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Inclusion of *Asparagopsis spp.* in Dairy Cow Diets to Mitigate Enteric Methane Emissions

Animal and microbial responses

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Abstract

Enteric methane (CH_4) emissions from dairy cows contribute significantly to greenhouse gas production; mitigating these emissions is a major challenge for sustainable livestock systems. This thesis evaluated the potential of *Asparagopsis spp.* to reduce enteric CH_4 emissions while considering animal performance, rumen and faecal microbiota, and the influence of algae cultivation conditions on bioactive compound concentrations.

Across three studies, *Asparagopsis spp.* consistently reduced CH_4 production, although the effects and persistence of mitigation depended on algae composition and feeding duration. The use of *Asparagopsis spp.* also caused microbial changes in the rumen. Supplementation influenced fermentation patterns, including volatile fatty acid profiles, and altered both ruminal and faecal microbial communities, highlighting the interaction between chemical inhibition and microbial shifts. The thesis also showed variability in algae quality and bioactive compound concentrations such as bromoform, underlining the need for standardized production and dosing strategies. Furthermore, halogen intake and accumulation were monitored to assess food safety implications.

While many of the observed responses are consistent with earlier studies, this thesis reveals new mechanistic insights into how temporal changes in the rumen and faecal microbiome, halogen accumulation patterns, and fluctuations in algae composition collectively influence the variability in animal responses. These findings highlight that *Asparagopsis spp.* cannot be regarded as a uniform or fixed feed; instead, its impact emerges from a highly dynamic interplay between algal chemistry, microbial adaptation, and metabolic regulation.

Overall, this thesis demonstrates that *Asparagopsis spp.* is a promising strategy for enteric CH_4 mitigation in dairy systems, but its practical application requires the careful consideration of algae quality and long-term efficacy.

Keywords: red algae, rumen microbiota, CH_4 mitigation, fermentation, halogen compounds, VFA

Inkludering av algen Asparagopsis i foderstater till mjölkkor för att minska metanbildning under fodersmältningen

Sammanfattning

Enterisk metan (CH_4) från mjölkkor, som bildas under fodersmältningen, bidrar i hög grad till utsläppen av växthusgaser, och att minska dessa utsläpp är en central utmaning för hållbar animalieproduktion. Denna avhandling utvärderade potentialen hos *Asparagopsis spp.* som en del av foderstaten för att reducera kornas enteriska CH_4-emissioner, samtidigt som djurens produktion, våm- och träckmikrobiota undersöktes. Vidare studerades hur odlingsförhållanden för alger påverkar koncentrationen av bioaktiva ämnen. I de tre studier som denna avhandling innefattar, så *minskade Asparagopsis spp.* konsekvent CH_4-produktionen, även om omfattningen och varaktigheten av denna reduktion berodde på algkomposition, inblandningsnivå samt åtföljande förändringar i våmmikrobiotan. Inblandning av alger påverkade fermentationsmönster, inklusive fermentationsprofilerna av flyktiga fettsyror (VFA), och förändrade både våm- och träckens mikrobiella samhällen, vilket belyser samspelet mellan kemisk inhibition och mikrobiell påverkan. Avhandlingen visade också att det fanns variationer i algkvalitet och koncentrationer av bioaktiva ämnen, vilket understryker behovet av standardiserade strategier både för produktion av alger och dosering. Dessutom undersöktes även kornas intag och ackumulering av halogener i mjölken för att bedöma potentiella livsmedelssäkerhetsaspekter. Även om många av de observerade responsmönstren överensstämmer med tidigare studier, ger denna avhandling nya insikter i hur tidsmässiga förändringar i våm- och träckmikrobiomet, mönster av halogenackumulering och variationer i algkomposition tillsammans påverkar, men även hur responsen varierar mellan individer. Dessa resultat visar att *Asparagopsis spp.* inte kan betraktas som ett statiskt eller oföränderligt fodermedel; dess effekt uppstår istället genom ett dynamiskt samspel mellan kemi, mikrobiell anpassning och metabol reglering. Sammantaget visar denna avhandling att Asparagopsis spp. är en lovande strategi för att minska enteriska CH_4-utsläpp inom mjölkproduktion, men dess praktiska tillämpning kräver noggrann hänsyn till algkvalitet, långtidseffektivitet och säkerhet vid hantering och utfodring.

Keywords: rödalger, våmmikrobiota, CH_4-reduktion, fermentation, halogenföreningar, flyktiga fettsyror

Inclusione di Asparagopsis spp. nelle diete delle bovine da latte per mitigare le emissioni enteriche di metano

Sommario

Le emissioni di metano enterico (CH_4) nelle bovine da latte contribuiscono in modo significativo alla produzione di gas serra, e la loro mitigazione rappresenta una sfida fondamentale per sviluppare sistemi zootecnici sostenibili. Questa tesi ha valutato il potenziale delle Asparagopsis spp. come mangime per ridurre le emissioni enteriche di CH_4, considerando al contempo le prestazioni animali, il microbiota ruminale e fecale, e l'influenza delle condizioni di coltivazione delle alghe sulla concentrazione dei composti bioattivi.

Nei tre studi condotti, *Asparagopsis spp*. ha ridotto in modo consistente il CH_4, sebbene l'entità e la persistenza dell'effetto dipendessero dalla composizione algale e dalla durata della somministrazione, e fossero accompagnate da cambiamenti fermentativi e microbici. La tesi ha inoltre evidenziato una marcata variabilità nella qualità delle alghe e nei composti bioattivi, sottolineando la necessità di una produzione e un dosaggio più standardizzati. Anche l'assunzione e l'accumulo di alogeni come bromo e iodio, sono stati monitorati per valutarne la sicurezza alimentare.

Pur confermando risultati precedenti, questa tesi fornisce nuove evidenze su come dinamiche microbiche temporali, accumulo di alogeni e variabilità delle algae contribuiscano alla diversa risposta animale, mostrando che *Asparagopsis spp.* non è un mangime statico ma parte di un sistema chimico-microbico complesso.

Nel complesso, *Asparagopsis spp*. emerge come una strategia promettente per mitigare il CH_4 enterico, ma la sua applicazione richiede attenzione alla qualità delle alghe, alla stabilità dell'effetto e alla sicurezza.

Keywords: alghe rosse, microbiota ruminale, mitigazione del CH_4, fermentazione, composti alogenati, VFA

To my father,
for his infinite curiosity and love of knowledge.

Wall painting with a human figure and a bovine, House of the Vettii, Pompeii, 1st century AD (Fourth Pompeian Style).
Source: RomanoImpero.com, "Casa dei Vettii (Pompeii)"

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. M. Angellotti, M. Lindberg, M. Ramin, S.J. Krizsan, R. Danielsson (2025). *Asparagopsis taxiformis* supplementation to mitigate enteric methane emissions in dairy cows — Effects on performance and metabolism. J.Dairy Sci, 108:2503-2516. https://doi.org/10.3168/jds.2024-25258

II. M. Angellotti, A. Singh, M. Lindberg, A. Schnürer, R. Danielsson. Impact of *Asparagopsis taxiformis* on the microbial composition and functional groups in dairy cows. (manuscript).

III. M. Angellotti, M. Lindberg, S.J. Krizsan, A. Demeter, R. Danielsson. Methane mitigation in dairy cows: *In vitro* assessment of *Asparagopsis spp*. grown under variable cultivation conditions. (submitted manuscript).

The contribution of Melania Angellotti to the papers included in this thesis was as follows:

I. Led the data curation, formal analysis, interpretation, visualization and investigation. Contributed to the methodology. Wrote the original draft and took the main responsibility for review and ending.

II. Led the data curation, formal analysis, interpretation, visualization and investigation. Contributed to the methodology Wrote the original draft and took the main responsibility for review and ending.

III. Led the data curation, formal analysis, interpretation, visualization and investigation. Wrote the original draft and took the main responsibility for review and ending.

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Abbreviations

AT	*A. taxiformis*
Br_2	Bromine
CH4	methane
$CHBr_3$	bromoform
CO_2	carbon dioxide
CON	0.0 % of AT on organic matter
CP	crude protein
DM	dry matter
DMI	dry matter intake
ECM	energy-corrected milk
EE	ether extract
FRAP	plasma antioxidant capacity
FW	fresh-weight basis
GHG	greenhouse gas
H-AT	0.3 % of AT on OM basis
I_2	iodine
iNDF	indigestible neutral detergent fibre
L-AT	0.15 % of AT on OM basis
MCR	methyl-coenzyme M reductase

MTL	maximum tolerable level
NADH	nicotinamide adenine dinucleotide hydride
NDF	neutral detergent fiber
OM	organic matter
TMR	total mixed ration
VFA	volatile fatty acids
VG1–VG5	five cultivated batches
WSC	water-soluble carbohydrates

1. Introduction

The sustainability of food and farming systems is an urgent global issue. According to the FAO (2023), agriculture, forestry, and other land use accounted for approximately 22% of global anthropogenic greenhouse gas (GHG) emissions between 2010 and 2019. Within this context, livestock production alone contributed 12-14.5% of global emissions (FAO, 2023). Enteric methane (CH_4) alone accounts for about 60-65% of livestock-related emissions, corresponding to 4% of the total global anthropogenic GHG emissions (FAO, 2023). Methane is particularly relevant for climate change mitigation due to its short atmospheric lifetime and high global warming potential, meaning that reducing CH_4 emissions can provide rapid and effective climate benefits.

Ruminants play a central role in this scenario. The global domestic ruminant population (cattle, buffalo, goats, sheep and camels) increased by 66% between 1960 and 2017 and is expected to continue growing, further amplifying livestock-related CH_4 emissions (World Food and Agriculture - Statistical Pocketbook, 2018). At the same time, ruminant products such as milk and meat remain critical sources of essential nutrients for human populations, particularly in regions with limited alternative protein sources. Ruminants have a unique ability to degrade fibrous plant material, thanks to their specialized rumen microbial community, which includes bacteria, protozoa, fungi, and archaea (Henderson et al., 2015). During ruminal fermentation, CH_4 is produced by methanogenic archaea as a product of microbial metabolism, particularly during the breakdown of cellulose and other complex carbohydrates. Methane production varies between individuals depending on species, breed, total feed intake, and diet composition. As a result, ruminants can lose between 2-12% of the gross energy consumed as enteric CH_4, with lower losses occurring on high-concentrate diets as compared to high-fiber diets, representing both an environmental concern and an inefficiency in energy utilization (Tapio et al., 2017; Beauchemin et al., 2020).

In recent years, several mitigation strategies have been proposed to reduce enteric CH_4 emissions. Among these, dietary interventions using red macroalgae of the genus *Asparagopsis*, such as *A. taxiformis* (AT) and *A. armata*, have emerged as one of the most promising approaches. Both *in vitro* and *in vivo* studies have demonstrated that *Asparagopsis spp.* can

reduce CH_4 emissions by up to 90% or more when included at 0.2- 0.5% of the diet dry matter (DM) (Machado et al., 2016; Roque et al., 2019; Kinley et al., 2020). The primary mechanism is linked to halogenated compounds, particularly bromoform ($CHBr_3$), which inhibit methanogenesis in the rumen (Stefenoni et al., 2021).

Despite these promising findings, several critical questions remain. Concerns include the risk of accumulation of $CHBr_3$, bromine (Br_2) and iodine (I_2) in animal tissues and milk, possible impacts on animal health and productivity, and the stability of the antimethanogenic effect over time (Muizelaar et al., 2021; Glasson et al., 2022). Additionally, changes in ruminal and faecal microbial communities following *Asparagopsis spp.* supplementation are not yet fully understood, raising questions about the long-term consequences for host–microbe interactions and overall gut functionality (Roque et al. 2021).

Another important dimension is the production and quality of the algae itself. *Asparagopsis spp.* naturally grows in coastal marine waters but has also been cultivated on land under controlled conditions. The concentration of bioactive compounds in these red algae can vary greatly depending on environmental and cultivation conditions, which may strongly affect its efficacy as a CH_4 inhibitor (Vucko et al., 2017; Paul et al., 2006a).

This highlights the importance of a multidisciplinary approach, bridging animal trials, microbiological studies, assessments of algal biomass quality, and sustainable cultivation systems.

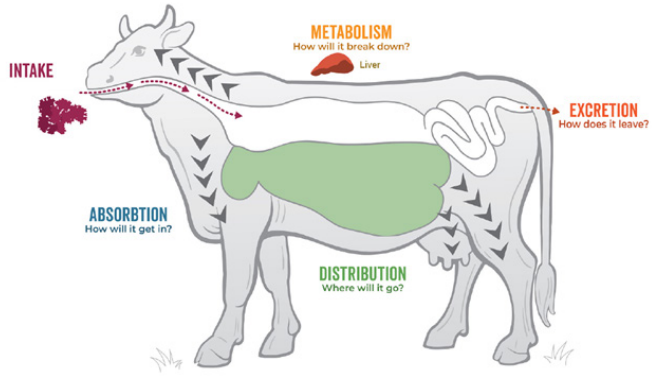

Figure 1. Key steps in how *A. taxiformis* is processed in the animal: absorption, distribution, metabolism and excretion. Reproduced from Glasson et al. (2022). Licensed under CC BY-NC-ND 4.0. no changes were made.

2. Background

2.1 Biochemical pathways in the rumen

Carbohydrates represent the primary source of energy in the dairy cow diet, typically accounting for about 70% of total dietary energy (Dann et al., 2014). Their central role helps explain why the rumen fermentation of these substrates is so critical for ruminant nutrition. Within the rumen, microorganisms ferment dietary carbohydrates, producing volatile fatty acids (VFAs), mainly acetate, propionate, and butyrate, which supply the majority of the cow's metabolizable energy. In parallel, microbial growth during fermentation produces microbial protein, which not only constitutes the principal protein source absorbed by the animal but also contributes to the overall energy supply available to the cow (Bergman, 1990; Janssen, 2010). This fermentation process also produces gases, including carbon dioxide (CO_2), hydrogen (H_2), and CH_4. Methanogenic archaea in the rumen mainly use H_2 to reduce CO_2 through hydrogenotrophic methanogenesis, forming CH_4. This process helps stabilise fermentation, but results in an energy loss of 2–12% of gross energy intake (Johnson and Johnson, 1995; Knapp et al., 2014). In addition to hydrogenotrophic methanogens, some methanogens, such as methylotrophic species that use methyl compounds like methanol or methylamines, and acetoclastic species that split acetate into CH_4 and CO_2, can also produce CH_4 (Jeyanathan et al., 2024). However, both methylotrophic and acetoclastic pathways contribute only a small proportion of total CH_4 production in the rumen.

Nutritional interventions aiming to alter VFA profiles or create alternative electron sinks are under active investigation. Approaches such as high-starch diets, supplementation with alternative electron acceptors (nitrate, fumarate), or feed additives including tannins, algae-derived compounds, and 3-nitrooxypropanol have shown promise in reducing methanogenesis without compromising fermentation (Hristov et al., 2015; Roque et al., 2021). These strategies shift VFA production towards a propionate decrease in H_2 availability for methanogens, lowering CH_4 emissions and enhancing energy efficiency.

Understanding the biochemical interplay between carbohydrate fermentation, H_2 turnover, and methanogenesis is therefore essential for improving ruminant productivity while mitigating greenhouse gas emissions.

2.1.1 Hydrogen and VFA production

During ruminal fermentation, carbohydrates from the feed are broken down and fermented by a diverse community of fibrolytic bacteria, amylolytic bacteria, protozoa and anaerobic fungi. The carbohydrates are metabolised into short-chain end products, mainly VFAs (Hagen et al., 2021; Li et al., 2021). A key feature of this process is the production of H_2, which acts as a central intermediate in microbial energy metabolism (Kelly et al., 2022). Fermentative microbes generate reduced cofactors, such as NADH, during glycolysis and other metabolic pathways, and these must be re-oxidised to maintain redox balance. One of the main disposal routes for these electrons is the formation of H_2, which is subsequently released into the rumen fluid (Janssen, 2010; Ungerfeld, 2015). If H_2 accumulates, it can increase the redox potential and specifically inhibit more oxidised fermentation pathways, such as those leading to acetate, thereby shifting fermentation towards more reduced end products rather than inhibiting fermentation (Ungerfeld, 2015). Hydrogenotrophic methanogens fulfil the main removal role for H_2, although alternative electron sinks can also contribute when available (Ungerfeld, 2015).

The volatile fatty acids are the major end-products of carbohydrate fermentation and represent the primary source of metabolizable energy for the ruminant (Bergman, 1990). Acetate, the most abundant, is essential for lipogenesis and milk fat synthesis (Sutton et al., 2003). Propionate serves as the main gluconeogenic precursor, supporting glucose homeostasis and lactose synthesis, while butyrate is largely metabolised by rumen epithelial cells and supports epithelial development and energy supply (Sutton et al., 2003). The proportion of VFAs produced vary with diet, with acetate generally being the main VFA. Forage-based diets tend to promote acetate production, whereas concentrate-rich diets increase the relative proportion of propionate, affecting energy partitioning and CH_4 output (Kessel and Russell, 1996).

The relationship between VFAs and hydrogen metabolism is tightly interconnected. Acetate and butyrate formation generate reducing equivalents, thereby releasing H_2, whereas propionate formation consumes reducing equivalents and serves as an alternative electron sink (Ungerfeld, 2015). As a result, the balance of VFA profiles has direct implications for CH_4 production.

2.1.2 Methanogenesis in the rumen

In the rumen, methanogens are carried out predominantly by archaeal species within the phylum *Euryarchaeota*. These hydrogenotrophic methanogens utilise H_2 and CO_2 as their main substrates for CH_4 formation, although some rumen species are also capable of using methylated compounds or acetate to a lesser extent (St-Pierre et al., 2015; Balch et al., 1979). The dominant genera in the rumen are *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera*, which mainly utilize H_2 and CO_2, while *Methanosarcina*, less common in this environment, can also metabolize methylated compounds (Thauer et al., 2008; Hook et al., 2010; Tapio et al., 2017).

Within these genera, *Methanobrevibacter* is typically the most abundant in the rumen, and recent phylogenomic analyses have revealed that *Methanobrevibacter* species cluster into two major clades: Ruminantium and Gottschalkii. These clades differ in several genomic and physiological traits, including hydrogen affinity, cell-surface structures, and the composition of *mcr*A gene variants (Danielsson et al., 2017). Such clade-level distinctions are thought to reflect adaptation to different ruminal hydrogen niches, with Ruminantium-associated species prevailing under lower H_2 availability, while members of the Gottschalkii clade may be better suited to conditions where H_2 concentrations are higher.

