

Review

Not peer-reviewed version

Insights into the Feed Additive Inhibitor and Alternative Hydrogen Acceptor Interactions: A Future Direction for Enhanced Methanogenesis Inhibition in Ruminants

[Ibrahim Ahmad](#)*, [Richard Rawnsley](#), [John P. Bowman](#), [Rohan Borojevic](#), [Apeh A Omede](#)

Posted Date: 23 October 2025

doi: 10.20944/preprints202510.1716.v1

Keywords: enteric methane emission; methane inhibitor; alternative hydrogen acceptor; rumen fermentation; ruminants



Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This open access article is published under a Creative Commons CC BY 4.0 license, which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

Review

Insights into the Feed Additive Inhibitor and Alternative Hydrogen Acceptor Interactions: A Future Direction for Enhanced Methanogenesis Inhibition in Ruminants

Ibrahim Ahmad *, Richard P. Rawnsley, John P. Bowman, Rohan Borojevic and Apeh A. Omede

Tasmanian Institute of Agriculture, University of Tasmania, Tasmania, Australia

* Correspondence: ibrahim.ahmad@utas.edu.au

Abstract

Enteric methane (CH₄) emissions from ruminants contribute significantly to agricultural greenhouse gases. Anti-methanogenic feed additives (AMFA), such as *Asparagopsis* spp. and 3-nitrooxypropanol (3-NOP), reduce CH₄ emissions by inhibiting methanogenic enzymes. However, CH₄ inhibition often leads to dihydrogen (H₂) accumulation, which can impact rumen fermentation and decrease dry matter intake (DMI). Recent studies suggest that co-supplementation of CH₄ inhibitors with alternative electron acceptors, such as phloroglucinol, fumaric acid, or acrylic acid, can redirect excess H₂ during methanogenesis inhibition into fermentation products nutritionally beneficial for the host. This review summarises findings from rumen simulation experiments and *in vivo* trials that have investigated the effects of combining a CH₄ inhibitor with an alternative H₂ acceptor to achieve effective inhibition of methanogenesis. These trials demonstrate variable outcomes depending on additive combinations, inclusion rates, and adaptation periods. The use of phloroglucinol *in vivo* consistently decreased H₂ emissions and altered fermentation patterns, promoting acetate production, compared to the co-supplementation with fumaric acid or acrylic acid as an alternative electron acceptor. As a proof of concept, phloroglucinol shows promise as a co-supplement for reducing CH₄ and H₂ emissions and enhancing volatile fatty acid profiles *in vivo*. Optimising microbial pathways for H₂ utilisation through targeted co-supplementation and microbial adaptation could enhance the sustainability of CH₄ mitigation strategies using feed additive inhibitors in ruminants. Further research using multi-omics analyses is needed to understand microbial dynamics and improve the efficacy of CH₄ inhibitor interventions with an alternative H₂ acceptor co-supplementation *in vivo*.

Keywords: enteric methane emission; methane inhibitor; alternative hydrogen acceptor; rumen fermentation; ruminants

Background

Several strategies have been explored in recent years to mitigate enteric methane (CH₄) emissions. Among these, the use of anti-methanogenic feed additives (AMFA) is increasingly recognised as the most potent abatement strategy to manipulate ruminal fermentation and decrease enteric CH₄ emissions from ruminants (Honan et al., 2021; Arndt et al., 2022; Beauchemin et al., 2022). Numerous feed additives, including CH₄ inhibitors and rumen modifiers, hold the potential to significantly reduce enteric CH₄ emissions primarily by direct inhibition of the enzymes that are unique to methanogens, indirectly by modulating rumen fermentation, or through both effects (del Prado et al., 2025). Notably, two additive inhibitors, red macroalgae *Asparagopsis* spp. and the synthetic compound 3-nitrooxypropanol (3-NOP), have consistently reduced enteric CH₄ emissions

by more than 20% in ruminants (del Prado et al., 2025). The CH₄ reduction effect of *Asparagopsis* is attributed to bromoform (CHBr₃), a major active ingredient found in concentrations up to 100-fold higher than the next most abundant component, dibromochloromethane (Machado et al., 2016). The mode of action of CHBr₃ occurs via competitive inhibition of coenzyme M methyltransferase and methyl coenzyme M reductase (MCR) involved in the reduction of one- or two-carbon substrates with dihydrogen (H₂) by methanogenic archaea to produce CH₄ (Wasson et al., 2022). Similarly, synthetic CH₄ inhibitors such as iodoform (Wood et al., 1968; Glasson et al., 2022), 2-bromoethanesulfonate (BES; Gunsalus et al., 1978), and 3-NOP (Duin et al., 2016) also specifically target MCR involved in the CH₄ production pathway of rumen archaea. These potent inhibitors block the action of the enzyme MCR, which is shared by all methanogenic pathways, and thus specifically targeted to inhibit all different methanogens (Belanche et al., 2025). Nitrate is also an effective synthetic feed additive for mitigating CH₄, both *in vitro* and *in vivo* (Olijhoek et al., 2016; Liu et al., 2017). Dietary nitrate supplementation reduces CH₄ emission in *in vivo* studies (van Zijderveld et al., 2011; Olijhoek et al., 2016), and the effect has been attributed to the direct toxic effect of the ingredient, resulting in an inhibited microbial activity of methanogens (Liu et al., 2017). In addition to the impact on CH₄ reduction, dietary feed additive inhibitors have potential effects on dry matter intake (DMI), weight gain, or milk production in ruminant livestock (del Prado et al., 2025).

The inhibition of enteric methanogenesis with CH₄ inhibitors affects the metabolism of the rumen microbial ecosystem (Belanche et al., 2025). This results in increased H₂ emissions *in vivo* (Ungerfeld et al., 2022), increased accumulation of H₂ *in vitro* (Ungerfeld, 2015) and *in vivo* (Melgar et al., 2020; Cowley et al., 2024), and sometimes increased ruminal levels of intermediate electron carriers such as ethanol, formate (Martinez-Fernandez et al., 2016; Melgar et al., 2020), and lactate (Amgarten et al., 1981). For example, H₂ accumulation is one of the marked indirect effects of CH₄ inhibitor supplementation, which has positive and negative impacts on hydrogenogenic and hydrogenotrophic microorganisms in the rumen (Ungerfeld, 2020). The concentration of ruminal H₂ centrally regulates the proportions of fermentative end-products, as indicated by the metabolic interactions among fermentative bacteria and between hydrogenogenic bacteria and hydrogenotrophic methanogens (Mackie et al., 2024). Enteric methanogenesis is an evolutionary adaptation that limits the accumulation of ruminal H₂ resulting from fibre degradation (Wolin et al., 1997), as high H₂ partial pressure in the rumen would otherwise impair the ability of microbial populations to digest carbohydrates (McAllister and Newbold, 2008; van Lingen et al., 2016). Under typical conditions, methanogenic archaea utilise H₂ to produce CH₄ (Belanche et al., 2025), which indirectly sustains the fermentation process and mitigates the negative feedback inhibition of excess H₂ on the degradation of dry matter in the rumen (McAllister and Newbold, 2008). Thus, the removal of H₂ enables the re-oxidation of NADH to NAD⁺, which occurs through the transfer of electrons released from the oxidation of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate during glycolysis, a process essential for the continuation of hexose catabolism in the rumen (Wolin, 1979; Russell and Wallace, 1997). The health and productivity of animals can be negatively impacted by disruptions to the rumen ruminal microbiome, which facilitate these reactions (Mackie et al., 2024). Therefore, to preserve feed conversion and animal productivity, dietary intervention strategies aimed at manipulation of the rumen microbial environment for methanogenesis inhibition require careful consideration (Choudhury et al., 2015).

Mitigation strategy using feed additives to reduce rumen methanogenesis requires a consistent and sustained reduction in CH₄ emissions from ruminants without negatively affecting animal health, welfare, or productivity (Honan et al., 2021; Belanche et al., 2025; Durmic et al., 2025). However, studies have shown that feed additive inhibitors reduce enteric methanogenesis and often increase emission of ruminal H₂ (Olijhoek et al., 2016; Krizsan et al., 2023; Thorsteinsson et al., 2023a; Maigaard et al., 2024a), which is suggested to be a consequence of direct inhibition of rumen CH₄ production (Thorsteinsson et al., 2023b). Increased enteric emission of H₂ indicates higher levels of dissolved H₂ in the rumen associated with a reduced redox state (Kjeldsen et al., 2024). A surplus of electron donors from H₂ accumulation during the inhibition of rumen methanogenesis can impact

