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Effects of *Aspragopsis taxiformis* on enteric methane emission, rumen fermentation and lactational performance of (Norwegian Red) dairy cows

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Animal Science

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Abstract

Ruminants with their rumen microbial fermentation contribute to greenhouse gas emissions when producing enteric methane. Feed supplements able to inhibit the formation of methane and thereby reduce the amount of methane emitted from rumen fermentation have been of great interest among researchers. *Asparagopsis taxiformis*, a red seaweed producing a diverse range of methane analogs, have recently proven its potential as a methane mitigator.

The aim of this thesis was to further evaluate the potential mitigating effects of *A. taxiformis* supplemented to Norwegian red dairy cattle, including its possible effects on rumen fermentation parameters and lactational performance. This study confirms a dose-dependent mitigating potential of *A. taxiformis*, where methane production (g/day) by dairy cattle fed 0.25% *A. taxiformis* on an organic matter basis were significantly reduced by 22% ($P = 0.037$). Including *A. taxiformis* in the diet also affected feed palatability and significantly reduced dry matter intake ($P < 0.001$) and further milk yield ($P < 0.001$). Cows fed *A. taxiformis* had a significant decrease in rumen acetate-to-propionate ratio ($P = 0.028$) and displayed a significant ($P = 0.026$) increase in milk lactose contents (%). While a methane mitigating effect of *Asparagopsis taxiformis* was obtained, further work is required to assess possible adverse and long-term effects on animal health and productivity.

Sammendrag

Drøvtyggere og deres mikrobielle vomfermentering bidrar til drivhusgassutslipp når metan produseres. Fôrtilskudd som kan hemme dannelsen av metan og dermed redusere mengden metan frigitt fra vomfermentering har vært av stor interesse blant forskere. *Asparagopsis taxiformis*, en rødalge som produserer et stort utvalg av metanaloger, har bevist sitt potensiale som en metaninhibitor.

Målet med denne oppgaven var å videre evaluere mitigeringspotensialet til *Asparagopsis taxiformis* supplert til melkekyr av rasen Norsk rødt fe, inkludert mulige effekter på vomfermentering og laktasjonsytelse. Denne studien bekrefter et doseavhengig reduserende potensial for *Asparagopsis taxiformis*, der metanproduksjonen (g/dag) hos melkekyr fôret med 0,25 % *Asparagopsis taxiformis* basert på organisk materiale ble signifikant redusert med 22 % ($P = 0,037$). Inkludering av *Asparagopsis taxiformis* i dietten påvirket også fôrets smak og reduserte tørrstoffinntaket betydelig ($P < 0,001$) samt melkemengde ($P < 0,001$). Kyr fôret med *Asparagopsis taxiformis* hadde en signifikant reduksjon i forholdet av produsert vomacetat og propionat ($P = 0,028$), i tillegg vistes en signifikant ($P = 0,026$) økning i innholdet av melkelaktose (%). Samtidig som det ble oppnådd en metanreduserende effekt av *Asparagopsis taxiformis*, kreves det ytterligere arbeid for å vurdere mulige skadelige og langsiktige effekter på dyrehelse og produktivitet.

Abbreviations

3-NOP	3-nitrooxypropanol
Ace	Acetate
AT	Asparagopsis taxiformis
HCO ₃ ⁻	Bicarbonate
But	Butyrate
BW	Body weight
CAZymes	Carbohydrate active enzymes
CH ₄	Methane
CO ₂	Carbon dioxide
CP	Crude protein
CPD	Crude protein digestibility
CPI	Crude protein intake
DIM	Days in milk
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
ECM	Energy corrected milk
FFA	Free fatty acids
GHG	Green house gas
H ₂ O	Water
He	Helium
IsoBut	Isobutyrate
IsoVal	Isovalerate
MCR	Methyl-coenzyme M reductase
MS	Milk samples
MUFA	Monounsaturated fatty acids
MUN	Milk urea nitrogen
MY	Milk yield
N ₂	Nitrogen
NDF	Neutral detergent fiber
NDFI	Neutral detergent fiber intake
NDFom	Neutral detergent fiber corrected for ash

NDFomD	Neutral detergent fiber corrected for ash digestibility
NEB	Negative energy balance
NH ₃ -N	Ammonia nitrogen
NRF	Norwegian Red
OM	Organic matter
OMD	Organic matter digestibility
OMI	Organic matter intake
OST	Esophageal tube
Pro	Propionate
PUFA	Polyunsaturated fatty acid
RS	Rumen samples
SCC	Somatic cell count
SF ₆	Sulfur hexafluoride
SFA	Total saturated fatty acid
StarchD	Starch digestibility
StarchI	Starch intake
TG	Total gas
TMAH	Tetramethylammonium hydroxide
TMR	Total mixed ration
TRF	Time relative to feeding
tVFA	Total volatile fatty acids
Val	Valerate
VFA	Volatile Fatty acids

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1. Introduction

Reducing the impact on global environmental changes is one of the human population's most significant commitments in modern times. By signing the Paris agreement in 2015 (Agreement, 2015), all signing parties recognized the need to stagger the increasing average global temperature and reduce greenhouse gas (GHG) emissions. At the same time, each party agreed to increase the availability of green solutions and adapt strategies to their unique circumstances without threatening national food production (Agreement, 2015). Furthermore, an initiative to reduce global methane (CH₄) emissions by 30% before 2030 was launched at the UN climate change conference in November of 2021 (COP26, 2021), increasing the pressure on the development and utilizing of green solutions in all industries.

This has also resulted in an intensified focus on strategies to reduce the carbon footprint from the agricultural sector, particularly from cattle. To meet its energy requirement, the ruminant depends on byproducts of microbial metabolism in the form of volatile fatty acids (VFA) (Chwalibog & Hvelplund, 2003). A major consequence of this microbial activity is the synthesis of enteric methane (CH₄), a potent GHG, in a process where byproducts carbon dioxide (CO₂) and hydrogen (H₂) are used to form CH₄, commonly referred to as methanogenesis. Methane production represents a significant loss of gross energy by the ruminant (Wolin et al., 1997). Inhibiting rumen methanogenesis and redirecting the H₂ produced during rumen microbial fermentation away from CH₄ and towards alternative hydrogen sinks are seen as a potential mitigation strategy but could potentially affect the rumen environment through alterations in the microbial population and its metabolites (Öztürk & Gur, 2021). These changes can, in turn, alter the ruminant's energy supply, and ultimately animal productivity (Wolin et al., 1997).

Although the agricultural sector in Norway reduced its GHG emissions by 6% from 1990 to 2019, agriculture practices still contribute to 8.8% of the national emissions (Miljødirektoratet, 2020). On a global scale, CH₄ by itself is responsible for 10-12% of the anthropogenic emissions, and agriculture is again responsible for about 44% of these (IPCC, 2007). Klimakur 2030, a report prepared by several Norwegian professional bodies assessing the potential of mitigating GHG emissions in Norway, points out feed additives in addition to increased feed quality and animal health as ways to mitigate GHG emissions from the agricultural sector (Enova, 2020). Numerous strategies have been tested to mitigate enteric CH₄. Among feed supplements, few have shown

tendency to a higher mitigating potential than macroalgae containing bromoform and other halomethanes, in particular, *Asparagopsis taxiformis* (Honan et al., 2021; Min et al., 2021), which have proven its mitigating potential *in vitro* (Kinley et al., 2016; Machado et al., 2016a; Machado et al., 2016b) and *in vivo* (Kinley et al., 2020; Li et al., 2018; Stefenoni et al., 2021).

2. Background

2.1 The rumen and role of microbial fermentation

The ruminant digestive system is unique with its compartmentalized forestomach divided into the rumen, reticulum, omasum, and abomasum. The two former, named as the reticulorumen, serve as one great fermentation chamber for plant materials, ensuring decomposition of feeds while making nutrients available for the ruminant (Sjaastad, 2010). Plant material carbohydrates are composed of structural and non-structural fractions. The structural carbohydrates, cellulose, hemicellulose, and lignin, a phenolic compound, are major polymers found in the plant cell wall (Weisbjerg, 2003). Carbohydrates as a whole contribute around 75% of the total organic matter (OM) in a typical ruminant diet (Weisbjerg, 2003), providing up to 85% of the gross energy (Sjaastad, 2010). Ruminants and monogastric animals do, however, not produce the enzymes needed to degrade plant fibers into utilizable energy directly. Instead, the ruminant's success in utilizing plant fibers is owed to the rumen microbial community, and their carbohydrate active enzymes (CAZymes), including cellulases and hemicellulases, which are able to break the beta-1,4-glucosidic bonds (Bohra et al., 2019) found within cell wall polymers (Weisbjerg, 2003).

2.1.1 The rumen microbiota

With the microbial community inhabiting the reticulorumen, feeding the ruminant means providing the microbes first and the ruminant second (Hungate, 1966). With a stable physiological temperature around 39°C, a pH usually fluctuating between 6.0-6.8, and depletion of oxygen, the rumen environment is stable and well suited for its microbial inhabitants (Sjaastad, 2010). The rumen microbiota consists of anaerobic bacteria, protozoa, fungi, and viruses living in a symbiotic relationship with the host (Figure 1) (Huws et al., 2018; Söllinger et al., 2018). Within this community, primary microbes utilize feed entering the rumen directly, whereas secondary microbes exploit metabolites produced by the former (Sjaastad, 2010).

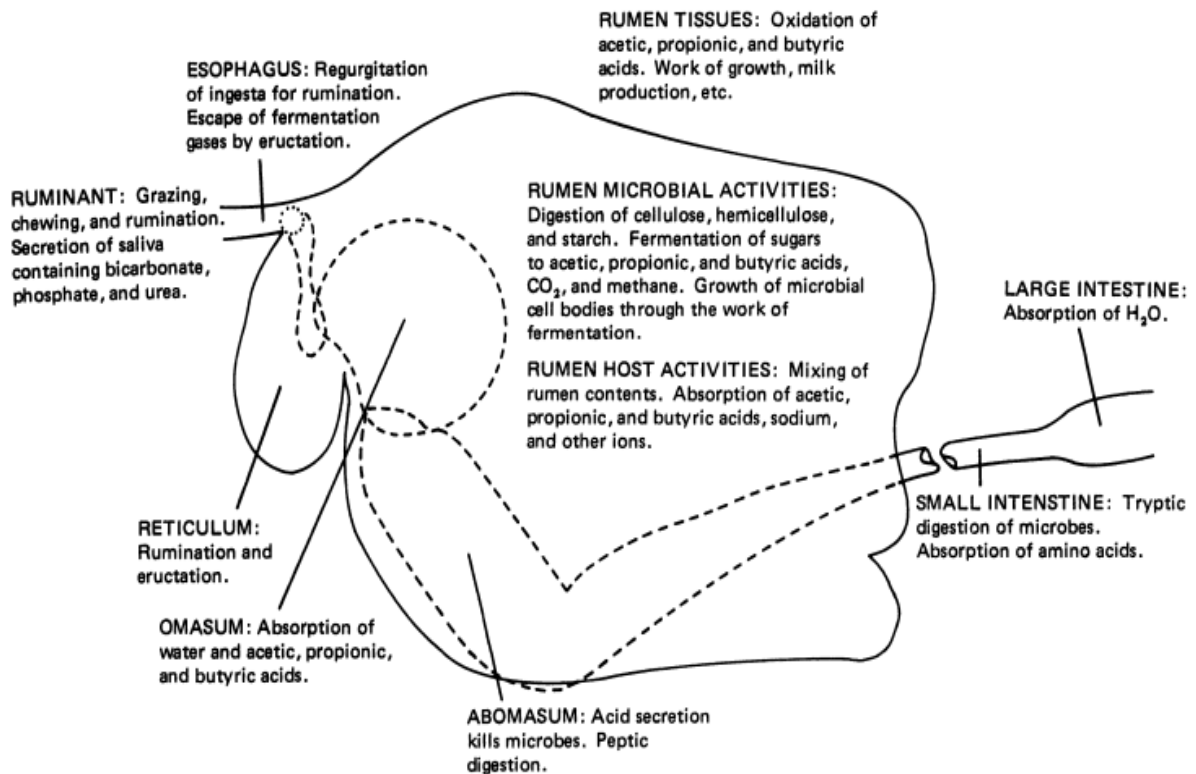


Figure 1. Overview of ruminant and microbiome relations during rumen biomass degradation and nutrient absorption. Adapted from Hungate (1985)

While the rumen microbial community is far from fully explored, a core bacterial microbiome consisting of *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* has been described across a variety of ruminant species (Henderson et al., 2015). These microbes can have different roles within the microbiome, from decomposition of recalcitrant plant fiber using CAZymes, to fermentation of soluble sugars into metabolites taken up by the host. Although the often neglected eukaryotic population, namely protozoa and fungi, are less numerous than the bacteria, they make up for it by size. Around 40% of the total rumen microbial biomass consists of Protozoa and 8% of fungi (Thirumalesh & Krishnamoorthy, 2013). Rumen eukaryotes are involved in plant fiber degradation (Huws et al., 2018), where especially rumen fungi contribute a diverse range of fiber degrading CAZymes (Hagen et al., 2021; Solomon et al., 2016).

The methane-producing population collectively referred to as methanogens are anaerobic archaea with a more specialized metabolism. *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium*, together with less abundant *Methanosphaera sp.* and *Methanomassiliicoccaceae* groups, are identified as the most predominant archaeal methanogens among several ruminants (Henderson et al., 2015).

2.1.2 Energy gained and energy lost through microbial fermentation

Carbohydrate degradation through microbial fermentation yields substrates available for the ruminant to utilize as energy in the form of VFA's (e.g., acetate, propionate, and butyrate) (Figure 2). These are absorbed (60-70%) across the reticulorumen epithelium, driven by the concentration difference between rumen and extracellular fluids in undissociated form, or as anions through passive transport, exchanged with bicarbonate (HCO_3^-) (Sjaastad, 2010). About 50-70% of the VFA produced is acetate, while butyrate and propionate make up around 5-20 and 15-40%, respectively. Acetate is synthesized through decarboxylation of pyruvate and phosphorylation of ADP to ATP. Butyrate synthesis via acetyl-coenzyme A (CoA) is similar to acetate synthesis, which generates 8H^+ per glucose molecule. However, synthesis of butyrate requires 4H^+ for the reduction of acetoacetyl-CoA to butyryl-CoA per glucose molecule and do therefore not yield more H^+ than used. Propionate is produced from pyruvate via lactate or succinate which, contrary to acetate, consume 8H^+ per glucose molecule used (Kristensen, 2003).

Hydrogen generated via VFA synthesis is the limiting factor for methanogenesis (Hungate, 1967), which serves as a hydrogen sink, reducing wasted carbon sources from microbial feed degradation via three pathways: the hydrogenotrophic, the methylotrophic, and the acetoclastic pathways (Galagan et al., 2002). The pathway used depends on the substrate of choice by different methanogenic archaea (Balows et al., 2013). While the initial substrates for each pathway are different, the last step involving the cobamine-dependent transfer of methyl groups by methyl-coenzyme M reductase (MCR) is common for all three (Ermler et al., 1997), and a desired target for inhibition of CH_4 formation via feed supplements, including *A. taxiformis*. In the case of CH_4 inhibition, there is a need to utilize alternative hydrogen sinks, including propionate synthesis, reductive acetogenesis, and reduction of sulfates and nitrates (Figure 2) (Pereira et al., 2022). These are, however, energetically unfavorable under normal rumen conditions (Ellis et al., 2008).

