Investigating the Effects of *Asparagopsis* Seaweed on Enteric Methane Emissions and Animal Productivity in Ruminants

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Abstract

Enteric fermentation represents the largest single source of anthropogenic methane (CH_4) emission in the United States, accounting for 30% and 27% of the total CH_4 emitted in California (CARB, 2020) and nationwide (US EPA, 2019), respectively. Due to the significant impact of CH_4 on climate change and the negative correlation of animal productivity and enteric CH_4 production, there is great interest in identifying feed additives that might mitigate CH_4 synthesis in the rumen ecosystem. The first aim of this dissertation is to support enteric CH_4 mitigation efforts by identifying novel feed additives that have significant potential to reduce enteric CH_4 production from ruminant livestock.

Red pigmented macroalgae, specifically members from the genus *Asparagopsis*, have previously been identified as feed additive that possess high potential of anti-methanogenic properties, however, has not yet been tested widely. The second aim of this dissertation was to test the efficacy of *Asparagopsis taxiformis (A. taxiformis)*, a red macroalgae that has shown to reduce enteric CH_4 when used in an Australian production environment, using an *in vitro* rumen simulation technique (RUSITEC) system to quantify the effects on enteric CH_4 production and volatile fatty acid (VFA) production under feed regimes specific to California. When supplemented at a 5% organic matter (OM) dosage *in vitro*, *A. taxiformis* supplementation of CA specific cattle feed resulted in a 95% reduction in CH_4 coupled with an increase in propionate to acetate ratio (Roque et al., 2019a). The third aim focused on the effects of the red macroalgae *Asparagopsis armata* (*A. armata)* on enteric CH_4 synthesis and animal production parameters when fed to lactating dairy cows. A Latin square design was used with 3 treatment groups with 4 cows

within each treatment and three 2-week treatment phases with 1-week washout periods between each treatment phase. Two doses of macroalgae were used (0.5% OM and 1.0% OM) in addition to the control group (0% OM) . Results from this study showed 26% and 67% decrease in CH_4 production for 0.5% OM and 1.0% OM treatment groups, respectively. However, dry matter intake (DMI) was reduced by or to ? 10 kg/d between the control and 1.0% OM treatments, ultimately representing a reduction in milk yield of approximately 4 kg/d. Due to sorting behaviors observed in the dairy cows, we hypothesized that there was a taste aversion to high levels of macroalgae in the diet (Roque et al., 2019b). The fourth aim tested *A. taxiformis* macroalgae, similar to what was used in the *in vitro* study, fed to growing beef steers for 147 days. A randomized block design was used with 3 treatment groups (7 steers in each treatment). All steers were blocked by weight then randomly assigned to one of three treatment groups; control, 0.25% OM, and 0.5% OM. The objectives of this study were to determine the long-term (147 days) efficacy of macroalgae on reducing enteric CH_4 and to determine if there were any effects on animal production. Results from this study indicate that *A. taxiformis*' ability to reduce enteric CH_4 persisted was more effective at reducing enteric CH_4 compared to the (Roque et al., 2019b) dairy study and these effects persisted throughout the duration of the 147-day study. Additionally, reductions in DMI were also found in this study however, no changes in average daily weight gain (Roque et al., 2021). To further investigate macroalgae as a methane reducing feed additive, rumen fluid was collected from both *in vivo* studies and will be used to discover if there are changes happening to the rumen microbiome diversity and activity using metagenomic and metatranscriptomic approaches. The proposed objectives for future research are to observe the role that

different rumen microbes play in methanogenesis as well as enteric fermentation in the rumen environment. Additionally, we will compare gene expression profiles of the rumen microbiome to determine which molecular processes are affected in the presence and absence of macroalgae.

Chapter 1: Enteric methane production and the role of methane-reducing feed additives in ruminant livestock systems

1.1 Methane and its role in the greenhouse gas effect

The climate system is regulated and balanced through incoming solar energy and outgoing thermal radiation. About half of solar shortwave radiation produced is absorbed by the Earth's surface, whereas longwave radiation is mainly absorbed through atmospheric elements such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), water vapor, and various halocarbons (Cubasch et al., 2013). Gases that absorb longwave radiation are known as greenhouse gases (GHG). Increasing levels of GHG contribute to the greenhouse gas effect, which is defined as heat added to the lower levels of the Earth's atmosphere. Naturally occurring GHG are regulated through negative and positive feedback effects, which provide cooling and warming effects to the Earth's climate (Cubasch et al., 2013). However, human (anthropogenic) activity has created an imbalance of these climate balancing effects through increased emissions of CO_2, CH_4, and N_2O.

As mitigation strategies to reduce anthropogenic GHG emissions are underway, CH_4 mitigation is of particular interest due to its abundance and ability to promote short term solutions toward reducing the effect of climate change. Methane is a potent GHG that has the ability to trap heat in the atmosphere at approximately 86 times the rate of CO_2 on a 20-year scale (IPCC, 2007). Atmospheric CH_4 concentrations alone have increased 248% from pre-industrial values of 715 ppb to 1774 ppb in 2005 (IPCC, 2007). Additionally, CH_4 has a relatively short life span (~12 years) in the atmosphere and should

be evaluated as a “flow gas”, meaning that if CH_4 emissions are held constant over a 12-year period, approximately the same amount of CH_4 is degraded to CO_2 each year (Allen et al., 2018; Cain et al., 2019). The implications are if, at the very least, CH_4 emissions are kept constant it would not contribute to additional atmospheric warming and reduced emissions will result in a negative, or cooling, effect on the climate. Figure 1-1 shows the contribution of enteric CH_4 as a proportion of total anthropogenic CH_4 and total anthropogenic GHG emissions on global, US, and California scales, respectively. Total GHG emissions are estimated at 48,970 million metric tons (MMT) CO_2eq globally, 6,677 MMT CO_2eq in the U.S., and 425 MMT CO_2eq in California and total CH_4 emissions are estimated at 7,045 MMT CO_2eq globally, 635 MMT CO_2eq in the U.S. and 40 MMT CO_2eq. in California (IPCC, 2007; CARB, 2020; EPA, 2021). Ruminant livestock, such as cattle, sheep, and goats are a large contributor of CH_4 production due to the formation of enteric CH_4 during digestive fermentation of plant matter. Enteric CH_4 emissions are approximately 2,769 MMT CO_2eq. globally (Gerber et al., 2013), 178 MMT CO2eq. in the US (EPA, 2021), and 11 MMT CO_2eq. in California (CARB, 2020). Enteric CH_4 accounts for 39% of total CH_4 emissions globally, 28% of U.S. total CH_4, and 28% of California total CH_4. As a percentage of total GHG emissions, enteric CH_4 emissions accounts for 5.7% of total global GHG, 2.7% of U.S total GHG, and 2.6% of California GHG (Table 1-1).

1.2 CH_4 production in ruminants

The term ‘enteric CH_4’ refers to CH_4 produced by microbial fermentation related activities in the gastrointestinal tract of ruminant and non-ruminant animals. The vast majority of enteric CH_4 production is from ruminant livestock mainly in the rumen

compartment of the stomach. The rumen is a diverse and densely populated ecosystem, and it harbors a complex microbial community that is the main driver of feed degradation in ruminant livestock. Most microbial fermentation processes in the rumen are beneficial to the host animal by breaking down feed particles into energy dense substrates that are then taken up by the host animal. Ruminants obtain energy in the form of short-chain fatty acids (SCFA) produced by microbial fermentation and account for 50-70% of the animal's total energy intake (Millen et al., 2016). Substrates produced by microbial fermentation that are not taken up by the host animal can have negative effects on fermentation, such as reduced fiber degradation, if they are allowed to accumulate within the rumen (McAllister and Newbold, 2008; Janssen, 2010). Carbon dioxide and hydrogen are released as end products of feed degradation; however, the host animal is unable to utilize such substrates. Symbiotic relationships between microbial populations in the rumen have evolved over time to reduce and remove such end products. Methanogenic archaea are able to utilize both CO_2 and hydrogen as well as other end products such as acetate, methanol, and methyl-amines all of which can accumulate in the rumen over time. Not all methanogens, however, are able to utilize all the substrates mentioned and are typically classified based on their specific substrate utilization pathway; hydrogenotrophic (CO_2 reduction), acetoclastic (acetate reduction), and methylotrophic (methanol and methyl amine reduction) (Costa and Leigh, 2014; Buan, 2018). Hydrogenotrophic methanogens have been identified as the core methanogenic community in the rumen with *Methanobrevibacter ruminantium* and *Methanobrevibacter gotschalkii* making up approximately 74% of total abundance (Henderson et al., 2015). Methanogens serve an important role in the rumen, with CH_4 production accounting for

animal energy losses between 6 – 12% (Johnson and Johnson, 1995; Beauchemin, 2009; Hristov et al., 2013b; Moraes et al., 2014), thus strategies to mitigate enteric CH_4 emissions could not only decrease ruminant livestock's contribution to atmospheric warming but also could contribute to increased energy availability to the animal. It is therefore not surprising that there is significant interest from the agricultural industry and policymakers to develop strategies to reduce enteric CH_4 emissions from ruminant livestock. This chapter focuses on feed additives that have been shown to significantly reduce enteric CH_4 and are either currently available or are in the process of becoming available in the near future.

1.3 Feed additives to reduce enteric CH_4 emissions

1.3.1. Dietary lipids

Dietary lipids are commonly used feed additives for cattle to increase energy density of the diet and improve overall animal productivity. However, at levels over 6% fat inclusion, reductions in dry matter intake (DMI) tend to reduce productivity. Impacts on methanogenesis through dietary lipid supplementation occur through direct suppression of methanogens and their symbionts such as protozoa, inhibition of ruminal organic matter fermentation, and through redirecting hydrogen utilization through biohydrogenation and propionate production (Johnson and Johnson, 1995). Several meta-analyses and review papers have been published on the efficacy of dietary lipids on reducing enteric CH_4 production and brief summaries of these results are presented here and in Table 1-1. Beauchemin et al. (2007) reviewed 17 studies that utilized dietary lipids to reduce enteric CH_4 emissions and concluded that a dietary fat increase of 1%

decreases CH_4 emissions in the range of 5-6%, depending on the form, source, and fatty acid composition of the supplementation. Medium-chain fatty acids sourced from products such as coconut oil, myristic oil, canola oil, and palm kernel oil have been shown to be some of the most effective lipids that reduce enteric CH_4 (Machmüller and Kreuzer, 1999; Machmüller et al., 2003; Rasmussen and Harrison, 2011). Meta-analyses from Grainger et al. (2011), a review of 27 studies, and (Patra, 2014), a review of 10 studies, showed that at 10 g/kg DMI inclusion of medium chain and long chain fatty acids reduced CH_4 production by approximately 1- 5%. Arndt et al. (2021) reviewed over 65 dietary oil studies and concluded that oils sourced from coconut, canola, sunflower, and linseed as well as oilseeds such as sunflower seeds, cottonseed, linseed, and canola seed were the main dietary fat supplements with the highest potential for CH_4 reduction.

1.3.2 Ionophores

Carboxylic ionophores are commonly applied to livestock feed to improve overall health and feed efficiency of animals. This subclass of ionophores work by forming ion shuttling complexes through hydrophobic lipid-bilayers of microorganisms (Novilla et al., 2017). To date (August 2021) there are seven ionophores on the market that were approved by the FDA. Monensin is the most common of these ionophores in cattle diets and it has been shown to increase feed efficiency. It has been propose that monensin shifts microbial populations away from gram-positive bacteria, which produce hydrogen as a by-product, thus allowing for more colonization of gram-negative bacteria, which are the predominant producers of propionate in the rumen (McGuffey et al., 2001). With the reduction of hydrogen and increase in propionate production, monensin has been

identified to be a potential feed additive to reduce CH_4 production in the rumen (Beauchemin et al., 2008). In a meta-analysis, across 13 studies, conducted by (Appuhamy et al., 2013), monensin reduced CH_4 production between 12-14% at an inclusion level of 21-32 mg monensin per kg DMI. However, actual efficacy of monensin on CH_4 reduction have been variable between individual studies and there is some indication that rumen microbial populations can adapt to monensin supplementation after 4 weeks (Guan et al., 2006).

1.3.3 Synthetic Inhibitors

1.3.3.1 Nitrooxypropanol

Nitrooxypropanol (3-NOP) is a synthetic compound that was developed to target and inhibit specifically methyl-coenzyme M reductase (MCR); an enzyme that catalyzes the last step in methanogenesis in all of the four known CH_4 producing pathways (Thauer, 2019). 3-NOP diffuses easily through cell membranes where it then directly targets MCR's nickel-active binding site, $cofactor_{430}$, where the nickel is oxidized from Ni^{+} to Ni^{2+}, thus effectively inactivating MCR (Duin et al., 2016). 3-NOP has been tested extensively *in vivo* and almost all studies showed its potential to reduce enteric CH_4 emissions. A meta-analysis conducted by (Dijkstra et al., 2018) reviewed a total of 11 studies using 3-NOP and found it reduced CH_4 production by 22 – 39% at an inclusion level of 123 mg 3-NOP per kg DMI. It has recently been reported that dietary fiber levels, more specifically neutral detergent fiber (NDF), have a negative impact on the efficacy of 3-NOP to reduce enteric CH_4 (Vyas et al., 2018). This suggests that diets containing high dietary NDF levels will need to be supplemented with more 3-NOP (g/kg DM) compared to a diet with lower NDF

levels to achieve similar reductions in enteric CH_4. One theory that may explain this observation is that methanogens are more active when a more fibrous diet is fed, due to the increase in CO_2 and H_2 substrate availability, which increases the levels of the MCR enzyme needed for the last step in methanogenesis (Vyas et al., 2018). Because 3-NOP specifically targets the MCR enzyme, any increases in MCR concentration levels in the rumen will also require increased 3-NOP supplementation. Additionally, Hristov et al. (2015) tested the persistence of 3-NOP over time and found no adaptation to this compound over a 12-week period, which suggests that this may be a viable long-term solution to reduce enteric CH_4 emissions. To date, there are no known adverse health effects of 3-NOP for ruminants and is currently awaiting regulatory approval for use as a livestock feed additive.

