoa counting, DNA extraction, and RNA extraction as described before (Chapter 3). Briefly, 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 then analyzed by gas chromatography (PerkinElmer Clarus 580, Waltham, USA). For ammonia analysis, 1 mL filtrate was mixed with 0.1mL of 5% orthophosphate solution and analyzed according to the phenol-hypochlorite reaction (Weatherburn, 1967). For protozoa counting, 1 mL of filtrate was mixed with 1 mL of methyl green–formalin solution, and stored away from direct light until counting. The pH value was measured directly by pH meter (pH 538, WTW). The rumen fluid samples from Tuesday and Thursday were incorporated for VFA, ammonia analysis, and protozoa counting during the covariate and experimental periods, respectively.

For total rumen microbes DNA extraction, 1 mL of filtrate was collected and centrifuged at 16 000 g for 10 min at 4 °C. The supernatant was discarded, and the pellet was stored at -20 °C until DNA extraction using DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's instructions. Additionally, around 10 mL filtrate was collected, cooled in liquid nitrogen immediately, and stored at -80 °C until total rumen microbes RNA extraction using the RNeasy Mini kit (Qiagen, Germany) following the manufacturer’s instructions. Turbo DNA-free kit (Thermo Fisher, Ambion, USA) was used to digest residual DNA, and RNA Clean & Concentrate kit (Zymo Research, Orange, CA, USA) was used to purify the digested total RNA. The rumen microbes DNA samples were

used for metataxonomic and metagenomic analysis, while the rumen microbes total RNA samples were used for metatranscriptomic analysis. In this thesis, we only reported the preliminary results from the metataxonomic analysis as the sequencing data are currently under analyzing.

Blood samples were collected from the jugular vein of each animal on Monday in week 4 during the covariate period, and on Wednesday in week 11 during the experimental period. Blood (9 mL) were sampled into a heparinized (BD vacutainer, Plymouth, UK) and an EDTA vacutainer (BD vacutainer, Plymouth, UK), respectively. After sampling, the heparinized and EDTA vacutainers were centrifuged at 3 000 g for 15 min at 4 °C. Plasma sample (1.2 mL) from the EDTA vacutainer was collected into a 2 mL Eppendorf tube. Then the tubes were cooled by liquid nitrogen immediately, and stored at -20 °C for subsequent analysis of urea, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), and glucose using biochemical analyser (Arene 20XT, Thermo Scientific). Plasma samples (1.2 mL X 2) from the heparinized vacutainer were collected into two 2 mL Eppendorf tubes, respectively. Then the tubes were cooled by liquid nitrogen immediately. One sample was stored at -20 °C for subsequent analysis of aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP) using biochemical analyser (Arene 20XT, Thermo Scientific). The other sample was stored at -80 °C for metabolomic analysis.

To perform the metabolomic analysis, plasma samples were analysed by two analytical platforms using the MxP® Quant 500 kit (Biocrates, Innsbruck, Austria): Lipids

and hexoses were measured by flow injection analysis-tandem mass spectrometry (FIA-MS/MS) using a Xevo TQ XS (Waters, Vienna, Austria) with an electrospray ionization source, and small molecules were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a 5500 QTRAP (AB Sciex, Darmstadt, Germany). In total, 630 metabolites from 26 biochemical classes were detected.

2.6 Statistical analysis

The raw data from the metabolomic analysis were analyzed by software SIMCA 14.1 (Göttingen, Germany). The primers used were 515F and 806R for V4 region of 16S rRNA gene amplification. The resulting amplicons were sequenced on an Illumina MiSeq platform using a MiSeq 2x250 pair-end sequencing kit at University of Illinois at Urbana-Champaign (Illinois, America). Downstream processing was performed using QIIME 2 (Bolyen et al., 2019). Specifically, demultiplexing was performed using Qiime demux emp-paired plugin; while denoising, quality control, and ASV generation were performed using the DATA2 plugin. Representative sequences from all ASVs were aligned against SILVA 138.1. The following analysis were performed by software R (Version R 4.2.2). For the analysis of alpha diversity, the Kruskal-Wallis test was performed to obtain *P*-values associated with treatment variations, while the Pairwise Wilcoxon test was performed to facilitate multiple comparisons between treatments. For beta-diversity analysis, permutational multivariate ANOVA was performed using the Adonis test (with 9999 permutations) to obtain *P*-values associated with treatment variations, and the Pairwise Adonis2 test was performed to enable multiple comparisons among treatments, also

employing 9999 permutations.

To analyze the data, normality of all datasets except sequencing and blood metabolomic data was assessed for normality by the UNIVARIATE procedure (version 9.4, SAS Institute Inc., Cary, NC.). The MIXED procedure of SAS then used for statistical analysis. The statistical analysis of SCC and protozoa counting data were log_{10}-transformed prior to statistical analysis. The statistical model included *Asparagopsis* (AT and MIX versus CON and GA), gallic acid (GA and MIX versus CON and AT), block, treatment x block interaction, *Asparagopsis* x gallic acid interaction, and covariate response. Block and treatment x block interaction were considered as random effects; *Asparagopsis, gallic acid, and Asparagopsis* x gallic acid interaction were considered as fixed effects.

Methane, dihydrogen, and carbon dioxide, milk yield, body weight, and body condition score data were averaged by a cow during covariate and experimental periods, respectively. And the average values were used for statistical analysis. The data of DMI, milk composition, milk composition production, energy corrected milk production (ECM), feed efficiency, ECM feed efficiency, and SSC were first averaged by week for covariate and experimental period, respectively. The ECM was calculated as described (Sjaunja, 1990). Data of pH value was averaged by Tuesday and Thursday for covariate and experimental periods, respectively. The degree of freedom was calculated by the Satterth statement, the *P* value was calculated by PDIFF and adjusted by the Turkey statement. Differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

3 Results

3.1 Enteric gas emissions

A. armata decreased methane production ($P = 0.0121$), methane yield ($P = 0.0010$), methane intensity (g/kg milk yield) ($P = 0.0068$), accompanied by a corresponding increase in dihydrogen emission (g/d) ($P < 0.0001$). Specifically, the AT group demonstrated a 13% decrease in methane production, a 25% decrease in methane yield, and a 16% decrease in methane intensity (g/kg milk yield), along with a fourfold increase in dihydrogen emission (g/d), compared to the CON group (Table 8).

Gallic acid did not have a significant effect on either methane or dihydrogen emissions. Furthermore, there was no interaction effect between gallic acid and *A. armata* on methane and dihydrogen (g/d) emissions. Carbon dioxide emissions were not affected by *A. armata* or gallic acid, however, an interaction effect between *A. armata* and gallic acid on carbon dioxide emission was observed.

Table 8 Effects of *A. armata*, gallic acid, and their combination on enteric gas emissions in lactating dairy cows.

Item	Treatment				SEM	P-Value[1]		
	CON	AT	GA	MIX		Asparagopsis	Gallic	AsparagopsisxGallic
CH_4, g/d	432	375	421	340	21.4	0.0121	0.4333	0.3152
CH_4, g/DMI	24.0	17.9	21.3	15.9	1.34	0.0010	0.1389	0.7290
CH_4, g/MY	24.0	20.1	22.8	17.1	1.49	0.0068	0.1898	0.5528
CH_4, g/ECM	27.9	23.6	27.0	21.8	1.40	0.0059	0.4846	0.5006
H_2, g/d	1.29	4.86	1.17	5.27	0.442	<0.0001	0.7685	0.3947
H_2, g/kg DMI	0.07	0.22	0.06	0.26	0.021	<0.0001	0.6974	0.0851
H_2, g/kg MY	0.08	0.25	0.06	0.29	0.024	0.0005	0.3625	<0.0001
H_2, g/kg ECM	0.08	0.30	0.03	0.33	0.027	0.0012	0.6750	0.4061
CO_2, g/d	11425	10663	11210	11276	183.2	0.0681	0.2970	0.0388

Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; DMI = dry matter intake; MY = milk yield; ECM = energy corrected milk yield.

[1]Asparagopsis represents the main effect of *A. armata* (CON and GA versus AT and MIX); Gallic represents the main effect of gallic acid (CON and AT versus GA and MIX); AsparagopsisxGallic represents the interaction between main effects of *A. armata* and gallic acid.

3.2 Rumen fermentation

A. armata decreased (*P* = 0.0259) total VFA concentration, acetate proportion (*P* = 0.0010), while it increased (*P* = 0.0163) propionate and butyrate proportion, thus acetate to propionate (A/P) ratio was decreased (*P* = 0.0003) (Table 9). Specifically, in AT group, total VFA concentration was decreased by 11%, acetate proportion was decreased by 6%, while propionate and butyrate proportion were increased by 8% and 18, respectively, thus A/P ratio was decreased, compared to the CON group (Table 9).

Gallic acid did not affect total VFA concentration or VFA profile, and there was no interaction effect of *A. armata* and gallic acid on total VFA concentration and VFA profile. There were no *A. armata* or gallic acid effects or their interaction effects on pH value and ammonia concentration. Furthermore, there were no *A. armata* or gallic acid effects or

their interaction effects on total protozoa counting or different rumen protozoa species counting, with the exception of an increased number of *Dasytricha* by *A. armata* (P = 0.0317) (Table 10).

Table 9 Effect of *A. armata*, gallic acid, and their combination on rumen fermentation in lactating dairy cows.

Item	Treatment				SEM	*P*-Value[1]		
	CON	AT	GA	MIX		Asparagopsis	Gallic	AsparagopsisxGallic
pH	6.81	6.88	6.80	6.86	0.100	0.7755	0.4958	0.9088
NH3, 100 mg/dL	2.26	2.92	3.19	3.42	0.667	0.5331	0.2118	0.6813
Total VFA, mmol/L	157.88	140.59	152.01	136.88	7.219	0.0259	0.3331	0.8355
VFA composition, mol/100 mol								
Acetate	69.70	65.28	69.29	63.05	1.40	0.0010	0.3434	0.5163
Propionate	17.73	19.20	17.77	21.34	0.779	0.0163	0.2029	0.2114
Butyrate	10.09	11.94	10.48	11.77	0.469	0.0018	0.7947	0.4808
Iso butyrate	0.61	0.78	0.60	0.74	0.042	0.0072	0.4719	0.6081
Valerate	0.79	1.11	0.78	1.17	0.154	0.0314	0.8747	0.8118
Iso valerate	0.61	1.07	0.64	1.73	0.228	0.0046	0.1739	0.1728
Caproate	0.29	0.65	0.27	0.49	0.091	0.0026	0.2933	0.4244
A/P	3.94	3.48	3.92	3.02	0.161	0.0003	0.1330	0.1646

Abbreviations: CON = control diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; A/P = acetate/propionate.

[1]Asparagopsis represents the main effect of *A. armata* (CON and GA versus AT and MIX); Gallic represents the main effect of gallic acid (CON and AT versus GA and MIX); AsparagopsisxGallic represents the interaction between main effects of *A. armata* and gallic acid.

Table 10 Effect of *A. armata*, gallic acid, and their combination on protozoa counting in lactating dairy cows.

Item	Treatment				SEM	*P*-Value[1]		
	CON	AT	GA	MIX		Asparagopsis	Gallic	AsparagopsisxGallic
Total protozoa, log_{10} / mL	5.01	4.99	5.03	5.22	0.078	0.2700	0.1263	0.2029
Entodiniomorphs, log_{10} / mL								
Small (< 100 μm)	4.97	4.93	4.97	5.16	0.080	0.3374	0.1468	0.1620
Large (> 100 μm)	3.64	4.25	3.92	3.86	0.210	0.1395	0.7604	0.0933
Holotrichs, log_{10} / mL								
Dasytricha (< 100 μm)	3.11	3.26	2.86	3.49	0.170	0.0317	0.9361	0.1307
Isotricha (> 100 μm)	3.08	3.14	3.04	3.24	0.182	0.4772	0.8874	0.6986

Abbreviations: CON = control diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet.

[1]Asparagopsis represents the main effect of *A. armata* (CON and GA versus AT and MIX); Gallic represents the main effect of gallic acid (CON and AT versus GA and MIX); AsparagopsisxGallic represents the interaction between main effects of *A. armata* and gallic acid.

