Assessment of molasses-based additives for methane mitigation in beef cattle

Maria Nikoloric

A thesis submitted in fulfil requirements for the degree of Master of Philosophy (Science)

School of Life and Environmental Sciences

The University of Sydney

2026

"This research reported in this thesis was supported by the award of a Research Training Program scholarship to the MPhil Candidate."

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Maria Nikoloric

Abstract

Enteric methane (CH_4) mitigation is a priority for pasture-based beef systems, where practical delivery of antimethanogenic additives to grazing cattle remains a key constraint. In Australia, enteric methane contributes a substantial share of the agricultural emissions profile, underscoring the need for scalable solutions suited to extensive systems. Molasses lick blocks (MLB) have been proposed as a feasible vehicle to regulate intake and enable more consistent dosing in grazing ruminants.

This thesis aimed to evaluate novel molasses-based additive formulations intended for incorporation into molasses lick blocks (MLBs) by (i) screening their in vitro antimethanogenic effects and (ii) quantifying in vivo responses in growing beef cattle over a 70-day feeding period. Specific ingredient identities are not disclosed due to commercial confidentiality and pending intellectual property protection; therefore, emphasis is placed on evaluating the performance of the formulated products rather than their proprietary components.

In vitro, four candidate formulations were assessed for their effects on rumen fermentation and methane production. One formulation ("Product 3") displayed the strongest response. At 6% inclusion, CH_4 production was reduced by >90%, although this was accompanied by reduced in vitro dry matter digestibility. Additional testing showed that at 4% inclusion, Product 3 reduced CH_4 production by 62% without impairing digestibility, highlighting the importance of dose optimisation.

The in vivo experiment evaluated Product 3 delivered as a grain-based pellet to growing steers over 70 days, with target intakes of 0, 100, or 200 g per head per day. Methane emissions were measured using a GreenFeed system. Compared with steers receiving pellets without the product, CH_4 production tended to decrease by up to 10.7% ($P = 0.07$), CH_4 yield (g CH_4/kg DMI) was reduced ($P < 0.01$) by 11.7% and 7.5% at 100 and 200 g/day, respectively, and CH_4 intensity tended ($P = 0.06$) to decline by 16.8%. No detrimental effects were detected on dry matter intake, liveweight gain, or feed efficiency. Product intake was negatively correlated with CH_4 yield and intensity and positively correlated with water intake and average daily gain ($P < 0.05$), indicating acceptable palatability and compatibility with forage-based diets.

Collectively, these findings demonstrate a clear dose-sensitive antimethanogenic effect for Product 3, with >90% inhibition observed in vitro and approximately a 12% reduction in methane emissions in vivo, achieved without compromising animal performance. Several factors, however, limit direct extrapolation of these results to grazing systems. These include the additive stability and the structured feeding environment imposed by the experimental design.

In conclusion, Product 3 reduced methane yield by up to 11.7% in vivo over 56 days without adverse effects on intake or growth and achieved >90% inhibition in vitro at high inclusion, with substantial reductions at lower doses that preserved fermentation. These findings identify a promising dietary strategy for low-emission beef production while emphasising the need for optimisation of inclusion rates, assessment of response persistence, and confirmation of performance using final MLBs in grazing herds.

Acknowledgments

I would like to express my sincere gratitude to my supervisors, Professor Chaves, A. V. and Professor González, L.A., for their invaluable guidance, support, and constructive feedback throughout the course of this research. Their expertise, encouragement, and patience were essential to the development and completion of this thesis.

I am also grateful to the entire research team for their assistance, collaboration and willingness to share their knowledge, which greatly enriched this work.

Finally, I would like to thank my family for their constant support, understanding, and encouragement throughout my studies. Their belief in me made this achievement possible.

Author Attribution Statement

I certify that this thesis is the result of my own work. I was primarily responsible for the research ideas, organisation, data analysis, and writing of all chapters and publications included in this thesis. The work was conducted independently under the supervision of Professor A. V. Chaves, Professor L. A. González, and Dr G. I. Simanungkalit.

Chapter 2 has been published in a peer-reviewed journal (Frontiers in Animal Science), and the published version is included in this thesis. All contributions by others to the research and preparation of this thesis have been appropriately acknowledged, either through authorship on the publication or in the acknowledgements section.

I certify that the intellectual content of this thesis is original and, to the best of my knowledge and belief, contains no material previously submitted, in whole or in part, for the award of any other degree or diploma at this or any other tertiary institution.

Maria Nikoloric

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Professor Chaves, A. V.

Generative AI Statement

During the preparation of the thesis, the author used ChatGPT to assist in the understanding of data analysis methods and Microsoft Copilot to support improvements in grammar, spelling, and overall readability, as well as to enhance the clarity and quality of the manuscript redaction. The author confirms that no text was directly generated or modified by generative AI during the preparation of the thesis. The author takes full responsibility for the submitted thesis, ensures the work is their own, and has used generative AI in accordance with university guidelines and policies (refer to the University of Sydney generative AI guide for researchers).

Grants

Postgraduate Scholarship in Livestock Production (2022)

We are extremely appreciative of the financing provided by the University of Sydney, the Commonwealth Government of Australia, and Meat and Livestock Australia. Their assistance during the entire investigation was essential to the accomplishment of our study.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

Contents

List of Tables and Figures

Tables

Chapter 1:

Chapter 2:

Figures

Chapter 2:

Abbreviations

The following abbreviations are used throughout this thesis and have been defined at first use in each chapter.

A

ADF – Acid Detergent Fibre

ADG – Average Daily Gain

A:P – Acetate-to-propionate ratio

B

BCVFA – Branched-chain volatile fatty acids

Bovaer® – Commercial formulation of 3-NOP

C

CH_4 – Methane

CP – Crude Protein

CO_2 – Carbon Dioxide

CT – Condensed Tannins

D

DM – Dry Matter

DMD – Dry Matter Digestibility

DMI – Dry Matter Intake

DHA – Docosahexaenoic Acid

DFM – Direct-Fed Microbials

E

EID – Electronic Identification Number

EFSA – European Food Safety Authority

EO – Essential Oils

EPA – Eicosapentaenoic Acid

e.g. – For example

F

FAO – Food and Agriculture Organisation

F:C ratio – Forage-to-Concentrate Ratio

G

GC–MS – Gas Chromatography–Mass Spectrometry

GHG – Greenhouse Gases

GreenFeed® – Automated gas measurement system

H

H_2 – Hydrogen

HT – Hydrolysable Tannins

HP5-MS – GC column type

I

IVDMD – In vitro dry matter digestibility

In vivo – Experiments performed in live animals

In vitro – Experiments performed outside the organism

Intergado® – Electronic feeding system

L

LW – Live Weight

M

MCFA – Medium-Chain Fatty Acids

MCR – Methyl-coenzyme M Reductase

MLB – Molasses Lick Blocks

MPhil – Master of Philosophy

MRV – Measurement, Reporting and Verification

O

OM – Organic Matter

OMI – Organic Matter Intake

O_2 – Oxygen

P

P – Statistical significance (probability value)

PUFA – Polyunsaturated Fatty Acids

PTV – Programmed Temperature Vaporisation inlet

R

RFID – Radio-frequency identification

Rusitec – Rumen Simulation Technique

S

SRB – Sulphate-Reducing Bacteria

T

TCA cycle – Tricarboxylic Acid Cycle

TDU – Thermal Desorption Unit

Tenax TA – Adsorbent polymer for GC-MS

3-NOP – 3-Nitrooxypropanol

V

VFA – Volatile Fatty Acids

W

WSC – Water-Soluble Carbohydrates

Introduction

Enteric methane emitted by ruminant livestock is a major contributor to agricultural greenhouse gas emissions and remains a significant constraint for climate-smart livestock production (Beauchemin et al., 2022). In countries with large grazing-based cattle industries, such as Australia, enteric methane accounts for about 10% of national agricultural emissions (Greenwood et al., 2018) and represents an energy loss to the animal (Johnson and Johnson, 1995; Morgavi et al., 2010). Because methane is produced by ruminal anaerobic fermentation, particularly via methanogenic archaea, effective mitigation strategies must reduce emissions without disrupting rumen function, animal performance, or welfare (Hristov et al., 2022).

Nutritional manipulation has emerged as one of the most practical approaches for methane mitigation because dietary interventions can directly influence rumen fermentation pathways without requiring major changes in infrastructure, genetics, or management (Hristov et al., 2013). A wide range of dietary compounds, including synthetic inhibitors (e.g., 3-nitrooxypropanol), lipids, plant secondary metabolites, probiotics, enzymes and electron acceptors have been investigated for their capacity to modify fermentation and reduce methanogenesis (Beauchemin et al., 2022). Among these, plant-derived compounds such as essential oils, tannins, and saponins have attracted significant interest due to their natural origin, potential safety, and consumer acceptance (Patra and Saxena, 2011). These compounds can inhibit methanogens, modify protozoa, and redirect hydrogen towards alternative metabolic sinks (Benchaar and Greathead, 2011). Essential oils (EO) in particular, have shown potential to alter rumen fermentation and improve feed efficiency (Calsamiglia et al., 2007), and the combinations of EO may also increase microbial efficacy due to additive or synergistic effects between EO components (Benchaar and Greathead, 2011). Commercial essential-oil-based feed additives such as Crina® and Agolin® Ruminant have been developed to provide standardised blends of bioactive plant compounds for livestock. These products have demonstrated modest methane-mitigating effects —typically in the range of 5–10%—in controlled feeding trials without compromising dry matter intake or animal performance (Belanche et al., 2020). However, translating these effects into consistent in vivo methane reductions remains challenging, as responses depend on diet composition, additive dose, the length of supplementation and the delivery method (Benchaar and Greathead, 2011), reflecting the broader inconsistency often observed in responses to plant secondary metabolites (Calsamiglia et al., 2007; Jayanegara et al., 2012).

One of the greatest challenges for methane mitigation in extensive grazing systems is the reliable and consistent delivery of feed additives (Beauchemin et al., 2022). Unlike feedlot systems, grazing cattle cannot be individually dosed, making controlled intake difficult to achieve. As a result, additives must be incorporated into supplements such as pellets, molasses mixes, or lick blocks (Hegarty et al., 2021). However, relatively few studies have evaluated the methane-reduction potential of bioactive compounds delivered through such practical feeding systems. Molasses lick blocks, in particular, have been proposed as a promising vehicle for additive delivery because they can regulate intake and provide a more consistent supplementation pattern under grazing conditions (Hristov et al., 2025).

This thesis proposes that incorporating methane-inhibiting additives into molasses lick blocks could provide a practical mitigation strategy for pasture-based beef systems. The primary objective was to assess the formulation of novel MLB-based products designed to improve cattle productivity while reducing enteric CH_4 emissions. These products contained known anti-methanogenic compounds; however, specific ingredients cannot be disclosed due to commercial confidentiality and patent restrictions. Therefore, the emphasis is placed on the performance of the formulated product rather than individual compounds. Importantly, this study evaluated the MLB-based formulations themselves—not hardened commercial lick blocks— which require further validation under grazing conditions.

Chapter 1 presents a literature review of safe dietary compounds with demonstrated or potential methane mitigation effects in ruminants. It examines the mechanisms by which these compounds influence rumen fermentation, hydrogen dynamics, methanogenic archaea, and overall fermentation pathways. Particular attention is given to the limitations and practical challenges associated with applying dietary strategies in grazing systems, including difficulty to measure intake, palatability concerns, and challenges in delivering consistent doses to free-ranging cattle. The literature review also identifies MLB technology as a promising vehicle for targeted additive delivery.

Chapter 2 evaluates four novel molasses-based additives formulations in vitro to assess their effects on fermentation and CH_4 production. Based on this result, the most effective formulation (Product 3) was subsequently incorporated into grain-based pellets and tested in vivo with growing steers. Results demonstrated reductions in methane yield and tendencies for lower CH_4 production and intensity, without adverse effects on dry matter intake or growth performance. These findings highlight Product 3 as a promising strategy; however, high inclusion levels posed potential risks for rumen fermentation, underscoring the need for dose optimisation. Chapter 3 provides a general discussion and conclusion that synthesises insights from both the literature and the experimental findings. It also highlights the limitations of the study and suggests possible directions for future research, along with the main conclusion of the thesis.

This thesis contributes to the growing body of work exploring nutritional strategies for low-emission beef production. Combining laboratory screening with controlled in vivo experimentation provides insight into the dose-dependent responses, practical constraints, and potential applications of molasses-based additive formulations. The finding also highlights areas requiring further investigation to explore higher dosages and extended feeding periods to better understand the trade-off between methane abatement, digestibility and overall productivity. It is also important to note that the products assessed were the formulations intended for manufacturing MLB, rather than the final commercial blocks. Therefore, the final blocks must be tested under grazing conditions to ensure optimal intake, followed by evaluating their effects on growth performance and methane emissions in practical production systems.

Chapter 1: Literature review on safe dietary compounds for mitigating enteric methane emissions in ruminants: mechanisms, efficacy, and future perspectives

1.1. Introduction

The search for safe, practical, and scalable strategies to mitigate enteric methane (CH_4) emissions has increasingly focused on dietary compounds that can be incorporated into conventional feeding systems. Among these, commercial feed additives have gained substantial attention because they can be integrated into existing production practices and provide measurable, reproducible reductions in CH_4 output (Beauchemin et al., 2022; Hristov et al., 2022). Prominent examples include the synthetic inhibitor 3-nitrooxypropanol (3-NOP; marketed as Bovaer®), which directly inhibits the methyl-coenzyme M reductase enzyme in methanogenic archaea (Hristov et al., 2022) and essential oil blends such as *Agolin®* and *Crina®*, which reduce the abundance of hydrogen-producing bacteria and protozoa that are symbiotically associated with methanogenic archaea (Benchaar et al., 2008). Other promising approaches include electron acceptors and alternative hydrogen sinks, lipid supplementation, and probiotic or enzyme formulations that redirect hydrogen metabolism toward alternative sinks such as propionate formation (Ku-Vera et al., 2020; Roques et al., 2024).

Although many of these additives have demonstrated methane-reducing potential, their efficacy and safety vary considerably depending on ruminant species, diet composition, management system, and environmental conditions (Hegarty et al., 2021; Miller et al., 2023). Furthermore, microbial adaptation and the long-term persistence of CH_4 mitigation remain key uncertainties that may affect durability of response (Beauchemin et al., 2022; Hristov et al., 2022). These challenges underscore the need for a comprehensive synthesis of recent evidence to determine which dietary compounds offer the most reliable and sustainable mitigation potential under commercial conditions.

This review provides an integrated assessment of safe dietary compounds for mitigating enteric CH_4 emissions in ruminants. Safety is defined based on the absence of adverse effects on animal health, feed intake, nutrient digestibility, rumen fermentation characteristics, and overall animal performance, as well as minimal negative impacts on the environment. Emphasis is placed on additives with demonstrated or emerging commercial applicability, comparing their mechanisms of action, efficacy, dose–response relationships, potential side effects on the animal and the environment, and regulatory or economic considerations (see Table 1). The review also highlights critical knowledge gaps and future research priorities to support the development of integrated, science-based, and sustainable strategies for methane abatement in diverse livestock systems.

1.2. Methanogenesis in the rumen

1.2.1. Rumen microbial ecology

The rumen is a specialised anaerobic fermentation chamber that hosts a complex and dynamic microbial ecosystem vital for digesting fibrous plant materials in ruminants. This ecosystem mainly consists of bacteria, archaea, protozoa, fungi, and viruses, which work together to break down structural carbohydrates such as cellulose and hemicellulose, as well as starch, proteins, and lipids (Morgavi et al., 2010; Huws et al., 2018). The fermentation process produces volatile fatty acids (VFA)—primary acetate, propionate, and butyrate—which are the major energy source for the host animal. It also generates gaseous by-products, including carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2).Under typical rumen conditions, CO_2 accounts for the majority of the gas produced (approximately 60–70%), followed by CH_4 (around 25–35%), while H_2 is present only in small amounts due to its rapid utilisation by methanogenic archaea (Hook et al., 2010).

Rumen bacteria group, represents nearly 50-75% of the total microbial population (Minato et al., 1966). They play key roles in carbohydrate degradation, proteolysis, and lipid metabolism. Among them, fibrolytic species such as *Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes* are responsible for the enzymatic hydrolysis of plant cell wall components, releasing sugars that are subsequently fermented to VFAs and gases. Other groups, such as amylolytic (*Streptococcus bovis*, *Selenomonas ruminantium*) and proteolytic bacteria (*Prevotella* spp.), specialise in starch and protein degradation, respectively.

Ciliate protozoa contribute significantly to feed particle breakdown and starch storage, but are also associated with the production of hydrogen and form close symbiotic relationships with methanogens (Finlay et al., 1994). Anaerobic fungi aid in fibre digestion by penetrating plant cell walls, facilitating bacterial access to structural polysaccharides (Gruninger et al., 2014). Bacteriophages and viruses regulate microbial populations through lytic and lysogenic interactions, influencing community stability and nutrient turnover (Anderson et al., 2017).

During fermentation, the production of acetate and butyrate by cellulolytic and saccharolytic by increasing the redox potential; therefore, efficient disposal of H_2 is critical for maintaining rumen fermentation balance. This disposal occurs through interspecies hydrogen transfer, where methanogenic archaea use H_2 to reduce CO_2 to CH_4 (Morgavi et al., 2010). Other microbes, including fumarate- or nitrate-reducing bacteria and propionate-producing species, can also serve as alternative hydrogen sinks, though methanogenesis is typically the dominant pathway under normal rumen conditions.

Methanogenic archaea and their roles

Methanogenic archaea represent approximately 1–4% of the rumen microbial population but play a pivotal role in maintaining anaerobic stability by consuming excess H_2. The predominant methanogens belong to the genera *Methanobrevibacter, Methanomicrobium, Methanosphaera,* and *Methanobacterium*, all of which utilise the CO_2 reduction pathway with H_2 as the primary electron donor (Janssen and Kirs, 2008; Hook et al., 2010). This pathway contributes to approximately 90% of total CH_4 formation, although minor contributions arise from the methylotrophic pathway, where methanol or methylamines serve as substrates (Poulsen et al., 2013). *Methanosphaera stadtmanae*, for instance, utilises methanol derived from pectin fermentation, producing CH_4 via the reaction.

Methanogens are closely associated with protozoa and fungal hyphae, often attaching to their surfaces or residing in symbiosomes, enabling efficient H_2 transfer (Finlay et al., 1994). These associations explain why defaunation (the removal of protozoa) can reduce CH_4 production in some systems, as it limits local H_2 availability to methanogens (Newbold et al., 2015).

Molecular studies using 16S rRNA sequencing and metagenomics have shown that *Methanobrevibacter ruminantium* and *Methanobrevibacter gottschalkii* clades dominate in cattle and sheep (Henderson et al., 2015). However, community composition can vary with diet, animal species, and geographical region, influencing CH_4 emission potential. For example, high-concentrate diets generally reduce total methanogen abundance due to lower rumen pH and greater propionate formation (Tapio et al., 2018).

Microbial interactions and methane yield

Methanogenesis is intricately linked to the fermentation pattern of the rumen. When acetate and butyrate are the dominant VFAs produced, more reducing equivalents (H_2) are generated, favouring CH_4 formation. In contrast, propionate production acts as a competitive hydrogen sink, consuming H_2 and thereby reducing CH_4 emissions (Ungerfeld, 2020). Therefore, dietary interventions that shift fermentation towards propionate generally achieve partial CH_4 mitigation.

The total CH_4 yield (g CH_4/kg dry matter intake) is thus a reflection of microbial community composition, substrate availability, and hydrogen flow dynamics. Maintaining a stable, functional microbiota is essential for animal performance; hence, any CH_4 mitigation strategy must balance the reduction of methanogenic activity with preservation of overall fermentation efficiency and nutrient digestibility (Hristov et al., 2013).

1.2.2. Biochemical pathways of methane production

Methanogenesis in the rumen is primarily driven by hydrogenotrophic archaea, which consume molecular H_2 generated during microbial fermentation of carbohydrates to reduce carbon dioxide CO_2 into methane CH_4, thereby maintaining low H_2 partial pressures and enabling efficient rumen fermentation (Hook et al., 2010; Ungerfeld, 2020). Alternative hydrogen sinks also contribute to ruminal H_2 metabolism, including reductive acetogenesis, propionate synthesis, and the reduction of nitrate or sulphate, which can divert H_2 from methanogenesis and reduce CH_4 emissions under specific dietary or microbial conditions (van Zijderveld et al., 2011). Emerging evidence indicates that interventions targeting these alternative hydrogen sinks—through dietary supplementation, feed additives, or microbial modulation—can effectively mitigate enteric methane emissions while maintaining or even enhancing fermentation efficiency and animal productivity (Beauchemin et al., 2022).

1.2.3. Factors influencing methane production

Diet composition

The composition and quality of the diet are among the most influential factors determining enteric CH_4 emissions in ruminants. Diets rich in structural carbohydrates, particularly cellulose and hemicellulose from forages, stimulate the production of acetate and butyrate during ruminal fermentation—volatile fatty acids that generate metabolic hydrogen as a by-product (Beauchemin et al., 2020). This H_2 serves as a key substrate for methanogenic archaea, facilitating methane synthesis via the hydrogenotrophic pathway. Consequently, high-forage diets typically lead to elevated methane emissions due to the dominance of fibre-degrading bacteria and acetogenic fermentation routes (Knapp et al., 2014).

In contrast, diets with a greater proportion of concentrates (starch-based feeds) tend to reduce methane output by enhancing propionate formation—an alternative hydrogen sink that competes directly with methanogens for available H_2 (Hristov et al., 2013; Kumar et al., 2014). High-concentrate feeding can lower ruminal pH, suppressing the activity of methanogenic archaea and protozoa while favouring amylolytic bacterial populations (Kumar et al., 2014). Also, excessive concentrate inclusion can increase the risk of ruminal acidosis and compromise animal health, emphasising the need for balanced diet formulations (Hristov et al., 2022).

Beyond basal diet manipulation, supplementation with targeted feed additives—such as plant secondary metabolites, lipids, or synthetic inhibitors—has demonstrated substantial mitigation potential. These compounds can inhibit methanogenesis directly or alter fermentation patterns to divert H_2 flow away from methane formation (Beauchemin et al., 2020). Thus, precision diet formulation integrating optimised nutrient composition and additive use represents a key systems-level strategy for sustainable methane abatement.

Animal species and feeding systems

Feeding systems further modulate methane output by influencing feed availability, rumen fermentation dynamics, and animal behaviour. Total mixed rations, which provide a consistent mixture of forages and concentrates, tend to promote stable rumen conditions and can reduce methane yield compared to systems where forages and concentrates are offered separately (Beauchemin et al., 2020). In contrast, grazing systems exhibit greater variability in methane emissions due to differences in forage maturity, nutrient composition, and seasonal feed availability. High-quality pastures with elevated digestibility can improve energy utilisation and reduce emissions per unit of product (Charmley et al., 2015). Pasture management strategies such as rotational grazing, maintaining optimal sward height, and supplementing with high-energy feeds can further reduce methane intensity through improved forage quality and increased animal productivity (Gerber et al., 2013a).

Beyond dietary and management influences, genetic variation among and within species represents an emerging avenue for methane mitigation. Heritability estimates for methane production on an absolute basis (g CH_4/day) are moderate—around 0.29 for sheep and 0.40 for cattle—suggesting that methane output has a genetic component that could be exploited through selective breeding (Pickering et al., 2015). However, when expressed relative to feed intake (g CH_4/kg dry matter intake, DMI), heritability declines substantially (0.13 - 0.19), indicating that methane yield is more strongly influenced by environmental and management factors (Pickering et al., 2015).

Despite the potential for genetic selection, practical challenges limit its application. Methane emissions fluctuate over time due to variation in DMI, feed fermentability, and animal physiological status, making it difficult to measure representative long-term phenotypes (Beauchemin et al., 2020).

Efforts are also underway to identify reliable proxies for CH_4 emissions that could enable large-scale genetic selection, including rumen microbial markers, milk fatty acid profiles, and specific metabolites (Negussie et al., 2017). However, the genetic correlations between CH_4 output and key production traits remain ambiguous. Breider et al. (2019) reported positive correlations between CH_4 production and milk yield, implying that animals genetically predisposed to higher productivity may also emit more methane. This relationship complicates the potential benefits of direct selection for lower methane output, as it could inadvertently reduce production efficiency. Consequently, indirect selection via productivity and feed efficiency traits may be a more practical route for mitigating emissions, since improving growth and milk yield typically reduces CH_4 intensity per unit of output (Beauchemin et al., 2020).

Overall, both feeding systems and genetic variation play critical roles in shaping methane emissions. While dietary strategies offer immediate and flexible mitigation potential, integrating genetic selection for feed efficiency and low-emitting phenotypes provides a long-term opportunity to achieve sustained reductions in enteric methane emissions across ruminant production systems.

Rumen pH, passage rate, and forage-to-concentrate ratio

Rumen environmental conditions, particularly pH, passage rate, and the forage-to-concentrate ratio, exert a profound influence on ruminal fermentation patterns and consequently on enteric methane production. Rumen pH plays a central regulatory role in shaping microbial community composition and activity. Optimal rumen pH (6.0–7.0) supports fibrolytic bacteria responsible for cellulose and hemicellulose degradation, leading to acetate production and higher hydrogen availability for methanogenesis (Kumar et al., 2014). When diets are rich in concentrates, increased fermentation of starches leads to greater production of lactic and propionic acids, which in turn lowers ruminal pH. A moderately reduced pH suppresses the growth and activity of methanogenic archaea and protozoa, both major contributors to H_2 generation and CH_4 formation (Hook et al., 2010). However, excessively low pH (<5.8) can induce subacute ruminal acidosis, impairing fibre digestion and overall feed efficiency (Beauchemin et al., 2020). Therefore, methane mitigation through dietary acidification must be balanced with maintaining an environment conducive to rumen health and productivity.

The rate of digesta passage through the rumen is another important determinant of methane yield, as it dictates the residence time available for microbial fermentation. Faster passage rates, often associated with higher feed intake and greater feeding frequency, reduce the exposure of substrates to methanogens, thereby decreasing total CH_4 output per unit of feed (Janssen, 2010). Shorter retention times can also limit protozoal colonisation and the establishment of methanogenic consortia on feed particles. In contrast, slower passage rates—typical of high-forage diets - favour extended microbial degradation of fibrous material, leading to enhanced acetate production and higher methanogenesis potential (Knapp et al., 2014). Moreover, physical factors such as particle size and effective fibre content influence rumen motility and liquid turnover, indirectly modulating fermentation kinetics and methane emission intensity (Hristov et al., 2013).

The forage-to-concentrate ratio (F: C) fundamentally alters ruminal fermentation pathways and the distribution of volatile fatty acids (VFAs). Diets with high forage inclusion stimulate cellulolytic fermentation and promote the formation of acetate and butyrate, which generate reducing equivalents H_2 that serve as substrates for methanogenesis (Hook et al., 2010; Knapp et al., 2014). Conversely, higher proportions of concentrates favour amylolytic fermentation, leading to increased propionate synthesis—a major alternative hydrogen sink that competes with methanogenesis for available H_2 (Morgavi et al., 2010). As a result, decreasing the F: C ratio typically reduces methane production per unit of feed intake. Nonetheless, excessively high concentrate levels may compromise rumen function and fibre utilisation, emphasising the importance of achieving an optimal dietary balance.

The relationship between forage proportion and methane yield is also influenced by forage quality and digestibility. Highly digestible forages, such as young temperate grasses or legumes, can enhance passage rate and reduce methane yield compared with mature, lignified forages (Benchaar, 2016). In mixed diets, incorporating moderate levels of rapidly fermentable carbohydrates (e.g., grains or molasses) can improve microbial efficiency and shift fermentation toward propionate without inducing acidosis (Hristov et al., 2013). Additionally, forage processing techniques, such as chopping or pelleting, may indirectly affect methane emissions by altering rumen retention time and fermentation site dynamics (Beauchemin et al., 2020).

Overall, maintaining optimal rumen pH, balancing forage and concentrate proportions, and managing feed particle size and turnover are critical strategies for regulating methanogenesis. These factors are interdependent: diet composition influences rumen pH and passage rate, while microbial adaptation modulates how efficiently energy is partitioned between methane production and alternative hydrogen-utilising pathways. Hence, an integrated understanding of rumen physicochemical dynamics is essential for designing effective, sustainable methane mitigation strategies in ruminant nutrition.