1.3.3.2 Synthetic halogenated compounds

Halogen compounds, such as bromide, iodide, and chloride, often form one carbon structures that are similar to CH_4 and are therefore considered to be CH_4 analogues. Halogenated CH_4 analogues (HMA), especially brominated- and chlorinated- analogues such as bromoform (Lanigan, 1972), bromochloromethane (BCM) (Tomkins et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012), and chloroform (Knight et al., 2011; Martinez-Fernandez et al., 2016), have been shown to significantly reduce enteric CH_4 formation. HMAs bind and sequester the prosthetic group required by MCR, similar to 3-NOP, thus effectively inactivating methanogenesis (Smith et al., 1962; Wood et al., 1968; Johnson et al., 1972). While halogenated CH_4 analogues seem to be effective at reducing total CH_4 production, it appears that the amount of CH_4 reduced is specific to the type of

halogenated CH_4 analogue used. Ungerfeld et al. (2004) suggested that methanogenic species may be differentially sensitive to these halogenated CH_4 analogues, thus these HMAs may not only suppress MCR but inhibit multiple reactions within the diverse methanogenesis pathways (Bauchop, 1967). Lanigan (1972) tested the effectiveness of pure bromoform in fistulated sheep and found that inclusion rates of only 0.03 g/day/animal was sufficient for near complete CH_4 reductions. BCM has been tested in ruminants and found to have a 93% efficiency at reducing CH_4 production in beef cattle at an inclusion rate of 0.30 (g BCM/ kg DMI) with no negative impacts on feed intake, weight gain, feed efficiency or carcass quality (Tomkins et al., 2009). Additionally, Abecia et al. (2012) found that an inclusion rate of 0.13 g BCM per kg DMI in lactating dairy goat diets resulted in 33% reduction of CH_4 as well as a 36% increase in milk production. Long-term efficacy of synthetic CH_4 analogues in the rumen remains to be confirmed. For example, Tomkins et al. (2009) report over 93% CH_4 reduction over 28 days, however, CH_4 production increased over the 90-day experimental period thus, when averaged over time, the overall decrease in CH_4 was only 57%. Chloroform similarly is effective at reducing enteric CH_4 production through reduced abundance and activity of methanogenic archaea in fistulated cattle, but only over a 42-day period (Knight et al., 2011).

1.3.4 Alternate Hydrogen sinks

1.3.4.1 Nitro Compounds

Nitro compounds, mainly nitrate (NO_3^-), are highly competitive hydrogen (H^+) acceptors that are thermodynamically favorable compared to methanogenesis. Briefly,

nitrates are first reduced to nitrite, then to ammonia which yields approximately 86% more Gibbs free energy (-125.5 kJ/mol H_2) than the reduction of CO_2 to CH_4 (-16.9 kJ/mol H_2) (Ungerfeld and Kohn, 2006). If fed enough nitrates, this pathway could shift the primary route of H_2 removal away from methanogenesis and may also provide additional ammonia to be fermented and used for microbial protein synthesis (Dijkstra et al., 1998). There have been a few reviews (Lee and Beauchemin, 2014; van Gastelen et al., 2019) that suggest nitrate is a viable feed additive to reduce enteric CH_4 emissions in ruminants. A recent meta-analysis showed that nitrates can reduce CH_4 production 10 – 20% at inclusion rates of 16.7 mg nitrate per kg DMI (Feng et al., 2020). However, the risks of feeding nitrate might outweigh the benefits of reducing CH_4. When nitrates are reduced to nitrites, the animal may absorb some of the nitrites within the rumen, which has the capability of converting blood hemoglobin into methemoglobin which then renders the cell incapable of transporting oxygen to the animal's tissues (Morris et al., 1958). This can be combatted through slow nitrate introduction into the animal's feed thus allowing for rumen adaptation to excess nitrites. However, this will be a limiting factor of nitrate supplementation on-farm as a tight regulation of the nitrate update by individual animals is not practical.

1.3.5 Plant Bioactive Compounds

1.3.5.1 Macroalgae (seaweed)

Macroalgae, particularly members belonging to the genus *Asparagopsis,* have shown great potential to reduce enteric CH_4 production. The reduction of enteric methanogenesis has been linked to seaweed's ability to synthesize and encapsulate

HMA within their cell wall structures (Paul et al., 2006b; Machado et al., 2016a). *Asparagopsis taxiformis*, has been shown to be one of the most promising species in reducing enteric CH_4 production *in vitro* (Machado et al., 2014). Although comprehensive *in vivo* trials are currently still limited, there is evidence that *Asparagopsis* reduced enteric CH_4 production by up to 80% in sheep fed 53.9 g / kg DMI (Li et al., 2018), 67% in dairy cows fed 18.4 g/kg DMI cows (Roque et al., 2019b), 82% in growing beef steers fed 9.1 g/kg DMI (Roque et al., 2021), and up to 98% in finishing stage beef steers fed 3.7 g/kg DMI (Kinley et al., 2020). Shelf life of *Asparagopsis* may be of concern depending on storage conditions, however different studies have conflicting results on shelf life and how it relates to CH_4 reduction. Stefenoni et al. (2021) found as much as 80% reductions in CH_4 production in lactating dairy cows fed 5 g/ kg DMI (0.5% of total DMI) during the initial inclusion of *Asparagopsis* into the diet, however changes in efficacy were observed in the different experimental periods of their Latin-square design. For example, average CH_4 reductions for cows fed 0.5% DM *Asparagopsis* was 65% for period 1 and 55% for period two, and the last two periods had no differences in CH_4 production when compared to control. The authors contributed this to a degradation of HMA within the seaweed during storage. In contrast, a study conducted over 21-weeks concluded no degradation in HMA concentrations as well as persistent CH_4 inhibition with increased feed efficiency in beef cattle (Roque et al., 2021). Safety concerns surrounding HMA, mainly bromoform, residues have briefly been explored in the aforementioned studies with no known bromoform residues found in meat, milk, fat, kidney, liver, or feces. Additional concerns include mineral residues such as iodine, particularly in milk (Stefenoni et al., 2021), and needs to be addressed before supplementation can happen large scale.

1.3.5.2 Tannins

Tannins are secondary plant metabolites, categorized into either hydrolysable or condensed tannins, which are distinct from other metabolites in their ability to form complexes and bind to proteins Hagerman (2012). Tannins are known to have beneficial effects in ruminant livestock such as increased post-rumen protein that is directly available to the animal for absorption (Patra and Saxena, 2011). However, tannins can also have detrimental effects to essential fermentative microbial populations in the rumen, depending on the type and concentration of tannins applied (Makkar, 2003; Lorenz et al., 2014). Condensed tannins in particular have been shown to have an inhibitory effect on CH_4 production in the rumen, although their mode of action is not yet clear. Naumann et al. (2017) suggested three main hypotheses: 1) condensed tannins work by binding to substrates in the rumen thus decreasing the degradation of these products into substrates needed by methanogens to produce CH_4; 2) condensed tannins directly inhibit rumen protozoal populations, thus interrupting an important pathway of interspecies hydrogen transfer between protozoa and methanogens in the rumen, and 3) condensed tannins may also act as a competitive hydrogen sink to redirect H_2 away from methanogenesis. It is likely that a combination of these three hypothesized mechanisms work synergistically to reduce overall enteric CH_4 production. Animut et al. (2008) found that inclusion rates between 50-150 g of condensed tannins per kg DMI resulted in CH_4 reductions between 32-54%. Additionally, a meta-analysis reviewing the effects of various types of tannins on CH_4 production indicates that while there are differences in the

biological activity of the various types of tannins, they all share their methane reducing ability (Jayanegara et al., 2012).

1.3.5.3 Saponins

Saponins are natural detergent compounds and are defined based on their structural components, categorized either as triterpene or steroid glycosides linked to one or more oligosaccharide sugar chains (Vincken et al., 2007). The hydrophobic nature of the steroid glycosides and hydrophilic nature of the attached oligosaccharides allow saponins to bind to surrounding compounds and microorganisms which causes foaming or emulsification (Shi et al., 2004). In some cases, this can be beneficial. For example, saponins are known to actively bind to cholesterol and prevent oxidation in the colon of humans, thus creating a positive antioxidant property (Reshef et al., 1976). However, such powerful binding effects can also reduce enzymatic activity in the digestive tract of monogastric animals and result in reducing weight gain of the animal when fed in excess (Rao and Sung, 1995). Saponins are thought to specifically target protozoal populations in the rumen by binding to sterols located within their cell membranes (Cheeke, 2000; Goel and Makkar, 2012). Rumen methanogens are known to form symbiotic relationships with rumen protozoa, mainly through interspecies hydrogen transfer, which explains why saponins suppress CH_4 emissions. Many edible legume plants contain saponins, however concentrations depend on age, type, and specific portions of plant material (Shi et al., 2004). Sources of saponins for enteric CH_4 reduction are tea [8-27% CH_4 reductions] (Yuan et al., 2007; Mao et al., 2010; Zhou et al., 2011), yucca extract [4-15% CH_4 reduction] (Lila et al., 2005; Wang et al., 2009), fruits [4-13% CH_4 reduction] (Hess

et al., 2004) or quillaja extract [3-8% CH_4 reduction] (Holtshausen et al., 2009). There appears to be a general CH_4 reduction effect with increasing levels of feed additives rich in saponins, however potential of saponins is highly variable probably due to the individual variability of saponin types, plant types, and maturity of plant (Jayanegara et al., 2014).

1.3.5.4 Essential Oils and Blends

Essential oils (EOs) are considered plant secondary metabolites and are typically volatile, aromatic compounds with a wide range in chemical structure and activity. The major EOs are organized into three categories: terpenes, phenylpropenes, and organosulfur compounds. Terpenes are further classified by the number of 5-carbon-based structures ranging from C_5-C_{40} (Benchaar et al., 2007) and are typically found in higher concentrations than phenylpropanoids and organosulfur compounds in plant material. More specifically, monoterpenes such as limonene, citral, menthone, thymol, carvacrol, and geranyl acetate can represent up to 90% of total EO concentrations (Cobellis et al., 2016), but may contain less antimicrobial activities when compared to phenylpropenes such as eugenol, menthol, cinnamaldehyde, and vanillin or when compared to organosulfur compounds such as allicin or its hydrolyzed compounds such as diallyl sulfide, diallyl di-sulfide, or diallyl tri-sulfide. Anti-microbial activity of monoterpenes is suggested to directly break down the lipid bilayer of microbial cytoplasmic membranes causing inhibition of growth and energy metabolism as well as cell death (Benchaar and Greathead, 2011), a mechanism of action similar to ionophores. This activity may be applied to methanogens themselves or would produce a downstream effect on rumen methanogens through targeting microorganisms that provide substrates

to methanogens. Metabolites containing oxygen (phenylpropenes) and sulfur (organosulfides) have been found to show stronger affinity toward antimicrobial activity when compared to terpenes (Burt, 2004). It has been suggested that this higher antimicrobial activity comes from disruption of cytoplasmic ion transport and inactivation of microbial enzymes (Benchaar and Greathead, 2011). Essential oils extracted from plant material typically are not isolated into their individual components but are usually testing in their complex form for their effect on the ruminant animal, additionally there may be additive effects of feeding terpenes, phenylpropenes, and organosulfur compounds together as one additive. Here an overview of individual EOs and EO blends, organized by extraction source or production company, is provided:

Garlic Essential Oil

Garlic essential oil has shown great promise in vitro, with CH_4 reductions as high as 91% (Soliva et al., 2011). However, there is little evidence *in vivo* to corroborate these findings. For example, five studies were reviewed testing multiple garlic-based compounds as well as isolated diallyl disulfide and allicin products and none were found effective *in vivo.* Extracted diallyl disulfide was fed to lactating dairy cows at 0.056 g/ kg DMI and at 0.20 g / k DMI with no effects found on CH_4 production (van Zijderveld et al., 2011a). Extracted allicin from dried garlic bulbs was fed to bulls at inclusion rates of 12 g / kg DMI with no change in CH_4 production (Staerfl et al., 2012). Additionally, (Meale et al., 2014) fed lactating dairy cows, in coordination with Yang et al. (2007), at 0.24 g garlic oil / kg DMI and allicin concentrations of 0.002 g / kg DMI with no change in CH_4

production. Patra et al. (2011) fed garlic bulbs to sheep at 3.89 g / kg DMI and total EO content of 0.19 g / kg DMI with no changes in CH_4 production. Klevenhusen et al. (2011) fed garlic EO to sheep at concentrations of 4.85 g / kg DMI and diallyl disulfide content of 1.94 g/ kg DMI, again with no changes in CH_4 production. While in-vitro experiments do reveal promising results, the corresponding *in vivo* studies have not shown and reductions in CH_4 production to date. The lack of evidence surrounding CH_4 reductions in vivo could be due to low inclusion levels of garlic and garlic-compounds in the animals' diet and higher inclusion rates might be more advantageous. However, caution should be taken on how much garlic is fed to dairy cows specifically since some of these compounds may affect the flavor, color, odor, and texture of dairy products at garlic bulb inclusion rates of 17.3 g / kg DMI (Rossi et al., 2018). Additionally, the authors did conclude that similar diallyl disulfide concentrations as the previous 17.3 g/kg DMI garlic bulbs were added to a separate treatment group [0.08 g diallyl disulfide per kg DMI] with no effect on dairy products, thus perhaps active anti-methanogenic compounds may need to be further extracted. A few studies have noted reductions in CH_4 output when scaled to different factors that total production (g/day) and yield (g CH_4 / kg DMI). For instance, Klevenhusen et al. (2011) reported reductions in CH_4 when standardized by digested NDF intake when diallyl disulfide was added to the diets of sheep however no differences in total CH_4 output or yield were found.