Methanogens possess unique cofactors, including coenzyme F_{420}, which functions in electron transfer, and coenzyme M (HS–CoM) and coenzyme B (HS–CoB), which participate in the final methane producing step catalysed by the methyl-coenzyme M reductase (MCR). The MCR contains the nickel-tetrapyrrole cofactor F_{430}(Glasson et al., 2022).

In the rumen, methanogenesis proceeds mainly through the hydrogenotrophic pathway:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

This process involves several enzymatic steps; hydrogenases oxidize H_2 and transfer electrons to F_{420}, which reduces methanopterin-bound C1 units. The methyl group is then transferred to coenzyme M, forming methyl-coenzyme M (CH_3–S–CoM), which is finally reduced to CH_4 by the MCR complex (Thauer et al., 2008; Ferry, 2011).

Alternative pathways, such as acetoclastic methanogenesis (splitting acetate into CH_4 and CO_2) and methylotrophic methanogenesis (using methanol or methylamines), are known to exist but appear to play only a minor role in the rumen compared with hydrogenotrophic methanogenesis (Hook et al., 2010).

Methanogens are not the only potential hydrogenotrophs in this environment. Other microbes, including reductive acetogens, sulfate-reducing bacteria, and nitrate- or nitrite-reducing bacteria, can also consume H_2, thereby competing with methanogens for reducing equivalents. From a thermodynamic perspective, several of these alternative pathways (e.g. sulfate reduction, denitrification) are more favourable electron-accepting processes than CO_2 reduction to CH_4. However, in the rumen, their overall contribution to H_2 consumption is usually limited, mainly because suitable alternative electron acceptors (e.g. sulfate, nitrate, nitrite, fumarate) are present at low or irregular concentrations, whereas CO_2 is abundant and continuously produced (Ungerfeld, 2015).

Under these conditions, hydrogenotrophic methanogenesis becomes the dominant sink for H_2, allowing ruminal microbes to maintain low H_2 partial pressures that favour fermentative metabolism and VFA production (Morgavi et al., 2010).

2.2 Halogen compounds in *Asparagopsis spp.* and their effects on rumen function and animal health

2.2.1 Bromine, iodine and bromoform

Halogenated molecules such as Br_2 and I_2 occur naturally in marine macroalgae, where they play important ecological and physiological roles (Dembitsky, 2006). In addition to these elemental halogens, macroalgae also produce halogenated organic compounds, including $CHBr_3$ and many other brominated and iodinated metabolites.

Bromoform has received particular attention due to its potential to inhibit methanogenesis in ruminants. Species of the red seaweed genus *Asparagopsis* (family *Bonnemaisoniaceae*), notably AT and *Asparagopsis armata*, are especially rich in halogenated compounds, including $CHBr_3$ (Machado et al., 2018). However, $CHBr_3$ concentration in *Asparagopsis spp.* is highly variable and depends on several factors.

The biosynthesis of halogenated compounds requires halogen availability, primarily bromide (Br^-) and iodide (I^-) in seawater. Bromine is directly involved in $CHBr_3$ production (Paul et al., 2006a), while I_2 contributes to the synthesis of other halogenated metabolites, such as methyl iodide (Dembitsky, 2006). Environmental conditions during growth, including temperature, light intensity, salinity, and nutrient concentrations, strongly influence halogen uptake and subsequent metabolite biosynthesis (Werner et al., 2004; Zhu et al., 2021; Stefenoni et al., 2021; Hutchings et al., 2024).

The mineral and halogen composition of *Asparagopsis spp.* also varies between wild and cultivated populations. Open-water cultivation systems face challenges due to fluctuating environmental conditions, which affect both growth performance and metabolite composition (Werner et al., 2004; Zhu et al., 2021). Controlled aquaculture offers a potential solution, but maintaining consistent $CHBr_3$ levels remains difficult. Parameters such as culture depth, biomass density, and nutrient supplementation are critical in shaping both the mineral profile and the halogenated metabolite content of the biomass (Stefenoni et al., 2021; Hutchings et al., 2024).

2.2.2 Effects on rumen and faecal microbiome

Halogenated alkanes such as $CHBr_3$, the main bioactive compound in *Asparagopsis spp*., shows its antimethanogenic effect through the inhibition of methanogenic archaea. Bromoform interferes with key metalloenzymes involved in methanogenesis, blocking both methyl group transfer and the final reduction step leading to CH_4 formation (Glasson et al., 2022*). In vitro* and *in vivo* trials have shown a strong reduction in methanogenic activity, accompanied by a decrease in methanogen abundance in the rumen, without a complete influence on the overall microbial community (Machado et al., 2018; Roche et al., 2019). This inhibition results in a reduction of enteric CH_4 emissions and a concomitant accumulation of H_2 in the rumen (Roque et al., 2019), which may alter the metabolic balance within the rumen microbiome.

The increase in ruminal H_2 can influence the activity of other microbial groups and fermentation pathways. When methanogenesis is suppressed, alternative H_2 sinks may be stimulated, including reductive acetogenesis and increased propionate formation (Denman et al., 2015). Several studies have

reported alterations in VFA profiles, with a consistent reduction in the acetate:propionate ratio due to a relative increase in propionate production (Machado et al., 2018; Krizsan et al., 2023). This shift is of particular interest, as propionate production acts as a major electron sink and provides gluconeogenic precursors, thereby potentially enhancing the efficiency of energy utilization by the ruminant. Butyrate concentrations have shown variable responses, sometimes increasing slightly or remaining unaffected (Roche et al.,2019).

At the microbial level, the inhibition of methanogens by $CHBr_3$ not only reduces their abundance but may also indirectly affect hydrogen-producing bacterial populations. For example, in studies on AT supplementation, a reduction in the relative abundance of *Ruminococcus* (a key fibrolytic genus) has been observed under elevated H_2 partial pressure (Li et al., 2025; Romero et al., 2023), whereas propionate-producing bacteria such as *Prevotella* tend to increase in relative abundance under these conditions, thereby enhancing propionate formation (Betancur-Murillo et al., 2022; Li et al., 2025). Despite these variations, overall rumen bacterial diversity does not appear to be drastically reduced, indicating that the microbial ecosystem adapts by redistributing metabolic roles in response to altered H_2 dynamics.

In addition to its effects on the ruminal microbiota, dietary inclusion of *Asparagopsis spp.* could also have implications for post-ruminal fermentation and the faecal microbiome. Modifications occurring in the rumen, such as altered fibre degradability, VFA profiles, and microbial populations, can influence the amount and composition of undigested organic matter reaching the hindgut and, subsequently, excreted (Huhtanen et al., 2021).

Changes in substrate availability for hind-gut fermentation may alter the diversity and metabolic activity of faecal microorganisms. Although direct studies on the effects of AT in the faecal microbiome are lacking, evidence from the rumen indicates that this red alga alters microbial composition and fermentation patterns, such as decreasing methanogens and altering the VFA profile in rumen (Roque et al., 2019).

2.2.3 Effects on animal health and performance

Supplementation with *Asparagopsis* spp. has been associated with the transfer of halogenated compounds into animal-derived products. Krizsan et al. (2023) reported increased concentrations of Br_2 and I_2 in the milk of cows

receiving 0.5% of AT on OM based, indicating that dietary halogenated molecules can be absorbed and secreted into milk. However, information on their broader metabolic fate remains limited, particularly in terms of the excretion through faeces or urine, leaving it unclear whether these halogens accumulate systemically or are largely eliminated via digestive or urinary pathways.

Similarly, Romero et al. (2023) reported that most $CHBr_3$ is rapidly degraded in the rumen, undergoing microbial conversion to dibromomethane with a half-life of approximately 26 minutes, implying limited systemic absorption and minimal tissue accumulation. Muizelaar et al. (2021) observed that small amounts of unmetabolized $CHBr_3$ may be excreted in urine and milk, with no detectable accumulation in muscle or liver tissues. These findings highlight the need to monitor supplementation levels to ensure food safety and animal health.

The inclusion of *Asparagopsis spp*. in dairy cow diets has been tested across a range of levels. At low inclusion rates (~0.12 – 0.25% OM), most studies report no consistent systemic adverse effects (e.g., limited changes in measured health parameters), but these low-dose treatments often do not substantially reduce enteric CH_4 and are sometimes associated with reduced dry matter intake (DMI), altered rumen fermentation, and, in some studies, local rumen lesions or adverse production effects (Muizelaar et al., 2021; Stefenoni et al., 2021). Moreover, Histological examinations in Muizelaar et al. (2021) identified rumen wall abnormalities (granulomatous and keratotic changes) in some animals, suggesting that even at relatively low inclusion levels there may be sub-clinical tissue effects.

Li et al. (2018) observed that supplementation with AT in sheep led to increases in blood cholesterol, urea, and total bilirubin concentrations over time, although all values remained within normal clinical limits. Liver enzymes and other standard blood metabolites were not affected, and no systemic toxicity was reported. However, parameters related to oxidative status or plasma antioxidant capacity (e.g., FRAP) in dairy cows were not assessed, underscoring the need for further investigation into systemic metabolic effects.

Regarding product quality, elevated I_2 and Br_2 levels in milk may influence human dietary intake and are therefore important considerations for ruminant nutrition. In dairy cows, the maximum tolerable level (MTL) of I_2 is approximately 5 mg/kg DM, while recommended dietary concentrations

range from 0.5 to 1 mg/kg DM to maintain normal physiological function without approaching toxic levels (EFSA, 2013). Although no dietary requirement has been established for Br_2, AT supplementation can increase Br_2 levels in milk; however, the concentrations reported so far remain below levels considered harmful for cattle.

From a human nutrition perspective, the recommended daily I_2 intake is 90–150 µg/day, with a tolerable upper intake level of 600 µg/day (EFSA 2013; Blomhoff et al., 2023). For Br_2, no official dietary requirement has been established, but the acceptable daily dose for humans is estimated at 0.4 mg/kg body weight, corresponding to ~28 mg/day for a 70 kg adult (Van Leeuwen et al., 1987). The elevated I_2 and Br_2 in milk from AT-supplemented cows may contribute to human intake, but current evidence indicates that levels remain within established safety limits. Importantly, no consistent changes in milk fat or protein content have been observed with AT supplementation (Krizsan et al., 2023).

2.3 Algal biomass for CH_4 mitigation

2.3.1 Variations in bioactive compound concentration

The antimethanogenic potential of *Asparagopsis spp.* is closely linked to its $CHBr_3$ content, which inhibits key enzymes involved in ruminal methanogenesis (Glasson et al., 2022). While $CHBr_3$ concentration is highly variable due based on species, growth stage, and environmental conditions (Camer-Pesci et al., 2023; Vucko et al., 2017), these variations directly impact the consistency of CH_4 mitigation when the algae are used as a feed additive.

Bromoform and other halogenated compounds are synthesized and stored within specialized gland cells in the algal thallus. The abundance and activity of these cells are strongly influenced by environmental stressors and mineral availability (Hargrave et al., 2024). For example, sufficient Br_2 supply is required for the halogenation process, whereas Br_2 limitation significantly reduces $CHBr_3$ biosynthesis (Paul et al., 2006). Similarly, light intensity has been shown to affect both the number and functionality of gland cells, with excessive or insufficient irradiance leading to lower $CHBr_3$ accumulation (Hargrave et al., 2024).

Temperature and nutrient balance also play key roles, as suboptimal conditions may alter metabolic fluxes and enzyme activity involved in halometabolite synthesis (Resetarits et al., 2024). As a result, *Asparagopsis spp.* grown under variable cultivation or environmental regimes often exhibits substantial differences in $CHBr_3$ concentrations, sometimes exceeding an order of magnitude between algae batches. This biochemical variability translates directly into inconsistent CH_4 mitigation efficacy when red algae is used as a feed additive. Understanding and controlling these factors is therefore critical to achieving reliable and predictable antimethanogenic performance in livestock systems.

2.3.2 Sustainability and scalability challenges

While wild harvesting has provided valuable material for research, it is ecologically unsustainable and cannot meet the biomass demand for large-scale livestock supplementation (Roque et al., 2021). Controlled cultivation systems, both land-based and offshore, are being developed to enable year-round production, yet each presents technical and environmental challenges. Land-based raceways and tanks allow precise control of growth parameters but require substantial energy, nutrient, and water inputs, whereas open-water systems are more resource-efficient but subject to fluctuating environmental conditions that affect yield and compound concentration (Zhu et al., 2021). Moreover, maintaining stable populations of *Asparagopsis spp.* in aquaculture remains difficult due to its complex life cycle and sensitivity to abiotic stress (Werner et al., 2004). These factors raise questions about the long-term sustainability, scalability, and cost of production necessary to support commercial application in ruminant systems.

2.3.3 Implications for large-scale adoption in ruminant production systems

Ensuring a stable and high-quality supply of *Asparagopsis spp*. biomass is crucial for ruminant feed applications, emphasizing the need for controlled cultivation systems (Glasson et al., 2022). However, variations in $CHBr_3$ and other halogenated compounds can cause inconsistent CH_4 reduction results and may raise concerns around animal health, product safety, and regulatory approval (Stefenoni et al., 2021; Krizsan et al., 2023). Furthermore, the environmental impact of large-scale cultivation may result in the release of nutrients such as nitrogen and phosphorus compounds into the surrounding

environment. If not properly managed, these nutrient emissions can contribute to eutrophication, algal blooms, and oxygen depletion in coastal ecosystems, potentially offsetting the intended climate benefits of the intervention. Therefore, environmental impacts, including nutrient management and high energy use, must be carefully evaluated to ensure that large-scale cultivation remains sustainable (Nilsson and Martin, 2022). Life cycle assessments of land-based *Asparagopsis spp.* cultivation systems indicate that maintaining optimal temperature and salinity in land-based systems can produce significant greenhouse gas emissions, emphasizing the need for process improvements and the use of renewable energy (Nilsson and Martin, 2022). Given these challenges, it is important to understand how cultivation conditions affect the biochemical composition and methane-reducing capacity of *Asparagopsis spp*.

In addition to cultivation parameters, post-harvest handling can markedly affect the stability and efficacy of halogenated metabolites. The concentration of $CHBr_3$ and related compounds may decrease during drying, storage, and processing, depending on the method and duration used. High-temperature drying or prolonged storage has been associated with substantial losses of $CHBr_3$ from *Asparagopsis spp.* biomass, with a loss of around 74% at 40°C over 24 weeks in freeze-dried material (Tan et al., 2023). Moreover, handling practices during feed preparation and delivery to animals can further influence the stability and bioavailability of these halogenated compounds. Exposure to air, heat, or delays between mixing and feeding may lead to volatilizing of $CHBr_3$ and a reduction in its antimethanogenic potential.

Because $CHBr_3$ is heat-sensitive, it is not suitable for incorporation into concentrates that are pelleted or otherwise exposed to high temperatures. Instead, it is best mixed directly into total mixed rations (TMR) or fed immediately after preparation to preserve its bioactivity. Proper incorporation of the algae into the diet immediately before feeding, or stabilization with a carrier matrix such as oil, has been shown to improve the retention and consistency of $CHBr_3$ (Magnusson et al., 2020).

3. Aim of the Thesis

The overall aim of this thesis is to provide an integrated assessment of the use of *Asparagopsis spp*. as an inclusion to the feed ration for dairy cows. Specifically, this thesis evaluates its potential to reduce enteric CH_4 emissions, considering animal performance and health (Paper **I**), rumen and faecal microbiota (Paper **II**), and the influence of cultivation and handling on algal $CHBr_3$ content and antimethanogenic efficacy (Paper **III**).

Specifically, this thesis addresses the following objectives:

- Evaluate the effects of *A. taxiformis* on enteric CH_4 emissions in dairy cows through a 12-week *in vivo* study, while assessing performance and metabolic parameters, as well as quantifying Br_2 and I_2 concentrations in milk and Br_2 excretion in urine and faeces to evaluate potential residue transfer and environmental excretion. These outcomes have not been fully addressed in previous short-term or *in vitro* studies.

- Investigate the impact of *A. taxiformis* supplementation on ruminal and faecal microbial communities and assess the implications for host–microbe interactions and the microbial mechanisms underlying CH_4 mitigation, providing insights beyond simple microbial enumeration or short-term fermentation studies.

- Determine how cultivation and handling conditions influence the concentration of bioactive compounds in *Asparagopsis spp.* and its antimethanogenic efficacy, linking environmental and handling factors to $CHBr_3$ content and practical methane-reducing performance in ruminant nutrition.

4. Materials and Methods

For Papers **I** and **II**, the materials and methods have been merged to provide a unified description of the *in vivo* experiment, including both the effects of AT supplementation on animal performance and metabolism, and the assessment of ruminal and faecal microbial composition and functional groups. Paper **III**, which describes an *in vitro* evaluation of *Asparagopsis spp*. cultivated under varying environmental conditions, is presented in a separate subsection.

4.1 *Asparagopsis taxiformis* effects on dairy cow performance, metabolism, and the microbiomes of rumen and faeces

4.1.1 Animals and Experimental design

The *in vivo* experiment (Paper **I** and **II**) was conducted at the Swedish University of Agricultural Sciences (SLU), Umeå, Sweden, between January and April 2022. All animal procedures were approved by the regional ethics committee in Umeå, Sweden, and conducted in accordance with Swedish legislation on animal studies. Thirty Nordic Red dairy cows (9 primiparous and 21 multiparous), averaging 61 ± 25 days in milk (DIM) and producing 32.7 ± 8.7 kg of energy-corrected milk (ECM) per day, were blocked by parity and DIM and randomly assigned to one of three dietary treatments: control (CON; 0 % AT), low AT inclusion (L-AT; 0.15 % of OM), or high AT group (H-AT; 0.3 % of OM). Cows were housed in a free-stall barn with an automated feeding system, offered a TMR formulated according to the Nordic feed evaluation system (NorFor) with 50:50 grass/clover silage and concentrate on DM basis, and milked twice daily in a parlour. The AT, collected in the Azores, preserved through freeze-drying and milled, was added and mixed into the TMR using a custom-built system that ensured precise dosing. The experiment lasted 13 weeks, including an adaptation period of two weeks prior to the study, during which the cows adapted to the basal diet, became familiar with the assigned feed bins and were introduced to the GreenFeed methane measurement system (C-Lock Inc., Rapid City, SD), as described by Huhtanen et al. (2015).

4.1.2 Sample collection

During the trial, feed samples (silage and concentrate) were collected twice a week, to monitor diet composition and adjust rations according to DM content. Silage and concentrate samples were then pooled over a two-week period. Milk yield was automatically recorded at each milking for all cows. Milk samples for milk composition analysis were collected in the morning and afternoon milkings on two consecutive days every second week, and additional samples were taken at weeks 0, 4, and 12 for analysis of Br_2 and I_2 associated with the algae inclusion. Enteric CH_4 and H_2 emissions were continuously recorded using the GreenFeed system throughout the

experiment. From a randomized subset of six cows per treatment, rumen fluid was collected after the morning milking at weeks 0, 2, 4, and 12. Faeces and urine were sampled at weeks 0, 2, 4, 8, and 12, while blood samples from the same subset were obtained during the morning milking at weeks 0, 4, and 12.

