International Master in Animal Nutrition

IX Edition

EFFECT OF *ASPARAGOPSIS ARMATA* ON RUMINAL FERMENTATION PROFILE AND METHANE PRODUCTION IN VITRO

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June, 2025

“The rigid and inflexible will surely fail, while the soft and flowing will prevail”

Lao Tzu

ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude to the Mediterranean Agronomic Institute of Zaragoza (IAMZ) and its esteemed director, Mr. **Raúl COMPÉS**, for granting me the opportunity to participate in this Master's program. I am equally grateful to Dr. **Andrés SCHLAGETER**, our Master's coordinator, for his attentiveness and support throughout the whole program.

My heartfelt appreciation goes to the Faculty of Veterinary Medicine at the University of Zaragoza (UNIZAR) and its esteemed Chair of Animal Nutrition, Professor **Manuel FONDEVILA CAMPS**, for his unwavering dedication to education. I also wish to express my sincere gratitude to the Animal Nutrition and Welfare Service (SNiBA) of the Autonomous University of Barcelona (UAB), notably its Chair of Animal Nutrition, Professor **José Francisco PÉREZ HERNÁNDEZ**, and **Montserrat SALA ROVIRA** for the warm welcome and for providing the resources and assistance needed to accomplish my thesis.

A special acknowledgment is owed to my supervisor, Dr. **Lorena CASTILLEJOS VELÁZQUEZ**, whose insightful feedback and unwavering support have been invaluable throughout this project. Her expertise and guidance greatly contributed to the development of my work during this process.

I would like to recognize the invaluable contributions of the exceptional lab team, including **Carmen MARTINEZ CANO**, **Anna GARRIT ANTÚNEZ**, and **Esther ALÒS**. Additionally, my gratitude goes to my colleagues in the ruminant team at SNiBA, who generously offered their assistance and support throughout the experiment, with special recognition to **Adriana SIURANA**, **Maria Roda**, **Laia Garrido Pérez**, and **Fernando Reimann Skonieski**.

I also wish to express my gratitude to the members of the jury for their time and expertise in evaluating my review. Their valuable insights and feedback significantly enhanced this work.

Finally, I am deeply thankful to my friends and family. Their encouragement and unwavering belief in me have been a source of strength and determination throughout this journey. I could not have achieved this without their support.

Thank you!

ABSTRACT

Methane (CH_4) emissions from ruminal fermentation represent both an environmental challenge and an energy loss in ruminant production. *Asparagopsis armata*, a red seaweed rich in halogenated compounds such as bromoform, has been proposed as a natural feed additive for methane mitigation. This study evaluated its effects on in vitro ruminal fermentation using rumen fluid (RF) from dairy cows and beef steers. Two diets were tested: high-concentrate (90:10, C) and high-forage (60:40, F), with *A. armata* included at 0% (Control), 0.3%, 0.6%, 1.2%, and 2.4% OM. Methane production, total gas production (TGP), pH, ammonia nitrogen, in vitro degradability, and volatile fatty acid (VFA) concentrations were analyzed. Results revealed a significant, dose-dependent reduction in CH_4 production across both experiments and diets ($p < 0.05$). In the dairy RF, CH_4 production decreased by 62.5% and 59.2% at a 0.6% inclusion level in Diet C and Diet F, respectively, with complete suppression (100%) achieved at 1.2% and 2.4% inclusion levels. In the beef RF, similar CH_4 suppression was observed at 1.2% inclusion in Diet C and at 2.4% in Diet F. Although TGP remained unaffected with the dairy RF, it showed a dose-dependent reduction with the beef RF at higher inclusion levels. Total VFA production was stable in the dairy RF but declined significantly in the beef RF at higher doses. No significant effect on in vitro degradability was observed in both experiments within diets. A decrease in acetate-to-propionate ratio with increasing *A. armata* inclusion indicated a shift in hydrogen utilization toward propionate production. These findings demonstrate that *A. armata* supplementation at 0.6% OM can reduce CH_4 production by over 50% in dairy RF without negatively impacting fermentation parameters, though further studies are needed to assess its broader applicability in different production systems.

Key words: methane mitigation, *asparagopsis armata*, in vitro ruminal fermentation, halogenated compounds, bromoform.

RESUMEN

Las emisiones de metano (CH_4) durante la fermentación ruminal representan un desafío ambiental y una pérdida energética en la producción de rumiantes. *Asparagopsis armata*, una macroalga roja rica en compuestos halogenados como el bromoformo, se presenta como un aditivo natural para mitigar la producción de CH_4. Este estudio evaluó su efecto sobre la fermentación ruminal in vitro utilizando líquido ruminal (LR) de vaca lechera y de ternero de engorde. Se evaluaron dos dietas: una alta en concentrado (90:10, C) y otra alta en forraje (60:40, F), con inclusión de *A. armata* en 0% (Control), 0,3%, 0,6%, 1,2% y 2,4% MO. Se analizaron la producción de CH_4, la producción total de gas (PTG), el pH, el nitrógeno amoniacal, la degradabilidad in vitro y la concentración de ácidos grasos volátiles (AGV). Los resultados mostraron una reducción significativa y dependiente de la dosis en la producción de CH_4 en ambos ensayos y dietas ($p < 0.05$). En el LR de vaca lechera, la inclusión de 0,6% MO de *A. armata* redujo la producción de CH_4 disminuyó en un 62,5% y 59,2% en las dietas C y F, respectivamente. A niveles de 1,2% y 2,4% MO de *A. armata*, la producción de CH_4 se redujo completamente (100%). En el LR de ternero, se observó una reducción de CH_4 similar a partir de una inclusión del 1,2% MO de *A. armata* en la dieta C y 2,4% en la dieta F. Aunque la PTG no se vio afectada en LR de vaca, sí se redujo significativamente en LR de ternero a niveles altos de inclusión. La producción total de AGVs se mantuvo estable en el LR de vaca lechera, pero disminuyó significativamente en el LR de ternero a dosis más elevadas (1,2% y 2,4%). No se observaron efectos significativos sobre la degradabilidad in vitro a ninguna de las dosis de inclusión de *A. armata* dentro de las dietas. La reducción de la relación acetato:propionato con el aumento de la inclusión de *A. armata* sugiere un cambio en la utilización del hidrógeno, favoreciendo la producción de propionato. Estos hallazgos indican que la suplementación con *A. armata* al 0,6% MO puede reducir la producción de CH_4 en más del 50% en el LR de vaca sin afectar negativamente los parámetros de fermentación. No obstante, se requieren más estudios in vivo para evaluar su aplicabilidad en diferentes sistemas productivos.

Palabras clave: mitigación del metano, *Asparagopsis armata*, fermentación ruminal in vitro, compuestos halogenados, bromoformo.

RÉSUMÉ

Les émissions de méthane (CH_4) issues de la fermentation ruminale constituent à la fois un défi environnemental et une perte énergétique dans la production des ruminants. *Asparagopsis armata*, une macroalgue rouge riche en composés halogénés tels que le bromoforme, a été proposée comme additif naturel pour l'atténuation de la production de CH_4. Cette étude a évalué ses effets sur la fermentation ruminale in vitro en utilisant du liquide ruminal (LR) provenant de vaches laitières et de taurillons. Deux régimes alimentaires ont été testés : un régime riche en concentré (90:10, C) et un régime riche en fourrage (60:40, F), avec une inclusion de *A. armata* à 0 % (Contrôle), 0,3 %, 0,6 %, 1,2 % et 2,4 % MO. La production de CH_4, la production totale de gaz (PTG), le pH, l'azote ammoniacal, la dégradabilité in vitro et les concentrations en acides gras volatils (AGV) ont été analysés. Les résultats ont révélé une réduction significative et dose-dépendante de la production de CH_4 dans les deux expériences et régimes alimentaires ($p < 0,05$). Dans le LR de vache laitière, la production de CH_4 a diminué de 62,5 % et 59,2 % à une inclusion de 0,6 % MO dans les régimes C et F, respectivement, avec une suppression totale (100 %) atteinte aux niveaux de 1,2 % et 2,4 % MO. Dans le LR de taurillon, une réduction similaire a été observée à partir de 1,2 % MO dans le régime C et à 2,4 % MO dans le régime F. Bien que la PTG soit restée stable dans le LR de vache, elle a diminué de manière dose-dépendante dans le LR de taurillon à des niveaux d'inclusion plus élevés. La production totale d'AGV est restée stable dans le LR de vache laitière, mais a diminué significativement dans le LR de taurillon aux doses les plus élevées. Aucun effet significatif sur la dégradabilité in vitro n'a été observé dans les deux expériences au sein des régimes alimentaires. La diminution du rapport acétate:propionate avec l'augmentation de l'inclusion de *A. armata* a indiqué un changement dans l'utilisation de l'hydrogène en faveur de la production de propionate. Ces résultats démontrent que la supplémentation en *A. armata* à 0,6 % MO peut réduire la production de CH_4 de plus de 50 % dans le LR de vache sans affecter négativement les paramètres de fermentation, bien que des études supplémentaires soient nécessaires pour évaluer son applicabilité dans différents systèmes de production.

Mots-clés: atténuation du methane, *asparagopsis armata*, fermentation ruminale in vitro, composés halogénés, bromoforme.

TABLES

FIGURES

ABBREVIATIONS

3-NOP	3-nitrooxypropanol
BCM	Bromochloromethane
CH4	Methane
$CHBr_3$	Bromoform
CoB	Coenzyme B
CoM	Coenzyme M
DM	Dry matter
DMdeg	Dry Matter degradability
DW	Dry weight
GHG	Greenhouse Gas
H2	Hydrogen
MCR	Methyl-coenzyme M reductase
NH_3	Ammonia
NH_3-N	Ammonia Nitrogen
OM	Organic Matter
OMdeg	Organic Matter degradability
RF	Rumen fluid
TGP	Total gas production
TVFA	Total volatile fatty acid
VFA	Volatile fatty acid

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INTRODUCTION

INTRODUCTION

Global population dynamics and their associated trends in food demand have placed unprecedented pressure on agricultural systems worldwide. The global population, currently estimated at 8.2 billion in 2024, is projected to grow significantly, reaching approximately 9 billion by 2037 (**Figure 1**), and reaching a peak of 10.3 billion by the mid-2080s (United Nations, 2024).

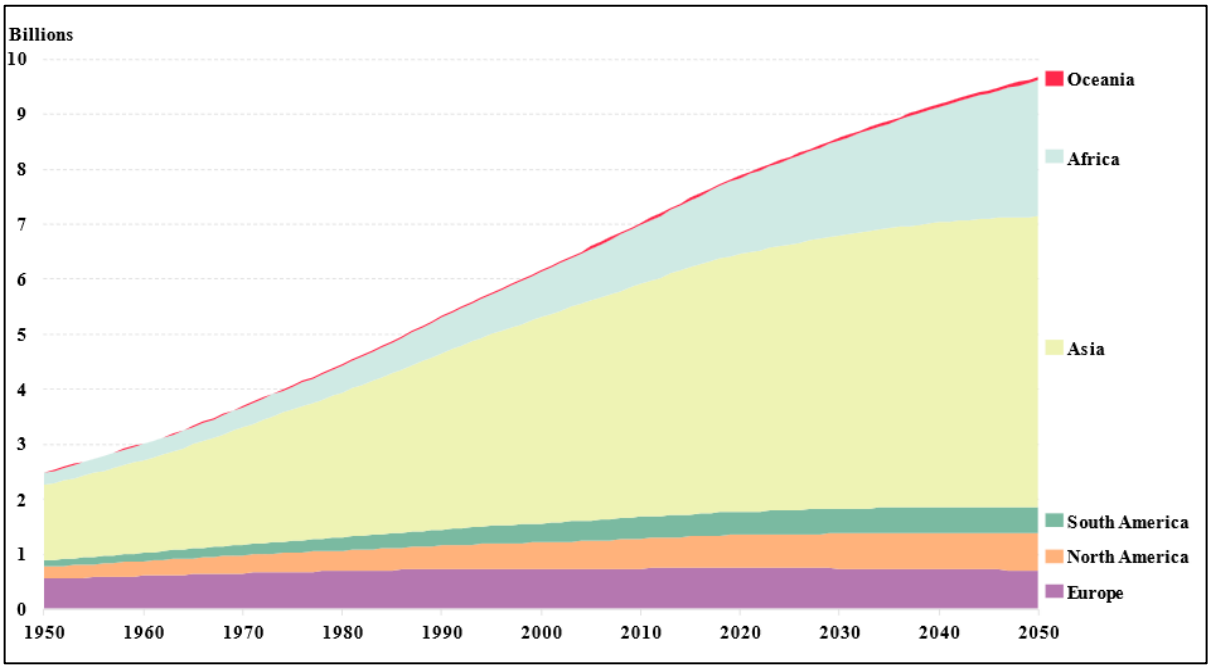

Figure 1. Population by world region (1950-2023) and projections based on medium variant (2023-2050) (adapted from HYDE (2023); Gapminder (2022); UN WPP (2024) – with major processing by Our World in Data).

Developing countries collectively drive the majority of global population growth, with Asia as a key contributor and Africa emerging as the fastest-growing region (Gerland et al., 2014), both expected to significantly influence the demand for animal products. The ruminant production sector within the livestock industry significantly contributes to food security by transforming inedible plant material into meat and milk, which provide essential nutrients and high-quality protein vital for human health (Ahmed et al., 2023).

The global demand for meat and milk is expected to grow significantly by 2050, driven by population growth, urbanization, and increasing incomes, particularly in developing regions (Gerber et al., 2013). **Figure 2** depicts the steady increase in global milk and meat production from 1961 to 2021, with milk production rising from approximately 350 million tons to 930

million tons and meat production increasing from 70 million tons to 340 million tons. Projections indicate that milk production will surpass 1,020 million tons by 2030, while meat production is expected to reach 450 million tons by 2050, with ruminant meat playing a critical role in meeting future protein demands (OECD, 2024).

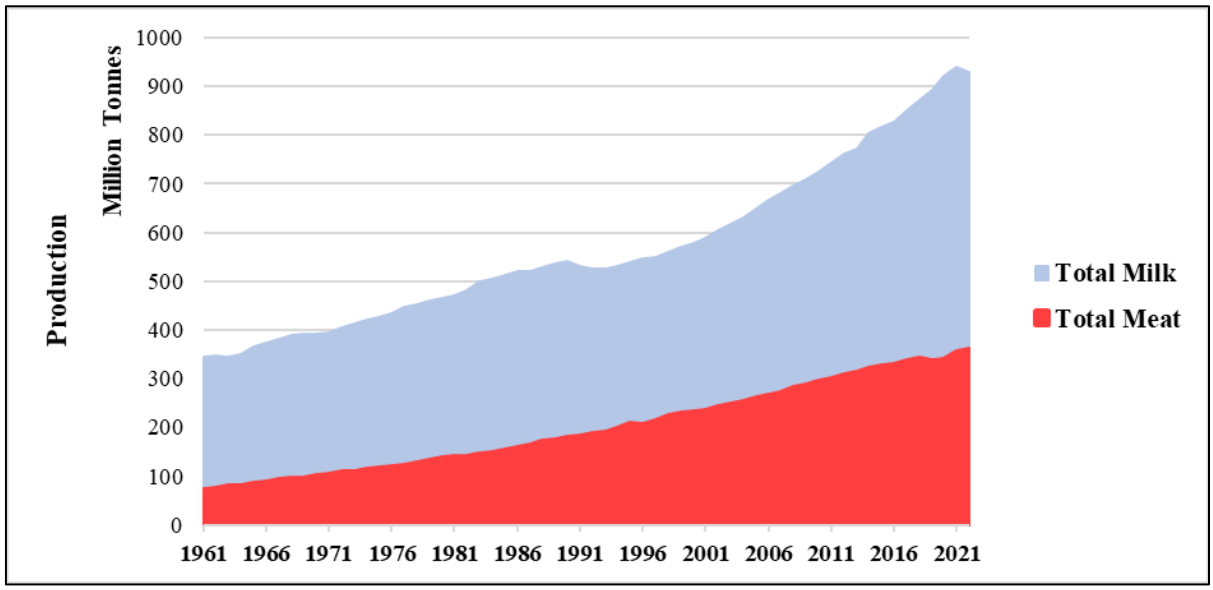

Figure 2. Global Trends in Milk and Meat Production (1961–2021) (Food and Agriculture Organization of the United Nations (FAO), 2024).

The rising demand for animal-source foods presents environmental challenges, notably greenhouse gas (GHG) emissions from livestock production. Total global GHG emissions from livestock supply chains; including animals, manure, feed production, and land-use changes; are estimated to account for 14.5% of total anthropogenic emissions (Gerber et al., 2013).

The three major GHGs emitted from animal production are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). They differ in their heat trapping effects and their lifetimes in the atmosphere (Ungerfeld et al., 2022). Specifically, enteric CH_4 from ruminants contributes approximately 6% of global emissions (Beauchemin et al., 2020). As illustrated in **Figure 3**, enteric fermentation alone accounts for about 40% of all livestock emissions, with beef and dairy production notably contributing 41% and 20% of the sector's total emissions, respectively (Gerber et al., 2013).

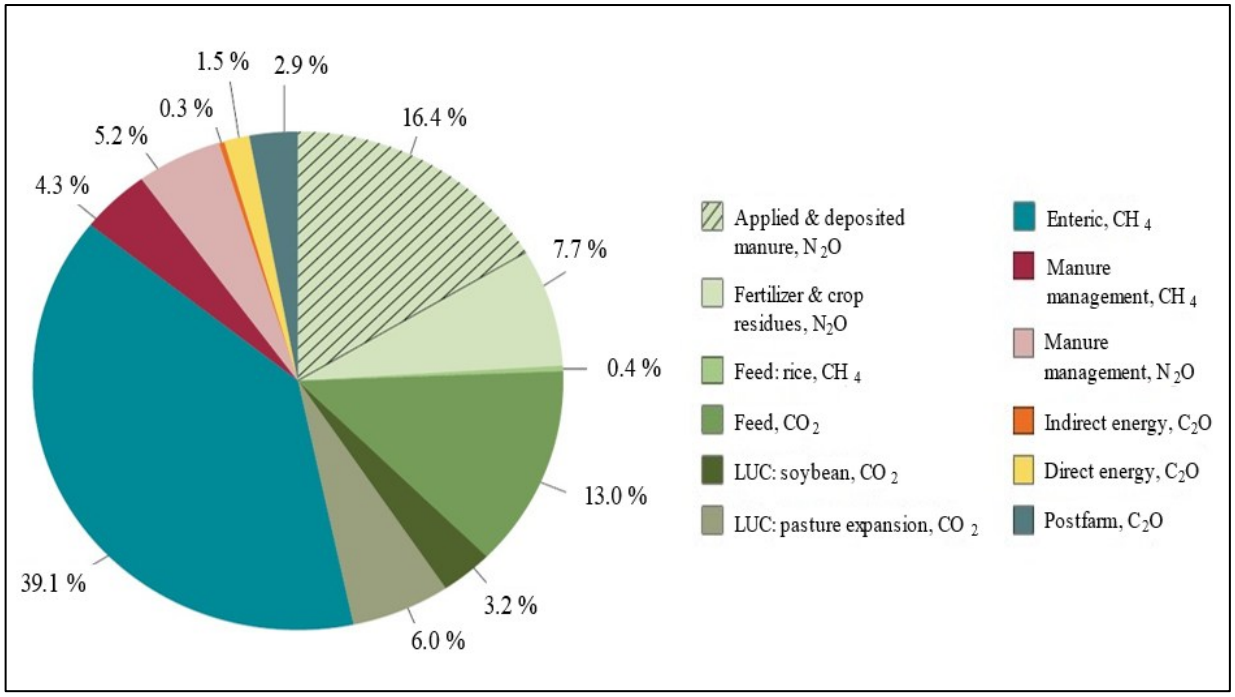

Figure 3. Global emissions from livestock supply chains by category of emissions (Gerber et al., 2013).