Oregano Essential Oil

Oregano oil effects on methanogenesis are extremely variable and seems to depend on concentration of the active ingredient carvacol within the plant. (Hristov et al., 2013a) found up to 35% reductions in CH_4 production when feeding oregano leaves at 18.18 g / kg DMI with carvacol concentrations at 0.21 g / kg DMI. Additionally, Tekippe et al. (2011) found that supplementation of oregano leaves at 19.2 g per kg DMI with a carvacrol content of 0.27 g / kg DMI resulted in a 39% decrease in CH_4 production. However, no effects on CH_4 production and small reductions in CH_4 yield were found *in vivo* when feeding 0.57 g / kg DMI oregano extract with a carvacrol content of 0.456 g / kg DMI in lactating dairy cows (Kolling et al., 2018). Olijhoek et al. (2019) fed two types of oregano leaves, high and low EO concentrations, and found no effects on CH_4 emissions with low EO containing oregano leaves fed at 17.8 – 53.3 g / kg DMI with carvacrol concentrations of 0.006 – 0.019 g / kg DMI and with high EO containing oregano leaves fed at 7 – 20.9 g / kg DMI with carvacrol concentrations between 0.10 – 0.31 g / kg DMI. Outcomes of the reported data indicate that more in vivo studies need to be done, particularly with oregano leaves, with high essential oil dosages over 0.30 g / kg DMI.

Essential Oil Blends

Agolin® Ruminant is a commercially available product that is historically used to improve rumen fermentation parameters in dairy cattle. Agolin® Ruminant is a proprietary blend of EOs including extract from coriander seed including eugenol, geranyl acetate, geraniol, and fumaric acid. A meta-analysis conducted on 23 studies using Agolin®

Ruminant at a dose of 0.05 g/kg DMI (1 g/ day) showed a 8.8-10% reduction in CH_4 production (g/day) and 12.9% reduction in CH_4 yield (g CH_4 / kg DMI) with an average 4% increase in overall milk yield during over 4-8 weeks of feeding (Belanche et al., 2020). However, there seems to be some uncertainty around Agolin® Ruminant as a CH_4 mitigating feed additive. For instance, Carrazco et al. (2020) found a numerical reduction in CH_4 emissions of about 6% but the estimate is not statistically significant. The authors do report an 11% difference in CH_4 intensity (g CH_4 / kg energy corrected milk). More *in vivo* studies should be conducted to resolve the variability between these two projects however, Agolin® is readily commercially available for use which makes it a promising feed additive to reduce enteric CH_4.

Mootral™ combines the CH_4 mitigating potential of organosulfur compounds in garlic with polyphenols from bitter orange citrus to produce an additive reduction in enteric CH_4 emissions. Mootral™ was first used *in vitro* with almost complete reductions in CH_4 production along with a direct reduction in methanogen populations (Eger et al., 2018). Limited studies have been conducted *in vivo* but results suggest Mootral to be a promising feed additive for CH_4 reduction. For instance, Vrancken et al. (2019) conducted a commercial farm study with lactating Jersey and Holstein cows fed 1.13 g / kg DMI and 0.67 g/ kg DMI (15 g/day), respectively, and found 38% and 20% reductions in CH_4 production. Mootral™ given to feedlot steers at 15 g/day [1.58 g / kg DMI] resulted in CH_4 reductions of approximately 23% over a 15-week period (Roque et al., 2019c). It was noted that reductions in this study seemed to compound over time and this may be due to additive effects of feeding Mootral™ daily or an adjustment of eating more of the offered

supplement since these animals were fed ad libitum. Most recently, Mootral™ was fed to young bull calves about 5 months in age at concentrations of 1.37 g / kg DMI which resulted in a 22% reduction in CH_4 production and 24% reduction in CH_4 yield (g CH_4 / kg DMI) (Brand et al., 2021).

1.3.6 Other compounds

1.3.6.1 Biochar

Pyrolyzed carbon compounds such as biochar have been shown to reduce CH_4 emissions in rice paddies (Feng et al., 2012) as well as during in vitro rumen fermentation (Saleem et al., 2018). Only a limited number of studies have been conducted *in vivo* and will be explained here. Reductions in enteric CH_4 have been found in two of the four total studies, the first was conducted by Leng et al. (2012) in cattle using a biochar, specifically made from rice husks, at inclusion rates of 6 g / kg DMI and found a 22% reduction in CH_4 production. The second study tested the effects of biochar, made from an unspecified type of pine tree, in growing and finishing beef steers and found reductions up to 10% for CH_4 production and 18.4% CH_4 yield (g CH_4 / g DMI) (Winders et al., 2019). While these two studies seem promising, two other studies have also shown no effects of biochar in reducing enteric CH_4. Terry et al. (2020) fed biochar made from southern yellow pine tree at levels of 5, 10, and 20 g / kg DMI with no significant changes in enteric CH_4 emissions. Additionally, in a study feeding 4.7 g / kg DMI of unspecified biochar to goats found no effects on enteric CH_4 production (Silivong and Preston, 2015). More information is needed about the potential differences in biochar types depending on their source, e.g. pine versus rice husks, as well as their overall carbon content. For example, Winders et

al. (2019) used biochar with 85% carbon content whereas Terry et al. (2020) biochar only contained 75% carbon. It could be possible that higher carbon content could induce a higher CH_4-mitigating effect.

1.4 Tables and Figures

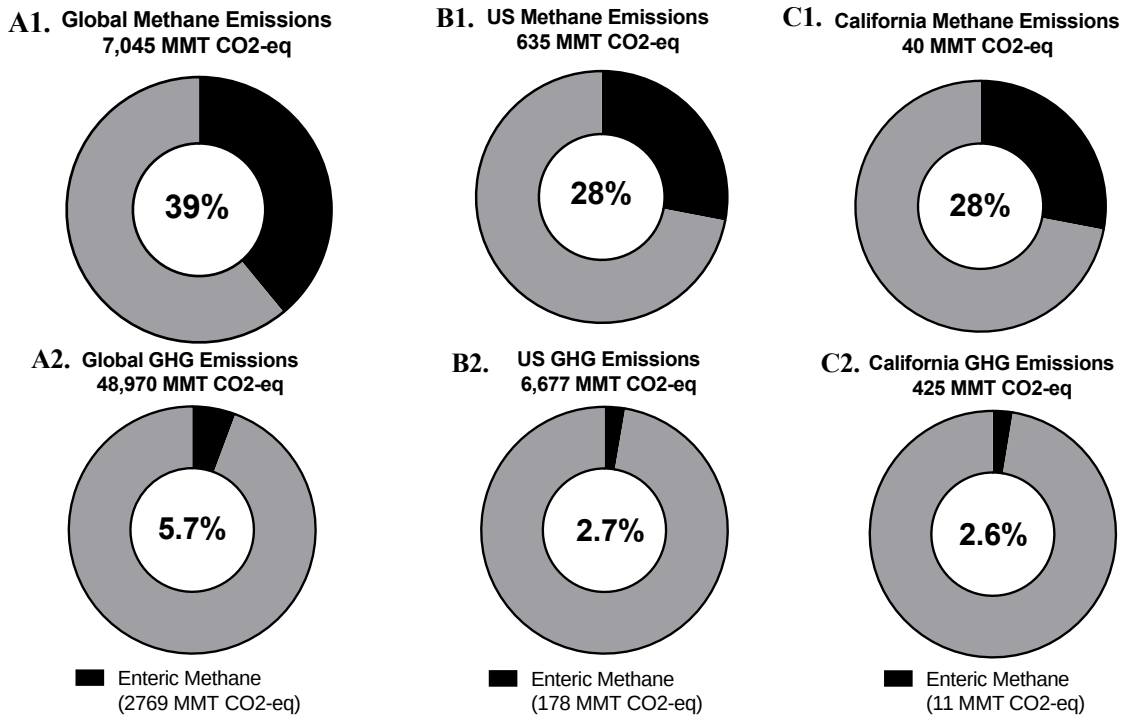


Figure 1-1. Proportion of enteric methane emissions compared to global, US, and California total emissions. Fig 1A: Contribution of enteric methane emissions on total anthropogenic methane emissions, global greenhouse gas (GHG) emissions (**Fig 1B**), total anthropogenic United States (US) methane emissions (B1), US greenhouse gas (GHG) emissions (B2), total anthropogenic California methane emissions (C1) and California GHG emissions (C2). Global emissions data (A1 & A2) was based on Gerber (2013), US emissions data was based on US EPA (2021), and CA emissions data was based on CARB (2020).

Table 1-1. Summary of peer reviewed research conducted with various feed additives and their potential for methane mitigation.

Category	Methane Reduction*	Dosage (g/kg DMI)**	No. studies	Source	Approved	Availability
Dietary Lipids	**Medium Potential**	**High Dosage Levels**				
Oils	19%	NA	50	Ardnt 2021(Review)	Yes	Commercially Available
Coconut Oil	25%	38.44 (13.6 - 71.0)	4	Jordan 2006a, Jordan 2006b, Lovett 2013, Machmuller 2000		
Canola Oil	13%	154 (46 - 309)	4	Beauchemin & McGinn 2006, Pinares-Patino 2016, Gidlund 2015, Moate 2011		
Linseed Oil	27%	687 (67 - 956)	3	Machmuller 2000, Martin 2008, Benchaar 2015		
Sunflower Oil	18%	278 (34 - 522)	2	Beauchemin 2007, McGinn 2004		
Oilseeds						
Sunflower Seed	39%	123	2	Ardnt 2021(Review)		
Cottonseed	15%	166 (150 - 182)	2	Grainger 2008, 2010		
Linseed	24.96	18.55	10	Ardnt 2021(Review)		
Canola Seed	13%	9	3	Ardnt 2021(Review)		
Ionophores	**Low Potential**	**Low Dosage**				
Monensin**	2-14%	0.021 - 0.032	13	Appuhamy 2013(Meta-analysis)	Yes	Commercially Available
Synthetic Inhibitors	**High Potential**	**Low Dosage**				
3-NOP	22 - 39%	0.123 (0.027 - 0.345)	11	Dijkstra 2018 (Meta-analysis)	No	Clinical Trials Underway
Bromoform	100%	0.03	1	Lanigan 1972	No	Banned
BCM	45%	0.215	2	Tomkins 2009(results averaged), Abecia 2012	No	Banned
chloroform	94%	1.5 mL /day	1	Knight et al 2011	No	Banned
Alternative [H] sinks		**Medium Dosage Levels**				
Nitrates	10.1 - 20.4%	16.7 (6 - 27)	24	Feng 2020*		

Category	Methane Reduction*	Dosage (g/kg DMI)**	No. studies	Source	Approved	Availability
Plant Bioactive Compounds	**Variable**	**High - Medium Dosage Levels**		Patra 2010		
Macroalgae	67 - 99%	6.58 (0.93 - 18.4)		Roque 2019a, Kinley 2020, Roque 2021, Stefenoni 2021	No	Clinical Trials Needed
Tannins	32 - 54%	50 -150	30	Animut et al 2018, Jayanegara 2011	Yes	forages contain tannins
Saponins	10%	71 (4 - 250)	6	Lila et al 2005, Hess 2004, Wang 2009, Poornachandra 2019, Mao 2009, Zhou et al 2011, Holtshausen 2009	Yes	legume forages contain saponins
Essential Oils						
Oregano (carvacrol)	0 - 36%	0.006 - 0.456	5	Stefenoni 2021, Kolling 2018, Tikippe 2011, Olijhoek 2019, Hristov 2013	Yes	Commercially Available
Garlic	0%	6 (0.0056 - 15)	5	Van Zijderveld 2011, Staerfl 2012, Meale 2014, Patra 2011, Klevenhousen 2011	Yes	Commercially Available
EO blends						
Mootral	26%	1.19 (0.67 - 1.58)	3	Vrancken 2019, Roque 2019b, Brand 2021	No	Clinical Trials Needed
Agolin	10%	0.05 (1g/day)	24	Belance 2020* , Carrazco 2020	Yes	Commercially Available
Other						
Biochar	0 - 22%	12 (4.7 - 20)	4	Leng 2012, Winders 2019, Terry 2019, Silivong & Preston 2015	Yes?	Experimental Trials Underway

* Methane Reduction refers to percent reduction in total methane on a grams per day basis.
** Values with only one dosage number are based on meta-analyses that concluded a % methane reduction at the appropriate dosage given multiple peer-review studies. Values with ranges do not yet have meta-analyses, therefore one single dose is not appropriate. Instead ranges from lowest dose to highest dose with methane reductions are provided.

Chapter 2: Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage.

Roque et al. (2019a) Animal Microbiome

2.1 Abstract

Recent studies using batch-fermentation suggest that the red macroalgae *Asparagopsis taxiformis* has the potential to reduce CH_4 production from beef cattle by up to ~99% when added to Rhodes grass hay (Kinley et al., 2020); a common feed in the Australian beef industry. These experiments have shown significant reductions in CH_4 without compromising other fermentation parameters (i.e. volatile fatty acid production) with *A. taxiformis* organic matter (OM) inclusion rates of up to 5%. During work performed as part of this thesis, *A. taxiformis* was evaluated for its ability to reduce CH_4 production from dairy cattle fed a mixed ration widely utilized in California, the largest milk producing state in the US. Fermentation in a semi-continuous in-vitro rumen system suggests that *A. taxiformis* can reduce CH_4 production from enteric fermentation in dairy cattle by 95% when added at a 5% OM inclusion rate without any obvious negative impacts on volatile fatty acid production. High-throughput 16S ribosomal RNA (rRNA) gene amplicon sequencing showed that seaweed amendment effects rumen microbiome consistent with the Anna Karenina hypothesis, with increased β-diversity, over time scales of approximately 3 days. The relative abundance of methanogens in the fermentation vessels amended with *A. taxiformis* decreased significantly compared to control vessels, but this reduction in methanogen abundance was only significant when averaged over the course of the experiment. Significant reductions of CH_4 in the *A. taxiformis* amended vessels was measured already in the early stages of the experiment, suggesting that *A.*

taxiformis has an immediate effect on the metabolic functionality of rumen methanogens whereas its impact on microbiome assemblage, specifically methanogen abundance, is delayed. The CH_4 reducing effect of *A. taxiformis* during rumen fermentation makes this macroalgae a promising candidate as a biotic CH_4 mitigation strategy for dairy cattle. But its effect in-vivo (i.e. in dairy cattle) remains to be investigated in animal trials. Furthermore, to obtain a holistic understanding of the biochemistry responsible for the significant reduction of CH_4, gene expression profiles of the rumen microbiome and the host animal are warranted.