4.1.3 Laboratory analyses

4.1.3.1 Biochemical analysis - Paper **I**

Feed and algae samples were analysed for DM and nutrient composition at the Department of Applied Animal Science and Welfare, the Swedish University of Agricultural Sciences, Uppsala, Sweden, for digestibility at the same department in Umeå, Sweden, and fermentation quality at the Department of Molecular Sciences, the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Milk samples were assessed for fat, protein, and lactose using infrared spectroscopy (MilkoScan FT120; Foss, Hillerød, Denmark) at the Department of Applied Animal Science and Welfare, SLU, Uppsala, Sweden. Additional milk samples were analysed for I_2 and Br_2 at Eurofins Food and Feed Testing, Lidköping, Sweden, following DS-EN 15111m:2007. Urine and faecal samples were also analysed for I_2 and Br_2 at Eurofins.

For indigestible neutral detergent fibre (iNDF) determination, feed and faecal subsamples were incubated in fistulated cows and subsequently analysed at the Department of Applied Animal Science and Welfare, SLU, Umeå, Sweden. Blood samples were analysed for metabolites and oxidative stress markers at the Department of Animal Sciences, Food and Nutrition, the Faculty of Agriculture, Food and Environmental Science, Università Cattolica del Sacro Cuore, Piacenza, Italy.

4.1.3.2 Microbial community analysis - Paper **II**

For microbial community analyses, DNA was extracted from rumen fluid and faecal samples using the FastDNA® Spin Kit for Soil (MP Biomedicals, United States). Microbial composition was characterized through 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform at SciLifeLab (Uppsala, Sweden). The V4 region of the 16S rRNA gene was amplified using the universal primers 515′F and 806′R (Hugerth et al. 2014), while archaeal community validation was performed with the primer pair 519′F

and 806′R at Novogene (Cambridge, UK). Bioinformatic analyses were conducted using established pipelines (dada2, phyloseq, and DESeq2) in R. Quantitative PCR (qPCR) was used to estimate total methanogen abundance. Amplification employed the primer set Met630′F3 and Met803′R3(Yu et al., 2005) on a QuantStudio™ 5 thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA). Standard curves (10^1–10^9 gene copies) showed linearity (r^2 = 0.993–0.999) with efficiencies of 90.8–99.6%.

4.1.4 Statistical analyses

For production, nutrient, and physiological data (Paper **I**), linear mixed-effects models were used, including the treatment and experimental week as fixed effects, block as a random effect, and week 0 as a covariate. Repeated measures were modelled using an autoregressive correlation structure AR (1) to account for within-cow temporal autocorrelation. Least square means were estimated, and treatment differences were evaluated using Tukey adjustment at a significance level of $p < 0.05$. Welch's two-sample t-test was applied to compare Br_2 concentrations in faeces and urine between CON and H-AT cows.

For microbial community data (Paper **II**), alpha and beta diversity metrics, PERMANOVA, and differential abundance analyses were conducted to evaluate the effects of treatment and time. Pearson′s Correlation between total methanogen abundance and enteric CH_4 emissions was also assessed. All the statistical analyses were performed with R (version 4.3.3).

4.2 *In vitro* assessment of *Asparagopsis spp.* grown under variable cultivation conditions

4.2.1 Sample collection and preparation

Six samples of *Asparagopsis spp*. macroalgae were evaluated for their potential to reduce CH_4 emissions: five cultivated batches (VG1–VG5) produced by Volta Greentech (Gothenburg, Sweden) under controlled tank-based conditions, and one wild-harvested batch (AT) from the Azores, Portugal, which had been used in the previous *in vivo* study (Paper **I**). Cultivated batches were grown under varying depth, light, and biomass density (Table 1), maintaining constant water quality, temperature (16–

20 °C), nutrient levels, salinity (>30 ppt), and pH (7.5–8.0). After harvest, the algae was rinsed, centrifuged, freeze-dried, milled, and stored at −20 °C until use. The experimental substrate consisted of 60% grass-clover silage and 40% commercial concentrate on DM basis. Algae was included at 0.5% of feed OM, and a control diet (CTR) without algae was also included. Feed ingredients were dried, milled (1 mm), and stored at room temperature before incubation.

Table 1. Specific cultivation parameters applied to the five tank-grown batches (VG1–VG5), including culture depth, light intensity, and biomass density.

Cultivated batches	Culture depth (cm)	Biomass density (g/L)	Light intensity (PPFD)
VG 1	50	1	80
VG 2	34	1	640
VG 3	34	3	640
VG 4	30	1	350
VG 5	30	3	sunlight

4.2.2 *In vitro* experimental setup and sample collection

Rumen fluid was collected from two cannulated dairy cows fed the same basal diet. Fluids were mixed, filtered, and combined with a buffer solution (rumen fluid-to-buffer ratio 1:4, v/v); they were continuously stirred and flushed with CO_2. Feed ingredients and algae were added to serum bottles, which were incubated in a water bath at 39 °C for 48h with continuous agitation. All treatments were performed in triplicate and replicated in two separate runs, each including blank bottles (rumen buffer only). Total gas was recorded using a Gas Production Recorder (GPR-2), while CH_4, and H_2 concentrations were measured with gas chromatography. Liquid samples were collected at 0 and 48h for VFA analysis by HPLC.

4.2.3 Chemical Analyses

Feed and algae samples were analysed for DM, crude protein (CP), starch, ash, water-soluble carbohydrates (WSC), neutral detergent fiber (NDF), ether extract (EE), and *in vitro* OM digestibility. Bromoform content and

mineral composition (macro-, microelements, heavy metals) were analysed at Scantox, Scandinavia AB, Sweden, at Scandinavia AB.

4.2.4 Calculation and Statistical analysis

Gas production values were blank-corrected and the predicted *in vivo* CH_4 emissions were calculated following the equation from Ramin and Huhtanen (2013), assuming a mean retention time of 50h. Predicted gas and VFA production were analysed using linear mixed-effects models in R (lmer, lme4 package). CH_4 and H_2 kinetics were analysed including time as a fixed effect with the AR (1) correlation structure to account for temporal autocorrelation. Relationships among fermentation parameters ($CHBr_3$ vs CH_4; Br_2 vs $CHBr_3$) were assessed via Pearson correlation and visualized with scatterplots and regression lines using ggplot2 and related R packages.

5. Results

This chapter presents the main findings from Papers **I** and **II,** which evaluated the effects of AT supplementation on CH_4 mitigation, rumen fermentation, microbial dynamics, and systemic metabolism in dairy cows. Paper **III** specifically reports the outcomes of an *in vitr*o experiment designed to assess how *Asparagopsis spp.* cultivated under variable environmental conditions influences its inhibitory effect on methanogenesis, through differences in halogenated compound concentrations.

5.1 Paper **I**: Effects of *Asparagopsis taxiformis* on dairy cow performance and metabolism

There was supplementation with AT affected gaseous emissions, particularly CH_4, which was reduced by approximately 30% in H-AT and by 7.6% in L-AT compared with the CON. The reduction in CH_4 was accompanied by a substantial increase in H_2 production, rising by 383% and 70% in the H-AT and L-AT groups, respectively. Notably, the interaction between treatment and week indicated that the methane-suppressing effect began to diminish by week 9 of the experiment. By the final sampling point at week 12, the reduction in CH_4 was no longer evident. Thus, while the overall inhibition of CH_4 averaged 30%, the magnitude of the effect varied across weeks.

Dry matter intake and ECM yield were both negatively affected in the H-AT group, with reductions of 7% in DMI and 2% in ECM compared with the CON. The effect on CH_4 production, feed intake, and milk yield is shown in Figure 1, highlighting the significant CH_4 reduction in H-AT compared with the CON and L-AT. Similarly, in the H-AT group, NDF digestibility was reduced by 8% compared with the CON, while overall OM digestibility was unaffected by treatment. Milk composition remained largely unchanged, although milk fat was slightly lower in the H-AT group compared with the L-AT and CON.

Rumen fermentation profiles reflected the effects of AT supplementation. The total concentration of VFA was reduced in the H-AT group, with a lower acetate proportion and higher concentrations of propionate, butyrate, and valerate compared with the CON.

Throughout the experiment, the $CHBr_3$ content in pooled AT samples was measured, showing a reduction over the experimental time by 23% on a fresh-weight (FW) basis of the algae (Table 2). Supplementation with AT also increased Br_2 and I_2 concentrations in several biological matrices. Milk from cows fed the H-AT diet contained approximately five times more Br_2 and nine times more I_2 than milk from cows in the CON group. Similarly, Br_2 levels were four and nine times higher in faeces and urine samples respectively, from cows receiving the H-AT compared with the CON (Table 3). Metabolic profiling showed AT related effects on systemic metabolism. Cows in the H-AT group had reduced plasma cholesterol concentrations and lower ferric-reducing antioxidant capacity (FRAP), whereas plasma magnesium levels were elevated compared with the CON.

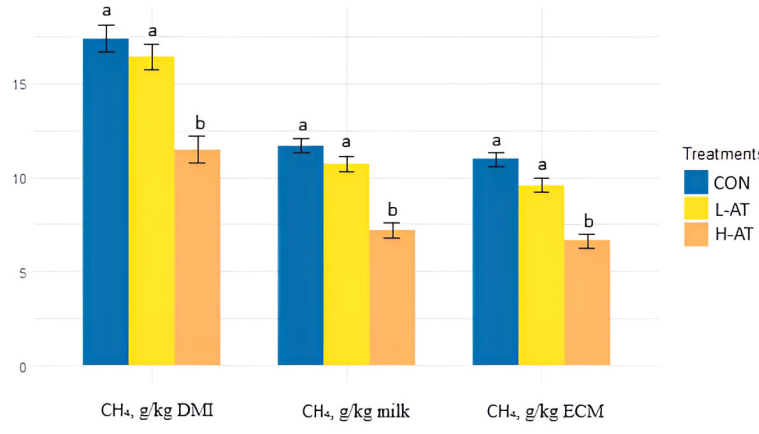

Figure 2. Effects of AT supplementation (CON 0 %, L-AT 0.15 %, H-AT 0.3 % AT on organic matter basis of the diet) on CH_4 production relative to intake (CH_4, g/kg DMI) and milk production parameters (CH_4, g/kg milk; CH_4, g/kg ECM). Values represent the estimated marginal means (Emmeans) per treatment group and week. Different letters above and within bars (a, b) indicate significant differences ($p < 0.05$) between treatments.

Table 2. Concentration of bromoform ($CHBr_3$) in *Asparagopsis taxiformis* (AT) pooled samples (A, B, C) collected at week 1, 4, 8, and 12 (n = 3). Values are expressed as mg/g on a fresh-weight basis (FW), mg/g dry matter (DM), and mg/g organic matter (OM).

AT samples	Week	$CHBr_3$, mg/g FW	$CHBr_3$, mg DM AT/kg OM feed	$CHBr_3$, mg OM AT/kg OM feed
A	2	6.6	18.1	35.5
B	4	7.2	19.8	39.3
C	12	6.0	16.5	36.6

Table 3. Concentrations of bromine (Br_2), iodine (I_2) and bromoform ($CHBr_3$) in *Asparagopsis taxiformis* (AT) and biological outputs from control group without AT inclusion (CON) and group with 0.3 % inclusion of AT at diet organic matter basis (H-AT) group.

		CON			H-AT		
	Input (AT)[1]	Output (milk)[2]	Output (faces)[3]	Output (urine)[3]	Output (milk)[2]	Output (faces)[3]	Output (urine)[3]
Br_2, g/kg	59 ± 5.9	3.9±1.5	6.1 ±0.6	3.3 ± 0.58	20.8±1.5	28.5 ± 3.0	29.7 ± 5.0
I_2, mg/kg	5.9 ± 0.4	0.1±0.8	ND	ND	0.9±0.8	ND	ND
$CHBr_3$, mg/kg	6.44 ± 0.5	ND	ND	ND	ND	ND	ND

[1]Pooled sample collected at week 1, 4, 8, and 12 (n = 4)

[2] Least squares mean ± SEM (n = 6); milk samples collected in weeks 0,4, and 12

[3]Samples collected in week 12 (n = 6); ND= not determined

5.2 Paper **II**: Effects of *Asparagopsis taxiformis* on rumen and faecal microbiome

In rumen fluid, archaeal diversity and composition were affected by supplementation with 0.3% AT on OM basis (H-AT). Alpha diversity decreased in the H-AT group compared with the CON, and beta diversity analysis revealed a clear separation between groups, as confirmed by PERMANOVA. Although *Methanobrevibacter spp*. remained the dominant genus in both treatments, species-level differences were evident.

M. olleyae showed higher relative abundance in H-AT, while *M. millerae* and *M. boviskoreani* had lower relative abundance compared with the CON. The relative abundance of *Methanomethylophilus* and UBA71 (order *Methanomassiliicoccales*) also decreased in H-AT (Figure 3), indicating a species-specific response of methanogens to AT supplementation. Quantification by qPCR showed a significant reduction in total methanogen abundance (SQ/ng DNA) in H-AT compared with the CON, and enteric CH_4 production (g/day) was correlated with the methanogenic abundance of the community profile in the H-AT group ($R^2 = 0.78$, $p < 0.001$).

The bacterial community in rumen fluid was also altered by AT supplementation. While alpha diversity was unaffected, beta diversity differed between H-AT and CON. The genus *Prevotella* dominated in both treatments. Several genera, including *Akkermansia*, *Acetomicrobium, Succinivibrio*, *Sharpea,* and *Romboutsia*, showed higher relative abundance in H-AT, whereas unclassified *Clostridia* and members of *Ruminococcaceae* had lower relative abundance compared with the CON (Figure.4). Correlation analyses revealed that, in H-AT, the relative abundance of *Prevotella*, *Anaerovibrio*, *Selenomonas A*, and *Cryptobacteroides* was positively associated with rumen propionate concentration ($0.01 \leq p \leq 0.001$).

In faecal samples, microbial diversity was not affected by 0.3% AT (OM based) supplementation; however, beta diversity and overall community composition differed between treatments. Genus *Lachnospiraceae* remained dominant and showed higher relative abundance in H-AT, while genera *Prevotella*, *Faecousia*, *Ruminococcus E*, *Clostridia*-NA, and *Cryptobacteroides* showed lower relative abundance (Figure.5). Differential abundance analysis confirmed that *Blautia A*, *Ruminococcus E*, and

Bifidobacterium had higher relative abundance in H-AT compared with the CON, whereas *Prevotella* and *Methanobrevibacter A* had lower relative abundance.

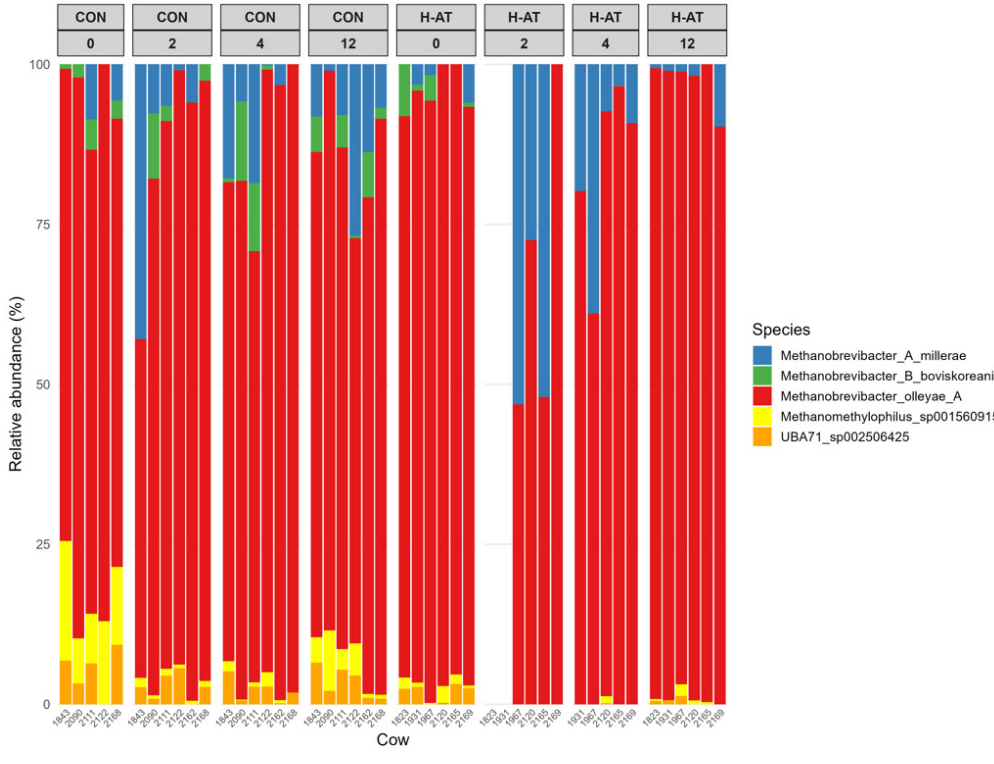

Figure 3. The stacked bar plot shows the relative abundance of archaeal species in rumen fluid samples from cows under the CON diet (0.0 % of AT on organic matter (OM) basis of the diet) and the H-AT diet (0.3 % of AT on OM basis of the diet) at weeks 0, 2, 4, and 12. Each bar represents an individual cow at a specific sampling time, and colours indicate different archaeal taxa annotated at the species level when possible.