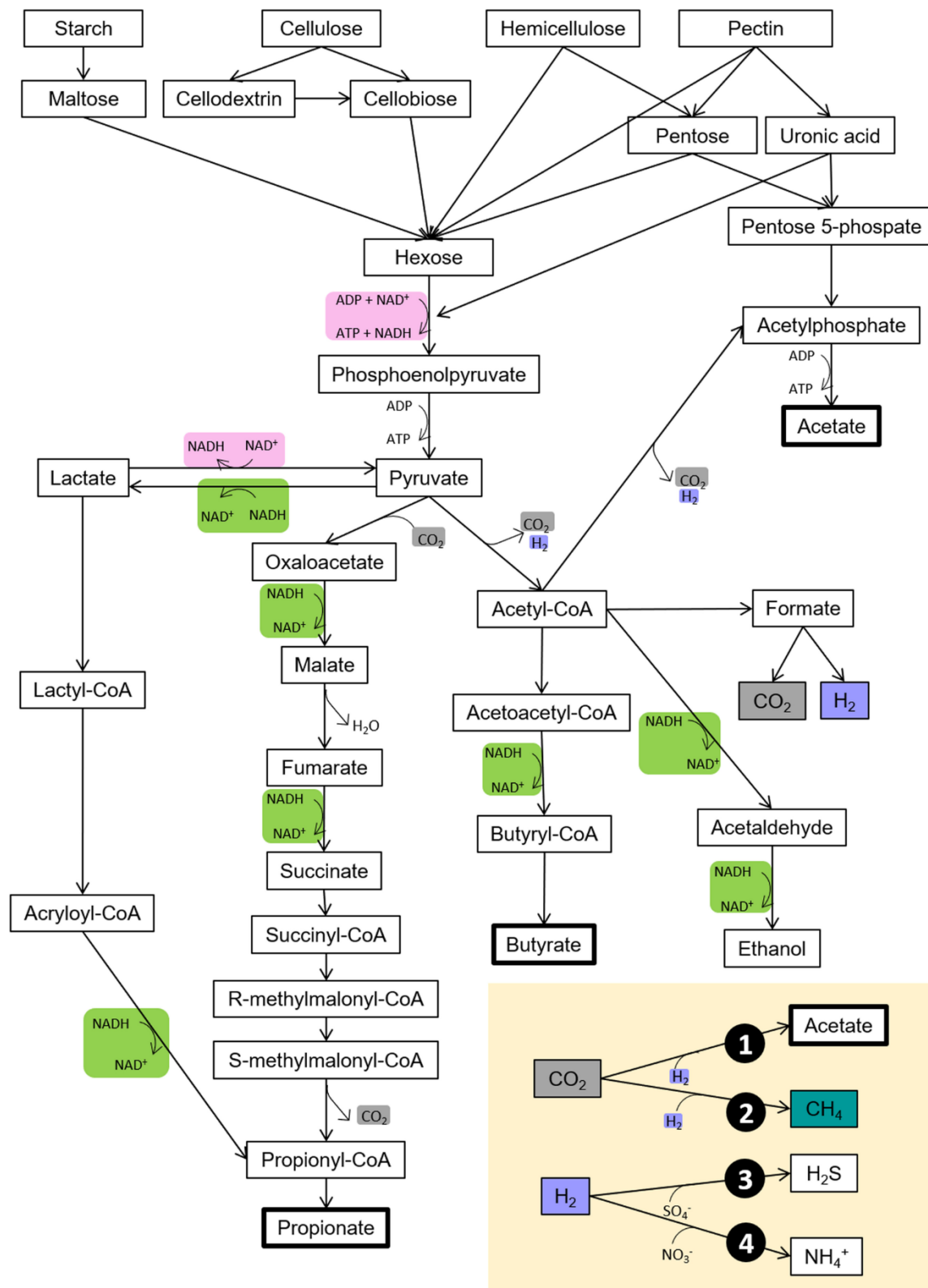


Figure 2. Pathways of volatile fatty acid (VFA) synthesis from rumen carbohydrate fermentation and rumen hydrogen sinks (yellow box) where 1 = reductive acetogenesis; 2 = methanogenesis, 3 = sulfate reduction; and 4 = nitrate reduction. Adapted from (Pereira et al., 2022).

2.3 *Asparagopsis taxiformis*

Macroalgae, also called seaweeds, are classified as protists and use chlorophyll for photosynthesis (Lobban et al., 1994). Classification of algae is usually based on pigmentation where *Asparagopsis taxiformis* belong to the Rhodophytas (taxonomy given by Guiry (2020); empire: Eukaryota, kingdom: Plantae, subkingdom: Biliphyta, phylum: Rhodophyta, subphylum: Eurhodophytina, class: Florideophyceae, subclass: Rhodymeniophycidae, order: Bonnemaisoniales, family: Bonnemaisoniaceae, genus: *Asparagopsis*), a group of red-pigmented algae. In addition to the Rhodophytas, two more main divisions are commonly used; the green Chlorophyta and the brown Phaeophyceae (Lobban et al., 1994). *Asparagopsis taxiformis* can be found in temperate and tropical waters (Chualáin et al., 2004) and have been included in the list of “the 100 ‘Worst Invasive’” Mediterranean marine species (Streftaris & Zenetos, 2006). A total of six lineages of *A. taxiformis* and two of *A. armata*, the closest relative, are presently recognized worldwide (Figure 4)(Dijoux et al., 2014; Zanolla et al., 2019).



Figure 3. “The red algae *Asparagopsis taxiformis* in Renion (lagoon of Saint-Leu)” Foto by: Quod (2013)

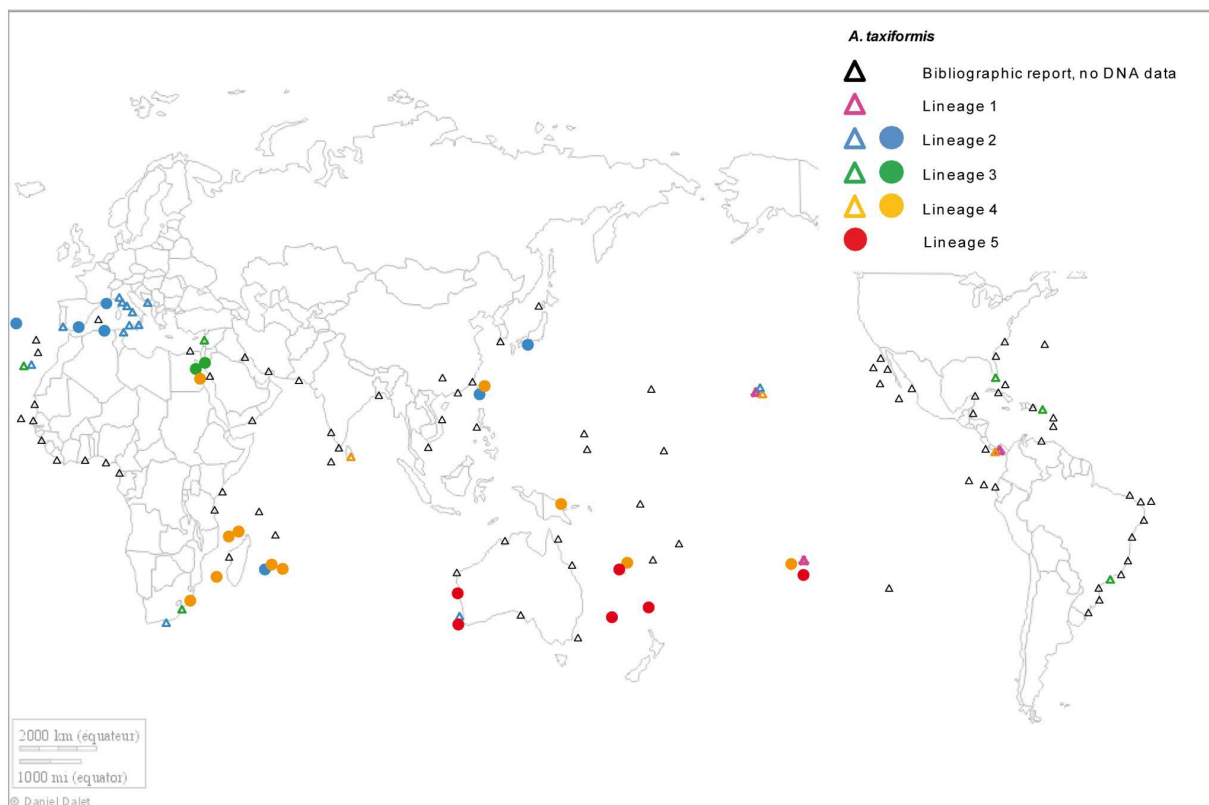


Figure 4. Five out of six recognized lineages of *Asparagopsis taxiformis* and their distribution around the world. Figure adapted from Dijoux et al. (2014).

Asparagopsis taxiformis, as well as *A. armata*, are described to have a haplo-diplontic life cycle with both gametophyte and tetrasporophyte phases (Dijoux et al., 2014). Although, with variation in developing stages depending on lineages and growing conditions (Zanolla et al., 2019). *Asparagopsis taxiformis* and *armata* differ morphologically by the spines possessed by *A. armata* during its gametophyte stage (Bonin & Hawkes, 1987), while being undistinguishable during the tetrasporophytic phase known as “Falkenbergia” (Chualáin et al., 2004). Importantly, both species have been found to produce bioactive plant metabolites with the potential to mitigate enteric methane in ruminants (Machado et al., 2016b; Paul et al., 2006; Roque et al., 2019b).

2.3.1 *Asparagopsis* spp. secondary plant metabolites

In 1976, Burreson et al. identified several iodinated and halogenated compounds in oil extracted from *A. taxiformis*, among them, bromoform. These bioactive plant metabolites are produced and released by the macroalgae as a chemical defense, hindering extensive microbial growth on the surface of the algae (Paul et al., 2006). Together with dibromochloromethane, bromochloromethane, and dibromoacetic acid, bromoform has been identified as the most

abundant bioactive compounds in *Asparagopsis spp.* (Machado et al., 2016b; Nørskov et al., 2021; Paul et al., 2014). The concentrations of these metabolites depend on the macroalgae growing conditions (Paul et al., 2014). Notably, the major halogenated compounds found serve as CH₄ analogs and are thought to be the main responsible for the CH₄ mitigating effect of *A. taxiformis* (Machado et al., 2016b). These will react with reduced cobamine (B₁₂) and compromise CH₄ formation (Goel et al., 2009; Rufener & Wolin, 1968) by inhibiting the cobamine-dependent transfer of methyl groups during the last stage in methanogenesis (Rufener & Wolin, 1968). The potential of halomethanes mitigating enteric CH₄ was previously recognized by Grass Jr et al. (1972) in their search for chemical substances which could improve feed efficiency and reduce enteric CH₄ emissions in ruminants. The potential of using *Aspragopsis spp.* as a methane inhibitor in the diet of ruminants has later been revealed through systematic evaluations of several seaweeds (Brooke et al., 2018; Machado et al., 2014; Machado et al., 2016b).

2.3.2 Practical challenges with the use of *Aspragopsis spp.*

The concentration of the highly volatile components in *A. taxiformis* depends on pre- and post-harvesting conditions where life stage and time of harvest (Vergés et al., 2008), in addition to preservative treatments, have proven to be influencing factors (Vucko et al., 2017). Samples containing more than 1 mg bromoform per g dry matter (DM) after *A. taxiformis* preservative processing reduced CH₄ production completely *in vitro*, independent of treatment, and with freeze-drying as the most successful in preserving bioactive compounds among treatments tested by Vucko et al. (2017). Also, storage time and storage conditions will affect the loss of volatile metabolites in freeze-dried *A. taxiformis*, according to Stefenoni et al. (2021). Bromoform concentrations have recently been shown to decrease by 75% and 84% after storing freeze-dried *A. taxiformis* for four months, when stored in the dark and exposed to light, accordingly (Stefenoni et al., 2021). Submerging *A. taxiformis* in oil has been presented as an alternative to freeze-drying by Magnusson et al. (2020), who demonstrated that the loss of bromoform preserved in oil was nonsignificant after three months of storage.

2.3.3 Previous findings

In vivo trials with *Asparagopsis spp.* first demonstrated a dose-dependent CH₄ mitigation effect in merino-cross wethers using ground milled and kiln-dried (45°C) *A. taxiformis* (Li et al., 2016). Li et al. (2016) observed up to 80% reduction in CH₄ production (g/day) when wethers consumed

around 3% *A. taxiformis* on organic matter (OM) basis. Roque et al. (2019b) included 0.5% and 1.0% freeze-dried *A. armata* on OM basis to dairy cattle reducing CH₄ by 67.2% with the highest inclusion level. They also evaluated the potential transfer of bromoform to milk and observed no significant increase between treatments (Roque et al., 2019b). Nevertheless, Stefenoni et al. (2021) reported a numerical increase in milk bromoform, together with a significant increase in milk bromide and milk iodine concentrations with *A. taxiformis* included at a 0.5% DM basis. These results advocates for the need of more research on the fate of bromoform in dairy cows. Studies has also shown that including *A. taxiformis* in the diet of steers did not affect meat quality and increased feed conversion efficiency significantly while reducing CH₄ by up to 98% (Kinley et al., 2020). Also, Roque et al. (2021) found *A. taxiformis* to have no effect on meat quality in beef cattle. The same study demonstrated that feed composition has an influencing effect on the mitigating potential of *A. taxiformis* by feeding three diets with increasing concentrate, simulating typical diets provided to growing steers through different life-stages (Roque et al., 2021).

Only two studies have so far provided data on the effect of *Asparagopsis* spp. on rumen fermentation parameters *in vivo*. These demonstrate a consistent decrease in rumen acetate and an increase in propionate production with various levels of *A. taxiformis* inclusion (Li et al., 2018; Stefenoni et al., 2021). Furthermore, the general effect of supplementing *Asparagopsis* spp. to dairy cattle is also scarcely described and with inconsistent results. Both Roque et al. (2019b) and Stefenoni et al. (2021) found feed intake to be compromised with increasing inclusion levels of *A. armata* and *A. taxiformis*, respectively. Roque et al. (2019b) did additionally observe increased feed efficiency and only minor changes in milk composition with a lower protein content (%) in cows receiving the highest inclusion level (1% on OM basis), whereas Stefenoni et al. (2021) found decreased milk fat and lactose contents.

2.6 Research aims and objectives

Asparagopsis taxiformis has the potential to mitigate enteric CH₄ from ruminants and reduce the climatic impact of livestock production through manipulation of the rumen microbiome. When reducing CH₄ formation, it is believed that excess hydrogen needs to be directed elsewhere to avoid inhibition of fermentation (van Lingen et al., 2016), and that subsequent changes in rumen microbiota will potentially affect rumen fermentation pattern and thereby energy substrates

available for the ruminant. Evaluating the effect feed additives have on rumen homeostasis, and animal physiological factors as a whole can add insight into potential challenges linked to animal welfare and health.

Data used in this thesis was generated during an animal experiment conducted at the Norwegian University of Life Sciences (NMBU) in the spring of 2021 where dairy cattle were fed *A. taxiformis* at different inclusion levels. The thesis aims to combine metadata from CH₄ measurements, rumen metabolites, and milk composition together with animal performance data to evaluate CH₄ production and animal productivity in Norwegian red dairy cattle (NRF) fed 0%, 0.125%, or 0.25% *A. taxiformis* on OM basis. In addition, data from an *in vitro* evaluation of the *A. taxiformis* used in the *in vivo* experiment is presented.