1.3. Classes of safe dietary compounds for methane mitigation

1.3.1. Plant secondary metabolites

Plant secondary metabolites are increasingly recognised as natural and safe feed additives for reducing enteric methane (CH_4) emissions in ruminants. They exert antimethanogenic effects primarily by inhibiting methanogens, modulating rumen protozoa, and redirecting hydrogen towards alternative pathways.

1.3.1.1. Tannins

Tannins are polyphenolic compounds that can be classified into condensed tannins (CTs), which are high molecular weight, and hydrolysable tannins (HTs), which have lower molecular weight. Condensed tannins primarily bind to dietary proteins, whereas hydrolysable tannins exert direct effects on rumen microbial populations (Aboagye and Beauchemin, 2019). Both types of tannins can reduce methane production. According to (Jayanegara et al., 2015), hydrolysable tannins are generally more effective in lowering methane concentrations than condensed tannins. The antimethanogenic effect of condensed tannins is largely attributed to a reduction in fibre digestion, while hydrolysable tannins primarily inhibit the growth and activity of methanogenic archaea.

In vitro studies demonstrated antimethanogenic activity of both HTs and CTs, by inhibition of methanogens or by targeting protozoa (Bhatta et al., 2009; Jayanegara et al., 2015). The in vivo effects depend on the dose, molecular weight, source and type (Jayanegara et al., 2012). A study by Jayanegara et al. (2011) found that the effects of tannins on methane emissions were more pronounced in in vitro settings compared to in vivo studies.

Grazing tannin-rich forages or adding extracts to the diet can also affect positive ruminants by preventing bloat, increasing N utilisation, lowering CH_4 production, acting as an antioxidant, controlling parasites, and improving animal production (Min et al., 2003; Gladine et al., 2007; Huang et al., 2018).

Acacia spp., particularly *Acacia mearnsii* and *Acacia nilotica*, are rich in condensed tannins and have been extensively studied for their antimethanogenic properties. Supplementation with Acacia leaf meal or extracts has been shown to reduce methane production in both in vitro and in vivo studies (Patra and Saxena, 2011; Aboagye and Beauchemin, 2019).

Quebracho is a tree native to South America and is a prominent source of condensed tannins. Quebracho tannins have demonstrated consistent reductions in methane emissions, likely due to their ability to bind dietary proteins and inhibit fibre-digesting microbes, indirectly limiting hydrogen availability for methanogenesis (Beauchemin et al., 2020). In vitro screening experiments report dose-dependent decreases in CH_4 production when quebracho CT is added to rumen incubations, with reductions often ranging from modest (10–20%) to large (>40%), depending on CT concentration and substrate (Battelli et al., 2023). Recent in vivo trials corroborate these findings but also illustrate important trade-offs: controlled dairy goat and cow experiments using commercial quebracho extracts (2–6% of diet DM) observed lowered methane yield and shifts in archaeal and bacterial community structure, yet some studies reported small decreases in fibre digestibility or dry matter intake at higher inclusion levels (Arik et al., 2024; Battelli et al., 2024). Comparative work suggests quebracho CTs tend to bind dietary protein more readily and may have a different impact on fibre-degrading populations than some other CT sources (e.g., Acacia) (Brutti et al., 2023). Very recent trials have begun pairing quebracho with other additives (e.g., essential oils or dietary lipids) to seek additive or synergistic methane mitigation while minimising negative effects on intake and productivity; early results are promising but indicate that reporting CT composition (molecular weight, degree of polymerisation) and standardising dose as % DM are essential for cross-study comparisons and practical recommendations (Foggi et al., 2022; Min et al., 2022).

Chestnut contains hydrolysable tannins and has been used in ruminant diets to suppress methane production. One proposed mechanism is that chestnut HTs act more directly on methanogenic archaea or their hydrogen supply, rather than predominantly inhibiting fibre-digesting bacteria as is often attributed to condensed tannins (Jayanegara et al., 2015). For example, an in vitro study comparing CTs and HTs found that while both reduced CH_4 production, the chestnut (HT) treatment was less effective than quebracho (CT) but did not reduce archaeal or protozoal counts in that short incubation (Battelli et al., 2023). Further in vitro experiments demonstrated that chestnut HT decreased methanogenesis and ammonia production while promoting acetate formation (Jayanegara et al., 2012; Cappucci et al., 2021) In vivo, supplementation with chestnut extract (4% DM) reduced methanogen abundance in lambs (da Costa, 2017) and decreased CH_4 emissions while improving milk performance in dairy cows (Prodanović et al., 2025), supporting its potential as a practical mitigation strategy.

In addition to conventional tannin sources, agro-industrial by-products such as grape marc have gained increasing attention as a methane mitigation strategy, particularly in Australia where the wine industry produces substantial quantities of this residue. Grape marc, consisting of grape skins, seeds, and stems remaining after wine production, is rich in condensed tannins and lipids, both of which contribute to its antimethanogenic properties. In vivo studies in dairy systems have demonstrated that dietary inclusion of grape marc can reduce enteric CH_4 emissions by approximately 10–20%, primarily through a combination of direct inhibition of methanogenic archaea and shifts in rumen fermentation pathways, including reduced hydrogen availability (Moate et al., 2014). Similarly, in vitro studies have reported that the efficacy of grape marc is influenced by its chemical composition, particularly tannin structure and fatty acid profile, which can vary depending on grape variety and processing conditions (Hixson et al., 2018). Despite these benefits, high inclusion levels may reduce diet digestibility and metabolisable energy content, highlighting the importance of optimising inclusion rates to balance methane mitigation with animal performance. Given its availability and dual role as a feed supplement and waste valorisation strategy, grape marc represents a promising and regionally relevant option for sustainable methane mitigation in Australian ruminant systems.

Several other tropical and subtropical leguminous plants, such as *Leucaena leucocephala*, *Flemingia macrophylla*, and *Calliandra calothyrsus*, are rich in condensed tannins. These plants have been incorporated into ruminant diets to reduce methane emissions while providing protein-rich feed (Patra, 2013).

Despite their potential, the use of tannins as methane mitigation agents presents some limitations. Palatability: high concentrations of tannins can reduce feed intake due to their astringent taste, impacting animal performance (Beauchemin et al., 2020); Nutrient Digestibility: excessive tannin levels can bind to proteins and other nutrients, reducing their availability and digestibility. This binding action can lead to reduced nutrient absorption and may impair the efficiency of feed utilisation (Besharati et al., 2022).The safe inclusion level of hydrolysable tannins in ruminant diets is generally considered to be below 20–30 g/kg DM, while concentrations exceeding 50 g/kg DM may lead to adverse effects, including reduced intake, impaired digestibility, and potential toxicity (Makkar, 2003; Jayanegara et al., 2012). At very high levels (e.g., >100 g/kg DM), tannins can exert toxic effects such as liver and kidney damage, particularly hydrolysable tannins (Makkar, 2003); Animal adaptation: over time, rumen microbes may adapt to the presence of tannins, potentially diminishing their effectiveness. reducing their impact on methane production (Makkar, 2003).

While these plants are effective for methane mitigation (typical reduction 10-30%) (Patra and Saxena, 2011; Jayanegara et al., 2012), their inclusion in diets must be carefully managed to avoid negative effects on feed intake and nutrient digestibility. The type of tannin (condensed vs. hydrolysable), its concentration, and the method of processing (e.g., drying, ensiling) all influence the extent of methane reduction and animal performance (Makkar, 2003; Jayanegara et al., 2015; Besharati et al., 2022).

1.3.1.2. Saponins

Saponins are glycosides, which means they consist of a hydrophobic component called sapogenin linked to hydrophilic sugar molecules (Goel and Makkar, 2012). Growing evidence indicates that saponins can help reduce enteric CH_4 emissions in ruminants, primarily by influencing rumen protozoa and the methanogenic archaea associated with them (Goel and Makkar, 2023; Pepeta et al., 2024; Yanza et al., 2024).

One of the primary mechanisms by which saponins reduce methane emissions is defaunation—the suppression or elimination of rumen protozoa. Rumen protozoa play an important role in methane production because they harbor symbiotic methanogens on their surfaces and within their cells, providing H_2 through the fermentation of carbohydrates (Newbold et al., 2015). By reducing protozoal populations, saponins indirectly limit the availability of H_2 for methanogenesis (Goel and Makkar, 2012). Additionally, Saponins interact with sterols present in protozoal cell membranes, causing increased membrane permeability, leakage of intracellular components, and ultimately cell lysis (Kholif, 2023). This membrane-disrupting effect is selective, as ruminal bacteria generally lack sterols in their membranes and are less affected (Wallace, 2004). The reduction in protozoal biomass leads to a decline in methane yield due to the disrupted protozoa–methanogen symbiosis (Goel and Makkar, 2012).

Several plant species are rich in saponins and have been investigated for their antimethanogenic potential. Two major commercial and research-relevant sources include *Yucca schidigera* and *Quillaja saponaria*.

Yucca schidigera (a desert plant native to North America) produces steroidal saponins that have been shown to reduce rumen protozoa populations and methane production in vitro and in vivo (Makkar et al., 1998; Wang et al., 2012). Supplementation with Yucca extract has achieved methane reductions of 10–30%, depending on dose and diet type (Goel and Makkar, 2012).

Quillaja saponaria, a South American tree, contains triterpenoid saponins with similar properties. Quillaja extracts have demonstrated protozoal inhibition and reduction in methane yield without major negative effects on total volatile fatty acid production, suggesting that fermentation efficiency is largely maintained (Pen et al., 2006; Holtshausen et al., 2009).

Other saponin-containing plants such as *Sapindus mukorossi*, Tea (*Camellia sinensis*), *Trigonella foenum-graecum* (fenugreek), and *Enterolobium cyclocarpum* have also been explored for similar purposes (Jayanegara et al., 2014). The antimethanogenic efficacy of saponins is highly variable, depending on the saponin type, concentration, diet composition, and rumen microbial adaptation (Jayanegara et al., 2014). Some studies report that the methane-suppressing effect diminishes over time, likely due to microbial adaptation—rumen microorganisms can degrade saponins into sapogenins, which lack defaunating activity (Wang et al., 2012). This adaptation may occur within weeks of supplementation, suggesting that the persistence of the antimethanogenic effect is limited unless saponin sources or concentrations are varied periodically.

In addition, excessive inclusion of saponins may reduce feed intake or fibre digestibility, particularly when protozoal suppression is extensive (Patra and Saxena, 2009). Therefore, identifying optimal inclusion levels (often 0.5–1.5 g saponin/kg DM) and delivery methods remains critical for practical application (Goel and Makkar, 2012).

Beyond their role in methane mitigation, saponins may offer additional health benefits in ruminant production systems. Their antimicrobial and antioxidant properties can enhance rumen microbial balance, gut health, and immune function (Cheeke, 2000). By reducing protozoal populations, saponins may also help control rumen ammonia concentrations, improving nitrogen utilisation efficiency (Patra and Saxena, 2009; Wang et al., 2012). Moreover, saponins have been reported to reduce incidences of bloat, acidosis, and parasitic infections, contributing to improved animal welfare and productivity (Wallace, 2004; Goel and Makkar, 2012). However, these health-promoting effects are dose-dependent and can be reversed at excessive inclusion levels, underscoring the need for careful formulation and monitoring.

Saponins present a promising natural strategy for methane mitigation (typical reduction 5-25%) (Pen et al., 2006; Goel and Makkar, 2012) through selective inhibition of rumen protozoa and disruption of methanogen–protozoa associations. Extracts from *Yucca schidigera* and *Quillaja saponaria* are the most widely studied and commercially viable options. However, the transient nature of their effects due to microbial adaptation and variable responses across diets highlights the need for continued research into formulation stability, dosage optimisation, and potential synergies with other feed additives.

1.3.1.3. Essential oils

Essential oils (EOs) are complex mixtures of volatile secondary metabolites extracted from aromatic plants. Their primary active components—such as thymol, eugenol, and cinnamaldehyde—belong to chemical groups including phenols, aldehydes, and terpenes, which confer strong antimicrobial and antioxidant properties (Calsamiglia et al., 2007; Benchaar and Greathead, 2011). Thymol and carvacrol are commonly derived from thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*), eugenol from clove (*Syzygium aromaticum*), and cinnamaldehyde from cinnamon (*Cinnamomum zeylanicum*). These compounds have been investigated for their potential to modulate rumen fermentation and reduce enteric methane emissions through selective inhibition of rumen microorganism (Benchaar and Greathead, 2011).

Commercial EO-based feed additives have been developed to provide standardised and safe inclusion rates for livestock. Crina® Ruminants (DSM-Firmenich) and Agolin® Ruminant (Agolin SA/Alltech) are two of the most widely studied EO blend. Studies have reported modest but consistent reductions in methane emissions, generally between 5–10%, when these blends are administered at recommended dosages without negative effects on feed intake or animal performance (Belanche et al., 2020). Crina® Ruminants (DSM-Firmenich) is an EO-based additive formulated with thymol, eugenol, and vanillin. The combination is aimed at improving rumen fermentation and feed utilisation while reducing methane and nitrogen losses. Experimental work has evaluated CRINA® for effects on rumen fermentation, animal performance, and enteric methane. For instance, in a controlled Latin-square study with Brahman steers fed Rhodes grass hay, Crina® was compared to Monensin and an untreated group at a low inclusion rate. Crina® was supplemented at approximately 1 g/head per day, while monensin was included at around 200 mg/head per day. While Crina® altered fermentation profiles, it produced only modest or inconsistent reductions in methane production and changes in the methanogen community metrics under the tested conditions (Tomkins et al., 2015). Several in situ studies observed that the blend of essential oils inhibited protein degradation, although the reported changes were small and varied depending on the type of feed being degraded, the specific ration fed to the animals, and the duration of the adaptation period (Molero et al., 2004; Newbold et al., 2004). McIntosh et al. (2003) further suggested that this combination of essential oils likely inhibits a specific group of gram-positive bacteria known as hyper-ammonia-producing bacteria. However, most studies assessing Crina® have found no significant impact on microbial protein synthesis in the rumen (Wallace et al., 2002; Castillejos et al., 2005).

Agolin® Ruminant is a standardised EO blend containing coriander oil (*Coriandrum sativum*), geraniol, geranyl acetate, and eugenol. These bioactive substances have been documented to exhibit a wide range of antimicrobial effects, including activity against rumen microorganisms. (Benchaar and Greathead, 2011) and was certified by Carbon Trust Assurance LTD for reduction of CH_4 emissions and improvements of feed efficiency (Belanche et al., 2020). In a meta-analysis of in vivo studies, Belanche et al. (2020) found that Agolin supplementation reduced methane emissions by an average of 8.8% and increased feed efficiency by 2.5%, with consistent effects across diet types and production systems; However, based on additional research, the effects of Agolin supplementation are only temporary (Klop et al., 2017) or don't have an effect at all (Miller et al., 2023; Silvestre et al., 2023;Benchaar and Hassanat, 2025). The causes of these discrepancies are not well understood but might involve variations in experimental conditions (such as the composition of the diet), the feeding rate conveyed on a dry matter intake basis, and the traits of the cows (Cobellis et al., 2016; Benchaar and Hassanat, 2025). Aoglin's effects appear largely indirect, through reduced hydrogen availability and selective inhibition of certain bacterial populations (e.g., *Ruminococcus albus* and *Methanobrevibacter spp.*). The blend's composition ensures efficacy at low inclusion rates (0.5–1 g/day per cow), minimising risks to palatability or rumen digestibility (Belanche et al., 2020).

The antimicrobial activity of EOs is attributed to their ability to disrupt microbial cell membranes, alter proton gradients, and interfere with enzymatic activity, particularly in Gram-positive bacteria (Benchaar et al., 2008). In the rumen, these effects can lead to shifts in the microbial ecosystem, reducing the abundance of hydrogen-producing bacteria and protozoa that are symbiotically associated with methanogenic archaea (Patra and Yu, 2012). Consequently, fermentation patterns may change toward a greater proportion of propionate and lower acetate, thereby decreasing the availability of H_2 required for methane formation (Calsamiglia et al., 2007).

Agolin and Crina specifically have been shown to enhance fermentation efficiency by stabilising rumen pH and reducing ammonia accumulation (Benchaar et al., 2008). However, excessive EO doses may depress overall fermentation and reduce fibre degradation (Patra and Yu, 2012). Prolonged exposure to EOs can also lead to microbial adaptation, as rumen microbial communities may adjust to the presence of EO compounds, diminishing their efficacy over time (Calsamiglia et al., 2007). Encapsulation technologies and rotation or blending of EO compounds have been proposed to mitigate adaptation effects and sustain long-term activity (Belanche et al., 2020). Essential oils used in feed are generally recognised as safe (GRAS) when administered at low concentrations (U.S. Food and Drug Administration, 2023). Nonetheless, palatability issues and potential toxicity at high doses require careful consideration (Burt, 2004). Commercial products such as Crina® and Agolin® Ruminant are formulated to ensure safety, stability, and effective delivery, and are approved for use in ruminant nutrition in several jurisdictions.

Overall, EOs represent a promising natural feed additive for methane mitigation (typical reduction 5-20%) (Calsamiglia et al., 2007; Benchaar et al., 2008) and enhancement of rumen efficiency, though their effects are variable across studies due to differences in EO composition, diet type, and duration of exposure. Further long-term and large-scale in vivo trials are needed to fully understand the persistence of effects and the economic feasibility of implementation in grazing systems.

1.3.2. Electron acceptors and alternative hydrogen sinks

In the rumen, methanogenesis serves as the primary pathway for H_2 disposal, maintaining redox balance during fermentation. Alternative hydrogen sinks—compounds capable of accepting electrons generated during carbohydrate metabolism—can redirect reducing equivalents away from CO_2 reduction to CH_4, thereby lowering enteric methane emissions (Hristov et al., 2013; Ungerfeld, 2020). Among these, sulphate and fumarate are two of the most extensively studied electron acceptors.

Sulphate as an alternative electron acceptor

Sulphate (SO_4^{2-}) reduction competes directly with methanogenesis for available H_2 in the rumen. Sulphate-reducing bacteria (SRB), such as *Desulfovibrio* spp. and *Desulfotomaculum* spp., utilise H_2 to reduce sulphate to hydrogen sulphide (H_2S). This process effectively diverts H_2 from methanogens, resulting in lower methane production (Li et al., 2013). In vitro studies have shown that adding sulphate to rumen cultures can significantly reduce CH_4 production, with inclusion levels typically ranging from approximately 5 to 20 mM sulphate in batch culture systems (Wu et al., 2015; Wu et al., 2021). Additionally, in vivo research using sulphate supplementation has demonstrated a reduction in methane emissions of 10–20%, depending on the dosage and sulphur content of the basal diet. (van Zijderveld et al., 2010; Silivong et al., 2011; Arif et al., 2016). Furthermore, it has been shown that incorporating sulphate into the ruminant diet increases the activity of fibre-digesting bacteria and rumen fungi (Wang et al., 2021).

However, the practical application of sulphate is constrained by the potential for hydrogen sulphide toxicity. Excessive production of H_2S can lead to polioencephalomalacia and respiratory distress in ruminants (Gould, 1998). Therefore, sulphate inclusion must be carefully managed to maintain total dietary sulphur below recommended limits (typically <0.4% of DM). Furthermore, high sulphate levels exceeding these dietary limits may impair palatability and affect mineral balance (Spears et al., 2011). Despite these limitations, strategic use of sulphate - particularly in low-sulphur diets - remains a promising, albeit safety-limited, methane mitigation strategy.

Fumarate as an alternative hydrogen sink

Fumarate, an intermediate of the tricarboxylic acid (TCA) cycle, serves as another effective electron acceptor in the rumen (Stewart et al., 1997). Through microbial reduction, fumarate is converted to succinate and subsequently to propionate, a VFA that acts as an alternative H_2 sink and provides an energy benefit to the host animal (Castillo et al., 2004). This pathway not only consumes reducing equivalents but also shifts fermentation toward propionate production, reducing hydrogen availability for methanogenesis (Ungerfeld, 2015a). In vitro and in vivo studies have shown that fumarate supplementation can reduce methane emissions depending on concentration and microbial adaptation (Lopez et al., 1999; Bayaru et al., 2001; García-Martínez et al., 2005; Newbold et al., 2005). Despite its mechanistic efficiency, the practical use of fumarate is limited by cost and dose requirements. Effective methane mitigation often requires several grams of fumarate per kilogram of feed, which may be economically unfeasible for large-scale livestock operations (Hristov et al., 2013). Moreover, rapid fermentation of fumarate may decrease rumen pH, posing risks of subacute acidosis when combined with high-concentrate diets (Ungerfeld, 2015a). Consequently, fumarate is primarily considered useful as a research tool or component of combined mitigation strategies, rather than a standalone additive for commercial use.

Both sulphate and fumarate function by diverting hydrogen away from methanogenesis, thereby lowering methane yield. However, their practical application differs. Sulphate offers stronger competition for H_2 but carries toxicity risks, whereas fumarate provides an additional nutritional benefit through propionate formation but requires higher and costlier doses. The success of these compounds is highly dependent on diet composition, sulphur background, and microbial adaptation (van Zijderveld et al., 2010; Ungerfeld, 2015a).

Sulphate and fumarate are promising alternative hydrogen sinks that can decrease enteric methane emissions (typical reduction 5-15%) (Ungerfeld et al., 2007) by providing competing pathways for hydrogen utilisation. Sulphate reduction offers high theoretical potential but is constrained by toxicity risks, while fumarate reduction provides both mitigation and energetic benefits but is limited by economic and practical feasibility. Future research should explore controlled-release formulations to enhance their safety and cost-effectiveness in ruminant production systems.

1.3.3. Lipids and fatty acids

Lipids and fatty acids have been extensively studied as dietary interventions to mitigate enteric methane emissions in ruminants (Patra, 2013; Arndt et al., 2022). Their efficacy arises primarily through two mechanisms: direct inhibition of methanogenic archaea and indirect effects on rumen fermentation, particularly fibre digestion.

Lipids can reduce methane production by directly affecting methanogens, the archaea responsible for CH_4 synthesis. Unsaturated fatty acids can disrupt the cell membranes of methanogens, leading to reduced growth and activity (Patra, 2013). Additionally, the presence of lipids in the diet can decrease fibre digestion by coating feed particles and altering microbial populations, thereby reducing the availability of hydrogen, a substrate for methanogenesis (Beauchemin et al., 2008).

Medium-chain fatty acids (MCFAs), such as lauric and myristic acids, are particularly effective at inhibiting methanogens due to their strong antimicrobial properties. Unsaturated oils, including linoleic and α-linolenic acids, also contribute to methane mitigation by hydrogenation reactions in the rumen, which compete with methanogenesis for hydrogen (Jenkins et al., 2008).

Coconut oil, palm kernel oil, and their derivatives are major sources of medium-chain fatty acids, particularly lauric and myristic acids. These fatty acids exhibit direct inhibitory effects on methanogenic archaea and rumen protozoa, reducing methane formation (Patra, 2013). Coconut oil is among the most extensively studied lipid sources for mitigating enteric methane, with reported reductions ranging from 13% to 73%, depending on dosage, diet composition, and ruminant species. Its lauric-to-myristic acid ratio (2.6:1) aligns with the most effective ratios identified for methane inhibition in vitro (Soliva et al., 2004), supporting its potential for substantial in vivo efficacy. Palm kernel oil, which has a slightly higher lauric-to-myristic acid ratio (3:1), may provide even greater methane abatement potential; however, in vivo evidence is currently lacking (Hook et al., 2010). Nevertheless, supplementation with either oil frequently leads to reduced dry matter intake, which can confound methane reduction results by lowering total feed fermentation (Jordan et al., 2006; Liu et al., 2011).

Linseed (*Linum usitatissimum*) is rich in α-linolenic acid (C18:3 n-3), which has shown potential to reduce methane emissions through suppression of methanogens and biohydrogenation pathways (Martin et al., 2010; Grainger and Beauchemin, 2011). Linseed oil or whole linseed supplementation at moderate levels typically decreases CH_4 yield (Martin et al., 2016). Similar effects have been observed with other omega-3-rich oils such as chia and perilla oil, indicating that n-3 fatty acids play a role in reducing hydrogen availability for methanogenesis (Grainger and Beauchemin, 2011). However, the efficacy of omega-3 supplementation varies depending on the form (oil vs. seed), dosage, diet composition, and adaptation period (Guyader et al., 2015). Excessive inclusion of unsaturated fats may impair fibre fermentation and reduce animal performance, highlighting the importance of balancing methane mitigation potential with production outcomes (Grainger and Beauchemin, 2011). Additionally, omega-3 enrichment of ruminant products (milk, meat) offers potential health benefits for human consumers, creating dual advantages of environmental and nutritional value (Shingfield et al., 2013).

Sunflower and canola (rapeseed) oils provide unsaturated fatty acids—mainly linoleic (C18:2 n-6) and oleic (C18:1 n-9)—which can act as alternative hydrogen sinks during rumen fermentation, thereby lowering methane production. Studies have shown reductions in CH_4 emissions including oil in the diet (Beauchemin et al., 2020). The inclusion of these lipids in ruminant diets has consistently demonstrated mitigation effects on CH_4 emissions, although the magnitude of reduction depends on the form and level of supplementation (Silveira et al., 2019). Canola and sunflower by-products, such as cakes and meals, provide these lipids in a matrix that is generally less inhibitory to fibre digestion than free oils, allowing for a more balanced approach between methane mitigation and rumen function (Arndt et al., 2022). Supporting this, (Brask et al., 2013) observed that methane intensity (L/kg organic matter intake) was 12.6% lower in cows fed rapeseed cake compared with those receiving rapeseed meal, likely due to differences in lipid content and fermentability between the two feed forms. Similarly, (Beauchemin et al., 2009) reported a 16% reduction in CH_4 emissions in dairy cows when dietary supplementation with canola meal. These findings collectively indicate that oilseeds and their by-products can effectively suppress ruminal methanogenesis through multiple mechanisms—such as the biohydrogenation of unsaturated fatty acids, reduced fibre degradation, and limited substrate availability for methanogens—while maintaining overall feed efficiency and animal performance.

Fish oils are rich in long-chain n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have potent antimethanogenic properties. These fatty acids can reduce methanogenesis directly through microbial inhibition and indirectly by decreasing ruminal protozoa populations. Inclusion of small amounts (up to 2–3% DM) of fish oil has achieved methane reductions, although high inclusion levels may impair fibre degradation (Kouřimská et al., 2014; Vargas et al., 2017).

Whole or processed oilseeds (e.g., flaxseed, cottonseed, sunflower seed) provide a slower lipid release than free oils, reducing potential negative effects on rumen fermentation. Grinding or crushing oilseeds enhances lipid bioavailability in the rumen (Beauchemin et al., 2020). Arndt et al. (2022) reported that both oil and oilseed supplementation reduced methane yield, indicating comparable mitigation effects when properly managed.

The antimethanogenic effects of dietary lipids in ruminants are highly variable (10-30%) (Benchaar et al., 2008) and depend on multiple factors, including lipid type, inclusion level, and animal species.

Medium-chain fatty acids (e.g., myristic acid) and polyunsaturated fatty acids (PUFAs) from fish, sunflower, linseed, and canola oils are generally the most effective at reducing methane emissions (Patra, 2013).

While lipids can significantly reduce methane emissions, excessive inclusion may impair fibre digestion and reduce dry matter intake, negatively affecting animal productivity (Eugène et al., 2008). Therefore, dietary strategies must balance the level and type of lipid supplementation to optimise methane reduction while maintaining feed efficiency and animal performance (Palmquist and Jenkins, 2017). Inclusion of fats at 4–6% of dietary dry matter has been reported to improve milk production in ruminants. This beneficial effect is largely attributed to the additional energy provided by lipids and their potential influence on milk fat synthesis. However, feeding diets with higher fat concentrations can disrupt rumen microbial activity, inhibit fibre-digesting bacteria, and reduce nutrient digestibility, ultimately impairing rumen fermentation and overall animal performance (Patra, 2013). The negative effects are particularly pronounced when fats are supplemented in forms that are readily bioavailable in the rumen, highlighting the importance of careful selection of fat type and inclusion level in ration formulation (Arndt et al., 2022).