Keywords: 16S rRNA community profiling, *Asparagopsis taxiformis*, Feed supplementation, Greenhouse gas mitigation, In-vitro rumen fermentation, Macroalgae, Rumen microbiome

2.2 Introduction

Methane (CH_4) is a major greenhouse gas with a global warming potential 28-fold greater than that of carbon dioxide (CO_2) on a 100-year scale (Smith et al., 2014) and it accounts for approximately 11% of the greenhouse gas (GHG) emissions in the US (Myhre et al., 2013). Enteric fermentation from ruminant animals alone accounts for approximately 25% of the total CH_4 emissions in the US; representing the largest anthropogenic source of CH_4 (NASEM et al., 2018). Increasing emphasis on reducing GHG emissions from the livestock industry requires advanced methods for reducing and controlling CH_4 production. Identifying efficient strategies to lower enteric CH_4 production could result in a significantly reduced carbon footprint from animal production and provide

the cattle industry with a way to meet legislative requirements; calling for a reduction of CH_4 emission of ~40% by 2030.

The biological production of CH_4 in the rumen is the product of symbiotic relationships between fiber degrading bacteria, hydrogen (H_2) producing protozoa and methanogenic archaea(Henderson, 1980; Czerkawski, 1986). Besides being converted into CH_4, metabolic H_2 may also be incorporated into volatile fatty acids (VFA), such as acetate, propionate, and butyrate which are then used as energy by the ruminant animal. Theoretically, inhibiting methanogenesis could free molecular H_2 for use in pathways that produce metabolites (i.e. VFAs) that are more favorable to the host animal, thus creating potential for increased feed efficiency. Since production of enteric CH_4 can account for up to 12% of the total energy consumed by the animal (Beauchemin and McGinn, 2006; Hristov et al., 2013b) even a small reduction of CH_4 production and redirection of carbon molecules into more favorable compounds has the potential to result in significantly more economically and ecologically sustainable production practices in the ruminant industry.

Extensive research has been performed on the effectiveness of feed supplements to reduce enteric CH_4 emissions through inhibition of microbial methanogenesis within the rumen system (Patra et al., 2017). Results have been reported for a number of feed supplements including inhibitors, ionophores, electron receptors, plant bioactive compounds, dietary lipids, exogenous enzymes, and direct-fed microbials indicating reductions on CH_4 production (Gerber et al., 2013). While several of these compounds have been shown to inhibit ruminal methanogenesis, some have been shown to decrease VFA production (Machado et al., 2016b), which decreases overall nutrient availability to the animal, and is therefore a non-desirable side effect.

Algae are a stable component of the human diet in some cultures (Nanri et al., 2017) and have also been used as feed for agricultural products such as abalone (Bansemer et al., 2016) and shrimp (Elizondo-González et al., 2018). The ability of algae to promote well-being and health is mediated to a great extent by highly bioactive secondary metabolites (Yang et al., 2010; Abdul et al., 2016; Corona et al., 2016) that are synthesized by some algal species (Blunt et al., 2005). Additionally, some of the brown and red macroalgae have shown to inhibit microbial methanogenesis when tested in-vitro (Machado et al., 2014) and a similar response of the animal microbiome has been proposed. These findings suggest that macroalgae could promote higher growth rates and feed conversion efficiencies in ruminants (Hansen et al., 2003; Marín et al., 2009). Macroalgal supplementation shows great promise as a CH_4 mitigation strategy during enteric fermentation (Wang et al., 2008; Dubois et al., 2013; Machado et al., 2014; Machado et al., 2016b). Macroalgae feed supplementation may therefore be an effective strategy to simultaneously improve profitability and sustainability of cattle operations.

Various types of algae have antibacterial, antiviral, antioxidant, anti-inflammatory, and anti-carcinogenic properties (González del Val et al., 2001; Yuan and Walsh, 2006; Chandini et al., 2008; Kang et al., 2008). Most recently, macroalgae has been tested in-vitro and in-vivo to determine if there are anti-methanogenic properties within selected types of macroalgae. *Asparagopsis taxiformis*, a red macroalgae, seems to be the most effective species of macroalgae to reduce CH_4 production.

A recent study identified *Asparagopsis taxiformis*, as a highly efficient feed supplement for CH_4 mitigation during enteric fermentation (Machado et al., 2014). In this work, the effect of a large variety of macroalgal species including: freshwater, green, red,

and brown algae on CH_4 production during in-vitro incubation was compared. Results showed *A. taxiformis* amendment yielded the most significant reduction (~ 98.9%) of CH_4 production. Moreover, *A. taxiformis* supplementation at inclusion rates up to 5% organic matter (OM) revealed CH_4 reduction by 99% without significant negative impact on VFA profiles and OM digestibility, in-vitro (Machado et al., 2016b). Furthermore*, A. taxiformis* was determined to contain an abundance of anti-methanogenic compounds including: bromoform, dibromocholoromethane, bromochloroacetic acid, dibromoacetic acid, and dichloromethane (Machado et al., 2016a). Bromoform, a halomethane, is the most abundant anti-methanogenic compound found in *A. taxiformis*, and has been shown to inhibit enzymatic activities by binding to vitamin B12 (Wood et al., 1968); which chemically resembles coenzyme F430 a cofactor needed for methanogenesis (Allen et al., 2014). Additionally, it has been shown that *A. taxiformis* reduces CH_4 production during enteric fermentation more effectively than highly concentrated halogenated CH_4 analogs (Machado et al., 2018). It has been suggested that the increased efficiency of *A. taxiformis* may be due to multiple anti-methanogenic bioactives working synergistically (Machado et al., 2018). While it is clear that *A. taxiformis* contains anti-methanogenic compounds, actual concentrations of these compounds seem to vary and what causes these variations remain unclear.

In the work presented here, we studied the effect of *A. taxiformis* (5% OM inclusion rate) on the rumen microbiome assemblage and function during in-vitro fermentation over the duration of four days. A better understanding of how this macroalgae affects CH_4 production from dairy cows fed a diet commonly used in California should provide insight into the value of an *A. taxiformis*-based CH_4 mitigation strategy for the dairy industry in

California. Additionally, high-throughput 16S rRNA amplicon sequencing was used to provide new insights of the effects of *A. taxiformis* supplementation on the rumen microbiome assemblage. To our knowledge this is the first time that this highly efficient procedure was employed to dissect the changes of the rumen microbiome in dairy cattle in response to *A. taxiformis* as a feed supplement and CH_4 mitigator.

2.3 Materials and Methods

2.3.1 Animals, diets and rumen content collection

All animal procedures were performed in accordance with the Institution of Animal Care and Use Committee (IACUC) at University of California, Davis under protocol number 19263. Rumen content was collected from two rumen fistulated cows, one Jersey and one Holstein, housed at the UC Davis Dairy Unit. Animals were fed a dry cow total mixed ration (50% wheat hay, 25% alfalfa hay/manger cleanings, 21.4% almond hulls, and 3.6% mineral pellet (Table 2-1). Three liters of rumen fluid and 60 g of rumen solids were collected 90 min after morning feeding. Rumen content was collected via transphonation using a perforated PVC pipe, 500 mL syringe, and Tygon tubing (Saint-Gobain North America, PA, USA). Fluid was strained through a colander and 4 layers of cheesecloth into two 4 L pre-warmed, vacuum insulated containers and transported to the laboratory.

2.3.2 In-vitro feed and feed additive composition and collection

Due to its wide utilization in the dairy industry for cows during lactation, super basic ration (SBR) was used as feed in the in-vitro experiment. SBR was composed of 70%

alfalfa pellets, 15% rolled corn, and 15% dried distillers' grains (Table 2-1). Individual components were dried at 55 °C for 72 h, ground through a 2 mm Wiley Mill (Thomas Scientific, Swedesboro, NJ) and manually mixed. *Asparagopsis taxiformis* used as feed additive was provided in kind from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Australia. The macroalgae was in its filamentous gametophyte phase when collected near Humpy Island, Keppel Bay, QLD (23o13'01"S, 150o54'01"E) by MACRO (Center for Macroalgal Resources and Biotechnology) of James Cook University (JCU) in Townsville, QLD. The collected biomass was frozen and stored at −15 °C then shipped to Forager Food Co. in Red Hills, Tasmania, AUS, where it was freeze dried and milled (2–3 mm) to ensure a uniform product. Chemical composition of SBR and of *A. taxiformis* were analyzed at Cumberland Analytical Services (Waynesboro, PA) (Table 2-2).

2.3.3 Engineered (in-vitro) rumen system

An advanced semi-continuous fermentation system, with six 1L vessels with peristaltic agitation, based on the rumen simulation technique (RUSITEC) developed by Czerkawski and Breckenridge (Czerkawski and Breckenridge, 1977) was used to simulate the rumen in the laboratory.

2.3.4 Experimental design

Equilibration (Day 0): Temperature, pH and conductivity of the rumen fluid and solids were recorded using a mobile probe (Extech Instruments, Nashua, NH). Rumen fluid, 3 L, from each cow were combined with 2L of artificial saliva buffer (Oeztuerk et al.,

2005) homogenized and then split into two 3 L aliquots. Rumen solids, 15 g, from each animal were sealed in Ankom concentration bags (Ankom, Macedon, NY) and added to each equilibration vessel (30g of rumen solids per vessel total). Three concentrate bags containing 10 g of SBR each were added to each vessel. One of the equilibration vessels was amended with 5% (w/w) of *A. taxiformis* 24 h prior to the start of the experiment (Figure 2-1). Content of the equilibration vessel without *A. taxiformis* was used to inoculate control vessels of the in-vitro system, whereas content of the equilibration vessel with *A. taxiformis* was used to inoculate the treatment vessels (Figure 2-1). SBR was ground in a 2 mm Wiley Mill before being added to each concentrate bag to increase substrate availability and therefore producing similar particle sizes that which the mastication function in-vivo provides to the animal. The two vessels were then placed in a 39 °C water bath and stirred with a magnetic stir bar for a 24 h equilibration period.

Fermentation (Days 1–4): After 24 h of equilibration, temperature, pH, and conductivity of the rumen fluid were recorded to determine stability of the vessels and their content. Each of the 6 in-vitro rumen vessels were randomly designated as either treatment or control vessel and filled with 750 mL of the corresponding fluid from the equilibration vessels. Location of the vessels within the in-vitro platform were randomly allocated. Each vessel received one concentrate bag of SBR from its respective equilibration vessel and one new concentrate bag. Control concentrate bags contained 10 g SBR. Treatment concentrate bags contained 10 g SBR plus 5% (OM) *A. taxiformis*. To simulate rumen retention time, each of the feedbags were incubated in the allocated fermentation vessel for 48h. Temperature, pH, and conductivity were measured every 24 h prior to exchanging one of the concentrate bags (feeding). After each feeding, all vessels were flushed with

N2 to maintain anaerobic conditions within the reactors. Individual reactor vessels of the artificial rumen system were connected to a reservoir containing artificial saliva buffer. A peristaltic pump delivered 0.39mL/min of buffer to each vessel throughout the course of the experiment. Gas bags (Restek, USA) and overflow vessel were used to continuously collect generated gas and effluent fluid. Effluent vessels were chilled with ice to mitigate residual microbial activity. An outline of the experimental set-up and the preparation of the treatment and control vessels is provided in Figure 2-1.

2.3.5 Sample collection and analysis

Liquid and gas sample collections took place at 3 time points every 24 h for 4 days. Time point intervals were 4, 12, and 24h post-feeding each day. Fluid samples were collected in 1.5 mL tubes, flash frozen in liquid nitrogen, and stored at − 20 °C until processed. Gas bags were collected at each time series interval for analysis of total gas production, CO_2 and CH_4 concentrations. Gas volume was measured with a milligas flow meter (Ritter, Germany) by manual expulsion of the collection bag.

2.3.6 Volatile fatty acid and greenhouse gas analysis

To determine VFA profiles, Gas Chromatography-Flame Ionization detection (GC-FID) was used. Fermentation fluid was prepared for VFA analysis by mixing with 1/5th volume 25% metaphosphoric acid, and centrifugation. Supernatant was filtered through a 0.22 µm filter and stored in amber autosampler vials at 4 °C until analysis. The GC conditions were as follows: analytical column RESTEK Rxi® – 5 ms (30 m × 0.25 mm I.D. × 0.25 µm) film thickness; the oven temperature was set to 80 °C for 0.50 min, and

followed by a 20 °C/min ramp rate until 200 °C, holding the final temperature for 2min; carrier gas was high purity helium at a flow rate of 2.0 mL/min, and the FID was held at 250 °C. A 1 µL sample was injected through Split/Splitless Injectors (SSL), with an injector base temperature set at 250 °C. Split flow and split ratio were programmed at 200 and 100 mL/min respectively. To develop calibration curves, certified reference standards (RESTEK, Bellefonte, PA) were used. All analyses were performed using a Thermo TriPlus Autosampler and Thermo Trace GC Ultra (Thermo Electron Corporation, Rodano Milan, Italy).

CH_4 and CO_2 were measured using an SRI Gas Chromatograph (8610C, SRI, Torrance, CA) fitted with a 3'×1/8″ stainless steel Haysep D column and a flame ionization detector with methanizer (FID-met). The oven temperature was held at 90 °C for 5 min. Carrier gas was high purity hydrogen at a flow rate of 30 ml/min. The FID was held at 300 °C. A 1 mL sample was injected directly onto the column. Calibration curves were developed with an Airgas certified CH_4 and CO_2 standard (Airgas, USA).