optimal microbial fermentation and synthesis (McAllister and Newbold, 2008; Thorsteinsson et al., 2023b), potentially leading to negative effects on animal performance as reflected in decreased feed intake (Maigaard et al., 2024a). The challenge with using inhibitors to reduce enteric CH₄ emissions is the inefficiency of the rumen microbiome to capture and redirect excess H₂ spared from methanogenesis inhibition into alternative metabolic pathways for the synthesis of metabolites usable by ruminants (Ungerfeld et al., 2022; Romero et al., 2023). This indicates that the rumen system can still be optimised to improve animal productivity by feeding novel substrates to modify rumen microbiome and redirect H₂ towards the production of beneficial fermentation products (Romero et al., 2024). Thus, co-supplementation of an alternative electron alternative sink with higher H₂ affinity during methanogenesis inhibition would be a promising strategy for a robust mitigation of enteric CH₄ emissions in ruminants. As an example, microbial reduction of phloroglucinol or fumaric acid could provide a pathway to incorporate increased H₂ produced under methanogenesis inhibition conditions into the production of acetate or propionate, an energy-yielding product used by the animal (Romero et al., 2023; Thorsteinsson et al., 2023b). The metabolic fate and significance of the absorbed alternative electron sink may impact the beneficial effects on animal productivity during the inhibition of enteric methanogenesis. This depends on the nutritional requirements of each animal species and the energy savings achieved through H₂ incorporation (Ungerfeld, 2013). Previous literature postulated that co-supplementation of a feed additive inhibitor with an alternative electron sink would significantly reduce CH₄ production without a concomitant increase in H₂ accumulation or emissions, thereby mitigating the negative effects of methanogenesis inhibition on fermentation and animal performance (Huang et al., 2023; Romero et al., 2023; Battelli et al., 2025). Hence, this current review summarised recent findings from rumen simulation experiments and *in vivo* trials that have investigated the effects of combining a CH₄ inhibitor with an H₂ acceptor to achieve effective inhibition of methanogenesis. Although the data were insufficient to conduct a meta-analysis, direct comparison of variances between the trials was used to highlight significant findings.

Integrated Approach: Co-Supplementation of a Methane Inhibitor with an Alternative H₂ Acceptor In Vitro and In Vivo

Only a small number of laboratory experiments and animal studies have explored the synergistic effects of methanogenesis inhibitors and alternative electron acceptors on fermentation parameters, CH₄ production, and H₂ accumulation or emissions (Table 1). The *in vitro* rumen simulation experiments by Huang et al. (2023) and Romero et al. (2023) evaluated the effects of CH₄ inhibitors (*Asparagopsis* or BES) used alone or with an alternative electron acceptor (phloroglucinol) on CH₄ production, H₂ accumulation, and fermentation parameters. These *in vitro* experiments confirmed the inhibitory potential of individual CH₄ inhibitors at different concentrations, which increased H₂ accumulation and substantially decreased CH₄ production, total VFA, and the acetate-to-propionate (A:P) ratio (Huang et al., 2023; Romero et al., 2023). Huang et al. (2023) showed that phloroglucinol supplemented alone and in combination with BES decreased CH₄ production, propionate production, and H₂ accumulation, as well as increased acetate production, total VFA and A:P ratio compared to the CH₄ inhibitor-only treatment. In Huang et al. (2023), the intensity of the impacts of co-supplementation on CH₄ production, fermentation parameters, and H₂ accumulation increased with the accelerated inclusion of phloroglucinol to 36 mM. Similarly, Romero et al. (2023) demonstrated the effects of CH₄ inhibitor-only (*Asparagopsis*) and co-supplementation with the increasing doses of phloroglucinol (6, 16, 26 and 36 mM) on CH₄ production, H₂ accumulation, and fermentation parameters. Supplementation of *Asparagopsis* at 2% DM of substrate with increasing doses of phloroglucinol (6, 16, 26 and 36 mM) linearly decreased CH₄ production, propionate production, and H₂ accumulation, while increased acetate production, total VFA, and A:P ratio (Romero et al., 2023). Thorsteinsson et al. (2023b) examine the effects of nitrate or *Asparagopsis* with and without fumaric acid as an alternative electron acceptor on CH₄ production and H₂ accumulation *in vitro*. Thorsteinsson et al. (2023b) found that supplementation with nitrate alone reduced CH₄ production without resulting in detectable levels of H₂ production. The combination of nitrate and

fumaric acid has not further decreased CH₄ production compared to the nitrate-only treatment, thus making it impossible to evaluate the effect of nitrate with fumaric acid co-supplementation on H₂ production (Thorsteinsson et al., 2023b). Thorsteinsson et al. (2023b) speculated that all available dissolved H₂ was used for nitrate reduction to ammonia or other competing pathways, explaining the lack of increased H₂ levels in the nitrate treatment. However, Thorsteinsson et al. (2023b) reported that supplementation of *Asparagopsis* with fumaric acid significantly reduced CH₄ production resulting in no significant change in H₂ concentration above the detection level (Table 1). There was no synergistic effect of supplementing *Asparagopsis* with fumaric acid on H₂ production in Thorsteinsson et al. (2023b). Severe inhibition of methanogenesis by *Asparagopsis*, with or without fumaric acid, had no effect on dry matter degradability at any incubation period compared to the control, suggesting that the excess H₂ produced may have been redirected in the production of other metabolites, such as propionate and butyrate, through pathways that have not been analysed in Thorsteinsson et al. (2023b). In a recent *in vitro* study, Battelli et al. (2025) observed a different interaction where none of the combinations of CH₄inhibitor (Iodoform or Quercetin) with alternative H₂-acceptor (Activated charcoal powder, phloroglucinol, or vitamin E) demonstrated a synergistic effect to reduce H₂ production and improve fermentation parameters (Table 1). However, a co-supplementation of CH₄ inhibitors, including Iodoform and Quercetin (Table 1), significantly decreased CH₄ production and numerically reduced H₂ production without impacts on the total VFA concentrations compared to the control (Battelli et al., 2025). The synergistic effect between these CH₄ inhibitors could be due to complementary mechanisms of action, where Quercetin reduces protozoan abundance (Kim et al., 2015) and modulates microbial fermentation (Battelli et al., 2023), while Iodoform targets and inhibits specific methanogenic enzymes (Glasson et al., 2022; Thorsteinsson et al., 2023a). The inclusion of Quercetin could indirectly limit CH₄ production by reducing the abundance of protozoa and thus decreasing interspecies H₂ transfer to methanogens for methanogenesis (Battelli et al., 2025). Microbial degradation of Quercetin to 3,4-dihydroxyphenylacetate in the rumen also consumes H₂ or formate, which acts as a competing electron sink and reduces the availability of H₂ for CH₄ production (Krumholz and Bryant, 1986; Berger et al., 2015).

Furthermore, the publications by Maigaard et al. (2024b) and Romero et al. (2024) reported effects of combined supplementation of CH₄ inhibitors and alternative H₂ acceptors on rumen fermentation, enteric CH₄ and H₂ emissions, and animal performance *in vivo* (Table 1). Maigaard et al. (2024b) reported that co-supplementation of 3-NOP (60 mg/kg of DM) with fumaric acid (390 g/d), acrylic acid (242 g/d), or phloroglucinol (480 g/d) as alternative electron acceptor, significantly reduced CH₄ emissions and numerically decreased H₂ emissions without pronounced effects on total VFA in lactating dairy cows fed high-forage diets. The co-supplementation of 3-NOP (60 mg/kg of DM) with phloroglucinol (480 g/d) had no pronounced effect on the DMI, but co-supplementation with fumaric acid (390 g/d) or acrylic acid (242 g/d) significantly increased propionate production and significantly reduced A:P ratio (Maigaard et al., 2024b). Consistently, supplementation of fumaric acid-only reduced CH₄ emissions and shifted rumen fermentation patterns towards a higher proportion of propionate, lower A:P ratio, and decreased total VFA in goats fed low and high forage diets (Li et al., 2018), as also found by Maigaard et al. (2024b). However, the anti-methanogenic co-supplementation of nitrate (15 g/kg of DM) with fumaric acid (390 g/d), acrylic acid (242 g/d), or fumaric acid (195 g/d) plus acrylic acid (121 g/d) had no significant effects on CH₄ and H₂ emissions, but improved DMI except for co-supplementation with acrylic acid in dairy cows (Maigaard et al., 2024b). The numerical reduction in H₂ emissions *in vivo*, due to the co-supplementation of nitrate with fumaric acid in Maigaard et al. (2024b), agrees with the previous findings using similar ingredients *in vitro* (Thorsteinsson et al., 2023b). In addition, Romero et al. (2024) demonstrated that inclusion of *Asparagopsis* with phloroglucinol up to 20 g/kg DM/d, a rumen equivalent concentration of 36 mM/d, in high-forage diets reduced CH₄ and H₂ emissions compared to the control without negative impacts on rumen fermentation and DMI in dairy goats. Moreover, the *in vivo* supplementation of phloroglucinol with or without *Asparagopsis*, promoted a shift in the fermentation

patterns towards a higher proportion of acetate and increased A:P ratio, and a lower proportion of propionate (Romero et al., 2024). Comparatively, a previous rumen simulation study (Romero et al., 2023) used *Asparagopsis* (2% DM) with phloroglucinol (36 mM) and found a more pronounced decrease in CH₄ production (-100%) and H₂ accumulation (-46%) compared to the reductions observed (Table 1) in dairy goats fed similar ingredients at the same concentrations in Romero et al. (2024). This discrepancy could be due to the differences between *in vitro* and *in vivo* experimental conditions. Overall, there is a paucity of data related to the *in vivo* evaluation of the co-supplementation of a CH₄ inhibitor with an alternative H₂ acceptor.