Methane has a 100-year global warming potential 32 times that of CO_2, making it a critical target for climate change mitigation efforts. With an average atmospheric lifetime of about 9-12.5 years in the troposphere, effectively reducing CH_4 emissions can significantly impact atmospheric conditions. This rapid response is essential for meeting the global warming limits set by international agreements like the Paris Agreement (Nisbet et al., 2020; Arndt et al., 2022).

Among the various strategies for CH_4 mitigation, dietary adjustments and direct manipulations of the rumen microbiota in livestock have been identified as the most immediate and potentially impactful methods (Martin et al., 2010; Beauchemin et al., 2020).

Despite the promising outcomes of nutritional and management strategies, their practical application encounters several obstacles. These include inconsistent effectiveness across different livestock species and farming systems, higher economic costs and less profitability, the need for adjustments to fit diverse agricultural contexts, and possible effects on animal health and product quality, such as milk or meat (Martin et al., 2010). Furthermore, feeding ruminants grains that are suitable for human consumption is often considered inefficient, as it overlooks the unique capacity of ruminants to transform fibrous, non-human-edible feed into valuable products (Beauchemin et al., 2020).

Biotechnological approaches, such as the selection of low-CH_4 producing animals, archaeal phages, and the development of vaccines targeting methanogenic archaea, have shown some efficacy but are still in the early stages of research and application. The complexity of the rumen ecosystem and the adaptability of its microbial communities pose significant barriers to the long-term success of these methods (Hristov et al., 2013; Beauchemin et al., 2020).

Recent approaches for CH_4 mitigation in ruminants have focused on various feed additives, including chemical inhibitors of rumen methanogenesis, ionophores, and plant secondary compounds (Hristov et al., 2013; Knapp et al., 2014; Beauchemin et al., 2020). These additives aim to reduce CH_4 emissions by targeting specific aspects of rumen microbial activity and methanogenesis, such as 3-nitrooxypropanol (3-NOP) targeting methyl-coenzyme M reductase (MCR), a key enzyme in the CH_4 production pathway (Martinez-Fernandez et al., 2018).

Recent research has explored *Asparagopsis spp.*, a red seaweed, as a potential natural feed additive for CH_4 mitigation in ruminants due to its natural origin and high content of halogenated secondary metabolites, particularly bromoform ($CHBr_3$) (Roque et al., 2019a; Vijn et al., 2020). Bromoform acts as a competitive inhibitor of MCR, the terminal enzyme in the methanogenesis pathway of archaea, effectively halting CH_4 synthesis in the rumen (Machado et al., 2016; Roque et al., 2019a). This inhibition reduces hydrogen (H_2) availability for methanogens, redirecting it towards alternative fermentation pathways.

In vitro studies have demonstrated the potential of *Asparagopsis* species, including *Asparagopsis taxiformis* and *Asparagopsis armata*, to significantly reduce CH_4 emissions in ruminants. At a 2% OM inclusion rate, *A. taxiformis* reduced CH_4 production by 99% (mL CH_4/g OM) within 24 hours of fermentation (Machado et al., 2016). Similarly, the inclusion of *A. armata* at 5% OM reduced CH_4 production by approximately 34% (mL CH_4/g DM of substrate), while *A. taxiformis* at 5% OM achieved reductions of approximately 86% (Nunes et al., 2024). In vivo studies have demonstrated the potential of *A. armata* to significantly reduce CH_4 emissions in ruminants. For example, Roque et al., (2019b) found that including *A. armata* at 0.5% and 1% OM in the diets of lactating dairy cows reduced CH_4 production (g/day) by 26.4% and 67.2%, respectively. This thesis aims to address the existing knowledge gaps by conducting an in vitro assessment of the use of *A. armata* as a CH_4 mitigation strategy in ruminant production systems.

LITERATURE REVIEW

LITERATURE REVIEW

1. Rumen metabolism

1.1. Rumen ecosystem and microbial composition

The rumen, the largest compartment of the ruminant digestive system, functions as a specialized anaerobic fermentation chamber maintained at approximately 39°C and a pH range of 6–7 (Nathani et al., 2015; Perez et al., 2024). This environment supports a diverse microbial ecosystem, including bacteria, archaea, viruses, bacteriophages, and eukaryotes such as fungi and protozoa, each with specific roles within the rumen microbiome (Keum et al., 2024). The sequential colonization of the rumen begins with bacteria as the first colonizers, followed by archaea, anaerobic fungi, and protozoa, facilitating the gradual adaptation of the microbiome to the animal's changing dietary composition during early growth (Amin and Seifert, 2021).

The microbial community in the rumen is highly diverse, with bacterial populations ranging from 10^{10} to 10^{11} organisms per mL, archaea from 10^{8} to 10^{9} cells per mL, protozoa from 10^{5} to 10^{6} cells per mL, and fungi from 10^{3} to 10^{4} cells per mL (**Table 1**) (Keum et al., 2024). Molecular analyses estimate that rumen bacteria alone include 300–400 distinct phylotypes, with many still uncultivated (Morgavi et al., 2010). The dominant bacterial phyla are Firmicutes (41.22%), Bacteroidetes (33.51%), and Proteobacteria (12.15%), although their proportions vary depending on factors such as diet and age (Keum et al., 2024). Firmicutes are more abundant in forage-based diets, whereas Bacteroidetes dominate in concentrate-based diets (Nathani et al., 2015).

Archaea account for less than 4% of the microbial community, with concentrations ranging from 10^{6} to 10^{8} cells per mL (Hook et al., 2010; Keum et al., 2024). These highly diverse archaea and bacterial consortia are categorized based on their functions, such as fiber-degrading bacteria, lactic acid utilizers, sulphate-reducing bacteria, acetogens, starch utilizers, and methanogens. Methanogens, predominantly represented by the Methanobacteriaceae family, are strictly anaerobic microorganisms that play a crucial role in the rumen by serving as H_2 sinks and maintaining fermentation efficiency (Martínez-Álvaro et al., 2020). There are about 20 species of methanogens isolated from the rumen and characterized, whereas other isolates remain uncharacterized (Khairunisa et al., 2023).

Protozoa, while not necessary for survival, play significant roles in the rumen ecosystem, notably in fiber digestion and volatile fatty acid (VFA) production. They contribute to 30–40% of overall fiber digestion and increase CH_4 production through interactions with hydrogenotrophic methanogens, producing H_2 via hydrogenosomes (Solomon et al., 2022).

Anaerobic fungi are an integral component of the microbiome and have been known to contribute to plant cell wall digestion. Fungi make up 8–12% of the biomass of the rumen microbiota, although their biomass varies greatly depending on the diet. Their superior decomposition ability plays a central role in breaking down lignocellulolytic tissues, granting bacteria greater access to fiber (Krause et al., 2013). Under anaerobic conditions, fungi, like protozoa, produce H_2 via hydrogenosomes, providing substrates for methanogens (Keum et al., 2024).

Table 1. Composition and general characteristics of rumen microorganisms (Keum et al., 2024).

			Eukaryotes		
	Bacteria	**Archaea**	**Protozoa**	**Fungi**	**Bacteriophage**
Populations (organisms/ml)	10^{10-11}	10^{8-9}	10^{5-6}	10^{3-4}	10^{7-9}
Size (μm)	0.3-50	0.7-4	1-100	25-250	0.024-0.2
Generation interval	20 mins	25 min-6 h	8-36 h	24 h	-
Oxygen requirements	Facultative anaerobes	Strict anaerobes	Strict anaerobes	Strict anaerobes	Strict anaerobes
Predominant microorganisms in the bovine rumen	Gram negative (-) bacteria species	Methanogens (Genus *Methanobrevibacter*)	Genus *Entodinium*	Genera *Piromyces, Anaeromyces, Cyllamyces, Neocallimastix,* and *Orpinomyces*	Relative to the bacterial dominance

1.2. Rumen fermentation and its products

The rumen microbiome plays a critical role in feed digestion by breaking down structural carbohydrates from plants, which are otherwise inaccessible or non-hydrolyzable, into bioavailable nutrients (Perez et al., 2024). It ferments carbohydrates (excluding lignin), proteins, and lipids, producing VFAs, metabolizable nitrogen as microbial protein, and other essential metabolites (Keum et al., 2024).

The fermentation process begins with the degradation of dietary polysaccharides such as cellulose, hemicellulose, pectin, and starch by microbial enzymes into monomers like glucose,

hexoses, and pentoses. These monomers undergo further catabolism, leading to the production of VFAs (primarily acetate, propionate, and butyrate), CO_2, and H_2 (Ungerfeld, 2020).

Volatile fatty acids play distinct roles in energy metabolism: acetate supports lipogenesis, propionate serves as a substrate for gluconeogenesis, and butyrate provides energy to the rumen epithelium. These VFAs are absorbed through the rumen wall, metabolized and contributing to 70% of the ruminant's energy demands, or further metabolized in the rumen (Morgavi et al., 2010; Choudhury et al., 2015). Minor VFAs such as valerate, iso-butyrate, and iso-valerate are also produced, contributing to overall fermentation efficiency (Perez et al., 2024).

Hydrogen exists in two phases within the rumen: dissolved in the liquid phase and in the gaseous phase, with only dissolved H_2 being available for microbial utilization (Beauchemin et al., 2020). This H_2 serves as a substrate for various metabolic pathways that play critical roles in maintaining rumen fermentation efficiency and microbial balance. Among these pathways, methanogenesis is the primary H_2 sink, where methanogenic archaea reduce CO_2 to CH_4, effectively maintaining low H_2 partial pressure and facilitating electron flow (Morgavi et al., 2010).

However, H_2 is also consumed by other competing pathways within the rumen. For instance, as shown in **Figure 4**, H_2 is utilized in propionate synthesis, where intermediates such as lactate or fumarate are reduced to succinate and subsequently converted to propionate, contributing to gluconeogenesis (Ungerfeld, 2020). Reductive acetogenesis represents another pathway, using H_2 to convert CO_2 into acetate, which is absorbed by the host and used in lipogenesis (Tapio et al., 2017; Beauchemin et al., 2020). Additionally, nitrate reduction consumes H_2 to produce ammonia (NH_3), serving as a nitrogen source for microbial protein synthesis (McAllister and Newbold, 2008).

In addition to VFAs, rumen fermentation generates NH_3 as an essential nitrogen metabolite. This ammonia can be incorporated into microbial protein, providing metabolizable nitrogen for the host, or absorbed through the rumen wall into the bloodstream. Once absorbed, ammonia is converted into urea by the liver, which is either excreted via urine or recycled back to the rumen through saliva or diffusion, thus contributing to nitrogen availability within the rumen (Beauchemin et al., 2020). The balance of ammonia production and utilization is influenced by

dietary protein levels and microbial activity, playing a central role in nitrogen metabolism within the rumen ecosystem.

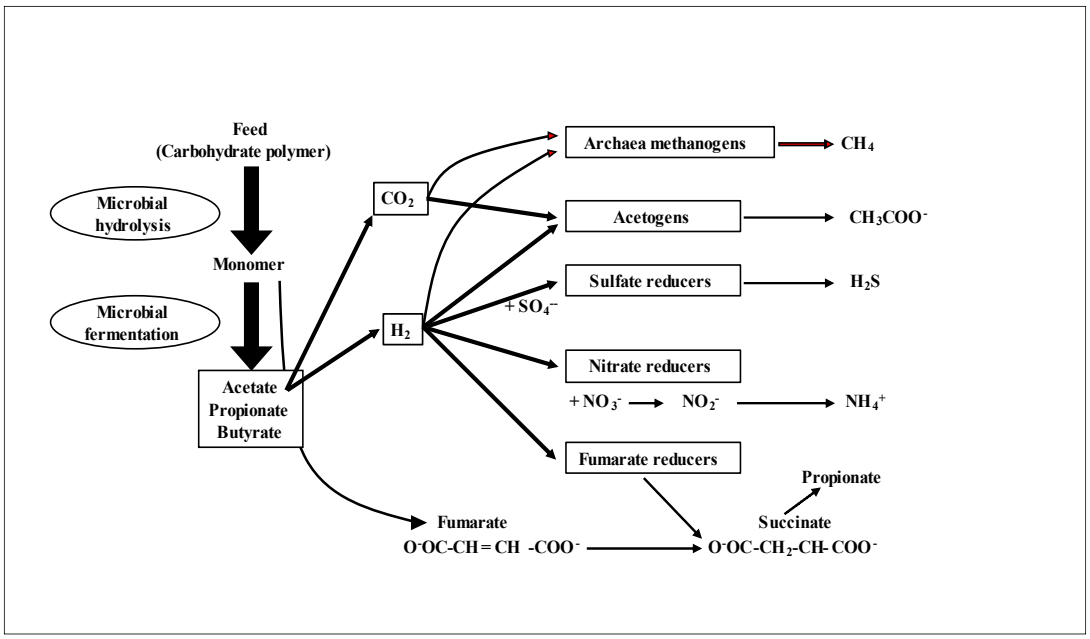

Figure 4. Schematic of microbial fermentation of feed polysaccharides and H_2 reduction pathways in the rumen (Morgavi et al., 2010).

1.3. Enteric methane and methanogenesis

The rumen's anaerobic and methanogenic environment, maintained at near-neutral pH and mesophilic temperatures, fosters optimal conditions for methanogens. These microorganisms have a low threshold for H_2 partial pressure and a rapid doubling time, as short as one hour, ensuring efficient H_2 utilization (Keum et al., 2024).

Methanogenic archaea produce CH_4 by utilizing CO_2 and H_2 through three distinct metabolic pathways: hydrogenotrophic, methylotrophic, and acetoclastic. The methylotrophic pathway uses substrates containing methyl groups, such as methanol, methylamines, and methylsulfides, as precursors for CH_4 synthesis. In the acetoclastic pathway, the carboxyl group of acetate is oxidized to CO_2 while the methyl group is reduced to CH_4 (Glasson et al., 2022).

Among these pathways, the hydrogenotrophic pathway predominates in the rumen, where CO_2 serves as the carbon source and H_2 acts as the primary electron donor. This pathway is responsible for the majority of CH_4 production in ruminants (Martínez-Álvaro et al., 2020). Additionally, formate plays a significant role as an alternative electron donor for many rumen

methanogens and may account for up to 18% of the CH_4 produced in the rumen (Morgavi et al., 2010).

The hydrogenotrophic pathway involves the stepwise reduction of CO_2 through eight enzymatically catalyzed reactions, collectively referred to as the Wolfe cycle (**Figure 5**). Each reaction is mediated by specific enzymes and cofactors, ensuring the precise conversion of intermediates into CH_4 (Glasson et al., 2022).

One of the key steps in this pathway is catalyzed by methyl-tetrahydromethanopterin: coenzyme M methyltransferase (MTR), which transfers a methyl group to coenzyme M (CoM), generating methylated coenzyme M (methyl-CoM). This cobamide-dependent reaction represents the sixth step in ruminal methanogenesis and is conserved across most methanogens (Belanche et al., 2025).

The seventh step in the Wolfe cycle is mediated by MCR, an enzyme containing cofactor F_{430}, a nickel-containing tetrapyrrole. MCR utilizes coenzyme B (CoB) as an electron donor to reduce the methyl group in methyl-CoM to CH_4 (Glasson et al., 2022). Importantly, MCR is a shared enzyme across all three methanogenesis pathways, making it a critical target for inhibitors (Belanche et al., 2025).

Finally, the eighth and final step of the Wolfe cycle is catalyzed by heterodisulfide reductase, which regenerates coenzyme M (CoM-SH) and coenzyme B (CoB-SH). This regeneration ensures the cycle's continuity and maintains methanogenic activity (Glasson et al., 2022).

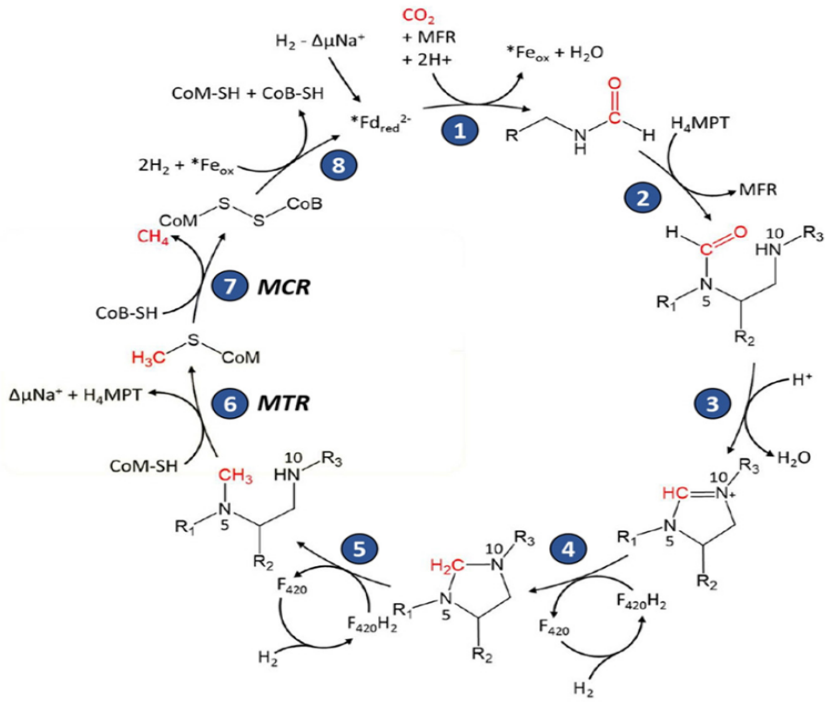

Figure 5. The Wolfe cycle for the reduction of CO_2 to CH_4 in hydrogenotrophic methanogenesis. Steps: (1) CO_2 reacts with methanofuran (MFR) to produce formyl-MFR; (2) The formyl group is transferred to tetrahydromethanopterin (H4MPT); (3–5) Intramolecular imine formation and successive reductions; (6) Methyl transfer from methyl-H4MPT to CoM-SH catalyzed by coenzyme M (CoM) methyl-transferase (cobalamin); (7) Methyl group reduced to CH4 catalyzed by methyl-CoM reductase (cofactor F430); (8) Ferredoxin mediated regeneration of CoM. (Belanche et al., 2025).