3.3 Effect of *A. armata* and gallic acid on animal performance

Table 11 summarizes the effect of *A. armata*, gallic acid, and their interaction effects on animal performance. Body condition score was not affected by *A. armata* and gallic acid, but *A. armata* significantly ($P = 0.0187$) decreased body weight. AT group decreased body weight by 3%, compared to the CON group. *A. armata* also decreased forage intake ($P = 0.0252$), concentrate intake ($P = 0.0067$), and total feed intake ($P = 0.0175$). AT group decreased forage intake, concentrate intake, and total feed intake by 11%, 12%, and 10%, respectively, compared to the CON group. In contrast, gallic acid did not affect feed intake and there was no interaction effect of *A. armata* and gallic acid on feed intake.

Milk yield decreased ($P < 0.0001$) by *A. armata,* however it was slightly increased ($P = 0.0320$) by gallic acid. AT group decreased milk yield by 18% and the GA group increased milk yield by 0.5%, compared to the CON group. There was an interaction effect of *A. armata* and gallic acid ($P = 0.0156$) on milk yield. In the MIX group, milk yield

increased by 9%, compared to AT group.

A. armata decreased milk fat concentration ($P = 0.0116$), milk fat production ($P < 0.0001$), milk true protein concentration ($P = 0.0007$), milk true protein production ($P < 0.0001$), and lactose production ($P < 0.0001$). However, gallic acid did not affect milk fat, milk true protein, and lactose concentration or production. Also, there was no interaction effect of *A. armata* and gallic acid on milk fat, milk true protein, and milk lactose concentration or production, except for milk true protein production ($P = 0.0398$). Milk true protein production increased by 12% in the MIX group, compared to AT group.

There was no effect on feed efficiency for *A. armata* or gallic acid, or their interaction, although ECM feed efficiency trended towards an increase (P = 0.0718) by *A. armata*. There was no treatment effect or interaction effect on milk urea nitrogen concentration (MUN) or SCC. However, gallic acid decreased ($P = 0.0437$) MUN concentration. In the GA group, MUN concentration decreased by 4%, compared to the CON group. Milk from CON, AT, and MIX groups did not contain bromoform. The bromoform concentration in the 3% *A. armata* containing pellet was 0.312 mg/g DM at week 7, 0.240 mg/g DM at week 9, and 0.171 mg/g DM at week 11 (Figure 10).

Table 11 Effects of *A. armata*, gallic acid, and their combination on lactating dairy cows' performance.

Item	Treatment				SEM	P-Value[1]		
	CON	AT	GA	MIX		Asparagopsis	Gallic	AsparagopsisxGallic
Forage intake, kg/d	16.4	14.6	16.4	15.2	0.51	0.0252	0.5927	0.6284
Concentrate intake, kg/d	5.7	5.0	5.6	5.0	0.19	0.0067	0.9624	0.9600
Total feed intake, kg/d	22.0	19.7	22.0	20.3	0.52	0.0175	0.4534	0.4528
Milk yield, kg/d	20.7	17.0	20.8	18.5	0.33	<0.0001	0.0302	0.0156
Feed efficiency[2], kg/kg	0.92	0.95	0.98	0.93	0.028	0.6736	0.5074	0.2127
Milk fat, %	3.64	3.48	3.62	3.35	0.077	0.0116	0.3357	0.4461
Milk fat, kg/d	0.75	0.58	0.76	0.62	0.020	<0.0001	0.2989	0.5518
Milk true protein, %	3.09	2.90	3.08	2.97	0.045	0.0007	0.6395	0.1575
Milk true protein, kg/d	0.64	0.49	0.64	0.55	0.015	<0.0001	0.1963	0.0398
Lactose, %	4.81	4.81	4.73	4.78	0.078	0.7595	0.5212	0.7755
Lactose, kg/d	1.00	0.82	0.99	0.89	0.024	<0.0001	0.3162	0.0879
ECM, kg/d	16.0	15.6	15.9	15.5	0.13	0.0025	0.5226	0.7177
ECM feed efficiency[3], kg/kg	0.75	0.82	0.73	0.77	0.026	0.0718	0.1959	0.4352
MUN, mg/L	70	76	67	62	3.8	0.8918	0.0437	0.1716
SCC	4.94	5.18	4.82	5.10	0.147	0.1253	0.4026	0.7848
BW, kg	652	630	656	640	7.2	0.0187	0.3809	0.6636
BCS	1.62	1.57	1.63	1.62	0.043	0.4378	0.6191	0.6093

Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; ECM = energy corrected milk yield; MUN = milk urea nitrogen; SCC = somatic cell count; BW = body weight; BCS = body condition score.

[1]Asparagopsis represents the main effect of *A. armata* (CON and GA versus AT and MIX); Gallic represents the main effect of gallic acid (CON and AT versus GA and MIX); AsparagopsisxGallic represents the interaction between the main effects of *A. armata* and gallic acid.

[2]Feed efficiency = milk yield / total feed intake.

[3]ECM feed efficiency = energy-corrected milk yield / total feed intake.

3.4 Blood biochemistry

A. armata and gallic acid did not affect blood biochemistry, except gallic acid decreased GGT and ALP concentration (P = 0.0288 and P = 0.0008, respectively) (Table 12). The interaction effects of *A. armata* and gallic acid were observed on AST, ALP, and ALT (P = 0.0429, P = 0.0425, and P = 0.0302, respectively).

Table 12 Effect of *A. armata*, gallic acid, and their combination on blood biochemistry in lactating dairy cows.

	Treatment				SEM	P-Value[1]		
Item	Con	AT	GA	MIX		Asparagopsis	Gallic	AsparagopsisxGallic
Glucose, g/L	0.50	0.50	0.39	0.52	0.039	0.1092	0.2637	0.1008
NEFA, mmol/L	0.10	0.08	0.04	0.05	0.030	0.9674	0.1489	0.6438
B-OH, mmol/L	0.45	0.43	0.38	0.48	0.032	0.3028	0.8951	0.0640
Urea, g/L	0.09	0.10	0.08	0.10	0.012	0.1573	0.6190	0.8121
AST, UI/L	66.2	53.7	49.0	54.4	6.62	0.4157	0.2637	0.0429
GGT, UI/L	21.3	18.0	15.3	16.8	2.26	0.6293	0.0288	0.1542
ALP, UI/L	59.9	47.9	38.3	41.3	5.38	0.2105	0.0008	0.0425
ALT, UI/L	24.5	16.4	17.7	17.8	2.56	0.0510	0.4431	0.0302

Abbreviations: CON = control diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; NEFA = non-esterified fatty acids; B-OH = beta-hydroxybutyrate; AST = aspartate transferase; GGT = gamma-glutamyl transferase; ALP = alkaline phosphatase; ALT = alanine transaminase.

[1]Asparagopsis represents the main effect of *A. armata* (CON and GA versus AT and MIX); Gallic represents the main effect of gallic acid (CON and AT versus GA and MIX); AsparagopsisxGallic represents the interaction between main effects of *A. armata* and gallic acid.

3.5 Metabolomic and metataxonomic analysis

The kit MxP® Quant 500 kit, capable of detecting a total of 630 metabolites, was used for metabolomic analysis. For the data analysis, only metabolites with a detection rate exceeding 50% in the 24 cows (only 24 samples were used) were retained. Consequently, 421 metabolites, representing 67% of the total detectable metabolites, were utilized for data analysis. Principal component analysis revealed that the first component accounted for 50% variance (Figure 9). Notably, samples from the CON treatment distinctly separated from samples from the AT treatment along component 1. Similarly, samples from the CON treatment also appeared to diverge from those samples from the GA treatment along the same component. We subsequently performed orthogonal partial least squares-discriminant analysis between the CON and AT treatments (Figure 10), identifying discriminant metabolites (Table 13). There were 148 differential metabolites identified ($P < 0.05$) and the concentrations of all these

metabolites in the CON treatment were higher than that of the AT treatment. The majority of these metabolites belong to the classes of phosphatidyl-cholines, cholesteryl esters, amino acid and amino acid related, dihexosylceramides, and triglycerides. An attempt at orthogonal partial least squares-discriminant analysis between CON and GA treatments was unsuccessful, with the model not achieving significance (P = 0.17743).

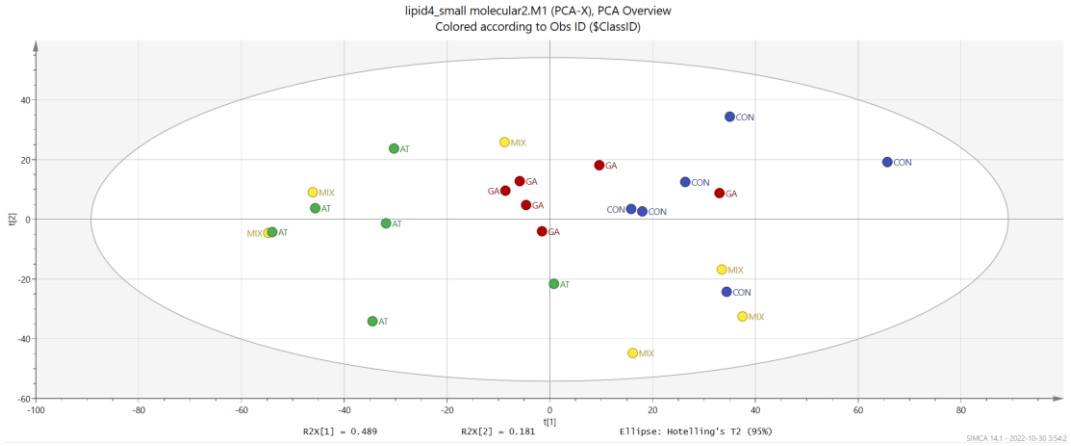


Figure 10 Score plots of principal component analysis of blood metabolites.

$R^2X[1]$ and $R^2X[2]$ represent component 1 (t[1]) and 2 (t[2]), respectively. Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet.

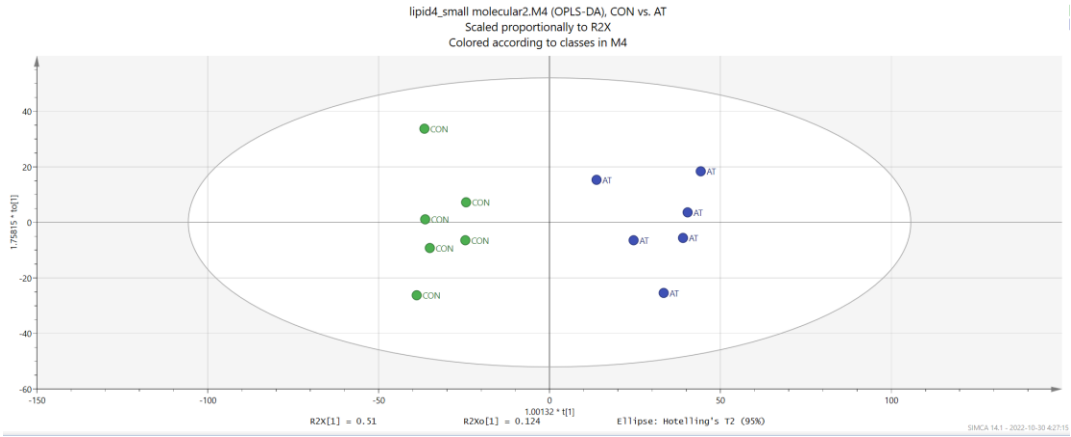


Figure 11 Orthogonal partial least squares-discriminant analysis between CON and AT treatments.

R2X[1] and R2Xo[1] represent predictive component (t[1]) and orthogonal component (to[1]), respectively. Components=1+0+0, R^2(cum)=0.817, Q^2(cum)=0.765, P-value=0.00147. Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet.

Table 13 Differential metabolites between CON and AT treatments.