Table 1. Summary of the experiments demonstrating effects of co-supplementation of methane inhibitors with alternative hydrogen acceptors on dihydrogen accumulation or emission during methanogenesis inhibition.

Refere nce	Type of study, duration	AMFA	Dose	Animals	Feed substrate	CH ₄	H ₂	Acetate	Propionate	A:P	Total	DMI
						production /emission (%)	accumulation /emission (%)	proportion (%)	proportion (%)	ratio (%)	VFA (%)	(%)
Huang et al., 2023	In vitro, 24 h	AT	1.5% DM	In vitro culture*	High fiber substrate	- 22 (c. C)	+ 104 (c. C)	- 13 (c. C)	+ 12 (c. C)	- 21 (c. C)	- 9 (c. C)	N.A
	In vitro, 24 h	AT + Phl	1.5% + 6 mM	In vitro culture*	High fiber substrate	- 38 (c. C)	+ 150 (n.s 1.5% AT)	+ 7 (n.s 1.5% AT)	- 11 (n.s 1.5% AT)	+ 35 (n.s 1.5% AT)	+ 17 (s.s 1.5% AT)	N.A
	In vitro, 24 h	AT	2.5% DM	In vitro culture*	High fiber substrate	- 76 (c. C)	+ 541 (c. C)	- 32 (c. C)	+ 35 (c. C)	- 47 (c. C)	- 22 (c. C)	N.A
	In vitro, 24 h	AT + Phl	2.5% + 6 mM	In vitro culture*	High fiber substrate	- 94 (c. C)	+ 10 (s.s 2.5% AT)	+ 41 (s.s 2.5% AT)	- 24 (s.s 2.5% AT)	+ 68 (s.s 2.5% AT)	+ 18 (s.s 2.5% AT)	N.A
	In vitro, 24 h	BES	3 μM	In vitro culture*	High fiber substrate	- 51 (c. C)	+ 5,733 (c. C)	- 26 (c. C)	+ 33 (c. C)	- 45 (c. C)	+ 2 (c. C)	N.A
	In vitro, 24 h	BES + Ph l	3 μM + 6 mM	In vitro culture*	High fiber substrate	- 46 (c. C)	- 36 (n.s BES)	+ 35 (s.s BES)	- 33 (s.s BES)	+ 102 (s.s BES)	+ 11 (s.s BES)	N.A
	In vitro, 96 h	BES	3 μM	In vitro culture*	High fiber substrate	- 70 (c. C)	+ 931 (c. C)	- 7 (c. C)	+ 7 (c. C)	- 21 (c. C)	- 6 (c. C)	N.A
	In vitro, 96 h	BES + Ph l	3 μM + 36 mM	In vitro culture*	High fiber substrate	- 100 (c. C)	- 72 (s.s BES)	+ 42 (s.s BES)	- 86 (s.s BES)	+ 752 (s.s BES)	+ 77 (s.s BES)	N.A
	In vitro, 94 h	Phl	36 mM	In vitro culture*	High fiber substrate	- 99.7 (c. C)	- 62 (s.s BES)	+ 38 (s.s BES)	- 85 (s.s BES)	+ 791 (s.s BES)	+ 69 (s.s BES)	N.A

Romero et al., 2023	In vitro, 24 h	AT	1% DM	In vitro culture ^a	High fiber substrate	- 94 (s.s C)	plus 779 (n.s C)	- 13 (s.s C)	+ 33 (s.s C)	- 34 (s.s C)	- 7 (n.s C)	N.A
	In vitro, 24 h	AT	2% DM	In vitro culture ^a	High fiber substrate	- 99 (s.s C)	+ 3,650 (s.s C)	- 14 (s.s C)	+ 33 (s.s C)	- 19 (s.s C)	- 4 (n.s C)	N.A
	In vitro, 24 h	AT	3% DM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	+ 5,793 (s.s C)	- 15 (s.s C)	+ 36 (s.s C)	- 38 (s.s C)	- 7 (n.s C)	N.A
	In vitro, 24 h	AT	4% DM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	+ 10,257 (s.s C)	- 15 (s.s C)	+ 35 (s.s C)	- 37 (s.s C)	- 7 (n.s C)	N.A
	In vitro, 24 h	AT	5% DM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	+ 7,007 (s.s C)	- 15 (s.s C)	+ 35 (s.s C)	- 37 (s.s C)	- 8 (n.s C)	N.A
	In vitro, 5 d	AT	2% DM	In vitro culture ^a	High fiber substrate	- 99 (c. C)	+ 1,450 (c. C)	- 12 (s.s C)	+ 15 (s.s C)	- 23 (c. C)	- 18 (c. C)	N.A
	In vitro, 5 d	AT + Phl	2% + 6 mM	In vitro culture ^a	High fiber substrate	- 100 (c. C)	- 37 (n.s 2% AT)	+ 8 (s.s 2% AT)	- 18 (s.s 2% AT)	+ 31 (s.s 2% AT)	+ 18 (s.s 2% AT)	N.A
	In vitro, 5 d	AT	2% DM	In vitro culture ^a	High fiber substrate	- 99 (s.s C)	+ 2,323 (s.s C)	- 13 (s.s C)	+ 14 (s.s C)	- 23 (s.s C)	- 14 (s.s C)	N.A
	In vitro, 5 d	AT + Phl	2% + 6 mM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	- 27 (s.s 2% AT)	+ 10 (s.s 2% AT)	- 17 (s.s 2% AT)	+ 33 (n.s 2% AT)	+ 27 (s.s 2% AT)	N.A
	In vitro, 5 d	AT + Phl	2% + 16 mM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	- 49 (s.s 2% AT)	+ 22 (s.s 2% AT)	- 44 (s.s 2% AT)	+ 121 (s.s 2% AT)	+ 71 (s.s 2% AT)	N.A
Thorsteinsson et al., 2023b	In vitro, 5 d	AT + Phl	2% + 26 mM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	- 63 (s.s 2% AT)	+ 32 (s.s 2% AT)	- 66 (s.s 2% AT)	+ 292 (s.s 2% AT)	+ 88 (s.s 2% AT)	N.A
	In vitro, 5 d	AT + Phl	2% + 36 mM	In vitro culture ^a	High fiber substrate	- 100 (s.s C)	- 46 (s.s 2% AT)	+ 34 (s.s 2% AT)	- 70 (s.s 2% AT)	+ 354 (s.s 2% AT)	+ 99 (s.s 2% AT)	N.A
	In vitro, 24 h	Nit	0.05 g	In vitro culture ^b	High fiber substrate	- 56 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 24 h	AT	0.05 g	In vitro culture ^b	High fiber substrate	- 98 (s.s C)	+ 202 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 24 h	Nit + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 47 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 24 h	AT + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 98 (s.s C)	+ 211 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 36 h	Nit	0.05 g	In vitro culture ^b	High fiber substrate	- 52 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A