Methanogens play a critical role in ruminal fermentation by acting as H_2 sinks, reducing partial H_2 pressure to sustain fermentation processes. Excess H_2, if unutilized, can disrupt microbial metabolism by inhibiting key enzymatic activities involved in electron transfer reactions, such as NADH dehydrogenase, leading to NADH accumulation and reduced fermentation efficiency (McAllister and Newbold, 2008). Methanogens maintain the metabolic balance in the rumen by converting H_2 and CO_2 into CH_4, but this process results in the loss of up to 10% of gross energy intake (Beauchemin et al., 2020).

Methanogenesis interacts with competing metabolic pathways that utilize H_2, such as reductive acetogenesis and sulfate reduction (Morgavi et al., 2010). These alternative pathways act as H_2 sinks, reflecting the metabolic adaptability of the rumen microbiome to redirect H_2 towards other end products, while maintaining fermentation efficiency when methanogenesis is constrained (McAllister and Newbold, 2008).

2. Mitigation strategies

A range of mitigation strategies has been proposed to address CH_4 emissions, focusing on directly inhibiting methanogenesis, redirecting fermentation to other pathways, or optimizing animal efficiency (Hristov et al., 2013; Beauchemin et al., 2022). Management-based approaches include long-term solutions such as selective breeding for animals with lower CH_4 emissions, as well as optimizing animal productivity to reduce CH_4 emission intensity (g CH_4/kg product) and adopting precision feeding technologies to align nutrient supply with animal requirements, minimizing waste and CH_4 production. Despite their potential, these strategies often face challenges related to cost and practical implementation (Terry et al., 2020).

Dietary manipulation is another key approach, as diet is the largest external factor influencing the rumen microbiome. Microorganisms gradually adapt to dietary changes, significantly impacting fermentation pathways (Sejian et al., 2011). Modifications such as altering the forage-to-concentrate ratio, incorporating lipids, improving forage quality, or selecting high-quality feeds can effectively reduce CH_4 emissions per unit of animal product (Knapp et al., 2014; Arndt et al., 2022).

Rumen microbial manipulation also shows promise in CH_4 mitigation by targeting the microbial ecosystem within the rumen. Strategies include vaccination against methanogens (Hook et al., 2010), the use of microbial inoculants such as probiotics and direct-fed microbials to alter fermentation pathways (Sejian et al., 2011), and defaunation, which reduces protozoa populations to decrease H_2 availability for methanogenesis (Hristov et al., 2013).

Non-specific approaches, such as ionophores, selectively inhibit gram-positive bacteria, shifting fermentation pathways toward propionate production (Hristov et al., 2013). Similarly, tannins reduce CH_4 emissions by binding to proteins and carbohydrates, reducing fermentability and indirectly suppressing protozoa and H_2-producing microbes (Patra et al., 2017). In contrast, more recent and specific strategies target methanogenesis directly by inhibiting key enzymes, such as MCR. Feed additives like 3-NOP, *Asparagopsis spp.*, and halogenated CH4 analogs have demonstrated significant potential in this regard, achieving significant CH_4 reductions under experimental conditions (Patra et al., 2017).

While numerous methane mitigation strategies have been proposed, their on-farm implementation remains challenging due to cost, scalability, and practical limitations (e.g.,

defaunation). Various approaches, including yeast-based additives, bacterial-directed microbials, saponins, tannins, and ionophores, have demonstrated variable efficacy in reducing CH_4 emissions (Beauchemin et al., 2020). Among these, tannins exhibit highly inconsistent effects on methane mitigation, with their impact strongly influenced by differences in molecular weight, source, dose, and type (condensed vs. hydrolysable tannins) (Aboagye and Beauchemin, 2019). Additionally, tannins have been reported to reduce H_2 production due to decreased fiber digestion (McGurrin et al., 2023). Nevertheless, while tannins remain a promising natural alternative for methane mitigation, their inconsistent effects and potential trade-offs necessitate further research (Beauchemin et al., 2020).

Ionophores, such as monensin, are feed additives commonly included in commercial feedlot diets to improve energy and nitrogen utilization, while also showing potential for reducing CH_4 emissions during enteric fermentation (Vyas et al., 2018). However, results regarding CH_4 reduction have been inconsistent across studies, highlighting variability in its overall effectiveness (Hristov et al., 2013; Vyas et al., 2018; Terry et al., 2020). Furthermore, ionophores like Monensin are banned in markets such as the European Union due to regulatory concerns (Martin et al., 2010). Other emerging strategies, such as bacteriocins and bacteriophages, remain in the early stages of development (Beauchemin et al., 2020).

This discussion will focus on strategies with the greatest potential for on-farm adoption in the short to medium term, including dietary manipulation and feed additives like 3-NOP, *Asparagopsis spp.*, and halogenated CH_4 analogs, which offer varying levels of effectiveness and feasibility across production systems.

2.1. Dietary manipulation

The efficiency of a particular dietary CH_4 mitigation strategy depends on its effects on ruminal H_2 flow and concentration, the microbial community, fermentation pathways, residence time of feed in the rumen and interactions among these factors (Beauchemin et al., 2020). The physico-chemical properties of ruminant diets have a significant impact in regulating enteric CH_4 production by influencing the factors mentioned above. Variations in feed digestibility and nutrient profiles affect microbial fermentation efficiency, which alters the production of VFAs and the formation of CH_4 (Knapp et al., 2014).

Modifying the physico-chemical properties of feed, through processing methods, such as grinding, pelleting, and steam flaking for concentrates, or chopping, and alkali treatment for forages can indirectly reduce CH_4 production (Knapp et al., 2014). For instance, a study found that feeding steers a steam-flaked corn-based diet reduced CH_4 yield by 17% compared to a dry-rolled corn-based diet (Hales et al., 2012). Reducing feed particle size enhances the surface area for microbial activity, accelerating feed breakdown and shifting fermentation patterns toward increased propionate production. This pathway utilizes H_2 that would otherwise support methanogenesis, consequently reducing CH_4 production (Hook et al., 2010).

However, reduced particle size also increases passage rates, which can reduce CH_4 production but may lead to lower digestibility due to decreased fermentation time in the rumen. This reduction in fermentation time also impacts patterns of VFA formation and microbial growth rates (Beauchemin et al., 2022). Faster passage of feed material through the rumen potentially reduces CH_4 production per unit of feed, depending on whether digestion is partially compensated in the small intestine (sugars and starches) or through fermentation in the hindgut (pectin, glucans, and neutral detergent fiber (NDF)) (Knapp et al., 2014). Increased passage rates also raise microbial energy requirements, as cells must divide more frequently to maintain rumen populations in the rumen (Knapp et al., 2014).

The components of the diet, play a significant role in CH_4 production, as they influence fermentation pathways, ruminal pH and subsequently alter the microbial community (Hook et al., 2010). Forages with high fiber content favor fibrolytic bacteria that produce acetate and more H_2, in contrast, high-concentrate diets promote starch-utilizing bacteria and propionate production, reducing CH_4 emissions (Vijn et al., 2020). Including concentrates in ruminant diets reduces CH_4 emissions per unit of animal product, particularly when concentrates exceed 40% of dietary dry matter (DM) (Hristov et al., 2013). For example, Sauvant and Giger-Reverdin, (2007) observed a curvilinear relationship between CH_4 production and the proportion of concentrate in the diet. Methane losses accounted for 6–7% of gross energy when concentrates constituted 30–40% of the diet but decreased to 2–3% of gross energy as the concentrate proportion increased to 80–90%.

Within the categories of concentrates and forages, feed ingredients vary significantly in fiber content, digestibility, and fermentability rates. Under most conditions, acetate is the predominant VFA produced, consistently present in the highest concentration relative to other

VFAs (McCauley et al., 2020). Differences in fermentability rates influence the molar proportions of VFAs, particularly in grain sources. Absolute CH_4 production and CH_4 yield are lowest with wheat and steam-flaked corn, followed by corn and barley. This ranking is highly dependent on grain composition and the extent of processing (Beauchemin et al., 2022). Within forages, differences in digestibility and fiber content significantly influence CH_4 production. Forages with high fiber content promote acetate fermentation, which increases H_2 production and consequently leads to higher CH_4 emissions (Grainger and Beauchemin, 2011). Methane emissions can be reduced when corn silage replaces grass silage in the diet, as corn silage contains less fiber and more starch, shifting fermentation toward propionate production. Similarly, feeding legume silages such as alfalfa or clover can further lower CH_4 emissions compared to grass silage, due to their lower fiber content, higher digestibility, and faster passage rates (Hristov et al., 2013).

While the use of more rapidly fermentable feed ingredients is an effective strategy for reducing CH_4 production, their rapid fermentation rate, especially cereal concentrates, can cause abrupt shifts in fermentation pathways and disrupt the ruminal microbiome, potentially leading to negative effects on animal health, productivity, and overall system costs (Hristov et al., 2013). This shift lowers ruminal pH and inhibit the growth of protozoa, which are partly responsible for CH_4 production (Janssen, 2010). Furthermore, diets rich in readily fermentable carbohydrates or preformed organic acids can result in the excessive accumulation of VFAs and lactic acid, which may cause ruminal acidosis. This condition compromises the rumen epithelial barrier and disrupts the balance of microorganisms within the gastrointestinal tract, potentially impairing animal health and productivity (McCauley et al., 2020). In dairy cows, feeding highly fermentable grains, such as wheat or oats, has been associated with reductions in milk protein and fat concentrations compared to feeding less fermentable grains like corn or barley (Moate et al., 2018). Additionally, higher concentrate intake raises feed costs and is often impractical or unfeasible in regions where pasture is a more economical and accessible feed source. Feeding ruminants grains that are also suitable for direct human consumption can be considered inefficient, as it undermines the unique ability of ruminants to convert fibrous feeds, which are unsuitable for human diets, into valuable products like milk and meat (Beauchemin et al., 2020).

Lipid supplementation is another dietary approach to mitigating CH_4 production, primarily by altering microbial populations and energy utilization in the rumen (Palangi et al., 2022). This

strategy typically involves the use of calcium salts of fatty acids, hydrogenated fats, animal-derived fats (e.g., tallow, grease), plant-based fats (e.g., soybean, canola, cottonseeds, sunflower seeds), and high-fat by-products such as brewers' grains and cold-pressed canola from food processing plants (Grainger and Beauchemin, 2011). Among fatty acids, lauric and linolenic acid, and other polyunsaturated fatty acids have been identified as more potent in suppressing CH_4 than saturated fatty acids (Patra et al., 2017). Their efficacy results from their role as H_2 acceptors during biohydrogenation, thereby limiting H_2 availability for methanogenesis (Terry et al., 2020).

A study by Patra, (2013) demonstrated that increasing dietary fat concentrations to a maximum of 6% of dietary DM could reduce CH_4 emissions by 15% in cattle, compared to the 2% fat levels typically present in standard diets. Additionally, fat supplementation at this level has been associated with improved milk production. Similarly, Rasmussen and Harrison, (2011) found that linseed oil at 3.3% DM reduced CH_4 emissions by 18% in lactating dairy cows. However, they also noted that higher inclusions of certain fats, such as sunflower oil, led to slight reductions in digestibility.

Despite its benefits, excessive fat inclusion above 6% DM can have unintended consequences. The addition of fats beyond this level has been shown to adversely affect lactation performance, feed intake, digestion, and overall rumen fermentation efficiency. Several factors, including degree of saturation of the added fat, fat inclusion rate, and the composition of the basal diet, influence the extent to which fat supplementation impacts CH_4 emissions and overall animal productivity (McCauley et al., 2020).

While most dietary approaches discussed earlier remain viable strategies for CH_4 mitigation, their application must be individually tailored to specific dietary conditions to ensure cost-effectiveness and maintain productivity with minimal negative impacts. Dietary intervention strategies must be carefully managed to optimize feed conversion efficiency, sustain animal productivity (both in terms of meat and milk yield), and preserve overall performance (Choudhury et al., 2015).

2.2. Feed additives

Compared to dietary management strategies, which rely on altering the composition or physical properties of the diet, feed additives offer a more direct approach to mitigating CH_4 emissions in ruminants. These additives target the microbial processes responsible for CH_4 production, either by directly inhibiting the growth and activity of methanogens or by redirecting metabolic pathways. Halogenated methane analogs, 3-NOP, and *Asparagopsis spp.* have been extensively studied for their potent antimethanogenic properties (Patra et al., 2017; Martinez-Fernandez et al., 2018; Roque et al., 2019a). The following sections will explore the mechanisms, efficacy, and challenges associated with each of these feed additives in detail.

2.2.1. Halogenated methane analogs

The anti-methanogenic effects of halogenated compounds have been widely explored, with substantial evidence supporting their efficacy in reducing CH_4 production. These compounds can be broadly classified into halogenated sulfonated compounds, halogenated aliphatic compounds, and other halogenated derivatives (Patra et al., 2017).

Several halogenated sulfonated compounds, including 2-bromoethanesulfonate, 2-chloroethanesulfonate, and 3-bromopropanesulfonate, are structural analogs of methyl-CoM. They competitively and specifically inhibit the activity of MCR, thereby lowering CH_4 production at relatively low concentrations (Patra et al., 2017). However, detailed experimental data on their in vitro or in vivo effects in ruminants remain limited.

Unlike halogenated sulfonated compounds, halogenated aliphatic compounds have undergone more extensive testing for their ability to reduce ruminal CH_4 production. Halogenated hydrocarbons are organic compounds composed of carbon and hydrogen atoms, with one or more halogen elements (fluorine, chlorine, bromine, or iodine) covalently bonded to the carbon backbone. Due to their high redox potential and structural similarity to intermediates in methanogenesis, these compounds inhibit CH_4 production by interfering with enzyme activity, cofactor availability, and electron flow (Lileikis et al., 2023).

Notable halogenated aliphatic compounds include chloroform, bromochloromethane (BCM), and $CHBr_3$. Other examples include bromodichloromethane, dibromochloromethane, carbon

tetrachloride, and various derivatives such as trichloroacetamide, trichloroethyl adipate, and trichloropivalate (Yu and Smith, 2000; Denman et al., 2007; Knight et al., 2011).

One key mechanism of inhibition involves the direct binding of halogenated hydrocarbons to essential cofactors, such as corrinoid and porphinoid enzymes, in methanogenic archaea. These enzymes, containing metal ions such as cobalt or nickel, play a fundamental role in methyl group transfer during methanogenesis. By binding to these enzymes, halogenated hydrocarbons disrupt cobamide-dependent methyl group transfer, a critical step in CH_4 production (**Figure 5**, step vi) (Lileikis et al., 2023). Additionally, halogenated hydrocarbons act as competitive terminal electron acceptors, diverting electrons away from methanogenesis pathways and further reducing CH_4 formation (Yu and Smith, 2000).

Among halogenated aliphatic compounds, BCM and chloroform are the most extensively documented for their ability to suppress CH_4 production in ruminants. In fact, BCM has been widely tested and is considered one of the most effective inhibitors of methanogenesis. reported that BCM administration in goats resulted in an 80% reduction in CH_4 emissions, with a corresponding increase in H_2 expelled, but without negatively affecting dry matter intake (DMI) or feed digestibility. In another study, a formulated BCM treatment reduced CH_4 production by up to 60% in steers fed grain-based diets over a 90-day feedlot period (Tomkins et al., 2009).

Similar to BCM, chloroform has been shown to significantly reduce CH_4 emissions without adversely affecting rumen fermentation. Studies indicate that chloroform reduces CH_4 production in dairy cows and in vitro systems to a similar extent as BCM (Knight et al., 2011). Knight et al., (2011) observed that in non-lactating dairy cattle, CH_4 production was initially strongly reduced within 4–5 days of chloroform administration but gradually recovered to 62% of baseline levels by the end of the trial.

Despite their strong anti-methanogenic effects, BCM and chloroform present several challenges that limit their long-term application. One major concern is microbial adaptation, as studies have shown that CH_4 production can gradually recover over time, thereby reducing the sustained efficacy of the inhibitors (McAllister and Newbold, 2008; Knight et al., 2011). Additionally, toxicity risks are associated with chloroform, which is known to be hepatotoxic and carcinogenic at high doses. However, low-dose short-term studies in ruminants have not

reported significant adverse effects (Knight et al., 2011). Furthermore, BCM is banned for commercial use due to environmental concerns, particularly its ozone-depleting properties, restricting its use to research settings (Knight et al., 2011).

2.2.2. 3-nitrooxypropanol (3-NOP)

3-Nitrooxypropanol is a potent and selective CH_4 inhibitor that has gained significant attention as a feed additive for reducing enteric CH_4 emissions in ruminants (Duin et al., 2016). The molecular structure of 3-NOP resembles methyl-CoM, enabling it to specifically target MCR. More specifically, 3-NOP inactivates MCR by oxidizing the Ni^{1+} atom in the F_{430} cofactor to Ni^{2+}, thereby preventing CH_4 formation (Duin et al., 2016).

This targeted mechanism allows 3-NOP to effectively suppress methanogenesis without significantly disrupting other microbial populations or fermentation processes in the rumen (Pitta et al., 2022). Van Lingen et al., (2021) reported that 3-NOP significantly reduced the methanogenic archaeal population, particularly hydrogenotrophic methanogens like *Methanobrevibacter spp.*, the primary CH_4 producers in the rumen. Methylotrophic methanogens like *Methanosphaera* appear less affected by 3-NOP, while having no major impact on the overall bacterial community (Pitta et al., 2022). Methanogenesis inhibition leads to H_2 accumulation, but this effect is transient as alternative electron sinks, such as propionate and butyrate production, become more active, reducing the acetate-to-propionate ratio (Xuan et al., 2024).