3. Materials and Methods

The experiment was conducted from mid-March until late May 2021 at the Teaching and Research Facility (etalism unit) of the Department of Animal and Aquacultral Sciences at the Norwegian University of Life Sciences (NMBU), Norway. All animal procedures were approved by the national animal research authority of the Norwegian Food Safety Authority (Mattilsynet; FOTS ID: 26318).

3.1 Experiment 1: *In vivo*

3.1.1 Animals and experimental design

Fifteen multiparous, early- to mid-lactation, NRF with an average starting bodyweight of 676 ± 55 kg (BW, mean \pm SD), 95 ± 27 days in milk (DIM), and average milk yield (MY) of 32.1 ± 3.7 kg milk were used in the experiment. The cows were divided into three groups based on MY, parity (2nd, 3rd, 4th), DIM, BW, and canulation status (yes/no). One of the treatment groups served as a control (no seaweed), while the two other groups were given either 0.125% or 0.25% inclusion levels of *A. taxiformis* on OM basis, hereby referred to as AT diets. Three cannulated (Bar Diamond Inc., Parma, Idaho, USA; \varnothing 10 cm) and two intact individuals were allocated to each group.

The total duration of the animal experiment was 74 days (Figure 5). Days -21 to 0 served as a covariance period, allowing cows to adapt to the common diet. Further, days 1 to 13 were used for adaptation to *A. taxiformis*, while days 14 to 52 were considered the experimental period.

All cows were housed in free stalls at the Livestock Production Research Centre (SHF) during the first 13 days of the covariate period. On day -8, experimental animals were moved to the Metabolism Unit at the faculty of Biosciences (BIOVIT). Here, they were placed in individual tie stalls (Figure 6) with rubber mats and sawdust bedding (Figure 7). Each individual had ad libitum access to clean drinking water in automated water bowls and feed in individual feed troughs allowing for individually controlled water and feed intake recordings.

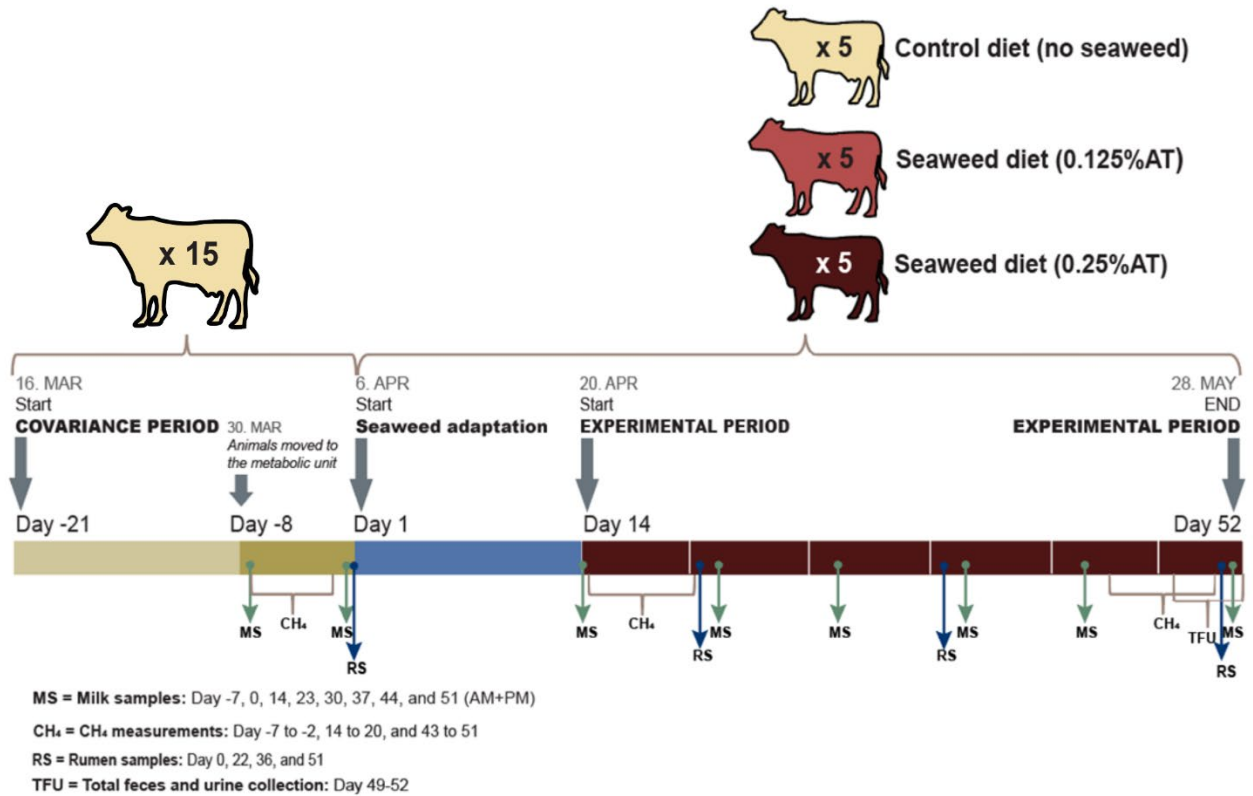


Figure 5. Experimental timeline including covariance period, seaweed adaptation period, and experimental period with sampling days.



Figure 6. Cows housed in the metabolic unit in individual tie stalls with sawdust bedding.



Figure 7. Experimental animals housed in the metabolic unit with individual feed trays.

3.1.2 Feed and feeding

A common diet in the form of a total mixed ration (TMR), consisting of conserved grass silage and concentrate (Drøv Energirik Høy, Norgesfôr) at a ratio of 65:35 (on DM basis) was prepared at least three times per week. The TMR was mixed using a Siloking Duo 1814 (Kverneland, Bryne, Norway) mixing machine with about 20 minutes of chopping the grass silage followed by 10 minutes of mixing the chopped silage and concentrate feed. The mixture was preserved using 6 L per ton wet feed of a propionic acid-based preservative (GrassAAT FEED, Addcon Nordic AS, Posgrunn, Norway) and kept in a cold room (4°C) until used up.

The cows were served the TMR three times daily, rationed as 40:30:30 (%) of the daily feed allowance at 7 a.m., 1 p.m., and 7 p.m., in respective order. The covariate period was used for gauging the level of individual cow feed intake. During the last eight days of this period, daily feed allowance (kg DM) was calculated individually, based on the previous day's DMI, expecting a minimum of 5% feed refusal. During both the adaptation and experimental periods, the mean daily allowance from the covariate period was used, with adjustments by 5% if a cow consumed more than what was offered. This guaranteed ad libitum access to feed by cows in all treatments and provided the opportunity to improve feed intake for the AT groups during the AT feeding period. Leftovers were removed daily before a.m. feeding and weighed for daily recording. After daily weighing, the TMR was stored at room temperature until feeding for a maximum of 24 hours.

Composite samples of TMR for chemical analysis were collected over the experimental period and analyzed in triplicates. The total mixed ration had a DM content of 360 g/kg feed with a composition of 924.1 g OM/kg DM, 75.9 g ash/kg DM, 401.1 g NDFom/kg DM, 175.3 g CP/kg DM, 119.9 g starch /kg DM and 40.5 g crude fat/kg DM.

3.1.3 *Asparagopsis taxiformis* – preparation and feeding

Asparagopsis taxiformis used as a feed additive in the experiment were provided by SeaExpert, Portugal. The seaweed was wild harvested during the spring of 2020 in the Azores, Portugal, and manually checked and cleaned for by-catch (other seaweed species, shells etc.). To avoid evaporation of volatile metabolites (i.e. bromoform), *A. taxiformis* was stored and shipped at -20°C, followed by freeze-drying (European Freeze Dry, Denmark). The freeze-dried *A. taxiformis* (Figure 8) was then milled using a cement mixer (Figure 9). Considering that the bioactive compounds in *A. taxiformis* are volatile, milling that generates heat had to be avoided.

Moreover, *A. taxiformis* contains possible carcinogenic compounds and since the freeze-dried seaweed is very brittle, grinding may cause a lot of small, possibly hazardous dusty particles. Therefore, milling was restricted to methods where heat generation and loss of *A. taxiformis* particles were limited. In brief, *A. taxiformis* was added to the cement mixer with three 1kg dumbbells (Figure 10). The cement mixer was sealed with a plastic tarp (Figure 11) to minimize material loss and avoid dust generation before mixing for approximately 20 minutes. Bromoform and other halomethanes are photosensitive, and the loss of these compounds depends on storage conditions. Therefore, the milled *A. taxiformis* was put back into the original, light-reflective plastic bags and stored at -20°C until daily weighing of individual portions. Individually weighed portions corresponding to the amount of TMR and inclusion level of *A. taxiformis* were stored at 4°C until feeding.



Figure 8. Freeze-dried *Asparagopsis taxiformis* prior to milling in the cement mixer.



Figure 9. Freeze-dried *Asparagopsis taxiformis* after milling in the cement mixer.

At each feeding point, the 0.125% AT and 0.25% AT groups received *A. taxiformis* with a fixed amount molasses-water-mix hand-mixed into the TMR. Molasses was mixed with water at a ratio of 1:1; 400 g of the mix were included in the daily feed ration of each individual to enhance the palatability of *A. taxiformis*. Control animals had the same daily amount of molasses-water-mix included in their daily diet to minimize any distortion in the nutrient composition of the TMR offered.

An aliquot of the *A. taxiformis* biomass was sent to Cawthron Institute, New Zealand, to quantify bromoform according to their analytical method described in Romanazzi et al. (2021). Iodine was

analyzed at the soil- and water laboratory at the Faculty of Environmental Sciences and Natural Resource Management (MINA), NMBU. An alkaline extraction was performed using Tetramethylammonium hydroxide (TMAH) solution in H₂O, added to 0.25g *A. taxiformis* before being placed in a heating cabinet at 90°C for a minimum of 3 hours. The sample was thereafter centrifuged and diluted with H₂O to 50 mL before being analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 8000 Triple Quadrupole mass spectrometer, Agilent Technologies Inc., Santa Clara, USA).



Figure 10. 1 kg dumbbells were added in the cement mixer together with *Asparagopsis taxiformis*.



Figure 11. The cement mixer sealed with a plastic tarp, fitted rubber tube, and loading strap.

3.1.4 Milk yield, milk sampling, and chemical analysis

The cows were milked twice daily, around 7 a.m. and 7 p.m. Milk yield was recorded individually at each time point using a DeLaval milk meter MM6 (DeLaval Inc., Tumba, Sweden). Milk samples were taken at days -7 and 0 as a covariate representative sample, in addition to days 14, 23, 30, 37, 44, and 51 during the experimental period. On these days, individual samples were collected at both a.m. and p.m. milking, preserved with Bronopol (2-bromo-2-nitro-1,3-propaediol, Broad Spectrum Microtabs[®] II), and stored at 4°C until sent for analysis. All milk

samples were analyzed by TINE SA for milk fat, protein, lactose, urea, somatic cell count (SCC), and free fatty acids (FFA) using an infrared milk analyzer (MilkoScan 6000; Foss Analytical, Hilleroed, Denmark). Milk fatty acid composition was estimated using Fourier transform infrared (FT-IR) spectra. Energy corrected milk (ECM) for each cow was calculated based on milk chemical composition and milk yield, according to Sjaunja (1990).

3.1.5 Enteric methane measurement

Enteric CH₄ production was measured during three periods from day -7 to -3, day 14 to 20, and day 43 to 49, using sulfur hexafluoride (SF₆) as described by Johnson et al. (1994). Individuals with faulty samplings for one or several days during a period of measurements were measured for an additional 24 hours (day -2, 21, and/or 50). For this, permeation tubes filled with SF₆ gas (mean \pm SD = 2486.1 \pm 34.7 mg) and predetermined release rate (mean \pm SD; 3.96 \pm 0.55 mg/d; $r^2 = 0.999$) were prepared by Agriculture and Agri-Food (Semiarid Prairie Agricultural Research Center, Saskatchewan, Canada) and inserted into the rumen of the experimental animals on day -14. All cows were mounted with a halter designed for gas collection (Figure 12) and a pressurized yoke (Figure 13) on days -7, 14, and 43. Two additional halter-yoke systems were placed on opposite ends of the room where the cows were housed to account for background gas concentrations. All yokes were replaced by freshly prepared yokes every 24h during the three periods with CH₄ measurements.

Yokes were prepared for mounting before each 24h sampling by cleaning in three cycles. First, the yokes were pressurized using ultra-pure nitrogen (N₂) for 12 sec at 1 bar; thereafter, N₂-gas were let out of the yokes, followed by evacuation using a vacuum pump for 12 sec at 2 bar. After the final cleaning cycle, the yokes were evacuated to create a sufficient negative pressure (ca -12 psi) for collecting gas sample for 24h from a nasal area of a cow through a capillary tubing of a halter. All yokes were mounted and demounted in the same order, at the same timepoint, each sampling day within a period to minimize the difference in sampling time between individuals.

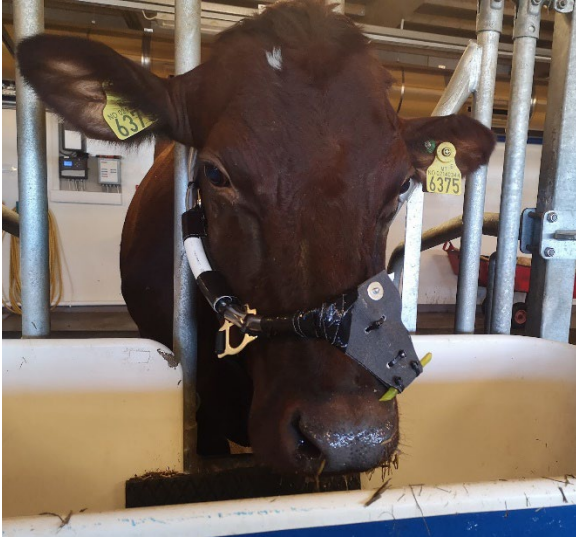


Figure 12. Cow mounted with halter designed for gas measurements.



Figure 13. Gas collection yoke being prepared for gas sampling.

After demounting, the pressure in the yokes was recorded, and N₂ was added for 6 sec at 0.5 bar to all yokes. The yokes were thereafter left for a minimum of 45 min, allowing for gasses to mix before gas sampling. Using a 20 ml syringe, 15-20 ml gas from each yoke was transferred into evacuated glass vials (10 ml) in triplicates.

Gas samples were analyzed for CH₄ and CO₂ by gas chromatography (GC, Model 7890A Agilent, Santa Clara, CA). Helium was used as a carrier gas with a Poraplot U capillary column to separate the gas under isothermic conditions at 50°C. For the detection of CH₄, a flame ionization detector was used, CO₂ was detected by a thermal conductivity detector, while an electron capture detector was used for SF₆; all detectors were calibrated to certified standards (Linde, Germany).