Long-term effects of dietary lipids are less well documented. Some studies indicate persistent antimethanogenic effects (Jordan et al., 2006), whereas recent research on grazing dairy cows suggests that the effects of oilseed supplementation may be transient (Muñoz et al., 2021). The inhibitory effect of lipids is generally greater in concentrate-based diets than in forage-based diets, potentially due to the lower rumen pH in concentrate-fed animals, which enhances the impact of fatty acids on methanogens (Beauchemin et al., 2022).

Additional research is required to determine economically viable and sustainable fat sources, as well as their optimal inclusion levels, that can mitigate methane emissions without compromising feed digestibility or animal performance. Furthermore, studies are needed to evaluate the long-term effects of fat supplementation on the persistence of methane reduction.

1.3.4. Synthetic compounds and feed additives

1.3.4.1. 3-Nitrooxypropanol (3-NOP)

3-Nitrooxypropanol (3-NOP) is a small synthetic molecule that specifically targets the terminal step of biological methanogenesis. Mechanistically, 3-NOP acts as an inhibitory substrate for methyl-coenzyme M reductase (MCR), the nickel-containing enzyme in methanogenic archaea that catalyses the final reduction of methyl-coenzyme M to CH_4, forming stable complex that prevent further turnover (Martinez-Fernandez et al., 2016). Because this mechanism acts directly on methanogens rather than on hydrogen-producing bacteria, it results in targeted suppression of CH_4 formation without major disruption of overall rumen fermentation (Hegarty et al., 2021).

Multiple controlled studies and meta-analyses have confirmed the robust efficacy of 3-NOP in reducing enteric methane emissions across dairy and beef cattle, sheep, and goats. The typical dosage reported in studies is 123 mg 3-NOP per kg of dry matter intake (DMI), which has been demonstrated to result in a 23% reduction in emissions for beef and a 39% reduction for dairy (Hegarty et al., 2021). A global meta-analysis reported average methane reductions of 29 % (Kebreab et al., 2023). Similar to the results found by (Dijkstra et al., 2018), with minimal effects on dry-matter intake and feed efficiency. These effects have been reproduced under both high-forage and high-concentrate diets. The composition of the diet can influence how effective antimethanogenic additives are. For instance, research shows that the ability of 3-NOP to reduce methane emissions diminishes as the concentration of NDF in the diet increases (Kebreab et al., 2023), probably because there is greater hydrogen production linked to acetate-type fermentation in the rumen (Hristov, 2023).

No strong evidence of microbial adaptation that restores methane production has been reported in studies (Hristov, 2023). Meta-regression analyses further indicate that baseline methane intensity and diet fermentability influence absolute reductions, but proportional effects are consistent across production systems (Kebreab et al., 2023).

Safety evaluations indicate that 3-NOP is rapidly metabolised in the rumen and animal tissues, and at recommended inclusion rates does not cause adverse effects on animal health, milk or meat safety, or consumer exposure (Bampidis et al., 2021). Several jurisdictions have completed regulatory evaluations and authorised the commercial product Bovaer® (active: 3-NOP) for use in ruminant feed under specified conditions. Regulatory approvals include—but are not limited to—the European Union (for certain dairy applications), Brazil, Chile, Canada, Australia, and the United States. Commercial rollout and on-farm implementation (via Bovaer® and equivalent formulations) are now documented in multi-site trials and company-sponsored large-scale trials showing real-world reductions consistent with experimental literature. However, labelling, permitted species, approved dose ranges, and target production categories vary by jurisdiction, and users should consult the relevant regulatory authorisation for precise use conditions.

Because 3-NOP acts by enzymatic inhibition, consistent daily intake is critical for maintaining efficacy; feed-delivery variability can attenuate its effect (Kebreab et al., 2023). Production responses are generally neutral. Research on grain-based diets indicated that incorporating 3-NOP did not provide any benefits for live weight gain, and there could be a decline in growth when high levels of 3-NOP are included (Hegarty et al., 2021). Long-term trials are still required to confirm persistence of efficacy, cost–benefit ratios, and potential interactions with other mitigation compounds such as nitrate or tannins (Hristov, 2023). Overall, 3-NOP currently represents the most technically validated and regulatorily approved chemical inhibitor of enteric methane formation (20-60%) (Hristov et al., 2015; Melgar et al., 2020) available. However, a practical limitation of this compound is its relatively short shelf-stability and susceptibility to degradation during feed processing and storage. Bampidis et al. (2021) reported losses of 16.7% of 3-NOP in pelleted feed after three months of storage at room temperature in sealed polyethylene bags. Although some exposure to oxygen and variable humidity was still possible during storage (Bampidis et al., 2021). Similarly, MLA-funded research has shown that the inherent volatility of 3-NOP can lead to variable losses during pelleting and storage, reducing the consistency of additive delivery to animals (Lawrence et al., 2024). These findings highlight the importance of careful formulation, processing, storage, and handling protocols to preserve the compound's stability and ensure its intended antimethanogenic efficacy under commercial feeding conditions.

1.3.4.2. Ionophores (e.g., Monensin) – very efficient to decrease CH_4 in vitro but not in vivo

Ionophores such as monensin are carboxylic polyether antibiotics that alter rumen fermentation by selectively increasing the permeability of microbial cell membranes to monovalent cations (Na^+, K^+), thereby favouring Gram-negative bacteria and shifting fermentation toward increased propionate production at the expense of acetate (Russell and Strobel, 1989). These shifts reduce hydrogen availability for methanogenesis and can depress methane formation in vitro and, under some conditions, in vivo. Ionophores commonly increase propionate: acetate ratios, reduce ruminal ammonia accumulation, and can improve feed efficiency in beef finishing systems (Hegarty et al., 2021).

Recent meta-analyses focusing on beef cattle show transient methane reductions that depend on diet composition (forage vs concentrate), ionophore dose, and duration of administration; sustained long-term mitigation is inconsistent (Cooke et al., 2024). Ionophore efficacy is typically greater in the short term or in high-grain diets where shifts to propionate are pronounced (Appuhamy et al., 2013), but adaptation of the rumen microbiome can reduce the magnitude of effect with prolonged exposure (Rychlik and Russell, 2002). A meta-analysis reported the most significant results during the first 79 days of monensin supplementation, particularly on high forage diets offered for *ad libitum* intake (Cooke et al., 2024). Consequently, monensin is regarded as nutritionally beneficial but not a reliable long-term methane mitigant under practical feeding conditions.

Ionophores are classified as veterinary antibiotics but are not used in human medicine; nevertheless, their use raises broader concerns about selection for antimicrobial resistance (AMR) determinants in the gut microbiome and potential co-selection of resistance to other antibiotic classes (McEwen & Fedorka-Cray, 2002). Experimental and observational studies demonstrate that ruminal bacteria can develop reduced sensitivity to ionophores, and shifts in bacterial community composition may select for tolerant strains (Rychlik and Russell, 2002). Several countries have limited or prohibited routine antibiotic use for growth promotion, but ionophores remain permitted in ruminant nutrition under veterinary supervision (Callaway et al., 2003). The potential antimicrobial resistance consequences and the limited, variable long-term methane mitigation observed with ionophores argue that they are a suboptimal strategy for sustained greenhouse-gas mitigation.

In summary, ionophores exert useful fermentation-modulating effects and transient methane suppression (5-10%) (McGinn et al., 2004; Guan et al., 2006), but microbial adaptation considerations restrict their role as a sustainable mitigation technology.

1.3.5. Probiotics, enzymes, and other emerging bioactives

Use of direct-fed microbials (DFM):

Direct-fed microbials (DFM; “probiotics” for ruminants) aim to alter rumen fermentation by introducing or enriching microbial taxa that (a) redirect metabolic hydrogen toward non-methanogenic products (e.g., propionate), (b) compete with hydrogenotrophic methanogens, or (c) stabilise rumen conditions (pH, redox) that disfavour methanogenesis (Newbold et al., 2015).

Key candidate organisms used are *Megasphaera elsdenii* and *Propionibacterium spp; M. elsdenii* consumes lactate and produces volatile fatty acids (VFA) such as butyrate and propionate; it has been proposed as a DFM to prevent acidosis during high-grain transitions and increase propionate formation (thereby acting as an H_2 sink relative to methanogenesis). Strain selection, dose, and dietary context strongly influence outcomes; reviews and experimental work report benefits for rumen stability and feed efficiency but inconsistent methane reductions and occasional associations with milk-fat depression in dairy cows (Beauchemin et al., 2020); Propionibacteria and other propionate-forming bacteria are of interest because propionate formation consumes reducing equivalents that would otherwise form CH_4 (Jeyanathan et al., 2016). Some in vitro and short-term in vivo studies show increased propionate and modest methane decreases when propionigenic DFMs are added (Vyas et al., 2014; Vyas et al., 2016), but effects are variable (Meale et al., 2014; Chen et al., 2020). Current data indicates that while propionibacteria can produce propionate to utilise hydrogen during fermentation, this process does not reduce CH_4 emissions (Hegarty et al., 2021).

Controlled in vitro studies and small-scale in vivo trials show that certain DFMs can shift fermentation toward propionate and reduce measured methane production under specific diets (low pH, high fermentability) (Oyebade et al., 2024). However, consistent meaningful methane reductions under commercial, long-term conditions are not yet well established; responses vary with basal diet, strain viability in the rumen, interaction with resident microbiota, and delivery format (capsules, boluses, feed premix) (Oyebade et al., 2024).

Enzyme supplements to alter fermentation pathways.

Exogenous feed enzymes (e.g., cellulases, xylanases, pectinases, amylases, and some dehydrogenases) are used to improve fibre and starch digestion and, in doing so, can alter fermentation kinetics and end-product profiles. Where enzymes increase the proportion of propionate or accelerate feed conversion, they may indirectly reduce H_2 availability for methanogens (Salem et al., 2023).

Experimental work and recent reviews show that tailored enzyme blends can improve nutrient digestibility and feed conversion in ruminants (Carrillo-Díaz et al., 2022). Reports of direct, reproducible methane reductions attributable solely to enzymes are limited (Palangi and Lackner, 2022). Many enzyme trials focus on performance (DMI, ADG, milk yield) and fibre digestibility rather than methane as a primary endpoint. Where methane was measured, reductions are often small and diet-dependent (higher potential in low-quality forages where enzyme-induced increases in fermentability shift VFA profiles) (Salem et al., 2023).

Enzyme effectiveness depends on substrate accessibility, enzyme specificity, and rumen residence time; encapsulation or targeted delivery may increase efficacy.

The industrial-scale production of anaerobic bacteria remains technically demanding and substantially more expensive than the conventional cultivation of microorganisms under aerobic conditions (McAllister et al., 2011). This constraint currently limits the range of DFM that can be developed for practical, on-farm application (Gallo et al., 2015; Ban and Guan, 2021). Moreover, maintaining the viability of obligate anaerobes during storage and distribution poses a significant challenge, as these organisms are highly sensitive to oxygen exposure and environmental stress (Hegarty et al., 2021). Continued technological innovation in microbial formulation and stabilisation is therefore critical to broaden the potential use of anaerobic strains in methane mitigation strategies. For novel DFM candidates, in vivo evaluation is particularly important (Doyle et al., 2019), as microbial interactions and rumen responses observed in live animals often differ from those detected in vitro. Such studies are essential to assess potential shifts in the rumen microbiome and to ensure that no unintended effects occur on animal health, productivity, or product safety. Feed additive approval requires detailed evidence of safety, efficacy, and stability, and considerable variation exists in product quality and strain identity among commercial DFM preparations (Hegarty et al., 2021). In addition, the persistence and consistency of the antimethanogenic response must be validated through long-term animal trials, given that microbial adaptation or loss of efficacy may occur over time. Like other nutritional mitigation approaches, DFM require continuous daily administration (Alazzeh et al., 2012) to maintain their beneficial effects, highlighting the need for cost-effective, stable, and user-friendly formulations suited to commercial production systems (Beauchemin et al., 2022).

Overall, while DFM represent a promising biological tool for improving rumen efficiency and mitigating enteric CH_4 emissions, their widespread use will depend on developing more robust strains, clarifying mechanisms of action, and demonstrating consistent economic returns under commercial production systems (Jeyanathan et al., 2014; Beauchemin et al., 2022).

1.4. Integrated approaches and synergies

The combination of feed additives may produce additive or synergistic effects. Combining compounds can target different steps or groups in ruminal H_2/CH_4 production (e.g., inhibit methanogenesis directly vs redirect H_2 to alternative sinks), lower the dose required of any single additive (reducing cost or side-effects), and reduce the likelihood of microbial adaptation to one single mode of action. However, combinations can also be antagonistic (no extra benefit) or produce negative effects on intake, digestibility, animal production or animal health (del Prado et al., 2025; Hristov et al., 2025). Trials combining 3-NOP with other additives (different feed types, cottonseed, monensin) show mixed outcomes—some additive gains, some neutral interactions—indicating diet context matters (Kebreab et al., 2023). Studies of tannin + saponin blends report greater CH_4 reductions than either alone in some trials (e.g., reduced CH_4 per kg digested NDF), suggesting beneficial interactions between plant secondary metabolite classes (Cardoso-Gutierrez et al., 2021; Cuervo et al., 2024).

Ionophores alter fermentation (shift VFA profile toward propionate, a hydrogen sink). Combining ionophores with direct methanogenesis inhibitors can be additive, but results are inconsistent — some studies show no extra benefit when combined with 3-NOP or monensin+3-NOP (Romero-Pérez et al., 2017; Muñoz et al., 2024). Probiotics that enhance propionate producers or enzymes that alter fibre degradation may complement chemical inhibitors by improving H_2 fluxes, but evidence is early and variable (del Prado et al., 2025). Thus, optimal additive combinations should be matched to diet type to maximise mitigation efficiency without impairing animal performance.

In grazing-based systems, where direct feed supplementation is often impractical, the delivery method becomes a critical factor influencing the consistency and efficacy of additive intake (Beauchemin et al., 2022). Molasses-based lick blocks have emerged as a practical strategy for administering methane-mitigating compounds under extensive conditions (Imaz et al., 2020). These blocks provide a controlled-release vehicle that promotes steady consumption and uniform exposure across animals, while their molasses base enhances palatability and energy supply (Zhao et al., 2022). The lick block approach also allows the integration of multiple additive types, potentially facilitating additive or synergistic methane reduction effects when formulated appropriately for the prevailing diet composition and production system (Hristov et al., 2025).

Interaction of feed additives with diet composition

The efficacy of methane-mitigating additives is strongly influenced by diet composition, particularly the forage-to-concentrate (F: C) ratio, which determines ruminal fermentation pathways, hydrogen (H_2) availability, and microbial community structure (Hook et al., 2010; Patra and Yu, 2012). Diets rich in forage promote fibrolytic fermentation and acetate production, generating more H_2, whereas high-concentrate diets enhance propionate formation, a natural hydrogen sink, thereby lowering baseline methane yield (Beauchemin et al., 2020). Consequently, the same additive can produce markedly different outcomes depending on the diet matrix; The effectiveness of plant secondary metabolites depends on both their chemical form and the substrate fermented. In high-forage diets, condensed tannins can form complexes with fibre and proteins, potentially reducing digestibility but also inhibiting methanogens and protozoa (Jayanegara et al., 2015). In concentrate-based diets, their effects on methane are often smaller because the baseline methanogenesis is already reduced. Saponins show stronger protozoa-suppressing activity in forage-based diets where protozoal populations are higher (Patra and Yu, 2015). Similarly, essential oils (EO) tend to show greater inhibition of methanogens in high-forage diets due to longer rumen retention time, allowing sustained antimicrobial contact (Cobellis et al., 2016). Electron acceptors such as sulphate, nitrate, and fumarate compete with CO_2 for available H_2. Their response depends heavily on ruminal H_2 partial pressure, which is generally higher in forage-based systems (Ungerfeld, 2020). Consequently, fumarate or nitrate supplementation tends to be more effective under high-forage conditions, where hydrogen availability is abundant. In contrast, in high-concentrate diets with inherently low H_2 production, these compounds may yield limited additional CH_4 reduction or may cause redox imbalances if excessive doses are used. The inclusion of unsaturated fatty acids can reduce CH_4 through biohydrogenation and inhibition of protozoa and methanogens. However, the magnitude of inhibition depends on diet composition. Lipid supplementation is generally more effective in high-forage diets, where hydrogen availability and microbial diversity allow greater scope for redirection of reducing equivalents (Beauchemin et al., 2020). In concentrate-rich diets, high lipid levels can depress fibre digestion and animal performance, offsetting the CH_4 benefits (Grainger and Beauchemin, 2011). The response to 3-nitrooxypropanol (3-NOP) is diet-dependent. Studies show higher efficacy in high-concentrate diets, where the additive is more stable and less degraded in the rumen (Romero-Perez et al., 2015). In forage-based systems, 3-NOP effectiveness can decline due to increased rumen passage time and microbial adaptation (Melgar et al., 2020). Conversely, ionophores (e.g., monensin) are more effective in concentrate-based diets, where they favour propionate-producing bacteria and suppress hydrogen formation, although responses are moderate when H_2 availability is already low (Russell and Houlihan, 2003). Probiotic and enzyme supplements interact with diet fibre content. Fibrolytic enzymes and certain propionate-producing bacterial strains exhibit greater methane-mitigating effects in high-forage diets by enhancing fibre breakdown while redirecting reducing equivalents (McAllister and Newbold, 2008). In concentrate diets, such additives may have minimal CH_4 effects but can improve feed efficiency and rumen health, indirectly influencing emissions intensity.

Additive or synergistic effects among compounds often depend on diet composition. For instance, 3-NOP combined with fumarate or lipids shows additive reductions in methane emissions in high-forage diets due to complementary hydrogen sinks, while tannin–saponin mixtures have greater effects under grazing systems where plant diversity supports microbial modulation (Guyader et al., 2015). Conversely, combinations involving ionophores and 3-NOP in concentrate diets may yield no additive effect, reflecting overlapping mechanisms (Romero-Perez et al., 2015). Thus, optimal additive combinations should be matched to diet type to maximise mitigation efficiency without impairing animal performance.

Systems-level mitigation: feed formulation, grazing management, and precision nutrition.

Methane mitigation in ruminant systems requires a holistic or systems-level approach, integrating nutritional, management, and technological strategies. Beyond individual feed additives, feed formulation, grazing management, and precision nutrition can collectively optimise rumen function, reduce methanogenesis, and improve productivity efficiency per unit of emission (Beauchemin et al., 2020). By improving feed conversion efficiency, animal productivity, and resource use, total methane output can be reduced even where absolute emissions per animal remain similar. Integration across scales—from rumen microbiome to whole-farm management—is essential to achieve sustainable, long-term mitigation compatible with animal welfare and profitability (Arndt et al., 2022).

1.5. Challenges and knowledge gaps

Microbial adaptation and long-term efficacy.

Many additives (EOs, saponins, 3-NOP, ionophores) show rapid methane reductions in short trials, but sustained efficacy over months or entire production cycles is poorly documented (Hegarty et al., 2021). The rumen microbiome is resilient and can adapt by selecting for resistant taxa or alternative H_2 pathways (Beauchemin et al., 2020). Microbial shifts and adaptation over time remain a major concern. Optimal dosing schedules, carrier matrices, and interactions with feed processing that influence persistence in the rumen are under-researched. Also, for some compounds (e.g., volatile essential oils), loss during feed processing or via rumen turnover reduces long-term exposure.

Variability among animal species and production systems.

The efficacy of methane-mitigating additives varies widely among animal species, diets, and production systems. Because methane production is closely linked to feed intake behaviour and diet composition, factors such as feed quantity, meal size, and feeding frequency can markedly influence additive performance (Hristov et al., 2025). Significant differences in both methane yield and the rumen microbiota responsible for methanogenesis have been observed between animals consuming high-concentrate diets and those maintained on forage-based rations (Miller et al., 2023). Much of the existing evidence is derived from controlled studies in sheep or dairy cattle, which may not reflect responses under extensive grazing or in beef production systems (Hegarty et al., 2021). Moreover, breed-related differences in rumen physiology and feeding behaviour may further alter additive responses (Hegarty et al., 2021).

Rumen microbial composition also shifts with age and physiological stage, influencing methane output and responsiveness to additives (Miller et al., 2023). Despite its potential importance, few studies have investigated whether early life antimethanogenic interventions can produce lasting reductions in methane emissions. Evidence suggests that supplementation initiated soon after birth could influence rumen microbial development and lower methane production later in life (Yáñez-Ruiz et al., 2015; Beauchemin et al., 2020). Key knowledge gaps remain in understanding additive responses across beef versus dairy systems, grazing versus confined feeding, and across breeds and developmental stages.

Safety, residue, and consumer perception.

Some feed additives can negatively affect feed intake or nutrient digestibility—for example, high levels of tannins or lipid inclusion may reduce fibre degradation or alter milk composition. However, systematic evidence linking methane mitigation to animal performance across diverse production contexts remains limited. For synthetic inhibitors and concentrated plant extracts, data on residue occurrence in milk, meat, or offal, as well as their metabolic fate, are incomplete (Ku-Vera et al., 2020), and maximum residue limits have not been fully established for many compounds. Ionophores, widely used as feed additives in several countries, pose additional challenges due to concerns about antimicrobial resistance and differing national regulations, contributing to uncertainty and consumer distrust (Ahmed et al., 2024). Furthermore, transparent information regarding additive safety, labelling, and environmental benefits is often lacking, and consumer willingness to pay for verified methane-mitigation claims remains poorly understood.

Regulatory and policy barriers.

Even where efficacy is established (3-NOP has strong evidence), the regulatory pathways are complex and vary by country (Hegarty et al., 2021). For many additives (plant metabolites, alternative hydrogen sinks), evidence is weaker, especially for field implementation. There is a lack of harmonised measurement, reporting and verification (MRV) systems for methane mitigation in livestock. Additionally, many grazing systems (extensive) have little on-farm evidence, limiting scale-up.

Additives hold substantial potential to reduce enteric methane, but their role at scale is constrained by microbial adaptation, context dependency (diet, species, system), safety/residue questions and fragmented regulatory and market mechanisms. Prioritising long-term, mechanistic, and system-level research together with harmonised regulatory pathways and credible MRV will be essential to move from promising trials to safe, repeatable, and widely adopted mitigation strategies.

1.6. Future directions

Genetic and microbiome-based approaches.

Combining host genetics and rumen microbiome selection offers a promising pathway for achieving permanent and cumulative reductions in methane emissions by breeding low-emitting animals and promoting the establishment of beneficial microbial communities. Recent genomic–microbiome association studies have highlighted the potential of integrating host genetic markers with microbial traits to enhance methane mitigation outcomes, although large-scale, multi-breed validation trials are still required to confirm the heritability and stability of these traits (Roques et al., 2024). Moreover, evidence suggests that early-life interventions -such as inoculating young ruminants with desirable microbial consortia or exposing them to low-emission microbiomes soon after birth - could facilitate the stable colonisation of beneficial microbes and reinforce the host's genetic predisposition toward lower methane production (Meale et al., 2021; Pérez-Enciso et al., 2021). Such strategies may help establish a persistent low-emission phenotype from the earliest stages of rumen development, complementing genetic selection approaches and increasing the long-term effectiveness of methane mitigation programs (Beauchemin et al., 2022).

Precision feeding technologies.

Sensors, automated feeders, and precision-livestock-farming systems can tailor additive dosing and diet composition to individual animals or cohorts, improving efficacy and cost-efficiency while minimising negative production trade-offs (van Empel et al., 2016; Morey et al., 2023). By continuously monitoring feed intake, rumen function, and animal performance, precision feeding technologies enable dynamic diet adjustments that optimise nutrient use and reduce excess fermentable substrates (Hristov et al., 2025), thereby limiting hydrogen production and subsequent CH_4 formation. These systems can also ensure the precise and consistent delivery of methane-mitigating additives at effective doses, preventing under- or over-supplementation. Furthermore, real-time data integration from sensors tracking rumen pH, gas emissions, and feed efficiency allows for early detection of inefficiencies or metabolic imbalances that could elevate CH_4 output (Morey et al., 2023). Collectively, these technologies enhance feed conversion efficiency, lower methane yield per unit of intake, and support adaptive management for sustained mitigation. Field studies and reviews increasingly identify precision-livestock-farming systems as a practical near-term strategy to scale methane reduction across commercial production settings (Papakonstantinou et al., 2024).

Integration with carbon credit and sustainability frameworks.

Linking verified on-farm methane reductions (e.g., from additives like 3-NOP/Bovaer) to credible carbon markets or in setting programs can create revenue streams for producers, but requires harmonised MRV, conservative baselines and robust regulatory oversight. Policy briefs and market analyses stress the need for science-based accounting to avoid double-counting. In this context, molasses-based lick block technologies could play a valuable role by enabling the practical deployment and quantifiable delivery of methane-mitigating additives under grazing conditions. Because lick blocks can provide consistent, traceable additive intake and are easily monitored at the herd and paddock level (Imaz et al., 2020), they may facilitate more reliable estimation of emission reductions, improving eligibility for carbon credit schemes in extensive ruminant systems (Callaghan et al., 2020). Integrating such feed-delivery strategies within verified emission-reduction methodologies could therefore enhance both the scalability and credibility of livestock-based mitigation initiatives.

1.7. Conclusion

Enteric methane mitigation remains a critical challenge for achieving climate-neutral livestock production. This review highlights that a wide range of dietary additives—plant secondary metabolites (tannins, saponins, and essential oils), lipids and fatty acids, electron acceptors, and synthetic compounds such as 3-nitrooxypropanol (3-NOP) and ionophores—can effectively reduce ruminal methane emissions through diverse mechanisms. However, their efficacy is influenced by diet composition, animal species, production system, and duration of use, with long-term adaptation of the rumen microbiome remaining a major uncertainty.

Among the evaluated options, 3-NOP and lipid supplementation currently show the most consistent methane-reducing potential without major negative effects on productivity. This is reflected in recent policy developments, such as Denmark, where regulatory frameworks from 2025 require larger dairy farms to adopt methane- reducing feed strategies—including 3-NOP additives and increased dietary fat—as part of the country's drive to meet ambitious greenhouse-gas targets (Landbrugsstyrelsen, 2024). In comparison, saponins, essential oils, tannin and lipid supplementation offer moderate but context-dependent benefits. Emerging strategies such as microbiome-based interventions, probiotics, and enzyme supplementation show promise but require further validation under field conditions (Yu et al., 2021).

Future progress will depend on integrated, systems-level approaches that combine additive use with optimised feed formulation, precision nutrition, improved animal genetics, and grazing management (Vargas et al., 2022). Sustained investment in long-term, multi-system research, harmonised regulatory frameworks, and credible carbon-accounting mechanisms is essential to ensure that methane mitigation strategies are both effective and sustainable, contributing to resilient and climate-smart livestock systems (Papakonstantinou et al., 2024).

In addition to the dietary strategies discussed throughout this review, the practical delivery of methane-mitigating compounds remains a key consideration for effective implementation under commercial conditions (Beauchemin et al., 2022). Among the emerging approaches, the use of molasses-based lick blocks represents a promising strategy to facilitate the controlled intake of feed additives in grazing ruminants (Imaz et al., 2020; Hristov et al., 2025). Lick blocks serve as a convenient and cost-effective vehicle for the sustained release of these compounds helping to ensure a consistent supply and uniform consumption among animals. This delivery method is particularly advantageous in extensive production systems, where conventional feed supplementation is often impractical (Beauchemin et al., 2020). By integrating nutritionally balanced ingredients with targeted additives, lick blocks can enhance both methane mitigation efficacy and animal productivity while supporting ease of adoption at the farm level (Hristov et al., 2025). Building upon the mechanistic understanding and safety evaluation of dietary compounds outlined in this review, the following chapter forms the experimental foundation of this thesis, presenting an in vitro and in vivo assessment of molasses-based lick block formulations containing methane-reducing additives.