	In vitro, 36 h	AT	0.05 g	In vitro culture ^b	High fiber substrate	- 99 (s.s C)	+ 82 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 36 h	Nit + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 46 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 36 h	AT + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 99 (s.s C)	+ 136 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 48 h	Nit	0.05 g	In vitro culture ^b	High fiber substrate	- 61 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 48 h	AT	0.05 g	In vitro culture ^b	High fiber substrate	- 99 (s.s C)	+ 124 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 48 h	Nit + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 55 (s.s C)	+ 0.00 (n.s C)	N.D	N.D	N.D	N.D	N.A
	In vitro, 48 h	AT + FA	0.05 g + 0.05 g	In vitro culture ^b	High fiber substrate	- 99 (s.s C)	+ 176 (n.s C)	N.D	N.D	N.D	N.D	N.A
Battelli et al., 2025	In vitro, 24 h	IOD	0.007% DM	In vitro culture ^b	High fiber substrate	- 62 (s.s C)	+ 4,803 (s.s C)	- 7 (s.s C) ^c	+ 23 (s.s C) ^c	- 25 (s.s C)	+ 3 (s.s C)	N.A
	In vitro, 24 h	QUE	3% DM	In vitro culture ^b	High fiber substrate	- 19 (s.s C)	- 19 (n.s C)	+ 2 (s.s C) ^c	- 2 (s.s C) ^c	+ 4 (s.s C)	- 3 (s.s C)	N.A
	In vitro, 24 h	ACP	5% DM	In vitro culture ^b	High fiber substrate	- 0.8 (s.s C)	- 35 (n.s C)	+ 2 (s.s C) ^c	- 6 (s.s C) ^c	+ 6 (s.s C)	- 0.9 (s.s C)	N.A
	In vitro, 24 h	Phl	23% DM	In vitro culture ^b	High fiber substrate	+ 4 (s.s C)	+ 3 (n.s C)	+ 17 (s.s C) ^c	- 37 (s.s C) ^c	+ 82 (s.s C)	+ 42 (s.s C)	N.A
	In vitro, 24 h	VitE	0.45% DM	In vitro culture ^b	High fiber substrate	+ 35 (s.s C)	+ 26 (n.s C)	+ 6 (s.s C) ^c	- 24 (s.s C) ^c	+ 39 (s.s C)	+ 9 (s.s C)	N.A
	In vitro, 24 h	IOD + ACP	0.007% + 5%	In vitro culture ^b	High fiber substrate	+ 0.00 (s.s C)	+ 835 (s.s C)	+ 1 (n.s C) ^c	+ 2 (n.s C) ^c	- 2 (n.s C)	+ 10 (s.s C)	N.A
	In vitro, 24 h	IOD + Phl	0.007% + 23%	In vitro culture ^b	High fiber substrate	- 68 (s.s C)	+ 8,416 (s.s C)	+ 9 (s.s C) ^c	- 19 (s.s C) ^c	+ 31 (s.s C)	+ 46 (s.s C)	N.A
	In vitro, 24 h	IOD + VitE	0.007% + 0.45 %	In vitro culture ^b	High fiber substrate	- 63 (s.s C)	+ 4,965 (s.s C)	- 5 (s.s C) ^c	+ 17 (s.s C) ^c	- 13 (s.s C)	+ 3 (n.s C)	N.A
	In vitro, 24 h	QUE + ACP	3% + 5%	In vitro culture ^b	High fiber substrate	- 6 (s.s C)	- 13 (n.s C)	+ 3 (s.s C) ^c	- 5 (s.s C) ^c	+ 6 (s.s C)	- 0.7 (s.s C)	N.A
	In vitro, 24 h	QUE + Phl	3% + 23%	In vitro culture ^b	High fiber substrate	- 32 (s.s C)	+ 2,019 (s.s C)	+ 16 (s.s C) ^c	- 31 (s.s C) ^c	+ 68 (s.s C)	+ 40 (s.s C)	N.A
	In vitro, 24 h	QUE + VitE	3% + 0.45%	In vitro culture ^b	High fiber substrate	+ 0.00 (s.s C)	+ 26 (n.s C)	+ 7 (s.s C) ^c	- 20 (s.s C) ^c	+ 31 (s.s C)	+ 5 (s.s C)	N.A
	In vitro, 24 h	IOD + QUE	0.007% + 3%	In vitro culture ^b	High fiber substrate	- 98 (s.s C)	+ 1,642 (s.s C)	- 2 (s.s C) ^c	+ 11 (s.s C) ^c	- 13 (s.s C)	+ 0.00 (n.s C)	N.A
	In vitro, 24 h	IOD + QUE + ACP	0.007 + 3 + 5%	In vitro culture ^b	High fiber substrate	- 82 (s.s C)	+ 952 (s.s C)	- 0.8 (n.s C) ^c	+ 7 (s.s C) ^c	- 9 (s.s C)	+ 3 (s.s C)	N.A

Maigaa rd et al., 2024b	In vitro, 24 h	IOD + QUE + Phl	0.007 + 3 + 23%	In vitro culture ^b	High fiber substrate	- 96 (s.s C)	+ 2,932 (s.s C)	+ 13 (s.s C) ^c	- 24 (s.s C) ^c	+ 46 (s.s C)	+ 42 (s.s C)	N.A
	In vitro, 24 h	IOD + QUE + VitE	0.007 + 3 + 0.45%	In vitro culture ^b	High fiber substrate	- 98 (s.s C)	+ 1,271 (s.s C)	- 2 (s.s C) ^c	+ 9 (s.s C) ^c	- 11 (s.s C)	+ 0.3 (n.s C)	N.A
	In vivo, N.S	Nit + FA	15 g/kg DM + 390 g/d	Lactatin g cows	High forage	- 2 (n.s C)	- 36 (n.s C)	- 2 (s.s C)	+ 6 (n.s C)	- 6 (n.s C)	+ 0.00 (n.s C)	+ 5 (s.s C)
	In vivo, N.S	Nit + AA	15 g/kg DM + 242 g/d	Lactatin g cows	High forage	- 4 (n.s C)	- 47 (n.s C)	+ 4 (s.s C)	+ 6 (n.s C)	- 0.4 (n.s C)	- 7 (s.s C)	- 11 (s.s C)
	In vivo, N.S	Nit + FA + AA	15 g/kg DM + 195 g + 121 g/d	Lactatin g cows	High forage	+ 3 (n.s C)	- 37 (n.s C)	+ 1 (n.s C)	+ 5 (n.s C)	- 3 (n.s C)	- 2 (s.s C)	+ 4 (s.s C)
	In vivo, N.S	3-NOP + FA	60 mg/kg DM + 390 g/d	Lactatin g cows	High forage	- 21 (s.s C)	- 11 (n.s C)	- 3 (n.s C)	+ 13 (s.s C)	- 16 (s.s C)	- 3 (n.s C)	- 6 (n.s C)
	In vivo, N.S	3-NOP + AA	60 mg/kg DM + 242 g/d	Lactatin g cows	High forage	- 50 (s.s C)	+ 18 (n.s C)	- 0.7 (n.s C)	+ 21 (s.s C)	- 18 (s.s C)	- 15 (s.s C)	- 24 (s.s C)
	In vivo, N.S	3-NOP + Phl	60 mg/kg DM + 480 g/d	Lactatin g cows	High forage	- 35 (s.s C)	- 3 (n.s C)	+ 0.6 (n.s C)	+ 0.00 (n.s C)	- 0.4 (n.s C)	- (n.s C)	- 5 (n.s C)
	In vivo, 14 d	Phl	20 g/kg DM/d	Dairy goats	High forage	- 7 (c. C)	- 34 (n.s C)	+ 9 (s.s C)	- 16 (s.s C)	+ 30 (s.s C)	- 4 (n.s C)	- 1 (n.s C)
	In vivo, 14 d	AT	5 g/kg DM/d	Dairy goats	High forage	- 40 (c. C)	+ 4,383 (s.s C)	- 11 (s.s C)	+ 33 (s.s C)	- 30 (s.s C)	- 5 (n.s C)	- 6 (n.s C)
	In vivo, 14 d	AT + Phl	5 g + 20 g/kg DM/d	Dairy goats	High forage	- 47 (c. C)	- 68 (s.s AT)	+ 11.8 (n.s AT)	- 22 (n.s AT)	+ 39 (n.s AT)	+ 6 (n.s AT)	+ 1 (n.s AT)

AMFA = anti-methanogenic feed additives; CH₄ = methane; H₂ = dihydrogen; A:P = acetate-to-propionate; VFA = volatile fatty acids; DMI = dry matter intake; AT = *Asparagopsis taxiformis*; Phl = phloroglucinol; BES = 2-bromoethanesulfonate; DM = dry matter substrate; Nit = Nitrate; FA = Fumaric acid; AA = Acrylic acid; IOD = Iodoform; QUE = Quercetin; ACP = Activated charcoal powder; VitE = Vitamin E; N.S = study duration not stated; d = day; h = hours; 3-NOP = 3-nitrooxypropanol; * = rumen fluid from dairy cows; ^a = rumen fluid from dairy goats; ^b = rumen fluid from nonlactating cows; c. C = compared to control treatment; s.s C = statistically significant compared to control treatment; n.s C = not statistically significant compared to control treatment; n.s 1.5% AT = not statistically significant compared to 1.5% *Asparagopsis taxiformis* treatment; s.s 2.5% AT = statistically significant compared to 2.5% *Asparagopsis taxiformis* treatment; n.s BES = not statistically significant

compared to BES treatment; s.s BES = statistically significant compared to BES treatment; s.s 2% AT = statistically significant compared to 2% AT treatment; n.s 2% AT = not statistically significant compared to 2% AT treatment; s.s AT = statistically significant compared to AT treatment; n.s AT = not statistically significant compared to AT treatment; N.D = not determined; ^c = the results for VFA after 48 h of fermentation; N.A = not applicable; + = increase; - = decrease; 1-5% AT: Inclusion of AT at the rate of 1-5% DM substrate.

Potentials of H₂ Acceptor Co-Supplementation for Enhanced Methanogenesis Inhibition