Var ID	*P*-value[1]	Average (CON)	Average (AT)	Std. dev. (CON)	Std. dev. (AT)	Fold change[2]
Phosphatidyl-choline ae C36:4	0.000	14.723	6.433	2.304	1.311	0.437
Phosphatidyl-choline ae C38:6	0.000	3.281	2.028	0.453	0.106	0.618
Phosphatidyl-choline ae C36:5	0.000	7.532	3.992	1.181	0.617	0.530
Phosphatidyl-choline ae C34:3	0.000	39.627	16.294	8.007	4.211	0.411
Sphingomyelin C16:1	0.000	10.599	7.178	1.065	0.804	0.677
Phosphatidyl-choline ae C38:5	0.000	5.123	3.328	0.635	0.299	0.650
Phosphatidyl-choline aa C36:5	0.000	9.645	6.388	1.011	0.789	0.662
Tryptophan	0.000	52.787	34.239	4.485	5.963	0.649
Sphingomyelin C18:1	0.000	5.605	3.703	0.589	0.537	0.661
Hydroxysphingo-myelin C14:1	0.000	12.823	9.432	1.156	0.926	0.736
Phosphatidyl-choline ae C34:2	0.000	31.980	18.370	5.023	3.278	0.574
Threonine	0.000	119.721	62.231	17.312	18.559	0.520
Tyrosine	0.000	66.557	41.622	10.523	4.040	0.625
Cholesteryl ester 15:0	0.000	2.853	1.732	0.409	0.328	0.607
Sphingomyelin C20:2	0.000	0.257	0.123	0.050	0.038	0.479
Hexosylceramide (d18:1/26:0)	0.000	0.042	0.020	0.008	0.006	0.475
Proline	0.000	95.887	59.964	9.779	13.842	0.625
Phosphatidyl-choline ae C36:3	0.000	22.648	11.999	3.816	3.315	0.530
Phosphatidyl-choline aa C36:2	0.000	323.277	184.663	57.210	32.756	0.571
Phosphatidyl-choline aa C34:3	0.000	36.898	21.833	4.891	5.237	0.592
Phosphatidyl-choline ae C32:1	0.000	5.182	3.153	0.788	0.558	0.608
Valine	0.001	238.923	162.187	29.737	22.557	0.679
Cholesteryl ester 18:2	0.001	1907.850	1088.800	310.542	253.864	0.571
Sphingomyelin C16:0	0.001	93.228	66.077	10.907	7.636	0.709
Alanine	0.001	309.315	189.263	53.769	26.147	0.612
Carnosine	0.001	13.842	7.720	2.571	1.648	0.558
Phosphatidyl-choline aa C34:2	0.001	174.517	100.381	34.526	13.735	0.575
Phosphatidyl-choline ae C42:4	0.001	0.305	0.177	0.051	0.040	0.580
Symmetric dimethylarginine	0.001	0.562	0.378	0.081	0.044	0.673
Phosphatidyl-choline ae C30:0	0.001	1.447	0.948	0.187	0.169	0.655
Phosphatidyl-choline ae C36:2	0.001	57.857	31.814	11.240	6.934	0.550
Phosphatidyl-choline aa C36:3	0.001	116.287	67.631	19.141	15.630	0.582
Phosphatidyl-choline ae C38:4	0.001	7.350	4.898	1.113	0.584	0.666
Hydroxysphingo-myelin C16:1	0.001	12.046	8.783	1.068	1.307	0.729
Phosphatidyl-choline aa C36:4	0.001	31.432	19.046	5.471	3.352	0.606

Cholesteryl ester 20:3	0.001	24.128	14.226	3.765	3.557	0.590
Phosphatidyl-choline ae C42:2	0.001	0.346	0.230	0.054	0.034	0.663
Leucine	0.001	133.700	81.838	27.483	8.020	0.612
Sphingomyelin C18:0	0.001	12.741	9.193	1.718	0.951	0.722
Phosphatidyl-choline aa C38:6	0.001	3.597	2.626	0.489	0.227	0.730
Asparagine	0.001	56.621	34.464	8.592	8.907	0.609
Phosphatidyl-choline ae C38:3	0.001	11.560	6.720	2.224	1.541	0.581
Lysophosphatidyl-choline a C28:1	0.001	1.310	0.869	0.158	0.189	0.664
Phosphatidyl-choline ae C32:2	0.001	11.395	6.825	2.012	1.601	0.599
Ceramide (d18:2/16:0)	0.001	0.041	0.029	0.006	0.004	0.705
Phosphatidyl-choline ae C30:1	0.002	23.765	12.998	4.747	3.857	0.547
1-Methylhistidine	0.002	4.987	3.238	0.782	0.639	0.649
Phosphatidyl-choline aa C38:4	0.002	38.575	28.978	4.783	2.797	0.751
Phosphatidyl-choline ae C44:3	0.002	0.122	0.077	0.022	0.014	0.633
Triacylglyceride (18:0_36:5)	0.002	0.237	0.158	0.039	0.025	0.663
Phosphatidyl-choline ae C34:0	0.002	6.434	4.076	0.941	1.027	0.634
Triacylglyceride (18:1_38:5)	0.002	0.793	0.497	0.155	0.081	0.627
Phosphatidyl-choline aa C42:2	0.002	0.226	0.141	0.043	0.027	0.623
Cholesteryl ester 17:0	0.002	10.822	6.563	1.818	1.757	0.606
alpha-Amino-butyric acid	0.002	22.860	15.762	2.313	3.582	0.690
Phosphatidyl-choline ae C42:1	0.002	0.247	0.170	0.042	0.020	0.686
Cholesteryl ester 14:0	0.002	19.192	13.173	2.953	2.131	0.686
Phosphatidyl-choline aa C38:3	0.002	54.458	36.111	7.402	8.342	0.663
Cholic Acid	0.002	23.486	10.384	7.657	2.287	0.442
Phosphatidyl-choline aa C34:4	0.002	12.485	6.683	2.695	2.300	0.535
Phosphatidyl-choline ae C42:3	0.002	0.447	0.271	0.078	0.074	0.607
Triacylglyceride (18:1_36:5)	0.002	0.289	0.183	0.048	0.043	0.635
Trihexosylceramide (d18:1/16:0)	0.003	0.709	0.486	0.122	0.061	0.686
Phosphatidyl-choline ae C38:2	0.003	8.105	5.232	1.239	1.250	0.646
Ceramide (d18:1/18:0)	0.003	0.096	0.069	0.016	0.004	0.723
Cholesteryl ester 17:1	0.003	49.152	34.194	5.164	7.597	0.696
Tetradecenoyl-carnitine	0.003	0.059	0.037	0.011	0.009	0.620
Phosphatidyl-choline ae C40:4	0.003	2.101	1.435	0.381	0.156	0.683
Lysophosphatidyl-choline a C24:0	0.003	0.157	0.093	0.024	0.032	0.593
Phosphatidyl-choline ae C40:3	0.003	1.782	1.256	0.241	0.227	0.705
Phosphatidyl-choline ae C34:1	0.003	19.576	13.502	3.150	2.219	0.690
Cholesteryl ester 18:3	0.004	828.111	520.620	111.951	169.915	0.629

Cholesteryl ester 20:5	0.004	113.959	85.918	14.916	11.084	0.754
Deoxycholic acid	0.004	1.234	0.590	0.369	0.225	0.478
Lysophosphatidyl-choline a C28:0	0.004	1.242	0.710	0.258	0.247	0.572
Hexosylceramide (d18:1/18:1)	0.005	0.087	0.062	0.013	0.011	0.718
Hexosylceramide (d18:1/16:0)	0.005	0.548	0.384	0.084	0.076	0.700
Lysophosphatidyl-choline a C26:0	0.005	0.452	0.234	0.103	0.109	0.519
Lysophosphatidyl-choline a C14:0	0.005	6.196	4.225	0.771	1.022	0.682
Lysophosphatidyl-choline a C18:2	0.005	30.289	17.483	3.337	8.246	0.577
Lysine	0.006	95.845	60.046	20.460	14.182	0.626
Cholesteryl ester 16:0	0.006	116.640	75.667	24.068	15.271	0.649
Ceramide (d18:1/16:0)	0.006	0.251	0.196	0.026	0.027	0.782
Phosphatidyl-choline aa C32:3	0.006	54.400	31.783	11.972	10.242	0.584
Taurine	0.006	39.863	23.257	9.508	6.864	0.583
Phosphatidyl-choline aa C28:1	0.006	3.381	2.467	0.529	0.376	0.730
Hexosylceramide (d18:1/24:1)	0.008	1.565	1.091	0.301	0.176	0.697
Dihexosylceramide (d18:1/14:0)	0.008	0.145	0.090	0.015	0.038	0.619
Hippuric acid	0.008	152.096	104.654	23.299	26.552	0.688
Trihexosylceramide (d18:1/20:0)	0.008	0.027	0.013	0.009	0.002	0.474
Phosphatidyl-choline ae C30:2	0.009	0.878	0.580	0.175	0.142	0.660
Kynurenine	0.009	8.096	4.615	2.298	1.298	0.570
Phosphatidyl-choline aa C40:2	0.009	0.371	0.213	0.089	0.081	0.573
Glycine	0.010	370.503	273.606	38.688	63.519	0.738
Phosphatidyl-choline ae C40:6	0.010	1.035	0.782	0.175	0.085	0.755
Cysteine	0.010	34.457	27.298	5.101	2.068	0.792
Cholesteryl ester 20:0	0.010	3.381	2.274	0.612	0.593	0.673
Lysophosphatidyl-choline a C20:3	0.010	3.209	1.863	0.467	0.928	0.581
Lysophosphatidyl-choline a C26:1	0.010	0.229	0.122	0.056	0.062	0.533
Cholesteryl ester 16:1	0.010	59.059	44.213	7.523	8.771	0.749
Phosphatidyl-choline aa C42:0	0.012	0.079	0.057	0.015	0.006	0.724
Phosphatidyl-choline aa C32:1	0.012	8.221	6.010	1.285	1.236	0.731
Phosphatidyl-choline aa C42:1	0.012	0.110	0.080	0.019	0.014	0.730
Methionine	0.013	33.171	20.287	9.333	4.559	0.612
Triacylglyceride (18:1_38:6)	0.013	0.381	0.267	0.062	0.068	0.703
beta-Alanine	0.013	2.804	2.035	0.323	0.535	0.726
Phosphatidyl-choline aa C32:0	0.013	8.779	6.316	1.392	1.445	0.719
Dihexosylceramide (d18:1/18:0)	0.014	0.200	0.133	0.046	0.031	0.664
Phosphatidyl-choline aa C32:2	0.014	17.098	12.126	2.901	2.923	0.709
Cholesteryl ester 20:1	0.016	1.446	1.103	0.207	0.202	0.763