2.3.7 DNA extraction

DNA extraction was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) with ~500 mg of sample according to the manufacturer's protocol. DNA was subsequently purified with a Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA) following the manufacturer's instructions. Extracted DNA was stored at −20 °C until subsequent PCR amplification and amplicon sequencing.

2.3.8 PCR amplification, library preparation, and sequencing

The V4-V5 hypervariable region of the 16S rRNA gene was sequenced on Illumina's MiSeq platform using the 515yF (3′-GTG YCA GCM GCC GCG GTA A-5′) and 926pfR (3'-CCG YCA ATT YMT TTR AGT TT-5′) primer pair (Research and Testing, Lubock Texas; (Caporaso et al., 2012; Walters et al., 2016) For sequencing, forward and reverse sequencing oligonucleotides were designed to contain a unique 8 nt barcode (N), a primer pad (underlined), a linker sequence (italicized), and the Illumina adaptor sequences (bold).

Forward primer: **AATGATACGGCGACCACCGAGATCTACAC-**NNNNNNNN-TATGGTAATT-*GT*-GTGY-CAGCMGCCGCGGTAA

Reverse primer: **CAAGCAGAAGACGGCATACGAGAT-**NNNNNNNN-AGTCAGTCAG-*GG*-CCGYCAATTYMTTTRAGTTT

Each PCR reaction contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems, Boston, MA), 1.5 mM MgCl2, 10 pmol of each primer, and 1 µL of DNA. The PCR was performed using the following conditions: 95 °C for 2 min, followed by 30 cycles at 95°C for 10s, 55°C for 15s, 72°C for 15s and a final extension step at 72 °C for 3 min. Amplicons were quantified using a Qubit instrument with the Qubit High Sensitivity DNA kit (Invitrogen, Carlsbad, CA). Individual amplicon libraries were pooled, cleaned with Ampure XP beads (Beckman Coulter, Brea, CA), and sequenced using a 300 bp paired-end method on an Illumina MiSeq at RTL Genomics in Lubbock Texas. Raw sequence reads were submitted to NCBI's Sequence Read Archive under the SRA ID: SRP152555.

2.3.9 Sequence analysis

Sequencing resulted in a total of 1,251,439 raw reads, which were analyzed using mothur v1.39.5 (Schloss et al., 2009) using the MiSeq SOP accessed on 3/10/2018 (Kozich et al., 2013). Using the make.contigs command, raw sequences were combined into contigs, which were filtered using screen.seqs to remove sequences that were > 420 bp or contained ambiguous base calls to reduce PCR and sequencing error. Duplicate sequences were merged with unique.seqs, and the resulting unique sequences were aligned to the V4-V5 region of the SILVA SEED alignment reference v123 (Quast et al., 2013) using align.seqs. Sequences were removed if they contained homopolymers longer than 8 bp or did not align to the correct region in the SILVA SEED alignment reference using screen.seqs. To further denoise the data, sequences were pre-clustered within each sample allowing a maximum of 3 base pair differences between sequences using pre.cluster. Finally, chimeric sequences were removed using VSEARCH (Edgar et al., 2011).

Quality filtered sequences were grouped into OTUs based on 97% sequence identity and classified using the Bayesian classifier and the Greengenes database (August 2013 release of gg_13_8_99) (DeSantis et al., 2006) with classify.seqs. Sequences that classified as mitochondria, chloroplasts, eukaryotic, or of unknown origin were removed using remove.lineage. Samples were rarefied to 6467 sequences per sample, the smallest number of sequences across all collected samples. Singleton abundances were calculated with filter.shared. Chao1 diversity (Chao, 1984), Good’s

coverage (Good, 1953), Shannon (Shannon, 1948), and inverse Simpson indices were calculated using summary.single to quantify coverage and α-diversity.

2.3.10 α-Diversity

To estimate the microbial diversity within each group, first, rarefaction analyses were performed and species richness and diversity indices were calculated. Variance of the microbial community between and among the different vessels were quantified using a θYC distance matrix (Yue and Clayton, 2005).

2.3.11 β-Diversity

To investigate slow-acting effects of seaweed addition on microbiome communities, we computed Bray-Curtis dissimilarity (β-diversity) (Yue and Clayton, 2005) between pairs of samples, both within vessels at different time points, and between vessels at identical time points. We also considered Jaccard dissimilarity which only reflects community composition and not relative abundance, but found similar results and so only report the results for Bray-Curtis dissimilarity. We independently computed β-diversity at the genus, family, order, class, and phylum level to assess whether the observed patterns were dependent on taxonomic resolution. For regression statistics, we computed 95% confidence intervals using non-parametric bootstrap resampling, and significance values using permutation tests. Both of the latter approaches gave qualitatively similar results. All analyses were performed using custom written Java, SQL, and Bash code available at https://github.com/jladau.

2.3.12 Statistical analysis

Analysis of molecular variance (AMOVA) (Bray and Curtis, 1957) was used to identify significant differences in community structure be- tween treatment and control vessels using a θ_{YC} distance matrix for the amova command in Mothur. The complete results of these statistical tests between each time interval combination is included in the supplementary data. Gas, VFA, and *Euryarchaeota* abundance data were analyzed using the linear mixed-effects model (lme) procedure using the R statistical software (version 3.1.1) (Excoffier et al., 1992; Team R, 2014). The statistical model included treatment, day, time point, treatment×day×time point interactions, treatment×day interactions, treatment×time point interactions, day×time point interactions and the covariate term, with the error term assumed to be normally distributed with mean = 0 and constant variance. Orthogonal contrasts were used to evaluate treatments vs. control, linear, and quadratic effects of treatments. Significant differences among treatments were declared at $p \leq 0.05$. Differences at $0.05 < p \leq 0.10$ were considered as trend towards significance.

2.4 Results

2.4.1 In-vitro standard measurements remained stable throughout the experiment

Rumen fluid and rumen solids were collected from two fistulated dairy cattle. Rumen contents were homogenized and equilibrated for 24 h and subsequently inoculated into the artificial gut system following the experimental design outlined in Figure 2-1. Temperature, pH, and mV remained relatively constant (37 °C ± 2, 6.8 pH ±0.03, 21 mV ±3) throughout the entire experiment and between individual vessels.

2.4.2 A. taxiformis contains an elevated mineral profile but less organic matter compared to SBR

A higher OM content for SBR was found (92.8% DM) when compared to *A. taxiformis* (53% DM). Crude protein amounts were relatively similar for SBR (20% DM) and *A. taxiformis* (17.8% DM). Neutral detergent fiber composition of SBR and *A. taxiformis* were also similar with 38.1 and 36.9% DM, respectively. Differences in starch content between SBR and *A. taxiformis* were prominent with 12.6 and 0.7% DM, respectively. Lignin content for SBR was determined with 6% DM and 4.4% DM for A. taxiformis. Total digestible nutrient content (TDN) for *A. taxiformis* was approximately half (33.8% DM) of the TDN determined for SBR (66.2% DM). *Asparagopsis taxiformis* contained elevated mineral profiles compared to SBR. More specifically, *A. taxiformis* exhibited higher calcium, sodium, magnesium, iron, and manganese concentrations. Zinc was present at 23.7 ppm in both SBR and *A. taxiformis*. The detailed composition of SBR and *A. taxiformis* is shown in Table 2-2.

2.4.3 A. taxiformis decreases CH_4 production and increases propionate:acetate ratio

Total gas production (TGP) and CH_4 production were significantly affected by the inclusion of *A. taxiformis* ($p < 0.05$, Table 2-3). Average total gas production for the *A. taxiformis* treatment group was 14.81 ml/(g OM) whereas the control group was 28.54 ml/(g OM), representing a 51.8% reduction in TGP with *A. taxiformis*. Average CH_4 production for the *A. taxiformis* treatment group was 0.59ml/(g OM), whereas the control group produced 12.08ml/(g OM), representing a 95% reduction of CH_4 being synthesized. No significant difference was found in CO_2 production between the *A. taxiformis* treatment and the control groups. Figure 2-2 illustrates how total gas (i.e. CH_4 and CO_2) was

affected over the duration of the experiment. It appears that *A. taxiformis* is effective at reducing TGP and CH_4 almost immediately, beginning at 12 h after the beginning of the experiment, and continues to inhibit CH_4 production over 24 h just prior to when new bioactive is provided during the feeding process (at 24 h 48 h, and 72 h). Inhibition of methanogenesis was also measured just prior to the termination of the experiment (96 h).

Slightly higher total VFA concentrations were recorded for the control group when compared to the *A. taxiformis* treatment group [2332.52 ppm vs. 2105.11 ppm ± 269.20 ppm respectively (means ± SE)], however this difference was not statistically significant ($p = 0.45$, Table 2-3). Additionally, no significant differences were found when comparing concentrations of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate (Table 2-3) between control and *A. taxiformis* treatment group. Although, valerate was not found to be statistically different between groups ($p < 0.05$), it was observed that the *A. taxiformis* treatment group tended to have lowered concentrations of valerate when compared to the control group ($p = 0.06$). Statistical differences were found between groups when comparing the propionate:acetate ratio, with a higher proportion of propionate to acetate within the *A. taxiformis* treatment groups ($p = 0.001$). Differences observed at each timepoint between control and *A. taxiformis* treatment groups were determined to be not significant (Figure 3-3).

2.4.4 Sequencing and quality filtering

A total of 1,251,439 reads were generated from a total of 77 samples, with a mean (± SD) of 16,275 (±1879) reads per sample. After quality filtering, 757,325 (60.5%) high

quality sequences remained. Operational taxonomic units (OTU) based analysis (at 97% sequence identity) revealed 32,225 unique OTUs across all samples. Singletons contributed 23,043 (3%) unique reads to the total filtered read count, and were removed prior further analysis. The mean Goods' coverage for all samples was 88 ± 3%, suggesting that the sequencing effort recovered a large proportion of the microbial diversity in each of the samples under investigation.

2.4.5 α-Diversity measurements show microbial communities diverged slightly over the course of the experiment

The microbial communities of the control and *A. taxiformis* amended vessels were compared at each incubation time. Significant differences in the microbial community between the two conditions appeared transiently at only two time points, the 12 h time point on the first day of the experiment and again at the 24 h time point on the fourth day (96h after the start of the experiment, AMOVA, $p \leq 0.02$, and $p \leq 0.04$ respectively). Comparison of the microbial communities from the start and end of the experiment within each group suggested that the microbial communities changed over the course of the experiment (AMOVA, $p \leq 0.06$ and $p \leq 0.05$, treatment and control respectively). The communities associated with treatment and control are very similar at the beginning but started to diverge immediately after the initiation of the experiment (4 h). While the diverging trajectory becomes more apparent throughout the experiment (i.e, 96 h), the first two axes of the PCoA plot account for a low fraction (13.5%) of the total variation that is observed between the samples, which coincides with the observation that the communities associated with the two vessel groups were largely similar. Microbial

communities respond to *A. taxiformis* as a stressor but recover quickly. Although the effects of seaweed amendments on CH_4 production were immediate (≤ 12 h), amendments may also affect microbial populations on a longer time scale. Over the duration of the experiment, β-diversity between pairs of control vessels remained constant (permutation test for non-zero slope: $p > 0.001$). In contrast, β-diversity between pairs of treatment vessels and between treatment and control vessels gradually changed. More specifically, β-diversity between treatment vessels increased and then decreased, with highest difference measured at ~ 72 h after the start of the experiment, while β-diversity between treatment and control vessels increased essentially monotonically until the end of the experiment (Figure 2-4a; permutation test for non-zero slope: $p < 0.001$). These slow shifts in community composition were evident regardless of the taxonomic level at which β-diversity was considered, including at coarse taxonomic resolutions (Figure 2-4b). Examination of the genus-level β-diversity within vessels across different time lags also indicated that the microbial communities continued to shift throughout the duration of the experiment (Figure 2-4c). Essentially, sample pairs collected at more distant times were on average more dissimilar than those collected at similar times. This trend was most pronounced for pairs of samples that had seaweed amendments.

2.4.6 Average methanogen abundance decreased, but not in concert with CH_4 reduction

Across all samples, one archaeal and 21 bacterial phyla were identified. The ten most abundant phyla recruited > 98% of the reads generated from the microbial communities of both the control and *A. taxiformis* amended vessels (Figure 2-5). Microbiomes throughout the experiment, regardless of experimental condition or time,

were dominated by *Bacteroidetes, Firmicutes,* and *Proteobacteria*. The *Bacteroidetes:Firmicutes* ratio decreased in both conditions over the course of the experiment, suggesting influence due to the experimental system (Figure 2-5). With the drastic decrease in CH_4 in mind, the differences between the two groups were investigated at a finer resolution by exploring the abundance dynamics of the Archaeal phylum *Euryarchaeota*, which include the methanogenic Archaea. Based on the 16S rRNA gene profiles, five genera of methanogenic Archaea were identified in all stages of the experiment. The five genera: *Methanobrevibacter*, *Methanosphaera*, *vadin CA11* of the *Methanomassiliicoccacaea* family, *Methanoplanus* and *Methanimicrococcus* accounted for all reads recruited by the *Euryarchaeota*. *Methanobrevibacter* and *Methanosphaera* accounted for > 99% of the reads assigned to methanogens. While CH_4 production decreased in the *A. taxiformis* amended vessels 12 h after the first feeding event, abundance of methanogenic Archaea in the two conditions did not differ significantly at individual time points (Figure 2-6). However, the average relative abundance of *Euryarchaeota* over the duration of the experiment were lower in the *A. taxiformis* amended vessels compared to control vessels (1.38 and 1.79% respectively, $p \leq 0.03$).