The effectiveness of a CH₄ inhibitor with an alternative H₂ acceptor has shown a wide variation across *in vitro* and *in vivo* studies due to differences in inclusion rates of the feed additives, ration formulation, duration of adaptation to the ingredients, and animal species or source of the rumen fluid inoculum for *in vitro* experiments (Table 1). Several metabolic pathways have been identified as alternative electron sinks for excess H₂ from methanogenesis inhibition, including co-supplementation with fumaric acid, acrylic acid (Maigaard et al., 2024b), or phloroglucinol (Romero et al., 2024). The dietary co-supplementation with alternative electron sinks stimulates rumen microbial groups that use H₂ as a reductant to metabolise these additives into nutritionally usable products for the animal, and as such decreases excess H₂ accumulation. This minimises digestible energy losses from greenhouse gas production while avoiding fermentation inhibition and thus is probably an excellent methanogenesis-inhibition strategy (Lan and Yang, 2019). For example, co-supplementation with fumaric acid and acrylic promote increased proportion of propionate, while microbial degradation of phloroglucinol promote acetate production *in vivo* (Table 1), which are energy yielding products nutritionally beneficial for the host. Succinate-forming bacteria in the rumen use H₂ or formate as an electron donor to reduce fumaric acid into succinic acids, which is further decarboxylated to propionate via the succinate-propionate pathway. This process limits the availability of H₂ for CH₄ production. In this reduction, one mole of H₂ is consumed for every mole of fumaric acid converted to succinic acid (Ungerfeld et al., 2007). The reduction of fumaric acid occurs when population of the succinate-forming bacteria is sufficiently large and the partial pressure of H₂ is high, as these ruminal microbes have a lower affinity for H₂ compared to methanogenic archaea (Asanuma et al., 1999). Similarly, acrylic acid is reduced to propionate via a different mechanism, the acrylate pathway, and one mole of H₂ is used for every mole of acrylic acid during the reduction process (Newbold et al., 2005). Maigaard et al. (2024b) observed an increased proportion of propionate in nitrate (15 g/kg DM/d) or 3-NOP (60 mg/kg DM/d) co-supplementation with fumaric acid (390 g/d) or acrylic acid (242 g/d), which suggests that some H₂ was redirected to the reduction of fumaric acid and acrylic acid to propionate (Table 1). Moreover, inclusion of phloroglucinol facilitated the incorporation of accumulated H₂ during biotransformation of the additive into VFA, particularly acetate (Huang et al., 2023). Huang et al. (2023) also found that the molar proportions of isobutyrate and isovalerate, which are produced from the deamination of branched-chain amino acids and function as a carbon skeleton in the production of branched-chain amino acids (Chen and Russell, 1988), were reduced by the supplementation of phloroglucinol, with or without BES. Increased synthesis or reduced degradation of branched-chain amino acids in the rumen may enhance the amount of these amino acids available for productive functions in the small intestine of the host ruminant (Huang et al., 2023). Rumen microbes such as *Eubacterium oxidoreducens* spp. and *Coproccoccus* spp. can use formate or H₂ as an electron donor to reduce phloroglucinol, producing VFA, particularly acetate (Krumholz et al., 1987; Tsai and Jones, 1975). Tsai et al. (1976) indicated that one molecule of H₂ is utilised to degrade one molecule of phloroglucinol to produce two molecules each of acetate and CO₂. Notably, different phloroglucinol reductases from anaerobic fermenting bacteria, classified within the NADPH-dependent short-chain dehydrogenases/reductases family, catalyse the reduction of phloroglucinol to dihydrophloroglucinol (DHP), which is followed by hydrolytic ring cleavage of DHP to form 3-hydroxy-5-oxohexanoate and then subsequently converted to acetate and butyrate as end products (Krumholz et al., 1987; Conradt et al., 2016). When supplemented at sufficient concentration,

phloroglucinol serves as an alternative H_2 acceptor, allowing excess H_2 produced from methanogenesis inhibition to be redirected into useful products for the host ruminant (Huang et al., 2023). Huang et al. (2023) demonstrated that, under equivalent CH_4 inhibition using BES (3 μM), co-supplementation with a higher dose of phloroglucinol at 36 mM resulted in a more pronounced reduction of H_2 accumulation compared to a lower dose (6 mM). The low H_2 recovery, concomitant with an increased A:P ratio due to higher proportion of acetate in accelerated dose of an alternative electron acceptor in BES (3 μM) + Phloroglucinol (36 mM) treatment, indicated a reduction of phloroglucinol to acetate (Huang et al., 2023). Microbial degradation of phloroglucinol by *Coproccoccus* spp. results in the production of acetate and CO_2 (Tsai and Jones, 1975), while *Eubacterium oxidoreducens* reduces phloroglucinol to acetate and butyrate as end products (Krumholz et al., 1987). However, the bacteria that catabolise phloroglucinol as a substrate, including *Coproccoccus* spp., *Eubacterium oxidoreducens*, and *Streptococcus ovis* (Tsai and Jones, 1975; Krumholz and Bryant, 1986), are present at low abundance in the rumen (Huang et al., 2023). Hence, long adaptation of rumen microbiota is necessary to promote the growth of such bacteria for the effective degradation of phloroglucinol using H_2 to deliver reducing equivalents. Published *in vitro* studies showed that a sequential batch incubation for a longer duration (3-5 d) using phloroglucinol as an alternative H_2 acceptor and BES or *Asparagopsis* as the CH_4 inhibitor decreased metabolic hydrogen recovery and increased total VFA and acetate production compared to shorter (24-h) incubations (Huang et al., 2023; Romero et al., 2023). Romero et al. (2024) adapted dairy goats to the diets containing increasing concentration of phloroglucinol (5, 10, 15, and 20 g/kg DM) for 10 d, which resulted in no significant difference in H_2 emissions. Similarly, the study by Maigaard et al. (2024b) found no interaction between co-supplementation of 3-NOP (60 mg/kg DM/d) with phloroglucinol (480 g/d) and H_2 emissions, potentially due to the 7-d short period for ingredient adaptation in the rumen. Therefore, a long adaptation (> 10 d) to a supplemented feed *in vivo* or longer incubation times *in vitro* could favour optimal growth of bacteria able to metabolise phloroglucinol or any other compound acting as an alternative electron acceptor to reduce H_2 accumulation during methanogenesis inhibition.

Although different combinations of CH_4 inhibitors and alternative H_2 acceptors have been recently explored *in vitro* and *in vivo* (Table 1), including co-supplementation of *Asparagopsis* with phloroglucinol, BES with phloroglucinol (Huang et al., 2023; Romero et al., 2023), nitrate with fumaric acid or acrylic acid (Maigaard et al., 2024b), iodoform with activated charcoal powder, vitamin E, or phloroglucinol (Battelli et al., 2025), and 3-NOP with phloroglucinol (Maigaard et al., 2024b). Dietary co-supplementation with alternative electron acceptors such as fumaric acid or acrylic acid increases ruminal propionate (Figure 1), while phloroglucinol mainly increases ruminal acetate production (Figure 2). These changes in VFA profiles only decrease excess H_2 during methanogenesis inhibition but also lead to an increase in energy-yielding precursors in animals, which consequently improves feed efficiency. Propionate serves as the main precursor for gluconeogenesis and is the primary energy source for weight gain and lactose production, while acetate provides the carbon required for milk fat synthesis in ruminants (Reddy and Hyder, 2023). The low efficacy in H_2 reduction from fumaric acid or acrylic acid co-supplementation in lactating dairy cows (Maigaard et al., 2024b) seems to result from incomplete conversion of these organic acids, which is typical of reduced efficiency in slow-growing, low-numbers ruminal microbiota after a short adaptation period. The use of phloroglucinol *in vivo* consistently decreased H_2 emissions compared to dietary co-supplementation with fumaric acid or acrylic acid as an alternative electron acceptor, as shown in Table 1. This suggests that phloroglucinol can redirect excess H_2 into beneficial fermentation products more effectively than fumaric acid and acrylic acid. However, animals supplemented with phloroglucinol alone showed a higher decrease in H_2 emissions than those fed a co-supplementation of CH_4 inhibitor with phloroglucinol, which suggests that further optimisation of this nutritional strategy may still be possible (Romero et al., 2024). The response of H_2 emissions in dairy goats (Romero et al., 2024) and lactating dairy cattle (Maigaard et al., 2024b) from co-supplementation with phloroglucinol, is a proof of concept and suggests that combining CH_4 inhibitor and phloroglucinol as dietary supplements can

be used across different ruminant species, although this would need further confirmation using animal experiments in different production settings. Future research is warranted to elucidate the H_2 transfer pathways and ecological dynamics within the rumen microbiome. This research should focus on the molecular interactions between alternative microbial H_2 consumers during supplementation of an alternative electron acceptor (e.g., phloroglucinol or any potential additive), with and without a CH_4 inhibitor. This requires using multi-omics analyses, such as metagenomics and metatranscriptomics, to allow precise identification of microbial communities and characterisation of genes for hydrogenases and other enzymes involved in H_2 utilisation within the rumen. These investigations will provide critical insights to engineer robust microbial communities that can sustainably metabolise phloroglucinol to redirect H_2 flow when methanogenesis is inhibited.