Enteric CH₄ and CO₂ emissions were calculated for each sampling day (g/day) according to the equation given by McGinn et al. (2006):

$$Q_{CH_4} = \frac{C_{CH_4} - C_{CH_4}^b}{C_{SF_6} - C_{SF_6}^b} Q_{SF_6} \frac{MW_{CH_4}}{MW_{SF_6}}$$

Where Q_{CH_4} is the daily CH₄ emission (g/day); Q_{SF_6} is the predetermined release rate of SF₆ (g/day) from permeation tubes; C_{CH_4} and C_{SF_6} represents CH₄ and SF₆ mixing ratios within the yoke

($\mu\text{mol/mol}$); $C_{CH_4^b}$ and $C_{SF_6^b}$ accounts for the background concentration of CH_4 and SF_6 ; while the molecular weight ratio, MW_{CH_4}/MW_{SF_6} , corrects for differences in gas density.

3.1.6 Rumen fermentation

Ruminal fluid samples for short-chain volatile fatty acids (VFA) and ammonia nitrogen (NH_3-N) analysis were taken on day 0, as a covariate, and during the experimental period at days 22, 36, and 51. For all cannulated cows, a total of five sampling points corresponding to 0, 2, and 6 hours relative to feeding (TRF)(Figure 14) were taken through the cannula, while intact cows were sampled with an esophageal tube (OST) at only a single time point, two hours after a.m. feeding (TRF = 2). When sampling via an OST, more than 500 mL fluid was discarded before samples were taken, and the OST was rinsed between the sampling of each animal. Each sample, regardless of sampling method, was filtered through strainer bender bags (Stomacher[®] 400 Seward BA 6041, Worthing, UK) before pH was measured (WTW, pH 3310, Germany with a Hamilton Bonaduz AG, Polyplast Pro, Switzerland, pH sensor), and 9.5 mL rumen fluid was transferred to a 10 mL centrifuge tube prepared with 0.5 mL of 98% formic acid (v/v) for preservation. Samples were stored at 4°C pending analysis.

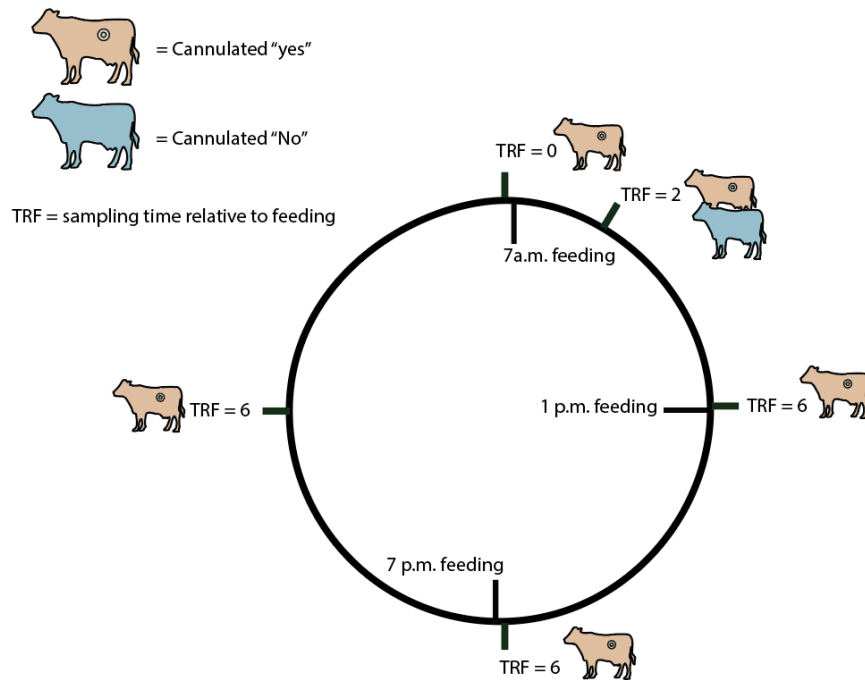


Figure 14. Five rumen fluid sampling time points, divided by time relative to feeding (TRF, = 0, 2, 6), displaying sampling schedule for cannulated (yes) and intact (no) individuals.

The VFA analysis was performed by LabTek (BIOVIT) using gas chromatography (TRACE 1300 Gas Chromatograph with Stabliwax-DA column 30m, 0.25 mm i.d., 0.25 μ m; Thermo Fischer Scientific S.p.A., Milan, Italy) whereas NH₃-N was analyzed with Kjeltec 8400 (Foss Analytical, Hilleroed, Denmark) using Method 2001.11 (AOAC., 2002) as described by Thiex et al. (2002), without block digestion.

3.1.7 Nutrient digestibility

Determination of nutrient digestibility was done by total collection of feces over 72h, from day 49 to 52 - eight cows, where two from control and three from each of the AT inclusion groups were used. These cows were placed at the same side of the barn in elevated tie stalls designed with fitted collecting trays to avoid loss of manure to the sewage system. Feces were primarily collected directly in buckets and were weighed each morning. After weighing, 10% of the feces collected over 24 hours were taken out, and pooled into one combined sample for each individual for the 72 hour collection period. These samples were kept at -20°C. The composite samples were later thawed at 4°C, thoroughly mixed using a cement mixer, and sub-sampled for analysis. Fecal samples for chemical analysis were freeze-dried, milled (0.5 screen for starch and 1.0 mm screen for other analysis) using Retsch cutting mill SM 200 (Retsch GmbH, Germany). The samples were analyzed by LabTek (NMBU, Norway) for dry matter (DM), organic matter (OM), neutral detergent fiber corrected for ash (NDFom), Kjeldahl-N for dietary crude protein (CP), and starch.

Estimated total tract digestibility (%) for each analyzed nutrient fraction were calculated as:

$$\text{Digestibility (\%)} = \frac{N_a - N_b}{N_a}$$

Where N_a is the amount of nutrient ingested; and N_b is the amount of nutrient recovered in feces.

3.1.8 Statistical analysis

Data collected over the sampling days were analyzed using Proc Mixed procedure of SAS (SAS Institute Inc. 2002-2012, SAS for Windows 9.4; Cary, 237 NC, USA). Data on individual cows over different days (e.g., DMI, Milk yield, milk composition) and at different time points over the sampling days (e.g., rumen fermentation products) were assumed correlated, hence modeled with autoregressive [AR(1)] or ante-dependence [Ante(1)] covariance structure based on Schwarz's Information Criterion (BIC) as described in Wang and LA Goonewardene (2004). For all

measurements, data collected during the common feeding period until day 0 were used as covariate in the statistical analysis to account for baseline differences in individual parameters tested. For example, the full model for the effect of AT inclusion on rumen fermentation parameters was tested using:

$$Y_{ijkl} = \mu + Diet_i + Day_j + TRF_l + (Diet \times Day)_{ij} + (Diet \times SM)_{ik} + (Diet \times TRF)_{il} + covX + e_{ijkl}$$

Where Y_{ijkl} = response variable (e.g. total VFA); μ = overall mean; $Diet_i$ = effect of the Diet (control, 0.125% AT, 0.25% AT); Day_j = effect of sampling day ($j = 22, 36, 51$); SM_k = effect of sampling method (k =esophageal or rumen cannula); TRF_l = time of sampling (i.e., hours) relative to feed delivery ($l = 0, 2, 6$); $covX$ = effect of baseline data as covariate for each cow within a Diet and sampling method (e.g. covariate period Total VFA); e_{ijkl} = residual error, and the other terms are the interaction effects of Diet within sampling day, time relative to feeding and sampling method.

Enteric methane production was measured at three distinct time points of the experiment: adaptation on a common Diet (later used as a covariate), days 14 to 21 (i.e., D_{14-21}), and days 43 to 50 (i.e., D_{43-50}) of the AT feeding period. Data collected over the latter two periods were analyzed using Proc Mixed procedure of SAS using a cow within dietary treatment as the experimental unit and days in each period as repeated factor using the following model:

$$Y_{ijkl} = \mu + Diet_i + Day_j + Period_k + (Diet \times Day)_{ij} + (Diet \times Period)_{ik} + covX + e_{ijkl}$$

Where Day_j = the effect sampling day within a period ($j = 14$ to 21 in Period 1; 43 to 50 in Period 2); $Period_k$ = Period effect ($k = 1$ and 2); $(Diet \times Day)_{ij}$ is the interaction effect of Diet and sampling day; $(Diet \times Period)_{ik}$ is the interaction effect of Period and Diet; and the others terms are as expressed above.

3.2 Experiment 2: *In vitro*

3.2.1 Experimental design

An *in vitro* trial was designed to test and evaluate the potential CH_4 mitigating effect of the *Asparagopsis taxiformis* biomass used in the *in vivo* experiment described above. The bioactive components of *A. taxiformis* are highly volatile and affected by storage time and conditions. In addition, *in vivo* results are based on voluntary intake of feeds enriched with *A. taxiformis*. Therefore, the *in vitro* assay was performed to compare *in vivo* and *in vitro* mitigating potential of

the same AT biomass using the same TMR fed in the *in vivo* experiment as substrate for the *in vitro* fermentation. The amount of *A. taxiformis* included was based on inclusion levels used *in vivo* and a higher level corresponding to a previously performed *in vitro* assay using the same *A. taxiformis* biomass, which initially was an intended level for *in vivo* inclusion. As such, two batches with the following substrates were tested in triplicates: TMR (1g DM) (chemical composition as described in chapter 3.1.2 Feed and feeding) and TMR enriched with 0.125%, 0.25%, or 0.5% OM *A. taxiformis*, corresponding to 2.5, 4.9, and 9.8 mg DM, respectively. The first batch of *in vitro* fermentation was carried out from day 51-52, whereas the second batch was performed at day 55-56 relative to the experimental timeline previously described for the *in vivo* experiment (Figure 5).

3.2.2 Sampling and chemical analysis

An ANKOM system (Ankom^{RF}GP System, Ankom Technology®, NY, USA) with 250 mL bottles (Figure 15) was used for *in vitro* fermentation. The ANKOM units were randomly allocated to treatment type for each batch. Bottles were prepared with ca 1g DM of feed the day prior to the experimental start and incubated at 39°C. Buffer solutions were prepared and mixed according to Goering and Van Soest (1970) as described in the ANKOM “Operator’s Manual.” (ANKOM, 2018).

Equal portions of rumen fluid from three cannulated cows fed the control diet (no seaweed) from the *in vivo* experiment previously described was collected 4 hours after morning feeding. The rumen fluid was collected into two pre-warmed thermos flasks and filtered through a nylon cloth (SEFAR NITEX, Sefar AG, Heiden, Switzerland) with a pore size of 200µm into glass bottles maintained in a 39°C water-bath. Afterward, the filtered rumen fluid (33.3 mL) and reduced buffer solution (66.7 mL) were dispensed into each ANOKOM bottle. The overhead space of each bottle was immediately flushed with CO₂ for 5 seconds and sealed with a pressure recording head unit. All bottles were incubated for 24 hours at 39°C with a continuous gentle shaking on Stuart SSL3 3D gyro-rocker (Cole-Palmer Ltd, Staffordshire, UK) simulating *in vivo* rumen motility and mixing. During incubation, a computer registered cumulative gas pressure at every 10-minute interval through wireless communication with individual modules.

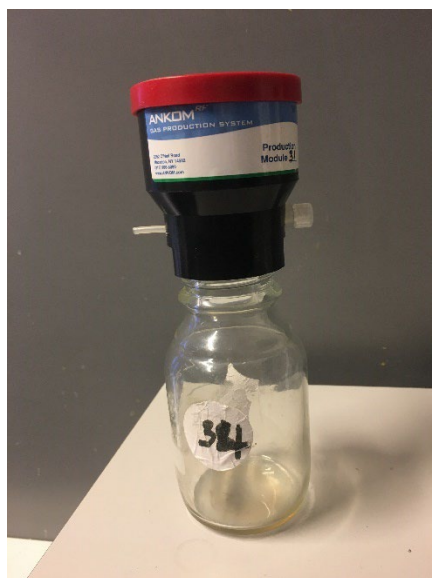


Figure 15. 250mL ANKOM bottle with gas pressure recording head unit.

Gas recordings were stopped after 24h, and gas samples were taken directly. Evacuated glass vials (10 mL) were prepared with 10 mL nitrogen (N₂) or helium (He) before 1 mL of the sampled gas was added, using a 1 mL syringe with a gas lock.

Gas samples were analyzed for concentrations of CH₄ and CO₂ by gas chromatography (GC, Model 7890A Agilent, Santa Clara, CA), using Helium as a carrier gas and a Poraplot U capillary column to separate the gas under isothermic conditions at 50°C. For the detection of CH₄, a flame ionization detector was used while a thermal conductivity detector was used for CO₂; both detectors were calibrated to certified standards (Linde).

3.2.3 Calculations and Statistical analysis

Gas pressure (GP) was recorded automatically, and total gas volume was calculated according to ANKOM (2018). Further, CH₄ and CO₂ production within each bottle were calculated based on average concentrations (vol%) of CH₄ and CO₂ in the analyzed gas samples, and data collected were analyzed using R statistical software (R Core Team 2021; version 4.1.1). A linear mixed-effects model (lmer) procedure where treatment was included as a fixed effect with batch and bottle within a batch as random effects were used. In addition, post hoc multiple comparison analyses were obtained using the emmeans package with Tukey's procedure.

4. Results

4.1 Experiment 1: *in vivo*

4.1.1 Feed intake and ad hoc adjustments to diets

Asparagopsis taxiformis biomass used in the experiment contained a 4.02 mg/g DM bromoform concentration and 7.1 g Iodine/g DM prior to the experimental period. Initially, inclusion levels of *A. taxiformis* were set to 0% (control), 0.25%, and 0.5% based on organic matter intake. As a result of feed aversion by the group provided 0.5% AT, and following health concerns, the 0.5% AT group had their amount of *A. taxiformis* decreased to 0.125% AT at day 11, allowing cows to recover their feed intake. The following inclusion groups were therefore used throughout the experimental period: 0% AT (control) 0.125% AT, and 0.25% AT. It was also observed that individuals fed seaweed were able to sift out and exclude the molasses-seaweed-mix. *Asparagopsis taxiformis* was therefore mixed with a molasses-water-mix and further mixed into the TMR until day 36. From day 37 *A. taxiformis* and the molasses-water-mix were included separately in the TMR, attempting to increase the homogeneity of the total mix.

Average feed and nutrient intake responses to *A. taxiformis* inclusion in diet for each treatment group from day 14-52 adjusted for covariate data are presented in Table 1. Feed and nutrient intake are based on daily feed allowance (kg DM/day) corrected for feed refusal (kg DM/day). Dry matter intake for the 0.25% AT inclusion group was reduced significantly ($P < 0.001$) compared to control and 0.125%AT (Table 1). A mean difference of 3.7 kg DM/day (16.2%) between control and 0.25%AT was observed, while there was no significant difference between control and 0.125%AT. The mean DMI (kg/day) for each group during the experimental period is shown in Figure 16 with mean DMI for the covariate period at day 0.

As for DMI, the intake of organic matter (OMI), neutral detergent fiber (NDFI), starch (StarchI) and crude protein (CPI) decreased with the 0.25%AT inclusion, and was significantly different from control and 0.125%AT ($P < 0.001$). In comparison, no significant difference was found between control and 0.125%AT inclusion. Correlation between all feed and nutrient intake parameters, Day and Diet*Day interactions were significant ($P < 0.001$).

Table 1. Effect of *Asparagopsis taxiformis* inclusion on feed and nutrient intake (kg/d) of early- to mid-lactation NRF dairy cows fed a total mixed ration.