2.5 Discussion

A significant reduction in CH_4 production was found when evaluating the effects of *A. taxiformis* on ruminal fermentation characteristics, in-vitro, at a 5% OM inclusion rate. Results from the overall experiment show an approximate decrease in TGP by ~ 50% and in CH_4 production by ~ 95%, which is similar to multiple studies conducted on the

effects of *A. taxiformis*, both in-vivo and in-vitro (Machado et al., 2014; Machado et al., 2016b; Li et al., 2018; Machado et al., 2018).

Carbon dioxide production remained similar between the control and *A. taxiformis* amended vessels. Comparison of total and individual VFA between vessels did not suggest any difference in VFA production at any specific time point with the 5% OM inclusion rate. A significant reduction of CH_4 was measured 12 h after *A. taxiformis* amendment (Figure 2-2), while CO_2 production and VFAs profiles remained unchanged throughout the fermentation process (Figures 2-2 and 2-3). This suggests that the amendment of SBR supplemented with *A. taxiformis*, inhibits methanogenesis but not CO_2 production, which is often used as a measurement for microbial growth. This targeted effect on a specific metabolic function, and hence a functional group within the microbiome, was also elucidated from the 16S rRNA profiles of the in-vitro rumen system. he overall assemblages of the microbiome associated with the treatment and control fermentation vessels remained rather similar throughout the duration of the fermentation process (Figure 2-5). Changes in the relative abundance of members belonging to the *Euryarchaeota*, the taxonomic group that encompasses the main rumen methanogens, could be observed as early as 36h after the initiation of the experiment. Although a semi-continuous batch fermentation system, as utilized for this study, is capable of maintaining more rumen like conditions, mainly through maintaining adequate pH and nutrient levels, when compared to a simple batch fermentation process, a wash-out of the more sensitive rumen microbes (i.e. protozoa) is inevitable (Cabeza-Luna et al., 2018). It is well known that there is a mutualistic relationship between protozoa and methanogens (Belanche et al., 2014; Holmes et al., 2014), and it has been shown before that the removal of rumen

protozoa results in a reduction of the methanogen population and methanogenesis during enteric fermentation (Newbold et al., 1995; Morgavi et al., 2010). Hence, the decrease in relative abundance of *Euryarchaeota* observed for the control vessels at later time points of the experiment is most likely an artifact caused by the inability of the in-vitro systems to maintain protists over an extended period of time.

2.5.1 Propionate:Acetate ratio increased in treatment vessels

Over the course of the experiment, the propionate:acetate ratio increased ($p < 0.001$) in treatment vs control groups. The first step of the formation of acetate in the rumen releases metabolic hydrogen which acts as a hydrogen donor to methanogenic archaea and therefore facilitates the production of CH_4 in the rumen (Wolin et al., 1997). In contrast, propionate acts as a competing hydrogen sink (Henderson, 1980; Janssen, 2010). The increased propionate:acetate ratio suggest that hydrogen is, at least in some part, being redistributed to propionate, which may help explain a portion of the CH_4 reduction seen here. In the context of dairy cattle and milk production, the increased propionate:acetate ratio seen in vessels amended with *A. taxiformis* may forecast an altered milk composition in-vivo. A decreased propionate:acetate ratio is associated with increased milk fat, and total milk yield is positively associated with butyrate and propionate in the rumen (Seymour et al., 2005). Under this paradigm, *A. taxiformis* supplementation has the potential to increase total milk yield, however may also negatively impact milk fat content.

2.5.2 Microbial communities overcame the stress of treatment

We observed that *A. taxiformis* has affects consistent with the Anna Karenina Hypothesis, which posits that disturbances act to increase differentiation of microbial communities (Zaneveld et al., 2017). Specifically, we found that communities in treatment vessels differentiated increasingly from each other up to hour 72, after which they reconverged (Figure 2-4a). This finding suggests that, the rumen microbial community undergoes changes that are both slow and variable in response to *A. taxiformis*. However, these changes do not appear to be associated with variability in reduction of gas production. While *A. taxiformis* may pose an initial stress on the rumen microbial community, measured by the increased differentiation between treatment vessels, the β-diversity between communities in amended vessels stabilized after only 72 h under recurrent daily stress (feeding).

2.5.3 A. taxiformis is a potential mineral supplement

Nutritional analysis of *A. taxiformis* revealed that *A. taxiformis* has high levels of important minerals including calcium, sodium, iron, and manganese (Table 2-2) suggesting that in addition to its CH_4 reduction potential, *A. taxiformis* may also be used to increase mineral availability to basic rations. In-vivo studies directed towards monitoring mineral transfer from feed into product should be conducted next to facilitate a better understanding of whether or not minerals, or other compounds, present in seaweed can be found in milk or meat of the consuming animals. While halogen compounds have been reported as important players in the bioactive process of CH_4 reduction, previous studies using seaweed as a feed supplement found that iodine, which

is abundant in brown algae, is found in the milk of cows to which it is fed (Rey-Crespo et al., 2014).

2.6 Conclusions

The CH_4 reducing effect of *A. taxiformis* during rumen fermentation of feed makes this macroalgae a promising candidate as a biotic CH_4 mitigation strategy for California dairy producers. The organic matter inclusion required to achieve such a drastic decrease in CH_4 is low enough to be practically incorporated in the rations of average dairy operations. Significant limitations to the implementation of *A. taxiformis*, and potentially other algae, include the infrastructure and capital necessary to make these products commercially available and affordable. Furthermore, our understanding of the host microbe interactions during seaweed supplementation are limited. In order to obtain a holistic understanding of the biochemistry responsible for the significant reduction of CH_4, and its potential long-term impact on ruminants, gene expression profiles of the rumen microbiome and the host animal are warranted.

2.8 Tables and Figures

Table 2-1. Composition of dry cow diet and super basic ration (SBR).

Dry Cow Diet		SBR	
Ingredient			
Alfalfa	25%	Alfalfa	70%
Wheat	50%	Dried Distillers Grain	15%
Almond Hulls	21.40%	Rolled Corn	15%
Mineral Pellets	3.60%		

Table 2-2. Composition of SBR and *Asparagopsis taxiformis*

	SBR[a)]	*A. taxiformis*
Chemical Composition		
% Dry Matter		
Organic Matter	92.8	53.0
Crude Protein	20.0	17.8
Neutral Detergent Fiber	38.1	36.9
Acid Detergent Fiber	27.3	11.6
Starch	12.6	0.7
Fat	2.7	0.4
Total Digestible Nutrients	66.2	33.8
Lignin	6.0	4.4
Calcium	0.9	3.8
Phosphorus	0.4	0.2
Sodium	0.1	6.6
Magnesium	0.5	0.8
Parts per million		
Iron	632.7	6241.0
Manganese	41.7	112.7
Zinc	23.7	23.7
Copper	11.0	8.7

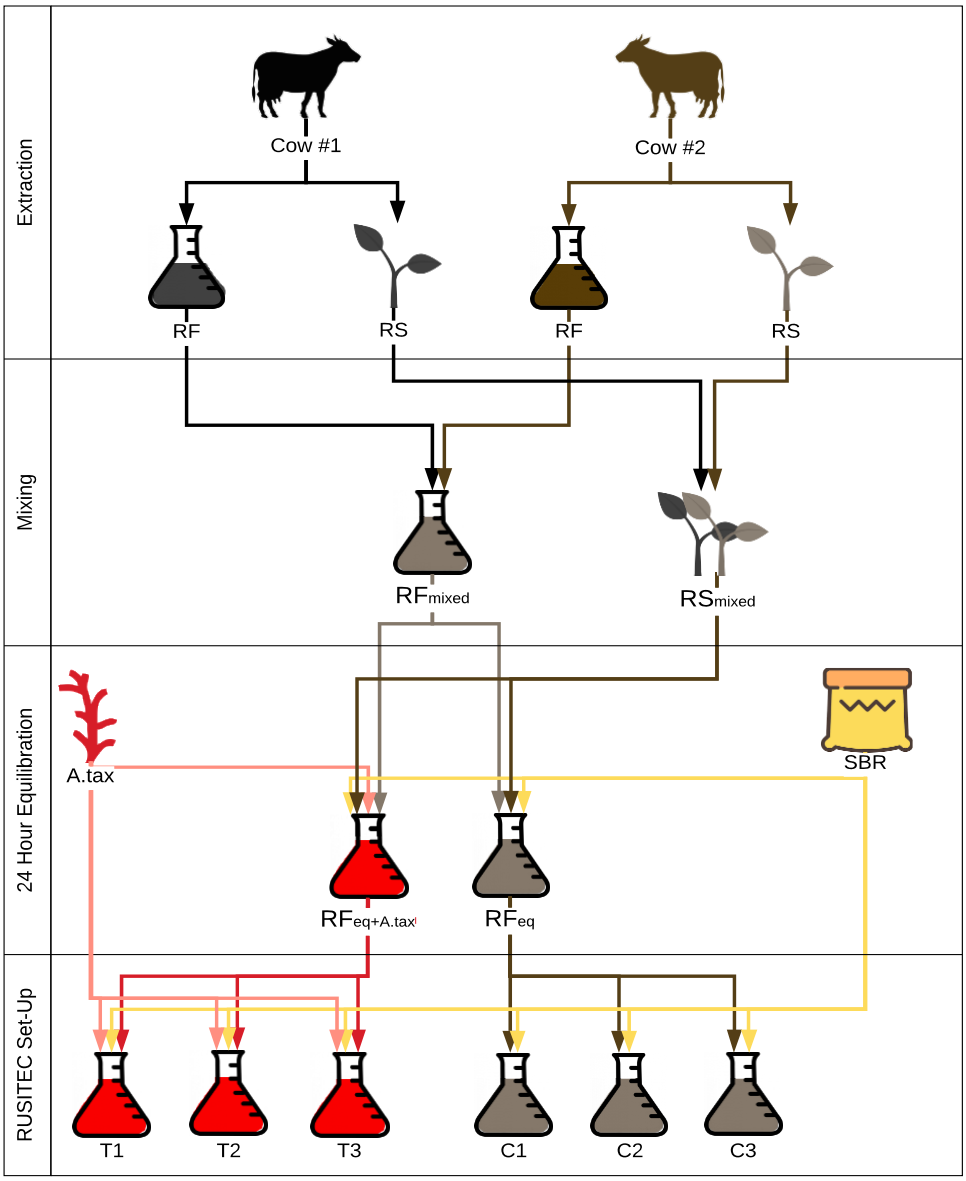
[a)]Super Basic Ration

Table 2-3. Effects of *Asparagopsis taxiformis* on total gas production and total volatile fatty acid production.

	Control	*A. taxiformis*	Standard error	*P*-value
Gas Production [mg/g/OM]				
CH_4	12.08	0.59	0.59	<0.0001
CO_2	15.67	14.24	3.82	0.73
Total Volume[1]	28.54	14.81	3.85	0.02
Volatile Fatty Acid Production [ppm]				
Total VFA	2332.52	2105.11	269.2	0.45
Acetate	1056.99	856.77	135.08	0.21
Propionate	481.12	490.54	58.36	0.88
Propionate : Acetate[2]	0.48	0.6	0.01	<0.001
Butyrate	394.35	423.01	53.55	0.62
Iso-Butyrate	84.81	79.83	4.32	0.31
Valerate	212.79	168.72	16.99	0.06
Iso-Valerate	102.44	86.21	14.49	0.33

[1] Total volume reported in ml/g OM

[2] reported as a ratio of respective VFA concentrations



RF = Rumen Fluid; RFmixed = Rumen Fluid from Cows 1 & 2; RFeq = Equilibrated Rumen Fluid; RFeq+A.tax = Equilibrated Rumen Fluid + Asparagopsis taxiformis; RS = Rumen Solids; RSmixed = Rumen Solids from Cows 1 & 2; SBR = Super Basic Ration; A.tax = *A. taxiformis; T* (1,2,3) = Treatment Vessels; C(1,2,3) = Control Vessels.

Figure 2-1. In-vitro rumen system set-up. Extraction: Rumen fluid and rumen solids were collected from 2 dairy cows. Mixing: Rumen fluid was homogeneously mixed and rumen solids were homogeneously mixed. After mixing, rumen fluid was separated into two Erlenmeyer flasks, where treatment was then assigned. 24 Hour Equilibration: The control flask received 30 g of mixed rumen solids and 30 g of SBR and the treatment flask received 30 g of mixed rumen solids, 30 g of SBR, and 1.5 g of *A. taxiformis*. After each flask received their treatment, the 24 h equilibration period began. After the