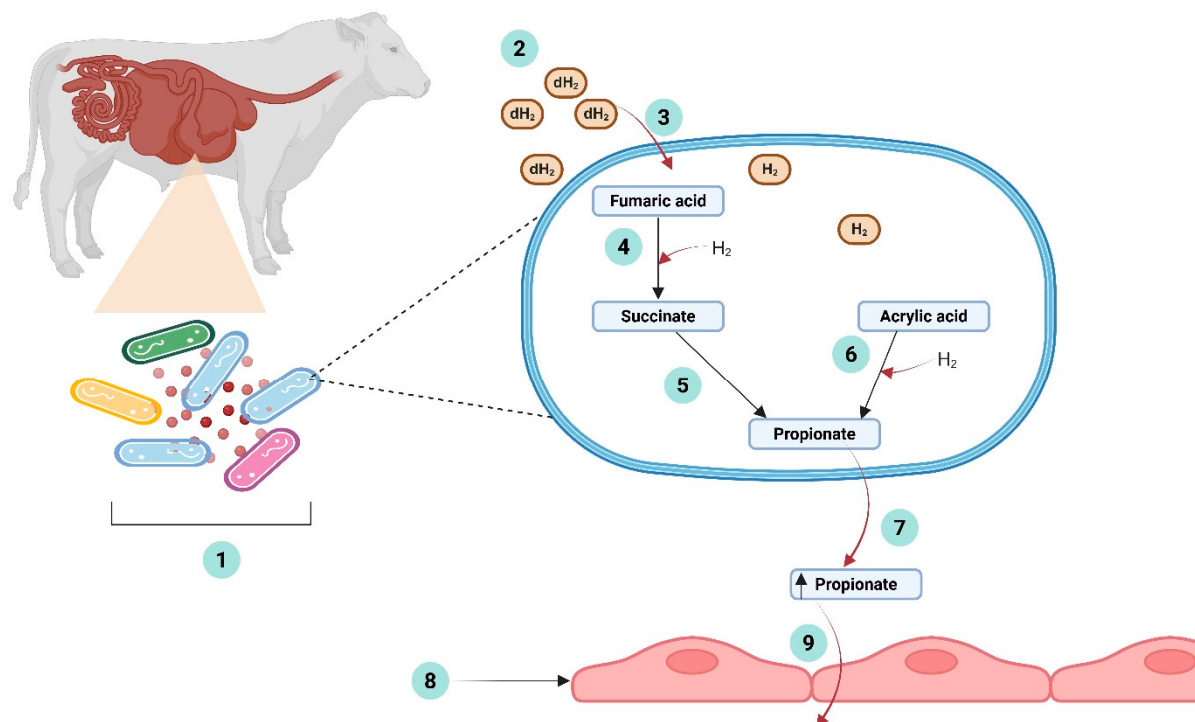


Figure 1. A schematic illustrating the pathways involved in the degradation of fumaric acid and acrylic acid, highlighting the redirection of excess dihydrogen by organic acid-utilising bacteria during the inhibition of methanogenesis in the rumen. (1) Microbial degradation of fumaric acid and acrylic acid by organic acid-utilising bacteria in the rumen. (2) Increased concentrations of dissolved dihydrogen (dH_2) during the inhibition of methanogenesis. (3) Capture of excess dihydrogen (H_2) for the reduction of organic acids (fumaric acid and acrylic acid) by ruminal bacteria. (4) Reduction of fumaric acid to succinate using incorporated dihydrogen (H_2) as an electron donor by organic acid-utilising bacteria in the rumen. (5) Decarboxylation of succinate to propionate via succinate-propionate pathway. (6) Reduction of acrylic acid to propionate using incorporated dihydrogen (H_2) as an electron donor, via the acrylate pathway. (7) Increased propionate production. (8) Rumen wall. (9) Absorption of propionate through the rumen wall. Created in BioRender: <https://BioRender.com/cmqqasgs>.

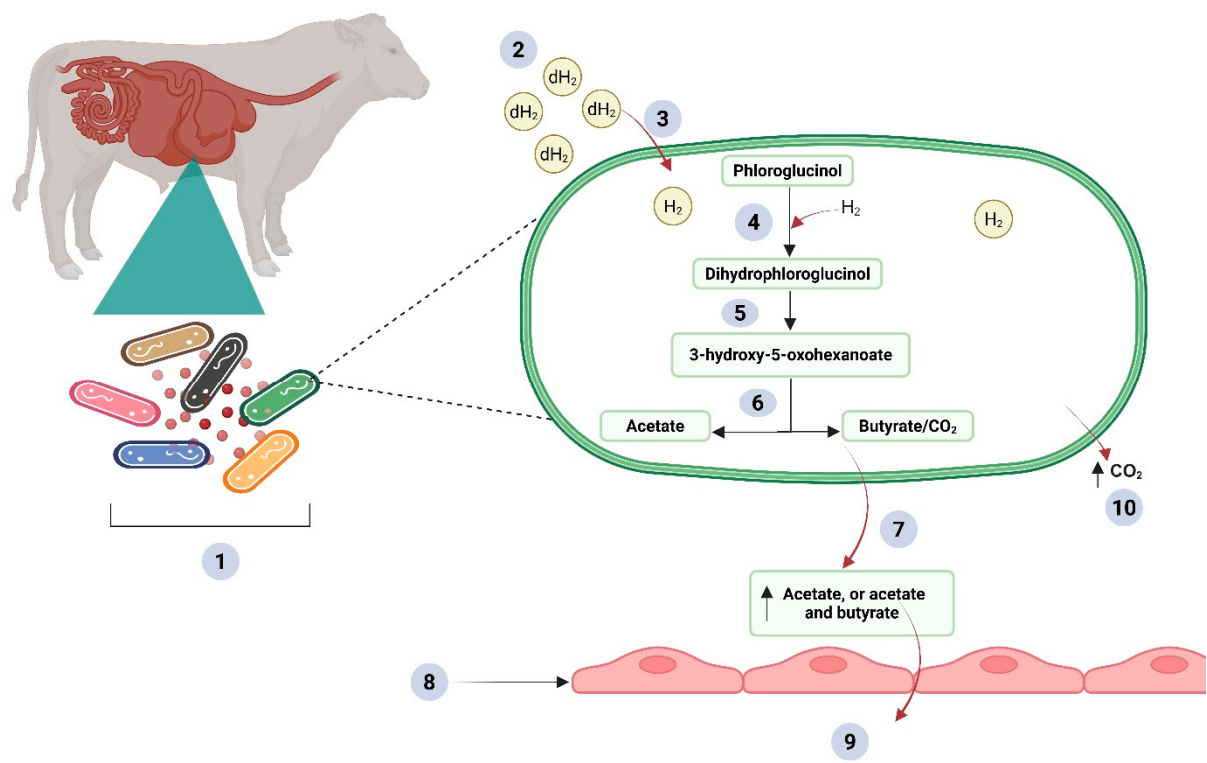


Figure 2. A schematic illustrating the pathways involved in the degradation of phloroglucinol, highlighting the redirection of excess dihydrogen by phloroglucinol-utilising bacteria during the inhibition of methanogenesis in the rumen. (1) Microbial degradation of phloroglucinol by bacteria in the rumen. (2) Increased concentrations of dissolved dihydrogen (dH₂) during the inhibition of methanogenesis. (3) Capture of excess dihydrogen (H₂) for the reduction of phloroglucinol by ruminal bacteria. (4) Reduction of phloroglucinol to dihydrophloroglucinol using incorporated dihydrogen (H₂) as an electron donor, catalysed by NADPH-dependent short-chain dehydrogenases/reductases. (5) Hydrolysis of dihydrophloroglucinol to 3-hydroxy-5-oxohexanoate. (6) Conversion of 3-hydroxy-5-oxohexanoate primarily results in acetate and carbon dioxide (CO₂) production when degraded by *Coprococcus* spp., or acetate and butyrate when degraded by *Eubacterium oxidoreducens*. (7) Increased acetate or acetate and butyrate production. (8) Rumen wall. (9) Absorption of acetate or acetate and butyrate through the rumen wall. (10) Increased carbon dioxide (CO₂) production. Created in BioRender: <https://BioRender.com/cfd68sv>.

Conclusions

Anti-methanogenic feed additive inhibitors effectively reduce enteric CH₄ emissions but often lead to H₂ accumulation, which can impair rumen fermentation and animal performance. Thus, an approach to stimulate enteric microbial groups capable of capturing excess H₂ via alternative energy-yielding metabolic pathways in the rumen should be considered when inhibiting CH₄ emissions from livestock. Dietary co-supplementation with alternative electron acceptors such as phloroglucinol, fumaric acid, or acrylic acid offers a promising strategy to redirect excess H₂ into beneficial fermentation products during methanogenesis inhibition. However, outcomes vary across studies due to differences in additive combinations, dosages, and adaptation periods. Phloroglucinol used as a co-supplement with *Asparagopsis*, or 3-NOP shows strong potential to reduce H₂ emissions and improve VFA profiles *in vivo*. Future research should explore long-term microbial adaptation, and the use of multi-omics analyses to gain a comprehensive understanding of the relationship between electron sink-utilising bacteria abundances and responses in the rumen, with the aim of optimising H₂ utilisation pathways through the co-supplementation of alternative H₂ acceptors. Developing a

tailored supplementation strategy of a CH₄ inhibitor with an alternative H₂ acceptor to methanogenesis could enhance CH₄ mitigation while preserving or improving animal health and feed efficiency. While co-supplementation with alternative H₂ acceptors, particularly phloroglucinol, may enhance mitigation of enteric CH₄ emissions in ruminant livestock, the cost of the quantities required to achieve a robust daily reduction in methanogenesis will be impractical for on-farm adoption.

Author Contributions: Conceptualisation: Ibrahim Ahmad; methodology, data curation, and writing-original draft preparation, Ibrahim Ahmad; writing-review and editing, Ibrahim Ahmad, Richard P. Rawnsley, John P. Bowman, Rohan Borojevic, and Apeh A. Omede, supervision, Richard P. Rawnsley, John P. Bowman, and Apeh A. Omede; funding acquisition, Richard P. Rawnsley. All authors have read and agreed to the published version of the manuscript.

Funding: This project is supported by the Department of Natural Resources and Environment Tasmania Agricultural Development Fund, Hobart, Australia. Ibrahim Ahmad also receives a Grant in the form of a top-up stipend from the Tim Healey Memorial Scholarship, awarded by The AW Howard Memorial Trust Inc., in recognition of his contributions to promoting sustainable agriculture in Australia (UTAS Ref: 00030728).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing is not applicable as no new data were generated or analysed during this study. .