Several in vitro studies have demonstrated the dose-dependent effects of 3-NOP on CH_4 reduction. In a rumen simulation study, Romero-Pérez et al., (2015) reported that increasing 3-NOP concentrations (0, 5, 10, and 20 mg/day) led to a significant CH_4 reduction of up to 86.2% at the highest dose without adversely affecting VFA concentrations. Similarly, Schilde et al., (2021) observed progressive CH_4 inhibition of up to 97% in a Rusitec system supplemented with 3-NOP at 73, 160, and 1200 mg/kg DM, accompanied by increased H_2 accumulation and a shift in fermentation from acetate toward propionate production. Xuan et al., (2024) further confirmed the dose-dependent inhibition of CH_4 production, with reductions ranging from 26% to 76%, particularly in lambs, where optimal inhibition was observed at 0.05 mg/g DM.

As for in vivo studies, Vyas et al., (2018) reported significant CH_4 reductions in beef cattle receiving 100 mg/kg DM or more, with reductions of 26–45% in high-grain diets and 16–23%

in high-forage diets. Dijkstra et al., (2018) confirmed this trend through a meta-analysis, showing that every 10 mg/kg DM increase in 3-NOP dose resulted in a CH_4 reduction of 2.56% in beef cattle and 2.48% in dairy cattle, while higher dietary fiber content weakened the effect. Pitta et al., (2022) demonstrated that long-term supplementation with 3-NOP reduced CH_4 emissions by 23–37% in dairy cattle and 27–57% in beef cattle, with no significant negative effects on DMI or animal productivity. Similarly, in a study on sheep, Martínez-Fernández et al., (2014) found that 3-NOP supplementation at 100 mg/day reduced CH_4 emissions by 21–25% after 30 days, with no adverse effects on DMI or overall rumen fermentation.

Although 3-NOP has demonstrated strong efficacy in reducing CH_4 emissions, its inhibitory effects vary among ruminant species (Duin et al., 2016). This variability suggests that structural differences in MCR influence the sensitivity of different methanogen populations to 3-NOP, leading to species-specific differences in CH_4 reduction (Pitta et al., 2022). Additionally, the inhibition of methanogenesis by 3-NOP is not permanent, as its effects are short-lived and fully reversible upon cessation of supplementation. This highlights the necessity for continuous dietary inclusion to sustain CH_4 reduction over time (Van Lingen et al., 2021).

3. Red seaweed

Marine algae, commonly known as seaweed, are non-flowering, photosynthetic macrophytes in their early developmental stages. Seaweed is typically classified based on pigmentation into three main taxonomic groups: green algae (*Chlorophyta*), red algae (*Rhodophyta*), and brown algae (*Phaeophyta*) (Wanapat et al., 2024). Among these, red seaweed, particularly *Asparagopsis spp.*, has gained increasing attention due to its ability to reduce enteric CH_4 emissions in ruminants.

The distribution of *Asparagopsis spp.* is strongly influenced by environmental factors such as temperature, salinity, and human-mediated transport. Their floating reproductive structures allow them to spread rapidly, facilitating their presence in non-native regions (Schaffelke et al., 2006). *Asparagopsis taxiformis* is native to tropical and warm-temperate regions, primarily found in the Atlantic and Indo-Pacific Oceans. It has also been introduced to the Mediterranean Sea, with the earliest record near Alexandria, Egypt. This species thrives in warm waters, with a lower survival limit of 10–13°C and an optimal growth range of 11–15°C (Andreakis et al., 2004). *Asparagopsis armata*, originally from Australia and New Zealand, has expanded into the Atlantic and Mediterranean. Unlike *A. taxiformis*, *A. armata* prefers more temperate waters

and is more tolerant of colder conditions. It is predominantly found in the western Mediterranean, thriving in areas where summer seawater temperatures do not exceed 25°C (Andreakis et al., 2004).

3.1. Biochemical properties of *Asparagopsis spp.*

Macroalgae have long been recognized for their applications in the nutraceutical and health markets due to their antibacterial, antiviral, antioxidant, and anti-inflammatory properties (O'Sullivan et al., 2010). Recently, interest has grown in their application to livestock nutrition, particularly *Asparagopsis spp.* for their potential to mitigate CH_4 emissions, as *A. taxiformis* and *A. armata* contain bioactive compounds that naturally disrupt methanogenesis, making them a promising solution for reducing CH_4 emissions in ruminants (Glasson et al., 2022).

In fact, *Asparagopsis spp.* produces over a hundred low molecular weight natural products, including halogenated compounds such as halomethanes, haloalkanes, haloketones, and haloacids (Machado et al., 2016). Some of these compounds predominantly stored in specialized gland cells function as a natural defense mechanism against disease and marine herbivory (Paul et al., 2006a). The most abundant and functionally significant of these compounds is $CHBr_3$, followed by dibromochloromethane, bromochloroacetic acid, and dibromoacetic acid (Paul et al., 2006b).

However, the halogenated compound profile of *Asparagopsis spp.* is influenced by several factors, including the site of collection and seasonal variations if harvested from natural environments (Kinley et al., 2016a), as well as growing conditions (Paul et al., 2014). Additionally, post-harvest processing and storage methods play a crucial role in preserving bioactive compounds (Paul et al., 2006a).

For instance, Stefenoni et al., (2021) reported that drying procedures significantly affect bioactive compound concentrations, with freeze-drying being the most effective. However, the methanogenic activity of *Asparagopsis spp.* declines over four months when incorporated into total mixed rations.

The effectiveness of CH_4 reduction appears to be closely linked to $CHBr_3$ concentration. However, other yet unidentified bioactive compounds may also contribute to its

antimethanogenic properties (Vijn et al., 2020). Bromoform levels in seaweeds could serve as a reliable indicator of their potential to reduce CH_4 emissions and could be used to standardize seaweed inclusion in livestock feed. For example, Min et al., (2021) established a polynomial correlation between $CHBr_3$ concentration and CH_4 emissions (in vitro), demonstrating that CH_4 production declines linearly when $CHBr_3$ exceeds 0.25 mg/g organic matter (OM), reaching near-zero emissions at ~0.8 mg/g.

The antimethanogenic mode of action of $CHBr_3$ closely resembles that of its synthetic analog BCM. In the Wolfe cycle (**Figure 5**), steps vi and vii are catalyzed by MTR and MCR respectively, which are particularly susceptible to competitive and oxidative inhibition (Glasson et al., 2022). The sixth step of the Wolfe cycle, catalyzed by a cobamide-dependent methyltransferase, requires vitamin B_{12} (cobalamin) as a cofactor, linking the inhibition process to cobalamin metabolism (Wood et al., 1968). It has been proposed that halogenated compounds, including $CHBr_3$, bind to B_{12}, thereby disrupting the cobamide-dependent methyltransferase reaction, which prevents methyl transfer from methyl-CoM to coenzyme B (CoB). This inhibition ultimately blocks the activity of MCR in step vii, preventing CH_4 formation (Machado et al., 2016).

3.2. Effect of *Asparagopsis spp.* on methane emissions in vitro

In vitro studies have extensively evaluated the CH_4-reducing potential of *Asparagopsis spp.*, although most research has focused on *A. taxiformis*, with limited data available for *A. armata*. While both species exhibit antimethanogenic properties in vitro, their efficacy varies depending on inclusion rate, bioactive compound concentration, substrate, and rumen fermentation conditions.

Nunes et al., (2024) reported that at a 5% OM inclusion rate, *A. armata* achieved a 34% reduction in CH_4 emissions, whereas *A. taxiformis* reduced CH_4 by 86% at the same dose. However, the study did not report $CHBr_3$ concentrations, making it difficult to determine whether the differences were directly related to $CHBr_3$ content or other factors influencing the reduction response. Nevertheless, Machado et al., (2016) reported that *A. taxiformis* completely inhibited CH_4 production at 2% OM inclusion, demonstrating a strong dose-response effect and a positive correlation with $CHBr_3$ concentration.

Other in vitro studies have also demonstrated significant CH_4 reduction effects. Chagas et al., (2019) reported a 99.5% reduction in CH_4 production after 48 hours when supplementing *A.*

taxiformis at 1% OM in a forage-based diet using rumen inoculum from dairy cattle. Similarly, Andreen et al., (2023) observed near-total CH_4 suppression at 48 hours under comparable conditions, using a slightly lower inclusion rate of 0.8% OM. Both studies utilized *A. taxiformis* with similar $CHBr_3$ contents of 0.68% and 0.71% dry weight (DW), reinforcing the relationship between $CHBr_3$ concentration and CH_4 reduction efficacy. In contrast, Kinley et al., (2016a) reported no significant CH_4 reductions at 1% OM inclusion when using *A. taxiformis* with a $CHBr_3$ content of only 0.17% DW in beef cattle fed a forage-based diet. This discrepancy further supports the notion that CH_4 mitigation is strongly correlated with $CHBr_3$ concentration in *A. taxiformis*.

Although classified into two broad categories, the diets in the in vitro studies from **Table 2** varied widely. Despite this, *Asparagopsis spp.* consistently demonstrated antimethanogenic effects across different substrates, albeit with varying efficacy. While no direct comparative study has quantified these differences statistically, *A. taxiformi*s achieved 99% and 100% CH_4 reduction at 2% and 5% OM inclusion, respectively, when fermented with a Rhodes grass hay substrate (Machado et al., 2016). Similarly, Roque et al., (2019a) reported a 95% reduction at 5% OM inclusion in a total mixed ration system for dairy cattle.

Beyond suppressing CH_4, *Asparagopsis spp.* alters ruminal fermentation dynamics in vitro by modulating total gas production (TGP), VFA proportions, and OM degradability (OMdeg). Total gas production consists primarily of H_2, CO_2, and CH_4, the main byproducts of rumen fermentation (Ungerfeld, 2015). Reductions in TGP have been observed across various studies. Brooke et al., (2020) reported that supplementing 5% DM of *A. taxiformis* resulted in a 28% reduction in TGP over 24 hours, accompanied by a 65% reduction in CH_4. Similarly, Roque et al., (2019a) observed a 95% reduction in CH_4 with a 51.8% decrease in TGP at 5% OM inclusion, though without significant negative effects on VFA profiles.

Moreover, these reductions appear to be dose-dependent. Andreen et al., (2023) observed a linear decrease in TGP with incremental additions of *A. taxiformis* (0.5%, 1.0%, and 1.5% DM), indicating a direct relationship between dosage and gas production. Similarly, (Kinley et al., 2016a) reported that while 1% OM inclusion of *A. taxiformis* reduced CH_4 with minimal impact on TGP, increasing the dosage to 2% OM led to sharp reductions in both TGP and fiber digestibility, indicating compromised fermentation efficiency. While data on *A. armata* are limited, its fermentation effects are expected to follow a similar trend to *A. taxiformis*, although outcomes could vary depending on its $CHBr_3$ content (Nunes et al., 2024).

Table 2. Effects of *A. taxiformis* and *A. armata* supplementation on methane production, total gas production, total volatile fatty acids, and organic matter degradability across different in vitro studies.

Algae		Dosage				CH4			TGP					
Strain	[CHBr3] %DW	%OM	%DM	Substrate	Rumen fluid	24h	48h	72h	24h	48h	72h	TVFA	OMdeg	Author
A. taxiformis	0.23	3	5	TMR	Dairy Cattle	ns	↓74%	-	ns	ns	-	-	-	Brooke et al., 2020
A. taxiformis	0.17	0.5	-	Forage-Based	Beef Cattle	ns	ns	ns	ns	ns	-	ns	ns	Kinley et al., 2016a
		1				ns	ns	ns	ns	ns	-	ns	ns	
		2				↓100%	↓100%	↓100%	↓30%	↓30%	↓30%	ns	ns	
		5				↓100%	↓100%	↓100%	↓	↓	↓	↓	ns	
		10				↓100%	↓100%	↓100%	↓	↓	↓	↓	↓	
A. taxiformis	-	0.07	-	Forage-Based	Beef Cattle	ns	ns	ns	ns	ns	ns	ns	ns	Machado et al., 2016
		0.125				ns	ns	ns	ns	ns	ns	ns	ns	
		0.25				ns	ns	ns	ns	ns	ns	ns	ns	
		0.5				ns	ns	ns	ns	ns	ns	ns	ns	
		1				↓	↓	↓84.7%	↓	↓	↓31.5%	ns	ns	
		2				↓	↓	↓99%	↓	↓	↓35.8%	↓25%	ns	
		5				↓	↓	↓100%	↓	↓	↓37.7%	↓40%	ns	
		10				↓	↓	↓100%	↓	↓	↓41.7%	↓47%	↓14%	
		16.7				↓	↓	↓100%	↓	↓	↓46.5%	↓55%	↓24%	
A. taxiformis	-	5	-	TMR	Dairy Cattle		↓95%			↓51.8%		ns	ns	Roque et al., 2019a
A. taxiformis	1.0	0.5	1	TMR	Dairy Cattle	↓98%	-	-	↓12.7%	-	-	-	-	Stefenoni et al., 2021
A. taxiformis	0.71	0.3	0.5	Forage-Based	Dairy Cattle	↓100%	↓100%	↓100%	↓	↓	↓	↓24.7%	ns	Andreen et al., 2023
		0.6	1			↓100%	↓100%	↓100%	↓	↓	↓	↓26.5%	ns	
		0.8	1.5			↓100%	↓100%	↓100%	↓	↓	↓	↓29%	ns	
A. taxiformis	0.68	1	-	Forage-Based	Dairy Cattle	↓	↓99.5%	-	↓	↓	-	ns	ns	Chagas et al., 2019
		2				↓	↓99.5%	-	↓	↓	-	ns	ns	

Table 2. (Continued)

Algae		Dosage				CH4			TGP					
Strain	[CHBr3] %DW	%OM	%DM	Substrate	Rumen fluid	24h	48h	72h	24h	48h	72h	TVFA	OMdeg	Author
A. taxiformis	-	2	-	Forage-Based	Beef Cattle	↓	↓99%	↓99%	↓	↓26%	↓29%	↓22%	ns	Kinley et al., 2019
A. taxiformis	-	1.7	1.25	Forage-Based	Dairy Cattle	↓38.7%	↓31.4%	↓28.6%	↓13.5%	↓12.8%	↓6%	-	-	Nunes et al., 2024
		3.5	2.5			↓51.8%	↓47.9%	↓43.5%	↓30%	↓26%	↓14.4%	-	-	
		6.9	5			↓86%	↓77.3%	↓74.2%	↓34.6%	↓34%	↓21.8%	-	-	
		13.8	10			↓76%	↓72.2%	↓68.3%	↓46.5%	↓39.4%	↓29.4%	-	-	
A. armata	-	1.7	1.25			ns	ns	ns	ns	ns	ns	-	-	
		3.5	2.5			↓17.28%	ns	ns	↓22.7%	ns	ns	-	-	
		6.9	5			↓34%	↓20.5%	↓17.3%	↓25.9%	ns	ns	-	-	
		13.8	10			↓40.65%	↓31%	↓24.6%	↓40%	↓18.6%	↓12.3%	-	-	

This table summarizes in vitro studies evaluating the effects of *A. taxiformis* and *A. armata* on methane (CH_4) production, total gas production (TGP), total volatile fatty acids (TVFA), and in vitro organic matter degradability (OMdeg). Dosages are presented as a percentage of organic matter (%OM) and dry matter (%DM). The studies used different substrates and rumen fluid sources (dairy and beef cattle). Methane and TGP reduction percentages are reported at 24h, 48h, and 72h, while TVFA and OMdeg were assessed at different incubation times. "ns" indicates no significant effect, "↓" represents a significant reduction, and "-" denotes that the parameter was not measured.

Reductions in TVFA production and OM degradability following *Asparagopsis* supplementation have been also observed to be affected in a dose-dependent manner across multiple in vitro studies. Andreen et al., (2023) reported a linear decrease in TVFA with increasing doses of *A. taxiformis* (0.5%, 1.0%, and 1.5% DM), with reductions of 24.7%, 26.5%, and 29%, respectively. Similarly, Machado et al., (2016) observed a dose-dependent decline in TVFA with increasing *A. taxiformis* inclusion, reporting reductions of 25%, 40%, 47%, and 55% at 2%, 5%, 10%, and 16.7% OM, respectively, indicating progressive suppression of rumen fermentation at higher doses.

Moreover, OMdeg was reduced by 14% and 24% at 10% and 16.7% OM inclusion levels. In addition to TVFA reductions, shifts in VFA proportions were reported. Kinley et al., (2016a) observed a decrease in the acetate-to-propionate ratio at 2% OM inclusion of *A. taxiformis*, indicating a shift towards a more H_2-efficient fermentation pathway. Similarly, Chagas et al., (2019) noted a reduction in acetate and an increase in propionate, consistent with redirected H_2 utilization due to methanogenesis inhibition.

Although the antimethanogenic effect of *Asparagopsis* is well-documented in vitro, long-term studies are essential to confirm its persistence and to determine whether rumen microbial adaptation or metabolic adjustments reduce its efficacy over time (Hristov et al., 2025).

3.3. Effect of *Asparagopsis spp.* on methane emissions in vivo

In vivo studies have reported reductions in CH_4 production following *Asparagopsis* supplementation. Roque et al., (2019b) investigated the effects of *A. armata* in dairy cattle and reported dose-dependent reductions in CH_4 production (g/d) of 26.4% and 67.2% at 0.5% and 1% OM inclusion rates, respectively. Similarly, Colin et al., (2024) reported reductions of 46% and 73% at inclusions of 0.8% and 1.3% OM, respectively. However, TGP is not measured in in vivo studies, although individual results for H_2 and CO_2 were reported.

An increase in H_2 production as a consequence of CH_4 reduction can be clearly observed in **Table 3**. Roque et al., (2019b) observed increases in H_2 emissions of 163% and 236% at 0.5% and 1% OM inclusion rates, respectively. Similarly, Stefenoni et al., (2021) reported H_2 production increases of 134% and 525% at 0.27% and 0.54% OM inclusion of *A. taxiformis*. However, no clear results indicated the extent to which H_2 was redirected to alternative fermentation pathways versus being expelled by eructation. In parallel, variations in CO_2

production were also observed. Roque et al., (2019b) reported a 13.9% reduction in CO_2 emissions with *A. armata* at 1% OM. In another study, Roque et al., (2021) observed no significant increases in CO_2 production (g/d) at 0.5% OM inclusion of *A. taxiformis*. However, when corrected for dry matter intake (DMI), significant increases in CO_2 emissions were reported, ranging from 9.73% to 15.4%.

The impact of *Asparagopsis* supplementation on organic matter digestibility was variable across studies. Colin et al., (2024) reported no significant changes in OM digestibility with *A. taxiformis* supplementation at either 0.8% or 1.3% OM inclusion. In contrast, Stefenoni et al., (2021) observed a 3.5% reduction at 0.54% OM inclusion, despite significant CH_4 reductions.