Parameters	Diet				Effect of: (P-Value)		
	Control	0.125%AT	0.25%AT	SE	Diet	Day	Diet*Day
DMI	22.9 ^a	22.0 ^a	19.2 ^b	0.37	<0.001	<0.001	<0.001
OMI	21.1 ^a	20.3 ^a	17.7 ^b	0.34	<0.001	<0.001	<0.001
NDFI	8.51 ^a	8.18 ^a	7.15 ^b	0.14	<0.001	<0.001	<0.001
StarchI	3.08 ^a	2.96 ^a	2.58 ^b	0.05	<0.001	<0.001	<0.001
CPI	4.07 ^a	3.92 ^a	3.42 ^b	0.07	<0.001	<0.001	<0.001

DMI = dry matter intake; OMI = organic matter intake; NDFI = neutral detergent fiber intake; StarchI = starch intake; and CPI = crude protein intake.

Means in a row carrying different superscripts are significantly different at $P \leq 0.05$.

As shown in Figure 16. Dry matter intake (mean \pm SE) of early- to mid-lactation NRF dairy cows given total mixed ration containing graded levels of red seaweed (Control= 0%; 0.125%AT = 0.125%; and 0.25% AT =0.25% *Asparagopsis taxiformis* on organic matter intake basis), mean DMI (mean \pm SE) for 0.25%AT was lower during the whole experimental period compared with control. Mean DMI of the group fed 0.125%AT, formerly fed 0.5%AT, were visibly affected by the change in AT inclusion, and mean DMI increased after changing inclusion level before stabilizing around day 20. Covariate DMI for each group is seen at day 0.

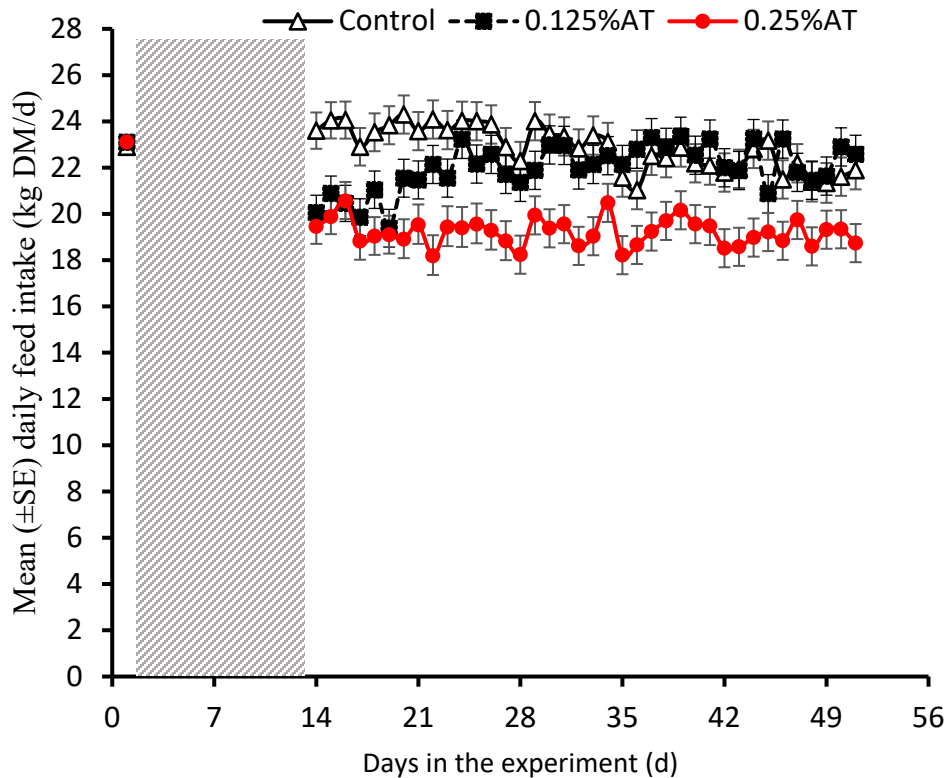


Figure 16. Dry matter intake (mean±SE) of early- to mid-lactation NRF dairy cows given total mixed ration containing graded levels of red seaweed (Control= 0%; 0.125%AT = 0.125%; and 0.25% AT =0.25% *Asparagopsis taxiformis* on organic matter intake basis). The shaded area represents the *A. taxiformis* adaptation period.

Nutrient digestibility based on total collection of feces from eight cows (2 control, 3 from 0.125%AT and 3 from 0.25%AT) is presented in Figure 17. Including graded levels of *A. taxiformis* had no significant effect on dry matter digestibility (DMD, $P = 0.403$), organic matter digestibility (OMD, $P = 0.350$), digestibility of neutral detergent fiber corrected for ash (NDFomD, $P = 0.128$), dietary crude protein digestibility (CPD, $P = 0.382$), or starch digestibility (StarchD, $P = 0.294$). However, a numerical increase in digestibility for all parameters was seen for 0.25%AT compared to Control and 0.125%AT. At the same time, 0.125%AT resulted in a numerically slightly lower nutrient digestibility compared to Control.

For the Control group DMD were 76.8%, with 0.125%AT at 76.1% and 0.25%AT at 77.6%. Further, OMD were 77.5%, 76.8% and 78.4% for Control, 0.125%AT and 0.25%AT, respectively.

The greatest difference was seen for NDFomD where Control, 0.12%AT and 0.25%AT ended at 69.8%, 67.2% and 71.8%, respectively. Resulting in a 4.6% difference between 0.125%AT and 0.25%AT. Crude protein digestibility was 75% for Control, 73.5% for 0.125%AT and lastly 76.2% for 0.25%AT. Starch digestibility ended at 99.5% (Control), 99.0% (0.125%AT) and 99.7% (0.25%AT).

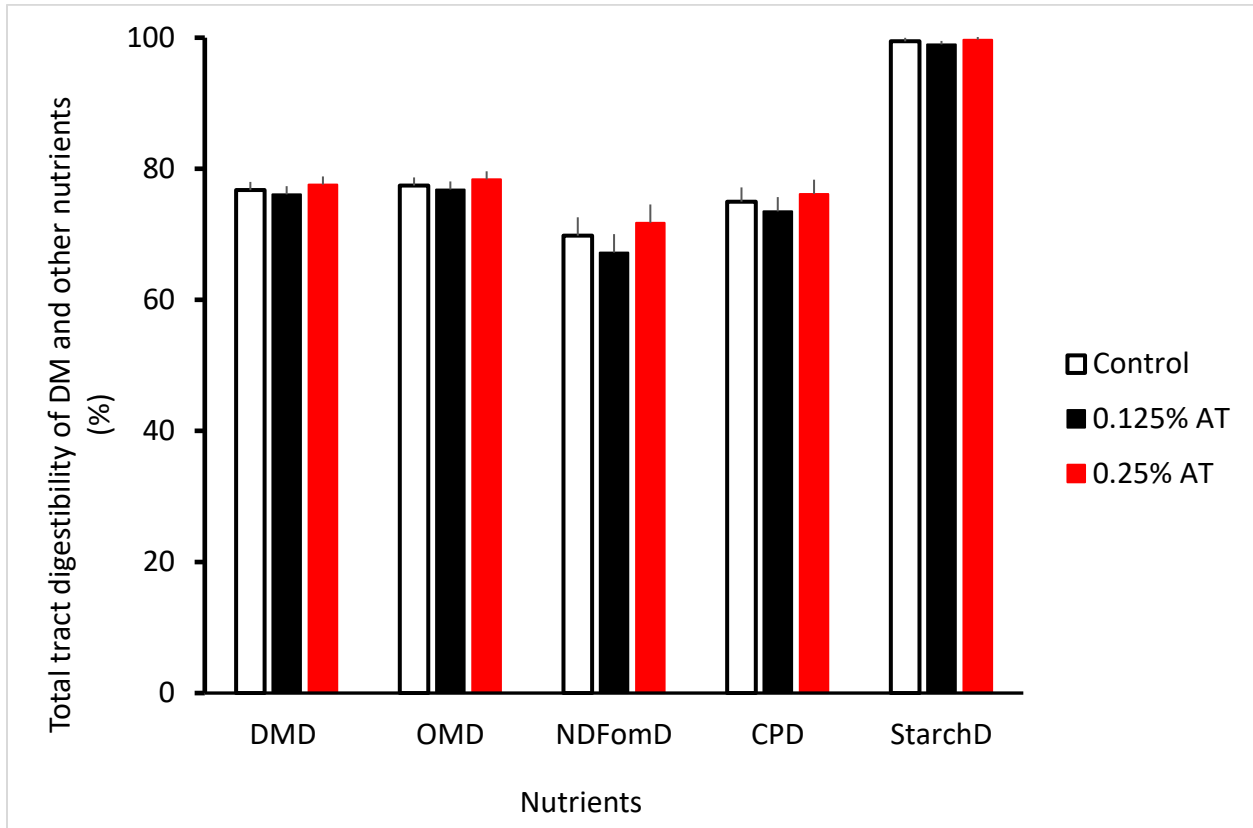


Figure 17. Effect of *Aspargopsis taxiformis* (mean \pm SE) inclusion with early lactation NRF cows fed total mixed ration on total tract dry matter and nutrient digestibility (DMD = dry matter digestibility; OMD = organic matter digestibility; NDFomD = digestibility of neutral detergent fiber corrected for ash; CPD= dietary crude protein digestibility and StarchD= starch digestibility).

4.1.2 Enteric gas measurements

Enteric CH₄ and CO₂ emissions, divided by diet and averaged per period, are presented in Table 2 as average production (g/day), intensity (g/kg ECM), and yield (g/kg DMI), followed by CH₄ to CO₂ proportions (CH₄:CO₂). During gas sample analysis, it was noted that SF₆ levels in background samples were below the method's detection limit. Consequently, SF₆ levels in the background were set to zero when calculating CH₄ and CO₂ concentrations for all samples.

Table 2. Effect of *Asparagopsis taxiformis* inclusion on enteric methane and carbon dioxide production in early- to mid-lactation NRF dairy cows fed total mixed ration

Parameter	Diet			SE	Period		SE	Effects of: (P-value)			
	Control	0.125%AT	0.25%AT		D ₁₄₋₂₁	D ₄₃₋₅₀		Diet	Day(Period)	Period	Diet*Period
<i>CH</i> ₄ , g/day	545.7 ^a	469.0 ^{ab}	425.3 ^b	29.2	486.3	473.7	22.7	0.037	<0.01	0.693	0.341
g <i>CH</i> ₄ /kg ECM	16.7	16.7	13.3	1.59	15.9	15.2	1.12	0.227	<0.01	0.607	0.651
g <i>CH</i> ₄ /kg DMI	25.0	22.4	20.9	1.41	22.9	22.6	1.10	0.155	<0.01	0.834	0.764
<i>CO</i> ₂ , g/day	18095	19730	22723	2113	20260	20105	1574	0.323	<0.01	0.939	0.430
g <i>CO</i> ₂ /kg ECM	550.8	695.6	715.8	76.7	660.5	647.7	55.7	0.293	<0.01	0.855	0.497
g <i>CO</i> ₂ /kg DMI	829.7	941.2	1129.5	103.8	965.0	968.9	78.0	0.158	<0.01	0.967	0.655
<i>CH</i> ₄ : <i>CO</i> ₂	0.031 ^a	0.025 ^b	0.020 ^c	0.002	0.025	0.025	0.001	0.002	0.016	0.936	0.328

Means in a row carrying different superscripts within a diet group are significantly different at $P \leq 0.05$

Inclusion levels of 0.125 and 0.25% OM *Asparagopsis taxiformis* resulted in a lowered mean CH₄ production of 14% and 22%, respectively, compared to control (Figure 18A). While 0.25%AT were significantly different from control ($P = 0.037$), 0.125%AT did not differ significantly from control or 0.25%AT. Although not statistically significant, an anti-methane effect was also observed for methane intensity, with a 20% reduction for the treatment group fed 0.25%AT compared to the two other groups (Figure 18B). Notably, the CH₄ intensity was the same for control and 0.125%AT. Methane yield for 0.125%AT was 10.4% lower, and 0.25%AT was 16.4% lower than the control (Figure 18C). Although CH₄ intensity and yield were not affected by diet or sampling period, a significant effect of sampling day on all CH₄ parameters was observed. At the same time, no correlation between diet and period was noticed for any parameters.

Carbon dioxide emissions, intensity, and yield increased numerically with increasing AT inclusion levels ($P > 0.1$). For 0.125%AT, CO₂ production, intensity, and yield increased by 8.3, 20.8, and 11.8%, while the same parameter increased by 20.4, 23.0, and 26.5% for 0.25%AT compared with control (Figure 18D, E, F). Similar to the CH₄ parameters, a significant correlation was seen between all CO₂ parameters and day within the period ($P < 0.001$), while all were unaffected by sampling period or diet-period interactions.

Lastly, the CH₄ to CO₂ ratio was significantly affected by diet, decreasing with increasing *A. taxiformis* inclusion (Figure 18G). All three groups were found to differ from each other ($P = 0.002$). From control to 0.125%AT, CH₄:CO₂ ratio decreased by 19.4% and 0.25%AT by 35.5%. The two AT inclusion groups differed from each other by 20%. In addition to the effect of diet, a correlation between CH₄:CO₂ ratio and sampling day within period was noticed ($P = 0.016$), while period and diet-period interactions were insignificant.

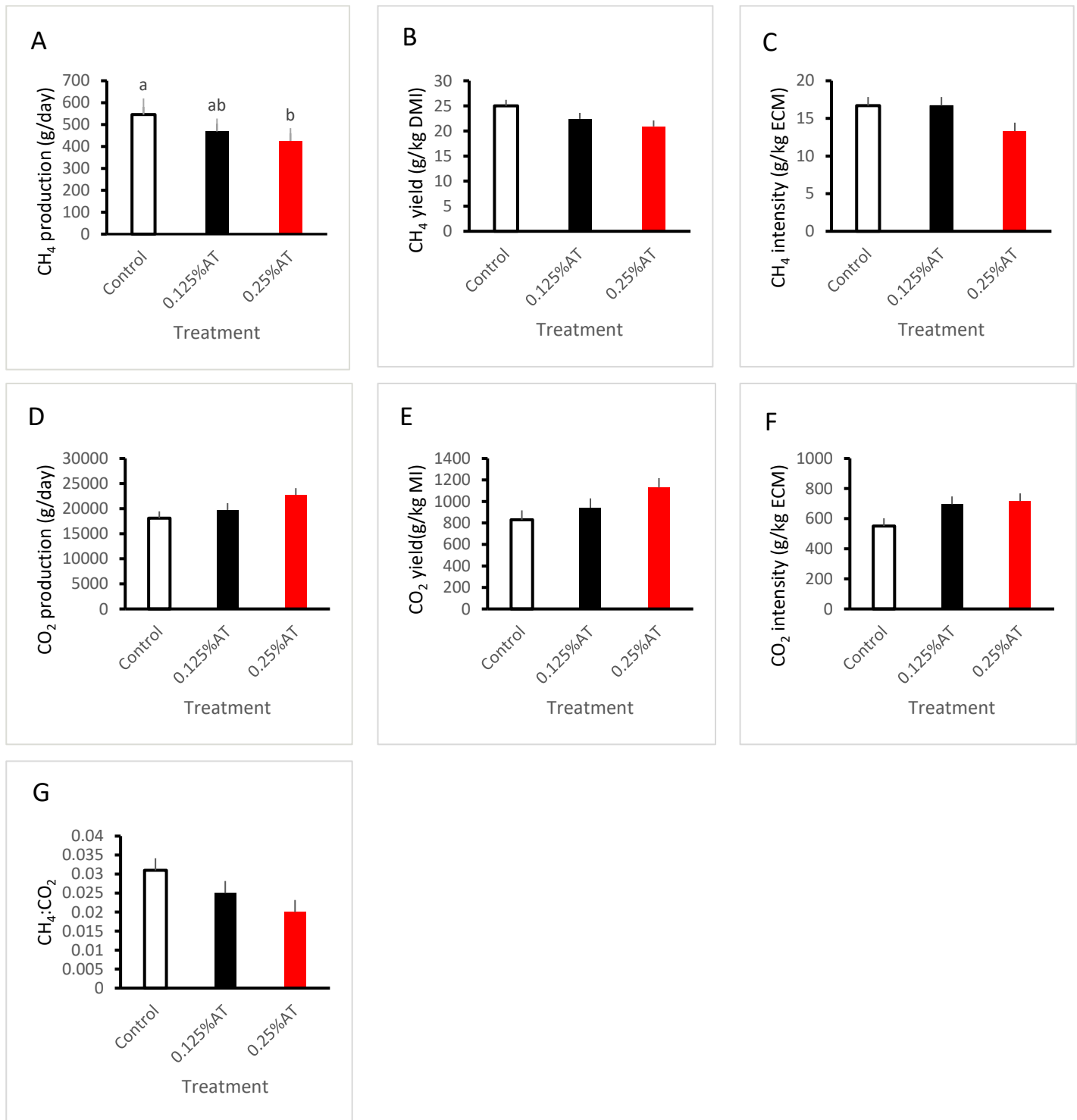


Figure 18. Effect of graded *Asparagopsis taxiformis* inclusion on mean (\pm SE) CH₄ and CO₂ production (g/day) (A,D), yield (g/kg DMI)(B,E) and intensity (g/kg ECM) (C,F), in addition to CH₄ to CO₂ ratio (CH₄:CO₂). Means within a graph carrying different superscripts within a diet group are significantly different at $P \leq 0.05$.