Conflicts of Interest: The authors declare that they have no conflicts of interest.

References

Amgarten, M., Schatzmann, H. J., & Wuthrich, A. (1981). ‘Lactate type’ response of ruminal fermentation to chloral hydrate, chloroform and trichloroethanol. *Journal of Veterinary Pharmacology and Therapeutics*, 4(3), 241–248. <https://doi.org/10.1111/j.1365-2885.1981.tb00736.x>

Arndt, C., Hristov, A. N., Price, W. J., McClelland, S. C., Pelaez, A. M., Cueva, S. F., Oh, J., Dijkstra, J., Bannink, A., Bayat, A. R., Crompton, L. A., Eugène, M. A., Enahoro, D., Kebreab, E., Kreuzer, M., McGee, M., Martin, C., Newbold, C. J., Reynolds, C. K., ... Yu, Z. (2022). Full adoption of the most effective strategies to mitigate methane emissions by ruminants can help meet the 1.5 °C target by 2030 but not 2050. *Proceedings of the National Academy of Sciences*, 119(20). <https://doi.org/10.1073/pnas.2111294119>

Asanuma, N., Iwamoto, M., & Hino, T. (1999). Effect of the Addition of Fumarate on Methane Production by Ruminal Microorganisms In Vitro. *Journal of Dairy Science*, 82(4), 780–787. [https://doi.org/10.3168/jds.S0022-0302\(99\)75296-3](https://doi.org/10.3168/jds.S0022-0302(99)75296-3)

Battelli, M., Maigaard, M., Lashkari, S., Nørskov, N. P., Weisbjerg, M. R., & Nielsen, M. O. (2025). Combination of methane-inhibitors and hydrogen-acceptors: effects on in vitro rumen fermentation. *Italian Journal of Animal Science*, 24(1), 2029–2040. <https://doi.org/10.1080/1828051X.2025.2555527>

Battelli, M., Nielsen, M. O., & Nørskov, N. P. (2023). Dose- and substrate-dependent reduction of enteric methane and ammonia by natural additives in vitro. *Frontiers in Veterinary Science*, 10. <https://doi.org/10.3389/fvets.2023.1302346>

Beauchemin, K. A., Ungerfeld, E. M., Abdalla, A. L., Alvarez, C., Arndt, C., Becquet, P., Benchaar, C., Berndt, A., Mauricio, R. M., McAllister, T. A., Oyhantçabal, W., Salami, S. A., Shalloo, L., Sun, Y., Tricarico, J., Uwizeye, A., De Camillis, C., Bernoux, M., Robinson, T., & Kebreab, E. (2022). Invited review: Current enteric methane mitigation options. *Journal of Dairy Science*, 105(12), 9297–9326. <https://doi.org/10.3168/jds.2022-22091>

Belanche, A., Bannink, A., Dijkstra, J., Durmic, Z., Garcia, F., Santos, F. G., Huws, S., Jeyanathan, J., Lund, P., Mackie, R. I., McAllister, T. A., Morgavi, D. P., Muetzel, S., Pitta, D. W., Yáñez-Ruiz, D. R., & Ungerfeld, E. M. (2025). Feed additives for methane mitigation: A guideline to uncover the mode of action of

- antimethanogenic feed additives for ruminants. *Journal of Dairy Science*, 108(1), 375–394. <https://doi.org/10.3168/jds.2024-25046>
- Berger, L. M., Blank, R., Zorn, F., Wein, S., Metges, C. C., & Wolfram, S. (2015). Ruminal degradation of quercetin and its influence on fermentation in ruminants. *Journal of Dairy Science*, 98(8), 5688–5698. <https://doi.org/10.3168/jds.2015-9633>
- Chen, G. J., & Russell, J. B. (1988). Fermentation of peptides and amino acids by a monensin-sensitive ruminal *Peptostreptococcus*. *Applied and Environmental Microbiology*, 54(11), 2742–2749. <https://doi.org/10.1128/aem.54.11.2742-2749.1988>
- Choudhury, P. K., Salem, A. Z. M., Jena, R., Kumar, S., Singh, R., & Puniya, A. K. (2015). Rumen Microbiology: An Overview. In *Rumen Microbiology: From Evolution to Revolution* (pp. 3–16). Springer India. https://doi.org/10.1007/978-81-322-2401-3_1
- Conradt, D., Hermann, B., Gerhardt, S., Einsle, O., & Müller, M. (2016). Biocatalytic Properties and Structural Analysis of Phloroglucinol Reductases. *Angewandte Chemie International Edition*, 55(50), 15531–15534. <https://doi.org/10.1002/anie.201607494>
- Cowley, F. C., Kinley, R. D., Mackenzie, S. L., Fortes, M. R. S., Palmieri, C., Simanungkalit, G., Almeida, A. K., & Roque, B. M. (2024). Bioactive metabolites of *Asparagopsis* stabilized in canola oil completely suppress methane emissions in beef cattle fed a feedlot diet. *Journal of Animal Science*, 102. <https://doi.org/10.1093/jas/skae109>
- del Prado, A., Vibart, R. E., Bilotto, F. M., Faverin, C., Garcia, F., Henrique, F. L., Leite, F. F. G. D., Mazzetto, A. M., Ridoutt, B. G., Yáñez-Ruiz, D. R., & Bannink, A. (2025). Feed additives for methane mitigation: Assessment of feed additives as a strategy to mitigate enteric methane from ruminants—Accounting; How to quantify the mitigating potential of using antimethanogenic feed additives. *Journal of Dairy Science*, 108(1), 411–429. <https://doi.org/10.3168/jds.2024-25044>
- Duin, E. C., Wagner, T., Shima, S., Prakash, D., Cronin, B., Yáñez-Ruiz, D. R., Duval, S., Rumbeli, R., Stemmler, R. T., Thauer, R. K., & Kindermann, M. (2016). Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proceedings of the National Academy of Sciences*, 113(22), 6172–6177. <https://doi.org/10.1073/pnas.1600298113>
- Durmic, Z., Duin, E. C., Bannink, A., Belanche, A., Carbone, V., Carro, M. D., Crüsemann, M., Fievez, V., Garcia, F., Hristov, A., Joch, M., Martinez-Fernandez, G., Muetzel, S., Ungerfeld, E. M., Wang, M., & Yáñez-Ruiz, D. R. (2025). Feed additives for methane mitigation: Recommendations for identification and selection of bioactive compounds to develop antimethanogenic feed additives. *Journal of Dairy Science*, 108(1), 302–321. <https://doi.org/10.3168/jds.2024-25045>
- Glasson, C. R. K., Kinley, R. D., de Nys, R., King, N., Adams, S. L., Packer, M. A., Svenson, J., Eason, C. T., & Magnusson, M. (2022). Benefits and risks of including the bromoform containing seaweed *Asparagopsis* in feed for the reduction of methane production from ruminants. *Algal Research*, 64, 102673. <https://doi.org/10.1016/j.algal.2022.102673>
- Gunsalus, R. P., Romesser, J. A., & Wolfe, R. S. (1978). Preparation of coenzyme M analogs and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. *Biochemistry*, 17(12), 2374–2377. <https://doi.org/10.1021/bi00605a019>
- Honan, M., Feng, X., Tricarico, J. M., & Kebreab, E. (2021). Feed additives as a strategic approach to reduce enteric methane production in cattle: modes of action, effectiveness and safety. *Animal Production Science*, 62(14), 1303–1317. <https://doi.org/10.1071/AN20295>
- Huang, R., Romero, P., Belanche, A., Ungerfeld, E. M., Yanez-Ruiz, D., Morgavi, D. P., & Popova, M. (2023). Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited in vitro – Part 1. Dairy cows. *Animal*, 17(5), 100788. <https://doi.org/10.1016/j.animal.2023.100788>
- Kim, E. T., Guan, L. L., Lee, S. J., Lee, S. M., Lee, S. S., Lee, I. D., Lee, S. K., & Lee, S. S. (2015). Effects of Flavonoid-rich Plant Extracts on In vitro Ruminal Methanogenesis, Microbial Populations and Fermentation Characteristics. *Asian-Australasian Journal of Animal Sciences*, 28(4), 530–537. <https://doi.org/10.5713/ajas.14.0692>
- Kjeldsen, M. H., Weisbjerg, M. R., Larsen, M., Højberg, O., Ohlsson, C., Walker, N., Hellwing, A. L. F., & Lund, P. (2024). Gas exchange, rumen hydrogen sinks, and nutrient digestibility and metabolism in lactating dairy