The supplementation of *Asparagopsis* was also reported to affect productive parameters such as DMI, feed conversion efficiency (FCE), body weight gain (BWG), and milk yield (MY). A consistent reduction in DMI was observed across multiple studies. Roque et al., (2019b) found that *A. armata* supplementation reduced DMI by 10.8% and 38% at 0.5% and 1% OM inclusion rates, respectively. Similarly, Colin et al., (2024) reported DMI reductions of 10.1% and 13.3% at 0.8% and 1.3% OM inclusion of *A. taxiformis*, respectively. Stefenoni et al., (2021) observed a 7.11% decrease at 0.54% OM inclusion.

The effect of *Asparagopsis* on FCE varied across studies Roque et al., (2019b) reported a 20.15% and 73.64% increase in FCE with *A. armata* at 0.5% and 1% OM inclusion, respectively, despite reductions in DMI. Similarly, Roque et al., (2021) found FCE increases of 7% to 15% with *A. taxiformis* at 0.5% OM across different forage diets. However, other studies observed no significant effect in FCE responses (Kinley et al., 2020; Stefenoni et al., 2021).

Regarding BWG, Kinley et al., (2020) observed a 22% and 26% increase in BWG at 0.1% and 0.2% OM of *A. taxiformis*, despite no significant increases in feed efficiency. However, Roque et al., (2019) reported contrasting results with *A. armata*, where BWG increased by 5.48% at 0.5% OM but decreased by 31.29% at 1% OM, indicating a dose-potential trade-off between CH_4 reduction and animal performance.

Additionally, Roque et al., (2021) observed no significant change in BWG across diets supplemented with 0.5% OM of *A. taxiformis*. As for milk production, Roque et al., (2019b) reported a slight increase of 2.76% in milk yield with *A. armata* at 0.5% OM inclusion; however, at 1% OM, milk yield decreased by 11.6%, coinciding with a 38% reduction in DMI （Roque et al., 2019). Similarly, Stefenoni et al. (2021) observed a 6.47% decrease in milk yield

Table 3. Effects of *A. taxiformis* and *A. armata* supplementation on methane reduction, gas emissions, digestibility, and animal performance in different in vivo studies.

Algae		Dosage									CH4			H2		CO2			
Strain	[CHBr3] %DW	%OM	%DM	Substrate	Rumen fluid	DMI	BWG	FCE	MY	g/d	g/kg DMI	g/kg ADG	g/d	g/kg DMI	g/kg ADG	g/d	g/kg DMI	OMdig	Author
A. taxiformis	-	0.8	0.77	TMR	Dairy Cattle	↓10.1%	-	-	-	↓46%	↓39%	-	-	-	-	↓6.94%	↓2.06%	ns	Colin et al. 2024
		1.3	1.2			↓13.3%	-	-	-	↓73%	↓64%	-	-	-	-	↓8.32%	↓2.51%	ns	
A. armata		0.5	-	TMR	Dairy Cattle	↓10.8%	↑5.48%	↑20.15%	↑2.76%	↓26.4%	↓20.3%	↓18.2%	↑163%	↑55%	↑33.3%	-	↑12.8%	-	Roque et al. 2019b
		1	-			↓38%	↓31.29%	↑73.64%	↓11.6%	↓67.2%	↓42.7%	↓60.1%	↑236%	↑78.9%	↑61.7%	↓13.9%	↑36.5%	-	
A. taxiformis	0.655	0.05	-	TMR	Beef Cattle	↓10.8%	ns	ns	-	-	ns	-	-	ns	-	-	-	-	Kinley et al. 2020
		0.1	-			↑7.5%	↑26%	ns	-	-	↓38%	-	-	↑380%	-	-	-	-	
		0.2	-			ns	↑22%	ns	-	-	↓98%	-	-	↑1700%	-	-	-	-	
A. taxiformis	0.78	0.25	-	Low Forage	Beef Cattle	ns	ns	ns	-	↓72.4%	↓69.8%	↓67.5%	↑419%	↑503%	↑566%	ns	ns	-	Roque et al. 2021
				Mid Forage		ns	ns	ns	-	↓51.8%	↓44.6%	↓54.4%	↑326%	↑404%	↑341%	ns	ns	-	
				High Forage		ns	ns	ns	-	↓36.4%	↓32.7%	↓36.9%	↑177%	↑198%	↑256%	ns	ns	-	
		0.5	-	Low Forage		↓6.95%	ns	↑	-	↓81.9%	↓80%	↓82.6%	↑618%	↑649%	↑559%	ns	↑9.73%	-	
				Mid Forage		↓18.11%	ns	↑	-	↓86.8%	↓79.7%	↓82.4%	↑535%	↑753%	↑626%	ns	↑16.1%	-	
				High Forage		↓18.44%	ns	↑	-	↓58.7%	↓51.9%	↓56.4%	↑360%	↑478%	↑524%	ns	↑15.4%	-	
A. taxiformis	-	0.27	0.25	TMR	Dairy Cattle	ns	ns	ns	ns	ns	ns	-	↑134 %	-	-	ns	-	↓3.5%	Stefenoni et al., 2021
		0.54	0.5			↓7.11%	ns	ns	↓6.47%	↓34.4%	↓29.4%	-	↑527 %	-	-	ns	-	ns	

This table summarizes in vivo studies evaluating the effects of *A. taxiformis* and *A. armata* on dry matter intake (DMI), body weight gain (BWG), feed conversion efficiency (FCE), milk yield (MY), CH_4 production (g/day), CH_4 yield (g/kg DMI), CH_4 intensity (g/kg ADG), H_2 production (g/day), H_2 yield (g/kg DMI), H_2 intensity (g/kg ADG), CO_2 production (g/day), CO_2 yield (g/kg DMI), and organic matter digestibility (OMdig). Dosages are presented as a percentage of organic matter (%OM) and dry matter (%DM). The studies utilized different substrates and rumen fluid sources (dairy and beef cattle). "ns" indicates no significant effect, "↓" represents a significant reduction, and "↑" denotes an increase in the measured parameter.

at 0.54% OM inclusion of *A. taxiformis* along with a decreased DM.

4. Potential tradeoffs

While *Asparagopsis* supplementation offers significant potential for CH_4 mitigation, several trade-offs must be considered, particularly regarding food safety, environmental impact, additive stability, and long-term efficacy.

As discussed previously, *Asparagopsis* supplementation has been associated with reductions in DMI and, at higher inclusion rates, declines in BWG and milk yield (Roque et al., 2019b; Stefenoni et al., 2021). Appropriate inclusion rates should be insured to reach significant reductions without adverse effects. Moreover, the persistence of the antimethanogenic effect from *Asparagopsis* may diminish over time due to rumen microbial adaptation. According to the newly reviewed article, long-term studies are essential, as many antimethanogenic feed additives lose efficacy when the rumen microbiome adjusts to their bioactive compounds. Similar concerns apply to *Asparagopsis*, where reduced potency from $CHBr_3$ degradation or microbial adaptation could compromise long-term CH_4 reductions (Hristov et al., 2025).

A critical limitation of *Asparagopsis*-based feed additives is the instability of $CHBr_3$ during storage. Stefenoni et al., (2021) reported an 84% reduction in $CHBr_3$ concentration in *A. taxiformis* after four months of storage, significantly diminishing its antimethanogenic efficacy. While similar data for *A. armata* are unavailable, it is likely subject to comparable degradation, raising concerns about its shelf life and long-term effectiveness in feed formulations.

Large-scale *Asparagopsis* farming could raise environmental concerns due to $CHBr_3$ emissions. However, Jia et al., (2022) estimated that annual *Asparagopsis* production of approximately 34,674 metric tons of dry weight in Australia would contribute ~27 metric tons of $CHBr_3$ per year, representing less than 0.9% of total coastal emissions and only 0.01% of global $CHBr_3$ emissions. Additionally, $CHBr_3$ degrades rapidly in the atmosphere, with a worst-case scenario increase in ozone depletion estimated at only 0.48%. Not to mention that *A. armata* can be found in the west mediterenean region (Andreakis et al., 2004), therefore taking advantage of its wild production for feed additive production could serve as a control method for its invasive growth.

Bromoform is classified as a potential carcinogen (Glasson et al., 2022). This raises concerns regarding its presence in animal products. Therefore, any detectable residues in milk or meat from cattle supplemented with *Asparagopsis* could pose a human food safety risk (Vijn et al., 2020). Consequently, stringent monitoring and regulatory compliance are essential to ensure that *Asparagopsis*-based additives meet feed safety standards (Glasson et al., 2022).

OBJECTIVES

OBJECTIVES

The primary aim of this study was to evaluate the potential of *A. armata* as a feed additive for ruminants to reduce CH_4 emissions during in vitro ruminal fermentation. Specific objectives included:

1. Determining whether the inclusion of *A. armata* reduces CH_4 production during ruminal fermentation.

2. Assessing its effects on ruminal fermentation parameters, such as VFA production, ammonia concentrations, and diet digestibility.

3. Identifying the optimal dose-response relationship to achieve significant CH_4 reduction without adversely affecting fermentation efficiency.

Hypothesis: Supplementing ruminant diets with less than 1% *A. armata* may significantly reduce methane production without compromising volatile fatty acid production or diet digestibility.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Red seaweed

Asparagopsis armata was supplied by Algabrava, S.L. (Tarragona, Spain). The seaweed was harvested during its gametophyte phase, freeze-dried, ground to a fine powder (1 mm² particle size) and stored. The bromoform concentration in the seaweed ranged from 5.3 to 10.5 mg/g of dry weight (DW). Four increasing doses of *A. armata* (Control: 0%, 0.30%, 0.60%, 1.2%, and 2.4% of organic matter) were selected based on concentrations tested in similar in vitro experiments with *Asparagopsis spp.*, which demonstrated significant response within a comparable concentration range (Kinley et al., 2016a; b; Machado et al., 2016; Chagas et al., 2019). *Asparagopsis armata* was added in such small doses in all treatment levels that no replacement of dietary ingredients was necessary.

2. Experimental design and diets

Two independent experiments were conducted, each repeated twice, with ruminal fluid sourced separately for each experiment.

The first experiment used rumen fluid (RF) collected from a dry, cannulated Holstein dairy cow (600 ± 5 kg) housed at the Autonomous University of Barcelona. The cow was adapted for two weeks to the diet later used as the substrate in the in vitro experiment. Feeding occurred once daily at approximately 0800 h, with free access to drinking water. The second experiment used RF collected from Angus × Friesian steers (536 ± 16 kg) at a slaughterhouse. These steers were adapted for two weeks to the finishing diet used as the substrate in the experiment.

The two diets used for adaptation and experimentation were representative of typical Spanish production systems. The first diet, reflecting a dairy-focused system, had a high forage-to-concentrate ratio (60% alfalfa: 40% concentrate, F) and consisted of alfalfa and a commercial dry cow concentrate. The second diet, representing a beef-focused system, had a low forage-to-concentrate ratio (90% concentrate: 10% barley straw, C) and included a commercial fattening concentrate and barley straw.

Composite samples of both diets were analyzed using wet chemistry at the Faculty of Veterinary Medicine of the Autonomous University of Barcelona. Parameters analyzed

included DM, OM, crude protein (CP), NDF, and acid detergent fiber (ADF). The chemical composition of the diets is summarized in **Table 4**.

Table 4. Chemical composition of the diets used in the experiment.

	Diet	
Chemical composition, %	**F**	**C**
Dry matter	93.24	89.40
Organic matter	90.24	95.39
Crude protein	15.57	11.56
NDF	43.06	25.17
ADF	25.96	10.23

All values are expressed as a percentage of dry matter (DM). F = Forage diet; C = Concentrate diet.

As **Figure 6** shows, in each of the four experimental periods, a total of 55 Wheaton bottles (60 mL capacity) were used, including five blanks. Each treatment was replicated five times. Both substrates were ground to a particle size of 1 mm², and 0.4 grams of each diet were added to each treatment bottle.

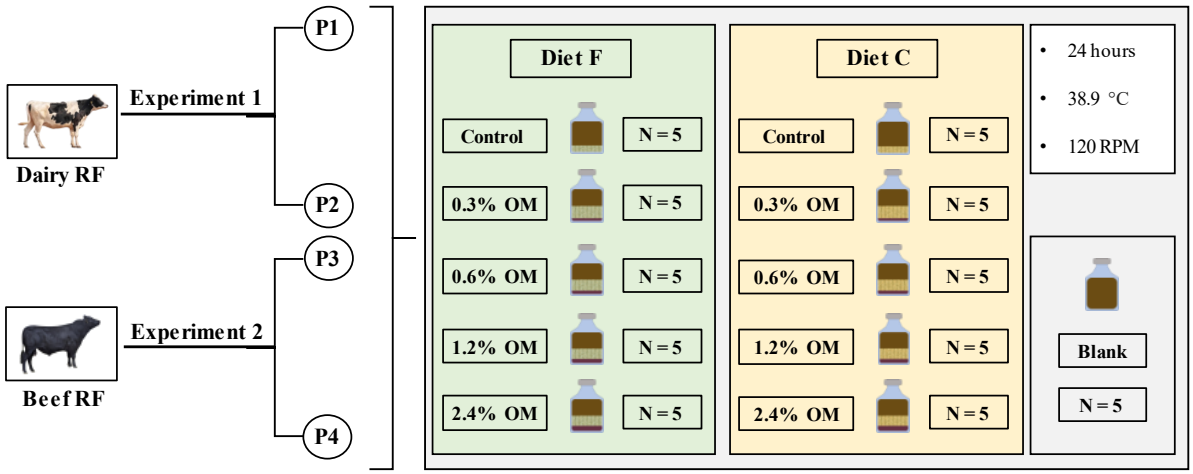

Figure 6. Schematic of the experimental design used in this experiment.

3. Preparation of ruminal fluid

For the first experiment, whole rumen content was collected before the morning feeding. Two liters were extracted from the four quadrants of the cannulated cow's rumen during each period using a probe connected to a vacuum extraction pump. For the second experiment, two liters of whole rumen content were collected directly at slaughter from two steers. In both cases, the

rumen content was filtered through double-layered cheesecloth and transported in thermos containers filled with hot water (40°C) to maintain the appropriate temperature. Upon arrival, the temperature and pH of the rumen fluid were recorded. The rumen content was then transferred to a mixing container, diluted with artificial buffer prepared as described by Menke et al., (1979) at a 4:1 ratio (buffer: rumen fluid), adjusted to a pH of 6.9, and flushed with high purity nitrogen gas (N2) to establish anaerobic conditions.

4. Incubation protocol

In each Wheaton bottle, 40 mL of the final diluted solution was allocated via a manual dosing dispenser along with 0.4 g of the respective substrate and the corresponding dosages of *A. armata*. After flushing with nitrogen gas, the bottles were sealed for fermentation. Before starting fermentation, the pressure in all Wheaton bottles was reduced to 0 kPa using a pressure transducer, ensuring accurate taring. The bottles were then incubated in a shaking incubator system according to Goering & Van Soest (1970) at 38.6°C for 24 hours, operating at 120 oscillations per minute.

5. Sampling

As shown in **Figure 7**, the number of replicates varied across the parameters measured. Gas production was recorded periodically from three replicates (n = 3) at 0, 2, 4, 8, 12, and 24 hours using a pressure transducer system, following the protocol described by Theodorou et al. (1994). At hour 24, replicates 4 and 5 were used to collect headspace CH_4 samples in 12 mL vacuum vials. The pH was recorded for all bottles, and the three replicates from each treatment were filtered through 25 µm filters. Samples of 4 mL and 5 mL were collected from the filtered effluent for ammonia nitrogen concentration (NH_3-N) and VFA analysis respectively.

The filtered residue was dried in an oven at 103°C for 24 hours to determine dry matter content. Subsequently, the dried residue was placed in a muffle furnace at 550°C overnight to measure ash content. Dry matter degradability (DMdeg) and organic matter degradability (OMdeg) were calculated from these values using standard formulas.

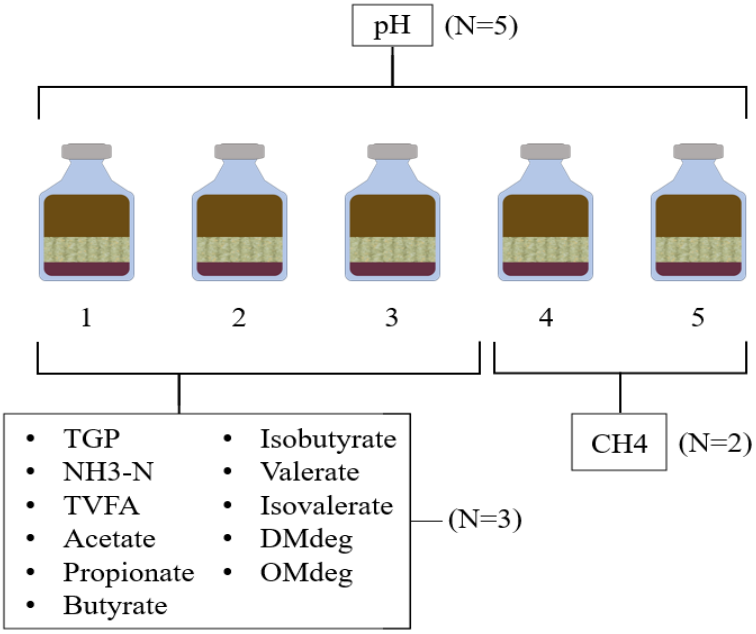

Figure 7. Replicate distribution for parameter measurements. Total gas production (TGP, mL/g OM); Ammonia nitrogen (NH_3-N, mg/dL); Total volatile fatty acids (TVFA, mmol); Acetate (% of total VFAs); Propionate (% of total VFAs); Butyrate (% of total VFAs); Isobutyrate (% of total VFAs), Valerate (% of total VFAs); Isovalerate (% of total VFAs); Dry matter degradability (DMdeg, %); Organic matter degradability (OMdeg, %); Methane (CH_4, mL/g OM).

6. Analysis and calculations

6.1. Gas production

Gas production values recorded during the experiment were converted from pressure (kPa) to volume (mL) using the following equation established by a linear regression developed under the specific conditions of our laboratory:

$V= (0.6369* P) + 0.2434$ ($n=40$, $R^2=0.999$)

where:

- V is the gas volume (mL),
- P is the measured pressure (kPa),
- 0.6369 (a) and 0.2434 (b) are constants defined by the regression curve.

The calculated gas volumes (mL) were corrected by subtracting the mean blank gas production. The cumulative gas production was then adjusted by the respective OM content of the substrate and expressed as mL of gas produced per g OM (mL/g OM).