Cholesteryl ester 18:0	0.016	14.336	6.929	5.685	2.615	0.483
Hexosylceramide (d18:1/23:0)	0.016	0.508	0.367	0.093	0.074	0.723
Ceramide (d18:2/24:0)	0.017	0.025	0.018	0.005	0.003	0.727
Cholesteryl ester 15:1	0.017	0.505	0.337	0.101	0.103	0.667
Ceramide (d18:2/18:0)	0.018	0.029	0.023	0.004	0.005	0.774
Triacylglyceride (20:2_36:5)	0.018	0.029	0.019	0.007	0.005	0.655
Phosphatidyl-choline ae C40:1	0.018	0.699	0.458	0.136	0.160	0.655
Methionine-Sulfoxide	0.019	3.021	1.834	0.700	0.766	0.607
Lysophosphatidyl-choline a C17:0	0.019	3.868	2.748	0.519	0.831	0.711
Triacylglyceride (20:3_36:3)	0.020	0.043	0.029	0.009	0.007	0.677
Isoleucine	0.020	140.436	105.229	18.171	25.272	0.749
Asymmetric dimethylarginine	0.020	0.788	0.610	0.141	0.070	0.774
Triacylglyceride (20:3_36:5)	0.021	0.098	0.057	0.034	0.012	0.589
Phosphatidyl-choline ae C36:1	0.021	29.548	22.343	4.536	4.626	0.756
Phosphatidyl-choline aa C38:5	0.024	20.120	16.218	1.769	3.125	0.806
Histidine	0.024	59.019	33.959	19.299	12.670	0.575
Lysophosphatidyl-choline a C16:1	0.025	1.529	1.072	0.187	0.382	0.702
Hexosylceramide (d18:1/26:1)	0.026	0.075	0.053	0.016	0.013	0.710
Triacylglyceride (18:0_38:6)	0.027	0.320	0.242	0.047	0.057	0.756
Creatinine	0.027	70.420	53.507	15.536	3.994	0.760
Phosphatidyl-choline aa C42:4	0.028	0.282	0.186	0.050	0.076	0.661
Cholesteryl ester 20:4	0.028	69.132	53.186	11.686	9.675	0.769
Trihexosylceramide (d18:1/22:0)	0.029	0.093	0.065	0.025	0.010	0.699
Citrulline	0.029	64.139	50.152	12.767	4.371	0.782
Phosphatidyl-choline aa C40:3	0.030	7.705	4.510	1.936	2.414	0.585
3-Methylhistidine	0.032	4.012	2.814	1.021	0.586	0.702
Cholesteryl ester 18:1	0.035	82.740	63.432	13.411	13.964	0.767
Triacylglyceride (18:1_35:3)	0.036	0.152	0.106	0.042	0.020	0.698
alpha-Aminoadipic acid	0.037	3.471	2.497	0.913	0.386	0.719
trans-4-Hydroxyproline	0.038	12.830	9.200	3.222	1.856	0.717
Lysophosphatidyl-choline a C16:0	0.041	21.050	15.784	2.066	5.081	0.750
Phosphatidyl-choline aa C34:1	0.041	66.061	54.456	9.805	7.091	0.824
Sphingomyelin C24:0	0.041	28.967	20.277	6.479	6.356	0.700
Triacylglyceride (20:4_36:3)	0.044	0.070	0.043	0.027	0.009	0.615
Lysophosphatidyl-choline a C18:0	0.044	25.032	19.019	2.280	5.990	0.760
Hexosylceramide (d18:1/18:0)	0.045	0.124	0.093	0.029	0.014	0.756
Phosphatidyl-choline aa C40:4	0.048	11.023	8.351	1.739	2.321	0.758
Phosphatidyl-choline aa C36:6	0.049	4.002	3.331	0.570	0.463	0.832

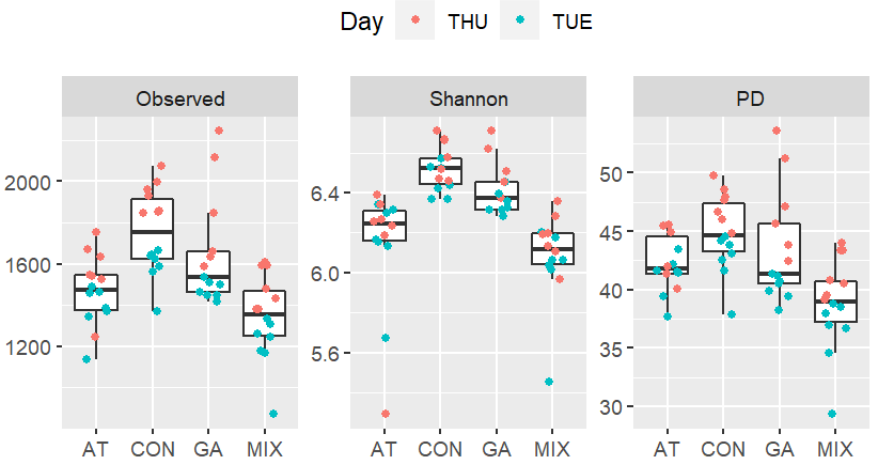
[1]This table shows the metabolites whose *P*-value < 0.05.

[2]Fold change = average (AT) / average (CON).

Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet.

For the rumen metataxonomic analysis, Figure 11 displays the alpha-diversity across treatments throughout the experimental period. The AT treatment significantly decreased alpha-diversity indexes compared to the CON treatment, and the MIX treatment significantly decreased alpha-diversity indexes compared to the GA treatment. Figure 12 displays the beta-diversity across treatments and sampling day throughout the experimental period. There was a significant difference between different treatments.

Figure 12 Alpha-diversity among treatments during the experimental period.



The *P*-values for observed ASV, Shannon, and PD were all < 0.0001, respectively. Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; TUE = Tuesday; THU = Thursday.

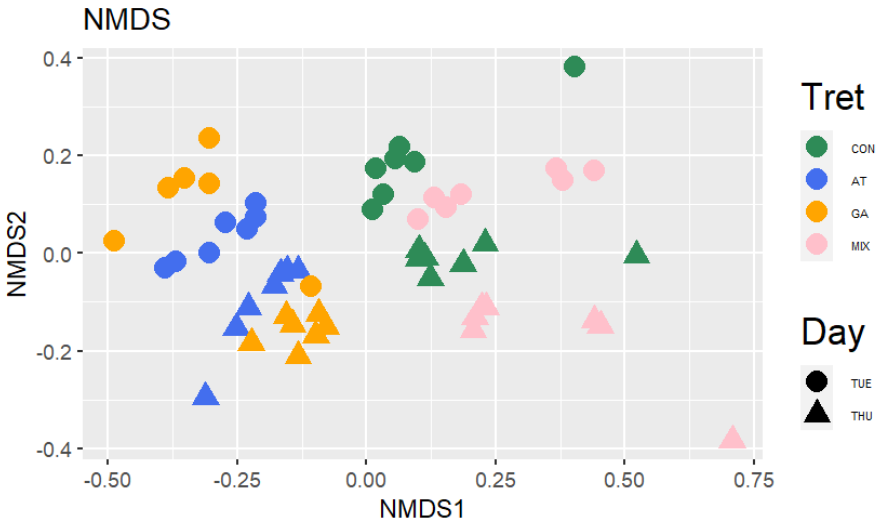


Figure 13 Beta-diversity among treatments during the experimental period.

P-value = 0.0001 for treatments, *P*-vaule = 0.006 for CON vs GA, *P*-value = 0.001 for CON vs MIX, *P*-value = 0.001 for CON vs AT, *P*-value = 0.001 for GA vs MIX, *P*-value = 0.013 for MIX vs AT. Abbreviations: CON = basal diet; AT = 0.25% *A. armata* in the basal diet; GA = 0.8% gallic acid in the basal diet; MIX = 0.25% *A. armata* and 0.8% gallic acid in the basal diet; Tret = treatment; TUE = Tuesday; THU = Thursday; NMDS = non-metric multidimensional scaling.

4 Discussion

Methanogenesis inhibition in the rumen results in dihydrogen accumulation. If we can capture this excessive dihydrogen use feed additives and convert it into nutrients such as VFA for the host animal, we could potentially improve animal production. In our previous *in vitro* experiments (Romero et at., 2023; Chapter 2), we tested 7 selected phenolic compounds as hydrogen acceptors and found that gallic acid and phloroglucinol were the most promising candidates (Romero et at., 2023; Chapter 2). In this study, we tested gallic acid as a hydrogen acceptor when methanogenesis was inhibited by *A. armata in vivo.* We chose gallic acid because it is the subunit of hydrolysable tannins which are present in common feedstuff, and it also in the feed additive list; *A. armata* is kinds of red seaweeds, which are potent methanogenesis inhibitors. We chose *A. armata* over *A. taxiformis* because our supplier was only providing *A. armata* at that time.

The AT group decreased methane yield by 25%, and increased dihydrogen emission (g/d) fourfold, compared to the CON group. These results suggest that *A. armata* is a promising model for inhibiting methanogenesis in dairy cows. Previous research has shown that the antimethanogenic activity of *A. taxiformis* was due to halogenated compounds, primarily bromoform (Machado et al., 2016). In this study, the concentration of bromoform in the *A. armata* was 15.5 mg/g DM, which is much higher than the concentration in reported in *Asparagopsis* by other researchers (Kinley et al., 2020; Roque et al., 2021). The high concentration of bromoform in *A. armata* likely accounts for the decrease methane yield by 25% observed in our study at a low inclusion level of

0.25% of *A. armata*. It should be noted that we added *A. armata* in the pellet, and the pelleting process may have resulted in bromoform loss, as bromoform is known to be unstable (Vucko et al., 2017). From week 5 to week 7, both the pelleting process and days of storage were identified as factors contributing to the decrease in bromoform. From week 7 to week 11, days of storage was the only factor contributing to the decrease in bromoform, and we found that days of storage associated with a linear decrease in bromoform, consistent with previous study (Stefenoni et al., 2021). According to the data from week 7 to week 11, we were able to calculate the rate of bromoform decrease by days of storage (Figure 10). Accordingly, we estimated that about 18% of the bromoform decrease observed from week 5 to week 7 was attributable to pelleting.

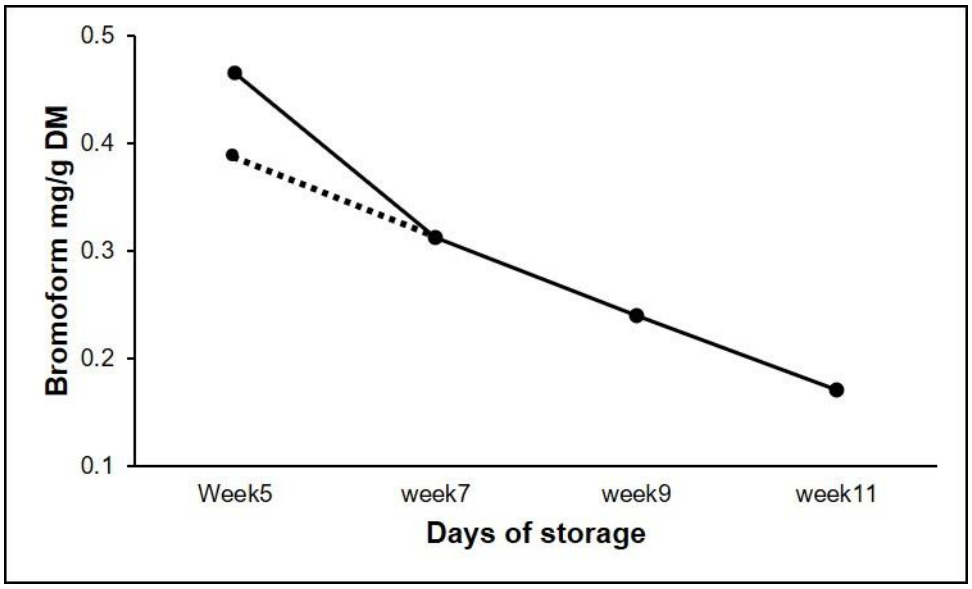


Figure 14 Effect of storage time and pelleting on concentration of bromoform (CHBr3) in 3% *A. armata* containing pellet. Pellet was made in week 5 and the bromoform concentration in week 5 (solid line) was calculated based on the inclusion level of *A. armata* in the pellet (3%), the bromoform concentration in the *A. armata* (15.5 mg/g DM), and assumed that there is no bromoform loss during pelleting. The bromoform concentration in week 5 (dashed line) was based on the linear relationship from week 7 to week 11.

In the MIX group, methane yield decreased by 34%, while in the AT group, methane yields only decreased by 25%, compared to the CON group. Thus, more dihydrogen emission (g/kg DMI) is expected in the MIX group, compared to the AT group. However, dihydrogen emission (g/kg DMI) in the AT group and MIX group were similar (0.22 vs. 0.26). Although dihydrogen emission (g/kg DMI) in the MIX group did not decrease, compared to AT group, it is possible that gallic acid still acts as a hydrogen acceptor due to the expected higher dihydrogen production the MIX group. The reason for the lower methane yield in the MIX group is not fully understood, particularly considering that both the AT and MIX treatments had the same concentrate intake (5.0 vs 5.0) and similar total feed intake (19.7 vs 20.3). Our previous work (Chapter 2) reported that phloroglucinol decreased methane production by decreasing the abundance of methanogens. Certainly, gallic acid can be converted into phloroglucinol in the rumen (McSweeney et al., 2001). However, methane yield was not decreased (P = 0.1389) by gallic acid in this study. Thus, the substantial decrease in methane yield observed in the MIX group cannot be attributed to gallic acid when compared to the AT group. Interestingly, the effect of gallic acid on methane yield contradicts findings from a previous publication. Aboagye et al. (2019) reported that gallic acid decreased methane yield, although it did not decrease dissolved dihydrogen concentration in the rumen fluid. The disparity in results between these two studies could be due

to the difference in gallic acid inclusion level, with 0.8% used in the current study and 1.5% in the study of Aboagye et al (2019).