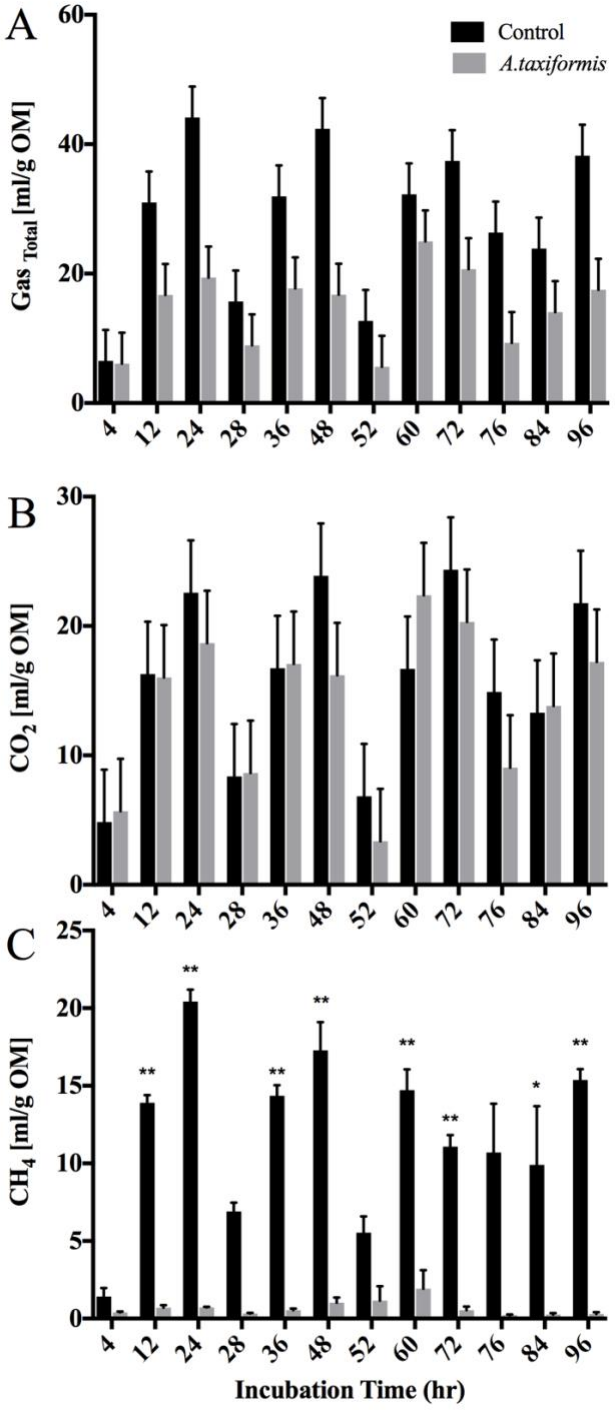


Figure 2-2. Total gas, CH_4, and CO_2 production during in-vitro fermentation. Production of total gas, CH_4 and CO_2 [ml/(g OM)] from vessels without (n = 3) and with (n = 3) *A. taxiformis* as additive at 4, 12, and 24 h over the course of the experiment. a Total gas production; b CH_4 production; c CO_2 production. Measurement were performed in triplicates. "**" indicates significant difference (p value ≤0.05), "*" indicates trend toward significance (0.05 > p value ≤0.1)

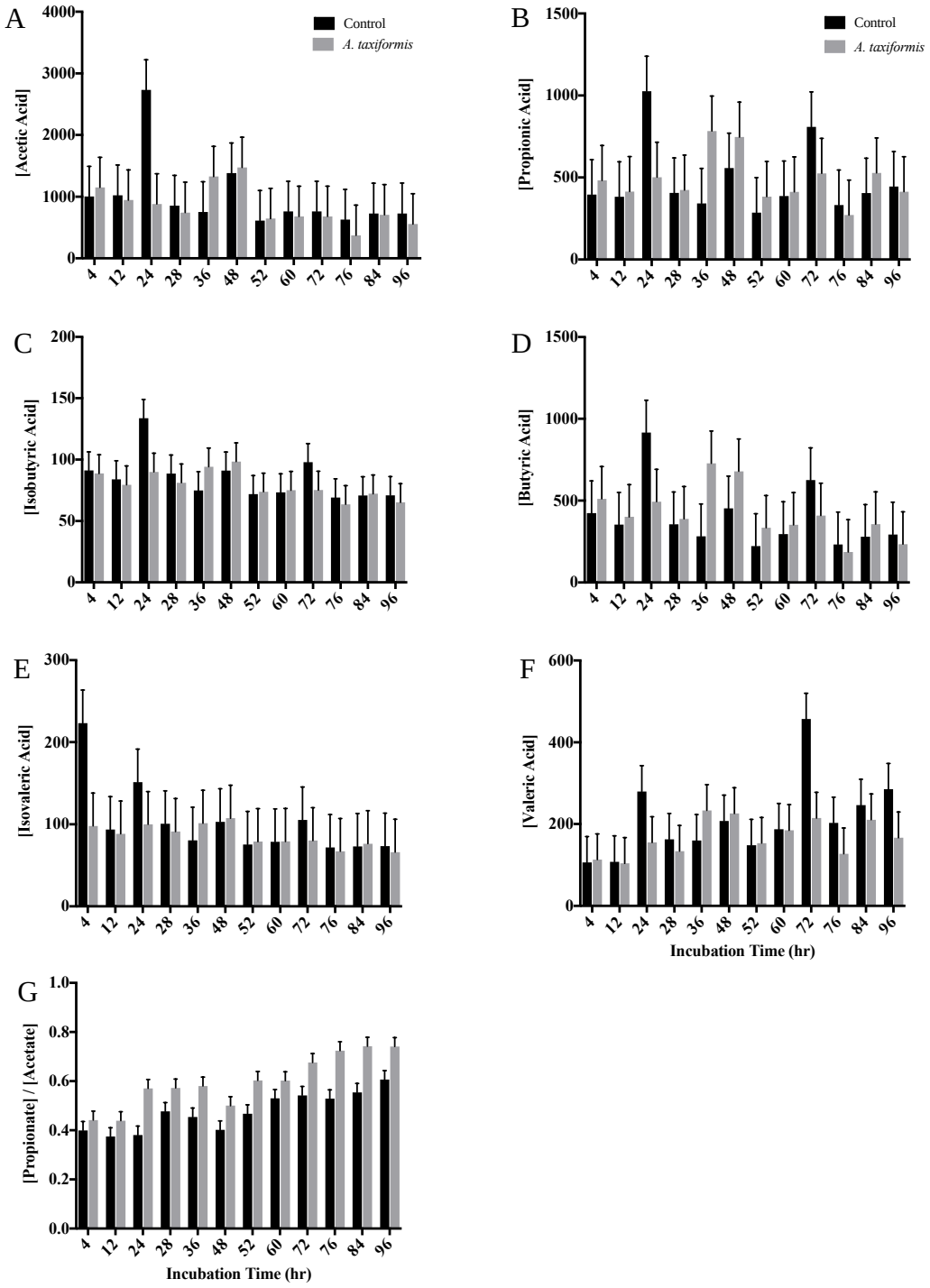


Figure 2-3. Volatile fatty acid production during in-vitro fermentation. Volatile fatty acid concentrations [ppm] of fermentation fluid of vessels without (n = 3) and with (n = 3) A. taxiformis as additive, determined 4, 12, and 24 h after feeding over 4 days. a Acetic acid; b Propionic acid; c Isobutyric acid; d Butyric acid; e Isovaleric acid f Valeric acid; g Propionate/Acetate Ratio. Measurement were performed in triplicates

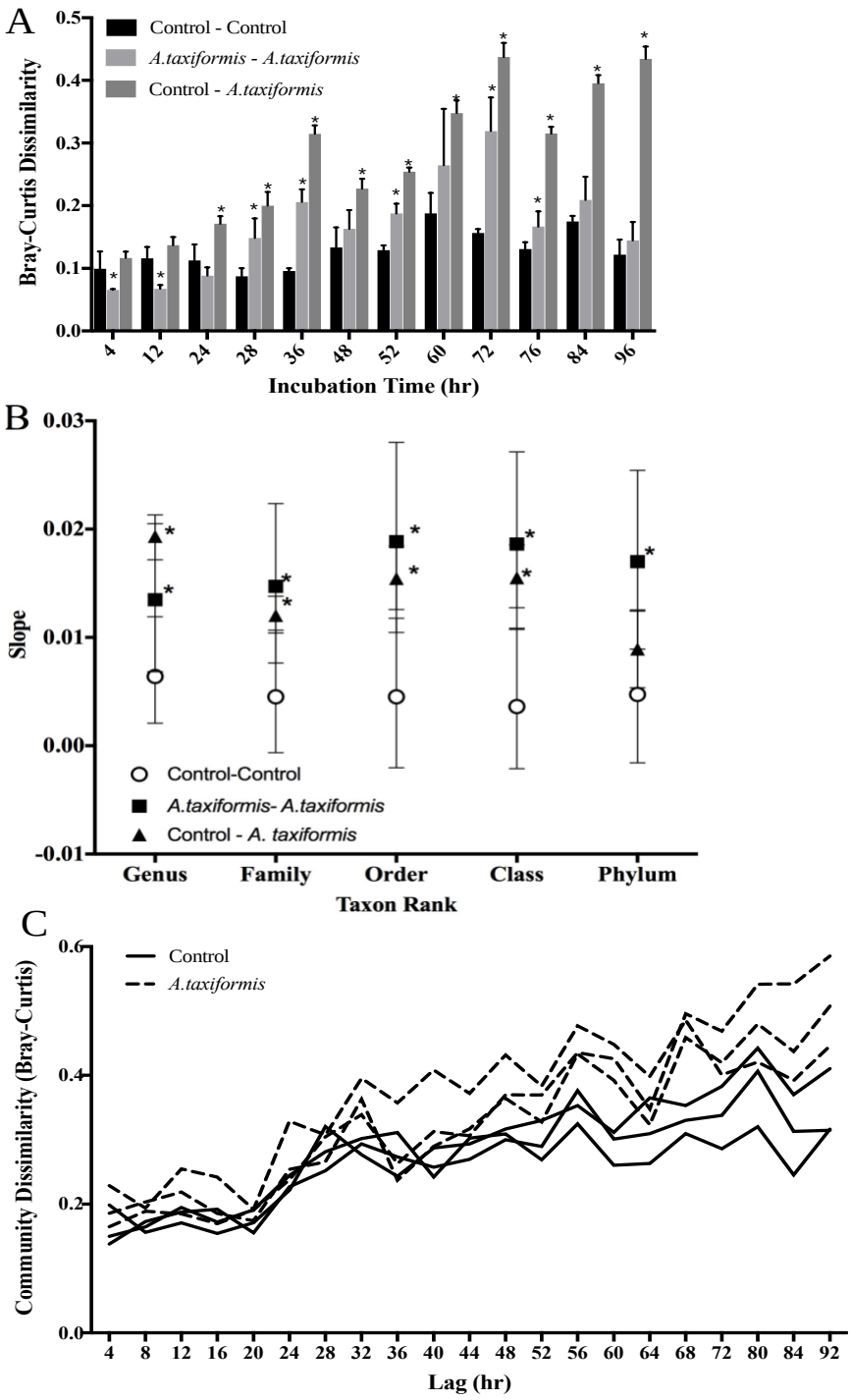


Figure 2-4. Effects of seaweed amendments on composition of in-vitro rumen microbiome. A) Genus-level β-diversity between pairs of vessels throughout the duration of the experiment. B) β-diversity across multiple taxonomic groups measured between pairs of samples versus sampling time for each of the 6 vessels. 95% bootstrap confidence intervals are shown. Regression slopes identified as significant ($p < 0.001$) by a permutation test are indicated with an asterisk. C) Genus-level β-diversity within individual vessels across different sampling times.

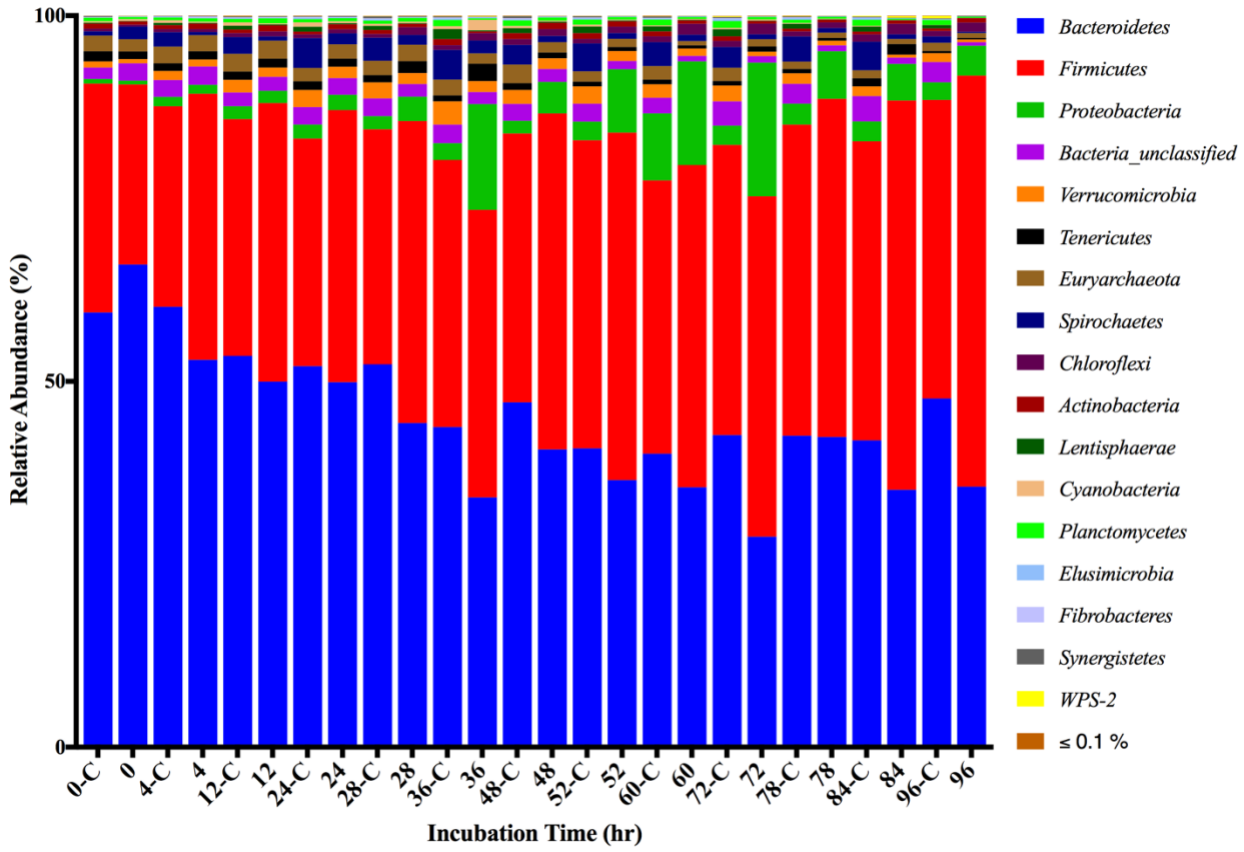


Figure 2-5. Relative abundance of phyla during in-vitro fermentation. Fermentations were performed in three in-vitro vessels (n = 3). Incubation times annotated with “C” represent control conditions