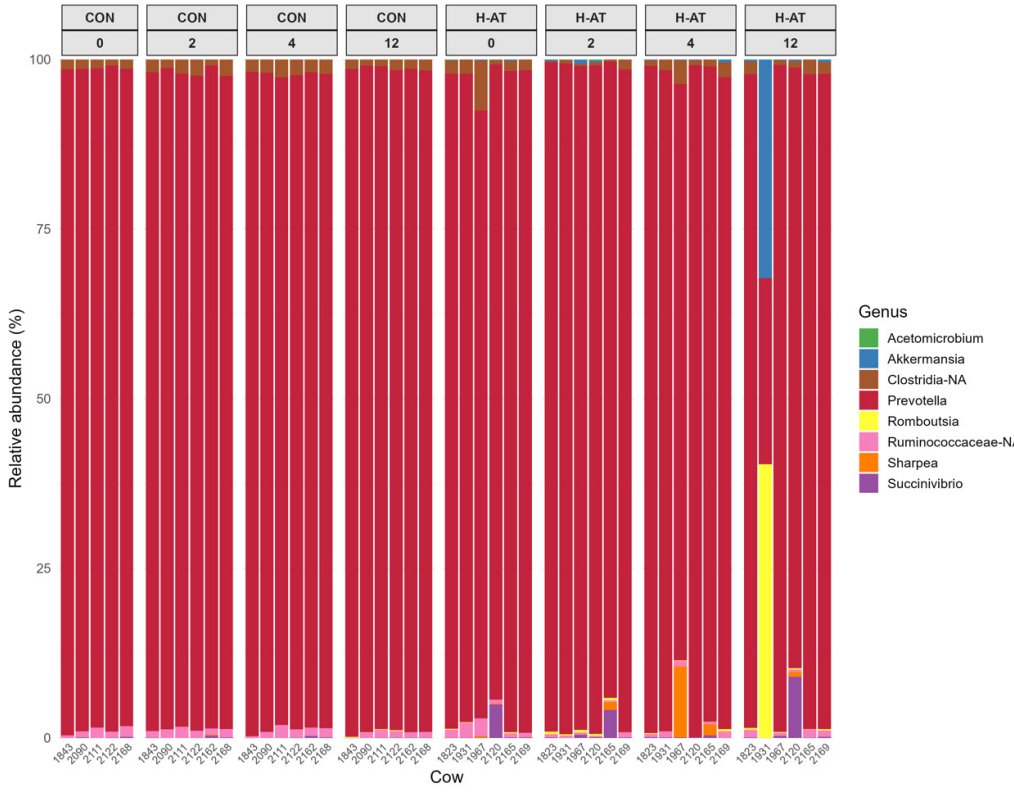

Figure 4. The stacked bar plot shows the relative abundance of bacterial genus in rumen fluid samples from cows under the CON diet (0.0 % AT on organic matter (OM) basis of the diet) and the H-AT diet (0.3 % AT on OM basis of the diet) at weeks 0, 2, 4, and 12. Each bar represents an individual cow at a specific sampling time, and colours indicate different archaeal taxa annotated at the genus level when possible.

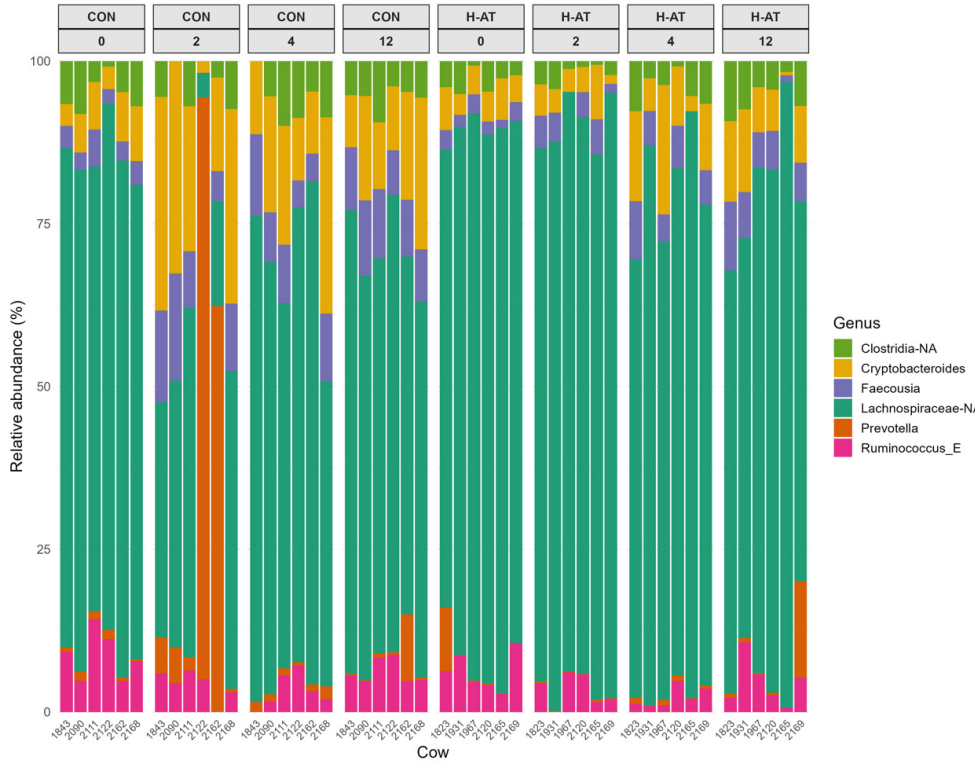

Figure 5. The stacked bar plot shows the relative abundance of bacterial genus in faecal samples from cows under the CON diet (0.0 % of AT on organic matter (OM) basis of the diet) and the H-AT diet (0.3 % of AT on OM basis of the diet) at weeks 0, 2, 4, and 12. Each bar represents an individual cow at a specific sampling time, and colours indicate different archaeal taxa annotated at the genus level when possible.

5.3 Paper **III:** Methane mitigation in dairy cows: *In vitro* assessment of *Asparagopsis spp*. grown under variable cultivation conditions

The inclusion of the different *Asparagopsis spp.* treatments in the *in vitro* fermentation system led to clear changes in CH_4 dynamics and fermentation profiles. The bromoform content of the algae varied substantially between algae samples. A Pearson linear correlation analysis showed a negative relationship between $CHBr_3$ (mg/g) concentration and CH_4 production (g/L) ($r = -0.86$, $R^2 = 0.74$). Moreover, Br_2 and I_2 concentrations also differed across algae samples, and Br_2 content was positively associated with $CHBr_3$ levels ($r = 0.75$, $R^2 = 0.56$). A broader mineral screening confirmed variability in mineral composition among algae samples.

Predicted *in vivo* CH_4 production (ml/g DM) was reduced by 83% and 28% in VG4 and VG5, respectively, compared with the CTR, whereas the other algae treatments did not differ from the CTR (Figure 6). While predicted total gas production (ml/g DM) remained unaffected, VG4 showed an increased rate of total gas production (/h). The proportion of CH_4 to total gas also varied substantially across treatments, with VG4 showing the lowest CH_4 production, approximately 85% lower than CTR.

The temporal profile of CH_4 production, during fermentation, showed different patterns. After 4 hours of incubation, all algae-supplemented treatments, except VG3, showed a reduction in CH_4 concentration relative to the CTR. By 8 hours, CH_4 suppression was evident for all algae treatments. At 24 hours, VG4 was the only treatment still showing a reduction in CH_4 production, both compared to CTR and to the other algae treatments, and this trend persisted through 48 hours. Hydrogen production was increased after 4 hours in VG1, VG4 and VG5.

Fermentation was also influenced by algae supplementation. After 48 hours, VFA concentrations differed among algae treatments and showed a treatment-by-run interaction. In the second run, all algae-supplemented groups increased their total VFA concentrations by approximately 28–43% compared with the CTR, whereas no differences were detected in the first run. All cultured algae treatments (VGs) led to a 11–26% reduction in the acetate-to-propionate ratio relative to the CTR, indicating a shift in fermentation towards propionate production.

Regarding the molar proportions of individual VFAs, VG3 and the AT exhibited slightly higher acetate proportions, while VG4 showed the lowest

acetate (9%) and the highest propionate (53%) proportions among the algae treatments. Butyrate was moderately increased in VG4 (10%) compared with the CTR, whereas valerate and isovalerate were reduced (13% and 31%, respectively) across all algae treatments.

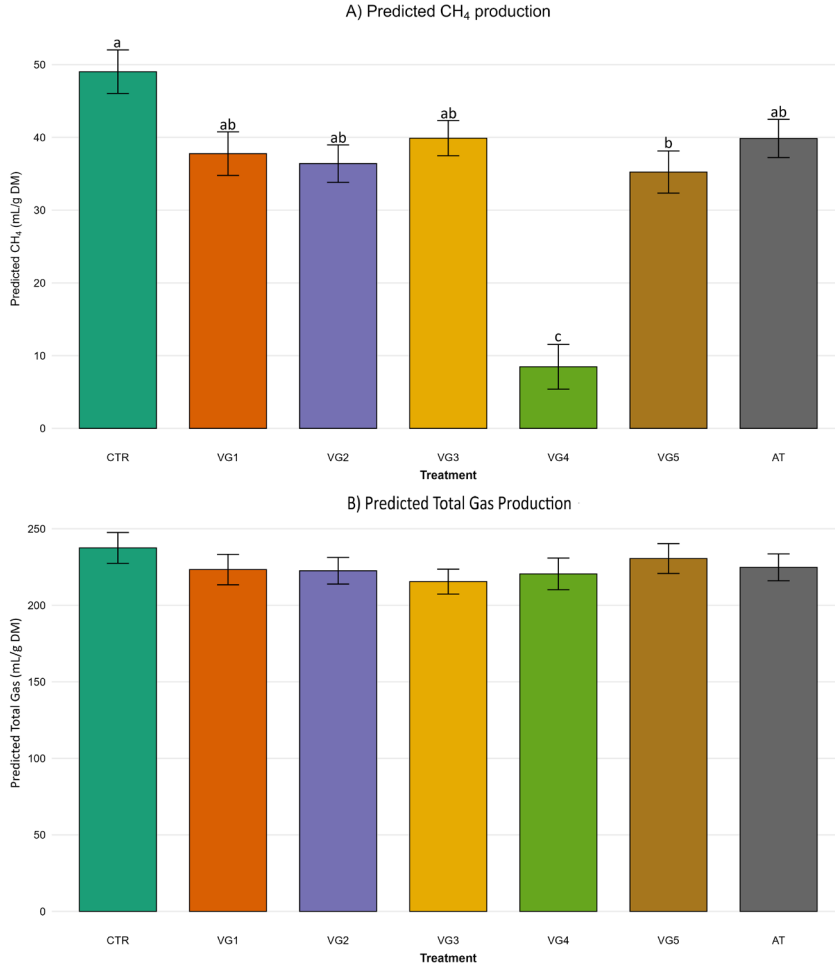

Figure 6. Effect of *Asparagopsis spp.* supplementation (0.5 % inclusion on OM) on predicted methane (CH_4) and total gas production (mL/g of incubated DM), mean retention time of 50h by following Ramin and Huhtanen (2013). Treatments included a control diet (CTR), *Asparagopsis spp.* cultured under different environmental conditions (VG1–VG5), and wild-harvested biomass (AT). Bars represent estimated marginal means ± standard error of the mean (SEM). Different superscript letters (a–c) indicate significant differences among treatments (Tukey's test, $P < 0.05$).

6. Discussion

Across the three papers presented in this thesis, supplementation with AT reduced enteric CH_4 formation, although the duration and efficacy of this effect varied depending on the experimental setup and the chemical composition of the AT biomass.

In Paper **I**, supplementation with AT effectively reduced enteric CH_4 emissions during the initial phase, although the effect declined over time, likely due to changes in rumen microbiota or the reduction of active compounds in the algae, or a combination of the two. Beyond CH_4 and H_2 dynamics, the study also reported alterations in rumen fermentation profiles, nutrient metabolism, and mineral balance, emphasizing how CH_4 mitigation is closely linked to broader metabolic and physiological responses in the cow.

Paper **II** provided mechanistic insights into these changes, showing variations in archaeal and bacterial community composition in response to 0.3% OM of AT supplementation, not only in the rumen but also in faecal samples. These findings highlight the importance of considering whole-gut microbial dynamics when evaluating the mechanisms underlying CH_4 mitigation and microbial adaptation.

Paper **III** demonstrated that variation in $CHBr_3$ content among red algae batches strongly influenced CH_4 inhibition, emphasizing how algae quality determines the overall anthropogenic effects of AT.

6.1 Methane mitigation and hydrogen dynamics in the rumen

6.1.1 Enteric CH_4 reduction and archaeal community

In Paper **I**, average reductions of up to 30% were observed, with the most pronounced decreases occurring during the first 2–4 experimental weeks, confirming the potential antimethanogenic capacity of AT. However, this effect declined after week 8, indicating a partial loss of efficacy over time. Such temporal patterns suggest that the rumen microbiota dynamically adjusts to the presence of halogenated compounds such as $CHBr_3$. Evidence from Paper **I**, **II**, and **III** indicates that this decline is driven by both microbial changes and the quality of AT biomass used.

In Paper **II**, CH_4 reduction was accompanied by restructuring of the archaeal community. *Methanobrevibacter spp.* remained the dominant genus in both groups, consistent with its role as the primary hydrogenotrophic methanogen in the rumen (Danielsson et al., 2017; Janssen and Kirs, 2008).

Members of the Gottschalkii clade, including *M. boviskoreani* and *M. millerae*, consistently reduce their relative abundance in the H-AT group, whereas the Ruminantium-associated *M. olleyae* increased in relative abundance. These contrasting responses likely reflect intrinsic physiological differences, such as variation in H_2 affinity and *mcrA* gene characteristics (Danielsson et al., 2017), which may influence sensitivity to $CHBr_3$ and altered ruminal redox conditions.

These community changes are broadly consistent with expected changes in ruminal H_2 dynamics under AT supplementation.

As $CHBr_3$ inhibits MCR, CH_4 formation is reduced and transient increases in H_2 have been documented in several *in vivo* and *in vitro* studies (Roque et al., 2019; 2021; Chagas et al., 2019). Elevated H_2 partial pressure can affect methanogen competitiveness, disadvantaging species with lower affinity for H_2 (Janssen, 2010). This may explain the decline of Gottschalkii-associated methanogens and the relative persistence of Ruminantium clade species, which appear better adapted to fluctuating H_2 conditions.

Quantitative PCR further supported these trends, showing a reduction of methanogen and a positive association between archaeal abundance and CH_4 output in the H-AT group. These results suggest that partial restoration of methanogenesis over time may be linked to clade-specific differences in H_2 use rather than full recovery of the original community.

In addition to microbial adaptation, differences in $CHBr_3$ content among *Asparagopsis spp.* batches likely contributed to the observed temporal decline in CH_4 suppression (Paper **III**), as well as the effects of storage time shown in Paper **I**. These aspects are discussed in Section 6.3.1.

Together, these findings demonstrate that the long-term efficacy of CH_4 mitigation by *Asparagopsis spp.* depends on both rumen's microbial changes and the stability of halogenated bioactive compounds. This dual influence provides an explanation for the temporal decline observed in CH_4 suppression across *in vivo* and *in vitro* systems (Papers **I**–**III**) and underscores the dynamic nature of the rumen's microbial and biochemical response to halogenated metabolites.

6.1.2 Hydrogen accumulation and bacterial community

Inhibition of methanogenesis by AT results in the accumulation of H_2 in the rumen, measured through increased H_2 concentrations in breath (Paper **I**). This occurs because the primary hydrogen sink (methanogenic CH_4 formation) is reduced (Paper **II**). The resulting increase in H_2 partial pressure acts as a metabolic signal that redirects electron flow towards alternative reductive pathways, primarily propionate and butyrate formation. Consequently, the VFA profiles reported in Paper **I**, with higher propionate and butyrate proportions and a lower acetate-to-propionate ratio in the H-AT group compared with the CON, indicate a shift in rumen fermentation aimed at maintaining redox balance under inhibited methanogenesis, as described in conceptual frameworks of electron partitioning (Ungerfeld, 2020). As described in Section 5.2, bacterial community responses reflected these hydrogen-driven changes in rumen metabolism. Although alpha diversity remained stable between treatments, suggesting moderate selective pressure at an AT inclusion level of 0.3% of OM, compositional shifts revealed functional adaptation to the altered redox environment. A higher relative abundance of taxa typically associated with succinate-propionate- pathways was observed in the H-AT group, including *Prevotella*, *Anaerovibrio*, *Selenomonas*, and *Succinivibrio*. Although these genera are metabolically diverse, several members are known to participate in fermentative routes that channel reducing equivalents towards propionate formation (Kelly et al., 2010; Henderson et al., 2015). Therefore, this increase may suggest a variation in community function towards alternative hydrogen-utilizing

pathways when methanogenesis is inhibited, consistent with the observed increases in propionate proportions in Paper **I**.

Propionate formation in the rumen can occur via three main routes: the succinate pathway, the acrylate pathway, and the propanediol pathway (Li et al., 2025), with succinate and lactate serving as key intermediates (Henderson et al., 2015; Mao et al., 2016). In contrast, the relative abundance of acetate-producing taxa, including unclassified *Clostridia* and *Ruminococcaceae* decrease, is consistent with lower acetate concentrations and a shift from fiber-driven acetate production towards pathways favouring propionate. Environmental fitting analyses further confirmed that propionate was the only VFA significantly correlated with bacterial community composition, particularly with *Prevotella*, *Anaerovibrio*, *Selenomonas A*, and *Cryptobacteroides*, supporting their key role in sustaining ruminal redox homeostasis under methanogenesis inhibition.

The increase in the relative abundance of *Acetomicrobium* in the H-AT group may reflect enhanced acetate- and hydrogen-producing fermentation, while the increased abundance of *Akkermansia*, typically uncommon in the rumen, suggests altered mucin degradation and epithelial nutrient turnover in response to AT supplementation (Li et al., 2018; Muizelaar et al., 2021). Conversely, RF16 and *Enterobacteriaceae* declined, indicating selective inhibition of sensitive functional groups by $CHBr_3$, consistent with *in vitro* observations reported by Machado et al. (2016).

6.2 Faecal microbiota and ecosystem implications

Compared with the rumen microbiota, which showed clear changes in response to methanogenesis inhibition, the faecal microbiota was less affected by AT supplementation (Paper **II**). Alpha diversity did not change between the H-AT and CON groups, but beta diversity showed distinct clustering, indicating a change in community composition without a loss of overall diversity. This suggests that changes in the substrates available for hindgut fermentation, induced by altered ruminal fermentation, can modify the diversity and metabolic activity of faecal microorganisms, even if overall diversity remains stable.

Differential abundance analysis showed that *Blautia*, *Ruminococcus*_E, *Bifidobacterium*, and members of the family *Lachnospiraceae,* increased in the H-AT group. These bacteria contribute to hindgut fermentation and VFA

production, which are important for colon health and provide a secondary source of energy to the host (Henderson et al., 2015). Although the contribution of hindgut fermentation to total energy is relatively small compared to the rumen, it can still account for approximately 5–10% of the host's energy intake (Henderson et al., 2015; Hoover, 1978). The increased abundance of hindgut-associated taxa may reflect the indirect consequences of ruminal changes, such as altered VFA proportions and shifts in substrate flow to the large intestine, rather than a direct response to ruminal H_2 accumulation. *Blautia* includes species that produce butyrate and acetate, and some can use the Wood–Ljungdahl pathway for acetogenesis (Trischler et al., 2022), possibly helping to utilize H_2 in the hindgut when ruminal H_2 is elevated.