A. armata decreased total VFA concentration is likely attributed to the reduced DMI. *A. armata* decreased acetate proportion, and increased propionate, butyrate, and valerate proportions. This is consistent with the fact that propionate, butyrate, and valerate production consumes dihydrogen compared to acetate production (Marty and Demeyer, 1973; Ungerfeld, 2020). Additionally, *A. Armata* led to an increase in isobutyrate, isovalerate, and caproate proportion, although only the increased proportion of isovalerate was reported (canola oil steeped with *A. Armata* with and without *A. Armata* biomass) (Alvarez-Hess et al., 2023).

In the rumen, gallic acid is supposed to be degraded to acetate and/or butyrate (Bhat et al., 1998; Conradt et al., 2016). However, the proportions of acetate and butyrate did not change by gallic acid supplementation. Our previous *in vitro* study reported that 6 mM gallic acid increased acetate proportion when methane production was inhibited by BES (Chapter 2), and an *in vivo* study reported that 1% gallic acid increased both total VFA concentration and butyrate proportion in preweaning dairy calves (Xu et al., 2022). Nevertheless, our results were consistent with the findings of Aboagye et al. (2019), who reported that acetate and butyrate proportions did not increase with 1.5% gallic acid, despite observing an increase in total VFA concentration in their study.

A. armata resulted in decreased forage intake, concentrate intake, and total feed intake. Several studies have reported a decrease in DMI with *A. taxiformis* supplementation (Muizelaar et al., 2021; Roque et al., 2021; Stefenoni et al., 2021). It has been speculated that the decrease in DMI might be attributed to the palatability of *A. taxiformis,* particularly due to its high ash content (Roque et al., 2019). However, it is worth noting that *A. taxiformis* also decreased forage intake when it was only mixed with concentrate and when forage and concentrate were offered separately, as observed in the current study and in another study (Alvarez-Hess et al., 2023). While some studies have reported no decrease in DMI with *A. taxiformis* supplementation (Li et al., 2016; Kinley et al., 2020).

Alvarez-Hess et al. (2023) speculated that the discrepancy in DMI response could be due to differences in the adaptation period. They suggested that a longer adaptation period, up to 30

days, may be necessary to minimize any potential negative effects on DMI with *A. taxiformis* supplementation. However, the studies reporting decreased DMI with *Asparagopsis* did include adaptation periods, such as 21 days in the study by Stefenoni et al. (2021), 13 days in the study by Nyløy et al. (2023), and 14 days in the present study. Additionally, *A. taxiformis* was found to affect cows' eating behaviors. Cows in the *A. taxiformis* group exhibited sorting behavior and showed increased rumination time, chewing time, and chew counts, as reported by Nyløy et al. (2023). Thus, it is likely that other factors contribute to the decrease in DMI observed with *Asparagopsis*. For example, halogenated compounds, primarily bromoform, present in *Asparagopsis* could be associated with the reduced DMI as these compounds are volatile and have a strong flavor. Studies have reported that canola oil containing bromoform decreased concentrate intake compared to the canola oil group, and there was no difference in concentrate intake between canola oil containing bromoform and canola oil containing both bromoform and *A. armata* biomass treatments (Alvarez-Hess et al., 2023). Notably, forage, concentrate, and total feed intake were not affected by gallic acid, which was consistent with previous findings (Aboagye et al., 2019).

The milk yield in the AT group showed a decrease, likely due to the lower feed intake observed in this group. This reduction in milk yield, accompanied by a decrease in milk fat and milk true protein concentrations, resulted in a decrease in milk fat, milk true protein, and milk lactose production in the AT group. These findings align with the study conducted by Stefenoni et al. (2021) using a 0.5% *A. taxiformis*. However, Stefenoni et al. (2021) did not observe any significant effects on milk fat and milk true protein concentrations, although they reported a decrease in milk lactose concentration in the 0.5% *A. taxiformis* group. Similar results were reported by Alvarez-Hess et al. (2023), who also observed a decrease in milk component production with *A. armata* supplementation.

In the present study, gallic acid supplementation slightly increased milk yield, and there was a positive interaction effect of gallic acid and *A. Armata* on milk production. Gallic acid mitigated the negative effect of *A. Armata* on milk production, in the MIX group, milk production increased by 9%, compared to the AT group.

Gallic acid supplementation resulted in a decrease in MUN concentration, while it had no

significant effect on ruminal ammonia concentration (Table 9) or plasma urea concentration (Table 12). To the best of our knowledge, studies investigating the effects of gallic acid on lactating dairy cows are scarce, therefore we are unable to directly compare our findings to those of other researchers. Bromoform concentration in the milk were below the limit of detection (0.001 mg/g) in the CON, AT, and MIX groups. Although bromoform has been detected in other studies, no significant differences were observed between the control and the *Asparagopsis* supplementation groups (Roque et al., 2019, Stefenoni et al., 2021). In study where bromoform concentration in milk was significantly increased with *Asparagopsis* supplementation, the concentration remained well below the safety limit (Alvarez-Hess et al., 2023).

Enzymes such as ALT, AST, ALP, and GGT are primarily found in the liver and are used as indicators to access liver cell damage. In our study, there was a noticeable trend towards a decrease (P = 0.0510) in ALT concentration in the plasmas with *A. Armata* supplementation, which was in line with Stefenoni et al. (2021) study. They also reported a decrease in ALT concentration in the plasma with 0.5% *A. taxiformis* supplementation. However, the specific mechanism behind the decrease in ALT concentration with *Asparagopsis supplementation* was not elucidated in either study. Furthermore, gallic acid supplementation resulted in a decrease in GGT (P = 0.0288) and ALT (P = 0.0008) concentration. This may be attributed to the antioxidant properties of gallic acid, as suggested in previous research by Xu et al. (2022). The exact mechanism by which gallic acid influences these enzyme concentrations requires further investigation.

The metabolomic analysis revealed the alterations in the blood metabolites of the AT treatment when compared to the CON treatment. Specifically, amino acids, amino acid-related, and lipid-related metabolites concentrations were higher in the CON treatments. This could be explained by less concentrate and less forage intake in the AT treatment. Less nutrients intake might decrease their concentration in the blood; however, this result is inconsistent with a previous publication (Guo et al., 2019). Guo et al. (2019) reported that feed intake restriction increased lipid related metabolites and some amino acid concentrations due to the energy negative balance. However, in our study the cows in the AT treatments were not in energy negative balance status because the B-OH and NEFA concentrations were not increased.

Previous studies suggested that *Asparagopsis* supplementation induce rumen inflammation (Li et al., 2016; Muizelaar et al., 2021). Although we could not collect rumen tissue samples in this study, there were metabolites measured in the blood that are known inflammation indicators. For example, dihexosylceramides whose concentration was reported to increase with inflammation (Mousa et al., 2019). However, in our study, we did not observe an increase in blood dihexosylceramide concentrations in cows subjected to the AT treatment. The effect of *Asparagopsis* on rumen inflammation needs more exploration. Currently, we are analyzing the pathways of the differential metabolites involved, anticipating that this exploration will yield further insights.

The AT and MIX treatments decreased microbial alpha diversity compared to CON treatment and also changed the beta-diversity. These results are in line with our *in vitro* study (Chapter 3). However, the results of the current study are inconsistent with those reported by Belanche et al. (2016), who found that seaweeds did not affect the biodiversity indices of rumen microbes. The discrepancy between the two studies may arise from the utilization of different classes of seaweed; while red seaweed was employed in the present study, Belanche et al. (2016) used brown seaweeds. We are currently further analyzing the metataxonomic, metagenomic, and metatranscriptomic data. In subsequent analysis, we will integrate this sequencing data with the data of rumen fermentation, gas emissions, and animal performance. These comprehensive approach aims to deeper our understanding of the effects of *A. armata* and gallic acid, both individually and in combination, on rumen microbiota and animal physiology.

5 Conclusion

Inclusion of 0.25% *A. Armata* in the diet of lactating dairy cows decreased methane yield and correspondingly increased dihydrogen emission. However, this inclusion level of *A. Armata* also decreased DMI, milk yield, and milk component production. Although there was no interaction effect of gallic acid and *A. armata* on dihydrogen and methane emission or acetate and butyrate proportion, they have a positive interaction effect on milk yield.

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CHAPTER 5

General discussion and conclusions

Methane emissions from the ruminant sector not only contribute to the greenhouse effect but also represent a loss of digestible feed energy. By using feed additives, it's now feasible to reduce methane production by 25% or more (Morgavi et al., 2023). Theoretically, the feed energy not wasted by methane production could be used to improve animal production; however, milk production was not increased when methane production was inhibited in dairy cows (Ungerfeld, 2018). Improving milk production is important when using methanogenesis inhibitors. This is because it could motivate farmers to purchase and implement methanogenesis inhibitors in their practice, leading to reduced greenhouse emissions from the ruminant sector. Also, it could help to improve feed utilization and address the challenges posed by a growing global population.

Methane production plays a crucial role as a primary dihydrogen sink in the rumen. Simultaneously, other hydrogenotrophic pathways, including propionate production, are naturally present (Ungerfeld, 2020). When methanogenesis is inhibited, dihydrogen released in the rumen increases. However, propionate production only utilizes a fraction of this excess dihydrogen, with a small amount being expelled by the animal (van Gastelen et al., 2020). In light of this, we hypothesized that supplementation with a hydrogen acceptor could enhance the capacity of other dihydrogen consuming pathways. This would allow to process the surplus dihydrogen more efficiently, converting it into valuable nutrients like VFA. Such a conversion could enhance rumen fermentation, providing additional energy to the host animal and thereby potentially boosting animal production.

In this thesis, we investigated the potential of selected phenolic compounds, some identified from previous publications (Tsai and Jones, 1975, Tsai et al., 1976, Krumholz and Bryant, 1986, Krumholz et al., 1987), and others based on their chemical structure, to act as hydrogen acceptors *in vitro*. Subsequently, based on our *in vitro* results, we selected an optimal phenolic compound from these candidates and evaluated its efficacy in capturing dihydrogen and

enhancing milk production in dairy cows.

The *in vitro* findings showed that gallic acid and phloroglucinol were promising hydrogen acceptors, whereas the *in vivo* experiment did not yield the same conclusion. Gallic acid or phloroglucinol degradation using dihydrogen produces VFA and carbon dioxide by rumen microbes in pure culture (Krumholz et al., 1987). In the *in vitro* experiment, 6 mM gallic acid increased the proportion of acetate by 23%, 6 mM phloroglucinol increased the proportion of acetate by 41%, and both gallic acid and phloroglucinol increased TGP by 8% when methanogenesis was inhibited by using 2.5% *A. taxiformis*. Moreover, in the long-term incubation, 36 mM phloroglucinol alone increased the proportion of acetate by 29% and butyrate by 58%, compared to the control treatment. Additionally, when combined with BES, 36 mM phloroglucinol increased the proportion of acetate by 42%, butyrate by 22%, and TGP by 16%, compared to BES treatment. However, in the *in vivo* experiment, 0.8% gallic acid did not affect the proportion of acetate or butyrate, and there were no interaction effects of gallic acid with methanogenesis inhibitor *A. armata* on VFA profile.

In the *in vitro* experiment, the results of gas emissions also suggested that phloroglucinol worked as hydrogen acceptor, while the results of gas emissions did not lead to the same conclusion for the *in vivo* experiment. Specifically, in the *in vitro* experiment, after adaptation, 36 mM phloroglucinol combined with BES decreased dihydrogen accumulation by 72%. This combination further decreased methane production, accompanied by a decrease in the abundance of methanogens in the sequential incubation. However, in the *in vivo* experiment, 0.8% gallic acid did not affect dihydrogen or methane emissions. Also, there were no interaction effects of gallic acid combined with *A. armata* on dihydrogen or methane emissions. The discrepancies observed between the *in vitro* and *in vivo* experiments could be attributed to the difference in the inclusion levels of phenolic compounds used in each study and the inherent variations between the two experimental methods.