Table 1. Safe dietary compounds for mitigating enteric methane in ruminants: a comparative overview

Compound/Class	**Mode of action**	**Typical CH_4 reduction (%)**	**Experimental system**	**Advantages**	**Limitations / risks**	**References**
Tannins (condensed, hydrolysable)	Bind proteins; inhibit methanogens and protozoa; reduce H_2 availability	10–30%	In vivo (cattle, sheep) and in vitro	Naturally occurring; improve protein utilization	May reduce fibre digestibility and intake at high doses	(Patra and Saxena, 2009;Jayanegara et al., 2012)
Saponins	Defaunation (protozoa reduction); disrupt microbial membranes	5–25%	In vitro and in vivo	Plant-derived; potential health benefits	Microbial adaptation; variable effects	(Pen et al., 2006;Goel and Makkar, 2012)
Essential oils (thymol, eugenol, cinnamaldehyde)	Antimicrobial activity; shift VFA pattern toward propionate	5–20%	Mainly in vitro; some in vivo	Multifunctional; natural preservative effect	Adaptation and inconsistent responses	(Calsamiglia et al., 2007;Benchaar et al., 2008)
Fumarate / malate	Alternative H_2 sink via propionate formation	5–15%	In vitro and in vivo	Safe organic acids	Limited effect in vivo; cost	(Ungerfeld et al., 2007)
Lipids / fatty acids (e.g., coconut oil, linseed oil)	Suppress methanogens; reduce fibre digestion	10–30%	In vivo (dairy and beef cattle)	Improve energy density	Depress fibre digestibility; possible milk fat depression	(Machmüller et al., 2003;Beauchemin et al., 2008)
3-Nitrooxypropanol (3-NOP)	Inhibits methyl-coenzyme M reductase in	20–60%	In vivo (cattle, sheep); supported	Proven efficacy; approved additive (Bovaer®)	Cost; stability under storage	(Hristov et al., 2015;Melgar et al., 2020)

Compound/Class	**Mode of action**	**Typical CH_4 reduction (%)**	**Experimental system**	**Advantages**	**Limitations / risks**	**References**
	methanogens		by in vitro studies			
Ionophores (Monensin)	Shift rumen fermentation toward propionate	5–10%	In vivo (feedlot cattle, dairy cows)	Improves feed efficiency	Reduced long-term effect due to adaptation; antibiotic concerns	(McGinn et al., 2004;Guan et al., 2006)
Probiotics / Direct-fed microbials (*Propionibacterium*, *Megasphaera elsdenii*)	Compete for H_2 via propionate pathway	5–15%	In vivo (rumen and feedlot trials)	Can enhance gut health	Variable persistence; diet-dependent	(Newbold et al., 2015)
Plant oils / extracts (emerging)	Disrupt methanogens; redirect H_2	5–25%	In vitro and pilot in vivo	Natural and renewable	Inconsistent efficacy	(Hook et al., 2010)

Chapter 2: Assessment of molasses products containing additives to reduce methane emissions from beef cattle

This chapter was published in full: Nikoloric, M., Simanungkalit, G., Possell, M., González, L. A., & Chaves, A. V. (2025). Assessment of molasses products containing additives to reduce methane emissions from beef cattle [Original Research]. *Frontiers in Animal Science*, *Volume 6 - 2025*. DOI: 10.3389/fanim.2025.1666818

Frontiers in Animal Science

TYPE Original Research
PUBLISHED 23 October 2025
DOI 10.3389/fanim.2025.1666818

Assessment of molasses products containing additives to reduce methane emissions from beef cattle

Maria Nikoloric[1], Gamaliel Simanungkalit[1], Malcolm Possell[2], Luciano Adrián González[1,2] and Alex V. Chaves[2]*

[1]Centre for Carbon Water and Food, The University of Sydney, Camden, NSW, Australia, [2]School of Life and Environmental Sciences, Faculty of Science, The University of Sydney, Sydney, NSW, Australia

OPEN ACCESS

EDITED BY
David L. Harmon,
University of Kentucky, United States

REVIEWED BY
Arda Yıldırım,
Gaziosmanpas¸a University, Türkiye
Olegario Herna´ndez,
National Institute of Agricultural Technology (INTA), Argentina

*CORRESPONDENCE
Alex V. Chaves
✉ alex.chaves@sydney.edu.au

RECEIVED 15 July 2025
ACCEPTED 16 September 2025
PUBLISHED 23 October 2025

CITATION
Nikoloric M, Simanungkalit G, Possell M, Gonza´lez LA and Chaves AV (2025) Assessment of molasses products containing additives to reduce methane emissions from beef cattle.
Front. Anim. Sci. 6:1666818.
doi: 10.3389/fanim.2025.1666818

Methane (CH_4) emissions from ruminants significantly contribute to agricultural greenhouse gases. Strategies to reduce these emissions without compromising productivity are urgently needed. This study evaluated the effectiveness of a novel product incorporated into molasses containing additives and grain-based pellets to reduce CH_4 emissions and improve productivity in beef cattle. Four products were screened *in vitro* for their efficacy in reducing CH_4 production. The most effective (Product 3) was then incorporated into grain-based pellets and offered to steers via GreenFeed units at target intakes of 0, 100, and 200 g/head per day. Dry matter intake (DMI), CH_4 production, CH_4 yield (g CH_4/kg DMI), CH_4 intensity, water intake, and average daily gain (ADG) were measured. Product 3 reduced *in vitro* CH_4 production by over 90% at a 6% inclusion rate ($P < 0.05$). *In vivo* study, steers supplemented with product-containing pellets tended ($P=0.07$) to reduce CH_4 production by 10.7% compared to the control. Methane yield was reduced ($P < 0.01$) by 11.7% and 7.5% for the 100 and 200 g/day supplementation levels, respectively. Methane intensity tended ($P=0.06$) to be 16.8% lower in supplemented groups. Product intake was negatively correlated with CH_4 yield and intensity and positively associated with water intake and ADG ($P < 0.05$). The inclusion of Product 3 in supplements beef cattle fed high forage diet significantly reduced methane yield and tended to reduce CH_4 production and intensity, without negatively impacting performance. Product 3 shows potential as a viable CH_4 mitigation strategy for high forage diet systems. However, further research is needed to assess the long-term effects and potential microbial adaptation associated with higher or prolonged dosing.

KEYWORDS

enteric methane, greenhouse gas, cattle, molasses, pellet supplementation

1 Introduction

Agriculture is responsible for 19.1% of Australia's total greenhouse gas (GHG) emissions, with livestock methane (CH_4) emissions contributing to 57% of the agriculture sector, approximately 10% of the country's overall emissions (Department of Climate Change, 2022) The vast Australian landscape spans 769 million hectares that vary in soil and plant types as well as temperature zones. This diverse environment supports the grazing of approximately 97% of Australia's beef cattle. Due to these environmental differences, there are variations in feed quality and availability across regions, which affect animal performance throughout the year. For instance, in certain regions, forages lack energy, crude protein, vitamins, and/or minerals (Dixon et al., 2020), leading to inefficient nutrient utilization (Leng, 1990) and, eventually, low animal performance. To address these challenges, improving nutritional management through efficient forage use has been identified as a viable strategy for reducing GHG emissions (Eugène et al., 2021). Improving the quality of the pasture is one of the most common approaches, however, it is not always easy or economically feasible to achieve. This context underscores a strategy for reducing the amount of CH_4 produced per unit of feed consumed, thereby mitigating GHG emissions through dietary supplementation of grazing animals. Numerous studies have explored the supplementation of additives to ruminant diets, aiming to decrease enteric CH_4 production. For instance, 3-NOP supplementation has been shown to reduce ruminant CH_4 emissions ranging from 18 to 39% (Almeida et al., 2021). *Asparagopsis* included from 0.05%-3% of OM intake) could reduce methane emissions between 9 and 98% (Wasson and Hristov, 2022). However, the implementation of additives as a mitigation strategy is currently challenged by multiple factors including consistency of results, lack of information of responses in different production systems, cost- benefit, price of additives, and policies and legislation, amongst others. For example, inconsistencies in CH_4 reduction across a range of studies, negative impacts on other fermentation parameters or DMI and challenges in integrating them into practical feeding regimes, particularly in grazing systems (Patra et al., 2017; O'Reilly et al., 2021).

Research has highlighted the potential of essential oils (EO) to modify rumen microbial fermentation and boost ruminant feed efficiency (Calsamiglia et al., 2007). Those are typically obtained from plants through steam distillation or solvent extraction techniques (Simon et al., 1990; Greathead, 2003) although the plant material can also be fed and included in diets (Ku-Vera et al., 2020). Essential oils have anti-nutritional and antimicrobial characteristics to protect plants, prevent herbivory and attract pollinators, and can also assist in modifying the rumen microbiome to reduce the production of CH_4 (Patra et al., 2017; Garcia et al., 2020; Honan et al., 2021). In addition, the combinations of various EO may also increase microbial efficacy due to additive or synergistic effects between EO components (Benchaar and Greathead, 2011). Despite these promising findings, integrating EO into grazing systems remains a challenge. Lick blocks (LB) technology could deliver anti-methanogenic additives in grazing animals, which is traditionally used as a livestock strategic supplementation around the world to provide energy, protein, minerals, or therapeutic substances to improve animal performance (Makkar et al., 2007). The LB is practical to deliver, easy to manufacture and store, capable of containing feed additives with anti-methanogenic properties, and economically feasible (Imaz et al., 2019). The objective of the present study was to evaluate LB formulation to reduce CH_4 emissions and enhance the performance of beef cattle fed high forage diet. An important aspect of LB is that the ingredients can be tailored for different regions, animal requirements, seasons, and production systems. Four proprietary formulations were initially evaluated *in vitro*, and the best candidate was tested *in vivo*.

2 Material and methods

2.1 Feed formulation

The products that have been used in the experiments of the present study comprised proprietary mixes of natural products and essential oils sourced from the *Myrtaceae, Rutaceae, Poaceae, Amaryllidaceae*, and *Lamiaceae*. The formulation is proprietary protected (Australian Provisional Patent Application No. 2023902943). All compounds are used in the human food chain as ingredients and are generally regarded as safe and the rate of feeding does not differ from concentrations used in food manufacturing. Free ranging cattle and sheep browse plants that contain the bioactive compounds identified in the products from the *Myrtaceae, Rutaceae* and *Poaceae*. The product is manufactured in accordance with excluded nutritional or digestive (ENDS) products and the Australian Stockfeed Regulation (Authority, 2024).

2.2 *In vitro* study

The *in vitro* trial was conducted to screen potential products for reducing CH_4 emissions. A commercial LB manufacturing company (AgCoTech Holdings Pty. Ltd., Crestmead, QLD, Australia) provided four different proprietary products that were assessed at different concentrations. The products evaluated in this project contain natural plant ingredients, including vegetable oil, essential oils, condensed tannins, and tea saponins. The DM and ash results of the 4 products is presented in Table 1.

All animals were cared for in accordance with the guidelines of the University of Sydney Research Integrity Animal Ethics Committee (approval #2022/2180).

Three cannulated adult non-lactating crossbred beef cows fed with grass hay (13% CP; 60% aNDF) at maintenance level were employed for rumen fluid collection. These collections took place between two and three hours after feeding in the morning. Rumen fluid samples were taken from the dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral rumen areas and put

TABLE 1 Dry matter and ash content for products 1, 2, 3 and 4 used to manufacture molasses products containing additives assessed *in vitro* for their anti-methanogenic properties.

	Dry matter content, % as-fed	Ash, %DM
Product 1	49.7	41.5
Product 2	33.9	10.3
Product 3	30.1	44
Product 4	53.5	40.9

into a prewarmed thermos filled to the lid to eliminate air in the headspace and immediately transported to the laboratory. The fluid was not purged with CO_2 because there was no headspace after closing the lid. Rumen fluid was anaerobically transferred into a 2-L flask mixed with two parts of a phosphate buffer and kept at 39°C until *in vitro* incubation (Forwood et al., 2019).

The incubations design was a completely randomized design (CRD) with three incubation bottles (replicates) per treatment per run and two incubation runs. The base substrate for the incubation was a ratio of 100% grass hay (60% aNDF). Products 1, 2, and 4 were dried in a forced air oven at 55°C for 72h and grounded to 1-mm. Product 1 and 2 powders were added into F57 ANKOM filter bags (Ankom Technology, Macedon, NY USA) in 0, 5, 25, 50, 10 and 200 mg, corresponding to concentrations of 0, 1, 5, 10, 20, and 40% respectively, replacing the substrate to make up 0.5g DM in total. The product 4 powder was added in 0, 50, 100, and 200 mg, corresponding to concentrations of 0, 10, 20, and 40%, respectively, replacing the substrate to make up 0.5g DM. Product 3 was evaluated in the original liquid form and was added to the substrate bag containing 0.5g DM before the addition of rumen inoculum in 0, 0.5,1.0, and 1.5 mL corresponding to 0, 2, 4, and 6% concentrations of total volume (rumen fluid and buffer). All treatments were incubated in triplicate bottles using the batch fermentation method described in (Meale et al., 2012). Each run also contained three blank bottles with no substrate or treatments. On the incubation day, amber serum vials were warmed in the incubator at 39°C for 60 minutes and gassed with CO_2 before adding 25 mL of a 2:1 mixture of buffer saturated with CO_2: rumen fluid (Forwoord et al., 2019), after that process, the bottles were immediately closed with rubber stoppers and placed in an incubator set at 39°C with a rotatory shaker at 120 oscillations per min.

Batch culture bottles were removed from the incubator after 24h. To determine CH_4 concentration at that timepoint, 25 mL of fermentation gas was collected from the headspace of bottle using a syringe and transferred into a 12 mL evacuated exetainer for later measurement by gas chromatography. A water displacement apparatus was used to measure the remaining gas in the bottle. Total gas production was calculated by adding the 25 mL of CH_4 sample to the reading from the water displacement instrument (Fedorah and Hrudey, 1983). Then serum bottles were opened and immediately placed on ice to stop microbial fermentation, and pH was immediately tested (Activon Model 209, Gladesville, NSW, Australia). A 1.5 mL subsample of each bottle culture was placed into a 2 mL Eppendorf tube with 300 mL of metaphosphoric acid (20% w/w) and refrigerated at -20°C for subsequent volatile fatty acid (VFA) measurement following procedures described by (Wang et al., 2000; Chaves et al., 2006). ANKOM bags were removed from bottles, washed twice using a washing machine at short cycle (10 minutes), and cleaned to remove culture media, then dried for 24h at 60 °C for the estimation of *in vitro* dry matter digestibility (IVDMD).

2.3 *In vivo* study

Animal ethics approval was obtained from the Animal Ethics Committee of The University of Sydney (Approval # 2293). This animal feeding trial was carried out with the product that had the greatest reduction in CH_4 production from all products tested in the *in vitro* trials.

2.3.1 Animal feeding

For 70-d period, a completely randomized design (CRD) experiment was conducted using 45 Angus steers with an initial live weight (LW) of 233 ± 41.3kg (mean ± SD). The cohort included 36 Angus and 9 Angus × Charolais steers. The 45 steers were stratified into three LW strata (15 steers each), and within each stratum, steers were randomly assigned to three groups of 15 steers each. Upon arrival, all steers were vaccinated against BVD virus, major clostridial diseases (Ultravac® 5 in 1, Zoetis Australia Pty Ltd., Rhodes, New South Wales, Australia), and BRD (Bovilis® MH + IBR, Intervet Australia Pty Ltd., East Bendigo, Victoria, Australia). The animals were fed an oaten hay-based diet for two weeks before the treatments were introduced (as an adaptation period) and throughout the entire experiment. Each of the 3 treatments had 15 animals assigned to it: 1) control group (supplemented with pellets without product), 2) pellets containing the product for a target intake of 100g of product per head per day and, 3) pellets containing the product for a target intake of 200g of product per head per day treatment of the product incorporated into pellets. All pelleted sources were delivered through the GreenFeed® system (C-Lock, 2023).

The three treatment groups were split into two pens (4m length × 20m width) groups, with one pen occupied by control and a second pen occupied by 100g and 200g treatments. The control pen was equipped with 4 and the treatment pen had 6 electronic feeders (Intergado® Ltd, Contagem, Minas Gerais, Brazil), to deliver the hay and measure individual feed intake. Each pen had a water through with two cattle weighing scales measuring individual LW and estimating water intake from the change in weight of the animals while drinking water (Intergado® Ltd, Contagem, Minas Gerais, Brazil) and a GreenFeed® system with dual hoppers (C-Lock, 2023) to measure individual daily CH_4, H_2, and CO_2 production rate (g/d) and O_2 consumption rate (g/d), and to deliver the target dose of pellet per animal. Electronic feeders are mounted on load cells to measure individual feed intake and are equipped with a radio-frequency identification (RFID) reader to detect the presence of an animal by recording its electronic identification (EID) number. The electronic feeders automatically assigned the amount of feed consumed to the unique EID number present at the feeder when the feed weight decreased (Chizzotti et al., 2015; Oliveira et al., 2018). Samples of the offered feeds were collected weekly and dried at 70°C for 24h for the determination of DM content. Animals were taken to the yard and weighed at the start of the experiment, at 14-d intervals throughout the trial, and on the last day of the experiment. The average daily gain (ADG) was calculated by dividing weight gain (Final– Initial weight) by 56 (days of the experiment).

2.3.2 Pellet manufacturing process

Pellets were manufactured in a low-temperature process to preserve the active ingredients of the product. Rice bran (66.7%) and canola meal (33.3%) were finely ground and proportionally blended with the liquid product to achieve the designated inclusion levels (0, 6.28%, and 11.74%) without adding the settling agent. Mechanical compression was used to form pellets via a 4.4mm × 12mm die with the maximum temperature maintained below 54°C. Following this, the pellets were air-dried and cooled under ambient conditions to reduce moisture content, improve pellet durability, and minimize volatilization of the active ingredients.

2.3.3 Product supplementation

The target intake was 0, 100, and 200g of product/d into a pellet amount of 1.5 kg/head.d. One ton of pellets was proportionally mixed with 0, 67, and 133kg of the liquid product, respectively. These pellets were fed at 1.5kg (0g product), 1.6kg (100g product), and 1.7kg (200g product) per head per day.

The GreenFeed system was configured to provide the target amount of pellets to entice the animals to visit the GreenFeed multiple times per day (C-Lock, 2023). The double hopper GreenFeed in pen with the two treatments provided the two doses of product incorporated into the pellets depending on the treatment allocated to the RFID identified by the system. The pellets were dropped into a tray in a semi-enclosed hood when the RFID tag of an animal was present within the hood of the GreenFeed. The visit frequency (visits/d), number of feed drops (drops/visit), and the interval between visits were adjusted to ensure that each steer across all groups received the targeted intake as follows:

Control: visit frequency = 6 visits/d (spaced at a minimum of 120min intervals); number of cups dropped/visit = 5 (at 40 s intervals); average cup drop = 42g.

100 g per head per day: frequency = 4 visits/day (120min interval); feed drops/visit = 6 (40 s interval); average cup drop = 70g.

200 g per head per day: visit frequency = 6 visits/d (spaced at a minimum of 120min intervals); number of cups dropped/visit = 7 (40 s interval); average cup drop = 41g.

2.3.4 Quantification of CH_4 emissions

Individual CH_4 emissions measured by the GreenFeed system are expressed as daily CH_4 production (g CH_4/d). Visit duration and the number of records per individual are critical for CH_4 measurement because CH_4 is typically belched at 40–120 s intervals (Hammond et al., 2016). The GreenFeed operation commences when the steer places its head inside the shroud (Hammond et al., 2015). Following this, the proximity sensor in the shroud will monitor the head position of the animal during each visit, which will also be used to dismiss all measures where animals stepped out from the GreenFeed. Air is continuously drawn through the shroud and past the neck of the animal at a precisely measured rate, and the CH_4 and carbon dioxide (CO_2) concentrations are quantified in the exhaust air stream (Velazco et al., 2016). As the GreenFeed system provides multiple short-term breath measures, 30 measurements over a two-week period (Koning et al., 2024) with a minimum of three min durations per visit are needed to achieve a minimal variance in CH_4 production rate per animal (Arthur et al., 2017). Data are logged and transmitted into the C-Lock Inc. data management system and can be downloaded through the C-Lock Inc. website interface (https://greenfeed.c-lockinc.com).

The chemical composition of the hay and treatment pellets is presented in Table 2, and the ingredients composition of pellets is shown in Table 3. Dry matter (DM) analysis was determined by oven-drying samples at 70°C for 48h, followed by hot weighing (Cunniff and Washington, 1995). Pellets were sent to be analyzed using AOAC methods of chemical analysis (Cunniff and Washington, 1995). Unless otherwise stated, all results are reported on a DM basis (Cunniff and Washington, 1995).

2.4 Compound odor analysis

Products compounds were identified through gas chromatography mass spectrometry (GC-MS) analysis of volatile compounds using a Gerstel Thermal Desorption Unit (TDU; Gerstel, Mülheim an der Ruhr, Germany). The samples were analyzed after drying for products 1, 2, and 4, and as received for product 4 as used for the *in vitro* studies. Milligram masses of each product were weighed into 200 µL micro vials, secured in glass thermal desorption liners and inserted into the TDU for analysis. When inserted in the TDU, the samples were purged with ultra-high purity helium (BOC Ltd, North Ryde, NSW, Australia) at 20°C for 1min to eliminate air from the sample and inlet. Samples were heated to 150°C by the TDU at a rate of 12°C/s with a helium flow of 75 mL/min. Thermal desorption (TD) products were carried by the helium through to a programmed temperature vaporization (PTV) inlet (CIS-4; Gerstel) installed in an Agilent 7890 GC (Agilent Technologies Pty Ltd, Mulgrave, Australia), which was used in solvent mode during the TD. The PTV inlet, containing a glass liner filled with Tenax TA, was held at 20°C during the TD using liquid CO_2 (BOC Ltd) as the cryogen. After 1min of TD, the CIS-4 was heated at 12°C/s to 300°C and held at that temperature for 5 minutes while the TD products were injected into the GC at either a 1:10 or 1:500 split ratio. TD products were separated on a HP5-ms capillary column (30m × 0.25mm × 0.25 µm film thickness; Agilent) for analysis by a mass selective detector (Model 5975C; Agilent). Ultra-high purity helium was used as carrier gas (flow rate through the HP5-ms column was 2.3 mL

TABLE 2 Chemical composition of Product 3 used to manufacture molasses products containing additives treatment pellets and oaten hay fed to growing steers during the *in vivo* trial.

	Unit	Product 3	Pellets			Oaten hay
			0 g	100 g	200 g	
Dry matter (DM)	%	38.4	91.3	92.4	90.3	92.0
Neutral detergent fiber (NDF)	% DM	NA	21.0	21.0	22.0	67.0
Acid detergent fiber (ADF)	% DM	NA	12.0	12.0	12.0	42.0
Crude protein (CP)*	% DM	14.4	23.1	22.8	22.7	5.9
Crude fat (ether extract)	% DM	25.3	15.7	15.5	15.3	–
Water soluble carbohydrates (WSC)	% DM	19.8	8.7	9.6	10.1	4.3
Dry matter digestibility (DMD)	% DM	NA	78.0	78.0	76.0	50.0
Inorganic ash	% DM	9.4	9.4	9.5	9.6	7.0
Organic matter (OM)	g/kg DM	906	906	905	904	930
Metabolizable energy (ME)**	MJ/kg DM	15.5	15.8	15.8	15.5	7.0
Total starch	% DM	NA	14.0	14.1	13.6	NA
Urea	g/100 g	2.79	<0.03	0.09	0.17	NA
Nitrate	mg/kg	29	13	42	46	NA
Aluminum	mg/kg	13	120	99	73	NA
Arsenic	mg/kg	<5	<5	<5	<5	NA
Boron	mg/kg	<4	10	11	10	NA
Calcium	%	0.66	0.30	0.37	0.44	NA
Cadmium	mg/kg	<0.2	<0.2	<0.2	<0.2	NA
Cobalt	mg/kg	0.52	0.20	0.21	0.24	NA
Chromium	mg/kg	0.23	0.67	0.62	0.54	NA
Copper	mg/kg	0.94	8.1	7.8	7.8	NA
Iron	mg/kg	55	200	160	150	NA
Potassium	%	0.70	1.6	1.6	1.7	NA
Magnesium	%	0.21	0.81	0.80	0.84	NA
Manganese	mg/kg	15	180	180	180	NA
Molybdenum	mg/kg	0.12	0.61	0.65	0.67	NA
Sodium	%	0.71	0.027	0.090	0.14	NA
Nickel	mg/kg	<0.7	0.91	0.88	1.0	NA
Phosphorus	%	0.031	1.9	1.8	1.9	NA
Lead	mg/kg	<2	<2	<2	<2	NA
Sulfur	%	0.12	0.34	0.33	0.34	NA
Selenium	mg/kg	<0.05	0.17	0.17	0.17	NA
Zinc	mg/kg	5.3	62	57	58	NA
Dietary cation-anion Difference *(calc)*	meq/kg	NA	200	200	220	NA

(Continued)

TABLE 2 Continued

	Unit	Product 3	Pellets			Oaten hay
			0 g	100 g	200 g	
Chloride	%	NA	0.028	0.13	0.22	NA
Monensin	mg/kg	<2	NA	NA	NA	NA

- *measured value from feed analysis using Dumas Combustion Method.
- **ME value for a liquid block was measured using bomb calorimeter; others were estimated using a following formula:
- ME = [(crude protein × 35) + (crude fat × 84.6) + (WSC × 35) kcal/kg] × 0.004184.

TABLE 3 Ingredients composition of control and treatment pellets used to manufacture molasses products containing additives fed to growing steers.

	Control	100 g	200 g
Daily allowance, kg per head per day	1.5	1.6	1.7
Rice, %	66.7	62.53	58.85
Canola, %	33.3	31.21	29.38
Product, % of total	0	6.25	11.76
Product, % in the pellet	0	6.66	13.33

min–1. The initial oven temperature of the GC was 35°C, held for 5 minutes, then heated at a rate of 5°C/min to 160°C, then heated at a rate of 25°C/min to 300°C and held isothermally for 4.4min. The temperature of the GC-MS interface was 280°C, the MS ion source 230°C and the quadrupole 150°C. The detector, in electron impact mode (70 eV), scanned the range of 35–300 m/z. Operation of the GC-MS was controlled by Agilent Chemstation (version E.02.01.117) and the TDU by Maestro (version 1.4.36.16; Gerstel).

The choice of 1:10 or 1:500 split ratio for a sample was determined by preliminary method testing the four products used for the *in vitro* study (Product 1, 2, 3 and 4), exhibiting significant quantities of VOCs. Hence, 2–5 mg of sample was used with a 1:500 split ratio. The remaining samples were injected at a 1:10 split ratio from 12–16 mg of sample.

Post-processing of the GC-MS data was performed as described in Lawson et al. (2020) except compound identification was made using the NIST14 database (Mikaia et al., 2014), and results were expressed as "present" or "not present".

2.5 Statistical analysis

2.5.1 *In vitro* study

Data obtained from *in vitro* fermentations were analyzed as a completely randomized design using PROC MIXED of SAS software (9.4 version, SAS Institute Inc., Cary, NC), using treatment, and dose as fixed effects whereas the random effects were defined as the incubation run (experimental unit). The incubation run was employed as an error term to assess treatment effects on CH_4, rumen fermentation parameters, and *in vitro* dry matter digestibility (IVDMD). Treatment effects were determined by comparing treatment dose replicates means with control, using least squares mean linear hypothesis test (LSMEANS/DIFF) with Dunnett adjustment, declaring significance at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

2.5.2 *In vivo* study

Data from the GreenFeed system and Intergado® electronic feeders were statistically processed using R (R Core Team, 2023). Intake data from feeders was deleted if DMI that were above 3.5% of individual LW from that period. Methane emissions, LW and feed intake data were used to calculate average values for each animal throughout the 56d of the treatment period and data from the 30-d adaptation period were not used for analysis. There were 15, 13, and 14 steers for treatment 0, 100, and 200 g/d that had more than 30 measurements for the statistical analysis. Pearson correlation analyses were performed among CH_4 production and feed and product intake after the data was averaged for each across the treatment period (Charmley et al., 2015). Average daily product intake was calculated by multiplying the pellet intake measured by GreenFeed by the concentration of product in the pellet according to treatment. A CRD with ANOVA was used with the treatment group as the fixed effect and 45 animals as the experimental units. Differences between treatments across the groups were considered significant when $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

3 Results

The results from odor analysis of the four products (Table 4) are consistent with those commonly found in blends of plant secondary metabolites such as essential oils (EO). Product 1 contained 3-carene, eucalyptol, D-limonene, and p-cymene. Product 2 contained 2,3-butanediol, 3-carene, eucalyptol, D-limonene, b-longipinene, (1aR,7R,7aS,7bR)-1,1,4,7-tetramethyl-1a,2,3,5,6,7,7a,7b-octahydro-1H-cyclopropa[e]azulene, and 2,3-dimethyl-nonadecane. Product 3 analysis found the presence of 2,3-butanediol, D-limonene, and trans-1-methyl-4-(1-methylethyl)- 2-cyclohexen-1-ol. Finally, the compounds found in product 4 were 3-carene, eucalyptol, and [1S, cis]-naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-[1-methylethyl].