Despite this increase in butyrate producers, *Prevotella* declined in the H-AT group. *Prevotella* is important for breaking down plant polysaccharides in the large intestine (Kou et al., 2024), so its reduction may lower fibre degradation and VFA production, potentially affecting gut health and energy supply (Macfarlane and Macfarlane, 2012). These changes are consistent with the reduced NDF digestibility and lower milk performance observed in Paper **I** and previous studies (Krizsan et al., 2023; Stefenoni et al., 2021), showing that AT induced changes in both the rumen and hindgut can affect energy use and milk fat synthesis.

The abundance of *Methanobrevibacter* A also decreased in faeces from the H-AT group, likely reflecting the indirect effects of rumen methanogen inhibition by $CHBr_3$. Residual bioactive compounds from AT, including halogenated metabolites such as Br_2, might persist in the digesta and exert minor selective pressure on the hindgut microbiota, potentially influencing methanogenesis and fermentation. However, empirical evidence confirming these downstream effects is still limited, highlighting the need for further studies linking ruminal and faecal microbiomes under *Asparagopsis spp.* supplementation.

6.3 Mineral and halogen dynamics

6.3.1 Compositional variability of *Asparagopsis spp.* and its effects on CH_4 inhibition and rumen fermentation

Paper **III** showed that the biochemical composition of *Asparagopsis spp.,* particularly its halogenated metabolites and mineral profile, plays a central role in determining both CH_4 inhibition and shifts in rumen fermentation. The variation in $CHBr_3$, Br_2, and I_2 concentrations among the VGs reflects environmental influences, particularly differences in light, nutrients, and oxidative conditions. These abiotic factors regulate haloperoxidase activity and hydrogen peroxide (H_2O_2) dynamics, thereby influencing the synthesis and stability of $CHBr_3$, a key antimethanogenic compound (Paul et al., 2006b; Kinley et al., 2020; Hargrave et al., 2024).

The variability in halogen content explains, at least in part, the inconsistent CH_4 suppression reported in both *in vitro* and *in vivo* studies (Paper **I**, Stefenoni et al., 2021; Roque et al., 2021). High $CHBr_3$ levels have been consistently associated with strong CH_4 inhibition, whereas storage or light-induced degradation reduces bioactivity, as was shown in Paper **I** and reported by Stefenoni et al. (2021). This highlights that *Asparagopsis spp*. is a chemically dynamic material whose antimethanogenic potency depends on growth conditions and post-harvest handling (Roque et al., 2021). Nevertheless, such variability does not preclude its use as a feed; rather, it underscores the need for appropriate quality control measures to ensure consistent $CHBr_3$ content and predictable efficacy.

In addition to affecting CH_4 inhibition, compositional variability was reflected in fermentation patterns. All VGs increased the total VFA compared with CTR, suggesting enhanced overall fermentation. At the same time, the acetate-to-propionate ratio decreased, indicating a metabolic shift toward pathways that increase propionate formation, an effect commonly linked to reduced H_2 availability for methanogenesis (Machado et al., 2018; Martins et al., 2024). However, these responses were not uniform across samples. Treatments derived from algae with higher $CHBr_3$ concentrations (VG4) showed the strongest increase in propionate, whereas VG2 and VG3, with lower halogen contents, produced weaker effects. These patterns may reflect both differences in $CHBr_3$ potency and potential microbial responses. Given that batches with lower halogen content showed weaker CH_4

inhibition, a reduced biochemical effect on methanogenesis alone could plausibly account for the attenuated shifts in VFA profiles.

Taken together, these results highlight the interconnectedness of algae chemistry and rumen function. Environmental and storage factors that alter halogen and mineral composition can modify fermentation efficiency, CH_4 formation and its downstream effects. For practical application, it will be essential to establish standardized cultivation and quality-control protocols that ensure stable levels of key compounds such as $CHBr_3$, Br_2, and I_2. Combining chemical profiling with fermentation bioassays could provide a practical framework for estimating the methane-reducing potential of *Asparagopsis spp*. before it is fed to animals (Roque et al., 2021; Martins et al., 2024).

6.3.2 Systemic and metabolic consequences of halogen Intake

In Paper **I**, dietary supplementation with AT led to the accumulation of Br_2 and I_2 in milk, as well as Br_2 in faeces and urine, and caused minor changes in plasma parameters in dairy cows. In the H-AT group, Br_2 and I_2 concentrations in milk were approximately seven- and thirteenfold higher than in the CON group, respectively. Concentrations were lower than in studies using higher inclusion rates of 0.5% AT of OM (Stefenoni et al., 2021; Krizsan et al., 2023), reflecting the relatively low AT dose in Paper **I** (0.3% of AT based on OM). It is important to note, however, that milk halogen concentrations are influenced not only by the inclusion rate but also by the halogen content of the algal biomass itself, which can vary substantially across batches and during storage (Paper **III**).

Excretion patterns revealed that cows excreted a portion of Br_2 via urine and faeces, with the average concentrations in the H-AT group up to fivefold higher in faeces and ninefold higher in urine compared with the CON. This indicates that Br_2 undergoes partial absorption and limited metabolic integration, with rapid clearance from the body (Paper **I**). These findings are important both for understanding bovine metabolism of halogens and for assessing potential environmental impacts through manure management.

From a food safety perspective, the measured halogen concentrations in milk translate into acceptable human intake levels under the conditions of the study in Paper **I**. For Br_2, milk from the H-AT group would allow a maximum daily intake of 1.3 kg milk per adult, assuming no other dietary sources (EFSA Scientific Committee et al., 2025). It should be noted,

however, that data on Br_2 consumption in humans is limited, and few studies exist to define safe intake levels. For I_2, safe daily milk consumption is estimated to range from 0.01 to 0.16 kg per person based on typical intake considerations, while the maximum allowable intake, considering the established upper intake limits, would be 0.65 kg per person (EFSA, 2013; Blomhoff et al., 2023). While these levels are generally within safe limits, the accumulation trend over time underscores the importance of monitoring halogen residues, particularly under higher inclusion rates or prolonged supplementation.

In addition to halogen accumulation, AT supplementation induced modest effects on systemic metabolism. Magnesium levels in the plasma increased slightly in the H-AT group but remained within normal ranges (Bertoni and Trevisi, 2013), reflecting the mineral richness of red macroalgae (MacArtain et al., 2008). Blood cholesterol concentrations were lower in the H-AT group, though still above normal ranges, and the total antioxidant capacity, measured by FRAP, decreased compared with the CON. This suggests a potential imbalance between free radical production and antioxidant defence, which could increase oxidative stress and affect immune function, milk quality, and overall animal performance (Benzie and Strain, 1996; Sordillo and Aitken, 2009).

Overall, these findings demonstrate that halogen intake from AT is partially absorbed and largely excreted, with systemic metabolic consequences that are generally minor under the studied conditions; they do, however, warrant careful monitoring.

6.4 Animal performance and nutrient utilization

In Paper **I**, dietary inclusion of AT affected feed intake and nutrient utilization depending on the inclusion level. While an inclusion level of 0.15% AT of OM (L-AT) did not alter DMI, an inclusion level of 0.3% OM (H-AT) reduced DMI by approximately 7% compared with the CON group. Similar declines have been observed in previous short- and medium-term trials at inclusion rates above 0.25% of OM (Eikanger et al., 2024; Krizsan et al., 2023), confirming intake depression as a response to AT supplementation. This lower DMI was accompanied by reductions in milk yield (4%) and ECM (2%) in the H-AT compared with the CON, indicating

that feed intake remains the primary driver of production losses under AT inclusion.

Alongside reducing feed intake, H-AT supplementation also decreased NDF digestibility by 8% compared with the CON. This contrasts with previous short-term findings (Stefenoni et al., 2021; Krizsan et al., 2023), suggesting that the longer duration of the experiment in Paper **I** allowed time- or adaptation-related changes in rumen fermentation to emerge. As VFAs provide up to 70% of metabolizable energy in ruminants (Bergman, 1990), reduced NDF digestibility and intake likely limited total VFA production and thus the energy available for milk synthesis. The concurrent 4.7% reduction in milk fat concentration aligns with a shift in ruminal fermentation from acetate towards propionate production (Palmquist and Beaulieu, 1993), consistent with the VFA patterns observed in Paper **I**.

However, ruminal VFA concentrations not only reflect production but also absorption dynamics. The inhibition of methanogenesis by $CHBr_3$ may impair epithelial function, potentially reducing VFA absorption. Because propionate is normally absorbed rapidly and supports gluconeogenesis, impaired uptake could elevate its ruminal concentration while limiting its contribution to milk synthesis. This possibility is supported by Muizelaar et al. (2021), who reported inflammation-related histological alterations in the rumen epithelium of cows fed AT, suggesting that mucosal integrity may be affected. Thus, increased propionate concentrations should not be interpreted solely as enhanced microbial production. Given the limited knowledge currently available regarding both VFA absorption and the potential toxicological effects of AT, further research is needed to clarify the mechanisms involved.

Results from Paper **II** offer mechanistic insight into this metabolic reorientation, showing that *Asparagopsis spp*. supplementation led to an increased relative abundance of specific bacterial taxa, involved in alternative H_2 utilization pathways, particularly those linked to succinate and propionate formation (*Prevotella*, *Selenomonas*, *Anaerovibrio*, and *Succinivibrio*). This microbial restructuring coincided with higher ruminal propionate concentrations in Paper **I**, supporting the concept of H_2 redirection away from methanogenesis towards propionate synthesis. Such a response likely represents an adaptive mechanism to maintain rumen redox balance under $CHBr_3$ exposure, but it also implies reduced acetate generation and lower energy efficiency for milk fat synthesis, as acetate formation

becomes increasingly constrained when methanogens are inhibited and electrons are no longer effectively removed via CH_4 production.

The downstream effects of these ruminal changes were reflected in hindgut fermentation patterns. As described in Section 6.2, moderate variation in the faecal microbiota suggested compensatory fermentative activity in the large intestine. However, these adjustments were insufficient to offset the reduction in total-tract fibre degradation, highlighting a systemic alteration in nutrient utilization rather than a localized effect within the rumen.

Taken together, the integration of ruminal, hindgut, and production data demonstrates that the reduction in CH_4 emissions induced by AT is closely tied to changes in fermentation pathways and energy partitioning. While redirecting reducing equivalents (electrons) towards propionate formation effectively mitigates methanogenesis, it also reduces acetate availability and fibre digestibility, leading to declines in milk yield and fat concentrations. This trade-off underscores the complexity of balancing environmental and productive outcomes in dietary CH_4 mitigation strategies.

6.5 Overall interpretation and implications

The results of this thesis demonstrate that the antimethanogenic efficacy of *Asparagopsis spp.* is strongly shaped by the interaction between algae composition, feeding duration, and rumen microbial adaptation. Across studies, AT consistently reduced enteric CH_4 emissions, but the magnitude and persistence of the effect varied according to changes in bioactive compound concentrations, changes in archaeal and bacterial communities, and the resulting alterations in H_2 metabolism and VFA patterns. While several of these responses align with previous findings, this thesis provides new evidence on how temporal microbial dynamics, halogen accumulation, and variability in algae quality contribute to heterogeneous outcomes. These insights emphasize that AT is not a static feed but part of a dynamic chemical–microbial system. The variability observed across studies highlights the need for longer-term trials and evaluations under practical farm conditions to assess the stability, products safety, and applicability of AT supplementation in dairy production.

In the European context, practical adoption will also depend on the progress of regulatory evaluation and scalable production of AT, both of which are

still developing. Moreover, dairy management systems differ greatly across member states, despite shared EU climate-mitigation targets, meaning that implementation strategies would necessarily require adaptation to local production conditions (European Environment Agency, 2024).

7. Conclusion

This thesis investigated the effects of AT supplementation in dairy cow diets on enteric CH_4 production, rumen fermentation, and dairy cow performance. Across the three studies, AT consistently reduced CH_4 emissions, confirming its antimethanogenic potential, although the magnitude and duration of the effect were influenced by algal composition and microbial adaptation (Paper **I**). The efficacy of CH_4 mitigation depends on the interaction between chemical inhibition and microbial shifts in the rumen, which also influence host metabolism and fermentation patterns (Paper **I** and **II**). Alterations in ruminal and faecal microbial communities contributed to changes in VFA profiles and H_2 utilization, highlighting the link between microbial ecology and antimethanogenic activity. Furthermore, cultivation conditions affect the concentration of bioactive compounds in *Asparagopsis spp.*, which in turn modulate its antimethanogenic efficacy (Paper **III**). Maintaining long-term efficacy requires stable algae quality, optimized inclusion levels, and the careful monitoring of halogen residues to ensure food safety. Overall, AT represents a promising strategy for reducing enteric CH_4 emissions in dairy systems, but its sustainable application depends on balancing environmental benefits with animal performance, health, and product safety.

8. Future perspectives

The results of this thesis highlight several areas where further research is needed to support the effective and sustainable use of *Asparagopsis spp*. in dairy production.

One key aspect is to improve the stability and predictability of antimethanogenic effects. The variability observed across studies indicates that future work should focus on optimizing cultivation, harvesting, and processing conditions to ensure consistent concentrations of bioactive compounds. Developing reliable indicators for algae quality would help reduce batch-to-batch variation and facilitate more targeted dosing strategies. A deeper understanding of rumen microbial adaptation is also required. Longer-term *in vivo* studies, extending experimental durations, are necessary to determine the persistence of CH_4 mitigation and to clarify the mechanisms underlying microbial resilience or the loss of efficacy over time. Integrating multi-omics approaches would provide a more comprehensive picture of how hydrogen metabolism, fermentation pathways, and microbial networks reorganize in response to AT supplementation.

Further research is needed to evaluate the systemic implications of halogen intake under practical feeding conditions. This includes detailed dose–response assessments, the characterization of long-term metabolic and endocrine effects, and the comprehensive monitoring of halogen residues in milk and dairy products to ensure consumer safety. At a broader level, the feasibility of large-scale application depends on the development of sustainable and scalable production systems for *Asparagopsis spp*. In Europe, commercial cultivation is still emerging, and future efforts should address supply reliability, environmental impacts, and cost-effectiveness. Life cycle assessments will be essential to determine whether *Asparagopsis spp*. delivers a net reduction in greenhouse gas emissions once production and processing are accounted for. Finally, applying *Asparagopsis spp.* as a feed additive in European dairy systems will require adaptation to the wide variety of management practices across member states. Because of this diversity, future research should test AT under different production conditions and in collaboration with industry stakeholders. Such efforts will be essential to assess whether *Asparagopsis spp*. could be a practical and reliable long-term CH_4 mitigation strategy that aligns with EU climate targets.

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Popular science summary

Enteric methane emissions from cattle are a major environmental challenge for livestock systems. Although invisible, methane produced during rumen fermentation is a potent greenhouse gas, and reducing these emissions is essential to ensure dairy production is sustainable. In recent years, a surprising potential solution has emerged from the ocean, the red seaweed *Asparagopsis spp.*

This thesis investigated the potential of *Asparagopsis spp*. as a feed additive to lower methane emissions from dairy cows, while also considering animal performance, changes in rumen and faecal microbiota, and the ways cultivation conditions influence the concentration of the bioactive compounds responsible for methane inhibition.

Across three independent studies, *Asparagopsis spp.* consistently reduced methane production. However, the magnitude and persistence of this mitigation depended on factors such as the chemical composition of the algae, the duration of supplementation, and the microbial adaptations that occurred in the rumen. The additive also altered fermentation patterns, including volatile fatty acid profiles, and influenced both ruminal and faecal microbial communities, pointing to a complex interplay between chemical inhibition and microbial shifts.

A critical issue highlighted by the research was a substantial variability in algae quality and in concentrations of key bioactive compounds. This underscores the need for standardized cultivation systems and precise dosing strategies. In parallel, halogen intake and accumulation were monitored to evaluate potential food-safety implications.

Populärvetenskaplig sammanfattning

Enteriskt metan är det metan som produceras under den naturliga fodersmältningsprocessen (fermenteringen i våmmen hos idisslare, som t.ex. mjölkkor. Dessa metanutsläpp från nötkreatur innebär en stor miljöutmaning för dagens animalieproduktion. Även om det är osynligt, är metan som bildas under våmfermentationen en kraftfull växthusgas, och att minska dessa utsläpp är avgörande för att göra mjölkproduktionen mer hållbar. Under de senaste åren har en oväntad möjlig lösning dykt upp från havet: rödalgen Asparagopsis spp.

Denna avhandling undersökte potentialen hos Asparagopsis spp. att sänka metanutsläppen från mjölkkor, genom att utgöra en del av fodret. Dessutom undersöktes påverkan på mjölkproduktionen och förändringar i mikrobiotan i våmmen och träcken. Hur olika odlingsförhållanden för algerna påverkar koncentrationen av de bioaktiva ämnen som ligger bakom metanhämningen analyserades också.

I tre oberoende studier minskade Asparagopsis spp. konsekvent metanproduktionen. Däremot varierade omfattningen och uthålligheten av denna minskning beroende på faktorer som algens kemiska sammansättning, inblandningsnivå och de mikrobiella förändringarna som uppstod i våmmen. Tillskottet förändrade även fermentationsmönstret, inklusive sammansättningen av flyktiga fettsyror, och påverkade både våm- och träckens mikrobiella samhällen, vilket tyder på ett komplext samspel mellan kemisk hämning och mikrobiella förändringar.

En viktig fråga som lyfts fram i forskningen var den stora variationen i algkvalitet och i koncentrationen av nyckelbioaktiva ämnen. Detta understryker behovet av standardiserade odlingssystem och noggranna doseringsstrategier. Forskningen visade också att intag och ackumulering av halogener i mjölken måste kontrolleras för att säkerställa att produkterna inte överskrider eventuella livsmedelssäkerhetsaspekter.