In the *in vitro* experiment, the inclusion level of gallic acid was 6 mM, equivalent to 1.7% based on DM, while the *in vivo* experiment utilized gallic acid at a level of approximately 0.8% based on DM. From another perspective, 0.8% gallic acid inclusion level *in vivo* equivalent to 7 mM in the rumen fluid, assuming a total of 85 L of rumen volume and 15% dry matter content.

However, it's important to recognize that the concentration of gallic acid in rumen fluid can be influenced by factors such as feed intake and passage rate. Notwithstanding, the inclusion levels *in vitro* and *in vivo* are difficult to compare because the systems, among other differences, have contrasting liquid to solid ratios. The inclusion methods for gallic acid and methanogenesis inhibitor *in vitro* and *in vivo* were also different. We added gallic acid and *A. taxiformis* directly into the fermentation bottle, while we added gallic acid and *A. armata* in the pellet. Moreover, the substrates for *in vitro* and the diet for *in vivo* were different. The *in vitro* experiment used 70% alfalfa hay as forage and 30% barley grain, while the *in vivo* experiment used around 75% mixed forage and 25% concentrate pellet (Table 7, Chapter 4).

Regarding VFA parameters, distinctions exist between the two experimental settings. In the *in vitro* experiment, VFA profiles reflect the production of different individual VFAs. On the other hand, in the *in vivo* experiment, the VFA profiles reflect the production and absorption by the rumen wall of different individual VFA (Dijkstra, 1994). Also, factors such as the rumen microbiota, and pressure in rumen and the bottle headspace are also a source of variation between the in vitro and *in vivo* experiments.

The methods used to measure dihydrogen and methane between in vitro and *in vivo* experiments were different. In the *in vitro* experiment, we used micro GC to measure dihydrogen and methane, while in the *in vivo* experiment, we used Greenfeed to measure dihydrogen and methane. The limit of quantitation for the micro GC is lower than 1 ppm (Micro GC Fusion, INFICON), while the limit of quantitation for the Greenfeed is 20 ppm for methane and lower than 1 ppm for dihydrogen (Hristov et al., 2015; C-Lock, 2023). Also, the *in vitro* experiment measured the concentration of dihydrogen and methane in the accumulated gas, conversely, the *in vivo* experiment measured the concentration of dihydrogen and methane in the breath of cow. During the *in vivo* experiment, cows were allowed to use the Greenfeed 6 times maximum per day with at least 4 hours between the consecutive visits.

We observed that 36 mM phloroglucinol supplementation increased in the abundance of bacteria potentially involved in phenolic compound degradation in cow and goat's sequential incubation experiments. These findings are in line with our fermentation results, phloroglucinol supplementation increased acetate proportion and total VFA production. In the *in vivo* experiment,

we collected rumen fluid samples for microbiota DNA and RNA sequencing. The sequencing data are currently undergoing metataxonomic, metagenomic, and metatranscriptomic analyses. The comprehensive omics data will provide insights into the response of the rumen microbiota to gallic acid and *A. armata* supplementation and the divergent results obtained from the *in vitro* and *in vivo* experiments. In this thesis, we only reported the preliminary results of the metataxonomic analysis.

While the role of gallic acid as a hydrogen acceptor in the *in vivo* experiment remains unknown, we observed that gallic acid supplementation alleviated the negative effect of *A. armata* on milk production. Moreover, there was a positive interaction effect between gallic acid and *A. armata* on milk production, given the conditions in this study. For future research, we recommend considering the following aspects:

- **Using different methods to measure dihydrogen**

As we discussed before, Greenfeed system measures methane and dihydrogen through spot sampling. Other methane and dihydrogen measurement devices such as respiration chambers might be more adequate. Respiration chambers continuously measure methane and dihydrogen emissions over a 24-hour period, accurately reflecting the emissions within a daily cycle. Nevertheless, the respiration chamber is costly, demands a significant workload, and has limitations regarding the number of animals used in the trial. The amount of dihydrogen erupted from rumen accounts for only 23% of the stoichiometric amount involved with the decrease in methane production (van Gastelen et al., 2020). The metabolic pathways of the remaining 77% dihydrogen are not fully understood. Research indicated that inhibiting methanogenesis leads to increases in dissolved dihydrogen (Janssen, 2010), propionate proportion (Janssen, 2010), formate concentration (Ungerfeld, 2015), and lactate concentration (Ungerfeld, 2015). Therefore, it is likely that these dihydrogen “metabolic pathways” could also be affected by supplementation of an external hydrogen acceptor. And we suggest to measure these parameters in future studies where feasible.

- **Using different methanogenesis inhibitor**

In our *in vivo* study, we employed *A. armata* as a methanogenesis inhibitor. We observed a 25% reduction in methane yield and a 200% increase in dihydrogen yield in the AT treatment

compared to the CON treatment. These findings suggest that *A. armata* effectively inhibited methanogenesis, corroborating the results of a previous study by Roque et al. (2019). We also found that gallic acid combined with *A. armata* had positive interaction effect on milk production. However, the effect of external hydrogen acceptors combined with other methanogenesis inhibitors on gas emissions and animal performance is not well known. Therefore, we suggest to test the effect of different methanogenesis inhibitor combined with hydrogen acceptor on milk production in future animal studies because this could accelerate the application of this technology. For example, 3-nitrooxypropanol was frequently reported that it could decrease methane production by around 30% without negative effects on DMI, feed digestion, animal production, and animal health (Thiel et al., 2019, van Gastelen et al., 2020). Moreover, this product is allowed to be used in many countries with large ruminant populations including the European Union and Brazil.

- **To test the effects of different inclusion levels of gallic acid supplementation on milk production**

Studies using gallic acid as feed additive are scarce. Xu et al. (2022) reported that gallic acid supplementation improved preweaning dairy calves daily weight gain, increased their rumen total VFA concentration, and improved their antioxidant ability. In line with this, Aboagye et al. (2019) demonstrated that gallic acid supplementation not only increased total VFA concentration but also reduced the proportion of urea nitrogen in urinary nitrogen and decreased methane yield in beef cattle. A similar decrease the proportion of urea nitrogen in urinary nitrogen in beef cattle following gallic acid supplementation was reported by Wei et al. (2016). In our *in vivo* experiment, we found that gallic acid alleviated the negative effect of *A. armata* on milk production. To the best of our knowledge, our study is the first to report that gallic acid had this positive effect. Given this initial finding, we believe further research is warranted to delve deeper into the impact of varying gallic acid inclusion levels on milk production and to elucidate the underlying mechanisms.

- **To test the effect of different inclusion levels of hydrogen acceptor combined with methanogenesis inhibitor on gas emissions and milk production**

Our *in vivo* experiment observed that gallic acid combined with *A. armata* had a positive interaction effect on milk production, given the conditions of this study. These results indicate that

using a methanogenesis inhibitor can improve animal production if combined with a hydrogen acceptor as feed additive. Nevertheless, there are several aspects for further research that we believe are crucial: 1) to find the optimal inclusion level of gallic acid to combine with methanogenesis inhibitor. Understanding the precise amounts can maximize the benefits while minimizing potential negative effects. 2) to study the diet effects on the interaction effect of gallic acid combined with *A. armata*. In our *in vivo* experiment, the forage to concentrate ratio is around 75 to 25. The interactions between gallic acid and *A. armata* could be influenced by the composition of the diet, especially since the efficacy of methanogenesis inhibitors like 3-nitrooxypropanol has been observed to vary with NDF concentration (Kebreab et al., 2023). 3) to study the methods used to add gallic acid and *A. armata* in the diet. In our *in vivo* experiment, we added gallic acid and *A. armata* in the concentrate, which was subsequently pelleted. Investigating different modes of inclusion, such as direct addition to the total mixed ration, is vital. The method of introduction might alter the interaction effect between gallic acid and *A. armata* on milk production.

- **To test different hydrogen acceptor**

Our *in vitro* experiment showed that gallic acid and phloroglucinol served as hydrogen acceptors, while we chose gallic acid for the *in vivo* experiment because it is in the feed additive list. However, we believe that phloroglucinol should also be tested *in vivo*. This belief is particularly strengthened by our findings in the sequential incubation experiment (Chapter 2), where phloroglucinol demonstrated a significant reduction in dihydrogen accumulation. Moreover, we could also test hydrolysable tannin-enriched by products as hydrogen acceptor due to hydrolysable tannin can be transferred into phenolic compound in the rumen.

In summary, our study provides evidence suggesting that both gallic acid and phloroglucinol have the potential to act as hydrogen acceptors within the rumen ecosystem. In the *in vitro* experiment, we observed that gallic acid and phloroglucinol acted as hydrogen acceptors, promoting alternative hydrogenotrophic pathways when methanogenesis was inhibited. Both gallic acid and phloroglucinol increased acetate proportion and total gas production. Importantly, after an adaptation phase, the supplementation of phloroglucinol with BES effectively reduced dihydrogen accumulation, methane production, and methanogen abundance. Furthermore, this

treatment increased proportions of acetate and butyrate. The combined supplementation of phloroglucinol or pyrogallol with a methanogenesis inhibitor also induced changes in the rumen microbiota of cows and goats, with an increase in the abundance of phenolic compound-degrading bacteria *in vitro*. In our *in vivo* experiment, although gallic acid had no effect and gallic acid combined with *A. armata* had no interaction effect on dihydrogen and methane emissions or VFA profiles, gallic acid supplementation alleviated the negative effect of *A. armata* on milk production, and gallic acid combined with *A. armata* had positive interaction effect on milk production. Our findings could accelerate the application of methanogenesis inhibitor in practice, aiming to decrease greenhouse gas emission from the ruminant sector. Also, they could help to address the challenge of world population growth by improving animal production.

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Appendix

1 Publications

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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 + methanogenesis 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

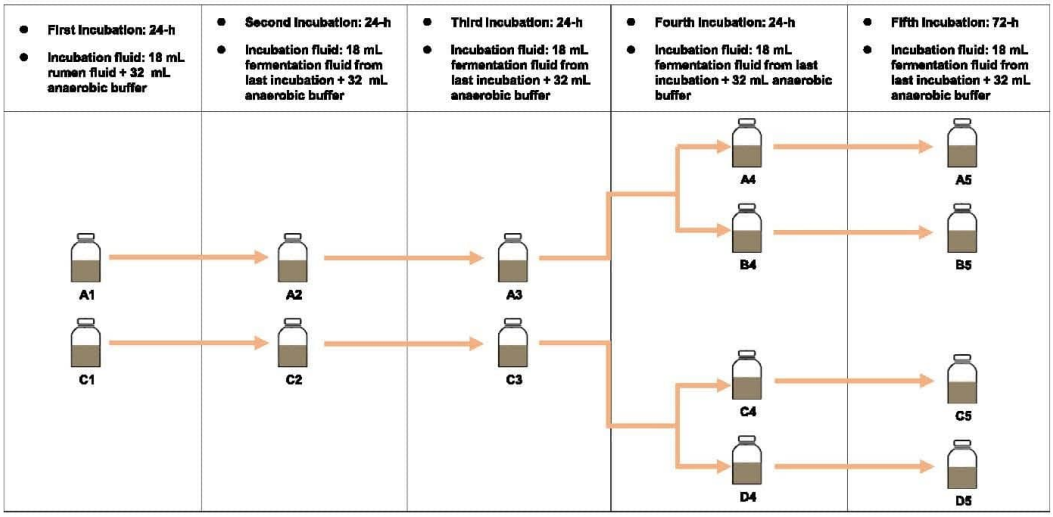

Fig. 1. Experimental design of the sequential batch incubation (Exp. 3) that used rumen fluid inocula from dairy cows (n = 8). Abbreviations: A = Substrate only, B = Substrate + 2-bromoethanesulfonic sodium, C = Substrate + phloroglucinol, D = Substrate + phloroglucinol + 2-bromoethanesulfonic sodium. 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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Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited *in vitro* – Part 2. Dairy goats