3.1 *In vitro* study

The results of *in vitro* trial evaluating the four products are shown in Table 5. All variables were affected by treatment ($P < 0.05$). Product 1 improved IVDMD at concentrations of 5% and above ($P < 0.05$)

TABLE 4 Gas chromatography mass spectrometry (GC-MS) analysis of products 1–4 for odor compounds potentially reducing methane emissions in cattle.

Mean retention time	Product 1	Product 2	Product 3	Product 4	Compound ID	Compound class	Synonym
1.75	Not present	Not present	Not present	Present	Dimethyl sulfide	Organosulfur	–
13.79	Not present	Present	Present	Not present	3-Carene	Monoterpene	Bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-
14.71	Present	Present	Not present	Present	Eucalyptol	Oxygenated monoterpene	1,8-Cineole
15.02	Present	Present	Not present	Present	D-Limonene	Monoterpene	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (R)-
15.24	Present	Present	Present	Not present	p-Cymene	Alkylbenzene (related to monoterpenes)	Benzene, 1-methyl-4-(1-methylethyl)-
17.13	Present	Not present	Not present	Not present	Cyclohexene, 1-methyl-4-(1-methylethylidene)	Monoterpene	Terpinolene
27.78	Not present	Not present	Present	Not present	b-Longipinene	Sesquiterpene	(1S,2R,7S,8S)-2,6,6-Trimethyl-9-methylenetricyclo $[5.4.0.0^{2},^{8}]$undecane
29.93	Not present	Present	Not present	Not present	1H-Cycloprop[e]azulene, octahydro-1,1,4,7-tetramethyl-, [1aR-(1aa,7a,7ab,7ba)]-	Sesquiterpene	(+)-Ledene
30.32	Not present	Present	Not present	Not present	Naphthalene, hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	Sesquiterpene	d-Cadinene
35.16	Not present	Not present	Not present	Present	1-Hexadecanol	Alcohol	–
37.39	Not present	Present	Not present	Not present	Unknown	Very noisy spectra – column bleed	–

and did not affect other *in vitro* fermentation parameters ($P > 0.10$). Product 2 increased IVDMD by 10% and above whereas gas production increased, and pH decreased by 40% of the product only ($P < 0.05$). Product 3 was the most effective in all *in vitro* fermentation parameters, reducing CH_4 production by over 90% at 6% dose of the product compared to the control ($P < 0.01$), and decreasing pH at 2% to 6% concentrations. In addition, 6% concentration of product 3 also reduced VFA concentration and IVDMD ($P < 0.05$). The lowest dose of product 3 enhanced fermentation due to a 27% numerical increase of total VFA concentration and 55% ($P < 0.01$) increase of gas production compared to the control treatment. A dose of 4% v/v of product 3 reduced methane production ($P < 0.01$) without negatively affecting fermentation as suggested by no differences in gas production and VFA against the control ($P > 0.05$). The molar percentage of acetate was drastically reduced with high doses of product 3 whereas propionate and butyrate were increased ($P < 0.01$). Product 4 improved IVDMD at all the concentrations assessed ($P < 0.05$), but all the other paraments were not affected ($P > 0.05$). Butyrate molar proportion increased with all doses of product 3 whereas branch-chained volatile fatty acids (BCVFA) increased by 6% of product 3 compared to the control treatment ($P < 0.05$; Table 5). None of the other treatments affected butyrate or BCVFA ($P > 0.05$).

3.2 *In vivo* study

Table 6 presents the performance and CH_4 emissions of growing beef steers over the 56-d experiment. Total feed, hay, and pellet intakes were similar among treatments ($P \geq 0.28$, Table 6). In addition, ADG was similar for all dietary treatments ($P=0.35$). Product intake (g/d) was significantly different across the groups, with the greatest intake in the 200 g/d group ($P < 0.01$).

The number of cups delivered was the fewest for steers fed 100 g/d, whereas the GreenFeed average visit duration was shortest for steers in the control treatment ($P < 0.05$; Table 6). This resulted in no differences in total daily time with good data in the GreenFeed between 0 and 100 g/d ($P > 0.05$), but the 200 g/d had the longest daily GreenFeed data compared to the other treatments ($P < 0.05$; data not shown).

Results indicated a tendency for CH_4 production to decrease in the 100 and 200 g/d groups compared to the control treatment, with reductions of 10.7% and 6.3%, respectively ($P=0.07$; Table 6). Methane yield (g CH_4/kg DMI) was lower ($P < 0.01$) in steers fed 100 and 200 g/d treatments, showing reductions of 11.7% and 7.5% compared to the control group (Table 6). Methane intensity (g CH_4/kg ADG) also tended to decrease in the 100 and 200 g/d groups by 16.8% and 14.9%, respectively ($P=0.06$) compared to the control.

TABLE 5 *In vitro* gas production and fermentation parameters of four different candidate products for the manufacturing of lick blocks.

	Control	Product 1					Product 2					Product 3			Product 4				P-value
	0%	1%	5%	10%	20%	40%	1%	5%	10%	20%	40%	2%	4%	6%	10%	20%	40%	SEM	Treat
Gas, mL/g DM	85	82	85.8	87.3	84.4	78.8	91.8	93.5	98	105.7†	117.0*	131.8*	102.4	76.5	91.7	92.4	96.1	8.42	<0.01
CH$_4$, %	14.8	15.1	15.4	15.5	13.2	12.7	15.3	14.4	14.7	15.2	14.6	15.6	5.6*	1.2*	15.2	14.8	14.3	1.4	<0.01
CH$_4$, mL/g DM	12.7	12.4	13.3	13.7	11.1	10	14.2	13.5	14.4	16.1	17.2	20.7*	6.8*	0.92*	13.9	13.7	13.8	2.25	<0.01
pH	6.89	6.89	6.88	6.84	6.86	6.9	6.86	6.81	6.74	6.69†	6.54*	6.48*	5.84*	5.00*	6.79	6.81	6.70†	0.05	<0.01
Total VFA, mM	66.4	68.9	66.9	65.3	67.3	58	72.8	69	67.9	75.7	88.2	84.5	61.2	16.5*	69.8	79.9	75.3	6.14	<0.01
% of total VFA																			
Acetate (A)	65.2	64.8	64.5	65	65.4	66.5	65.5	63.5	64.5	64.6	62.7	59	65.3	3.2*	67.6	65.8	64.9	2.01	<0.01
Propionate (P)	21.3	21.4	20.6	20.4	20.6	19.3	21.3	21.4	22	21.5	22.8	12.0*	11.5*	42.5*	20.5	20	20.9	2.15	<0.01
Butyrate	9.1	9.4	9.3	9.8	9.6	9.6	8.7	9.6	9.7	10.1	10.4	23.8*	20.6*	45.6*	8.2	10.5	10.7	1.87	<0.01
BCVFA	2.53	2.51	3.1	2.75	2.48	2.65	2.56	3.04	2.2	2.19	2.24	2.63	1.49	5.06*	2.18	2.12	2.03	0.707	<0.01
Ratio A:P	3.06	3.03	3.14	3.19	3.17	3.44	3.08	2.97	2.93	3	2.75	4.92	5.68	0.08	3.3	3.28	3.1	1.1	N/A
IVDMD, %	51.7	51.4	54.1*	54.4*	57.7*	63.9*	51.4	53.7	56.6*	59.7*	66.9*	49.5†	48.9*	44.7*	57.0*	60.2*	69.3*	0.72	<0.01

CH$_4$, methane; IVDMD, *in vitro* dry matter digestibility; VFA, volatile fatty acids; BCVFA, branched-chain VFA (iso-butyrate + iso-valerate); SEM, standard error of the means.
*Means differ (P ≤ 0.05) from control treatment, † means tend to differ from control treatment (P ≤ 0.10).

TABLE 6 Animal performance and greenhouse gases (GHG) emissions of growing beef steers after being offered pelleted with product 3 for 56 days of the experiment.

	Treatment g Product per head per day			SEM	*P*-value
	Control	100 g	200 g		
Total DMI, kg/day	6.93	6.90	7.01	0.272	0.96
Hay DMI, kg/day	5.74	5.60	5.85	0.251	0.78
Pellet DMI, kg/day	1.20	1.29	1.15	0.588	0.28
Targeted pellet intake, kg/day	1.50	1.60	1.70	–	–
Product intake, g/day	0.00^{a}	92.3^{b}	172.5^{c}	4.99	<0.01
ADG, kg/day	0.71	0.78	0.78	0.04	0.35
GreenFeed cup consumption, n#/day	31.8 ^{a}	20.0^{b}	32.2 ^{a}	1.03	<0.001
GreenFeed visits, n#/day	4.42^{a}	3.26^{b}	4.40^{a}	0.203	<0.001
GreenFeed visit length, sec/visit	257.9^{b}	331.8^{a}	361.6^{a}	15.69	<0.001
Time of GreenFeed visit, h in 24 h	9.0	11.3	12.0	0.230	<0.001
GreenFeed Total time visits, h	30.8	23.4	31.4	1.58	<0.001
GreenFeed total visits	358	234	308	12.6	<0.001
CH_4 production, g/day	159	142†	149 †	5.10	0.07
CH_4 yield, g/kg DMI	24.0^{a}	21.2^{b}	22.2^{ab}	0.55	<0.01
CH_4 intensity, log (g/kg ADG)	2.35	2.27†	2.28†	0.03	0.06
CH_4 intensity, g/kg ADG	223.9	186.2†	190.5†	1.07	0.06
CO_2 production, g/day	4492	4424	4479	146	0.94
H_2 production, g/day	0.217	0.236	0.225	0.01	0.42
O_2 consumption, g/day	3258	3449	3484	119	0.35
Water intake, L/day	28.9^{a}	37.0^{bc}	40.3^{cd}	2.31	<0.01

DMI, dry matter intake; ADG, average daily gain; SEM, standard error of the means.

^{a,b}means with different superscript differ at $P < 0.05$.

†means tend to differ from control treatment ($P \leq 0.10$).

No significant differences were observed between the 100g and 200 g/d treatments for any variable ($P > 0.10$). Additionally, there were no differences among groups ($P \geq 0.35$) in CO_2 and H_2 production (g/d) or oxygen consumption (g/d) as measured by the GreenFeed unit.

Daily water intake increased with the incremental dose of product supplementation, showing 28% and 39% increases on the steers treated with the 100 and 200 g/d treatments compared to the control steers, respectively ($P < 0.01$).

The correlation matrix among selected variables is illustrated in Figure 1. Average product intake throughout the trial was positively correlated with ADG ($P < 0.05$) and water intake ($P< 0.001$). Importantly, the average product intake was negatively correlated with CH_4 intensity ($P < 0.01$) and CH_4 yield ($P < 0.05$) and positive with O2 consumption ($P < 0.05$).

4 Discussion

The objective of the *in vitro* studies was to assess different formulations of products containing a range of essential oils. Some of these products are volatile as demonstrated in the odor analysis, and others can bind with sugar and lipid moieties. It is important to note that products 1, 2, and 4 were dried at 55°C to improve dose accuracy. In contrast, product 3 was used 'as received' because it could not be dried. Drying may have affected the activity or concentration of some essential oils, which prevents us from making a direct comparison between the products. However, the objective of the *in vitro* trial was to select the product with the greatest effect on CH_4 production. It is important to note that the analysis of volatile compounds of each product was done after drying products 1, 2, and 4 as used *in vitro*. In addition, both pellet

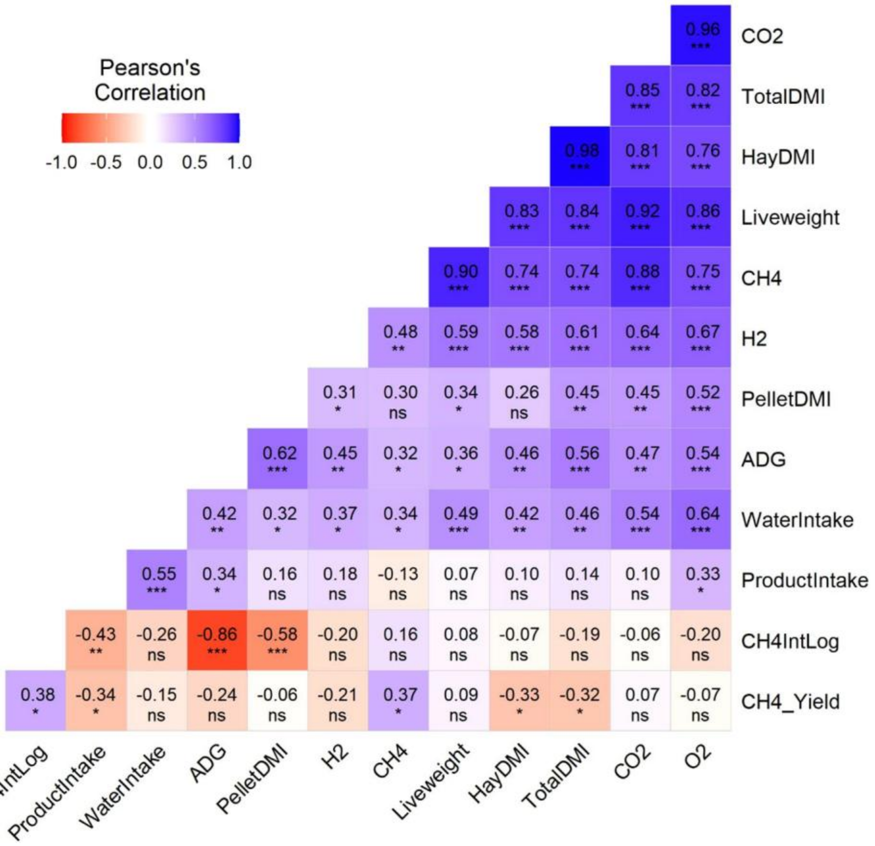

FIGURE 1
Correlation matrix of the performance and greenhouse gases (GHG) emissions of beef steers offered product supplement used to manufacture pellets with molasses containing additive during 56d of the experiment. ns P ≥ 0.05; *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001. TotalDMI, Total Dry Matter Intake; HayDMI, Total Dry Matter Intake from Hay; PelletDMI, Dry matter Intake from Pellets; ADG, Average Daily Gain.

and lick block manufacturing (where the products are added) also involve increased temperatures. Furthermore, the ash content values obtained in this study are higher than typically expected for organic feed products. The exceptionally high ash content, particularly in Product 3 (44% DM), raises concerns about the composition of the material. While Mix 3 is described as a liquid-solid admixture, the ash content suggests a significant presence of inorganic material, potentially making it similar in composition to a clay slurry or mineral-rich mixture. Given this, further investigation into the mineral composition of the products may be warranted to confirm the nature of the ash content and its potential impact on fermentation dynamics.

Product 3 emerged as the standout performer in reducing CH_4 emissions *in vitro*, demonstrating a remarkable efficacy across all evaluated *in vitro* parameters. Notably, at a 6% dosage of product 3, it reduced CH_4 production by over 90% in comparison to the control group, highlighting its potent effect albeit the significant reduction in IVDMD. This product was then selected for the *in vivo* trial, where results indicated that steers treated with the product tended to have up to 10.7% reduction in CH_4 production. Moreover, CH_4 yield from steers treated with the product were up to 11.7% lower than those without the product in the pellet. Furthermore, CH_4 intensity tended to be 16.8% lower for the animals fed Product 3 compared with the control, reflecting the efficiency of the product in reducing emissions.

Results from the volatile analysis showed compounds present in each product that are consistent with those commonly found in blends of plant secondary metabolites such as EO. Dimethyl sulfide was one of the compounds found to be present in Product 4, belonging to the organosulfur chemical group, which are common constituents of many plant species including those used in the present study and garlic oil (Honan et al., 2021). Organosulfur compounds have been suggested to reduce enteric CH_4 due to their toxic effects on methanogenic archaea (Soliva et al., 2011). In a study by Soliva et al. (2011), garlic oil was added to a mix of hay and soybean meal concentrate *in vitro* at a dose of 300 mg/L of incubation liquid and reported a 91% reduction in CH_4 production. Pawar et al. (2014) also screened garlic oil *in vitro* in a concentrate diet and reported reductions in CH_4 of up to 71.5% when included in a dose of 30 µL/mL of liquid incubation, though there was also a significant negative impact on overall fermentation noted. Patra and Yu (2015) used garlic oil in a forage-based diet *in vitro* and also reported a reduction in CH_4 but only by 29%, indicating that dimethyl sulfide may be more effective for use in concentrate diets compared to forage-based diets as used in the present study. However, Product 4 did not have a significant reduction in CH_4 and IVDMD had a significant increase in the present study. These differences may be due to low concentration of the active ingredients in the present compared to previous studies, but this cannot be confirmed because dimethyl sulfide concentration was not measured, and it is also unclear whether this or other compounds present in garlic oil may be responsible for a reduction of CH_4 production.

Compounds belonging to the monoterpene and oxygenated monoterpene chemical groups were identified in all four products screened in the present study. As classes of secondary plant metabolites commonly found in EO, some oxygenated monoterpenes have been found to inhibit the metabolism and growth of microbes in the rumen (Garcia et al., 2020). Eucalyptol, an oxygenated monoterpene was identified in product 1, 2, and 4. Results from *in vitro* analysis of those products did not indicate any significant change in CH_4, pH and VFA in the present study which aligns with the results found in the literature. Eucalyptol oil was screened *in vitro* in a study by Colombini et al. (2021). It was added to a total mixed ration substrate in a dose of 200 mg/L of inoculum and incubated with measurements taken at 24 and 48h time points. The study found that there was no observable impact on CH_4 production at either time point, or on CH_4 concentration after 24h. However, there was a 7% reduction in CH_4 concentration after 48h (Colombini et al., 2021). The latter study did not observe any impact on pH or total volatile fatty acids (VFA) production, despite the changes in the relative abundance of rumen microbes. In particular, the Firmicutes-to-Bacteroidetes ratio was higher when eucalyptol was added compared to the control, a trend that has been previously associated with reduced feed conversion (Jami et al., 2014; Colombini et al., 2021). Colombini et al. (2021) did not measure IVDMD, but the reduced fermentation may have been a result of impaired microbial proportions and explained the reduction of CH_4 at a later time point.

Comparatively, the hydrocarbon monoterpenes, such as 3-carene or D-limonene identified in products 1, 2, 3, and 4, have been reported to increase rumen microbial activity in some cases (Kahvand and Malecky, 2018), which aligns with results from our experiments assessing product 3. For instance, the *in vitro* evaluation of product 3 showed a significant reduction of CH_4 at 4 and 6% v/v concentration, but no significant changes at 2% concentration. This could be attributed to an insufficient dose. Furthermore, feeding product 3 *in vivo* confirmed that CH_4 yield decreased and CH_4 production and intensity tended to decrease with product 3. In contrast, no change was observed on CH_4 production when product 2 was assessed *in vitro*, but there was an increase in gas production and IVDMD. It has been suggested that monoterpenes may be broken down and utilized as a source of carbon by rumen microbes (Benchaar et al., 2008; Garcia et al., 2020). This may explain the significant increase in IVDMD with increasing dose of products 1, 2, and 4 in the present study, all of which contained monoterpenes. Nonetheless, Product 3 also contained monoterpenes but showed a significant decrease in IVDMD especially when a greater dose was added. This decrease could be attributed to the reduction in pH which will be further explained in this discussion.

Sesquiterpene compounds were also found in Products 2 and 3. (Nunes et al., 2023) evaluated the effects of three EO from exotic plants at different levels, with *Hedychium gardnerianum* containing essential oils from the sesquiterpene family. Results from that study indicated that both gas and CH_4 production decreased significantly compared to the control treatment after incubation at medium and high but not at the lowest concentration. Similarly, a significant reduction in CH_4 production was observed at medium and high concentration of product 3, but gas production was not affected at medium and high concentrations and increased at the lowest concentration of product compared to the control treatment. Product 2 increased gas production by 28.9 and 37% at 20 and 40% of the substrate, although $CH_{4\%}$ was not affected. The variation in outcomes observed from differing treatments and concentrations indicates an interaction between the type of EO and its quantity, which affects both total gas and CH_4 production. The presence of active compounds within each EO, and their combination, can lead to distinct synergistic, antagonistic, or additive impacts on the results (Burt, 2004). Also, possible differences exist in rates at which microbial populations degrade these secondary metabolites (Broudiscou et al., 2007). Given these factors, additional studies are required to ascertain the degree to which the essential oils used in the present project can be degraded or metabolized within the digestive system of ruminants.

Previous research that has demonstrated successful CH_4 mitigation from EO supplementation suggests that a trade-off exists between CH_4 inhibition and overall fermentation (Durmic et al., 2014; Jahani-Azizabadi et al., 2019; Parra et al., 2023).

Although modifications in VFA profiles are often deemed beneficial, EO can sometimes lead to undesirable shifts in individual VFA proportions. For instance, Castillejos et al. (2006) observed that eugenol at 500 mg/L diminished propionate proportion without altering total VFA concentration. Moreover, Cardozo et al. (2005) demonstrated that the impact of EO on VFA profiles is contingent upon pH. The 6% concentration of Product 3 decreased the medium's pH to 5, seemingly at odds with the lower VFA concentration. These pH values are of particular concern as pH < 5.5 is considered within the threshold for subacute ruminal acidosis, and pH < 5.0 is considered acute ruminal acidosis (Penner et al., 2007). However, this can be attributed to the product's inherent acidity which was measured at 4.82 (data not shown), indicating that the low ruminal pH may be due to the increasing concentration of Product 3 instead of a result of fermentation products. It is important to point out that the dose rate used in the present *in vitro* study may not yield a significant pH change *in vivo* because the animal produces bicarbonate buffer and the dose is low compared to total rumen turnover and volumes. In contrast, 4% of product 3 reduced $CH_{4\%}$ by 62% without negatively impacting fermentation, and medium pH was 5.84. This underscores the significance of product 3 dose optimization and suggests that as an optimum concentration.

In the *in vivo* study, water intake increased with product 3 supplementation, potentially due to its higher mineral content, prompting animals to drink more to sustain high pellet intake. This phenomenon has been well-documented: cattle consuming salt-enhanced supplements or saline water show increased water intake and enhanced renal diuresis (e.g., heifers consuming salty water exhibited water intake rising from 21.8 to 41.5 kg/day with increasing salt concentrations) (Visscher et al., 2013). Additionally, studies investigating the broader impacts of salt supplementation to low-quality forage diets found that increasing salt levels can alter forage intake, rumen fill, liquid kinetics, and fermentation patterns, further reflecting the physiological consequences of elevated salt intake (White et al., 2024). Despite this rise in water intake, no significant changes were noted in ADG or feed intake. This observation aligns with findings from various *in vivo* studies with EO fed to beef cattle where no effect was demonstrated on feed intake (Beauchemin and McGinn, 2006; Yang et al., 2010), suggesting no changes in rumen methanogenesis or adaptation of the microbes to EO. Cardozo et al. (2006) found that while a high dose of EO (a blend of 600 mg/day of cinnamaldehyde and 300 mg/d of eugenol) did not affect DMI, a lower dose (180 mg/d of cinnamaldehyde and 90 mg/d of eugenol) decreased DMI in beef heifers fed a high-concentrate diet. Similarly, Yang et al. (2010) reported that eugenol supplementation in growing heifers did not influence DMI. Conversely, Benchaar et al. (2006) observed that supplementation of 2–4 g/day of an EO mixture containing eugenol increased DMI in a digestion study, but this effect was not replicated in a growth study with beef cattle on a high-forage diet. Nonetheless, product 3 supplementation showed a trend for a reduction in CH_4 production and intensity, especially noticeable in 100 g/head.d and a significant reduction in CH_4 yield compared to the control group. Additionally, a negative correlation exists between the log of CH_4 intensity and ADG. This correlation may stem from the fact that Product 3 could be enhancing feed conversion, resulting in better weight gain without a proportional increase in methane production. Therefore, while the cattle gain more weight, the methane per unit of weight gain is reduced. These results indicate the potential for product 3 to contribute to lower CH_4 emissions from beef steers fed high-forage diets. The specificity of Product 3 in reducing CH_4 without affecting CO_2 and H_2 production further supports its targeted action, as corroborated in the literature on plant-based compounds, including EO, saponins, and tannins (Francis et al., 2002; Benchaar and Greathead, 2011; Wang et al., 2012; Honan et al., 2021).

5 Conclusion

This study evaluated four novel products *in vitro*, with Product 3 showing the strongest methane-reducing effect. At 6% (v/v), CH_4 production was reduced by over 90%, although digestibility was also compromised. At a lower inclusion level (4%), methane emissions were reduced by 62% without negatively affecting digestibility, emphasizing the importance of dose optimization.

In vivo, incorporation of Product 3 into beef steer diets modestly reduced methane yield (−11.7%) without affecting feed intake or growth performance. While these results are promising, they should be interpreted cautiously given the short duration and scale of the trial.

Further research at higher dosages, over extended periods, and with incorporation into lick blocks is needed to clarify the balance between methane abatement, digestibility, and overall animal performance.

Data availability statement

The raw data supporting the conclusions of this article are not publicly available because they are subject to confidentiality and contractual obligations. In accordance with the governing agreement, such data cannot be disclosed or distributed without the prior written consent of both Meat & Livestock Australia (MLA) and Agcotech. Requests to access the datasets should be directed to the corresponding author/s.

Ethics statement

The experimental protocols and use of animals were approved by the Animal Ethics Committee of the University of Sydney for both the *in vitro* (2022/2180) and *in vivo* (ARA No. 2023/2293) trials. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MN: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. GS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. MP: Formal Analysis, Investigation, Methodology, Writing – review & editing, Data curation. LG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. AC: Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research and/or publication of this article. This paper and the research behind it would not have been possible without the financial support from grant PPSH.1379 from Meat & Livestock Australia Donor Company, Commonwealth Government of Australia, The University of Sydney, and AgCoTech. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

We would like to thank Paul and Jeanette the managers at Pye Farm where the pivotal *in vivo* study took place. We are equally indebted to Milad, Mulisa and Alessio, whose commitment and enthusiasm help during the experiment set up played a crucial role in fruition of this study. Without their help and passion, this study would not have been possible. We also thank AgCoTech for providing the novel products evaluated in the present paper, inclusive of Products 1–4 as well as molasses products. Products 1–4 are protected under Australian Provisional Patent Application No. 2023902943.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

References

Almeida, A. K., Hegarty, R. S., and Cowie, A. (2021). Meta-analysis quantifying the potential of dietary additives and rumen modifiers for methane mitigation in ruminant production systems. *Anim. Nutr.* 7, 1219–1230. doi: 10.1016/j.aninu.2021.09.005

Arthur, P., Barchia, I., Weber, C., BirdGardiner, T., Donoghue, K., Herd, R., et al. (2017). Optimizing test procedures for estimating daily methane and carbon dioxide emissions in cattle using short-term breath measures. *J. Anim. Sci.* 95, 645–656. doi: 10.2527/jas.2016.0700

Authority, A.P.V.M.A (2024). *Stockfeed and pet food regulation* (Australian Government: Australian Pesticides and Veterinary Medicines Authority).