4.1.3 Rumen fermentation parameters

4.1.3.1 Comparisons of two rumen fluid sampling methods

Two out of five cows in each treatment group were not fitted with a rumen cannula; therefore, rumen fluids from these cows had to be sampled via esophageal tubing. Whereas this is normal practice when intact cows are used, the reticulorumen sampling site is unknown and might therefore not be the same for all cows, presenting a limitation to the study. Because of this, comparisons of the two sampling methods in regard to rumen fermentation parameters are presented in Table 3.

Notably, sampling via the rumen cannula was associated with a significantly higher total VFA concentration ($P < 0.001$) compared to sampling via the esophageal tube. Molar proportions of isovalerate were lower when rumen fluids were sampled through a rumen cannula versus esophageal tube ($P = 0.006$), as the only individual VFA showing a significant difference between sampling methods. Further, rumen $\text{NH}_3\text{-N}$ was higher and ruminal fluid pH was significantly lower in samples taken via the rumen cannula compared to the esophageal tube ($P < 0.001$).

Table 3. Effect of ruminal fluid sampling method (Esophageal tubing vs. rumen cannula) on ruminal fluid pH, ammonia nitrogen ($\text{NH}_3\text{-N}$, mg/L), total short chain volatile fatty acids (tVFA, mMol/L), molar proportions of specific VFA (% of tVFA) and acetate to propionate ratio (Ace:Pro) in early- to mid-lactation NRF dairy cows fed total mixed ration.

	$\text{NH}_3\text{-N}$	tVFA	Ace	Pro	But	IsoBut	Val	IsoVal	Ace:Pro	pH
Sampling										
Esophageal tube	149.9	80.7	54.0	23.5	16.8	1.13	1.69	1.86	2.23	6.77
Rumen cannula	225.4	106.8	53.9	26.6	17.0	1.10	1.86	1.58	2.27	6.04
SE	15.7	3.08	2.87	2.23	1.16	0.07	0.10	0.09	0.21	0.07
P-value	<0.001	<0.001	0.981	0.175	0.905	0.44	0.097	0.006	0.853	<0.001

Ace= Acetate, Pro = Propionate, But = Butyrate, IsoBut = Isobutyrate, Val = Valerate, IsoVal = Isovalerate

4.1.3.2 Effect of *Asparagopsis taxiformis* on rumen fermentation

Rumen fermentation parameters for all cows (regardless of sampling method) including rumen $\text{NH}_3\text{-N}$, total concentration and molar proportions of VFAs and pH, are presented in Table 4. There was no significant difference in rumen $\text{NH}_3\text{-N}$ concentration between the three treatment groups; however, a numerical decrease was seen with incremental *A. taxiformis* inclusion

levels. Notably, including *A. taxiformis* in the diets did result in several significant changes within the measured volatile fatty acids (VFAs).

Total rumen VFA (tVFA) concentrations (mMol/L) were greatest within the control group and significantly different ($P = 0.0027$) from the groups given the *A. taxiformis* supplement, decreasing 14.4 and 8.8% in the 0.125%AT and 0.25%AT groups, respectively. The rumen acetate concentrations also decreased significantly with the AT inclusion ($P = 0.019$), lowering molar proportions in 0.125%AT by 7.5% and 0.25%AT by 8% compared to control. In contrast to acetate, propionate concentrations increased by 12.1% and 9.3%, with 0.125%AT and 0.25%AT accordingly, being significantly higher than control ($P = 0.047$). Yet not statistically significant, the concentration of butyrate also increased with increasing dose of AT, leaving 0.125%AT with 8.1% and 0.25%AT with 13.1% greater molar concentrations than control. Isobutyrate concentrations differed between control and 0.25%AT by increasing 11.8%, while 0.125%AT was similar to both groups previously mentioned. The increase in valerate for AT inclusion groups (8.8 % and 11.9% for 0.125%AT and 0.25%AT, respectively) were insignificant, while isovalerate increased significantly ($P = 0.05$) by 12.2 and 20.1% for 0.125%AT and 0.25%AT.

With AT inclusion, lower acetate and greater propionate concentrations resulted in a significantly decreasing acetate to propionate ratio ($P = 0.028$). This ratio was 18.9% and 15.9% greater in the control group compared to the groups fed 0.125%AT and 0.25%AT, respectively. Notably, the treatment group given the highest AT dose (0.25%AT) also had a significantly ($P = 0.027$), while within the normally expected range, greater pH. Increasing by 4.39% compared to control.

Table 4. Effect of adding graded levels of red seaweed (*Asparagopsis taxiformis*) on ruminal fluid pH, ammonia nitrogen ($\text{NH}_3\text{-N}$, mg/L), total short-chain volatile fatty acids (tVFA, mMol/L), molar proportions of specific VFA (% of tVFA), and acetate to propionate ratio (Ace:Pro) in early- to mid-lactation NRF dairy cows fed TMR.

	NH ₃ -N	tVFA	Ace	Pro	But	IsoBut	Val	IsoVal	Ace:Pro	pH
Treatment										
Control	159.4	107.4 ^a	58.7 ^a	21.4 ^b	16.0	0.97 ^b	1.60	1.39 ^b	2.77 ^a	6.09 ^b
0.125%AT	136.4	96.4 ^b	54.3 ^b	24.0 ^a	17.3	1.00 ^{ab}	1.74	1.56 ^a	2.33 ^b	6.14 ^b
0.25%AT	145.3	90.5 ^b	54.0 ^b	23.4 ^{ab}	18.1	1.10 ^a	1.79	1.67 ^b	2.39 ^b	6.37 ^a
SE	10.27	2.35	1.06	2.20	0.55	0.03	0.22	0.05	0.10	0.06
P-value	0.409	0.0027	0.019	0.047	0.081	0.05	0.176	0.010	0.028	0.027

Ace= Acetate, Pro = Propionate, But = Butyrate, IsoBut = Isobutyrate, Val = Valerate, IsoVal = Isovalerate
Means in a column carrying different superscripts are significantly different at $P \leq 0.05$

4.1.4 Milk yield and composition

Notably, an overall effect of the seaweed supplementary diets was observed among all milk yield and milk composition parameters, as presented in Table 5. Both MY and ECM yield (kg/d) were significantly lower ($P < 0.001$) for the 0.125%AT and 0.25% AT diet groups compared to control. Daily MY over the experimental period (mean \pm SE) along with the covariate period MY (d = 0) for each treatment group is presented in Figure 19.

As to milk composition, both milk fat and protein contents were significantly lower in the AT groups. However, milk lactose was higher ($P = 0.021$) in the AT groups than the control group. As such, milk component yields differed significantly among all treatments ($P < 0.05$). For instance, milk fat yield decreased by 16.6% in the 0.125%AT group and 12.4% in the 0.25%AT group compared to the control group. Similarly, lactose yields were significantly different between all groups, decreasing by 5.5% for 0.125%AT and 8.9% for 0.25%AT. However, weighed lactose % increased with AT inclusion, resulting in a significant difference between control and the two inclusion groups. Milk lactose % increased by 2.7 and 1.9% for 0.125%AT and 0.25%AT, respectively. All milk yield parameters were statistically affected by sampling day, while no significant effect was observed for milk composition parameters regarding sampling day. Also, the interaction effects of diet by sampling day were not significant for both milk yield and milk composition parameters.

Among other milk variables, milk free fatty acids (FFA) were insignificantly affected by dietary treatment, while a significant correlation to sampling day ($P < 0.001$) was noticed. Further, milk urea nitrogen was significantly affected by diet ($P = 0.022$) as well as sampling day, whereas no significant effects were seen for Diet*Day interactions. There were not observed any indications of *A. taxiformis* affecting milk somatic cell count (SCC), which were statistically unaffected by diet, sampling day, and diet-sampling day interactions.

Table 5. Milk yield and milk composition from early- to mid-lactation NRF dairy cows given total mixed ration containing graded levels of red seaweed (Control= 0%; 0.125%AT = 0.125%; and 0.25%AT =0.25% *Asparagopsis taxiformis* on organic matter intake basis).

Yield, kg/d	Diet			SE	Effect of: (P-Value)		
	Control	0.125%AT	0.25%AT		Diet	Day	Diet*Day
Milk yield	31.7 ^a	29.2 ^b	28.4 ^b	0.32	<0.001	0.008	0.316
ECM [†]	34.4 ^a	30.0 ^b	30.5 ^b	0.30	<0.001	0.005	0.230
Fat	1.45 ^a	1.21 ^c	1.27 ^b	0.017	0.002	0.014	0.429
Protein	1.16 ^a	1.02 ^b	0.99 ^c	0.011	<0.001	0.007	0.281
Lactose	1.46 ^a	1.38 ^b	1.33 ^c	0.016	0.002	0.010	0.379
Composition, %							
Fat	4.65 ^a	4.15 ^b	4.18 ^b	0.110	0.018	0.522	0.977
Protein	3.65 ^a	3.51 ^b	3.49 ^b	0.028	0.010	0.099	0.934
Lactose	4.60 ^b	4.73 ^a	4.69 ^a	0.026	0.021	0.112	0.084
Other variables							
FFA [‡] , mmol/L	0.47	0.39	0.45	0.032	0.136	<0.001	0.501
MUN [‡] , mg/dL	13.9	12.3	12.4	0.360	0.022	<0.001	0.469
SCC ^{††} , log(count+1)/mL	4.83	4.75	4.65	0.087	0.439	0.526	0.763

[†] ECM= energy corrected milk yield according to Sjaunja et al. (1991)

[‡] FFA= milk free fatty acids

[‡] MUN= milk urea nitrogen

^{††} SCC= log transformed somatic cell count

Means in a row carrying different superscripts are significantly different at $P \leq 0.05$

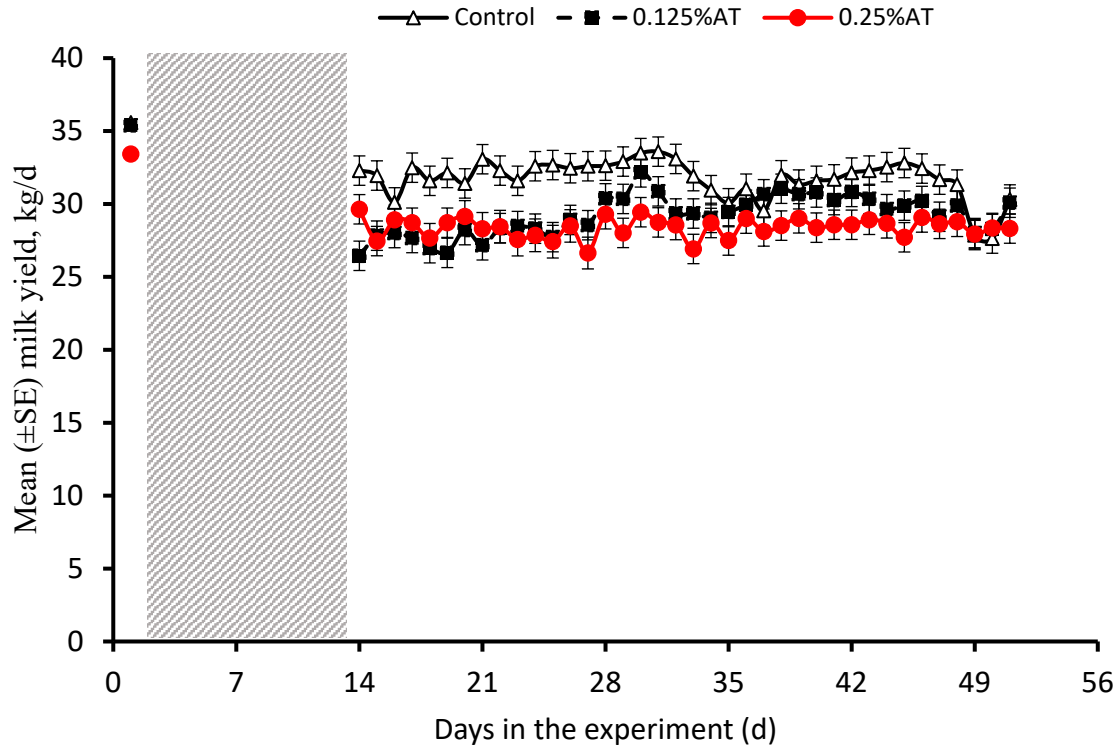


Figure 19. Milk yield of early- to mid-lactation NRF dairy cows given total mixed ration containing graded levels of red seaweed (Control= 0% ; 0.125%AT = 0.125%; and 0.25% AT =0.25% *Asparagopsis taxiformis* on organic matter intake basis). The shaded area represents the *A. taxiformis* adaptation period.

In Table 6, feed utilization efficiency calculated in four different ways are displayed; as milk yield corrected for DMI (MY_{eff}); energy corrected milk yield efficiency corrected for DMI (ECM_{eff}); as well as MY_{eff} and ECM_{eff} adjusted for body weight change ($adj.MY_{eff}$ and $adj.ECM_{eff}$) during the experiment. Independent of calculation method, feed utilization efficiency was numerically greater, however insignificantly, in 0.25%AT compared to control and 0.125%AT. For 0.125%AT MY_{eff} and $adj.MY_{eff}$ were numerically similar to control, while ECM_{eff} and $adj.ECM_{eff}$ decreased slightly.

Table 6. Effect of *Asparagopsis taxiformis* inclusion on feed utilization efficiency in early- to mid-lactation NRF dairy cows fed a total mixed ration.