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I

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Asparagopsis taxiformis supplementation to mitigate enteric methane emissions in dairy cows—Effects on performance and metabolism

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ABSTRACT

Methane emissions from ruminant digestion contribute significantly to global anthropogenic greenhouse gas emissions. Members of the phylum Rhodophyta (red algae), particularly *Asparagopsis* sp., have shown promising results in reducing methane emissions in ruminants, due to their high content of halogenated methane analog compounds. However, knowledge is lacking regarding the effects of red algae on animal performance and metabolism. This study investigated the effects of dairy cow diet supplementation with *Asparagopsis taxiformis* on enteric methane performance, metabolism of bromine and iodine, and health status of the cows. Thirty lactating Nordic Red dairy cows fed a TMR were blocked according to parity and DIM, and randomly assigned to 1 of 3 diets: a control diet with no *A. taxiformis* (CON), a diet with 0.15% *A. taxiformis* on an OM basis (L-AT), and a diet with 0.3% *A. taxiformis* on an OM basis (H-AT). The cows were fed the experimental diets continuously for 13 wk, beginning with a baseline week (wk 0), which served as covariate by week where all cows received the basal diet. Individual feed intake and milk yield were recorded automatically throughout the experiment. Milk composition was determined by collecting milk samples during each milking session on 2 consecutive days every experimental week. Enteric methane and hydrogen levels were measured continuously by the GreenFeed system. Feces grab samples were collected as spot samples from a subset of 6 cows per treatment after milking during sampling wk 0, 2, 4, 8, and 12. Urine spot samples were collected from the same subset of cows during the same weeks as fecal samples. One urine sample was taken per day on 2 consecutive days, and the samples were analyzed for wk 12. Rumen fluid was collected after morning milking using a stomach tube in wk 0, 2, 4, and 12. We observed a 30% reduction in methane production in the H-AT group, with a concomitant increase in hydrogen production by 383%. However, the interaction between treatment and week showed that the AT effect on methane reduction began to diminish by wk 9 of the experiment. In the L-AT group, methane was reduced by 7.6% and hydrogen production was increased by 70%. However, DMI was 7% lower and ECM yield was 2% lower in the H-AT group compared with the other 2 groups. Total concentration of volatile fatty acids in rumen fluid was lower in the H-AT group compared with CON, with a reduction in acetate concentration and an increase in propionate, butyrate, and valerate in the H-AT group. Bromine concentration was 5-fold higher, and iodine concentration was 9-fold higher in milk from the H-AT group compared with CON. Bromine concentration in feces and urine samples from H-AT cows was approximately 4-fold and 9-fold higher, respectively, than in samples from CON cows. Metabolic profiling revealed a reduction in cholesterol levels and a decrease in the ferric-reducing ability of plasma in the H-AT treatment group compared with CON, as well as an increase in plasma magnesium concentration in the H-AT group. In conclusion, using 0.3% *A. taxiformis* as an additive in dairy cow feed rations can mitigate enteric methane emissions, but this reduction was observed only during the first 8 wk of the experiment, with no effect on methane emissions from wk 9 to 12. Additionally, it may have negative effects on DMI and ECM yield. Further long-term studies on red algae as methane inhibitor is needed to examine its sustained inhibitory effects over time and its effect on various metabolic processes. The effects appear to decline after wk 8 and influence several metabolic mechanisms.

Key words: *Asparagopsis taxiformis*, bromine, environmental impact, iodine, seaweed supplementation

INTRODUCTION

Several strategies to reduce enteric methane (CH_4) emissions from ruminants have been examined globally.

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

Table 1. Chemical composition of dietary ingredients (g/kg DM unless otherwise stated; n = 5)[1]

Item	Grass silage (n = 6)	SD	Concentrate[2] Komplett 180	SD	Concentrate[3] Komplett 180 GF	SD	Concentrate[4] ExPro	SD
DM, g/kg	250	12.0	872	2.45	871	3.40	892	2.40
ME, MJ/kg DM	11.9	0.07	13.4	ND	13.4	ND	15.5	ND
Composition								
Ash	80.6	1.66	61.3	4.55	59.8	0.65	74.3	0.72
CP	185	4.86	187	3.63	187	4.26	343	3.05
NDF	530	10.1	218	4.32	226	20.10	293	73.28
iNDF	72.8	3.0	74.6	6.94	ND	ND	99	3.37
Starch	ND	ND	309	6.38	311	6.69	23.4	8
pH[5]	3.8	0.01	ND	ND	ND	ND	ND	ND
NH_3-N,[5] % DM	3.4	0.32	ND	ND	ND	ND	ND	ND

[1]iNDF = indigestible NDF; ND = not determined.
[2]Commercial concentrate, Komplett Norm 180 (Lantmännen), added to the TMR.
[3]Commercial concentrate, Komplett Norm 180 (Lantmännen), added to the GreenFeed (GF) system.
[4]Heat-treated rapeseed meal, added to the TMR. ExPro, AAK, Karlshamn, Sweden.
[5]Fermentation quality for silage.

The livestock sector is responsible for ~80% of annual carbon dioxide equivalent emissions related to anthropogenic CH_4, with ~90% of these emissions resulting from enteric fermentation in ruminants, particularly cattle and sheep (Reisinger et al., 2021). In this context, dietary supplementation with seaweed, particularly *Asparagopsis taxiformis* (**AT**) and *Asparagopsis armata*, has been tested as a CH_4 mitigation strategy. These species of algae are known for their high content of halogenated analog compounds, such as bromoform and di-bromochloro-methane (Paul et al., 2006), which are able to block the last step of the methanogenesis in the rumen (Machado et al., 2016).

Several short-term trials have demonstrated varying mitigation effects of *Asparagopsis* sp. on enteric CH_4 depending on dosage, seaweed storage conditions, and basal diet (Roque et al., 2019, 2021; Kinley et al., 2020; Stefenoni et al., 2021; Krizsan et al., 2023). A crossover study by Stefenoni et al. (2021) observed a reduction in enteric CH_4 emissions of 65% in vivo when adding AT to the diet of lactating dairy cows, whereas Roque et al. (2021) observed a reduction of 98% when adding AT to the diet of steers, with both studies utilizing an AT inclusion level of 0.5% of OM. However, supplementation with AT has also been shown to reduce voluntary feed intake and potentially damage the rumen wall in sheep and dairy cattle (Li et al., 2018; Muizelaar et al., 2021). Further, there is evidence of transfer of metabolites such as bromoform to milk, feces, and urine of lactating dairy cows (Muizelaar et al., 2021; Stefenoni et al., 2021). Red algae are known to accumulate iodine and bromine (Vinogradov, 1953), and the relationship between dietary intake of these elements and their presence in dairy cows shows a dose-response pattern (Stefenoni et al., 2021; Krizsan et al., 2023). Both iodine and bromine residuals play crucial roles in public health, as inadequate iodine levels can impair thyroxin synthesis in humans and animals (Leung and Braverman, 2014), whereas even small doses of bromine can affect inhibition processes in the nervous system (Saenko et al., 1978). Against this background, continuous, long-term studies are needed to acquire a comprehensive understanding of the overall effect of *Asparagopsis* sp. supplementation of ruminant diets. Most of the studies cited above use a high level of AT (0.5% of OM) in vivo. However, given the potential negative effects on metabolism and the digestive process in cows, which could influence productivity and animal health, it would be beneficial to delve deeper into the long-term effects and assess the effect of reducing the proportion of AT in the diet. The optimal dosage will be that which achieves a significant reduction in methane emissions while minimizing adverse effects. Our main aims of this study were therefore to examine the effect of long-term AT supplementation on methane emissions and production parameters in dairy cows, check for presence of bromine and iodine residues in milk, urine, and feces, and assess cow health through analysis of metabolic blood parameters. We hypothesize that long-term AT supplementation in dairy cows will lead to detectable residues of bromine and iodine in milk, urine, and feces and altered metabolic responses, indicating risk of impaired health in the cows.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

The study was conducted at the Swedish University of Agricultural Sciences, Röbäcksdalen Livestock Research Center, Umeå, Sweden (N63°45′; E20°17′) from January to April 2022. All animals were cared for according to the rules and guidelines proposed by the

Table 2. Intake and production parameters in dairy cows fed the control diet (CON, 0% AT) and diets containing 0.15% (L-AT) or 0.3% (H-AT) *Asparagopsis taxiformis* (AT) on an OM basis[1]

	Treatment				*P*-value		
Item per cow and day	CON	L-AT	H-AT	SEM	Treatment	Week	Treatment × Week
Intake							
Total DMI, kg	24.9[a]	24.7[a]	23.1[b]	0.35	<0.001	<0.001	0.09
Silage DMI, kg	12.4[a]	12.2[a]	11.4[b]	0.18	<0.001	<0.001	0.04
Concentrate DMI, kg	12.4[a]	12.3[a]	11.6[b]	0.17	<0.001	<0.001	0.23
CP, kg	4.6[a]	4.8[a]	4.3[b]	0.19	<0.001	<0.001	<0.001
Starch, kg	3.3[a]	3.4[a]	2.9[b]	0.15	0.02	<0.001	<0.001
NDF, kg	9.4[a]	9.7[a]	8.8[b]	0.38	<0.001	<0.001	<0.001
iNDF,[2] g/kg NDF	1.7	1.8	1.5	0.08	0.12	<0.01	0.05
Milk production							
Milk,[3] kg	37.1	37.6	36.2	0.43	0.48	<0.001	0.41
Milk,[4] kg	37.3[a]	37.7[a]	35.7[b]	0.50	0.03	<0.001	0.21
ECM, kg	39.2[a]	40.8[a]	38.3[b]	0.55	0.01	0.46	0.17
Fat, g/kg milk	45.0[a]	45.4[a]	42.9[b]	0.07	0.009	0.02	0.10
Protein, g/kg milk	37.2	36.9	35.9	0.07	0.64	<0.001	0.78
Lactose, g/kg milk	47.2	48.1	47.1	0.03	0.16	<0.001	0.60
ECM/DMI, kg/kg	1.60	1.63	1.64	0.50	0.13	0.52	0.13

[a,b]Different superscripts within a row indicate a significant difference ($P \leq 0.05$).

[1]Least squares means and SEM (n = 360).

[2]Analysis performed only for wk 0, 4, and 12; iNDF, indigestible NDF.

[3]Milk yield as mean for the entire experimental period.

[4]Milk yield as mean for the milk sampling weeks of the experiment.

Swedish University of Agricultural Sciences Animal Care and Use Committee and the National Animal Research Authority (Dnr: A6-2021).

We carried out a continuous study running for 13 experimental weeks, including an initial wk 0, where all cows were fed the basal diet, which served as covariate in the statistical analyses. Two weeks before the experiment, the cows were adapted to the basal diet, assigned feed bins, and introduced to the GreenFeed methane measurement system. In total, 9 primiparous and 21 multiparous Nordic Red dairy cows were recruited. At the start of the experiment, these cows were 61 ± 25.3 (mean ± SD) DIM, with average daily milk yield of 32.7 ± 8.7 (mean ± SD) kg of ECM, parity was 2.4 ± 1.36 (mean ± SD). Based on parity and DIM, we assigned the 30 animals to 10 blocks and randomly assigned each cow in the block to 1 of 3 dietary treatment groups. We randomly selected 6 of the blocks (i.e., 6 cows per treatment) as subgroups for sampling of feces, urine, blood, and rumen fluid. The 3 treatments comprised different inclusion levels of AT in the TMR: a control group with no AT (**CON**), a group with 0.15% AT on an OM basis (**L-AT**), and a group with 0.3% AT on an OM basis (**H-AT**). Accidently, the H-AT cows received a double dose of AT during the first week, and corrective measures were implemented immediately, and the correct dose was administered starting from d 1 of wk 2 of the experiment. To mitigate any effects of the initial incorrect dosage, the experimental period was extended by 4 wk (from 8 to 12).

The cows were housed in an insulated freestall barn equipped with an automatic feed intake recording system (Insentec RIC system bins, B.V., Marknesse, the Netherlands) and had free access to fresh water and salt licks. The stalls were bedded with sawdust on rubber mattresses. The cows were milked twice daily in a parlor, starting at 0500 and 1600 h. Milk yield at each milking was recorded using a gravimetric milk recorder (SAC Swing-over, S. A. Christensen and Co. Ltd., Kolding, Denmark).

The diets were composed of feeds commonly used in Swedish dairy production, fed as TMR ad libitum (Table 1). The ration was calculated according to the Nordic feed evaluation system (**NorFor**; Volden, 2011) to cover the energy and protein requirements for the cows based on the average ECM yield at the start of the experiment. The TMR had a forage:concentrate ratio of 50:50 on DM basis; 50% grass/clover silage, 47% commercial concentrate that contained 37% barley, 28% rapeseed meal, 12% corn, 7% sugar beet fiber, 5% oat husks, 4% distillers grains, 2% wheat bran, 2% molasses, 2% vegetable fat, 2% minerals and vitamins (Komplett Norm 180, Lantmännen, Malmö, Sweden), and 3% pure rapeseed meal (ExPro, AAK, Karlshamn, Sweden), plus an additional 100 g of mineral mixture (Effekt intensiv, Lantmännen, Malmö, Sweden) per cow and day. Iodine was excluded from the mineral mixture for the cows that received AT, due to high content of iodine in AT. The grass silage was harvested from a primary-growth perennial ley

Table 3. Apparent digestibility of the control diet (CON; 0% AT) and of the dairy cow diet containing 0.3% *Asparagopsis taxiformis* (AT) on an OM basis (H-AT) in experimental wk 4 and 12[1]

	Treatment			*P*-value		
Item	CON	H-AT	SEM	Treatment	Week	Treatment × Week
Digestibility, g/kg						
DM	745	734	6.1	0.08	0.63	0.08
OM	758	749	6.0	0.14	0.70	0.04
CP	732	739	8.0	0.43	0.74	0.35
NDF	713	657	20.7	0.04	0.32	0.24

[1]Least squares means and SEM (n = 24).

dominated by timothy (*Phleum pratense*), with <25% red clover (*Trifolium pratense*), and ensiled in bunker silos. The TMR mix was the same for all cows but differed in the amount of AT included. The AT supplement was added as such directly into the mixer wagon (Nolan A/S, Viborg, Denmark) through an automatic hand-made system, which consisted of a custom-designed apparatus equipped with the capability to automatically introduce different amounts of algae based on the AT inclusion level assigned to the treatment groups. In this system, AT was placed in small plastic cups, which were then positioned on an electric roller situated above the TMR mixer and linked to the automatic feeding system. When the mixer was activated, the cups were moved along the roller and emptied into the mixer. The TMR was delivered to the cows 3 times daily (at 0500, 1300, and 1800 h) by an automatic feeding wagon (Mullerup Smart Feeder M2000, Ullerslev, Denmark). The AT used was harvested at the gametophyte life cycle stage in the Azores, Portugal (N38°31′45″; W28°37′09″), during 2020 by the company SeaExpert (Feteira, Ilha Do Faial, Portugal). The algal biomass was collected as described by Stefenoni et al. (2021) and shipped by truck to the company European Freeze Dry A/S (Kirke Hyllinge, Denmark) for freeze-drying. It was then stored in hermetic plastic bags and sent frozen to the Swedish University of Agricultural Sciences. The AT bags were stored in a dark room at −20°C until the start of the experiment. During the experimental period, the AT bags were kept in a dark room at 0°C until use. To reduce the risk of variation between bags when feeding the cows, AT from 3 different bags was milled in a grain mill (Golia 4V, NOVITAL, Italy) once per day, pooled in vacuum bags, and stored at 4°C until feed mixing.

Sampling and Analyses

Diet and Feed Ingredients. Samples of silage were collected daily on 5 consecutive days per week. Samples of concentrate were collected 2 times a week. The DM content of the feed was assessed by drying 2 times a week, and feed proportions were adjusted relative to the changes in DM.

Daily silage samples were pooled into 1 sample for a 14-d period and then dried at 60°C for 48 h and milled to 1-mm size for chemical composition analysis. Additional samples from wk 0, 4, and 12 were milled to 2-mm size, using a cutter mill (SM 300, Retsch GmbH, Haan, Germany), for determination of indigestible NDF (**iNDF**). Separate samples of fresh silage were stored at −20°C for analysis of fermentation quality. Concentrate samples from the silos and the GreenFeed unit were pooled to 1 sample over a 14-d period and stored at −20°C until further analysis.

Chemical analyses were performed at the laboratory at the Department of Applied Animal Science and Welfare, Swedish University of Agricultural Sciences, Umeå, Sweden. Silage DM content was determined following the method described by Åkerlind et al. (2011), whereas concentrate DM was determined through overnight drying at 103°C (EC No. 152/2009). Starch content was analyzed according to Larsson and Bengtsson (1983). Ash content in all feeds was evaluated by ignition at 550°C for 3 h (EC No. 152/2009), whereas CP was analyzed for nitrogen (N) in an automated Kjeldahl procedure (Kjeltec 8400 Analyser unit and 8460 sampler unit, Foss, Hillerød, Denmark). Feed NDF content was analyzed following the procedure of Chai and Udén (1998), after applying a heat-stable amylase and excluding residual ash (see Danielsson et al., 2017, for details). Organic matter digestibility in vitro was determined following Bertilsson and Murphy (2003) and Volden (2011), from which ME in silage was calculated according to Lindgren et al. (1983). The ME content in concentrate was calculated based on feed table values from the Swedish Board of Agriculture (SJVFS, 2011).

In silage, pH and ammonia-N (NH_3-N) were evaluated. Analysis of ammonia-N was carried out using a flow injection analyzer technique (FIA star 5010 Analyzer, 5017 samples and 5032 controller, Tecator; Broderick and Kang, 1980). The concentrations of iNDF in silage, in concentrate, and in feces from 6 cows in CON and 6

Table 4. Methane (CH_4) and hydrogen (H_2) production during the entire experimental period in dairy cows fed the control diet (CON, 0% AT) and diets containing 0.15% (L-AT) or 0.3% (H-AT) *Asparagopsis taxiformis* (AT) on an OM basis[1]

	Treatment				*P*-value		
Per cow	CON	L-AT	H-AT	SEM	Treatment	Week	Treatment × Week
CH_4, g/d	433[a]	401[a]	281[b]	15.5	<0.001	<0.001	<0.001
CH_4, g/kg DMI	17.4[a]	16.4[a]	11.5[b]	0.70	<0.001	<0.001	<0.001
CH_4, g/kg milk	11.7[a]	10.7[a]	7.2[b]	0.40	<0.001	<0.001	<0.001
CH_4, g/kg ECM	10.9[a]	9.6[a]	6.6[b]	0.37	<0.001	<0.001	0.0006
H_2, g/d	1.2[b]	2.5[b]	5.8[a]	0.32	<0.001	<0.001	<0.001

[a,b]Different superscripts within a row indicate a significant difference ($P \leq 0.05$).

[1]Least squares means and SEM (n = 360).

cows in H-AT were determined in situ as described by Krizsan et al. (2015).

Five grams (approximately) of pooled AT was sampled weekly and stored at −20°C until analysis. The bromine content and iodine content in pooled AT were analyzed at Eurofins Food and Feed Testing, Lidköping, Sweden (DS EN 15111:2007), whereas the bromoform content was analyzed at Scantox, Mölndal, Sweden (Method/Ref 1635; BioventureHub, Mölndal, Sweden).