Beauchemin, K., and McGinn, S. (2006). Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. *J. Anim. Sci.* 84, 1489–1496. doi: 10.2527/2006.8461489x

Benchaar, C., and Greathead, H. (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Anim. Feed Sci. Technol.* 166–167, 338–355. doi: 10.1016/j.anifeedsci.2011.04.024

Benchaar, C., Calsamiglia, S., Chaves, A. V., Fraser, G., Colombatto, D., McAllister, T. A., et al. (2008). A review of plant-derived essential oils in ruminant nutrition and production. *Anim. Feed Sci. Technol.* 145, 209–228. doi: 10.1016/j.anifeedsci.2007.04.014

Benchaar, C., Duynisveld, J., and Charmley, E. (2006). Effects of monensin and increasing dose levels of a mixture of essential oil compounds on intake, digestion and growth performance of beef cattle. *Can. J. Anim. Sci.* 86 (1), 91–96. doi: 10.4141/A05-027

Broudiscou, L. P., Cornu, A., and Rouzeau, A. (2007). *In vitro* degradation of 10 mono- and sesquiterpenes of plant origin by caprine rumen micro-organisms. *J. Sci. Food Agric.* 87, 1653–1658. doi: 10.1002/jsfa.2863

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Calsamiglia, S., Busquet, M., Cardozo, P., Castillejos, L., and Ferret, A. (2007). Invited review: essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* 90, 2580–2595. doi: 10.3168/jds.2006-644

Cardozo, P. W., Calsamiglia, S., Ferret, A., and Kamel, C. (2005). Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. *J. Anim. Sci.* 83 (11), 2572–2579. doi: 10.2527/2005.83112572x

Cardozo, P., Calsamiglia, S., Ferret, A., and Kamel, C. (2006). Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. *J. Anim. Sci.* 84, 2801–2808. doi: 10.2527/jas.2005-593

Castillejos, L., Calsamiglia, S., and Ferret, A. (2006). Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. *J. Dairy Sci.* 89 (7), 2649–2658. doi: 10.3168/jds.s0022-0302(06)72341-4

Charmley, E., Williams, S. R. O., Moate, P. J., Hegarty, R. S., Herd, R. M., Oddy, V. H., et al. (2015). A universal equation to predict methane production of forage-fed cattle in Australia. *Anim. Prod. Sci.* 56, 169–180. doi: 10.1071/AN15365

Chaves, A. V., Thompson, L. C., Iwaasa, A. D., Scott, S. L., Olson, M. E., Benchaar, C., et al. (2006). Effect of pasture type (alfalfa vs. grass) on methane and carbon dioxide production by yearling beef heifers. *Can. J. Anim. Sci.* 86, 409–418. doi: 10.4141/A05-081

Chizzotti, M., MaChado, F., Valente, E., Pereira, L., Campos, M., Tomich, T., et al. (2015). Validation of a system for monitoring individual feeding behavior and individual feed intake in dairy cattle. *J. Dairy Sci.* 98, 3438–3442. doi: 10.3168/jds.2014-8760

C-Lock. (2023). *GreenFeed system: User manual* (USA: C-Lock Inc.). Available online at: https://www.c-lockinc.com.

Colombini, S., Graziosi, A. R., Parma, P., Iriti, M., Vitalini, S., Sarnataro, C., et al. (2021). Evaluation of dietary addition of 2 essential oils from Achillea moschata, or their components on *in vitro* ruminal fermentation and microbial community composition. *Anim. Nutr.* 7, 224–231. doi: 10.1016/j.aninu.2020.11.001

Cunniff, P., and Washington, D. (1995). *AOAC, Official methods of analysis of AOAC international* (Arlington, VA: AOAC).

Department of Climate Change, E., The Environment and Water (2022). *Quarterly Update of Australia's National Greenhouse Gas Inventory: September 2022* (Canberra, Australia: Department of Climate Change, Energy, the Environment and Water).

Dixon, R., Fletcher, M., Anderson, S., Kidd, L., Benvenutti, M., Mayer, B., et al. (2020). *Improved Management of Cattle Phosphorus Status through Applied Physiology* (North Sydney, NSW Australia: Meat and Livestock Australia).

Durmic, Z., Moate, P. J., Eckard, R., Revell, D. K., Williams, R., and Vercoe, P. E. (2014). *In vitro* screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. *J. Sci. Food Agric.* 94, 1191–1196. doi: 10.1002/jsfa.6396

Eugène, M., Klumpp, K., and Sauvant, D. (2021). Methane mitigating options with forages fed to ruminants. *Grass Forage Sci.* 76, 196–204. doi: 10.1111/gfs.12540

Fedorah, P. M., and Hrudey, S. E. (1983). A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. *Environ. Technol.* 4, 425–432. doi: 10.1080/09593338309384228

Forwood, D. L., Hooker, K., Caro, E., Huo, Y., Holman, D. B., Meale, S. J., et al. (2019). Crop sorghum ensiled with unsalable vegetables increases silage microbial diversity. *Front. Microbiol.* 10, 2599. doi: 10.3389/fmicb.2019.02599

Francis, G., Kerem, Z., Makkar, H. P., and Becker, K. (2002). The biological action of saponins in animal systems: a review. *Br. J. Nutr.* 88, 587–605. doi: 10.1079/bjn2002725

Garcia, F., Colombatto, D., Brunetti, M. A., Martínez, M. J., Moreno, M. V., Scorcione Turcato, M. C., et al. (2020). The reduction of methane production in the *in vitro* ruminal fermentation. *Animals* 10, 786. doi: 10.3390/ani10050786

Greathead, H. (2003). Plants and plant extracts for improving animal productivity. *Proc. Nutr. Soc* 62, 279–290. doi: 10.1079/pns2002197

Hammond, K., Humphries, D., Crompton, L., and Green, C. (2015). Methane emissions from cattle: Estimates from short-term measurements using a GreenFeed system. *Anim. Feed Sci. Technol.* 203, 41–52. doi: 10.1016/j.anifeedsci.2015.02.003

Hammond, K., Waghorn, G., and Hegarty, R. (2016). The GreenFeed system for measurement of enteric methane emission from cattle. *Anim. Prod. Sci.* 56, 181–189. doi: 10.1071/AN15316

Honan, M., Feng, X., Tricarico, J., and Kebreab, E. (2021). Feed additives as a strategic approach to reduce enteric methane production in cattle: modes of action, effectiveness and safety. *Anim. Prod. Sci.* 61, 1153–1163. doi: 10.1071/AN20295

Imaz, J. A., García, S., and González, L. A. (2019). Real-time monitoring of self-fed supplement intake, feeding behaviour, and growth rate as affected by forage quantity and quality of rotationally grazed beef cattle. *Animals* 9, 1129. doi: 10.3390/ani9121129

Jahani-Azizabadi, H., Durmic, Z., Vadhanabhuti, J., and Vercoe, P. (2019). Effect of some Australian native shrubs essential oils on *in vitro* rumen microbial fermentation of a high-concentrate diet. *J. Anim. Plant Sci.* 29, 8–15. doi: 10.36899/JAPS.2019.1.003

Jami, E., White, B. A., and Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS One* 9, e85423. doi: 10.1371/journal.pone.0085423

Kahvand, M., and Malecky, M. (2018). Dose-response effects of sage (Salvia officinalis) and yarrow (A. millefolium) essential oils on rumen fermentation in *vitro*. *Ann. Anim. Sci.* 18, 125–142. doi: 10.2478/aoas-2018-0009

Koning, L., van Gastelen, S., Burgers, E. E. A., Klootwijk, C. W., Bannink, A., and Dijkstra, J. (2024). Minimum number of spot measurements required for quantifying enteric methane production in dairy cattle. In: Why grasslands? Proceedings of the 30th General Meeting of the European Grassland Federation. Klootwijk, C. W., Bruinenberg, M. H., Cougnon, M., Hoekstra, N. J., Ripoll-Bosch, R., Schelfhout, S., et al (eds.), (Leeuwarden, Netherlands: Grassland Science in Europe) 29, 465–467.

Ku-Vera, J.C., Jiménez-Ocampo, R., Valencia-Salazar, S.S., Montoya-Flores, M.D., Molina-Botero, I.C., Arango, J., et al. (2020). Role of secondary plant metabolites on enteric methane mitigation in ruminants. *Front. Vet. Sci.* 7, 584. doi: 10.3389/fvets.2020.00584

Lawson, C. A., Seymour, J. R., Possell, M., Suggett, D. J., and Raina, J.-B. (2020). The volatilomes of Symbiodiniaceae-associated bacteria are influenced by chemicals derived from their algal partner. *Front. Mar. Sci.* 7, 106. doi: 10.3389/fmars.2020.00106

Leng, R. (1990). Factors affecting the utilization of 'poor-quality' forages by ruminants particularly under tropical conditions. *Nutr. Res. Rev.* 3, 277–305. doi: 10.1079/NRR19900018

Makkar, H. P., Sánchez, M., and Speedy, A. W. (2007). *Feed supplementation blocks: urea-molasses multinutrient blocks: simple and effective feed supplement technology for ruminant agriculture* (Rome, Italy: Food & Agriculture Org).Available online at: https://www.fao.org/4/a0242e/a0242e00.htm.

Meale, S. J., Chaves, A. V., Baah, J., and McAllister, T. A. (2012). Methane production of different forages in *in vitro* ruminal fermentation. *AsianAustralas. J. Anim. Sci.* 25, 86–91. doi: 10.5713/ajas.2011.11249

Mikaia, A., White, E. V., Zaikin, V., Zhu, D., Sparkman, O. D., Neta, P., et al. (2014). *NIST standard reference database 1A: NIST/EPA/NIH mass spectral library (NIST 14) and NIST mass spectral search program (version 2.2).* National Institute of Standards and Technology.

Nunes, H., Dias, C. M., and Borba, A. (2023). Bioprospecting essential oils of exotic species as potential mitigations of ruminant enteric methanogenesis. *Heliyon* 9, e12786. doi: 10.1016/j.heliyon.2022.e12786

O'Reilly, G. C., Huo, Y., Meale, S. J., and Chaves, A. V. (2021). Dose response of biochar and wood vinegar on *in vitro* batch culture ruminal fermentation using contrasting feed substrates. *Transl. Anim. Sci.* 5, txab107. doi: 10.1093/tas/txab107

Oliveira, B. Jr., Ribas, M., MaChado, F., Lima, J., Cavalcanti, L., and Chizzotti, M. (2018). Validation of a system for monitoring individual feeding and drinking behavior and intake in young cattle. *Animal* 12, 634–639. doi: 10.1017/S1751731117002002

Parra, M. C., Forwood, D. L., Chaves, A. V., and Meale, S. J. (2023). *In vitro* screening of anti-methanogenic additives for use in Australian grazing systems. *Front. Anim. Sci.* 4. doi: 10.3389/fanim.2023.1123532

Patra, A. K., and Yu, Z. (2015). Effects of adaptation of in vitro rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. *J. Appl. Microbiol.* 6, 1434. doi: 10.3389/fmicb.2015.01434

Patra, A., Park, T., Kim, M., and Yu, Z. (2017). Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *J. Anim. Sci. Biotechnol.* 8, 1–18. doi: 10.1186/s40104-017-0145-9

Pawar, M. M., Kamra, D. N., Agarwal, N., and Chaudhary, L. C. (2014). Effects of essential oils on in vitro methanogenesis and feed fermentation with buffalo rumen liquor. *Agric. Res.* 3 (1), 67–74. doi: 10.1007/s40003-014-0092-z

Penner, G., Beauchemin, K., and Mutsvangwa, T. (2007). Severity of ruminal acidosis in primiparous Holstein cows during the periparturient period. *J. Dairy Sci.* 90, 365–375. doi: 10.3168/jds.S0022-0302(07)72638-3

R Core Team (2023). *R: A Language and Environment for Statistical Computing.* (Vienna, Austria: R Foundation for Statistical Computing). Available online at: https://www.R-project.org/.

Simon, J. E., Quinn, J., and Murray, R. G. (1990). Basil: a source of essential oils. *Adv. New Crops* 1, 484–489. doi: 10.1007/BF02907824

Soliva, C. R., Amelchanka, S. L., Duval, S. M., and Kreuzer, M. (2011). Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). *Br. J. Nutr.* 106, 114–122. doi: 10.3168/jds.S0022-0302(07)72638-3

Velazco, J., Mayer, D., Zimmerman, S., and Hegarty, R. (2016). Use of short-term breath measures to estimate daily methane production by cattle. *Animal* 10, 25–33. doi: 10.1017/S1751731115001603

Visscher, C. F., Witzmann, S., Beyerbach, M., and Kamphues, J. (2013). Watering cattle (young bulls) with brackish water–a hazard due to its salt content? *Tierarztl. Prax. Ausg. G: Grosstiere – Nutztiere* 41, 363–370. doi: 10.1055/s-0038-1623198

Wang, Y., McAllister, T. A., Yanke, L. J., Xu, Z. J., Cheeke, P. R., and Cheng, K. J. (2000). *In vitro* effects of steroidal saponins from Yucca schidigera extract on rumen microbial protein synthesis and ruminal fermentation. *J. Sci. Food Agric.* 80, 2114–2122. doi: 10.1016/S0377-8401(98)00137-0

Wang, J. K., Ye, J. A., and Liu, J. X. (2012). Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance—a review. *Trop. Anim. Health Prod.* 44, 697–706. doi: 10.1007/s11250-011-9960-8

Wasson, D. E., and Hristov, A. N. (2022). Enteric methane mitigation through Asparagopsis taxiformis supplementation and potential algal alternatives. *Front. Anim. Sci.* 3. doi: 10.3389/fanim.2022.999338

White, H. C., Davis, N. G., Van Emon, M. L., DelCurto-Wyffels, H. M., Wyffels, S. A., and DelCurto, T. (2024). Impacts of increasing levels of salt on intake, digestion, and rumen fermentation with beef cattle consuming low-quality forages. *J. Anim. Sci.* 102, skae284. doi: 10.1093/jas/skae284

Yang, W., Benchaar, C., Ametaj, B., and Beauchemin, K. (2010). Dose response to eugenol supplementation in growing beef cattle: ruminal fermentation and intestinal digestion. *Anim. Feed Sci. Technol.* 158, 57–64. doi: 10.1016/j.anifeedsci.2010.03.019

Chapter 3: General Discussion

This chapter synthesises the key findings of this thesis, integrating results from both in vitro and in vivo experiments to evaluate the efficacy of MLB formulations containing anti-methanogenic ingredients. The discussion highlights practical constraints and identifies areas for future research to optimise the use of MLBs as a mitigation strategy in pasture-based beef systems.

3.1 Main findings

Reducing methane emissions on pasture-based systems remains a significant challenge. The literature review of this thesis (Chapter 1) evaluated several methanogenesis- inhibiting feed additives, while those show promise, practical and scalable delivery methods for grazing cattle are still lacking. Attempts to prolong the effectiveness of rumen methane inhibition -for example, the use of 3-NOP (Muetzel et al., 2019) formulations have been explored. Research has also examined ways to improve the stability of volatile additives within feed (Magnusson et al., 2020). Despite these advances, available evidence supporting reliable delivery of these additives remains limited and insufficient for widespread adoption in grazing production system (Hegarty et al., 2021). Among the emerging solutions, molasses-based carrier systems have been proposed as a practical means of improving additive stability and intake control under grazing conditions (Imaz et al., 2020; Hristov et al., 2025).

Chapter 2 of the thesis presents an experimental evaluation of novel molasses-based products, formulated as additive carriers containing essential oils and plant secondary metabolites, rather than molasses lick blocks per se. Results from the in vitro screening identified one formulation (Product 3) as the most promising additive. At the highest inclusion level (6%), Product 3 reduced methane production by more than 90% compared with the control group, although this was accompanied by a significant reduction in in vitro dry matter digestibility (IVDMD). At a lower inclusion level (4%), methane emissions were reduced by 62% without negatively affecting digestibility, emphasising the importance of dose optimisation. This initial screening effectively differentiated products based on anti-methanogenic potential and guided the selection of Product 3 for subsequent in vivo testing.

In contrast to the strong in vitro inhibition, the in vivo responses were more moderate but directionally consistent. Supplementation with the Product 3-pellet did not significantly reduce methane production, but it achieved reductions in methane yield (up to 11.7%) and tended to decrease methane intensity (16.8%). These reductions occurred without negative effects on dry matter intake, water intake, or average daily gain, indicating that the supplement was well-tolerated and compatible with forage-based diets. The positive association between supplement intake and both water consumption and weight gain suggests potential production benefits or, at minimum, no trade-off in growth performance.

3.2 Practical considerations and limitations

While the present study demonstrates that Product 3 has potential to reduce enteric methane emissions in beef cattle, several factors limit the interpretation and extrapolation of these findings. These limitations relate primarily to (i) additive stability and microbial adaptation, (ii) duration of supplementation and implications for long-term mitigation, and (iii) constraints associated with methane measurement using GreenFeed system.

3.2.1 Additive stability and microbial adaptation

The strong inhibition of methane production observed in vitro contrasted with the more modest reduction recorded in vivo, reflecting the fundamental differences between these two experimental systems rather than an inconsistency in additive efficacy. In vitro techniques are primarily intended as screening tools that allow rapid identification of compounds with antimethanogenic potential under tightly controlled conditions. These systems are closed, short-term incubations in which additives, particularly essential oils, tannins, and saponins, are typically applied at relatively high concentrations and experience minimal degradation or host-mediated modulation. As a result, in vitro assays often report pronounced methane inhibition that may overestimate responses achievable under practical feeding conditions (Patra and Yu, 2015; Beauchemin et al., 2020).

In contrast, in vivo experiments are designed to validate the efficacy of selected candidate (e.g.: Product 3) under biologically complex and dynamic conditions, where rumen fermentation is influenced by feed intake patterns, digesta passage rate, microbial adaptation, compound metabolism, and host-diet interactions. The in vivo dose response evaluation conducted in this thesis therefore represents a more realistic assessment of the mitigation potential of a single promising product identified during in vitro screening. Lower effective concentrations of EO-based additives in vivo, combined with microbial adaptation and potential detoxification mechanisms, are well documented to attenuate antimethanogenic responses relative to in vitro systems (Calsamiglia et al., 2007; Durmic et al., 2021). Consequently, the more moderate but production-relevant methane reductions observed in vivo are consistent with previous literature and reinforce the importance of interpreting in vitro findings as indicative rather than predictive of in vivo outcomes. Chemical characterisation of Products 2 and 3 indicated the presence of sesquiterpene compounds, a class of EO constituents previously associated with antimethanogenic activity (Nunes et al., 2023). In that study, sesquiterpene- rich EO extracted from exotic plants reduced total gas and methane production at medium and high inclusion levels, but not at low concentrations. A comparable dose-dependent response was evident in the present in vitro experiment. Product 3 significantly reduced CH_4 production at medium and high inclusion rates, whereas gas production was unaffected at these levels and increased at the lowest dose. In contrast, Product 2 increased total gas production substantially without altering CH_4 proportion, suggesting stimulation of fermentative activity without concurrent inhibition of methanogenesis. These divergent responses across products and inclusion rates indicate a strong interaction between EO composition and dosage, affecting both fermentation dynamics and methane output. The presence of multiple bioactive compounds within each EO blend may result in synergistic, antagonistic, or additive effects, leading to divergent outcomes depending on concentration and microbial context (Burt, 2004). Further variability likely arises from differences in the extent to which rumen microorganisms degrade, transform, or detoxify secondary metabolites (Broudiscou et al., 2007). Such processes may substantially reduce the persistence and bioavailability of EO constituents in vivo, thereby limiting their capacity to sustain methane inhibition. The absence of direct measurements of EO degradation or rumen metabolite profiles in the present study represents a limitation, as it constrains interpretation of whether reduced in vivo efficacy reflects chemical instability, microbial metabolism, or both.

Beyond chemical factors, the adaptive capacity of rumen microbial communities presents a critical challenge to the long-term effectiveness of antimethanogenic additives (Patra and Yu, 2012). Rumen microorganisms are highly resilient and capable of adapting to antimicrobial compounds through shifts in community structure, induction of detoxification mechanisms, and functional redundancy among hydrogen-utilising pathways (Calsamiglia et al., 2007; Morgavi et al., 2010). Such adaptations may progressively diminish the inhibitory effects of plant-based additives, leading to declining efficacy during prolonged supplementation (Hegarty et al., 2021) . The 56-day duration of the present in vivo trial may therefore have captured only the early phase of response, with longer-term exposure potentially resulting in further attenuation of the methane-reducing effect. Indeed, previous studies have reported time-dependent declines in efficacy for essential oil-based and tannin-based additives, highlighting the challenge of sustaining antimethanogenic activity in vivo (Durmic et al., 2014; Patra and Yu, 2015).

An additional limitation related to the narrow margin between effective methane inhibition and undesirable impacts on rumen function (Patra and Yu, 2012; Hristov et al., 2025). Studies reporting successful methane suppression with EO supplementation consistently emphasise a trade-off between antimethanogenic efficacy and overall fermentation (Durmic et al., 2014; Jahani-Azizabadi et al., 2019; Parra et al., 2023). Although alterations in VFA profiles are often interpreted as a favourable indicator of improved rumen efficiency, EO can also induce less desirable shifts depending on dose and rumen conditions. For instance, (Castillejos et al., 2006) demonstrated that eugenol at 500 mg/L reduced propionate proportion without affecting total VFA concentration, while (Cardozo et al., 2005) showed that EO effects on fermentation pathways are strongly pH-dependent, with divergent responses observed under acidic versus neutral conditions. In the present study, the highest inclusion (6%) of Product 3 decreased medium pH to approximately 5.0, a value within the threshold for acute ruminal acidosis (Penner et al., 2007). This appears inconsistent with the observed reduction in VFA concentration; however, this response is likely attributable to the inherent acidity of Product 3, which had a measured pH of 4.82 (data not shown), rather than to excessive fermentative acid production. It is important to recognise that this pH may overestimate the magnitude of response expected in vivo, given the substantial buffering capacity provided by salivary bicarbonate and dilution within the much larger rumen volume and continuous digesta turnover (Beauchemin, 2018). Nevertheless, this response illustrates a key practical constraint: dose escalation to maximise methane suppression may inadvertently compromise rumen stability, either directly through additive acidity or indirectly via altered microbial activity. Notably, a 4% inclusion of Product 3 reduced CH_4 proportion by 62% without negatively affecting fermentation, with medium pH maintained at 5.84. This finding highlights the importance of dose optimisation, demonstrating that effective methane mitigation can be achieved without compromising rumen function when inclusion levels are carefully calibrated (Patra and Yu, 2012; Belanche et al., 2020). Despite their potential, optimal dosing strategies, suitable carrier matrices, and feed-processing interactions that influence additive stability and persistence in the rumen remain under-researched (Hristov et al., 2025). Addressing these factors is essential to improve the consistency, safety, and scalability of EO-based methane mitigation strategies in ruminant production systems.

3.2.2 Duration of supplementation and implications for long-term mitigation

The relatively short duration of the in vivo trial also limits confidence in the persistence of the observed methane reductions. Methane mitigation strategies based on plant secondary metabolites are particularly susceptible to time-dependent declines in efficacy, as rumen microbes adapt to repeated exposure (Patra and Yu, 2012).

This limitation has important implications for carbon accounting and mitigation claims. For a feed additive to contribute meaningfully to national greenhouse gas inventories or carbon credit schemes, its effects must be consistent, predictable, and durable over time (Hristov et al., 2015; Beauchemin et al., 2022). Short-term reductions measured over several weeks may not translate into reliable annual mitigation if microbial adaptation leads to diminishing responses.

Accordingly, the present findings should be viewed as evidence of short-term mitigation potential rather than definitive proof of long-term effectiveness. Extended feeding trials across production cycles are required to assess the stability of the methane response and to quantify any adaptation-related attenuation of efficacy (Hristov et al., 2022).

3.2.3 Constraints associated with GreenFeed methane measurements

Methodological constraints related to methane quantification using the GreenFeed system must also be considered. GreenFeed relies on voluntary animal visits, meaning that methane estimates depend on visit frequency, timing, and duration (Hammond et al., 2016b). Although minimum thresholds for visit numbers improve precision, differences in animal behaviour inevitably introduce sampling bias (Hammond et al., 2016a).

In the present study, treatment differences in visit duration and frequency were observed, particularly for steers receiving the 200 g/day dose. While this may reflect increased motivation to consume pellets, it complicates the interpretation of methane production data because animals with more frequent or longer visits contribute disproportionately to the dataset (Velazco et al., 2016). If treatment affects motivation to visit GF (e.g., due to palatability or novelty), the emissions dataset becomes behaviour-conditioned. Protocols recommend standardising attractant type and delivery, auditing visit distributions, and verifying that treatment differences in visitation do not drive apparent CH_4 responses (Hammond et al., 2015; Hammond et al., 2016b).

Furthermore, GreenFeed captures emissions during short sampling windows rather than continuously. Which increases variability compared with whole-day enclosure techniques as respiration chambers. This limitation is well documented, even though GreenFeed remains a validated method for estimating enteric methane emission in cattle (Hammond et al., 2016b; Arthur et al., 2017). Therefore, modest treatment differences, particularly those approaching statistical tendencies, should be interpreted with appropriate caution.

Regardless of the method used, methane measurement is affected by both systematic and random errors, which together define the uncertainty of the results (Gates et al., 2009; Tedeschi et al., 2022). Because uncertainty has not been evaluated for all published emissions values, comparisons across studies, assessment of data quality, and certification of emission reductions remain challenging. Establishing standardised methodologies for uncertainty assessment will be a critical step forward (Hristov et al., 2018). Further research is required to validate individual measurement techniques, assess their performance across diverse production scenarios, and establish calibration and standardisation protocols for existing methodologies (Tedeschi et al., 2022).

3.3 Future research directions

Building on the present findings, future work should prioritise field-relevant validation, persistence of the antimethanogenic effect, dose optimisation under voluntary intake, and clarification of the mechanism of action. The following studies are recommended:

3.3.1 Validate Product 3 in hardened MLB under commercial grazing:

Conduct paddock trials using finalised, hardened blocks to quantify true voluntary intake behaviour under grazing, including between-animal variability, the proportion of non-consumers over consumers, and intake stability across differing forage conditions. High variability in MLB intake is well documented and represents a major challenge for ensuring consistent additive delivery at the herd level (Bowman and Sowell, 1997; Imaz et al., 2019; Callaghan et al., 2020).

To strengthen inference, block-level disappearance should be paired with individual intake monitoring technologies (e.g. electronic identification–linked feeders or marker-based approaches) (Imaz et al., 2019) to link intake distribution with methane outcomes and animal performance. Integrating individual intake data with in-paddock methane measurement systems (e.g., GreenFeed® or portable accumulation chambers) (Hammond et al., 2015) would allow assessment of how heterogeneous supplement consumption translates into variation in methane mitigation efficacy and growth responses at the animal and herd level (Charmley et al., 2015).

Collectively, such studies would provide critical evidence on the feasibility, robustness, and scalability of Product 3 as a practical methane mitigation strategy within commercial grazing systems, where self-regulated intake and environmental variability are unavoidable.

3.3.2 Assess persistence of the methane response over longer durations

Longer-term supplementation trials are required to determine whether the observed methane-reducing effect is maintained, attenuated, or lost over time as a result of rumen microbial adaptation. Although the present study extended over 56 days, adaptation of rumen microbial communities to antimethanogenic compounds—particularly plant secondary metabolites and essential oil–based additives—has been widely reported and may substantially reduce efficacy beyond short experimental periods (Patra and Yu, 2012; Cobellis et al., 2016).

Season-long studies conducted under production-relevant conditions would enable quantification of the temporal stability of methane suppression, as well as potential rebound effects following prolonged exposure. Such designs would also allow assessment of interactions between additive persistence, diet quality, and seasonal changes in pasture composition, which are known to influence both rumen fermentation and methane emissions (Hristov et al., 2013; Beauchemin et al., 2020; Hristov et al., 2022).

Critically, evaluating persistence over extended durations is essential to ensure that short-term mitigation responses translate into durable, cumulative emissions reductions that are compatible with national greenhouse gas emissions inventories and emerging carbon accounting frameworks. Without evidence of sustained efficacy, extrapolation of short-term methane reductions risks overestimating real-world mitigation potential (Gerber et al., 2013b; Hristov et al., 2015).

3.3.3 Clarify mechanisms of action

Complementary studies are required to elucidate how Product 3 influences rumen microbial ecology, hydrogen outputs, and fermentation pathways that ultimately regulate methanogenesis. Integrating microbial community profiling with metabolomic analyses would provide insight into shifts in rumen metabolic networks induced by supplementation (Morgavi et al., 2010; Ungerfeld, 2015b).

Such approaches would help determine whether methane suppression arises from direct inhibition of methanogens, redirection of metabolic hydrogen toward competing pathways or a broader restructuring of the rumen microbiome (Patra and Yu, 2012). This mechanistic resolution is particularly important for explaining the dose-dependent responses observed under controlled feeding and for determining whether any time-dependent attenuation of efficacy reflects microbial adaptation or functional redundancy within the rumen ecosystem (Beauchemin et al., 2020).