Parameters	Diet			Statistics	
	Control	0.125%AT	0.25%AT	SE	P-value
MY _{eff} ; kg/kg DMI	1.37	1.38	1.52	0.060	0.194
ECM _{eff} ; kg/kg DMI	1.50	1.42	1.59	0.060	0.154
Adj.MY _{eff} ; kg/kg DMI	1.39	1.37	1.49	0.058	0.296
Adj.ECM _{eff} ; kg/kg DMI	1.52	1.41	1.58	0.056	0.159

MY_{eff} = milk yield efficiency; ECM_{eff} = energy corrected milk yield efficiency; Adj.MY_{eff} = Body weight change adjusted milk yield efficiency (calculated according to Roque et al. (2019b)); Adj.ECM_{eff} = Body weight change adjusted energy corrected milk yield efficiency.

As shown in Table 7, including *A. taxiformis* in the diet of dairy cows showed no significant treatment effect on milk fatty acid composition. Despite insignificant results, increasing inclusion of *A. taxiformis* resulted in a slight increase in stearic acid and MUFA, while SFA, palmitic acid, and PUFA were associated with an insignificant decrease. However, a significant effect ($P < 0.001$) of sampling day were observed for all milk fatty acid composition parameters, whereas there was no significant correlation between treatment and sampling day (Diet*Day).

Table 7. Effect of *Asparagopsis taxiformis* inclusion in milk fatty acid composition (g/100 g milk fat) of early- to mid-lactation NRF dairy cows fed a total mixed ration.

Fatty acids	Diet			SE	Effect of: (P-Value)		
	Control	0.125%AT	0.25%AT		Diet	Day	Diet*Day
SFA	74.8	74.1	73.7	0.39	0.193	<0.001	0.094
Palmitic acid	29.9	29.5	29.5	0.11	0.114	<0.001	0.332
Stearic acid	11.1	11.1	11.3	0.04	0.150	<0.001	0.124
MUFA	18.0	18.5	18.9	0.30	0.125	<0.001	0.384
PUFA	2.75	2.74	2.73	0.016	0.822	<0.001	0.835

SFA = total saturated fatty acid in milk

MUFA = monounsaturated fatty acids

PUFA = polyunsaturated fatty acids

4.2 Experiment 2: *In Vitro*

The same inclusion levels of *A. taxiformis*, i.e., 0% (control), 0.125%, and 0.25% included at OM basis, were tested in the *in vitro* experiment. In addition, 0.5% AT at OM basis was included, representing the initially intended inclusion level of the 0.125%AT group. Gas measurements indicated that *A. taxiformis* had no significant effect on total gas (TG) production ($P = 0.81$). This can be seen in Figure 20A, where mean TG production (mL/g DM \pm SE) is presented for each inclusion level. However, the methane production decreased when the two highest levels of *A. taxiformis* were added (Figure 20B). Resulting in a significantly ($P < 0.001$) different mean CH₄ production between control, 0.125%AT, and 0.5%AT, where including 0.5% OM *A. taxiformis* reduced CH₄ production by 29.8% compared to control. While the 9.4% reduction by 0.25%AT compared to control was insignificantly different from all inclusion groups, including 0.5%AT. Adding 0.125%AT, however, did not reduce the CH₄ production compared to the Control. As with TG production, mean CO₂ production was not affected ($P = 0.44$) by *A. taxiformis* inclusion *in vitro* (Figure 20C), while a numerical difference between groups was observed. Carbon dioxide production was numerically greatest for 0.125%AT, and lowest for 0.25%AT, while control and 0.5%AT were numerically similar.

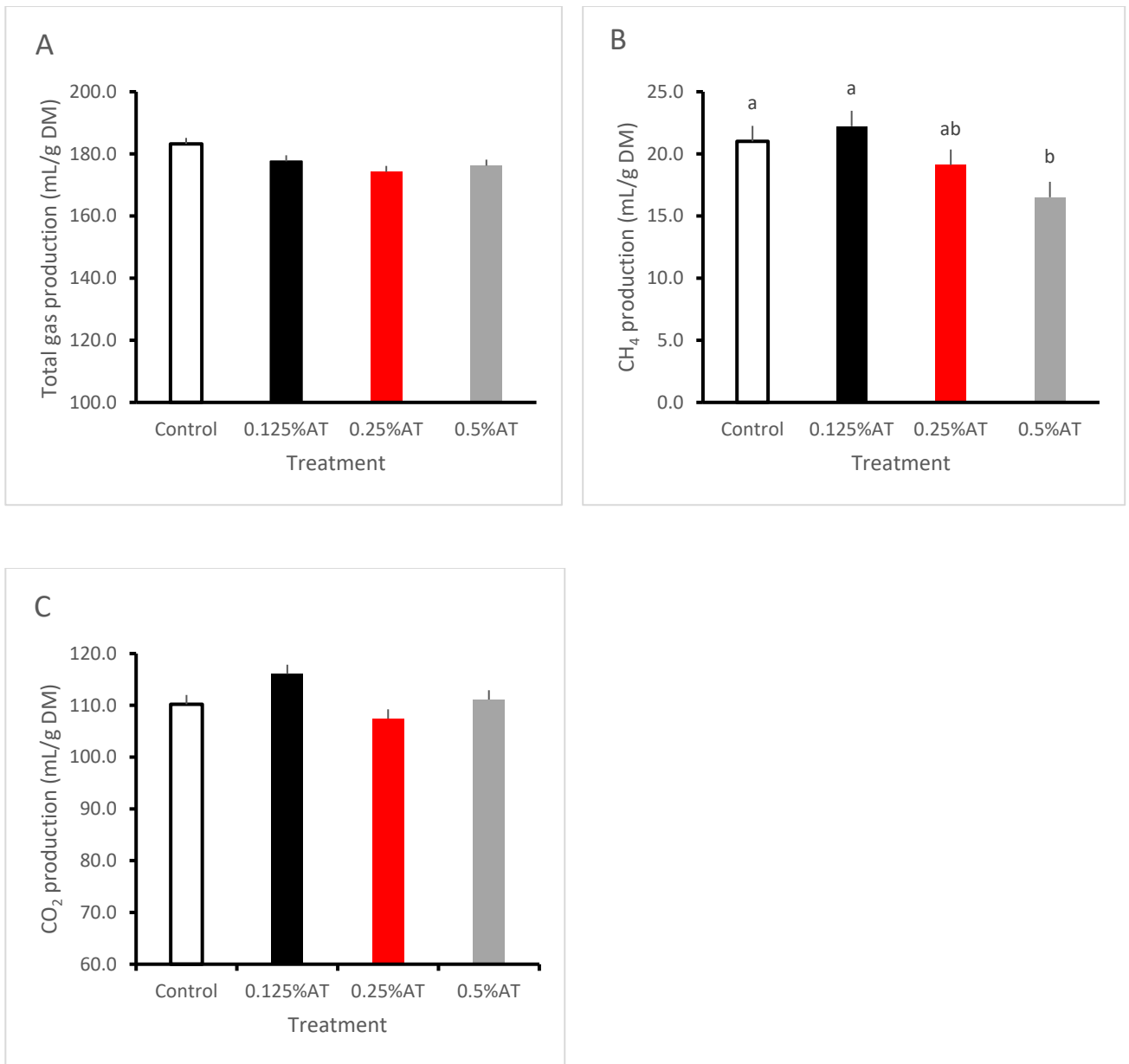


Figure 20. The effect of *Asparagopsis taxiformis* inclusion on mean mL/g DM (\pm SE) in vitro total gas (A), CH₄ (B), and CO₂ (C) production. Each bar represents mean values of three replicates within each in vitro batch for each inclusion level (Control= 0%; 0.125%AT = 0.125%; 0.25% AT =0.25%; and 0.5%AT = 0.5% *Asparagopsis taxiformis* on organic matter basis). Means carrying different superscripts are significantly different at $P \leq 0.05$.

5. Discussion

This study aimed to assess the CH₄ mitigating potential of the red seaweed *Asparagopsis taxiformis* in NRF dairy cows and, at the same time eventual changes in rumen fermentation and animal productivity associated with the nutritional supplementation of *A. taxiformis*. Findings in the present study suggest that *A. taxiformis* (AT) indeed possesses a highly dosage-dependent potential in mitigating enteric CH₄ emissions, presumably redirecting hydrogen to alternative sinks, affecting rumen fermentation and energy distribution in early- to mid-lactation dairy cattle. The results are discussed hereunder.

5.1 Feed intake and feed utilization

In this study, we observed that *A. taxiformis* affected DMI negatively when included at 0.25% based on OM, resulting in a 16.2% lower DMI intake. This aligns with previous studies including *Asparagopsis* spp. in dairy cattle by Roque et al. (2019b) and Stefenoni et al. (2021) where including *Asparagopsis* spp. affected DMI significantly. Roque et al. (2019b) observe that including 0.5% and 1.0% *A. armata* at OM basis reduced feed intake by 11% and 38%, while a more moderate decrease of 3% and 7% were seen by Stefenoni et al. (2021) with 0.25% and 0.5% *A. taxiformis* included at DM basis. On the other hand, Kinley et al. (2020) reported no negative impact on DMI by *A. taxiformis* fed to Brangus steers (0.05%, 0.10%, and 0.20% OM); also, DMI of Angus-Hereford steers decreased insignificantly ($P = 0.34$) with 0.25%, while 0.5% OM *A. taxiformis* gave a significantly reduced DMI compared with control over a 21 weeks period (Roque et al., 2021). Based on differences in inclusion levels and DMI between the studies mentioned, including the present one, a negative correlation between inclusion level and DMI might be indicated. The periodic decrease seen in the current study for the 0.125%AT group (Figure 16) is likely a consequence of starting feeding this group with 0.5% OM *A. taxiformis*, which resulted in severe feed refusal by all cows. This also explains the significant variation in Diet-Day interactions for DMI and nutrient intake parameters seen (Table 1) as day-to-day variations for control and 0.25%AT seem to follow the same pattern.

Roque et al. (2019b) suggested high mineral composition of *Asparagopsis* spp. (Selmi et al., 2020) to affect feed palatability and, therefore, a possible explanation for the compromised DMI. In the present study, OM% of *A. taxiformis* was 46.4%, comparable OM content was reported by Roque et al. (2019b) for *A. armata* (49.6%), as well as by Kinley et al. (2020) (~50%) and Roque et al. (2021)(50.9%) for *A. taxiformis*. Assuming the inorganic fraction of *Asparagopsis* spp. are representative of the total biomass mineral content (Berg, 2020), the

mineral content in AT biomass for the present study would be 53.6 g/100g DM, whereas the average mineral content for *Aspragopsis* spp. biomass used in the aforementioned studies would be 49.8 g/100g DM. With the potentially high mineral content in mind, *A. taxiformis* biomass used in the current study was analyzed for iodine which is seen as a potential pitfall in food- and feed safety when using seaweeds in animal- and human diets (Duinker et al., 2020). *Asparagopsis taxiformis* is previously reported to have a high iodine affinity (Palumbo, 1955). This was also observed by Mairh et al. (1989) and Nunes et al. (2017), who found *A. taxiformis* to have the highest iodine concentrations of all seaweeds tested in their studies. Roque et al. (2021) found their AT biomass to contain 2.27 mg Iodine/g, whereas the iodine content in our study was measured to be 7.04 mg Iodine/g DM. Iodine levels seen for the current study are somewhat in the mid-range of the greatest values (4.39 mg/g DM) seen by Mairh et al. (1989), who reported seasonal variations, and the 12.03 mg/g DM found by Nunes et al. (2017). Iodine is essential for thyroid hormone production in humans and other mammals, whereas both insufficient and excessive consumption can influence thyroid function (Chung, 2014). The adult cow requires less than 0.016 mg/kg BW per day to obtain normal thyroid hormone biosynthesis (Paulíková et al., 2002); given the mean BW for cows included in this study, the average requirement was 10.8 mg I/day, while the established dairy cows' tolerance limit is 50 mg I/kg DMI (Council, 2006). Iodism (i.e., iodine intoxication) in ruminants is described with a diverse range of symptoms, including anorexia (Paulíková et al., 2002). Based on the DMI seen in Table 1 and analyzed iodine content, *A. taxiformis* contributed around 17.49 and 31.95 mg I/kg DM per day for cows fed 0.125%AT and 0.25%AT, accordingly. While iodine inclusion in this study was above the daily requirement of cows, the levels are well within the tolerance limit. Elevated levels of iodine after AT supplementation have, however, been found in meat (Roque et al., 2021) and milk (Stefenoni et al., 2021). Based on these results, we believe attention should be directed towards further evaluating iodine levels in samples collected from this study as well as all future animal trials incorporating AT diets.

Other than the high mineral content and low palatability of *A. taxiformis*, it has been suggested that increased rumen H₂ partial pressure caused by the low turnover to CH₄ might suppress DMI (Janssen, 2010). While hydrogen emissions were not estimated during the current experiment, previous studies have demonstrated that H₂ emissions increases with decreasing CH₄ emissions (Kinley et al., 2020; Roque et al., 2021). Decreased DMI as a cause of increased rumen H₂ partial pressure is not supported when considering the most significant CH₄ mitigations obtained with *Aspragopsis* spp. were obtained without significantly reduced DMI (Kinley et

al., 2020; Roque et al., 2021). Additionally, decreased DMI has not been observed with reduced CH₄ achieved with bromochloromethane in dairy goats (Abecia et al., 2012) or 3-NOP in dairy cattle (Hristov et al., 2015; Melgar, A et al., 2020; Van Wesemael et al., 2019). This supports the likelihood of decreased DMI to be caused by low palatability of *Aspragopsis* spp.

Feed utilization increased numerically with the highest inclusion level of *A. taxiformis* (0.25%AT), agreeing with the significantly ($p < 0.05$) increased feed utilization seen by Roque et al. (2019b) and Kinley et al. (2020). Roque et al. (2019b) state that this increase was caused by a noticeable reduction in DMI while maintaining an MY not corresponding to the decreased DMI. In contrast, cows being fed 0.25%AT in the present study adjusted their MY according to the DMI, maintaining a reasonably stable MY throughout the experimental period. The numerically lower feed utilization seen for the 0.125%AT group, despite a higher DMI and lower MY compared to 0.25%AT, are probably a carryover effect from the change in AT inclusion levels from what they started on. This change is likely to have induced a negative energy balance (NEB) when DMI decreased. Cows used in this study were at 95 ± 27 DIM when *A. taxiformis* first was included in the diet. Comparably, Gross et al. (2011) induced NEB by restricting DMI (50%) in dairy cows at 98 ± 7 DIM for three weeks, where cows regained a positive energy balance within two weeks after feed restriction ceased. Animals in NEB will mobilize body fat, achieving a decrease in feed utilization. As seen in Figure 16 and Figure 19, the 0.125%AT group started the experimental period at day 14 with a slightly lower DMI and MY in the first two weeks of the experimental period while gradually increasing. This could indicate that these cows were recovering from an NEB.

In addition to the changes in inclusion levels, the feeding method of *A. taxiformis* was modified during the experimental period. It was noticed that by mixing *A. taxiformis* with the described molasses-water-mix before including the mix in the TMR, individuals were prominent to sift out and exclude *A. taxiformis* from the TMR. Mixing the seaweed directly into the TMR before pouring and again mixing in the molasses-water-mix increased homogeneity of the total TMR-mix. It was hypothesized that changing the way of mixing *A. taxiformis* into the TMR would increase intake of AT and thereby increase the CH₄ mitigation efficiency. However, no significant difference in mean CH₄ production was seen between the two periods of CH₄ measurements.