Fermentation kinetics were analyzed by fitting the cumulative gas production data to a nonlinear Gompertz model. The gas production parameters being the asymptotic gas production a (mL/g OM), the rate of gas production b (mL/g OM per h), and the lag phase c (h) were calculated using a nonlinear procedure (SAS Institute Inc., USA, version 9.4), and introduced into the following equation:

$$Y = a \times e^{(-b \times e^{(-c \times t)})}$$

where:

- Y is the cumulative gas volume (mL/g OM),
- a is the asymptotic gas production (mL/g OM),
- b is the rate of gas production (mL/g OM per h),
- c is the lag phase (h),
- t is the incubation time (h).

6.2. Methane

At the end of the fermentation, the 12 mL Exetainer tubes containing headspace gas samples were sent to the laboratory (Ingeniería Analítica, S.L.) for CH_4 analysis. The laboratory utilized micro gas chromatography with a thermal conductivity detector (µGC/TCD) for the analysis. The detection limit for CH_4 was 0.000312 mol/L (5 ppmv). No pre-treatment of the samples was required, as they were introduced directly into the analytical equipment under standard conditions. The CH_4 concentrations obtained from the gas chromatograph were initially expressed in mol/L and subsequently corrected for blanks. After blank correction, methane concentrations (mol/L) were converted to mass per volume (mg/mL) using the ideal gas law:

$$PV = nRT$$

where:

- P = pressure (in atmospheres, atm),
- V = volume (in liters, L),
- n = number of moles of gas (mol),

- R = ideal gas constant (L atm/K·mol),
- T = temperature (in Kelvin, K).

Following this conversion, values were corrected for standard temperature and pressure (STP) and normalized by headspace cumulated gas production for each respective treatment group. Finally, the corrected methane concentrations were further adjusted based on OM content and expressed as mL of CH_4 per g of OM.

6.3. Volatile fatty acids, pH, and ammonia nitrogen

After filtering all samples, 5 mL of the liquid phase were collected for VFA analysis using gas chromatography at the Chemical Analysis Service (SAQ) of the Autonomous university of Barcelona. Additionally, 4 mL samples were extracted for NH_3-N analysis, which was performed at the Faculty of Veterinary Medicine at the Autonomous university of Barcelona via spectrophotometry.

For volatile fatty acids, the analyzed parameters included total volatile fatty acids (TVFA, mM) and the individual profile components expressed as a percentage of the total. These components were acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate. Additionally, the acetate-to-propionate (A:P) ratio was calculated.

For NH_3-N, absorbance values were converted to concentration (mg/L) using a linear regression model developed under the specific conditions of our laboratory. From these concentrations, the NH_3-N content was calculated using the following equation:

$$[NH_3 - N] = \frac{[(NH_3 concentration \times 2) \times 14.0067]}{[14.0067 + 3(1.00797)]}$$

Where:

- NH_3-N: Ammoniacal nitrogen concentration, expressed in mg/dL,
- NH_3 concentration: Measured ammonia concentration in mg/L,
- 14.0067: Molar mass of nitrogen,
- 1.00797: Molar mass of hydrogen.

6.4. In vitro DM and OM degradability

The remaining solid content retained on the filters was used to determine the DM content by drying the samples in an oven at 103°C for 24 hours. To analyze ash content, the dried solid was further incinerated in a muffle furnace at 550°C overnight. Using the data from the DM and ash analyses, degradability coefficients for both DM and OM were calculated based on the method described by Tilley and Terry, (1963) using the following equations:

1) Dry matter degradability:

$$\text{DMdeg (\%)} = \frac{(\text{Initial substrate DM}) - (\text{Final residue DM} - \text{Blank final residue DM})}{\text{Initial substrate DM}} \times 100$$

2) Organic matter degradability:

$$\text{OMdeg (\%)} = \frac{(\text{Initial substrate DM}) - (\text{Final Ash residue} - \text{Blank final Ash residue})}{\text{Initial substrate OM}} \times 100$$

7. Statistical analysis

Data were analyzed using SAS software (SAS Institute Inc., USA, version 9.4). The experimental unit was the bottle. The statistical model used for the analysis was as follows:

$$Y_{ijk} = \mu + D_i + T_j + (D \times T)_{ij} + R_k + B_k + e_{ijk}$$

Where:

- Y_{ijk}: Observed value of the dependent variable,
- μ: Overall mean,
- D_i: Fixed effect of diet (i levels),
- T_j: Fixed effect of dose (j levels),
- $(D \times T)_{ij}$: Interaction effect between diet and dose,
- R_k: Random effect of period (k levels),
- B_k: Repeated effect of bottle,
- e_{ijk}: Residual error term.

The response variables analyzed included pH, total gas production (TGP, mL/g OM), methane (CH_4, mL/g OM), ammonia nitrogen (NH_3-N, mg/dL), total volatile fatty acids (TVFA, mM),

acetate (%), propionate (%), butyrate (%), isobutyrate (%), valerate (%), isovalerate (%), A:P ratio, in vitro DM degradability (DMdeg, %), and in vitro OM degradability (OMdeg, %).

The experimental design was factorial, considering the fixed effects of diet (F or C) and dose (0, 0.3, 0.6, 1.2, and 2.4% of organic matter) and their interaction (Diet × Dose). Data were evaluated using a mixed-effects model (PROC MIXED and PROC GLIMMIX), with bottle being included as the repeated variable, with the covariance structure for repeated measures modeled using compound symmetry (CS), and period was treated as a random effect. Least-squares means (LSMEANS) were compared using Tukey's test (adjust = tukey).

Model parameters were estimated using the Marquardt iterative method in PROC NLIN. All data are reported as standard error of means (SEM). Missing data were excluded from the analysis. Significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

1. Results

1.1. Experiment 1

1.1.1. Gas and methane production

Table 5 illustrates the effects of *A. armata* inclusion on CH_4 production, TGP, and kinetic parameters for the dairy rumen liquid for both diets at different inclusion levels. A significant and dose × diet interaction ($p < 0.0001$) indicated that the effect of *A. armata* on CH_4 production differed between Diet C and Diet F.

In Diet C, CH_4 concentrations peaked at 0.3% inclusion (C0.3), with no significant difference from the control (CCT), then decreased significantly by 62.5% at the 0.6% inclusion. In contrast, Diet F showed its highest CH_4 value at the control (FCT), a non-significant but notable drop at 0.3%, and a significant decrease of 59.2% at 0.6%, consistent with the interaction observed.

A significant dose × diet interaction ($p = 0.011$) was observed for total gas production (TGP), indicating that the response to *A. armata* inclusion differed between diets. Within diet C, the only significant change in TGP occurred between the 1.2% (C1.2) and 2.4% (C2.4) inclusion levels, decreasing from 462 to 390 mL/g OM. In contrast, TGP values in diet F showed minimal variation across doses, ranging from 352 to 368 mL/g OM, with no statistically significant differences among treatments.

Concerning kinetic parameters, **Figure 8** illustrates the effect of *A. armata* inclusion on the cumulative gas production curves for both concentrate (C) and forage (F) diets at different inclusion levels. A significant dose × diet interaction ($p = 0.006$) was detected. The only significant change in (a) occurred between the 1.2% (C1.2) and 2.4% (C2.4) inclusion levels, decreasing from 467 to 392 mL/g OM. The concentrate diet (C) treatments consistently showed higher values of (a), ranging from 392 to 467 mL/g OM, compared to the forage diet (F) treatments, which ranged from 355 to 376 mL/g OM.

For the gas production rate parameter (b), no significant dose effect ($p = 0.091$) or dose × diet interaction ($p = 0.189$) was observed. However, a significant diet effect ($p < 0.0001$) was detected, with diet C showing higher gas production rates (3.35 - 3.87 mL/g OM per h) compared to diet F, which ranged from 2.91 to 3.03 mL/g OM per h.

Table 5. Effect of different levels of *A. armata* inclusion on total gas production, methane production, and kinetic parameters in dairy rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
Gas production														
TGP (mL/g OM)	419^{ab}	426^{ab}	450^{ab}	462^{a}	390^{bc}	368^{c}	358^{c}	352^{c}	352^{c}	360^{c}	22.10	0.112	<.0001	0.011
Kinetic parameters														
a (mL/g OM)	424^{abc}	431^{ab}	456^{a}	467^{a}	392^{bcd}	376^{cd}	367^{d}	355^{d}	359^{d}	368^{d}	0.0238	0.110	<.0001	0.006
b (mL/g OM per h)	3.52^{ab}	3.35^{bc}	3.55^{ab}	3.51^{ab}	3.87^{a}	3.03^{bcd}	3.00^{cd}	2.91^{d}	2.96^{cd}	3.03^{bcd}	0.2125	0.091	<.0001	0.189
c (h)	0.22^{b}	0.22^{b}	0.20^{b}	0.22^{b}	0.27^{a}	0.20^{b}	0.19^{b}	0.20^{b}	0.19^{b}	0.19^{b}	0.010	0.003	<.0001	0.002
CH4 (mL/g OM)	72.5^{ab}	79.3^{a}	27.2^{d}	0.00^{e}	0.00^{e}	62.1^{bc}	49.5^{c}	25.4^{d}	0.00^{e}	0.00^{e}	3.965	<.0001	<.0001	<.0001

Values represent LSMeans ± SEM of TGP = Total gas production (mL/g OM), a = asymptotic gas production, b = gas production rate, c = lag phase, and CH_4 = methane production (mL/g OM). SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d, e) within the same row indicate statistically significant differences ($P < 0.05$).

For the lag phase parameter (c), a significant dose × diet interaction (p = 0.002) was detected. In the concentrate diet (C), the lag phase increased by 22.7% at the 2.4 % inclusion level, while in the forage diet (F), the lag phase was not significantly affected across doses. Indicating that the lag phase response varied with diet composition and dose, despite both diets having similar lag phase values, except for the notable increase at 2.4% in the concentrate diet.

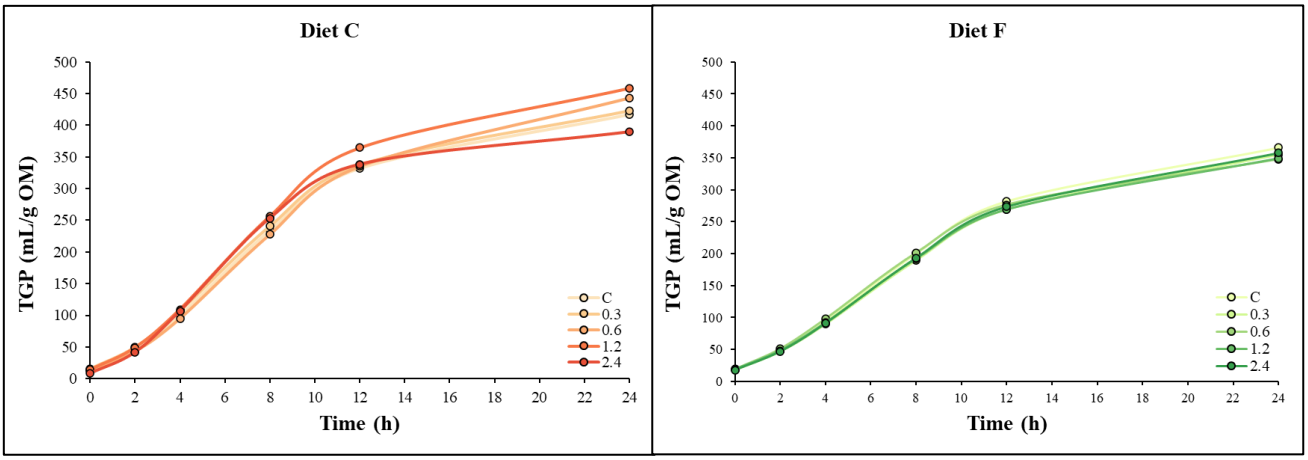

Figure 8. The time series effect of increasing dose of *A. armata* on in vitro total gas production in the concentrate diet (Diet C) and the forage diet (Diet F) in the dairy rumen fluid

1.1.2. pH, In Vitro Degradability, and Ammonia Nitrogen

Table 6 illustrates the effects of *A. armata* inclusion on pH, in vitro dry matter and organic matter degradability, and ammonia nitrogen for the dairy rumen liquid for both diets at different inclusion levels. No diet × dose interaction (p = 0.093) nor dose effect (p = 0.5688) was observed for pH. However, a clear diet effect was shown (p < 0.0001). The concentrate diet (C) treatments consistently showed lower pH values, ranging from 5.45 to 5.49, compared to the forage diet (F), which maintained pH values between 5.79 to 5.82, across all inclusion levels.

For dry matter degradability, no significant dose × diet interaction (p = 0.092) nor dose effect was observed (p = 0.8742), but a clear diet effect was evident (p = 0.006) . The concentrate diet (C) treatments consistently showed higher DM degradability values, ranging from 57.0 to 65.2 %, compared to the forage diet (F) treatments, which ranged from 50.3 to 60.6 % across all inclusion levels. This pattern was consistent with the pH results, where a more acidic environment aligned with higher in vitro degradability.

Table 6. Effect of different levels of *A. armata* inclusion on pH, in vitro degradability, and ammonia nitrogen in dairy rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
pH	5.48^{a}	5.48^{a}	5.45^{a}	5.46^{a}	5.49^{a}	5.80^{b}	5.82^{b}	5.81^{b}	5.82^{b}	5.79^{b}	0.017	0.5688	<.0001	0.093
In vitro Degradability														
DMdeg (%)	57.0	57.0	65.1	64.3	65.2	60.6	56.1	54.2	50.3	54.2	5.120	0.8742	0.006	0.092
OMdeg (%)	60.7	59.1	66.5	65.6	66.4	59.7	58.5	55.7	54.7	56.3	3.6715	0.9254	0.001	0.201
NH3-N (mg/dL)	9.38^{d}	10.5^{cd}	8.02^{d}	7.82^{d}	7.81^{d}	16.79^{ab}	15.5^{abc}	18.3^{a}	15.1^{abc}	14.6^{bc}	1.9859	0.0375	<.0001	0.084

Values represent least squares means (LSMeans) ± SEM for pH, dry matter degradability (DMdeg), organic matter degradability (OMdeg), and ammonia nitrogen (NH_3-N) concentration (mg/dL). SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d) within the same row indicate statistically significant differences ($P < 0.05$).

For OMdeg, no significant interaction ($p = 0.201$) nor dose effect ($p = 0.9254$) was observed, but significant differences were found for diet ($p = 0.0013$). Similar to DMdeg, a pattern consistent with pH results was observed in the OMdeg results. The concentrate diet treatments displayed higher OM degradability, ranging from 59.1 to 66.5 %, whereas forage diet treatments ranged from 54.7 to 59.7 %.

Regarding NH_3-N, no significant dose × diet interaction was found ($p = 0.084$); however, a significant dose effect was observed for ($p = 0.0375$). The inclusion of *A. armata* had minimal impact on ammonia nitrogen concentrations within each diet, as values remained relatively stable across doses in Diet C (9.38^{d} mg/dL in CCT versus 7.81^{d} mg/dL for C2.4) and Diet F (16.79^{ab} mg/dL in FCT versus 14.6^{bc} mg/dL for F2.4). Additionally, a significant diet effect was reported ($p < 0.0001$), with the diet C consistently showing lower NH_3-N concentrations, ranging from 7.81 to 10.5 mg/dL, compared to the diet F, which ranged from 14.6 to 18.3 mg/dL across inclusion levels.

1.1.3. Total volatile fatty acid production and VFA profile

Table 7 presents the effects of *A. armata* inclusion on total volatile fatty acid (TVFA) production and the percentage proportions of individual VFAs (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) for the dairy rumen liquid in both diets at different inclusion levels. For TVFA, no significant diet effect ($p = 0.787$) or dose × diet interaction ($p = 0.337$) was observed. A significant dose effect was observed for TVFA ($p = 0.001$). However, *A. armata* inclusion had minimal effect on TVFA within diets, as values remained statistically similar across doses. At 0.6% inclusion, TVFA production was non-significantly reduced by 5.4% in diet C and by 6.1% in diet F. At 1.2% inclusion, TVFA was again numerically reduced by 9.8% in diet C and by 12.8% in diet F. The only significant change in TVFA occurred between the C0.3 (58.4 mM) and C2.4 (50.1 mM) inclusion levels.

For all VFAs measured in experiment 1, a significant dose effect ($p < 0.05$) and diet effect ($p < 0.05$) were observed. Furthermore, a significant dose × diet interaction ($p = 0.005$) was detected for acetate. At 0.6% inclusion, acetate decreased by 10.4 percentage points in the concentrate diet (C) compared to CCT, whereas acetate decreased by 5.4 percentage points in diet F compared to FCT. Larger reductions were observed at 1.2% inclusion level, notably by 12.5 percentage points in diet C and 8.2 percentage points in diet F. When comparing diets, diet C consistently showed lower acetate concentrations compared to diet F.

Table 7. Effect of different levels of *A. armata* inclusion on total volatile fatty acids, and individual VFA proportions in dairy rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
Volatile fatty acids														
TVFA (mM)	57.3^{ab}	58.4^{a}	54.2^{ab}	51.7^{ab}	50.1^{b}	58.7^{a}	53.5^{ab}	55.1^{ab}	51.2^{ab}	51.8^{ab}	7.880	0.001	0.787	0.337
Acetate (%)	56.5^{cd}	53.6^{d}	46.1^{e}	44.0^{e}	42.2^{e}	65.5^{a}	62.7^{ab}	60.1^{bc}	57.3^{cd}	56.5^{cd}	2.115	<.0001	<.0001	0.005
Propionate (%)	22.6^{bc}	23.1^{b}	29.9^{a}	29.3^{a}	30.0^{a}	17.7^{c}	19.1^{bc}	20.3^{bc}	22.8^{bc}	21.7^{bc}	1.787	<.0001	<.0001	0.110
Butyrate (%)	17.7^{cd}	19.8^{bc}	20.3^{bc}	23.0^{ab}	23.9^{a}	12.9^{e}	14.2^{de}	15.4^{de}	15.7^{de}	17.6^{cd}	1.027	<.0001	<.0001	0.452
Isobutyrate (%)	0.85^{cd}	0.85^{cd}	0.74^{de}	0.72^{e}	0.72^{e}	1.06^{a}	1.04^{ab}	1.06^{a}	1.00^{ab}	0.92^{bc}	0.026	<.0001	<.0001	0.050
Valerate (%)	1.28^{c}	1.40^{c}	2.00^{ab}	2.03^{ab}	2.22^{a}	1.31^{c}	1.51^{bc}	1.58^{bc}	1.75^{abc}	1.72^{abc}	0.252	<.0001	0.011	0.070
Isovalerate (%)	1.20^{cde}	1.24^{bcd}	0.94^{e}	0.95^{de}	0.93^{e}	1.59^{a}	1.53^{a}	1.63^{a}	1.48^{abc}	1.52^{ab}	0.062	0.0091	<.0001	0.021
A:P	2.56^{b}	2.49^{b}	1.64^{c}	1.53^{c}	1.42^{c}	3.73^{a}	3.31^{a}	3.07^{ab}	2.53^{b}	2.62^{b}	0.286	<.0001	<.0001	0.297

Values represent least squares means (LSMeans) ± SEM for total volatile fatty acids (TVFA, mM), individual volatile fatty acids percentages (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) and A:P ratio. SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d, e) within the same row indicate statistically significant differences ($P < 0.05$).