A clearer understanding of the mechanism will strengthen confidence in the durability and predictability of Product 3 under variable grazing conditions and will inform rational optimisation of dose, formulation, and delivery strategy in future field applications.

3.3.4 Evaluate economic feasibility and adoption potential

Finally, evaluation of Product 3 should extend beyond biological efficacy to include whole-farm economic and adoption analyses. Integrating additive cost, delivery infrastructure, labour requirements, and any effects on animal productivity, with estimates of methane abatement value (e.g. carbon credits or emissions intensity reductions) (Callaghan et al., 2020) would provide a realistic assessment of commercial viability. Such whole-system approaches are essential to determine whether observed methane reductions translate into economically meaningful outcomes at the enterprise level (Beauchemin et al., 2020; Hegarty et al., 2021).

Equally important is understanding producer decision-making constraints, including variability in supplement intake, compatibility with extensive grazing management, perceived risk, and regulatory or market incentives. Previous studies highlight that adoption of methane mitigation technologies in extensive systems is strongly influenced by practicality, cost-effectiveness, and ease of integration into current management rather than mitigation potential alone (Almeida and Hegarty, 2021; Almeida et al., 2023).

Addressing these economic and behavioural dimensions, alongside technical performance, will be critical for scaling Product 3 beyond experimental settings and enabling durable, large-scale implementation within pasture-based livestock industries.

3.4 Conclusion

This thesis demonstrated that the molasses-based additive formulation Product 3 can reduce enteric methane emissions in beef cattle without negatively affecting performance. In vitro screening showed strong, dose-dependent inhibition of methane production, with reductions exceeding 90% at high inclusion levels, while moderate inclusion (4%) achieved a 62% reduction without impairing fermentation, supporting dose optimisation and guiding the selection of Product 3 for in vivo testing.

In the subsequent 56-day in vivo trial, Product 3 reduced methane yield by up to 11.7% under controlled feeding conditions, with no adverse effects on feed or water intake, or average daily gain. The positive associations between supplement intake, growth, and water consumption suggest good palatability and compatibility with forage-based diets. Overall, these findings indicate that Product 3 can effectively mitigate methane emissions while maintaining productivity, demonstrating its potential applicability in pasture-based beef production systems. Several methodological and practical limitations must be considered when interpreting these findings. The pellet-based delivery system does not reflect the intake variability and behavioural dynamics associated with free-access molasses lick blocks, the intended commercial delivery method. In addition, the known potential for rumen microbial adaptation to plant secondary metabolites highlights the need for longer-duration studies to assess persistence of the antimethanogenic effects over time.

Despite these limitations, this thesis provides valuable evidence supporting the potential of molasses-based additives containing essential oils as methane mitigation tools for grazing beef cattle. The demonstration of a clear dose-response relationship under controlled conditions provides a strong foundation for further formulation refinement.

Future research should prioritise longer-term in vivo evaluation, validation under grazing conditions using hardened lick blocks, and mechanistic investigations of rumen microbial and fermentation responses. These steps will be essential to confirm durability of mitigation effects and determine suitability for integration into emerging carbon accounting frameworks.

References

Aboagye, I.A., and Beauchemin, K.A. (2019). Potential of molecular weight and structure of tannins to reduce methane emissions from ruminants: a review. Animals 9, 856. DOI: 10.3390/ani9110856

Ahmed, M.G., Elwakeel, E.A., El-Zarkouny, S.Z., and Al-Sagheer, A.A. (2024). Environmental impact of phytobiotic additives on greenhouse gas emission reduction, rumen fermentation manipulation, and performance in ruminants: an updated review. Environmental Science and Pollution Research 31, 37943-37962. DOI: 10.1007/s11356-024-33664-5

Alazzeh, A.Y., Sultana, H., Beauchemin, K.A., Wang, Y., Holo, H., Harstad, O.M., and McAllister, T.A. (2012). Using strains of propionibacteria to mitigate methane emissions in vitro. Acta Agriculturae Scandinavica, Section A–Animal Science 62, 263-272. DOI: 10.1080/09064702.2013.773056

Almeida, A.K., Cowley, F.C., and Hegarty, R.S. (2023). A regional-scale assessment of nutritional-system strategies for abatement of enteric methane from grazing livestock. Animal Production Science 63, 1461-1472. DOI: 10.1071/AN22315

Almeida, A.K., and Hegarty, R.S. (2021). Managing livestock to reduce methane emissions: assessment of strategies for abatement of enteric methane, in: NSW Department of Primary Industries.

Anderson, C.L., Sullivan, M.B., and Fernando, S.C. (2017). Dietary energy drives the dynamic response of bovine rumen viral communities. Microbiome 5, 155. DOI: 10.1186/s40168-017-0374-3

Appuhamy, J.A.D.R.N., Strathe, A.B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., France, J., and Kebreab, E. (2013). Anti-methanogenic effects of monensin in dairy and beef cattle: a meta-analysis. Journal of Dairy Science 96, 5161-5173. DOI: 10.3168/jds.2012-5923

Arif, M., Sarwar, M., Hayat, Z., and Younas, M. (2016). Effect of supplementary sodium nitrate and sulphur on methane production and growth rates in sheep and goats fed forage based diet low in true protein. Journal of Animal & Plant Sciences 26.

Arik, H.D., Inanc, Z.S., Kahraman, O., Damar, S., and Akkaya, A.B. (2024). Effects of quebracho tannin supplementation in early lactation dairy cow rations on milk yield parameters, rumen fermentation, digestibility and blood parameters. Journal of Animal and Feed Sciences 33, 321-330. DOI: 10.22358/jafs/183438/2024

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., Schwarm, A., Shingfield, K.J., Veneman, J.B., Yáñez-Ruiz, D.R., and 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, e2111294119. DOI: 10.1073/pnas.2111294119

Arthur, P.F., Barchia, I.M., Weber, C., Bird-Gardiner, T., Donoghue, K.A., Herd, R.M., and Hegarty, R.S. (2017). Optimising test procedures for estimating daily methane and carbon dioxide emissions in cattle using short-term breath measures. Journal of Animal Science 95, 645-656. DOi: 10.2527/jas.2016.0700

Bampidis, V., Azimonti, G., Bastos, M.d.L., Christensen, H., Dusemund, B., Fašmon Durjava, M., Kouba, M., López-Alonso, M., and López Puente, S. (2021). Safety and efficacy of a feed additive consisting of 3-nitrooxypropanol (Bovaer® 10) for ruminants for milk production and reproduction (DSM Nutritional Products Ltd). Efsa Journal 19, e06905. DOI: 10.2903/j.efsa.2021.6905

Ban, Y., and Guan, L.L. (2021). Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. Journal of Animal Science and Biotechnology 12, 109. DOI: 10.1186/s40104-021-00630-x

Battelli, M., Colombini, S., Crovetto, G.M., Galassi, G., Abeni, F., Petrera, F., Manfredi, M.T., and Rapetti, L. (2024). Condensed tannins fed to dairy goats: effects on digestibility, milk production, blood parameters, methane emission, and energy and nitrogen balances. Journal of Dairy Science 107, 3614-3630. DOI: 10.3168/jds.2023-24076

Battelli, M., Colombini, S., Parma, P., Galassi, G., Crovetto, G.M., Spanghero, M., Pravettoni, D., Zanzani, S.A., Manfredi, M.T., and Rapetti, L. (2023). In vitro effects of different levels of quebracho and chestnut tannins on rumen methane production, fermentation parameters, and microbiota. Frontiers in Veterinary Science 10. DOI: 10.3389/fvets.2023.1178288

Bayaru, E., Kanda, S., Kamada, T., ITABASHI, H., ANDOH, S., NISHIDA, T., ISHIDA, M., ITOH, T., NAGARA, K., and ISOBE, Y. (2001). Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. Nihon Chikusan Gakkaiho 72, 139-146. DOI: 10.2508/chikusan.72.139

Beauchemin, K.A. (2018). Invited review: current perspectives on eating and rumination activity in dairy cows. Journal of Dairy Science 101, 4762-4784. DOI: 10.3168/jds.2017-13706

Beauchemin, K.A., Kreuzer, M., O’Mara, F., and McAllister, T.A. (2008). Nutritional management for enteric methane abatement: a review. Australian Journal of Experimental Agriculture 48, 21-27. DOI: 10.1071/EA07199

Beauchemin, K.A., McGinn, S.M., Benchaar, C., and Holtshausen, L. (2009). Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: effects on methane production, rumen fermentation, and milk production. Journal of Dairy Science 92, 2118-2127. DOI: 10.3168/jds.2008-1903

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., Oyhantcabal, W., Salami, S.A., Shalloo, L., Sun, Y., Tricarico, J., Uwizeye, A., De Camillis, C., Bernoux, M., Robinson, T., and Kebreab, E. (2022). Invited review: current enteric methane mitigation options. Journal of Dairy Science 105, 9297-9326. DOI: 10.3168/jds.2022-22091

Beauchemin, K.A., Ungerfeld, E.M., Eckard, R.J., and Wang, M. (2020). Review: fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. Animal 14, s2-s16. DOI: 10.1017/S1751731119003100

Belanche, A., Newbold, C.J., Morgavi, D.P., Bach, A., Zweifel, B., and Yáñez-Ruiz, D.R. (2020). A meta-analysis describing the effects of the essential oils blend Agolin Ruminant on performance, rumen fermentation and methane emissions in dairy cows. Animals 10, 620. DOI: 10.3390/ani10040620

Benchaar, C. (2016). Diet supplementation with cinnamon oil, cinnamaldehyde, or monensin does not reduce enteric methane production of dairy cows. Animal 10, 418-425. DOI: 10.1017/ S175173111500230X

Benchaar, C., Calsamiglia, S., Chaves, A.V., Fraser, G.R., Colombatto, D., McAllister, T.A., and Beauchemin, K.A. (2008). A review of plant-derived essential oils in ruminant nutrition and production. Animal Feed Science and Technology 145, 209-228. DOI:10. 1016/ j.anifeedsci.2007.04.014

Benchaar, C., and Greathead, H. (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology 166-167, 338-355. DOI: 10.1016/ j.anifeedsci.2011.04.024

Benchaar, C., and Hassanat, F. (2025). Diet supplementation with a mixture of essential oils: effects on enteric methane emissions, apparent total-tract nutrient digestibility, nitrogen utilisation, and lactational performance. Journal of Dairy Science 108, 3560-3572. DOI: 10.3168/jds.2024-25447

Besharati, M., Maggiolino, A., Palangi, V., Kaya, A., Jabbar, M., Eseceli, H., De Palo, P., and Lorenzo, J.M. (2022). Tannin in ruminant nutrition: review. Molecules 27, 8273. DOI:10.3390molecules27238273

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., and Kurihara, M. (2009). Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. Journal of Dairy Science 92, 5512-5522. DOI: 10.3168/jds.2008-1441

Bowman, J.G.P., and Sowell, B.F. (1997). Delivery method and supplement consumption by grazing ruminants: a review. Journal of Animal Science 75, 543-550. DOI: 10.2527/1997.752543x

Brask, M., Lund, P., Weisbjerg, M.R., Hellwing, A.L.F., Poulsen, M., Larsen, M.K., and Hvelplund, T. (2013). Methane production and digestion of different physical forms of rapeseed as fat supplements in dairy cows. Journal of Dairy Science 96, 2356-2365. DOI: 10.3168/jds.2011-5239

Breider, I.S., Wall, E., and Garnsworthy, P.C. (2019). Heritability of methane production and genetic correlations with milk yield and body weight in Holstein-Friesian dairy cows. Journal of Dairy Science 102, 7277-7281. DOI: 10.3168/jds.2018-15909

Broudiscou, L.P., Cornu, A., and Rouzeau, A. (2007). In vitro degradation of 10 mono-and sesquiterpenes of plant origin by caprine rumen micro-organisms. Journal of the Science of Food and Agriculture 87, 1653-1658. DOI: 10.1002/jsfa.2863

Brutti, D.D., Canozzi, M.E.A., Sartori, E.D., Colombatto, D., and Barcellos, J.O.J. (2023). Effects of the use of tannins on the ruminal fermentation of cattle: a meta-analysis and meta-regression. Animal Feed Science and Technology 306, 115806. DOI: 10.1016/j.anifeedsci.2023.115806

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology 94, 223-253. DOI: 10.1016/ j.ijfoodmicro.2004.03.022

Callaghan, M.J., Tomkins, N.W., Hepworth, G., and Parker, A.J. (2020). The effect of molasses nitrate lick blocks on supplement intake, bodyweight, condition score, blood methaemoglobin concentration and herd scale methane emissions in Bos indicus cows grazing poor quality forage. Animal Production Science 61, 445-458.DOI: 10.1071/AN20389

Callaway, T.R., Edrington, T.S., Rychlik, J.L., Genovese, K.J., Poole, T.L., Jung, Y.S., Bischoff, K.M., Anderson, R.C., and Nisbet, D.J. (2003). Ionophores: their use as ruminant growth promotants and impact on food safety. Current Issues in Intestinal Microbiology 4, 43-51.

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., and Ferret, A. (2007). Invited review: essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science 90, 2580-2595. DOI: 10.3168/jds.2006-644

Cappucci, A., Mantino, A., Buccioni, A., Casarosa, L., Conte, G., Serra, A., Mannelli, F., Luciano, G., Foggi, G., and Mele, M. (2021). Diets supplemented with condensed and hydrolysable tannins affected rumen fatty acid profile and plasmalogen lipids, ammonia and methane production in an in vitro study. Italian Journal of Animal Science 20, 935-946. DOI: 10.1080/1828051X.2021.1915189

Cardoso-Gutierrez, E., Aranda-Aguirre, E., Robles-Jimenez, L.E., Castelán-Ortega, O.A., Chay-Canul, A.J., Foggi, G., Angeles-Hernandez, J.C., Vargas-Bello-Pérez, E., and González-Ronquillo, M. (2021).Effect of tannins from tropical plants on methane production from ruminants: a systematic review. Veterinary and Animal Science 14, 100214. DOI: 10.1016/j.vas.2021.100214

Cardozo, P.W., Calsamiglia, S., Ferret, A., and Kamel, C. (2005). Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. Journal of Animal Science 83, 2572-2579. DOI: 10.2527/2005.83112572x

Carrillo-Díaz, M.I., Miranda-Romero, L.A., Chávez-Aguilar, G., Zepeda-Batista, J.L., González-Reyes, M., García-Casillas, A.C., Tirado-González, D.N., and Tirado-Estrada, G. (2022). Improvement of ruminal neutral detergent fibre degradability by obtaining and using exogenous fibrolytic enzymes from white-rot fungi. Animals 12, 843. DOI: 10.3390/ani12070843

Castillejos, L., Calsamiglia, S., and Ferret, A. (2006). Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. Journal of Dairy Science 89, 2649-2658. DOI: 10.3168/jds.S0022-0302(06)72301-3

Castillejos, L., Calsamiglia, S., Ferret, A., and Losa, R. (2005). Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. Animal Feed Science and Technology 119, 29-41. DOI: 10.1016/ j.anifeedsci.2004.12.008

Castillo, C., Benedito, J.L., Méndez, J., Pereira, V., Lopez-Alonso, M., Miranda, M., and Hernández, J. (2004). Organic acids as a substitute for monensin in diets for beef cattle. Animal Feed Science and Technology 115, 101-116. DOI: 10.1016/j.anifeedsci.2004.02.001

Charmley, E., Williams, S.R.O., Moate, P.J., Hegarty, R.S., Herd, R.M., Oddy, V.H., Reyenga, P., Staunton, K.M., Anderson, A., and Hannah, M.C. (2015). A universal equation to predict methane production of forage-fed cattle in Australia. Animal Production Science 56.DOI: 10.1071/an15365

Cheeke, P.R. (2000). Actual and Potential Applications of Yucca schidigera and Quillaja saponaria Saponins in Human and Animal Nutrition. In: W. Oleszek & A. Marston (Eds.), Saponins in Food, Feedstuffs and Medicinal Plants (Proceedings of the Phytochemical Society of Europe, Vol 45, pp. 241–254). DOI: 10.1007/978-94-015-9339-7_25

Chen, J., Harstad, O.M., McAllister, T., Dörsch, P., and Holo, H. (2020). Propionic acid bacteria enhance ruminal feed degradation and reduce methane production in vitro. Acta Agriculturae Scandinavica, Section A—Animal Science 69, 169-175. DOI: 10.1080/09064702.2020.1737215

Cobellis, G., Trabalza-Marinucci, M., and Yu, Z. (2016). Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: a review. Science of the Total Environment 545-546, 556-568.DOI: 10.1016/j.scitotenv.2015.12.103

Cooke, R.F., Eloy, L.R., Bosco, S.C., Lasmar, P.V.F., de Simas, J.M.C., Leiva, T., and de Medeiros, S.R. (2024). An updated meta-analysis of the anti-methanogenic effects of monensin in beef cattle. Translational Animal Science 8. DOI: 10.1093/tas/txae032

Cuervo, W., Gómez, C., Tarnonsky, F., Fernandez-Marenchino, I., Maderal, A., Podversich, F., Vargas, J.d.J., and DiLorenzo, N. (2024). Effect of cashew nutshell extract, saponins and tannins addition on methane emissions, nutrient digestibility and feeding behaviour of beef steers receiving a backgrounding diet. Animals 14, 3126. DOI: 10.3390/ani14213126

da Costa, M.M. (2017). Modulation of ruminal biohydrogenation in sheep through dietary tannins or energy sources. Doctoral Thesis, Universidade de Lisboa 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., and 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, 411-429. DOI: 10.3168/jds.2024-25044

Dijkstra, J., Bannink, A., France, J., Kebreab, E., and Van Gastelen, S. (2018). Antimethanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fibre content, and cattle type. Journal of Dairy Science 101, 9041-9047. DOI: 10.3168/jds.2018-14456

Doyle, N., Mbandlwa, P., Kelly, W.J., Attwood, G., Li, Y., Ross, R.P., Stanton, C., and Leahy, S. (2019). Use of lactic acid bacteria to reduce methane production in ruminants, a critical review. Frontiers in Microbiology 10, 2207. DOI: 10.3389/fmicb.2019.02207

Durmic, Z., Black, J.L., Martin, G.B., and Vercoe, P.E. (2021). Harnessing plant bioactivity for enteric methane mitigation in Australia. Animal Production Science 62, 1160-1172. DOI: 10.1071/AN21004

Durmic, Z., Moate, P.J., Eckard, R., Revell, D.K., Williams, R., and Vercoe, P.E. (2014). In vitro screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. Journal of the Science of Food and Agriculture 94, 1191-1196. DOI: 10.1002/jsfa.6396

Eugène, M., Massé, D., Chiquette, J., and Benchaar, C. (2008). Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. Canadian Journal of Animal Science 88, 331-337. DOI: 10.4141/CJAS07112

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., and Hirt, R.P. (1994). Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiology Letters 117, 157-161. DOI: 10.1111/j.1574-6968.1994.tb06758.x

Foggi, G., Terranova, M., Conte, G., Mantino, A., Amelchanka, S.L., Kreuzer, M., and Mele, M. (2022). In vitro screening of the ruminal methane and ammonia mitigating potential of mixtures of either chestnut or quebracho tannins with blends of essential oils as feed additives. Italian Journal of Animal Science 21, 1520-1532. DOI: 10.1080/1828051X.2022.2130832

Gallo, A., Giuberti, G., Frisvad, J.C., Bertuzzi, T., and Nielsen, K.F. (2015). Review on mycotoxin issues in ruminants: Occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. Toxins 7, 3057-3111. DOI: 10.3390/toxins7083057

García-Martínez, R., Ranilla, M.J., Tejido, M.L., and Carro, M.D. (2005). Effects of disodium fumarate on in vitro rumen microbial growth, methane production and fermentation of diets differing in their forage: concentrate ratio. British Journal of Nutrition 94, 71-77. DOI: 10.1079/BJN20051455

Gates, R.S., Casey, K.D., Xin, H., and Burns, R.T. (2009). Building emissions uncertainty estimates. Transactions of the ASABE 52, 1345-1351. DOI: 10.13031/2013.27784

Gerber, P.J., Henderson, B., and Makkar, H.P.S. (2013a). Mitigation of greenhouse gas emissions in livestock production: a review of technical options for non-CO2 emissions. Food and Agriculture Organisation of the United Nations (FAO).

Gerber, P.J., Hristov, A.N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A.T., Yang, W.Z., Tricarico, J.M., Kebreab, E., Waghorn, G., Dijkstra, J., and Oosting, S. (2013b). Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 7 Suppl 2, 220-234. DOI: 10.1017/S1751731113000876

Gladine, C., Rock, E., Morand, C., Bauchart, D., and Durand, D. (2007). Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. British Journal of Nutrition 98, 691-701. DOI: 10.1017/S0007114507742666

Goel, G., and Makkar, H.P.S. (2012). Methane mitigation from ruminants using tannins and saponins. Tropical Animal Health and Production 44, 729-739. DOI: 10.1007/s11250-011-9966-2

Gould, D.H. (1998). Polioencephalomalacia. Journal of Animal Science 76, 309-314. DOI: 10.2527/1998.761309x

Grainger, C., and Beauchemin, K.A. (2011). Can enteric methane emissions from ruminants be lowered without lowering their production? Animal Feed Science and Technology 166, 308-320. DOI: 10.1016/j.anifeedsci.2011.04.021

Greenwood, P.L., Gardner, G.E., and Ferguson, D.M. (2018). Current situation and future prospects for the Australian beef industry—a review. Asian-Australasian Journal of Animal Sciences 31, 992. DOI: 10.5713/ajas.18.0090

Gruninger, R.J., Puniya, A.K., Callaghan, T.M., Edwards, J.E., Youssef, N., Dagar, S.S., Fliegerova, K., Griffith, G.W., Forster, R., and Tsang, A. (2014). Anaerobic fungi (phylum neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. FEMS Microbiology Ecology 90, 1-17. DOI: 10.1111/1574-6941.12383

Guan, H., Wittenberg, K.M., Ominski, K.H., and Krause, D.O. (2006). Efficacy of ionophores in cattle diets for mitigation of enteric methane. Journal of Animal Science 84, 1896-1906. DOI: 10.2527/jas.2005-652

Guyader, J., Eugène, M., Meunier, B., Doreau, M., Morgavi, D.P., Silberberg, M., Rochette, Y., Gerard, C., Loncke, C., and Martin, C. (2015). Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. Journal of Animal Science 93, 3564-3577. DOI: 10.2527/jas.2014-8196

Hammond, K.J., Crompton, L.A., Bannink, A., Dijkstra, J., Yáñez-Ruiz, D.R., O'Kiely, P., Kebreab, E., Eugène, M.A., Yu, Z., and Shingfield, K.J. (2016a). Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. Animal Feed Science and Technology 219, 13-30. DOI: 10.1016/j.anifeedsci.2016.05.018

Hammond, K.J., Humphries, D.J., Crompton, L.A., Green, C., and Reynolds, C.K. (2015). Methane emissions from cattle: estimates from short-term measurements using a GreenFeed system compared with measurements obtained using respiration chambers or sulphur hexafluoride tracer. Animal Feed Science and Technology 203, 41-52. DOI: 10.1016/j.anifeedsci.2015.02.008

Hammond, K.J., Waghorn, G.C., and Hegarty, R.S. (2016b). The GreenFeed system for measurement of enteric methane emission from cattle. Animal Production Science 56, 181-189. DOI: 10.1071/AN15631

Hegarty, R.S., Passetti, R.A.C., Dittmer, K.M., Wang, Y., Shelton, S.W., Emmet-Booth, J., Wollenberg, E.K., McAllister, T., Leahy, S., and Beauchemin, K. (2021). An evaluation of emerging feed additives to reduce methane emissions from livestock. Wellington, New Zealand: Climate Change, Agriculture and Food Security (CCAFS); New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC); Global Research Alliance on Agricultural Greenhouse Gases.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P.H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Scientific Reports 5. DOI: 10.1038/srep14567

Holtshausen, L., Chaves, A.V., Beauchemin, K.A., McGinn, S.M., McAllister, T.A., Odongo, N.E., Cheeke, P.R., and Benchaar, C. (2009). Feeding saponin-containing Yucca schidigera and Quillaja saponaria to decrease enteric methane production in dairy cows. Journal of Dairy Science 92, 2809-2821. DOI: 10.3168/jds.2008-1843

Hook, S.E., Wright, A.-D.G., and McBride, B.W. (2010). Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010, 945785. DOI: 10.1155/2010/945785

Hristov, A.N. (2023). Perspective: could dairy cow nutrition meaningfully reduce the carbon footprint of milk production? Journal of Dairy Science 106, 7336-7340. DOI: 10.3168/jds.2023-23461

Hristov, A.N., Bannink, A., Battelli, M., Belanche, A., Cajarville Sanz, M.C., Fernandez-Turren, G., Garcia, F., Jonker, A., Kenny, D.A., Lind, V., Meale, S.J., Meo Zilio, D., Muñoz, C., Pacheco, D., Peiren, N., Ramin, M., Rapetti, L., Schwarm, A., Stergiadis, S., Theodoridou, K., Ungerfeld, E.M., van Gastelen, S., Yáñez-Ruiz, D.R., Waters, S.M., and Lund, P. (2025). Feed additives for methane mitigation: recommendations for testing enteric methane-mitigating feed additives in ruminant studies. Journal of Dairy Science 108, 322-355. DOI: 10.3168/jds.2024-25050

Hristov, A.N., Degaetano, A.T., Rotz, C.A., Hoberg, E., Skinner, R.H., Felix, T., Li, H., Patterson, P.H., Roth, G., and Hall, M. (2018). Climate change effects on livestock in the northeast US and strategies for adaptation. Climatic Change 146, 33-45. DOI: 10.1007/s10584-017-2023-z

Hristov, A.N., Melgar, A., Wasson, D., and Arndt, C. (2022). Symposium review: effective nutritional strategies to mitigate enteric methane in dairy cattle. Journal of Dairy Science 105, 8543-8557. DOI: 10.3168/jds.2021-21398

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., and Lee, C. (2013). Special topics—mitigation of methane and nitrous oxide emissions from animal operations: a review of enteric methane mitigation options. Journal of Animal Science 91, 5045-5069. DOI: 10.2527/jas2013-6583

Hristov, A.N., Oh, J., Giallongo, F., Frederick, T.W., Harper, M.T., Weeks, H.L., and Tricarico, J.M. (2015). An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proceedings of the National Academy of Sciences 112, 10663-10668. DOI: 10.1073/pnas.1504124112

Hixson, J.L. et al. (2018). Exploiting grape marc to modulate methanogenesis. Animal Feed Science and Technology. DOI: 10.1016/j.anifeedsci.2018.10.007

Huang, Q., Liu, X., Zhao, G., Hu, T., and Wang, Y. (2018). Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Animal Nutrition 4, 137-150. DOI: 10.1016/j.aninu.2017.09.004

Huws, S.A., Creevey, C.J., Oyama, L.B., Mizrahi, I., Denman, S.E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S.M., and Hess, M. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. Frontiers in Microbiology 9, 2161. DOI: 10.3389/fmicb.2018.02161

Imaz, J.A., García, S., and González, L.A. (2019). Real-time monitoring of self-fed supplement intake, feeding behaviour, and growth rate as affected by forage quantity and quality of rotationally grazed beef cattle. Animals 9, 1129. DOI: 10.3390/ani9121129

Imaz, J.A., García, S., and González, L.A. (2020). Application of in-paddock technologies to monitor individual self-fed supplement intake and liveweight in beef cattle. Animals 10, 93. DOI: 10.3390/ani10010093

Jahani-Azizabadi, H., Durmic, Z., Vadhanabhuti, J., and Vercoe, P.E. (2019). Effect of some Australian native shrubs essential oils on in vitro rumen microbial fermentation of a high-concentrate diet. Journal of Animal & Plant Sciences 29.