Gas Measurements. Enteric emissions of CH_4 and hydrogen (H_2) were recorded during the whole experiment by a GreenFeed emission monitoring system (C-Lock Inc., Rapid City, SD), as described by Huhtanen et al. (2015). Gas calibration (N_2 and mixture of CH_4, O_2, and CO_2) was performed following the procedures described in Krizsan et al. (2023). The cows were fed a commercial concentrate (Komplett Norm 180, Lantmännen, Malmö, Sweden) in the GreenFeed. The average number of drops per day was 19.8 ± 7.5 (mean ± SD) for the CON group, 21.4 ± 6.8 (mean ± SD) for L-AT, and 24.4 ± 3.7 (mean ± SD) for H-AT. The average number of visits per day was 8 ± 1.3 (mean ± SD) for the CON group, 8 ± 2.2 (mean ± SD) for L-AT, and 9 ± 1.3 (mean ± SD) for H-AT, during the experimental period.

Rumen Fluid. Rumen fluid was collected from the same subset of cows during wk 0, 2, 4, and 12. Collection was performed after morning milking using a stomach tube (Ruminator) as described by Geishauser and Gitzel (1996). To minimize saliva contamination, the first portion of rumen fluid (~500 mL) was discarded. Then ~500 mL was collected, filtered through 2-layer cheesecloth, and aliquots were transferred to one 50-mL Falcon tube (Falcon Conical Centrifuge Tube, Corning Inc., Corning, NY) and four 2-mL SafeSeal microtubes (Sarstedt Inc., Nümbrecht, Germany). All samples were stored at −20°C until analysis of VFA at the Department of Molecular Sciences SLU, Uppsala, Sweden. Rumen fluid samples for VFA analysis were prepared by pipetting 700 µL of sample into a microcentrifuge tube, adding 70 µL of H_2SO_4 (5 *M*), mixing, and centrifuging at 14,000 × *g* for 15 min at 20°C (room temperature). The supernatant was then filtered through a 0.2-µm syringe filter into an HPLC glass vial. Volatile fatty acids were analyzed by HPLC (Shimadzu 2050 Series HPLC, Shimadzu Corporation, Kyoto, Japan), where samples were separated on an ion exclusion column (Rezex ROA-Organic Acid H^+, 300 × 7.80 mm, Phenomenex) and detected by a UV detector at wavelength 210 nm. The mobile phase used was 5 n*M* H_2SO_4 with a flow rate of 0.6 mL/min.

Milk Production and Composition. Milk samples were collected from all 30 cows at each milking on 2 consecutive days biweekly, and stored in small cups with preservative (bronopol) at 4°C until analysis. The samples were sent to the laboratory at the Department of Applied Animal Science and Welfare (SLU, Uppsala, Sweden) for analysis of fat, protein, and lactose, which was performed using infrared spectroscopy (MilkoScan FT120, Foss, Hillerød, Denmark). Additional milk samples were collected during wk 0, 4, and 12 for analysis of iodine and bromine. These samples were pooled by cow and week and stored at −20°C until further analysis at Eurofins Food and Feed Testing, Lidköping, Sweden (DS EN 15111m: 2007).

Feces and Urine. Grab samples of feces (~300 g) were collected as spot samples (Mehtiö et al., 2016) from the subset of 6 cows per treatment in the parlor immediately after milking at 0500 and 1600 h on 2 consecutive days in sampling wk 0, 2, 4, 8, and 12. Feces samples were stored at −20°C, pooled by cow and week to obtain representative samples, dried in a forced-air oven at 60°C for 48 to 72 h, and ground using a mortar to pass through a 2.5-mm sieve. For iNDF analysis, samples from wk 0, 4, and 12 were analyzed at the Department of Applied Animal Science and Welfare (SLU, Umeå, Sweden). Additionally, small fresh (before drying) subsamples of feces collected during wk 12 were stored at −20°C for bromine analysis on the same subsets of 6 cows per treatment. Urine was sampled by stimulating the area below the vulva on the same subset of cows, with 1 sample per day taken on 2 consecutive days in wk 0, 2, 4, 8, and 12, collected in the morning in the freestalls in the barn. Urine samples were collected in plastic cups, filtered into

Table 5. Effect of the control diet (CON, 0% AT) and the diet containing 0.3% (H-AT) *Asparagopsis taxiformis* (AT) on an OM basis on VFA concentration in rumen fluid from lactating dairy cows in experimental wk 2, 4, and 12[1]

	Treatment			*P*-value		
Item	CON	H-AT	SEM	Treatment	Week	Treatment × Week
Total VFA, mmol/L	110	92.8	6.62	0.01	0.43	0.22
Molar proportion, mmol/100 mol						
Acetate	60.3	55.4	1.21	<0.001	<0.001	<0.001
Propionate	23.5	24.3	1.15	0.73	<0.001	<0.001
Butyrate	12.2	13.0	0.60	<0.01	<0.01	<0.01
Isobutyrate	1.8	1.7	0.30	0.99	0.37	0.17
Valerate	1.7	3.3	0.62	<0.01	0.09	0.01
Isovalerate	0.9	1.1	1.17	0.38	0.12	<0.01
Acetate/propionate, mmol/L	2.6	2.5	0.16	0.63	<0.01	<0.01

[1]Least squares means and SEM (n = 54).

a funnel with 2-mm sieve, and pooled within cow and week. The pooled urine samples were stored at −20°C. The urine samples from the CON and H-AT groups collected at wk 12 were subsequently analyzed for bromine concentration at Eurofins Food and Feed Testing, Lidköping, Sweden (DS EN 15111m: 2007).

Metabolic Profile Assessment. Blood samples were collected during morning milking from the same 6 cows/treatment, during wk 0, 4, and 12 of the experiment. Sampling was performed using evacuated tubes (13 × 75 mm BD Vacutainer, NJ) containing lithium heparin as an anticoagulant. The tubes were centrifuged for 15 min at 3,500 × *g* at 4°C (centrifuge model 48 R Rotina Hettich, Germany). Plasma was then divided into 3 aliquots and stored in 2-mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany) at −20°C until further analysis at the Department of Animal Sciences, Food and Nutrition, Faculty of Agriculture, Food and Environmental Science, Universitá Cattolica Del Sacro Cuore, Piacenza, Italy. The concentrations of glucose, urea, zinc, aspartate aminotransferase (**AST**), gamma-glutamyl transferase (**GGT**), total protein, haptoglobin, ceruloplasmin, albumin, cholesterol, and globulin were determined by a clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington, MA). Serum amyloid α (**SAA**) were determined by a commercial kit (SAA TP-802, Tridelta D.L., Ireland) and reactive oxygen metabolites (**ROMt**), paraoxonase, according to Mezzetti et al. (2019), whereas the ferric-reducing ability of plasma (**FRAP**) was determined as described by Premi et al. (2021).

Calculations and Considerations

Concentrations of milk constituents were calculated as the weighted mean of morning and afternoon milk yields. Daily ECM yield was calculated according to Sjaunja et al. (1990). Feed efficiency was determined as the ratio of ECM yield to DMI. Dietary chemical components and feeding values were estimated based on ingredient proportions and their corresponding values.

Total-tract apparent DM digestibility and OM, CP, and NDF digestibility were calculated with equations taken from Guinguina et al. (2021), using iNDF as an internal marker in both feeds and feces. Daily fecal DM output was calculated using NorFor (Volden, 2011), with iNDF used as an internal marker for these calculations. Daily nutrient excretion in feces, including OM, NDF, and CP, was determined by multiplying fecal nutrient concentration by daily fecal DM output.

Because CH_4 emissions did not differ between the CON and L-AT groups, analyses on urine, feces, blood, and rumen fluid were only performed on samples from the CON and H-AT subgroups of cows to save financial resources.

Statistical Analysis

Statistical analyses were conducted using the MIXED procedure in RStudio Team (4.2.1, RStudio: Integrated Development for R, RStudio, PBC, Boston, MA). The analyses involved estimating treatment effects on feed intake, milk yield, CH_4 and H_2 production, and digestibility. The statistical model included fixed effects of treatment and experimental week, and random effect of block. Autoregressive correlation structure of order AR (1) was applied to account for the temporal autocorrelation within each cow, ensuring that the correlation between measurements decreased as the time interval between them increased. Week was considered as repeated measurement. During analysis, nonsignificant parameters were removed. The model used was

$$Y_{ijkl} = \mu + T_i + B_j + W_k + P_l + (TW)_{ik} + \varepsilon_{ijkl}, \quad [1]$$

where Y_{ijkl} is the dependent variable, μ is the mean of all observations, T_i is the effect of treatment, B_j is the effect

of block, W_k is the effect of week, P_l is the pretreatment week (0, which was used as a covariate), $(TW)_{ik}$ is the interaction between treatment and week, and ε_{ijkl} is the random residual error.

A slightly different statistical model, without the covariate, was used for milk composition, bromine and iodine content in the milk, metabolic profile, ECM, and feed efficiency parameters

$$Y_{ijk} = \mu + T_i + B_j + W_k + (TW)_{ik} + \varepsilon_{ijk}, \quad [2]$$

where the variables have the same meanings as in Equation [1].

To calculate LSM, the LSMEANS/DIFF option was used. Statistical differences between treatments were determined using Tukey adjustment with a significance level of $P < 0.05$. Denominator df were obtained by the Kenward-Roger method. Welch 2-sample *t*-test was used to test the null hypothesis (H_0) that the mean bromine concentration in both feces and urine from CON and H-AT cows did not differ. The equation was as follows:

$$H_0: \overline{x_1} = \overline{x_2}; \; H_a: \overline{x_1} \neq \overline{x_2}, \quad [3]$$

$$t = \frac{\overline{x}_1 - \overline{x}_2}{\sqrt{\frac{s_1^2}{n^1} + \frac{s_2^2}{n^2}}},$$

where $\overline{x}_1$ and $\overline{x}_2$ are the sample means of group 1 and 2, s_1^2 and s_2^2 are sample variance of the 2 groups, and n^1 and n^2 are sample sizes of the 2 groups.

RESULTS

DMI and Digestibility

The grass silage was of high nutritional and hygienic quality (Table 1). The cows in the H-AT group had lower daily total DMI than cows in the other 2 groups ($P < 0.01$; Table 2). The NDF digestibility was reduced by 8% in the H-AT group compared with CON. Overall, there were no effect of treatment on OM digestibility, but an interaction between treatment and week was shown ($P = 0.04$; Table 3) in wk 12, with lower OM digestibility in the H-AT group compared with CON ($P = 0.03$), with estimated means of 744 and 762 g/kg, respectively.

Gas Emissions and Rumen Fermentation

The results revealed a reduction of CH_4 production gram per day in the H-AT group compared with the other 2 groups ($P < 0.01$). Additionally, CH_4 yield, measured as gram per kilogram of DMI, and emission intensity per kilogram milk and ECM were lower in the H-AT group compared with the other groups (Table 4). Significantly, during the initial week of the experiment, the H-AT group showed a substantial reduction in CH_4 emission compared with the other groups, indicating a treatment × week interaction ($P < 0.001$), as illustrated in Figure 2. This reduction initially diminished but then stabilized into a more consistent trend by wk 6. However, no differences in methane emissions were observed from wk 9 to 12. The CH_4 mitigation effect caused by AT was reinforced by a concomitant increase in H_2 production. Indeed, cows in the H-AT group showed the highest H_2 production, which was greater compared with both CON and L-AT ($P < 0.01$) and a treatment × week interaction, due to the higher inclusion rate of AT the first week, as shown in Table 4.

Cows in the H-AT treatment demonstrated lower total VFA concentration in rumen fluid compared with CON cows ($P = 0.05$; Table 5). Additionally, the concentration of acetate was lower in the H-AT group compared with CON ($P < 0.0001$), whereas the concentrations of butyrate and valerate were significantly higher in H-AT compared with CON ($P < 0.01$). Additionally, there was a treatment-by-week interaction effect for propionate, with the H-AT group showing a higher concentration in wk 2 compared with CON ($P < 0.05$), but no significant differences were observed between treatments in wk 4 or 12.

Milk Yield, Milk Composition, and Feed Efficiency

Overall, milk yield recorded over the whole study period did not differ between the treatment groups. Milk yield in the sampling weeks for milk composition was lower in the H-AT group than in the other groups ($P = 0.03$). Additionally, the H-AT group showed lower ECM yield and milk fat concentration compared with the other groups ($P = 0.01$). Our results showed no differences between the treatment groups in terms of feed efficiency during the sampling week (Table 2).

Bromine, Iodine, and Bromoform Content

The bromine concentration in the AT material added to the TMR was 59 ± 5.9 g/kg (mean ± SD), the iodine concentration was 5.9 ± 0.45 mg/kg, and the bromoform concentration was 6.44 ± 0.55 mg/kg. Throughout the experiment, the bromoform content in pooled AT samples was measured, showing a reduction over time by 23% (Figure 1). Figure 2 shows (A) enteric methane (CH_4) and (B) hydrogen (H_2) production (g/kg DMI) in cows fed the control diet (CON, 0% AT on an OM basis), L-AT (0.15% AT on an OM basis), and H-AT (0.3% AT on an OM basis).

Table 6. Bromine and iodine concentrations in milk from cows fed the control diet (CON, 0% AT) and the diet containing 0.3% AT (H-AT) on an OM basis during experimental wk 4 and 12[1]

	Treatment			*P*-value		
Item	CON	H-AT	SEM	Treatment	Week	Treatment × Week
Bromine, mg/kg milk	3.9	20.8	1.51	<0.001	<0.001	<0.001
Iodine, mg/kg milk	0.1	0.9	0.83	<0.001	<0.001	<0.001

[1]Least squares means and SEM (n = 24).

In milk, both bromine and iodine were higher in samples from the H-AT group compared with CON (Table 6). The concentrations of bromine and iodine in the milk were determined on 3 occasions (wk 0, 4, and 12) and showed an increase with time and an interaction between treatment and week ($P < 0.001$; Figure 3). A decrease in the concentrations of both bromine (5.2%) and bromoform (16.9%) was observed in the AT from wk 4 to wk 12. Furthermore, the concentration of bromine in milk during this same period showed a 25.2% reduction. The mean bromine concentration in feces samples during the final week of the experiment was 28.5 ± 3.01 mg/kg, whereas that in urine samples was 29.7 ± 5.00 mg/kg. The corresponding values for the CON group were 6.1 ± 0.67 mg/kg in feces and 3.3 ± 0.58 mg/kg in urine. The *t*-tests results revealed differences between the 2 groups for both feces (t [5.5] = −7.24, $P < 0.001$) and urine (t [5.1] = −5.17, $P < 0.01$; Figure 4).

Metabolic Profile

The concentrations of plasma parameters used as biomarkers of energy, protein, mineral metabolism, antioxidants, and inflammation are shown in Table 7. Inclusion of AT resulted in lower cholesterol levels and FRAP levels in plasma from H-AT cows compared with CON cows ($P < 0.001$). Magnesium concentration was slightly higher in plasma samples from the H-AT group compared with CON (mean ± SEM: 1.02 ± 0.02 mmol/L vs. 0.97 ± 0.02 mmol/L, respectively; $P = 0.03$).

DISCUSSION

Our study sought to address knowledge gaps regarding the potential physiological side effects and long-term effects of supplementing dairy cow diets with AT to mitigate enteric CH_4 emissions. We confirmed that AT supplementation effectively reduces enteric CH_4 production in ruminants, consistent with previous findings (Roque et al., 2021; Stefenoni et al., 2021; Krizsan et al., 2023). However, our results also showed that the efficacy of AT diminished over time, likely due to a reduction in bromoform content in AT, similar to what was observed in Stefenoni et al. (2021). By extending the experimental period to 13 wk and conducting a thorough evaluation of animal productivity and potential residues in biological fluids, our research offers novel insights into the long-term efficacy of AT in reducing methane emissions, as well as the metabolism of bromoform and its residue excretion.

DMI and Digestibility

In our study, an inclusion level of the L-AT group did not affect DMI compared with CON, whereas an inclusion level of H-AT decreased DMI by 7% compared with CON. Similarly, Eikanger et al. (2024) demonstrated in a 5-wk continuous experiment that an inclusion level of 0.25% AT on an OM basis resulted in a 16.2% reduction in DMI compared with the control group and a 12.7%

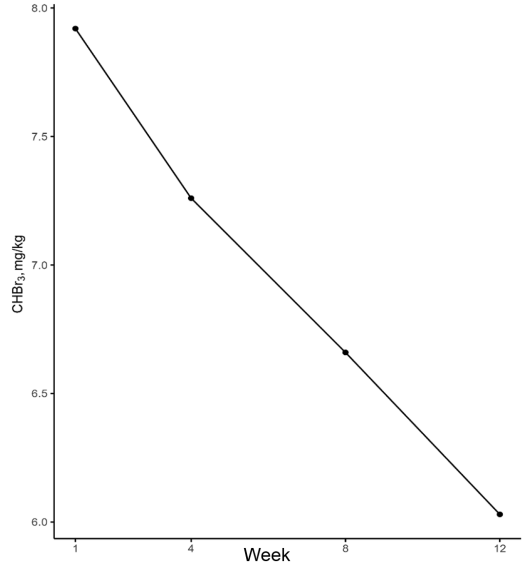

Figure 1. Concentration of bromoform ($CHBr_3$) in *Asparagopsis taxiformis* (AT) pooled samples collected at wk 1, 4, 8, and 12 (n = 4).

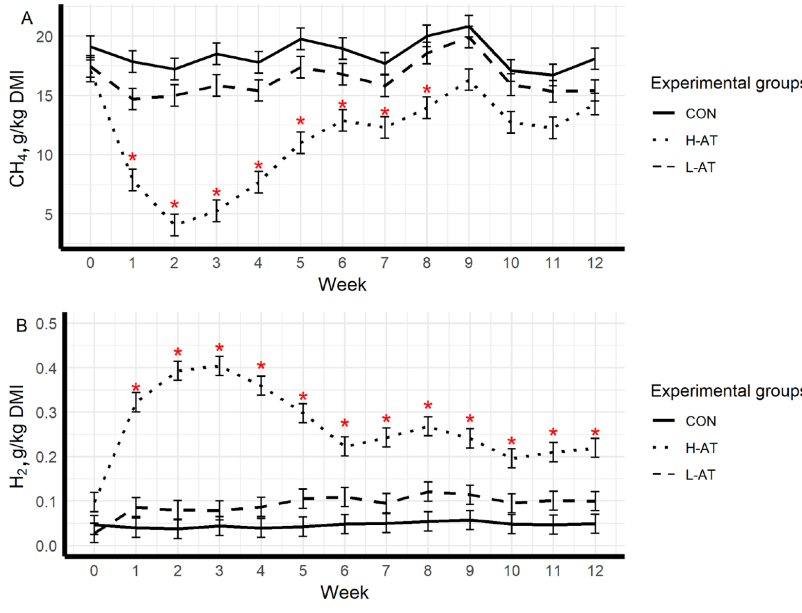

Figure 2. (A) Enteric methane (CH_4) production and (B) enteric hydrogen (H_2) production (both g/kg DMI) in cows fed the control diet (CON, 0% AT on OM basis), low additive treatment diet (0.15% AT on an OM basis; L-AT), and high additive treatment diet (0.3% AT on an OM basis; H-AT). Least squares means per treatment group and week are shown, with error bars representing the SEM. Each data point represents observations from 10 cows per treatment over 12 wk (n = 360). Statistical significance ($P < 0.001$) of the difference between CON and H-AT is indicated with an asterisk (*).

reduction compared with an inclusion level of 0.12% AT on an OM basis. Additionally, Krizsan et al. (2023) observed in a short-term changeover trial, a decline in DMI in cows fed a diet supplemented with 0.5% AT on an OM basis, with a reduction of 13.8% compared with the control group.