5.2 Enteric CH₄ emissions

While *A. taxiformis* inclusion at 0.25% OM reduced CH₄ production (g/day) significantly by 22%, CH₄ intensity (g/kg ECM, -20%) and yield (g/kg DMI, -16.4%) reductions were not significant. This contradicts previous studies where *A. taxiformis* inclusion resulted in decreased CH₄ daily production, yield, and intensity (Roque et al., 2021). While more reliable results were reported by Stefanoni et al. (2021), who found that including 0.25% and 0.5% *A. taxiformis* at DM basis only decreased CH₄ production, emission, and yield with the highest inclusion level. The insignificant variation in CH₄ intensity and yield is likely caused by the decrease seen in DMI for 0.25%AT and MY for both AT inclusion groups. It is worth mentioning that CH₄ intensity was similar for 0.125% and control, explained by the decreased ECM production by the former. Also, the slightly lower CH₄ yield aligns with lower CH₄ production and similar DMI as control, indicating that *A. taxiformis* had no mitigating effect when included at 0.125% OM basis. As earlier mentioned, cows fed 0.25%AT adjusted their MY according to the lowered DMI. The decrease in CH₄ production seen with 0.25%AT is, by other words, not fully explained by depressed DMI or MY. Indicating that the numerical decrease in CH₄ intensity and yield is derived from a CH₄ mitigating effect by *A. taxiformis*. Carbon dioxide emissions did not increase significantly with *A. taxiformis* inclusion, in agreement with Roque et al. (2021). However, a numeric increase was seen for all CO₂ parameters, where especially the >20% increases in CO₂ intensity and yield should be noticed. Roque et al. (2019b) saw, in contrast to the present study, no increase in CO₂ production with 0.5% OM *A. armata*, while their 1.0% OM inclusion level resulted in a significant decrease. The CO₂ yield did, however increase with increasing *A. armata* inclusion (Roque et al., 2019b), similarly to the results found by Roque et al. (2021). In accordance with previously reported results by Stefanoni et al. (2021), CH₄:CO₂ ratio decreased significantly. Rumen produced CO₂ is usually reduced together with H₂, producing CH₄ (Sjaastad, 2010) and the increase seen in the present study could indicate that less CO₂ were reduced while microbial activity were maintained. Daily CH₄ and CO₂ production depends on daily DMI (Jonker et al., 2014), the significant day within period variations in CH₄ and CO₂ emissions agree with the significant variation in DMI per day, the significant variation in CH₄ and CO₂ between days are therefore explained by fluctuations in DMI.

So far, *Asparagopsis ssp.* biomass used in the present and other studies mentioned have been wild-harvested. Before the experiment started, bromoform concentrations of the AT biomass used in the present study were measured to 4.02 mg/g DM, about three times higher than the

concentration (1.32 mg/g DM) in *A. armata* used by Roque et al. (2019b). These levels are, in comparison, remarkably lower than concentrations seen by Roque et al. (2021) and Kinley et al. (2020), who found bromoform to be 6.55 and 7.8 mg/g DM in *A. taxiformis*. The latter is the highest bromoform concentrations yet reported used *in vivo* with *Asparagopsis spp.* Similarly to previous studies with *Asparagopsis spp.* in cows, the seaweed in the current study were freeze-dried and incorporated into a TMR before offered to the animals (Kinley et al., 2020; Roque et al., 2019b; Roque et al., 2021; Stefenoni et al., 2021). Freeze drying was found to be a suitable form for processing, with low loss of bioactive compounds by Vucko et al. (2017). On the other hand, a more recent publication by Magnusson et al. (2020) implies that submerging *A. taxiformis* to conserve bioactive CH₄ mitigating compounds in oil should be further investigated as an alternative to freeze-drying. By submerging *A. taxiformis* in oil, it might be possible to increase concentrations of bioactive compounds and hence increase the mitigating effect per unit included in a feed ration (Magnusson et al., 2020).

Results obtained by Stefenoni et al. (2021) indicate that loss of the highly volatile plant metabolites might cause *A. taxiformis* to lose its mitigating effect over time, demonstrating that bromoform concentrations were reduced by up to 84% ($P < 0.001$) over four months, depending on storage conditions. Furthermore, Stefenoni et al. (2021) found that *A. taxiformis* only had an CH₄ mitigating effect through two of four experimental periods when tested *in vivo*. On the other hand, Roque et al. (2021) saw a consistent mitigating effect over 21 weeks. These contradicting results could be affected by the initial concentration of bromoform as well as the diet composition. For the present study, *A. taxiformis* biomass was harvested during April 2020, around one year prior to the experimental start. The *in vitro* trial described in this thesis (Exp. 2) was conducted as an attempt to confirm the mitigating potential seen in Experiment 1, as well as an earlier *in vitro* assay where the same *A. taxiformis* biomass was used in August of 2020. During the experiment in 2020, CH₄ was reduced by up to 99% when including 9 mg AT to 1g substrate (Alvarez, 2021), compared with a 29.8% reduction with a 9.8 mg AT enrichment (0.5% OM) in the present study - indicating, in line with Stefenoni et al. (2021), that storage time might affect the mitigating potential of *A. taxiformis*. When comparing these results, a limiting factor is implied by the fact that different substrates were used. In addition, rumen fluids for the present study were collected from control cows used in the *in vivo* trial (Exp. 1) while on the experimental diet (no seaweed), whereas rumen fluids for the 2020 experiment were obtained from dry cows fed a standardized diet, as described for the “NorFor in sacco standard protocol” by Volden (2011). These differences are believed to affect the comparability

of the *in vitro* results due to differences in substrate formulation and microbial variations in rumen fluids. Therefore, an actual change in mitigation potential by *A. taxiformis* between the two *in vitro* studies can not be concluded.

A potential explanation of the consistent CH₄ mitigation seen by Roque et al. (2021), could potentially be a combination of high initial bromoform concentrations and changes in the diet during the experiment towards a diet with less structural carbohydrates. Roque et al. (2021) tested three diets and found that *A. taxiformis* mitigating effect was more prominent with 18.6% NDF, reducing CH₄ yield by 69.9% compared to TMR with 33.1% NDF, reducing CH₄ yield by 32.7% when feeding 0.25% *A. taxiformis* at OM basis. Similarly, increased *in vitro* CH₄ inhibition by *A. taxiformis* with a lower roughage to concentrate ratio was also recently demonstrated by Kinley et al. (2021). Furthermore, during a meta-analysis, Dijkstra et al. (2018) found feed composition, especially NDF content, to affect the mitigating effect obtained with 3-NOP in dairy and beef cattle. A negative correlation between CH₄ emissions and NDF, together with a positive correlation of CH₄ and 3-NOP, were among their main findings (Dijkstra et al., 2018). Correlations between NDF content in feed and CH₄ emissions has also previously been emphasized by Storlien et al. (2014). The positive relationship between CH₄ and NDF is primarily caused by a slower fermentation of structural carbohydrates and higher CH₄ yield per g DM fermented than non-structural carbohydrates such as starch and sugars (Holter & Young, 1992). Structural carbohydrates are also associated with a longer rumen retention time, increasing CH₄ yield (Huhtanen et al., 2016; Pinares-Patiño et al., 2011). Given the slightly greater NDFomD seen for 0.25%AT, it could be theorized that a potentially higher retention time, if allocated to decreased DMI, influences the mitigating effect by *A. taxiformis*, overestimating the CH₄ yield obtained by 0.25%AT compared to control for the present study, by not taking total feed digestibility into account.

Roque et al. (2021) hypothesize that lower NDF content is linked to lower concentrations of methyl-coenzyme M reductase (MCR), an enzyme only produced by methanogens. Changes in rumen microbiota associated with *A. taxiformis* are yet not described *in vivo*, while *in vitro* changes are reported by Machado et al. (2018) and Roque et al. (2019a), who found *A. taxiformis* inclusion to be associated with lower methanogen abundance. In accordance with this, changes in methanogen abundance are described *in vivo* for 3-NOP (Martinez-Fernandez et al., 2018; Meale et al., 2021) and bromochloromethane in goats, where a decrease in rumen fungi also was described (Mitsumori et al., 2012). The common diet used in the present dairy trial contained 40.1% NDF, which is higher than in diets used by other studies feeding

Asparagopsis spp. to cattle (Kinley et al., 2020; Roque et al., 2019b; Roque et al., 2021). As such, the lower reduction seen in the current study might be partially caused by greater numbers of methanogens, compared with published studies where feeds with lower NDF contents have been used, achieving a higher mitigating result with *A. taxiformis*. In light of this, a need to further investigate how the mitigating effect of *A. taxiformis*, and other feed supplements, are affected by feed composition is highly needed to clarify and assess potential feed-supplement interactions which can optimize practical use.

5.3 Rumen fermentation

As expected, a shift in rumen VFA production was noticed with decreasing CH₄ production. This shift was seen in a decreased molar acetate:propionate ratio and were, in line with results found by Stefenoni et al. (2021), accompanied by lower total VFA production. Also, Li et al. (2016) described a decrease in tVFA when feeding >1% OM *A. taxiformis* to sheep, together with a shift towards decreased acetate and increased propionate molar percentage, as seen in the present study and by Stefenoni et al. (2021). The decrease in tVFA is believed to be caused by a lower DMI, resulting in less microbial VFA production, which again increases rumen pH. Although not statistically significant, a slight increase in pH was indeed measured with increased *A. taxiformis* inclusion levels. Lower tVFA production has also been previously observed *in vitro* in combination with decreased acetate and increased propionate and butyrate (Kinley, 2015; Machado et al., 2014; Machado et al., 2016a). The *in vitro* decrease in tVFA was in contrast to the present *in vivo* study explained by lower OM digestibility (Machado et al., 2016a). Notably, increased rumen pH is associated with increased cellulose degradation efficiency (Hu et al., 2004), which has been found to be most effective around pH 6.5 (Zhang et al., 2017). This aligns with the increased NDFomD and pH seen for the 0.25%AT group.

Moreover, the production of individual VFAs might be affected by the rumen hydrogen partial pressure (Ellis et al., 2008; Janssen, 2010; van Lingen et al., 2016). Assuming an increase in rumen hydrogen pressure caused by a suppressed CH₄ production, propionate production could potentially serve as an alternative hydrogen sink leading to increased rumen fluid propionate concentrations. Acetate production, on the other hand, may be suppressed by an increasing H₂ in the rumen, caused by inhibition of NADH oxidation (van Lingen et al., 2016). The observed numeric increase in butyrate might also be explained by an increase in H₂ partial pressure if butyrate is formed at the expense of acetate, which again yields less CH₄ per mol glucose (Janssen, 2010). Branched VFAs (e.g., isobutyrate and isovalerate) are products derived from oxidative deamination of branched amino acids (Andries et al., 1987) and are of minor

importance as an energy source for the ruminant itself. However, they are essential for most fiber-degrading rumen microbes (Bryant, 1973). The increase in isobutyrate and isovalerate obtained with increasing AT inclusion might therefore also have been a stimulating factor in regard to the increased NDFomD found for 0.125%AT.

5.3.1 Effect of sampling method

Comparison of samples collected using esophageal tubing vs. via rumen cannula strongly suggests that rumen NH₃-N, tVFA, and pH were influenced by the sampling method, and differences obtained in the present study might indicate that samples taken via OST were sampled from the cranial ventral rumen (Shen et al., 2012). The increase in NH₃-N and tVFA, together with decreased pH, coincides with the difference in fermentation pattern, which Shen et al. (2012) found between the central rumen and cranial ventral rumen while assessing the significance of OST insertion depth on rumen fermentation parameters. Variations in sampling site in regard to fermentation pattern were also evaluated by Alvarez (2021), where samples obtained via OST from the ventral and central rumen only differed in the abundance of branched VFAs, in accordance with Shen et al. (2012). The influence of sampling site on the profile of rumen fermentation parameters are important to keep in mind when comparisons are made across sampling methods.

5.4 Milk and Milk fat composition

The results of this study also shows that milk composition was affected when AT was included in the diet, leading to a reducing fat and protein content while increasing lactose. The decrease in milk yield and milk component yield is believed to be, in agreement with Stefenoni et al. (2021), a consequence of the decreased MY caused by reduced DMI by cows offered *A. taxiformis*. So far, scarce information about *Asparagopsis* spp. inclusion in dairy cows where CH₄ and milk composition are reported have been published. Except for a decrease in milk protein composition when including 1% *A. armata* at OM basis, no effect associated with seaweed inclusion was seen for other milk fractions or with lower inclusion levels by Roque et al. (2019b). Whereas, Stefenoni et al. (2021) only saw a significant decrease in milk lactose composition with 0.5% AT fed at DM basis. Supplementation of 3-NOP has yielded different results where Melgar, A. et al. (2020) found no effect on milk composition, while Van Gastelen et al. (2020) saw an increase in milk protein and fat in accordance with Lopes et al. (2016).

For the present study, the changes seen might partially be explained by the shift in rumen fermentation. Decreased molar proportions of acetate could cause a reduction in milk fat, as

acetate is the primary energy source of de novo milk fat synthesis (Urrutia et al., 2016). Furthermore, the lower milk protein content and milk urea nitrogen are likely a consequence of decreased CPI (Frank & Swensson, 2002; Ropstad et al., 1989). At the same time, the shift towards greater molar percentage propionate might indicate an increase in udder available glucose. Similarly, Abecia et al. (2012) assumed a increase in glucose availability to be the cause of increased MY seen when supplementing bromochloromethane to dairy goats. Lactose synthesis is stimulated by glucose availability (Lin et al., 2016) and an osmotic regulator in milk synthesis (Holsinger, 1988). Increases in milk lactose have formerly been described when feeding energy-dense diets to dairy cattle (Ouweltjes et al., 2007; Xue et al., 2011).

Although changes in milk fatty acids were not significantly affected by *A. taxiformis* inclusion, numerical variations between the diet groups do align with expected changes caused by the observed shift in rumen fermentation pattern. Engelke et al. (2018) suggest palmitic acid to be positively correlated with CH₄ emissions due to reduced de novo synthesis of palmitic acid from acetate. Monounsaturated fatty acids, including Oleic acid, are, on the other hand, believed to be negatively correlated with CH₄ emissions due to increased biohydrogenation in the rumen as an alternative hydrogen sink (Engelke et al., 2018). Despite the numerical difference in milk fatty acid composition seen in Table 7, the difference is not prominent enough to attribute this to AT inclusion and reduced CH₄ production. The results obtained between the present and the two aforementioned studies including *Asparagopsis* spp. in dairy cattle, do not present sufficient evidence towards the possible effect of AT on milk composition. Further work is needed to establish whether *A. taxiformis* affects milk composition as well as the milk fatty acid profile in dairy cattle.

5.7 Conclusion and future perspectives

This thesis confirms the potential CH₄ mitigating effects of *A. taxiformis* when included in the diet of NRF dairy cattle. However, feeding *A. taxiformis* reduced voluntary dry matter intake, and affected lactational performance. This study recognizes the potential practical challenges of using *A. taxiformis* as a feed supplement. Emphasizing the need of further investigating possible factors affecting the concentration of CH₄ mitigating compounds and explore alternative preservative treatments. Furthermore, attention should be directed towards the possible adverse effects concerning the observed palatability issues, animal health and performance, related to supplementing *A. taxiformis* in the diet of dairy cattle.

6. References

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