Janssen, P.H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Animal Feed Science and Technology 160, 1-22. DOI: 10.1016/j.anifeedsci.2010.07.002

Janssen, P.H., and Kirs, M. (2008). Structure of the archaeal community of the rumen. Applied and Environmental Microbiology 74, 3619-3625. DOI: 10.1128/AEM.02812-07

Jayanegara, A., Goel, G., Makkar, H.P.S., and Becker, K. (2015). Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. Animal Feed Science and Technology 209, 60-68. DOI: 10.1016/ j.anifeedsci.2015.08.002

Jayanegara, A., Kreuzer, M., Wina, E., and Leiber, F. (2011). Significance of phenolic compounds in tropical forages for the ruminal bypass of polyunsaturated fatty acids and the appearance of biohydrogenation intermediates as examined in vitro. Animal Production Science 51, 1127-1136. DOI: 10.1071/AN11059

Jayanegara, A., Leiber, F., and Kreuzer, M. (2012). Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. Journal of Animal Physiology and Animal Nutrition 96, 365-375. DOI: 10.1111/j.1439-0396.2011.01172.x

Jayanegara, A., Wina, E., and Takahashi, J. (2014). Meta-analysis on methane mitigating properties of saponin-rich sources in the rumen: influence of addition levels and plant sources. Asian-Australasian Journal of Animal Sciences 27, 1426. DOI: 10.5713/ajas.2014.14086

Jenkins, T.C., Wallace, R.J., Moate, P.J., and Mosley, E.E. (2008). Board-invited review: recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. Journal of Animal Science 86, 397-412. DOI: 10.2527/jas.2007-0588

Jeyanathan, J., Martin, C., and Morgavi, D.P. (2014). The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. Animal 8, 250-261. DOI: 10.1017/S1751731113002085

Jeyanathan, J., Martin, C., and Morgavi, D.P. (2016). Screening of bacterial direct-fed microbials for their antimethanogenic potential in vitro and assessment of their effect on ruminal fermentation and microbial profiles in sheep. Journal of Animal Science 94, 739-750. DOI: 10.2527/jas.2015-9682

Johnson, K.A., and Johnson, D.E. (1995). Methane emissions from cattle. Journal of Animal Science 73, 2483-2492. DOI: 10.2527/1995.7382483x

Jordan, E., Lovett, D.K., Monahan, F.J., Callan, J., Flynn, B., and O'mara, F.P. (2006). Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. Journal of Animal Science 84, 162-170. DOI: 10.2527/2006.841162x

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

Kholif, A.E. (2023). A review of effect of saponins on ruminal fermentation, health and performance of ruminants. Veterinary Sciences 10, 450. DOI: 10.3390/vetsci10070450

Klop, G., Van Laar-Van Schuppen, S., Pellikaan, W.F., Hendriks, W.H., Bannink, A., and Dijkstra, J. (2017). Changes in in vitro gas and methane production from rumen fluid from dairy cows during adaptation to feed additives in vivo. Animal 11, 591-599. DOI: 10.1017/S1751731116002019

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., and Tricarico, J.M. (2014). Invited review: enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. Journal of Dairy Science 97, 3231-3261. DOI: 10.3168/jds.2013-7234

Kouřimská, L., Vondráčková, E., Fantová, M., NovýP, ., Nohejlová, L., and Michnová, K. (2014). Effect of feeding with algae on fatty acid profile of goat’s milk. Scientia Agriculturae Bohemica 45, 162-169. DOI: 10.2478/sab-2014-0103

Ku-Vera, J.C., Jiménez-Ocampo, R., Valencia-Salazar, S.S., Montoya-Flores, M.D., Molina-Botero, I.C., Arango, J., Gómez-Bravo, C.A., Aguilar-Pérez, C.F., and Solorio-Sánchez, F.J. (2020). Role of secondary plant metabolites on enteric methane mitigation in ruminants. Frontiers in Veterinary Science 7, 584. DOI: 10.3389/fvets.2020.00584

Kumar, S., Choudhury, P.K., Carro, M.D., Griffith, G.W., Dagar, S.S., Puniya, M., Calabro, S., Ravella, S.R., Dhewa, T., and Upadhyay, R.C. (2014). New aspects and strategies for methane mitigation from ruminants. Applied Microbiology and Biotechnology 98, 31-44.DOI: 10.1007/s00253-013-5365-0

Landbrugsstyrelsen (2024). Obligatorisk krav til metanreducerende foder: Bekendtgørelsen er nu offentliggjort. [Online]. Copenhagen, Denmark. Available: https://lbst.dk/alle-nyheder/nyheder/2024/okt/obligatorisk-krav-til-metanreducerende-foder-bekendtgoerelsen-er-nu-offentliggjort [Accessed 16/01/2026].

Lawrence, R., Loxton, I., Lisle, A., Shirley, D., and Doyle, J. (2024). Effect of Bovaer®10 on performance, health, carcase characteristics and carbon footprint of Australian feedlot cattle. (Yeppoon, Queensland: Meat & Livestock Australia Limited). Available at: https://www.mla.com.au/contentassets/a2b1680c30de4babb532d3344cd3ef85/mla-final-report-p.psh.1375-180324-print.pdf

Li, L., Silveira, C.I., Nolan, J.V., Godwin, I.R., Leng, R.A., and Hegarty, R.S. (2013). Effect of added dietary nitrate and elemental sulphur on wool growth and methane emission of Merino lambs. Animal Production Science 53, 1195-1201. DOI: 10.1071/AN13222

Liu, H., Vaddella, V., and Zhou, D. (2011). Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. Journal of Dairy Science 94, 6069-6077.DOI: 10.3168/jds.2011-4508

Lopez, S., Valdes, C., Newbold, C.J., and Wallace, R.J. (1999). Influence of sodium fumarate addition on rumen fermentation in vitro. British Journal of Nutrition 81, 59-64. DOI: 10.1017/S000711459900015X

Machmller, A., Soliva, C.R., and Kreuzer, M. (2003). Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. British Journal of Nutrition 90, 529-540. DOI: 10.1079/BJN2003923

Magnusson, M., Vucko, M.J., Neoh, T.L., and de Nys, R. (2020). Using oil immersion to deliver a naturally-derived, stable bromoform product from the red seaweed Asparagopsis taxiformis. Algal Research 51, 102065. DOI: 10.1016/j.algal.2020.102065

Makkar, H.P., Sen, S., Blmmel, M., and Becker, K. (1998). Effects of fractions containing saponins from Yucca schidigera, Quillaja saponaria, and Acacia auriculoformis on rumen fermentation. Journal of Agricultural and Food Chemistry 46, 4324-4328. DOI: 10.1021/jf980269q

Makkar, H.P.S. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Research 49, 241-256. DOI: 10.1016/s0921-4488(03)00142-1

Martin, C., Ferlay, A., Mosoni, P., Rochette, Y., Chilliard, Y., and Doreau, M. (2016). Increasing linseed supply in dairy cow diets based on hay or corn silage: effect on enteric methane emission, rumen microbial fermentation, and digestion. Journal of Dairy Science 99, 3445-3456. DOI: 10.3168/jds.2015-10110

Martin, C., Morgavi, D.P., and Doreau, M. (2010). Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351-365. DOI: 10.1017/S1751731109990620

Martinez-Fernandez, G., Denman, S.E., Yang, C., Cheung, J., Mitsumori, M., and McSweeney, C.S. (2016). Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. Frontiers in Microbiology 7, 1122. DOI: 10.3389/fmicb.2016.01122

McAllister, T.A., Beauchemin, K.A., Alazzeh, A.Y., Baah, J., Teather, R.M., and Stanford, K. (2011). The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Canadian Journal of Animal Science 91, 193-211. DOI: 10.4141/CJAS10047

McAllister, T.A., and Newbold, C.J. (2008). Redirecting rumen fermentation to reduce methanogenesis. Australian Journal of Experimental Agriculture 48, 7-13. DOI: 10.1071/EA07218

McGinn, S.M., Beauchemin, K.A., Coates, T., and Colombatto, D. (2004). Methane emissions from beef cattle: effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. Journal of Animal Science 82, 3346-3356. DOI: 10.2527/2004.82113346x

McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.A., and Newbold, C.J. (2003). Effects of essential oils on ruminal microorganisms and their protein metabolism. Applied and Environmental Microbiology 69, 5011-5014. DOI: 10.1128/AEM.69.8.5011-5014.2003

Meale, S., Popova, M., Saro, C., Martin, C., Bernard, A., Lagree, M., Yáñez-Ruiz, D.R., Boudra, H., Duval, S., and Morgavi, D.P. (2021). Early life dietary intervention in dairy calves results in a long-term reduction in methane emissions. Scientific Reports 11, 3003. DOI: 10.1038/ s41598-021-82084-9

Meale, S.J., Ding, S., He, M.L., Dugan, M.E.R., Ribeiro Jr, G.O., Alazzeh, A.Y., Holo, H., Harstad, O.M., McAllister, T.A., and Chaves, A.V. (2014). Effect of Propionibacterium freudenreichii on ruminal fermentation patterns, methane production and lipid biohydrogenation of beef finishing diets containing flaxseed oil in a rumen simulation technique. Canadian Journal of Animal Science 94, 685-695. DOI: 10.4141/CJAS-2014-051

Melgar, A., Harper, M.T., Oh, J., and Hristov, A.N. (2020). Effects of 3-nitrooxypropanol on enteric methane emissions and productivity of dairy cows: a meta-analysis. Journal of Dairy Science 103, 6145-6161. DOI: 10.3168/jds.2019-17664

Miller, G.A., Bowen, J.M., Dewhurst, R.J., Zweifel, B., Spengler, K., and Duthie, C.-A. (2023). Enteric methane emissions from dairy–beef steers supplemented with the essential oil blend Agolin Ruminant. Animals 13, 1826. DOI: 10.3390/ani13111826

Min, B.R., Barry, T.N., Attwood, G.T., and McNabb, W.C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology 106, 3-19. DOI: 10.1016/s0377-8401(03)00041-5

Min, B.R., Willis, W., Casey, K., Castleberry, L., Waldrip, H., and Parker, D. (2022). Condensed and hydrolyzable tannins for reducing methane and nitrous oxide emissions in dairy manure—a laboratory incubation study. Animals 12, 2876. DOI: 10.3390/ani12202876

Minato, H., Endo, A., Higuchi, M., Ootomo, Y., and Uemura, T. (1966). Ecological treatise on the rumen fermentation I. The fractionation of bacteria attached to the rumen digesta solids. The Journal of General and Applied Microbiology 12, 39-52. DOI: 10.2323/jgam.12.39

Molero, R., Ibars, M., Calsamiglia, S., Ferret, A., and Losa, R. (2004). Effects of a specific blend of essential oil compounds on dry matter and crude protein degradability in heifers fed diets with different forage to concentrate ratios. Animal Feed Science and Technology 114, 91-104. DOI: 10.1016/j.anifeedsci.2003.11.011

Morey, L., Bach, A., Sabrià, D., Riau, V., Fernández, B., and Terré, M. (2023). Effectiveness of precision feeding in reducing N excretion in dairy cattle. Animal Feed Science and Technology 304, 115722. DOI: 10.1016/j.anifeedsci.2023.115722

Morgavi, D.P., Forano, E., Martin, C., and Newbold, C.J. (2010). Microbial ecosystem and methanogenesis in ruminants. Animal 4, 1024-1036. DOI: 10.1017/S1751731110000546

Muetzel, S., Lowe, K., Janssen, P.H., Pacheco, D., Bird, N., Walker, N., Vidoni, O., Schweikert, L., Clasadonte, L., and Kindermann, M. (2019). Towards the application of 3-nitrooxypropanol in pastoral farming systems, in: Poster presented at the New Zealand Agricultural Climate Change Conference.)

Muñoz, C., Muñoz, I.A., Rodríguez, R., Urrutia, N.L., and Ungerfeld, E.M. (2024). Effect of combining the methanogenesis inhibitor 3-nitrooxypropanol and cottonseeds on methane emissions, feed intake, and milk production of grazing dairy cows. Animal 18, 101203. DOI: 10.1016/ j.animal.2024.101203

Muñoz, C., Villalobos, R., Peralta, A.M.T., Morales, R., Urrutia, N.L., and Ungerfeld, E.M. (2021). Long-term and carryover effects of supplementation with whole oilseeds on methane emission, milk production and milk fatty acid profile of grazing dairy cows. Animals 11, 2978. DOI: 10.3390/ ani11102978

Negussie, E., Lehtinen, J., Mäntysaari, P., Bayat, A.R., Liinamo, A.-E., Mäntysaari, E., and Lidauer, M. (2017). Non-invasive individual methane measurement in dairy cows. Animal 11, 890-899. 10.1017/ S1751731116002718

Newbold, C.J., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N.R. (2015). The role of ciliate protozoa in the rumen. Frontiers in Microbiology 6, 1313. DOI: 10.3389/fmicb.2015.01313

Newbold, C.J., López, S., Nelson, N., Ouda, J.O., Wallace, R.J., and 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, 27-35. DOI: 10.1079/BJN20051445

Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R., and Wallace, R.J. (2004). Effects of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 114, 105-112. DOI: 10.1016/j.anifeedsci.2003.12.006

Nunes, H., Dias, C.M., and Borba, A. (2023). Bioprospecting essential oils of exotic species as potential mitigations of ruminant enteric methanogenesis. Heliyon 9. DOI: 10.1016/ j.heliyon.2022.e12786

Oyebade, A.O., Arriola, K., Queiroz, O., Cappellozza, B.I., and Vyas, D. (2024). Effects of direct-fed microbials supplementation on in vitro and ex vivo ruminal fermentation and nutrient degradability in lactating Holstein dairy cows. Translational Animal Science 8, 162. DOI: 10.1093/tas/txae162

Palangi, V., and Lackner, M. (2022). Management of enteric methane emissions in ruminants using feed additives: a review. Animals 12, 3452. DOI: 10.3390/ani12243452

Palmquist, D.L., and Jenkins, T.C. (2017). A 100-year review: fat feeding of dairy cows. Journal of Dairy Science 100, 10061-10077. DOI: 10.3168/jds.2017-12924

Papakonstantinou, G.I., Voulgarakis, N., Terzidou, G., Fotos, L., Giamouri, E., and Papatsiros, V.G. (2024). Precision livestock farming technology: applications and challenges of animal welfare and climate change. Agriculture 14, 620. DOI: 10.3390/agriculture14040620

Parra, M.C., Forwood, D.L., Chaves, A.V., and Meale, S.J. (2023). In vitro screening of anti-methanogenic additives for use in Australian grazing systems. Frontiers in Animal Science 4, 1123532. DOI: 10.3389/fanim.2023.1123532

Patra, A.K. (2013). The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: a meta-analysis. Livestock Science 155, 244-254.DOI: 10.1016/j.livsci.2013.05.023

Patra, A.K., and Saxena, J. (2009). The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. Nutrition Research Reviews 22, 204-219. DOI: 10.1017/S0954422409990163

Patra, A.K., and Saxena, J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. Journal of the Science of Food and Agriculture 91, 24-37. DOI: 10.1002/jsfa.4152

Patra, A.K., and Yu, Z. (2012). Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. Applied and Environmental Microbiology 78, 4271-4280. DOI: 10.1128/AEM.00309-12

Patra, A.K., and Yu, Z. (2015). Effects of garlic oil, nitrate, saponin and their combinations supplemented to different substrates on in vitro fermentation, ruminal methanogenesis, and abundance and diversity of microbial populations. Journal of Applied Microbiology 119, 127-138. DOI: 10.1111/jam.12819

Pen, B., Sar, C., Mwenya, B., Kuwaki, K., Morikawa, R., and Takahashi, J. (2006). Effects of yucca schidigera and quillaja saponaria extracts on in vitro ruminal fermentation and methane emission. Animal Feed Science and Technology 129, 175-186. DOI: 10.1016/j.anifeedsci.2006.01.002

Penner, G.B., Beauchemin, K.A., and Mutsvangwa, T. (2007). Severity of ruminal acidosis in primiparous Holstein cows during the periparturient period. Journal of Dairy Science 90, 365-375. DOI: 10.3168/jds.S0022-0302(07)72638-3

Pepeta, B.N., Hassen, A., and Tesfamariam, E.H. (2024). Quantifying the impact of different dietary rumen modulating strategies on enteric methane emission and productivity in ruminant livestock: a meta-analysis. Animals 14, 763. DOI: 10.3390/ani14050763

Pérez-Enciso, M., Zingaretti, L.M., Ramayo-Caldas, Y., and de Los Campos, G. (2021). Opportunities and limits of combining microbiome and genome data for complex trait prediction. Genetics Selection Evolution 53, 65. DOI: 10.1186/s12711-021-00658-7

Pickering, N.K., Oddy, V.H., Basarab, J., Cammack, K., Hayes, B., Hegarty, R.S., Lassen, J., McEwan, J.C., Miller, S., and Pinares-Patiño, C.S. (2015). Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. Animal 9, 1431-1440. DOI: 10.1017/S1751731115000968

Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R.M., Spang, A., Canibe, N., Højberg, O., Milinovich, G., Fragner, L., and Schleper, C. (2013). Methylotrophic methanogenic thermoplasmata implicated in reduced methane emissions from bovine rumen. Nature Communications 4, 1428. DOI: 10.1038/ncomms2432

Prodanović, R., Bošnjaković, D., Djordjevic, A., Simeunović, P., Arsić, S., Mitrović, A., Jovanović, L., Vujanac, I., Kirovski, D., and Nedić, S. (2025). Effects of chestnut tannin extract on enteric methane emissions, blood metabolites and lactation performance in mid-lactation cows. Animals 15, 2238. DOI: 10.3390/ani15152238

Romero-Pérez, A., Okine, E.K., Guan, L.L., Duval, S.M., Kindermann, M., and Beauchemin, K.A. (2017). Rapid Communication: evaluation of methane inhibitor 3-nitrooxypropanol and monensin in a high-grain diet using the rumen simulation technique (Rusitec)1,2. Journal of Animal Science 95, 4072-4077. DOI: 10.2527/jas.2017.1896

Romero-Perez, A., Okine, E.K., McGinn, S.M., Guan, L.L., Oba, M., Duval, S.M., Kindermann, M., and Beauchemin, K.A. (2015). Sustained reduction in methane production from long-term addition of 3-Roques, S., Martinez-Fernandez, nitrooxypropanol to a beef cattle diet. Journal of Animal Science 93G., 1780-1791.DOI: 10.2527/jas.2014-8726

Roques, S., Martinez-Fernandez, G., Ramayo-Caldas, Y., Popova, , 17M., Denman, 80-1791. S., Meale, S.J., and Morgavi, D.P. (2024). Recent advances in enteric methane mitigation and the long road to sustainable ruminant production. Annual Review of Animal Biosciences 12, 321-343. DOI: 10.1146/annurev-animal-021022-024931

Russell, J.B., and Houlihan, A.J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiology Reviews 27, 65-74. DOI: 10.1016/S0168-6445(03)00019-6

Russell, J.B., and Strobel, H. (1989). Effect of ionophores on ruminal fermentation. Applied and Environmental Microbiology 55, 1-6. DOI: 10.1128/AEM.55.1.1-6.1989

Rychlik, J.L., and Russell, J.B. (2002). The adaptation and resistance of clostridium aminophilum F to the butyrivibriocin-like substance of butyrivibrio fibrisolvens JL5 and monensin. FEMS Microbiology Letters 209, 93-98. DOI: 10.1111/j.1574-6968.2002.tb11115.x

Salem, A.Z.M., Hassen, A., and Anele, U.Y. (2023). Exogenous enzymes as feed additives in ruminants. Springer. DOI: 10.1007/978-3-031-27993-5

Shingfield, K.J., Bonnet, M., and Scollan, N.D. (2013). Recent developments in altering the fatty acid composition of ruminant-derived foods. Animal 7, 132-162. DOI: 10.1017/S1751731112001681

Silivong, P., Preston, T.R., and Leng, R.A. (2011). Effect of sulphur and calcium nitrate on methane production by goats fed a basal diet of molasses supplemented with mimosa (mimosa pigra) foliage. Livestock Research for Rural Development 23, 2011. Available at: https://lrrd.cipav.org.co/lrrd23/3/sili23058.htm (accessed 11 November 2025).

Silveira, S.R., Terry, S.A., Biffin, T.E., Maurício, R.M., Pereira, L.G.R., Ferreira, A.L., Ribeiro, R.S., Sacramento, J.P., Tomich, T.R., Machado, F.S., Campos, M.M., Gama, M.A.S., and Chaves, A.V. (2019). Replacement of soybean meal with soybean cake reduces methane emissions in dairy cows and an assessment of a face-mask technique for methane measurement. Frontiers in Veterinary Science Volume 6 - 2019. DOI: 10.3389/fvets.2019.00295

Silvestre, T., Martins, L.F., Cueva, S.F., Wasson, D.E., Stepanchenko, N., Räisänen, S.E., Sommai, S., Hile, M.L., and Hristov, A.N. (2023). Lactational performance, rumen fermentation, nutrient use efficiency, enteric methane emissions, and manure greenhouse gas-emitting potential in dairy cows fed a blend of essential oils. Journal of Dairy Science 106, 7661-7674. DOI: 10.3168/jds.2022-23181

Soliva, C.R., Meile, L., Cieślak, A., Kreuzer, M., and Machmüller, A. (2004). Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. British Journal of Nutrition 92, 689-700. DOI: 10.1079/BJN20041250

Spears, J.W., Lloyd, K.E., and Fry, R.S. (2011). Tolerance of cattle to increased dietary sulphur and effect of dietary cation-anion balance. Journal of Animal Science 89, 2502-2509. DOI: 10.2527/jas.2010-3265

Stewart, C.S., Flint, H.J., and Bryant, M.P. (1997). The rumen bacteria, in The Rumen Microbial Ecosystem, eds. P.N. Hobson & C.S. Stewart. 2 ed., Chapter 2. Blackie Academic & Professional.

Tapio, I., Bayat, A., Ahvenjärvi, S., Luukkonen, T., and Vilkki, J. (2018). The overall structure of rumen bacterial communities significantly differentiates animals by diet but not by their methane emission phenotype. Advances in Animal Biosciences 6.DOI: 10.1017/S2040470018000212

Tedeschi, L.O., Abdalla, A.L., Álvarez, C., Anuga, S.W., Arango, J., Beauchemin, K.A., Becquet, P., Berndt, A., Burns, R., De Camillis, C., Chará, J., Echazarreta, J.M., Hassouna, M., Kenny, D., Mathot, M., Mauricio, R.M., McClelland, S.C., Niu, M., Onyango, A.A., Parajuli, R., Pereira, L.G.R., del Prado, A., Paz Tieri, M., Uwizeye, A., and Kebreab, E. (2022). Quantification of methane emitted by ruminants: a review of methods. Journal of Animal Science 100. DOI: 10.1093/jas/skac197

Tomkins, N.W., Denman, S.E., Pilajun, R., Wanapat, M., Mcsweeney, C.S., and Elliott, R. (2015). Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. Animal Feed Science and Technology 200, 25-34. DOI: 10.1016/j.anifeedsci.2014.11.013

U.S. Food and Drug Administration (2023). Generally recognised as safe (GRAS) substances [Online]. Available: https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras [Accessed October 23, 2025].

Ungerfeld, E.M. (2015a). Limits to dihydrogen incorporation into electron sinks alternative to methanogenesis in ruminal fermentation. Frontiers in Microbiology 6, 1272. DOI: 10.3389/fmicb.2015.01272

Ungerfeld, E.M. (2015b). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Frontiers in Microbiology 6, 37. DOI: 10.3389/fmicb.2015.00037

Ungerfeld, E.M. (2020). Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. Frontiers in Microbiology 11. DOI: 10.3389/fmicb.2020.00589

Ungerfeld, E.M., Kohn, R.A., Wallace, R.J., and Newbold, C.J. (2007). A meta-analysis of fumarate addition on methane production in ruminal batch cultures. Journal of Animal Science 85, 2556-2563. DOI: 10.2527/jas.2006-666

van Empel, M.J., Makkar, H.P.S., Dijkstra, J., and Lund, P. (2016). Nutritional, technological and managerial parameters for precision feeding to enhance feed nutrient utilisation and productivity in different dairy cattle production systems. CABI Reviews, 1–27. DOI: 10.1079/pavsnnr201611037

van Zijderveld, S.M., Gerrits, W.J.J., Apajalahti, J.A., Newbold, J.R., Dijkstra, J., Leng, R.A., and Perdok, H.B. (2010). Nitrate and sulphate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. Journal of Dairy Science 93, 5856-5866. DOI: 10.3168/jds.2010-3281

van Zijderveld, S.M., Gerrits, W.J.J., Dijkstra, J., Newbold, J.R., Hulshof, R.B.A., and Perdok, H.B. (2011). Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. Journal of Dairy Science 94, 4028-4038. DOI: 10.3168/jds.2011-4236

Vargas, J., Ungerfeld, E., Muñoz, C., and DiLorenzo, N. (2022). Feeding strategies to mitigate enteric methane emission from ruminants in grassland systems. Animals 12, 1132. DOI: 10.3390/ani12091132

Vargas, J.E., Andrés, S., Snelling, T.J., López-Ferreras, L., Yáñez-Ruíz, D.R., García-Estrada, C., and López, S. (2017). Effect of sunflower and marine oils on ruminal microbiota, in vitro fermentation and digesta fatty acid profile. Frontiers in Microbiology 8, 1124. DOI: 10.3389/fmicb.2017.01124

Velazco, J.I., Mayer, D.G., Zimmerman, S., and Hegarty, R.S. (2016). Use of short-term breath measures to estimate daily methane production by cattle. Animal 10, 25-33. DOI: 10.1017/S1751731115001603

Vyas, D., Alazzeh, A., McGinn, S.M., McAllister, T.A., Harstad, O.M., Holo, H., and Beauchemin, K.A. (2016). Enteric methane emissions in response to ruminal inoculation of propionibacterium strains in beef cattle fed a mixed diet. Animal Production Science 56, 1035-1040. DOI: 10.1071/AN14801

Vyas, D., McGeough, E.J., McGinn, S.M., McAllister, T.A., and Beauchemin, K.A. (2014). Effect of Propionibacterium spp. on ruminal fermentation, nutrient digestibility, and methane emissions in beef heifers fed a high-forage diet. Journal of Animal Science 92, 2192-2201.DOI: 10.2527/jas.2013-7492

Wallace, R.J. (2004). Antimicrobial properties of plant secondary metabolites. Proceedings of the Nutrition Society 63, 621-629. DOI: 10.1079/PNS2004393

Wallace, R.J., McEwan, N.R., McIntosh, F.M., Teferedegne, B., and Newbold, C.J. (2002). Natural products as manipulators of rumen fermentation. Asian-Australasian Journal of Animal Sciences 15, 1458-1468. DOI: 10.5713/ajas.2002.1458

Wang, C., Han, L., Zhang, G.W., Du, H.S., Wu, Z.Z., Liu, Q., Guo, G., Huo, W.J., Zhang, J., and Zhang, Y.L. (2021). Effects of copper sulphate and coated copper sulphate addition on lactation performance, nutrient digestibility, ruminal fermentation and blood metabolites in dairy cows. British Journal of Nutrition 125, 251-259. DOI: 10.1017/S0007114520002986

Wang, J.K., Ye, J.A., and Liu, J.X. (2012). Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance: a review. Tropical Animal Health and Production 44, 697-706. DOI: 10.1007/s11250-011-9960-8

Wu, H., Li, Y., Meng, Q., and Zhou, Z. (2021). Effect of high sulphur diet on rumen fermentation, microflora, and epithelial barrier function in steers. Animals 11, 2545. DOI: 10.3390/ani11092545

Wu, H., Meng, Q., and Yu, Z. (2015). Effect of pH buffering capacity and sources of dietary sulphur on rumen fermentation, sulphide production, methane production, sulphate reducing bacteria, and total archaea in in vitro rumen cultures. Bioresource Technology 186, 25-33. DOI: 10.1016/j.biortech.2015.02.110

Yáez-Ruiz, D.R., Abecia, L., and Newbold, C.J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review. Frontiers in Microbiology 6, 1133. DOI: 10.3389/fmicb.2015.01133

Yanza, Y.R., Irawan, A., Jayanegara, A., Ramadhani, F., Respati, A.N., Fitri, A., Hidayat, C., Niderkorn, V., Cieslak, A., and Szumacher-Strabel, M. (2024). Saponin extracts utilisation as dietary additive in ruminant nutrition: a meta-analysis of in vivo studies. Animals 14, 1231. DOI: 10.3390/ani14081231

Yu, G., Beauchemin, K.A., and Dong, R. (2021). A review of 3-Nitrooxypropanol for enteric methane mitigation from ruminant livestock. Animals 11. DOI: 10.3390/ani11123540

Zhao, X., Degen, A., Hao, L., and Liu, S. (2022). Ruminant lick blocks, particularly in China: a review. Sustainability 14, 760. DOI: 10.3390/su14137620