Despite a high SEM (20.7), our data clearly demonstrate a reduction in NDF digestibility by 8% in the H-AT group compared with CON. This finding contradicts previous short-term observations reported by Stefenoni et al. (2021) and Krizsan et al. (2023).The extended duration of our study suggests that additional factors or interactions may contribute to the observed differences in NDF digestibility between the CON and H-AT groups. This underscores the importance of considering study duration and potential time-dependent effects in such experiments.

Gas Emissions and Rumen Fermentation

Methane yield was 30% lower for the H-AT group compared with CON (Table 4). A similar trend was observed by Stefenoni et al. (2021) when cows were fed AT at an inclusion level of 0.5% (OM basis), compared with 0.3% in our study. When expressed as gram of CH_4 per kilogram of DMI, the CON and L-AT groups showed similar levels of emissions over time, whereas H-AT showed a marked decrease in CH_4 emissions in wk 1 and then a gradual increase over time until the level stabilized at around 30% lower emissions compared with CON and L-AT from wk 6. However, no mitigating effect of AT was observed from wk 9. The rapid decrease in CH_4 production in H-AT cows in wk 1 was caused by the accidently higher (double) dose of AT than originally intended. Corrective measures were taken instantly, and the correct dose was distributed from d 1 in wk 2 of the experiment. To offset any effects of the incorrect dosage, the experimental period was extended by 4 weeks, from a planned 8 wk to 12 wk, to ensure time for the rumen microbiota to re-establish. Methane yield (g of CH_4/kg of DMI) was 34% lower in the H-AT group compared with CON, similar to what was observed in other studies (Roque et al., 2021; Stefenoni et al., 2021; Krizsan et al., 2023). We observed a decline in CH_4 in-

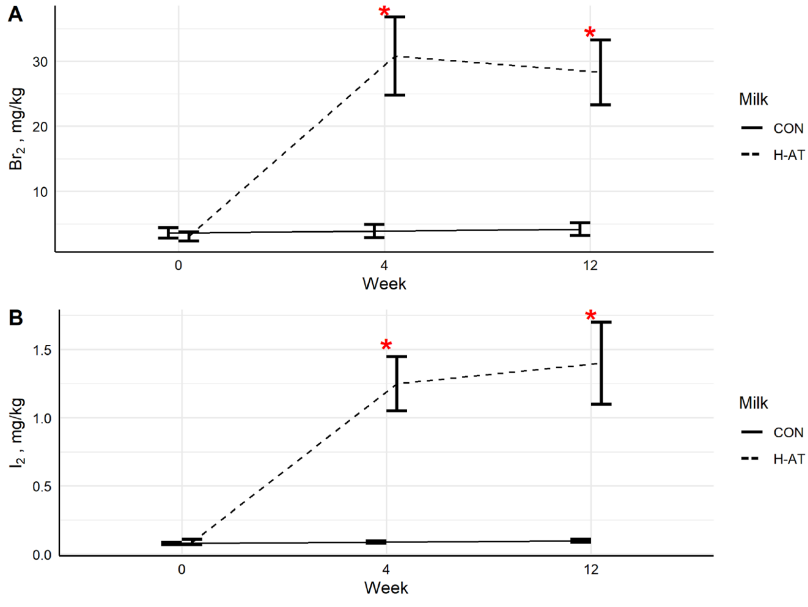

Figure 3. Concentration of (A) bromine (Br_2) and (B) iodine (I_2) in milk samples taken at wk 0, 4, and 12 from cows fed the control diet (CON, 0% AT) and the diet containing 0.3% AT (H-AT) on an OM basis. Least squares means per group and week (n = 36). Error bars represent the SD within each group. A significant ($P < 0.001$) difference between CON and H-AT is indicated with an asterisk (*).

tensity (g of CH_4/kg of ECM), with a 40% reduction in H-AT cows compared with CON. Similarly, Stefenoni et al. (2021) observed a 26% reduction in CH_4 intensity from dairy cows at an AT inclusion level of 0.5% of DM compared with a control diet, although the effect diminished over time. It seems that the reduction of the effect of AT was due to a reduction in the concentration of bromoform in AT during storage (Figure 1). The reduction effect in CH_4 in the H-AT group from wk 4 to 12 decreased by 42.2%; however, from wk 9 to 12, there was no interaction effect between treatment and week. This pattern may be compared with the 16.9% reduction in bromoform concentration in AT over the same period. This discrepancy may indicate a potential adaptation of rumen microbes to bromoform, potentially reducing its long-term effectiveness in CH_4 reduction, warranting further investigation. As highlighted by Kinley et al. (2020) and Roque et al. (2019), achieving reductions in CH_4 emissions requires a certain concentration of bromoform. These studies indicate that when bromoform levels are below a critical threshold, the effectiveness of CH_4 mitigation may be diminished. Specifically, Roque et al. (2019) found that a low inclusion rate of 12.1 mg of bromoform/kg of DMI fed to dairy cows resulted in a 20% methane reduction, whereas Kinley et al. (2020) observed an inclusion of 11.8 mg of bromoform/kg of DMI in beef steers leading to a 38% methane reduction. These results, as noted by Alvarez-Hess et al. (2024), suggest a minimum bromoform concentration of approximately 12 mg bromoform/kg DMI in both beef and dairy cattle TMR systems is needed for substantial CH_4 reduction, reinforcing the nonlinear response to lower bromoform levels. In our study, bromoform concentrations in L-AT were below 12 mg bromoform/kg DMI, whereas for H-AT, the levels were 20.7 mg bromoform/kg DMI at wk 4, decreasing to 16.8 mg bromoform/kg DMI by wk 12. Although the bromoform concentration in H-AT exceeded 12 mg/kg DMI by the end of this study, its inhibitory effect on CH_4 was reduced.

Early work by Chalupa (1977) showed that inclusion of halogenated compounds as feed additives for ruminants appears to have repercussions. It has been established that supplementation of dairy cow diets with halogenated compounds or other methanogen-inhibiting compounds, such as 3-nitroxypropanol, leads to an increase in metabolic H_2 concentration in the rumen (Hristov et al., 2015; Roque et al., 2019; Stefenoni et al., 2021). This increase, in turn, is associated with impaired microbial

production of vitamin B_{12} and ineffective metabolism of increased proportions of propionate and butyrate in rumen fluid caused by AT supplementation (Chalupa, 1977). In line with these findings, we observed an almost 5-fold increase in enteric H_2 concentration in the H-AT group compared with CON, and the elevated concentration of H_2 in H-AT, compared with CON, remained consistent across all weeks. This increase underscores the relevance of previous research demonstrating similar effects of methanogen-inhibiting compounds on rumen metabolism. Such findings support our observations and highlight the potential metabolic implications of the additive treatment in our study.

Methane inhibition in the H-AT group was accompanied by a decrease in total VFA concentration in rumen fluid, a reduction in acetate concentration, and an increase in propionate, butyrate, and valerate concentrations. Similar results have been reported by Stefenoni et al. (2021) and Krizsan et al. (2023), indicating a shift in fermentation pattern as a result of inhibited methanogenesis in the rumen. This shift may be associated with alterations in microbial metabolic activity.

The lack of effect of AT supplementation on CH_4 and H_2 production in the L-AT group indicates that the effect of AT as a CH_4-mitigating additive is dose-dependent, necessitating further investigations on the minimum level of addition required to achieve an inhibitory effect.

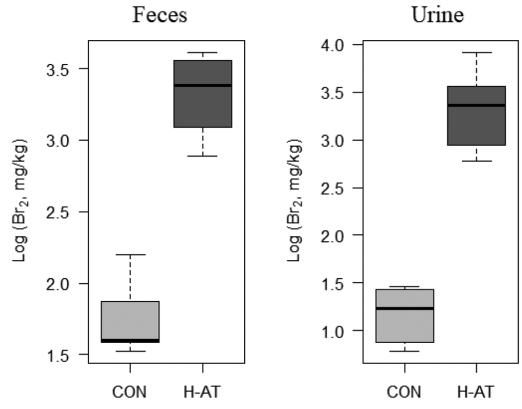

Figure 4. Box plot distribution of bromine concentration (Br_2, mg/kg; $P < 0.001$) in feces and urine samples collected in experimental wk 12 from cows fed the control diet (CON, 0% AT) and cows fed the diet containing 0.3% AT (H-AT) on an OM basis. Logarithmic values (n = 12). The line inside the box indicates the median concentration of bromine. The box represents the interquartile range (IQR) from the 25th to the 75th percentile of the data. The whiskers extend to the smallest and largest values within 1.5 times the IQR from the box.

Lactational Performance: Milk Yield and Composition

The H-AT group in our study had 4.7% lower fat concentration in milk compared with CON. This may be attributable to a shift in rumen fermentation pattern (Palmquist et al., 1993), resulting in increased propionate and reduced acetate in the rumen rather than directly affecting milk composition in the H-AT group. We observed a 2% and 4% reduction in ECM yield and milk yield, respectively, in the H-AT group compared with CON, during the study period. Similarly, Stefenoni et al. (2021) reported comparable reductions in both milk and ECM yields. Additionally, Krizsan et al. (2023) reported reduced DMI and ECM yields with 0.5% AT on OM supplementation, although milk yield did not differ. This links the observed differences in nutrient availability for milk production primarily to DMI. Additionally, differences were observed in rumen molar proportions of specific VFA across groups, as discussed previously. Given that up to 70% of the energy supplied to ruminants is derived from VFA produced in the rumen (Bergman, 1990), the observed variations in ruminal fluid total VFA concentrations and DMI among dietary groups in our study likely influenced the achieved milk yield and ECM yield. These findings highlight the importance of conducting long-term studies to understand the enduring effects of dietary interventions, such as AT, on milk production. The lower production outcomes observed in the current study underscore potential limitations of AT as a mitigation strategy, particularly in contexts where sustaining or enhancing milk output is critical for economic viability and production efficiency.

Bromine and Iodine in Milk, Feces, and Urine Samples

There were higher levels of bromine and iodine residues in milk from the H-AT group compared with CON. The bromine concentration was approximately 7-fold higher, whereas the iodine concentration was approximately 13-fold higher, which is in line with findings in previous studies where dairy cows were fed 0.5% AT (Stefenoni et al., 2021; Krizsan et al., 2023). Additionally, Eikanger et al. (2024) reported an increase in iodine in milk samples from cows fed an inclusion level of 0.25% AT on an OM basis. However, the bromine and iodine concentrations in milk in our study were lower than observed by Krizsan et al. (2023), due to our lower inclusion level of AT.

In contrast, a decline in both bromine in the milk and bromoform content in AT was observed from wk 4 to 12. This may be explained by the degradation of bromoform content in AT during storage. We observed a linear trend in bromine and iodine concentrations in milk, which

Table 7. Plasma levels of different metabolic parameters in lactating dairy cows fed the control diet (CON, 0% AT) and the diet containing 0.3% AT on an OM basis (H-AT) during experimental wk 4 and 12[1]

	Treatment			*P*-value		
Parameter[2]	CON	H-AT	SEM	Treatment	Week	Treatment x Week
Glucose, mmol/L	4.04	4.15	0.1	0.30	0.56	0.12
Total cholesterol, mmol/L	8.35	7.01	0.4	<0.01	0.47	0.54
Total protein, g/L	78.0	80.9	1.8	0.11	0.06	0.50
Albumin, g/L	38.3	38.1	0.5	0.70	0.40	0.94
Globulin, g/L	39.7	42.8	1.1	0.05	0.01	0.42
Urea, mmol/L	5.75	5.40	0.3	0.83	0.42	0.005
Calcium, mmol/L	2.49	2.54	0.05	0.29	0.20	0.27
Magnesium, mmol/L	0.97	1.02	0.02	0.03	<0.01	0.01
Zinc, µmol/L	11.4	10.3	0.5	0.06	0.06	0.92
AST, U/L	92.3	91.4	6.6	0.36	0.04	0.36
GGT, U/L	27.1	29.7	3.4	0.45	<0.001	0.76
Haptoglobin, g/L	0.09	0.10	0.05	0.87	0.85	0.18
Ceruloplasmin, µmol/L	2.30	2.28	0.1	0.91	0.16	0.39
Paraoxonase, U/L	85.7	87.2	8.6	0.86	0.34	0.27
ROMt, mg of H_2O_2/100 mL	14.4	14.6	0.65	0.85	0.42	0.36
FRAP, µmol/L	191	175	3.51	<0.001	0.43	0.24
SAA, µg/mL	75.4	70.0	21.4	0.80	0.09	0.18

[1]Least squares means and SEM (n = 24).

[2]AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ROMt, reactive oxygen metabolites test; FRAP, ferric-reducing ability of plasma; SAA, serum amyloid A.

accumulated over time in the H-AT group (Figure 3). Knowledge of the accumulation rate is important when evaluating the metabolism of these compounds in cows, and further studies are required.

There are limited recommendations regarding daily bromine intake for human consumption. According to van Leeuwen et al. (1987), the acceptable daily dose of bromine is 0.4 mg/kg BW, which corresponds to 28 mg daily in an adult weighing 70 kg. The bromine concentration in milk samples from the H-AT group was 20.8 mg/kg, allowing for a maximum daily intake of 1.3 kg of milk per day, assuming no other dietary sources of bromine. Maximum daily iodine intake in humans varies with age, with a generally accepted range of 90 to 150 µg/d (EFSA, 2017) and a maximum upper level of 600 µg/d (Blomhoff et al., 2023). The concentration of iodine in milk from the H-AT group was 0.92 mg/kg, allowing for a daily milk consumption of 0.01 to 0.16 kg per person. For an individual, the recommended maximum daily milk intake would be 0.65 kg. Our analyses showed that during the final week of the experiment, the H-AT group excreted almost 5-fold higher average bromine concentrations in feces and 9-fold higher concentrations in urine compared with CON. This finding represents a significant and novel contribution to the current understanding of bromine excretion patterns in dairy cattle receiving an AT supplement to reduce enteric CH_4 emissions. The elevated bromine levels in both feces and urine suggest that a substantial portion of the bromine from the AT supplement is not metabolized but rather excreted by the cows. This finding suggests that the cows' digestive systems and metabolic pathways may not fully process or use bromine, resulting in its excretion. The high excretion rates indicate that bromine, in the form of bromoform, may have limited metabolic integration within the bovine system, with the compound or its metabolites being rapidly cleared from the body. These elevated excretion levels raise concerns about the long-term effects on animal health, particularly the potential toxicity of accumulated bromine in tissues, as well as environmental contamination through manure management practices.

Potential Alteration of Metabolic Profile

Metabolic profile assessment revealed an effect of AT on magnesium level in the H-AT group, although the levels recorded in both groups were within the normal range (Bertoni and Trevisi, 2013). A similar finding was made by Li et al. (2018) in sheep fed various concentrations of AT for 21 d. Red macroalgae species, such as AT, are rich in minerals (MacArtain et al., 2007), and could be beneficial in addressing magnesium deficiency issues in cattle.

In contrast to our findings, Li et al. (2018) observed an increase over time in blood cholesterol concentrations in sheep fed an AT supplement. In our study, blood cholesterol was lower in the H-AT group, although values in both CON and H-AT were higher than the normal range (Bertoni and Trevisi, 2013).

The FRAP level decreased in the H-AT treatment group compared with CON, suggesting that AT addition to the diet may have reduced the antioxidant capacity of blood plasma. Antioxidants play a crucial role in neu-

tralizing harmful free radicals in the body (Benzie and Strain, 1996), and the decrease in FRAP may indicate an imbalance between free radical production and the body's ability to neutralize these, potentially leading to increased oxidative stress. This increased oxidative stress can impair immune function, making cows more susceptible to infections and diseases. Additionally, it can negatively affect milk yield, quality, and reproductive performance, as well as hinder growth and overall development in young cattle (Sordillo and Aitken, 2009). Ongoing research into the specific antioxidant needs and effective supplementation strategies for cows, particularly when using AT, can lead to improved guidelines and practices for cattle management.

CONCLUSIONS

Supplementation of dairy cow diets with 0.3% AT on an OM basis reduced enteric CH_4 production by 30%, whereas a lower dose (0.15% OM) had no effect. However, from wk 9 to 12, no further reduction in CH_4 was observed, emphasizing the need for stable storage conditions to preserve bromoform in AT. This diminished effect may also indicate rumen microbiome adaptation over time. Cows on the 0.3% AT treatment showed reduced DMI, milk yield, and milk fat content, along with altered rumen fermentation. Elevated bromine and iodine concentrations in milk, and high bromine levels in feces and urine, suggest metabolic and excretion processes. Plasma FRAP values were lower in the 0.3% AT group, indicating increased oxidative stress. Positive outcomes included reduced total cholesterol and increased plasma magnesium. Further studies on algae storage and rumen microbiota adaptations are needed to understand metabolic changes and ensure cow health.

NOTES

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Nonstandard abbreviations used: AST = aspartate aminotransferase; AT = *Asparagopsis taxiformis*; CON = control group with no AT; FRAP = ferric-reducing ability of plasma; GF = GreenFeed; GGT = gamma-glutamyl transferase; H-AT = 0.3% AT on an OM basis; iNDF = indigestible NDF; L-AT = 0.15% AT on an OM basis; ND = not determined; NorFor = Nordic feed evaluation system; ROMt = reactive oxygen metabolites; SAA = serum amyloid α.

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This thesis assesses *Asparagopsis spp.* as a strategy to mitigate enteric methane (CH_4) in dairy cows across three studies. Paper I shows that A. taxiformis reduces CH_4 while influencing performance, metabolism, and fermentation. Paper II demonstrates shifts in rumen and faecal microbiota linked to CH_4 inhibition. Paper III highlights how cultivation conditions affect algae composition and its in *vitro* CH_4-reducing potency. Together, the studies reveal that mitigation depends on feeding level and duration, microbial adaptation and algal composition emphasizing the need for standardized production and effective application.

Melania Angellotti received her doctoral education at the Department of Applied Animal Science and Welfare at the Swedish University of Agricultural Sciences. She received her M.Sc. in Animal Biotechnology at the University of Bologna, Italy.

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