For propionate, no significant dose × diet interaction was detected ($p = 0.110$). At 0.6% inclusion, propionate increased significantly by 7.3 percentage points in the concentrate diet (C). At the same inclusion level in diet F, a notable but non-significant increase of 2.6 percentage points was observed. Across all doses, diet C consistently exhibited higher propionate concentrations compared to diet F which were non-significant.

No significant dose × diet interaction was observed for the A:P ratio ($p = 0.297$). Nevertheless, the A:P ratio exhibited a dose-dependent ($p < 0.0001$) declining trend with increasing *A. armata* inclusion levels, as supported by the observed reductions in acetate and increases in propionate. In the concentrate diet (C), the ratio decreased by 35.9% at 0.6% inclusion level and by 40.2% at 1.2% inclusion level compared to the control (CCT). In diet F, a smaller but notable decrease of 32.2% was observed at 1.2% inclusion compared to the control (FCT). Across all inclusion levels, the concentrate diet maintained lower A:P ratios compared to the forage diet.

No significant dose × diet interaction was observed for butyrate ($p = 0.452$). However, at the 2.4% inclusion level, butyrate concentrations decreased by 6.2 percentage points compared to CCT in diet C and by 4.7 percentage points compared to FCT in diet F. Across all inclusion levels, diet C consistently showed higher butyrate concentrations compared to diet F. Isobutyrate concentrations showed reductions of 0.13 percentage points in diet C and 0.14 in diet F at 2.4% inclusion level. A non-significant dose × diet interaction was observed ($p = 0.050$). Nevertheless, concentrations ranged from 0.72 to 0.85 % in diet C and from 0.92 to 1.06 % in diet F, with diet C consistently exhibiting lower isobutyrate concentrations across all inclusion levels.

For valerate, no significant dose × diet interaction was observed ($p = 0.070$). Nonetheless, valerate concentrations increased in a dose-dependent manner ($p < 0.0001$) with higher *A. armata* inclusion levels. In diet C, an increase of 0.72 percentage points was observed at the 0.6% inclusion level, whereas a notable but non-significant increase of 0.27 percentage points was observed in diet F at the same inclusion level. Moreover, a significant diet effect ($p = 0.011$) was observed for valerate, where across all inclusion levels, diet C exhibited higher maximum values.

A significant dose × diet interaction ($p = 0.021$) was observed in isovalerate concentrations. Across all inclusion levels, diet C consistently exhibited lower isovalerate concentrations compared to diet F.

1.2. Experiment 2

1.2.1. Gas and methane production

Table 8 illustrates the effects of *A. armata* inclusion on CH_4 production, TGP, and kinetic parameters for the beef rumen liquid for both diets at different inclusion levels. For methane, a significant dose × diet interaction ($p < 0.0001$) was detected, indicating that CH_4 suppression varied depending on the dose-diet interaction. Methane was completely inhibited (100%) at 1.2% and 2.4% inclusion levels. However, in diet F, CH_4 production was only completely inhibited at 2.4%.

Regarding TGP, a significant dose × diet interaction ($p < 0.0001$) was observed following *A. armata* inclusion in both diets. In Diet C, TGP significantly decreased by 5.0% at 0.6% inclusion, 11.4% at 1.2%, and 12.5% at 2.4% compared to the control. In Diet F, TGP showed a reduction of 14.4% at 1.2% and 29.8% at 2.4% compared to the control. The concentrate diet (C) treatments consistently showed higher total gas production (TGP) across all inclusion levels, ranging from 470 to 537 mL/g OM, compared to the forage diet (F) treatments, which ranged from 322 to 376 mL/g OM.

Concerning kinetic parameters, **Figure 9** illustrates the effect of *A. armata* inclusion on the Cumulative Gas Production Curves for both concentrate (C) and forage (F) diets at different inclusion levels. **Table 8** shows a significant dose × diet interaction effect in the asymptotic gas production parameter (a) ($p < 0.0001$). In Diet C, (a) decreased by 10.4% at 1.2% inclusion and by 11.8% at 2.4% inclusion. Similarly, in Diet F, (a) decreased by 13.1% at 1.2% inclusion and by 30.3% at 2.4% inclusion. Furthermore, the concentrate diet (C) treatments showed higher values of (a), ranging from 457 to 518 mL/g OM, compared to the forage diet (F) treatments, which ranged from 260 to 373 mL/g OM.

For the gas production rate parameter (b), a significant dose × diet interaction was observed ($p < 0.0001$). Significant differences were only detected at the 0.6% inclusion level in both diets and at the 1.2% level in diet F. The remaining fermentation rates appeared unaffected, as (b) values remained relatively stable across treatments, ranging from 2.64 to 2.68 mL/g OM per h.

Table 8. Effect of different levels of *A. armata* inclusion on total gas production, methane production, and kinetic parameters in beef rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
Gas production														
TGP (mL/g OM)	537^{a}	527^{ab}	510^{b}	476^{c}	470^{c}	376^{d}	373^{d}	375^{d}	322^{e}	264^{f}	41.791	<.0001	<.0001	<.0001
Kinetic parameters														
a (mL/g OM)	518^{a}	507^{a}	496^{a}	464^{b}	457^{b}	373^{c}	368^{c}	373^{c}	324^{d}	260^{e}	0.050	<.0001	<.0001	<.0001
b (mL/g OM per h)	2.66^{b}	2.68^{b}	2.76^{a}	2.65^{b}	2.66^{b}	2.64^{b}	2.64^{b}	2.78^{a}	2.73^{a}	2.67^{b}	2.7789	<.0001	<.0001	<.0001
c (h)	0.31^{a}	0.32^{a}	0.29^{b}	0.28^{b}	0.29^{ab}	0.22^{cd}	0.22^{c}	0.21^{cd}	0.20^{d}	0.23^{c}	0.0335	<.0001	<.0001	0.026
CH4 (mL/g OM)	57.8^{a}	53.6^{a}	52.1^{ab}	0.00^{d}	0.00^{d}	34.2^{bc}	28.2^{c}	29.5^{c}	24.7^{c}	0.00^{d}	13.062	<.0001	0.0001	<.0001

Values represent LSMeans ± SEM of TGP = Total gas production (mL/g OM), a = asymptotic gas production, b = gas production rate, c = lag phase, and CH_4 = methane production (mL/g OM). SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d) within the same row indicate statistically significant differences ($P < 0.05$).

For the lag phase parameter (c), **Table 8** shows a significant dose × diet interaction ($p < 0.026$). The concentrate diet (C) consistently exhibited longer lag phases (0.28 to 0.32 h) compared to the forage diet (F), which ranged from 0.20 to 0.23 h. In diet C, significant differences were observed at the 0.6% and 1.2% inclusion levels compared to the control (CCT). In contrast, lag phase values in diet F did not differ significantly from the control (FCT) and remained relatively stable across inclusion levels.

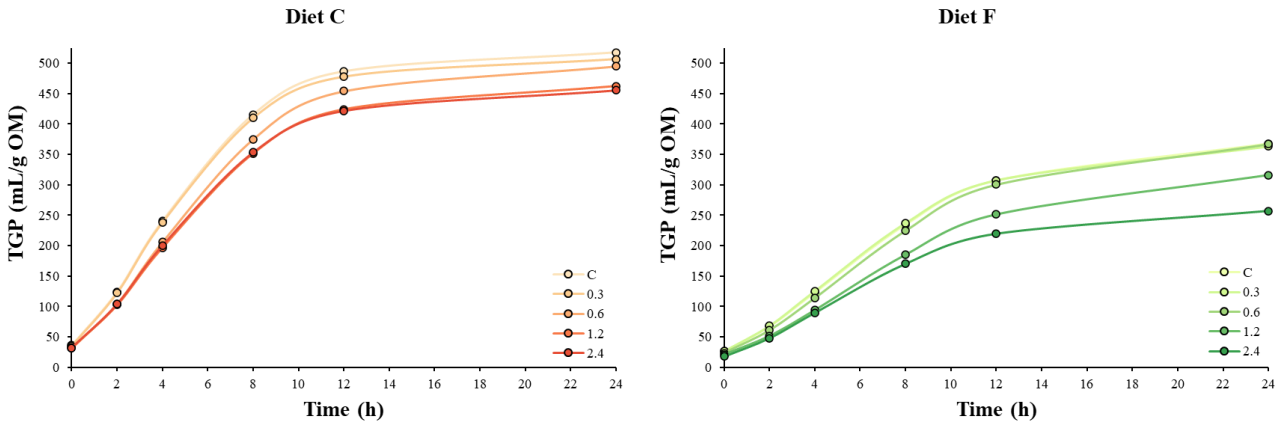

Figure 9. The time series effect of increasing dose of *A. armata* on in vitro total gas production in the concentrate diet (Diet C) and the forage diet (Diet F) in the beef rumen fluid.

1.2.2. pH, in vitro degradability, and ammonia nitrogen

Table 9 illustrates the effects of *A. armata* inclusion on pH, in vitro dry matter and organic matter degradability, and ammonia nitrogen in beef rumen liquid for both diets at different inclusion levels. For pH, no significant diet × dose interaction was observed ($p = 0.168$); however, a significant dose effect was detected ($p = 0.0271$). The only significant change in pH occurred in diet F at the 1.2% inclusion level compared to the forage control (FCT), with the remaining pH values not differing significantly across doses within each diet. Moreover, a clear diet effect was observed ($p < 0.0001$). The concentrate diet (C) consistently showed lower pH values, ranging from 6.34 to 6.37, compared to the forage diet (F), which maintained pH values between 6.50 and 6.55 across all inclusion levels.

Table 9. Effect of different levels of *A. armata* inclusion on pH, in vitro degradability, and ammonia nitrogen in beef rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
pH	6.35^{c}	6.35^{c}	6.37^{c}	6.36^{c}	6.34^{c}	6.50^{b}	6.52^{ab}	6.52^{ab}	6.55^{a}	6.54^{ab}	0.036	0.0271	<.0001	0.168
In vitro Degradability														
DMdeg (%)	48.7	44.0	41.6	40.4	40.6	52.9	38.7	37.3	47.6	37.5	9.056	0.0215	0.9182	0.410
OMdeg (%)	59.9^{a}	58.6^{ab}	57.8^{ab}	55.5^{ab}	54.8^{abc}	53.4^{abc}	49.3^{bc}	50.6^{abc}	50.8^{abc}	45.6^{c}	2.6828	0.0372	<.0001	0.757
NH3-N (mg/dL)	28.4^{c}	29.4^{c}	29.5^{bc}	23.1^{e}	23.1^{e}	31.8^{a}	31.6^{a}	31.7^{a}	31.2^{ab}	24.8^{d}	3.7065	<.0001	<.0001	<.0001

Values represent least squares means (LSMeans) ± SEM for pH, dry matter degradability (DMdeg), organic matter degradability (OMdeg), and ammonia nitrogen (NH_3-N) concentration (mg/dL). SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d, e) within the same row indicate statistically significant differences ($P < 0.05$).

No significant dose × diet interaction was observed for DMdeg. Nevertheless, a significant dose effect ($p = 0.0215$) was observed, indicating that *A. armata* inclusion influenced dry matter degradability across treatments. Although no significant pairwise differences were detected, the observed reduction in DMdeg as inclusion levels increased suggests a dose-dependent trend. No significant diet effect ($p = 0.9182$) was observed.

For OMdeg, no significant dose × diet interaction was observed; however, a significant dose effect was detected ($p = 0.0372$). The only treatments that differed significantly were the concentrate control (CCT), which showed the highest OMdeg, and the 2.4% inclusion in the forage diet (F2.4), which showed the lowest. The remaining treatments appeared unaffected and remained relatively consistent. A significant diet effect was also detected ($p < 0.0001$), with the concentrate diet (C) treatments exhibiting higher OM degradability values (54.8 to 59.9 %) compared to the forage diet (F) treatments (45.6 to 53.4 %) across all inclusion levels.

For NH_3-N, a significant dose × diet interaction was observed ($p < 0.0001$). Ammonia nitrogen concentrations decreased by 18.6% at the 1.2% inclusion level in diet C compared to the control CCT. In diet F, NH_3-N concentrations were not significantly affected at the same inclusion level but showed a significant 22.0% reduction at 2.4% inclusion compared to the control FCT. The concentrate diet (C) consistently resulted in lower NH_3-N concentrations, ranging from 23.1 to 29.5 mg/dL, compared to the forage diet (F), which ranged from 24.8 to 31.8 mg/dL across all inclusion levels.

1.2.3. Total volatile fatty acid production and VFA profile

Table 10 presents the effects of *A. armata* inclusion on total volatile fatty acid (TVFA) production and the percentage proportions of individual VFAs (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) for the beef rumen liquid in both diets at different inclusion levels. For TVFA, no significant dose × diet interaction was observed ($p = 0.644$), but significant main effects of diet and dose ($p < 0.0001$). At 1.2% inclusion level, TVFA was reduced by 8.5% in diet C compared to the control CCT, whereas in diet F, TVFA was reduced by 8.0% compared to the control FCT. The concentrate diet (C) treatments exhibited higher TVFA values (81.5 to 74.6 mM) compared to the forage diet (F) treatments (75.2 to 69.6 mM) across all inclusion levels.

Table 10. Effect of different levels of *A. armata* inclusion on total volatile fatty acids, and individual VFA proportions in beef rumen liquid.

	Diet * Dose													
	CCT	**C0.3**	**C0.6**	**C1.2**	**C2.4**	**FCT**	**F0.3**	**F0.6**	**F1.2**	**F2.4**	**SEM**	**Dose Effect**	**Diet Effect**	**Dose*Diet Interaction**
Volatile fatty acids														
TVFA (mM)	81.5^{a}	80.9^{ab}	81.1^{ab}	74.6^{c}	76.7^{bc}	75.2^{c}	72.7^{cd}	73.6^{cd}	69.2^{d}	69.6^{d}	15.46	<.0001	<.0001	0.644
Acetate (%)	49.7^{d}	49.6^{d}	48.6^{d}	44.3^{e}	44.3^{e}	56.7^{a}	56.5^{a}	56.0^{a}	53.1^{b}	51.1^{c}	3.306	<.0001	<.0001	0.001
Propionate (%)	31.5^{b}	31.6^{b}	32.3^{b}	34.8^{a}	35.1^{a}	26.4^{e}	26.4^{e}	26.6^{e}	28.3^{d}	30.1^{c}	3.488	<.0001	<.0001	0.001
Butyrate (%)	12.7^{bc}	12.8^{bc}	13.0^{b}	14.5^{a}	14.4^{a}	10.5^{e}	10.6^{e}	10.9^{e}	11.7^{d}	12.3^{c}	0.431	<.0001	<.0001	0.016
Isobutyrate (%)	1.18^{b}	1.17^{b}	1.18^{b}	1.16^{b}	1.09^{c}	1.40^{a}	1.40^{a}	1.40^{a}	1.39^{a}	1.19^{b}	0.077	<.0001	<.0001	<.0001
Valerate (%)	2.65^{c}	2.72^{c}	2.81^{c}	3.27^{ab}	3.28^{a}	2.59^{c}	2.62^{c}	2.63^{c}	3.06^{b}	3.25^{ab}	0.629	<.0001	0.000	0.299
Isovalerate (%)	2.18^{b}	2.18^{b}	2.16^{b}	1.99^{c}	1.84^{d}	2.47^{a}	2.46^{a}	2.45^{a}	2.42^{a}	2.10^{b}	0.295	<.0001	<.0001	0.000
A:P	1.61^{d}	1.59^{de}	1.52^{e}	1.28^{f}	1.26^{f}	2.26^{a}	2.25^{a}	2.19^{a}	1.95^{b}	1.73^{c}	0.343	<.0001	<.0001	<.0001

Values represent least squares means (LSMeans) ± SEM for total volatile fatty acids (TVFA, mM), individual volatile fatty acids percentages (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) and A:P ratio. SEM = Standard Error of the Mean, CT = Control. Dose Effect, Diet Effect, and Dose × Diet Interaction columns represent the P-values for the main effects and their interaction. Superscript letters (a, b, c, d, e, f) within the same row indicate statistically significant differences ($P < 0.05$).

A significant dose × diet interaction effect ($p < 0.05$) was observed for all VFAs measured in Experiment 2, except for valerate, which showed no significant interaction. For acetate, at 1.2% inclusion level, a reduction of 5.4 percentage points was observed in diet C compared to the control (CCT), while a 3.6 percentage points reduction was observed at the same inclusion level in diet F compared to its control (FCT). Across all inclusion levels, acetate concentrations ranged from 44.3% to 49.7% in diet C, consistently remaining lower than those in diet F, which ranged from 51.1% to 56.7%.

In addition, for propionate, increases of 3.3 percentage points were reported in diet C compared to CCT at 1.2% inclusion level. At the same inclusion level, an increase of 1.9 percentage points was observed in diet F compared to FCT. Across all inclusion levels, propionate concentrations ranged from 31.5% to 35.1% in diet C and from 26.4% to 30.1% in diet F, with diet C consistently showing higher propionate concentrations compared to diet F.

The A:P ratio showed a consistent decline with increasing *A. armata* inclusion levels, with a significant dose × diet interaction detected ($p < 0.0001$). In diet C, reductions of 5.59% at 0.6% inclusion level and 20.1% at 1.2% inclusion in diet C were observed. In diet F, a reduction of 13.7% at 1.2% inclusion was observed. Across all inclusion levels, the ratio ranged from 1.26 to 1.61 in diet C and from 1.73 to 2.26 in diet F, with diet C maintaining lower A:P ratios compared to diet F.

For butyrate, at 1.2% inclusion level, reductions of 1.8 percentage points in diet C and 1.2 percentage points in diet F were observed. Butyrate concentrations ranged from 12.7% to 14.5% in diet C and from 10.1% to 12.3% in diet F across all inclusion levels, with diet C maintaining consistently higher butyrate values than diet F.

Isobutyrate concentrations showed reductions of 0.09 percentage points at 2.4% inclusion in diet C and 0.21 percentage points at the same inclusion level in diet F. Concentrations for isobutyrate ranged from 1.16% to 1.18% in diet C and from 1.19% to 1.40% in diet F across all inclusion levels, with diet C maintaining lower isobutyrate concentrations.