- cows fed 3-nitrooxypropanol and cracked rapeseed. *Journal of Dairy Science*, 107(4), 2047–2065. <https://doi.org/10.3168/jds.2023-23743>
- Krizsan, S. J., Ramin, M., Chagas, J. C. C., Halmemies-Beauchet-Filleau, A., Singh, A., Schnürer, A., & Danielsson, R. (2023). Effects on rumen microbiome and milk quality of dairy cows fed a grass silage-based diet supplemented with the macroalga *Asparagopsis taxiformis*. *Frontiers in Animal Science*, 4. <https://doi.org/10.3389/fanim.2023.1112969>
- Krumholz, L. R., & Bryant, M. P. (1986). *Eubacterium oxidoreducens* sp. nov. requiring H₂ or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. *Archives of Microbiology*, 144(1), 8–14. <https://doi.org/10.1007/BF00454948>
- Krumholz, L. R., Crawford, R. L., Hemling, M. E., & Bryant, M. P. (1987). Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreducens* via 3-hydroxy-5-oxohexanoate. *Journal of Bacteriology*, 169(5), 1886–1890. <https://doi.org/10.1128/jb.169.5.1886-1890.1987>
- Lan, W., & Yang, C. (2019). Ruminant methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Science of The Total Environment*, 654, 1270–1283. <https://doi.org/10.1016/j.scitotenv.2018.11.180>
- Li, Z., Liu, N., Cao, Y., Jin, C., Li, F., Cai, C., & Yao, J. (2018). Effects of fumaric acid supplementation on methane production and rumen fermentation in goats fed diets varying in forage and concentrate particle size. *Journal of Animal Science and Biotechnology*, 9(1), 21. <https://doi.org/10.1186/s40104-018-0235-3>
- Liu, L., Xu, X., Cao, Y., Cai, C., Cui, H., & Yao, J. (2017). Nitrate decreases methane production also by increasing methane oxidation through stimulating NC10 population in ruminal culture. *AMB Express*, 7(1), 76. <https://doi.org/10.1186/s13568-017-0377-2>
- Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., & Tomkins, N. (2016). Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in vitro. *Journal of Applied Phycology*, 28(5), 3117–3126. <https://doi.org/10.1007/s10811-016-0830-7>
- Mackie, R. I., Kim, H., Kim, N. K., & Cann, I. (2024). — Invited Review — Hydrogen production and hydrogen utilization in the rumen: key to mitigating enteric methane production. *Animal Bioscience*, 37(2), 323–336. <https://doi.org/10.5713/ab.23.0294>
- Maigaard, M., Weisbjerg, M. R., Johansen, M., Walker, N., Ohlsson, C., & Lund, P. (2024a). Effects of dietary fat, nitrate, and 3-nitrooxypropanol and their combinations on methane emission, feed intake, and milk production in dairy cows. *Journal of Dairy Science*, 107(1), 220–241. <https://doi.org/10.3168/jds.2023-23420>
- Maigaard, M., Weisbjerg, M. R., Hellwing, A. L. F., Larsen, M., Andersen, F. B., & Lund, P. (2024b). The acute effects of rumen pulse-dosing of hydrogen acceptors during methane inhibition with nitrate or 3-nitrooxypropanol in dairy cows. *Journal of Dairy Science*. <https://doi.org/10.3168/jds.2023-24343>
- Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M., & McSweeney, C. S. (2016). Methane Inhibition Alters the Microbial Community, Hydrogen Flow, and Fermentation Response in the Rumen of Cattle. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.01122>
- McAllister, T. A., & Newbold, C. J. (2008). Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture*, 48(2), 7. <https://doi.org/10.1071/EA07218>
- Melgar, A., Harper, M. T., Oh, J., Giallongo, F., Young, M. E., Ott, T. L., Duval, S., & Hristov, A. N. (2020). Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. *Journal of Dairy Science*, 103(1), 410–432. <https://doi.org/10.3168/jds.2019-17085>
- Newbold, C. J., López, S., Nelson, N., Ouda, J. O., Wallace, R. J., & Moss, A. R. (2005). Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. *British Journal of Nutrition*, 94(1), 27–35. <https://doi.org/10.1079/BJN20051445>
- Olijhoek, D. W., Hellwing, A. L. F., Brask, M., Weisbjerg, M. R., Højberg, O., Larsen, M. K., Dijkstra, J., Erlandsen, E. J., & Lund, P. (2016). Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *Journal of Dairy Science*, 99(8), 6191–6205. <https://doi.org/10.3168/jds.2015-10691>
- Reddy, P. R. K., & Hyder, I. (2023). Ruminant Digestion. In *Textbook of Veterinary Physiology* (pp. 353–366). Springer Nature Singapore. https://doi.org/10.1007/978-981-19-9410-4_14

- Romero, P., Huang, R., Jiménez, E., Palma-Hidalgo, J. M., Ungerfeld, E. M., Popova, M., Morgavi, D. P., Belanche, A., & Yáñez-Ruiz, D. R. (2023). Evaluating the effect of phenolic compounds as hydrogen acceptors when ruminal methanogenesis is inhibited in vitro – Part 2. Dairy goats. *Animal*, 17(5), 100789. <https://doi.org/10.1016/j.animal.2023.100789>
- Romero, P., Ungerfeld, E. M., Popova, M., Morgavi, D. P., Yáñez-Ruiz, D. R., & Belanche, A. (2024). Exploring the combination of *Asparagopsis taxiformis* and phloroglucinol to decrease rumen methanogenesis and redirect hydrogen production in goats. *Animal Feed Science and Technology*, 316, 116060. <https://doi.org/10.1016/j.anifeedsci.2024.116060>
- Russell, J. B., & Wallace, R. J. (1997). Energy-yielding and energy-consuming reactions. In P. N. Hobson & C. S. Stewart (Eds.), *The Rumen Microbial Ecosystem* (pp. 246–282). Springer Netherlands. https://doi.org/10.1007/978-94-009-1453-7_6
- Thorsteinsson, M., Lund, P., Weisbjerg, M. R., Noel, S. J., Schönherz, A. A., Hellwing, A. L. F., Hansen, H. H., & Nielsen, M. O. (2023a). Enteric methane emission of dairy cows supplemented with iodoform in a dose-response study. *Scientific Reports*, 13(1), 12797. <https://doi.org/10.1038/s41598-023-38149-y>
- Thorsteinsson, M., Maigaard, M., Lund, P., Weisbjerg, M. R., & Nielsen, M. O. (2023b). Effect of fumaric acid in combination with *Asparagopsis taxiformis* or nitrate on in vitro gas production, pH, and redox potential. *JDS Communications*, 4(5), 335–339. <https://doi.org/10.3168/jdsc.2022-0259>
- Tsai, C.-G., Gates, D. M., Ingledew, W. M., & Jones, G. A. (1976). Products of anaerobic phloroglucinol degradation by *Coprococcus* sp. Pe15. *Canadian Journal of Microbiology*, 22(2), 159–164. <https://doi.org/10.1139/m76-022>
- Tsai, C.-G., & Jones, G. A. (1975). Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. *Canadian Journal of Microbiology*, 21(6), 794–801. <https://doi.org/10.1139/m75-117>
- Ungerfeld, E. M. (2013). A theoretical comparison between two ruminal electron sinks. *Frontiers in Microbiology*, 4. <https://doi.org/10.3389/fmicb.2013.00319>
- Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Frontiers in Microbiology*, 6. <https://doi.org/10.3389/fmicb.2015.00037>
- Ungerfeld, E. M. (2020). Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, 11. <https://doi.org/10.3389/fmicb.2020.00589>
- Ungerfeld, E. M., Beauchemin, K. A., & Muñoz, C. (2022). Current Perspectives on Achieving Pronounced Enteric Methane Mitigation From Ruminant Production. *Frontiers in Animal Science*, 2. <https://doi.org/10.3389/fanim.2021.795200>
- Ungerfeld, E. M., Kohn, R. A., Wallace, R. J., & Newbold, C. J. (2007). A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *Journal of Animal Science*, 85(10), 2556–2563. <https://doi.org/10.2527/jas.2006-674>
- van Lingen, H. J., Plugge, C. M., Fadel, J. G., Kebreab, E., Bannink, A., & Dijkstra, J. (2016). Thermodynamic Driving Force of Hydrogen on Rumen Microbial Metabolism: A Theoretical Investigation. *PLOS ONE*, 11(10), e0161362. <https://doi.org/10.1371/journal.pone.0161362>
- van Zijderveld, S. M., Gerrits, W. J. J., Dijkstra, J., Newbold, J. R., Hulshof, R. B. A., & Perdok, H. B. (2011). Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *Journal of Dairy Science*, 94(8), 4028–4038. <https://doi.org/10.3168/jds.2011-4236>
- Wasson, D. E., Yarish, C., & Hristov, A. N. (2022). Enteric methane mitigation through *Asparagopsis taxiformis* supplementation and potential algal alternatives. *Frontiers in Animal Science*, 3. <https://doi.org/10.3389/fanim.2022.999338>
- Wolin, M. J. (1979). *The Rumen Fermentation: A Model for Microbial Interactions in Anaerobic Ecosystems* (pp. 49–77). https://doi.org/10.1007/978-1-4615-8279-3_2

Wolin, M. J., Miller, T. L., & Stewart, C. S. (1997). Microbe-microbe interactions. In *The Rumen Microbial Ecosystem* (pp. 467–491). Springer Netherlands. https://doi.org/10.1007/978-94-009-1453-7_11

Wood, J. M., Kennedy, F. Scott., & Wolfe, R. S. (1968). Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry*, 7(5), 1707–1713. <https://doi.org/10.1021/bi00845a013>

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.