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

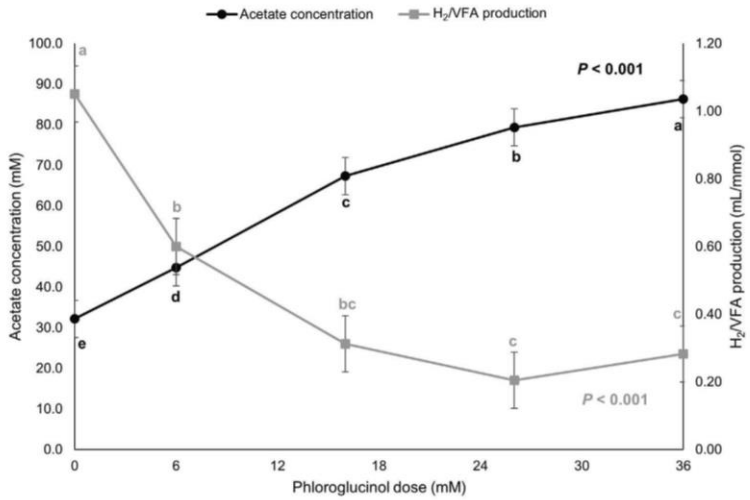

Fig. 1. Effect of phloroglucinol at different initial concentrations (0, 6, 16, 26, 36 mM) when combined with *A. taxiformis* at 2% DM on dihydrogen production per total volatile fatty acid production (mL/mmol) and acetate concentration (mM) after 5 days of incubation using rumen fluid from goats (Exp. 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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2 Poster presentation

EAAP 2023 – Lyon 2023 EAAP Annual Meeting

Gallic acid alleviates the negative effect of *Asparagopsis armata* on milk yield in dairy cows

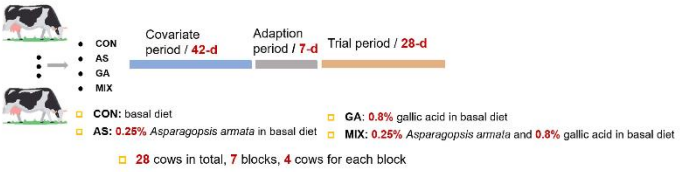
R. Huang[1], P. Romero[2], C. Martin[1], E.M. Ungerfeld[3], A. Demeter[4], A. Belanche[5], D.R. Yáñez-Ruiz[2], M. Popova[1], D. Morgavi[1]

[1]Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, Saint-Genès Champanelle, 63122, France
[2]Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, Granada, 18008, Spain
[3]Instituto de Investigaciones Agropecuarias INIA, Centro Regional de Investigación Carillanca, Vilcún, La Araucanía, 4880000, Chile
[4]Volta Greentech, AB Nanna Svartz väg 2-4, Solna, 17165, Sweden
[5]Universidad de Universidad de Zaragoza-CITA, Departamento de Producción Animal y Ciencia de los Alimentos, Miguel Servet 177, Zaragoza, 50013, Spain

INTRODUCTION

1. Enteric methane production can be decreased without negative effects on animal performance
2. However, feed energy not lost as methane is not used for production
3. H2 accumulates when methane production is inhibited
4. Gallic acid degradation by rumen microbes consumes H2 and produces acetate and butyrate
5. We **hypothesized** that gallic acid can improve animal production when methanogenesis is inhibited

MATERIALS AND METHODS

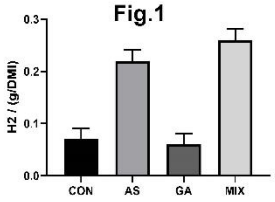


Parameters	Methods
CH4 and H2	Greenfeed
VFA	Gas chromatograph
Dry matter intake	Auto-feeder
Milk yield	Milking machine

RESULTS

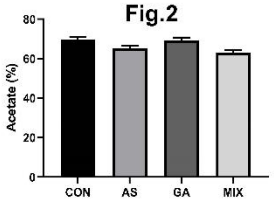
The effect of *Asparagopsis:* CON+GA **vs** AT+MIX
The effect of Gallic: CON+AT **vs** GA + MIX

Fig.1



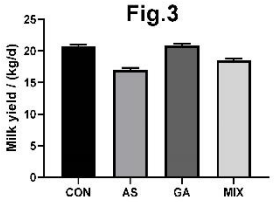
AS vs CON	200% ↑
MIX vs CON	300% ↑
Asparagopsis (*P*-value)	**<0.0001**
Gallic (*P*-value)	0.6974
AsparagopsisxGallic (*P*-value)	0.0851

Fig.2



Asparagopsis (*P*-value)	**0.0010**
Gallic (*P*-value)	0.3434
AsparagopsisxGallic (*P*-value)	0.5163

Fig.3



AS vs CON	18% ↓
MIX vs AS	9% ↑
Asparagopsis (*P*-value)	**<0.0001**
Gallic (*P*-value)	**0.0302**
AsparagopsisxGallic (*P*-value)	**0.0156**

- AS supplementation decreased methane yield (g/kg DM) by 25%, but it decreased DMI and milk yield (data not show)
- AS supplementation increased H2 emission, however, gallic acid supplementation did not decrease H2 emission (Fig.1) or increase acetate concentration (Fig.2)
- Gallic acid supplementation alleviated the negative effect of AS on milk production (Fig.3), and gallic acid with *Asparagopsis armata* had a positive interaction effect on milk production (Fig.3)

CONCLUSIONS

This study confirms the antimethanogenic effect of *A.armata* but also shows a negative effect on feed intake and milk yield. While gallic acid did not decrease dihydrogen loss, it partially alleviated the negative effect of *A. armata* on milk yield.

rongcai.huang@inrae.fr

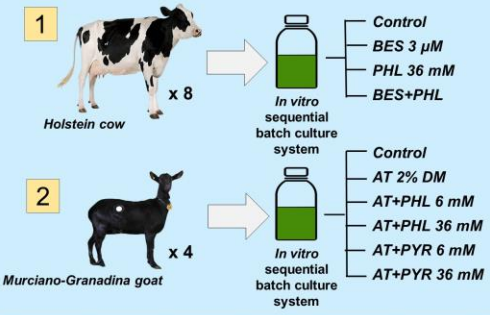
Phenolic compounds increased phenolic utilization bacteria abundance when methanogenesis was inhibited *in vitro*

R. Huang[1], P. Romero[2], E. Jiménez[2], A.I. Martín-García[2], P.E. Smith[3], E.M. Ungerfeld[4], M. Popova[1], A. Belanche[5], D. Morgavi[1], D.R. Yáñez-Ruiz[2]

[1]Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, Saint-Genès Champanelle, France; [2]Estación Experimental del Zaidín, CSIC, Granada, Spain; [3]Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Dunsany, Ireland; [4]Centro Regional de Investigación Carillanca, Instituto de Investigaciones Agropecuarias INIA, La Araucanía, Chile; [5]University of Zaragoza, Zaragoza, Spain.

Background

Two previous *in vitro* studies found that phloroglucinol (**PHL**) and pyrogallol (**PYR**) decreased rumen H_2 accumulation and increased volatile fatty acid production, in combination with a CH_4 inhibitor: 2-bromoethanesulfonate (**BES**) or *Asparagopsis taxiformis* (**AT**). The present work describes the effects of these combinations on the bacterial community when methanogenesis was inhibited with rumen inoculum from cows (1) and goats (2).



Methodology

Microbial DNA was extracted using DNeasy PowerSoil Pro Kit. The rumen fluid DNA was prepared for DNA library using Fluidigm amplification of the V3-V4 hypervariable region of 16s rRNA gene, using primers pairs MiSeq_F and MiSeq_R targeting both archaea and bacteria. Amplicons were sequenced on an Illumina MiSeq platform using a MiSeq 300-cycle sequencing kit. Downstream processing was performed using QIIME 2 Demultiplexing sequences were performed by Qiime demux plugin; the denoise, quality control, and amplicon sequence variants (ASVs) generation were performed by the DATA2 plugin, and the representative sequences from all ASVs were aligned against SILVA 138.1.

Results

Table 1. Alpha diversity metrics of ruminal bacteria from dairy cows.

	CTL	BES	PHL	BES+PHL	SEM	*P*-value
Observed ASVs	246a	224a	71b	83b	9.5	<0.0001
Shannon index	4.41a	4.37a	3.60b	3.48b	0.080	<0.0001
Simpson index	0.96	0.97	0.96	0.95	0.004	0.0375
Faith's Phyl. Div.	11.04a	10.48a	3.90b	3.85b	0.244	<0.0001

Table 2. Alpha diversity metrics of ruminal bacteria from dairy goats.

	CTL	AT	AT+PHL 6 mM	AT+PHL 36 mM	AT+PYR 6 mM	AT+PYR 36 mM	SEM	P-value
Observed ASVs	317a	315a	301a	120b	310a	129b	14.7	<0.0001
Shannon index	4.46a	4.47a	4.56a	3.79b	4.58a	3.40b	0.090	<0.0001
Simpson index	0.96a	0.97a	0.98a	0.96a	0.97a	0.90b	0.009	0.0005
Faith's Phyl. Div.	12.99a	11.95a	11.67a	6.18b	12.47a	6.28b	0.538	<0.0001

Fig 1. Beta diversity of ruminal bacteria from dairy cows (left) and dairy goats (right).

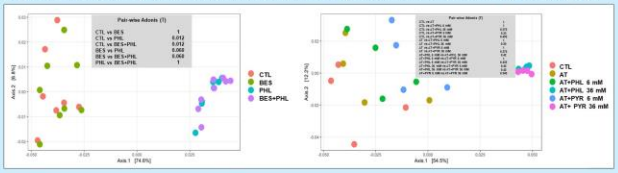
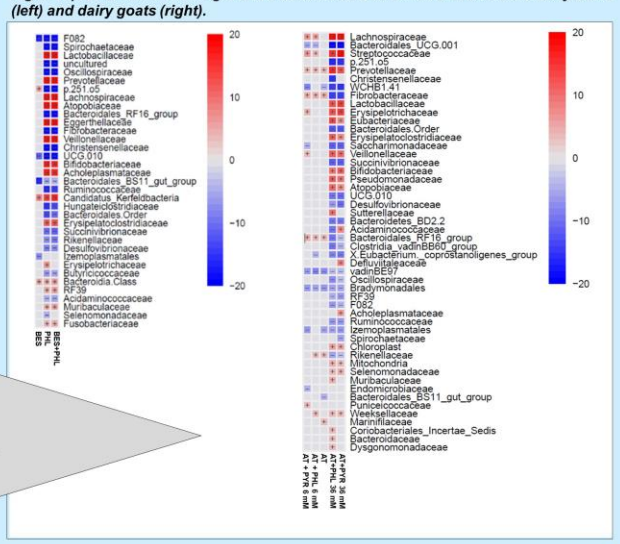


Fig 2. Top 50 features with significant associations of rumen bacteria from dairy cows (left) and dairy goats (right).



PHL and PYR supplementation at 36 mM:

- ⬆ **Bacteria related to phenolic compound metabolism** (*Lachnospiraceae, Streptococcaceae, Prevotellaceae*)
- ⬆ **Lactate producers** (*Lactobacillaceae, Veillonellacaeae*)
- ⬇ **Cellulolytic bacteria** (*Fibrobacteraceae, Succinivibrionaceae*)

Conclusions

Bacteria from cow's rumen fluid and bacteria from goat's rumen fluid response to phenolic compounds were similar. In both experiments, such compounds at high concentration promoted a shift in rumen bacterial community structure. Therefore, nutritional supplementation with phenolic compounds could modify the rumen bacterial community towards a more efficient rumen H_2 metabolism and ultimately as a CH_4 mitigation strategy.

Synergistic effects of natural hydrogen acceptors and a methanogenesis inhibitor on in vitro rumen fermentation

HUANG Rongcai[1], ROMERO MÁRQUEZ Pedro[2], BELANCHE Alejandro[2], UNGERFELD Emilio M.[3], YANEZ-RUIZ David [2], POPOVA Milka[1] and MORGAVI Diego P.[1]

[1] Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France; [2] Estación Experimental del Zaidín (CSIC), Profesor Albareda, 1, 18008, Granada, Spain; [3] Centro Regional de Investigación Carillanca, Instituto de Investigaciones Agropecuarias INIA, Temuco 4880000, Chile.