For valerate, significant effects of both diet and dose were detected ($p < 0.001$). At the 1.2% inclusion level, valerate concentrations decreased by 0.62 percentage points in diet C and by 0.47 percentage points in diet F. At 2.4% inclusion, reductions of 0.63 and 0.66 percentage points were observed in diets C and F, respectively. Valerate concentrations ranged from 2.65%

to 3.28% in diet C and from 2.59% to 3.25% in diet F, with diet C consistently exhibiting higher values across all inclusion levels.

For isovalerate, a reduction of 0.19 percentage points was observed in diet C at the 1.2% inclusion level. At the same inclusion level, no significant changes were detected in diet F; however, a reduction of 0.37 percentage points was observed at 2.4% inclusion. Isovalerate concentrations were consistently lower in diet C, ranging from 1.84% to 2.18%, compared to diet F, where values ranged from 2.10% to 2.47% across all inclusion levels.

2. Discussion

This study aimed to evaluate the potential of *A. armata* as a feed additive for mitigating CH_4 emissions in ruminant production using an in vitro ruminal fermentation approach. Specifically, the research sought to determine the extent to which *A. armata* supplementation reduces CH_4 emissions while assessing its effects on key ruminal fermentation parameters, including TGP, TVFA, NH_3-N, and in vitro degradability. Additionally, the study aimed to establish a dose-response relationship to identify an optimal inclusion level that balances methane mitigation with fermentation efficiency for a given diet and rumen fluid.

The two basal diets (C and F) used in this study were formulated to align with standard feeding practices in Spain's beef and dairy sectors. Fiber content, particularly NDF, plays a significant role in CH_4 production and can also impact the efficacy of anti-methanogenic compounds, such as 3-NOP and $CHBr_3$, which specifically target MCR (Roque et al., 2021). Diet F had an NDF content of 43%, which is lower than the NDF content of more forage-based diets typically used in in vitro studies, where values range from 62% to 75% (Machado et al., 2016; Kinley et al., 2016b; Andreen et al., 2023; Nunes et al., 2024). However, the NDF content in diet F is more in line with the basal diets used in in vivo studies, where NDF content ranges from 30% to 41% (Roque et al., 2019b; Muizelaar et al., 2023; Angellotti et al., 2025). For diet C, the NDF content was comparable to those reported in other in vivo studies, which range from 19% to 33% (Kinley et al., 2020; Roque et al., 2021).

Moreover, *A. armata* used in this experiment was cultivated in indoor facilities Algabrava, S.L.), whereas *Asparagopsis spp.* used in most in vitro studies was wild-harvested (Chagas et al., 2019; Brooke et al., 2020). Bromoform concentrations in *Asparagopsis spp.* reported in literature ranges from 0.17% DW (Kinley et al., 2016b) to 1.0% DW (Stefenoni et al., 2021),

if specified at all, which can complicate comparisons across studies, even at the same dietary doses.

2.1. Gas and methane production

The results from both experiments indicate a significant dose-dependent reduction in CH_4 emissions, suggesting that *A. armata* effectively inhibits hydrogenotrophic methanogenesis. This inhibition was observed at increasing doses across both experiments and diets. This dose response effect aligns with many similar studies (Machado et al., 2016; Kinley et al., 2016b).

In Experiment 1, a 0.6% OM inclusion level of *A. armata* resulted in partial but significant CH_4 reductions averaging 60%, whereas complete suppression of CH_4 production was achieved at higher inclusion levels (1.2% and 2.4% OM). It is well known that the antimethanogenic effect of *A. armata* is primarily attributed to $CHBr_3$ (Ungerfeld, 2020). The lower inhibition observed at the 0.6% dose could be attributed to the partial degradation of $CHBr_3$ at this lower concentration. This degradation is likely mediated enzymatically by methanogenic archaea, which are capable of reductive dehalogenation of $CHBr_3$, as previously described (Romero et al., 2023). In vivo, bromoform would be progressively removed from the rumen due to ruminal outflow, and unless it is continuously replenished via fresh *A. armata* biomass, its inhibitory effect on CH_4 production would diminish over time. However, this phenomenon was not comparable in the in vitro batch culture system, which lacks ruminal flow dynamics (Denman et al., 2007).

Interestingly, Andreen et al. (2023) reported similar total reductions in vitro at a lower inclusion level of 0.6% OM, suggesting a potential difference in the effective dose required for CH_4 inhibition between studies. Moreover, the similarity in inclusion levels, but a difference in $CHBr_3$ concentration between studies could partially explain the observed variation in methane reduction. In the present study, *A. armata* contained 0.53-1.05% DW of $CHBr_3$, whereas *A. taxiformis* used by Andreen et al. (2023) had a narrower range of 0.68–0.71% DW. While both studies used rumen fluid from dairy cattle, the variability in $CHBr_3$ concentrations may have influenced methane inhibition efficacy (Stefenoni et al., 2021). Moreover, factors such as diet composition and microbial adaptation may influence how effectively $CHBr_3$ inhibits methanogenesis (Roque et al., 2019b) which is furthermore reinforced by the significant diet effect in both experiments.

In Experiment 2, CH_4 reduction was less pronounced compared to Experiment 1 at lower doses of *A. armata*, indicating a delayed inhibitory effect. This could be explained by the adaptation of the beef RF to starch-rich diets enhances the activity of amylolytic bacteria and facilitates the redirection of H_2 toward alternative pathways, such as propionate synthesis, which may reduce the availability of hydrogen for methanogens (Li et al., 2022).

When comparing diets, diet C exhibited higher CH_4 production at low inclusion levels in both experiments. When looking at CH_4 in control groups of experiment 1, diet C resulted in a CH_4 production of 72.5 mL/g OM compared to 62.1 mL/g OM for diet F. Similarly, in experiment 2, higher CH_4 level of 57.8 mL/g OM was observed for diet C compared to diet F which resulted in a 34.2 mL/g OM. Li et al., (2022) suggests that a starch-rich diet can lead to higher levels of dissolved hydrogen in the rumen, as starches are more readily fermentable by amylolytic bacteria. This is supported by the significant diet effect for fermentation rate (b) in both experiments. Higher H_2 concentrations may increase methanogenesis in the rumen due to more H_2 being available for methanogens (Ungerfeld, 2015).

Although H_2 was not directly measured in this study, TGP can serve as a partial indicator of hydrogen accumulation, since TGP consists primarily of H_2, CO_2, and CH_4, which are the main gas byproducts of rumen fermentation (Ungerfeld, 2015). In Experiment 1, the inclusion of *A. armata* had no significant effect on TGP, which is consistent with findings from Brooke et al., (2020), who reported no significant changes in TGP even at a 3% OM inclusion of *A. taxiformis* in dairy cattle RF. However, in Experiment 2, increasing doses of *A. armata* significantly modulated TGP, with a clear dose-dependent effect. A significant reduction was observed at inclusion levels of 1.2% OM and above. These findings align with other in vitro studies using beef-derived rumen fluid, which reported TGP reductions of approximately 30% at 1.0% OM (Machado et al., 2016) and 31.5% at 2.0% OM (Kinley et al., 2016a). At sufficient inclusion levels, bromoform likely disrupts not only methanogenesis but also the activity of fibrolytic and amylolytic bacteria, leading to reduced fermentation efficiency, lower TGP, and impaired overall substrate utilization (Machado et al., 2016; Roque et al., 2019a).

Between diets, the rapid fermentation of starch may have driven the higher TGP values observed under diet C compared to diet F in both experiments, which is clearly supported by the significant diet effect in both experiments ($p < 0.0001$). However, incubation duration also plays a role, as faster-degrading substrates, such as starch, generate greater TGP in the short term, while slower-degrading substrates, like fiber, may produce similar TGP over extended

periods (Menke et al., 1979; Schofield et al., 1994; Van Soest, 1994). In an in vivo study, Roque et al. (2021) compared two inclusion levels of *A. taxiformis* (0.25% and 0.5% OM) against a control across three different diets, varying in forage content. The study reported that hydrogen (H_2) production (g/d) increased significantly, with the greatest increase (419%) observed in the low-forage diet, followed by the medium-forage diet (326%), and the high-forage diet (177%). This trend suggests that lower forage content enhances hydrogen accumulation. These findings could also explain the higher TGP observed in Diet C compared to Diet F, as this shift in fermentation pathways may have influenced overall gas production.

Hydrogen redirection into alternative pathways, such as propionate, butyrate, or valerate synthesis, acts as a compensatory mechanism; however, these pathways have limited capacity and may not fully compensate for the loss of CH_4 as a hydrogen sink. As a result, excess H_2 further disrupts microbial activity, reduces fermentation efficiency, and contributes to declines in parameters such as total TVFA and other fermentation parameters (Morgavi et al., 2010; Ungerfeld, 2020).

2.2. pH, in vitro degradability, and ammonia nitrogen

Previous studies have highlighted the effect of *Asparagopsis spp.* inclusion on rumen pH. Andreen et al. (2023) reported a linear increase in pH with increasing *A. taxiformis* inclusion. In the present study, a statistically significant effect of dose on pH was detected ($P = 0.0271$), but no consistent trend was observed across all inclusion levels. Notably, in Diet F, the 1.2% OM inclusion level showed a significantly higher pH (6.55) compared to the control (6.50), while other doses did not differ significantly. In all other treatment groups, pH values remained relatively stable. This finding aligns with Roque et al. (2019), who reported that pH remained relatively constant throughout the experiment and between individual vessels. However, a clear diet effect was evident, with higher amylolytic activity associated with Diet C leading to a lower pH in the fermentation medium compared to Diet F across both experiments.

In Experiment 1, NH3-N was not shown to be affected although clear difference between diets was observed. This was consistent with results reported in Andreen et al. (2023), where NH3-N was not affected even at 0.8% OM inclusion level. However, in experiment 2, a clear decrease was observed at inclusion levels where CH_4 was inhibited. Other studies observed NH3-N reductions with increasing inclusion of *Asparagopsis. Spp.* (Chagas et al., 2019),

indicating a possible inhibition of microbial activity by bromoform, leading to reduced deamination of amino acids and lower NH3-N concentrations (Machado et al., 2016). According to Li et al. (2022), fiber-rich diets which promote fibrolytic bacteria such as *Fibrobacter* and *Ruminococcus*, enhance proteolysis and deamination, ultimately increasing NH_3-N concentrations in the rumen compared to starch-rich diets. This is furthermore explained by the crude protein (CP) content of the diets, where Diet F had a higher CP content of 15.57% compared to Diet C with 11.56% CP. The observed differences in NH3-N concentrations between diets align with findings by Hristov et al., (2004), who reported that dietary CP and Rumen Degradable Protein levels significantly influence NH3-N concentrations in the rumen, which could explain the higher NH3-N values in Diet F.

The absence of a dose effect on DMdeg and OMdeg in Experiment 1 suggests that *A. armata* inclusion did not substantially alter degradation dynamics under dairy rumen conditions. This was consistent with Chagas et al. (2019), who observed no reductions in OMdeg at 1.0% or 2.0% inclusion levels of *A. taxiformis* in dairy cattle rumen fluid with a forage-based diet. Similarly, Roque et al. (2019) reported no significant effect on OMdeg when *A. taxiformis* was included at 5% OM in a TMR diet with dairy cattle rumen fluid. This contrasts with Experiment 2, where a dose-dependent decline was evident, likely due to differences in microbial populations and fermentation efficiency between dairy and beef rumen environments.

In Experiment 2, no significant pairwise differences were detected between doses within the same diet, though a numerical decline in dry matter degradation (DMdeg) was observed, with reductions of 16.7% at 1.2% inclusion in Diet C and 29.1% at 2.4% inclusion in Diet F, where CH_4 inhibition was complete. Similar numerical trends were observed for OM degradation (OMdeg), albeit with more moderate reductions (7.3% at 1.2% inclusion in Diet C and 14.6% at 2.4% in Diet F). However, these patterns cannot be interpreted as a dose-response relationship, since they were not statistically supported within diets. Varying results were reported with in vitro studies using beef RF. Kinley et al. reported no significant reductions in OMdeg at 2.0% OM inclusion of *A. taxiformis*. However, Kinley 2016 reported reductions at 10% OM of *A. taxiformis* with 0.17 % DW of bromoform. Similarly, Machado et al. (2016) reported reductions of 14% and 24% with 10% OM and 16.7% OM respectively.

The significant diet effect indicates that OMdeg was consistently higher in the concentrate diet than in the forage diet, which can be attributed to the greater fermentability of starch-based

diets. Starch is more readily hydrolyzed by amylolytic bacteria, leading to faster and more efficient microbial fermentation (Li et al., 2022).

2.3. Total volatile fatty acid production and VFA profile

In vitro studies have highlighted the potential negative effects of *Asparagopsis spp.* inclusion on TVFA production. Andreen et al. (2023) reported significant reductions in TVFA at 0.3% OM (24.7%), 0.6% OM (26.5%), and 0.8% OM (29%). In Experiment 1, while TVFA values decreased numerically with increasing *A. armata* inclusion, the only statistically significant difference was between C0.3 and C2.4 treatments ($p<0.05$). This single significant difference does not demonstrate a consistent dose-dependent effect on fermentation dynamics. Some studies propose that reductions in TVFA could be attributed to lower substrate availability due to reduced feed degradation (Kinley et al., 2016). This may explain the more pronounced TVFA reductions observed in Andreen et al. (2023) where the higher NDF content (61.7%) and lower OM degradability (ranging from 32.4% to 25.5%) likely limited fermentable substrate availability. In contrast, OMdeg values in our study were notably higher (54.7% to 66.5%), which may have buffered against more amplified TVFA reductions across treatment groups.

However, in Experiment 2, the dose-dependent reductions in TVFA became significant at 1.2% OM inclusion levels in both diets, reinforcing the hypothesis that higher inclusion rates consistently impact fermentation dynamics, as highlighted by Kinley et al. (2019). The observed decline in TVFA concentrations across both diets suggests that hydrogen accumulation did not fully compensate through alternative pathways, aligning with previous findings that highlight the limitations of these compensatory mechanisms (Ungerfeld, 2020; Morgavi et al., 2010). Similar studies have reported comparable dose-dependent reductions in TVFA. Kinley et al. (2016) reported significant TVFA reductions at 5% and 10% OM inclusion levels. Likewise, Machado et al. (2016) observed a 25% reduction in TVFA at 2.0% OM inclusion and a 40% reduction at 5.0% OM of *A. taxiformis*. The significant diet effect in Experiment 2 for OMdeg suggests that dietary composition also influenced TVFA results. The higher TVFA values for Diet C observed align with higher OMdeg values observed suggesting higher substrate availability in Diet C. Additionally, the higher TGP results may indicate higher H_2 availability to be redirected towards alternative pathways.

The redirection of H_2 into alternative pathways due to methanogenesis inhibition was evident in both experiments. This shift was primarily reflected in the increase of hydrogen sinks, mainly propionate, with smaller contributions from butyrate and valerate, while acetate decreased significantly. These findings align with previous studies on *A. taxiformis* (Andreen et al., 2023; Machado et al., 2016), which also observed a dominant shift toward propionate synthesis when methanogenesis was inhibited.

For example, Andreen et al. (2023) reported significant VFA profile changes at 0.6% and 0.8% OM inclusion levels, demonstrating a clear shift in hydrogen redirection. At both doses, propionate increased by 34%, while butyrate increased by 5.8% at 0.6% OM and 17.7% at 0.8% OM. Additionally, valerate increased dramatically, by 97.5% at 0.6% OM and 113% at 0.8% OM, confirming the role of these VFAs as alternative hydrogen sinks. These increases were accompanied by notable reductions in acetate and the A:P ratio. Specifically, acetate decreased by 16.1% at 0.6% OM and 18.2% at 0.8% OM, while the A:P ratio declined by 37.4% and 38.9%, respectively. Similarly, Machado et al. (2015) observed the same metabolic pattern at 1.0% OM inclusion, reporting a 47.5% increase in propionate, a 38.8% increase in butyrate, and a 25% increase in valerate. These changes were accompanied by a 28.6% reduction in acetate and a 30% decline in the A:P ratio.

Nevertheless, in Experiment 1, while a significant dose effect was observed, pairwise differences in propionate and valerate were not significant, and butyrate only showed a delayed response, with a significant increase occurring at the 2.4% inclusion level. The reduction of 12.5% in acetate at 1.2% inclusion was notable and likely explains the corresponding decline of 32.2% in the A:P ratio. However, the overall magnitude of these shifts was lower than those observed in diet C. This can be explained by the reduced TGP and OMdeg in Diet F, which likely limited hydrogen availability, constraining its redirection into alternative metabolic pathways such as propionate, butyrate, and valerate synthesis.

CONCLUSIONS

CONCLUSION

The findings of this study reinforce the potential of *Asparagopsis armata* as a promising natural feed additive for methane mitigation in ruminants. The in vitro experiments demonstrated a significant dose-dependent reduction in methane production, validating their direct inhibitory effect on methyl-coenzyme M reductase (MCR) and methanogenic archaea. However, the study also revealed some challenges associated with *Asparagopsis armata* supplementation. In Experiment 2, higher inclusion rates were associated with a decline in total gas production (TGP), organic matter degradability (OMdeg), ammonia nitrogen (NH_3-N), and total volatile fatty acids (TVFA).

It is of importance to optimize inclusion levels to maximize methane reduction while minimizing negative impacts on rumen fermentation. In the case of beef cattle rumen fluid (experiment two), the negative impact on ruminal fermentation prevented the identification of a viable inclusion dose. However, in dairy cow ruminal fluid (experiment one), a 0.6% OM inclusion of *Asparagopsis armata* proved effective in reducing methane emissions for both high forage and and high concentrate diets, without compromising ruminal fermentation.

FUTURE PERSPECTIVES

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Future research should focus on validating the long-term efficacy of *A. armata* under in vivo conditions. While significant methane reduction has been demonstrated in vitro, its sustained impact on animal performance, feed efficiency, and rumen microbiome adaptation remains uncertain. Evaluating its effectiveness across different feeding systems will be critical to understanding potential trade-offs.

Moreover, optimizing the dose and delivery mechanisms is essential. Lowering the required inclusion rate while maintaining efficacy could help mitigate adverse effects on rumen fermentation. Novel delivery methods, such as encapsulation or slow-release formulations, should be explored to enhance its stability in feed.

Additionally, combining *A. armata* with other active compounds, such as essential oils and tannins, may further enhance methane mitigation while maintaining fiber digestibility. From a commercial perspective, the harvesting, processing, and storage of *A. armata* need further optimization to ensure large-scale production.

By addressing these key challenges, *A. armata* has the potential to become a viable and scalable solution for methane reduction, contributing to more sustainable and climate-smart livestock systems.

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