INTRODUCTION

Inhibiting rumen methanogenesis often causes hydrogen accumulation, potentially negatively affecting the rumen fermentation process. We hypothesized that the use of hydrogen acceptors when methanogenesis is inhibited would reduce hydrogen accumulation and improve production of useful fermentation end-products.

The aims of this study are to evaluate the effect of seven phenolic compounds (phenol, catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol, and gallic acid) as hydrogen acceptors on hydrogen accumulation, methane production, rumen fermentation, and microbiologic composition by *in vitro* incubation.

MATERIALS AND METHODS

Exp.1

- Tret: 2, 4, and 6 mM of each phenolic compounds, control
- Incubation time: 24 h

Exp.2

- Tret: 3 μM BES, 6 mM of each phenolic compounds + 3 μM BES
- Incubation time: 24 h
- **Exp.3 (sequential incubation)**
- Tret: 36 mM phloroglucinol, 36 mM phloroglucinol+3 μM BES, 3 μM BES, and control for the fourth incubation.
- Incubation time: 24 h

Sample collection: gas and fermentation fluid were collected at end of each incubations.

RESULTS

Exp.1

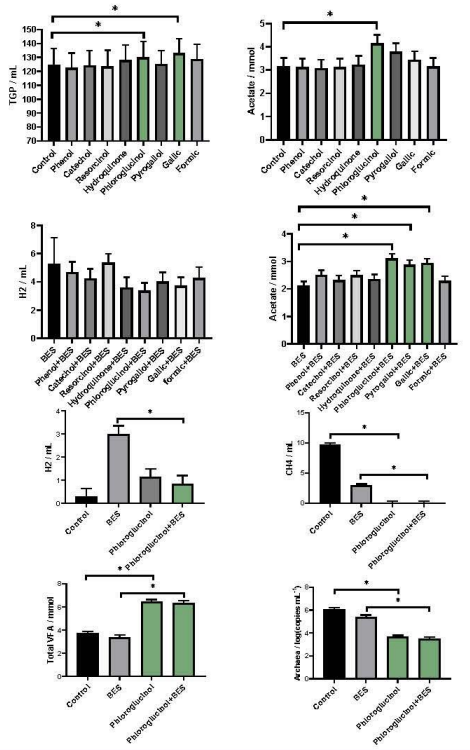
- 6 mM phenolic compounds didn't have negative effect on fermentation
- Phloroglucinol and gallic increased TGP, phloroglucinol increased acetate production

Exp.2

- 6 mM phenolic compounds combined with BES didn't decreased hydrogen accumulation
- Phloroglucinol, pyrogallol, and gallic acid combined with BES increased acetate production by 47%, 36%, and 39%, respectively

Exp.3

- Ploroglucinol+BES **V.S.** BES: decreased H2 accumulation by 64%
- Ploroglucinol+BES **V.S.** BES: decreased CH4 by100%
- Phloroglucinol **V.S.** Control: decreased CH4 by 99%
- Phloroglucinol+BES, and phloroglucinol treatments increased total VFA production by 87% and 74%, respectively
- Supplementation phloroglucinol decreased methanogens number, however didn't effect other microbes



CONCLUSIONS

Phloroglucinol is the most effective phenolic compound, which decreased hydrogen accumulation, methane production, and increased total VFA production after adaptation. Moreover, phloroglucinol decreased methanogens' numbers, however didn't have negative effect on other microbes

ACKNOWLEDGEMENTS

CONTACT

rongcai.huang@inrae.fr

Phloroglucinol reduced methane production and hydrogen accumulation in vitro

HUANG Rongcai[1], ROMERO MÁRQUEZ Pedro[2], BELANCHE Alejandro[2], UNGERFELD Emilio M.[3], YANEZ-RUIZ David [2], POPOVA Milka[1] and MORGAVI Diego P.[1]

[1] Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France; [2] Estación Experimental del Zaidín (CSIC), Profesor Albareda, 1, 18008, Granada, Spain; [3] Centro Regional de Investigación Carillanca, Instituto de Investigaciones Agropecuarias INIA, Temuco 4880000, Chile.

INTRODUCTION

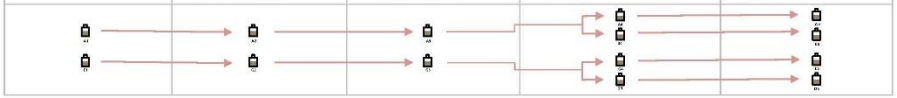
Nutritional strategies that inhibit rumen methanogenesis can result in hydrogen accumulation, a waste product that cannot be used by the animal. We hypothesised that the addition of an adequate electron acceptor can incorporate hydrogen into the production of useful metabolites and eventually improve rumen fermentation.

The aims of this study are to investigate phloroglucinol, a natural metabolite found in certain plants, on hydrogen accumulation, methane production, rumen fermentation, and microbiologic composition by using consecutive batch culture.

MATERIALS AND METHODS

- A: Substrate (alfalfa + barley) only; B: Substrate + BES; C: Substrate + phloroglucinol; D: Substrate + phloroglucinol + BES
- The inclusion levels of phloroglucinol and BES are 36 mM and 3 µM, respectively. Rumen fluids are from 8 dairy cows (n=8)
- The first to third incubations were used to let rumen microbes adapt to phloroglucinol.
- BES: 2-bromoethanesulfonic sodium used as methane inhibitor

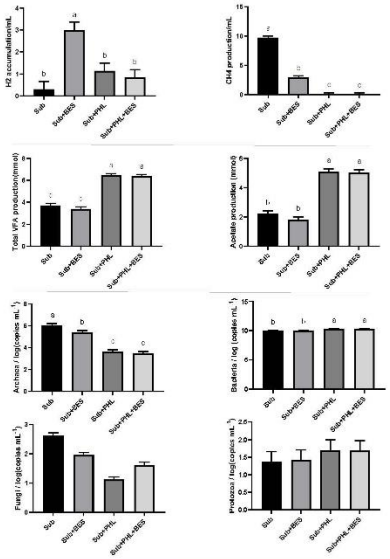
- First incubation: 24 h - Incubation fluid: 18 mL rumen fluid + 32 anaerobic buffer	- Second incubation: 24 h - Incubation fluid: 18 mL fermentation fluid from last incubation + 32 anaerobic buffer	- Third incubation: 24 h - Incubation fluid: 18 mL fermentation fluid from last incubation + 32 anaerobic buffer	- Fourth incubation: 24 h - Incubation fluid: 18 mL fermentation fluid from last incubation + 32 anaerobic buffer	- Fifth incubation: 72 h - Incubation fluid: 18 mL fermentation fluid from last incubation + 32 anaerobic buffer



RESULTS

- Phloroglucinol decreased H2 cumulation when methane production was inhibited by BES
- Phloroglucinol decreased methane production both when methane production was inhibited by BES or not.
- Phloroglucinol increased total VFA production mainly through increasing acetate production when methane production was inhibited by BES or not.
- Phloroglucinol decreased archaea abundance, increased bacteria abundance, and had no effect on fungi or protozoa abundance when methane production was inhibited by BES or not.

Sub, substrate; PHL, phloroglucinol. Above results are from the fourth incubation. Results from the fifth incubation are very similar to the forth incubation.



CONCLUSIONS

- In this work, we showed that phloroglucinol is effective in reducing hydrogen accumulation and methane production in vitro. Decrease in methane production was associated with a decrease in archaea numbers.
- Addition of phloroglucinol also improved rumen fermentation by increasing total VFA production.

ACKNOWLEDGEMENTS

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CONTACT

rongcai.huang@inrae.fr

3 Curriculum Vita

Email Address: huangrcai@foxmail.com

1 EDUCATION

- Ph.D. degree in Microbiology, UMR Herbivores INRAE and Université Clermont Auvergne, 2023.
- Master degree in Animal Nutrition and feed Science, Chinese Academy of Agricultural Sciences, 2019.
- Bachelor degree in Animal Nutrition and feed Science, South China Agricultural University, 2016.

2 ACADEMIC EXPERIENCES

- Visiting student in Pro. Chris Creevey's lab at Queen's University Belfast (December 2022 to April 2023).

3 AWARDS

- Recipient of China Scholarship Council and Agreenium joint scholarship.
- European scholarship Erasmus+ and local region scholarship CAP 20-25.
- Recognized as an outstanding researcher by CSC-Seminar in Germany and France committee. (2021)

4 PUBLICATIONS

- **Huang, R.**, P. Romero, A. Belanche, E. Ungerfeld, D. Yanez-Ruiz, D. Morgavi, and M. Popova. 2023. Evaluating the effect of phenolic compounds

as hydrogen acceptors when ruminal methanogenesis is inhibited *in vitro*– Part 1. Dairy cows. animal 17:1-9.

- Romero, P., **R. Huang**, E. Jiménez, J. Palma-Hidalgo, E. Ungerfeld, M. Popova, D. Morgavi, A. Belanche, and D. Yáñez-Ruiz. 2023. Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited *in vitro*–Part 2. Dairy goats. animal 17:1-11.
- **HUANG R.**, P. ROMERO, E. JIMÉNEZ, A. I. MARTÍN-GARCÍA, P. E. SMITH, E. M. UNGERFELD, M. POPOVA, A. BELANCHE, D. MORGAVI, D. R. YÁÑEZ-RUIZ. 2023. Phenolic compounds increased phenolic utilization bacteria abundance when methanogenesis was inhibited *in vitro*. 13. International Symposium on Gut Microbiology. (Abstract)
- **Huang R.**, P. Romero, C. Martin, E.M. Ungerfeld, A. Demeter, A. Belanche, D.R. Yáñez-Ruiz, M. Popova, D. Morgavi. Gallic Acid alleviates the negative effect of Asparagopsis armata on milk yield in dairy cows. EAAP meetings in Lyon for Annual Meeting. (Abstract)
- **Huang R.**, P. Romero, A. Belanche, E. M. Ungerfeldd, D. R. Yáñez-Ruiz, M. Popova, D. P. Morgavi, 2022. Synergistic Effects of Natural Hydrogen Acceptors and a Methanogenesis Inhibitor on *In Vitro* Rumen Fermentation. 8th International Greenhouse Gas and Animal Agriculture Conference. (Abstract)
- **Huang R**., P. Romero, A. Belanche, E. M. Ungerfeldd, D. R. Yáñez-Ruiz, M. Popova, D. P. Morgavi, 2021. Phloroglucinol reduced methane production and hydrogen accumulation *in vitro*. 12. International Symposium on Gut Microbiology. (Abstract)

5 ACADEMIC ACTIVITIES

- Poster presentation at EAAP 2023 – Lyon 2023 EAAP Annual Meeting.

  Poster title: Gallic acid alleviates the negative effect of *Asparagopsis armata* on milk yield in dairy cows.

- Presentation at MASTER project (https://www.master-h2020.eu/theproject.html) workshop in 2022. Presentation title: Combined effects of natural hydrogen acceptors and a methanogenesis inhibitor on rumen fermentation, gas emissions, and production in lactating dairy cow.

- Poster presentation at the 8th International Greenhouse Gas and Animal Agriculture Conference in 2022. Poster title: Synergistic effects of natural hydrogen acceptors and a methanogenesis inhibitor on *in vitro* rumen fermentation.
- Presentation at Journee des Doctorants et Post-Doctorants de L'UMRH INRAE in 2022. Presentation title: combined effects of natural hydrogen acceptors and a methanogenesis inhibitor on rumen microbiota, rumen fermentation, gas emissions, and production in lactating dairy cow.
- Presentation at JEDs 2022 Université Clermont Auvergne in 2022. Presentation title: Synergistic effects of natural hydrogen acceptors and a methanogenesis inhibitor on *in vitro*.
- Poster presentation at Journee des Doctorants et Post-Doctorants de L'UMRH INRAE in 2021. Poster title: Phloroglucinol reduced methane production and hydrogen accumulation *in vitro*.
- Presentation at CSC-Seminar in Germany and France in 2021. Presentation title: Capture Excessive Hydrogen Generated from Methanogenesis Inhibition to Improve Animal Production.