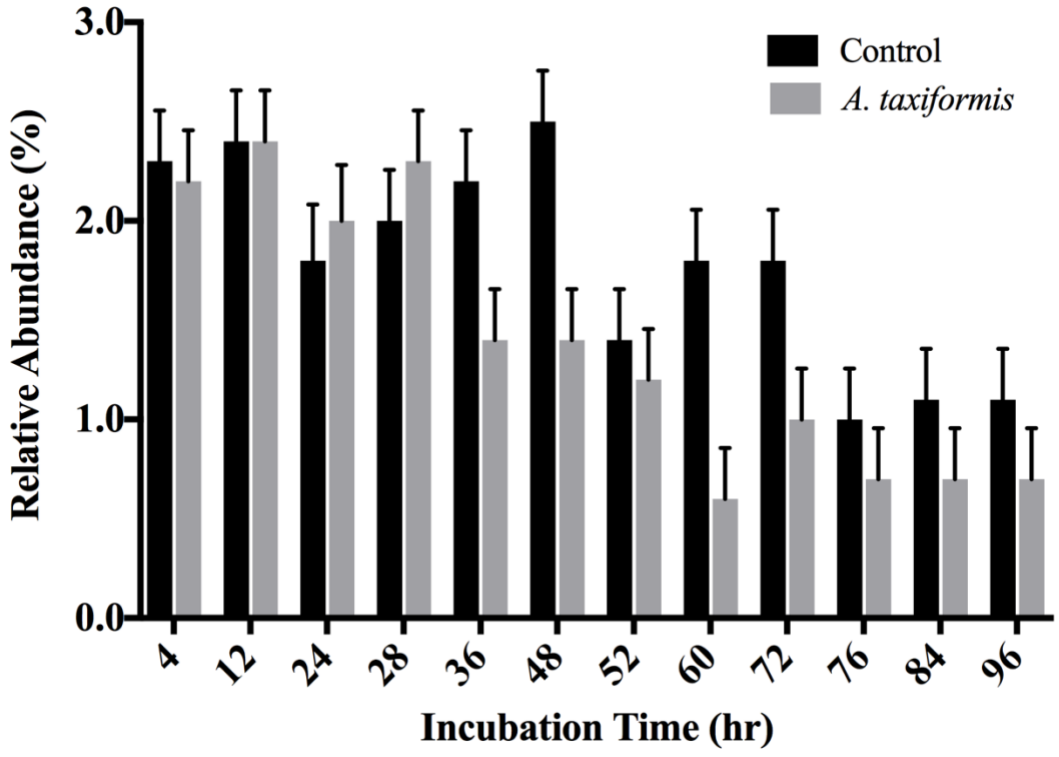


Figure 2-6. Relative abundance of *Euryarchaeota* during in-vitro fermentation. Fermentations were performed in three in-vitro vessels (n = 3). Error bars indicate standard error of the mean.

Chapter 3: Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane by over 50 percent.

Roque et al. (2019b) Journal of Cleaner Production

3.1 Abstract

Livestock production, particularly enteric CH_4 production, contributes to greenhouse gas emissions globally. Various mitigation strategies developed to reduce enteric emissions have limited success. Although in vitro studies have shown a considerable reduction in CH_4 emissions using *Asparagopsis* spp., no studies have been conducted to investigate the effect of any species of *Asparagopsis* in dairy cattle. Our objective was to evaluate quantitatively the response of cows consuming *Asparagopsis armata* on CH_4 production (g/kg), yield (g/kg feed intake) and intensity (g/kg milk yield). Twelve post-peak lactating Holstein cows were randomly assigned to three treatments (control, 0.5% and 1% inclusion levels of *A. armata* on organic matter basis) in a 3 x 3 Latin square design with three 21-day periods. Enteric CH_4 emissions were measured using the GreenFeed system. CH_4 production by cows decreased significantly by 26.4% at the low (0.5%) level of *A. armata* inclusion and 67.2% at the high (1%) level of inclusion. Feed intake was reduced by 10.8 and 38.0%, in cows fed the low and high level of macroalgae inclusion, respectively. CH_4 yield decreased significantly by 20.3 and 42.7% in cows fed diet including 0.5% and 1% *A. armata* inclusion levels, respectively (P ¼ <0.0001). CH_4 intensity significantly decreased by 26.8% from cows fed at 0.5% level and 60% at the 1.0% *A. armata* inclusion level. Bromoform concentrations in milk were not significantly different between treatments. Our in vivo results showed that *A. armata* has potential to be used as a feed additive to reduce enteric CH_4 emissions.

3.2 Introduction

The livestock sector contributes 14.5% of global GHG emissions (Gerber et al., 2013), with global CH_4 emissions contributing to about 2.1 Gt CO_2 equivalent in 2010 (Smith et al., 2014). There are considerable differences in contribution of enteric CH_4 in different regions and countries of the world. The main source of anthropogenic CH_4 emissions in the United States is generated by enteric fermentation of livestock (25%NASEM et al. (2018)). US EPA (2019) estimated the total CH_4 emissions from enteric fermentation in the United States to be 6.46 Tg in 2017, which is equivalent to 27% of the nation's anthropogenic CH_4 emissions. Enteric CH_4 is a natural by-product of microbial fermentation of nutrients in the digestive tract of animals. Enteric CH_4 emissions represent up to 11% of dietary gross energy consumed by ruminants and in North American dairy cattle it is estimated to be about 5.7% of gross energy intake (Moraes et al., 2014).

Hristov et al. (2013) reported that mitigation options including nitrates, ionophores, tannins, direct-fed microbials and vaccines may offer opportunities to reduce enteric CH_4 emissions; however, the results have been inconsistent. (Knapp et al., 2014) estimated that nutrition and feeding approaches may contribute to reducing CH_4 emission intensity (i.e., emissions per milk yield) by 2.5 - 15%, whereas rumen modifiers had little success in sustained CH_4 emissions without compromising milk production. CH_4 inhibitors may be a more successful approach in reducing emissions from enteric fermentation. For example, 3-nitrooxypropanol (3NOP) has been reported to substantially decrease CH_4 emissions from ruminants (Duin et al., 2016).

Seaweeds have been a traditional part of livestock diet and have been used since the recording of agricultural practices began (Evans and Critchley, 2014). There have been several studies on seaweeds to characterize their effects as livestock feeds and their potential to manipulate rumen fermentation and CH_4 production. Maia et al. (2016) evaluated several seaweeds and reported that their efficacy is impacted by the formulation of the basal feed of the livestock. The utility of seaweeds as feeds is impacted by the composition of the biomass which in turn is a result of many inherent factors such as species, growth stage, habitat, and external factors such as temperature, light, and nutrient availability. A key feature is the circumstantial production and accumulation of secondary metabolites (Paul et al., 2006a) that may have a bioactive impact on the animals or from the perspective of CH_4 production, on the microbial consortium that thrives in a rumen. Many seaweeds have been demonstrated to reduce CH_4 production by rumen methanogens but with variable effects on fermentative health and substrate digestibility (Machado et al., 2014). The effectiveness of the seaweeds has been shown to have a relationship with the level of inclusion in the diet (Kinley et al., 2016a; Machado et al., 2016b; Machado et al., 2016a; Li et al., 2018) and only *Asparagopsis* has been demonstrated to remain effective and dramatically anti-methanogenic without negative impacts on rumen function and at low inclusion levels in animal diets (Kinley et al., 2016a; Li et al., 2018). In the development of knowledge of *Asparagopsis spp*. effects on rumen microbial production of CH_4, there has been progression through multiple in vitro studies all of which have demonstrated significant if not total reduction of CH_4 emissions at levels of approximately 2% of diet substrates (Dubois et al., 2013; Machado et al., 2016b; Machado et al., 2016a). Even though this dietary level of the seaweed was low and

considered feasible for livestock production systems, Li et al. (2018) demonstrated in animals the potential for efficacy at lower intake levels. From their study in sheep using *Asparagopsis taxiformis*, Li et al. (2018) reported up to 80% reduction in CH_4 emissions. Although the project applied *A. taxiformis* offerings of 0.5, 1, 2, and 3% of the diet, the feed formulation provided for voluntary intake of the seaweed product which resulted in partial unavailability or refusal by the sheep. Nevertheless with reduced intake of the seaweed the results showed significant CH_4 reduction and was the first indication that in vitro studies had over predicted the levels of *Asparagopsis* intake required for effective CH_4 reduction in vivo. This created an exploratory research requirement for subsequent animal studies to characterize the optimal intake of *Asparagopsis* to significantly reduce CH_4 emissions which is proposed to be variable based on diet composition. In the systematic in vivo characterization of *Asparagopsis* as an anti-methanogenic feed additive for ruminant livestock this study is the first demonstration of the effects in lactating dairy cattle. Based on previous in vitro and in vivo work it was hypothesized that application of *Asparagopsis* as a feed additive in a total mixed ration (TMR) would significantly reduce enteric CH_4 emissions and improve productivity represented by increased milk production. The objectives of the study were to: (1) investigate the potential of the macroalgae *Asparagopsis armata* in reducing CH_4 emissions in vivo; and (2) quantify CH_4 production (g/day per cow), yield (g/kg dry matter intake (DMI)) and intensity (g/kg milk yield) as a result of inclusion of *A. armata* in the TMR of lactating dairy cattle.

3.3 Materials and Methods

3.3.1 Animals and experimental design

All animal procedures were approved by the UC Davis Institutional Animal Care and Use Committee. Twelve multiparous Holstein cows with an average weight of 729 ± 24.9 kg, 35.1 ± 2.19 kg/d milk yield, and 201 ± 37 days in lactation were housed in a freestall barn equipped with individual animal sensor electronic recognition Calan gates (American Calan, Northwood, NH) to measure individual animal feed intake. As this was the first reported study using dairy cattle, we conducted a pretrial to determine effective levels of *A. armata* inclusion in the ration. The inclusion was gradually introduced from 0.25 to 1% of dietary OM. During the pretrial, there was a linear reduction of CH_4 as the level of inclusion increased. However, at inclusion approaching 1%, the cows demonstrated moderately reduced feed intake. Based on these observations, the inclusion levels were set as follows: no *Asparagopsis*, (control), 0.5% (low) and 1% (high) inclusion levels on OM basis. Cows were randomly assigned to a 3x3 Latin square design within the three treatment groups and 3 periods. Cows were fed twice daily at 0600 and 1700 h at 105% of their individual previous day intake and had free access to water at all times. Cows were fed a total mixed ration over the course of the study (Table 3-1) that was formulated to meet or exceed their growth requirement according to the recommendations of the National Research Council Requirement for Dairy Cattle (NRC, 2001). The nutritional composition of the basal diet is given in Table 3-1. *Asparagopsis armata* in the sporophyte stage was mixed with 400 ml of molasses and water to increase palatability, then hand mixed into the total mixed ration. The *A. armata* biomass was harvested from Cloudy Bay, Bruny Island (43.44226S, 147.23773E) near Hobart, Tasmania, Australia. After harvest, the material was blast frozen to -25°C over

approximately 6h, then freeze dried under vacuum and temperature control for 30h before packing and shipping. The macroalgae used in the study contained 1.32 mg/g dry weight level of bromoform concentration.

3.3.2 Sample collection and analyses

CH_4, carbon dioxide, and hydrogen gases from each cow were measured using the automated emissions measurement of the GreenFeed Large Animal System (C-Lock, Inc., Rapid City, SD). Gases were measured for 7 days to develop a baseline followed by 14 days with each cow visiting the system at least 3 times a day. Breath gas samples were collected for 8e10 min followed by a 2 min background gas sample collection. Calibration of the GreenFeed gas monitor was performed once per week throughout the trial. Bait feed consisting of alfalfa pellets was offered at each sampling event and kept below 5% of the total DMI during each sampling period. Diets were formulated to account for bait feed consumption offered by the GreenFeed system. Milk production, bodyweight, blood, feces, orts, and milk components were measured for 14 days daily during the treatment period and 7 days daily post treatment. Milk production was recorded twice daily representing morning and afternoon milking throughout the trial. Milk bromoform concentrations were analyzed using an Agilent 7890B GC applied to Agilent 7000C triple quad Mass Spectrometer equipped with a ZB-5ms column (Agilent Technologies, Inc. Santa Clara, CA). The limit of detection and limit of quantification were 0.01 mg/L and 0.05 mg/L, respectively.

3.3.3 Statistical analyses

All statistical analyses were performed using R statistical software (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria). Gas measurements were averaged per cow and treatment period and the averaged data was used for statistical analysis. Additionally, gas measurements were presented (normalized) as emissions per kg DM intake (g/kg DMI). Dry matter intake, body weight, milk production, milk components, and feed conversion efficiency (milk production/DMI) were averaged per treatment period prior to statistical analysis. Statistically significant differences were declared at $0.05<P<0.10$.

3.4 Results

3.4.1 Gas parameters

The average CH_4, hydrogen, and carbon dioxide production for the three treatment groups is given in Figure 3-1. CH_4 production (g/d) by cows decreased significantly by 26.4% at the low (0.5%) level of *A. armata* inclusion and 67.2% at the high (1%) level (Figure 3-1A). Hydrogen production increased 163 and 236% by cows fed diets with low and high levels of macroalgae inclusion. Carbon dioxide production was similar between control and low level of inclusion; however, there was a significant 13.9% decrease in total carbon dioxide production between control and high level of inclusion. When normalized for amount of feed intake, CH_4 yield (g/kg DMI) decreased significantly by 20.3% in cows offered 0.5% *A. armata* and at 1% inclusion level decreased further to 42.7% ($P < 0.0001$). Hydrogen yield significantly increased with the addition of *A. armata* with the average hydrogen yield for cows fed 0.5% *A. armata* increasing by 55.5% and further increasing by 78.9% at the highest macroalgae inclusion level (P 1⁄4 <0.0001).

Similar to hydrogen yield, carbon dioxide yield significantly increased with the addition of *A. armata*. Average carbon dioxide yield increased by 12.8 and 36.5%, at low and high levels of *A. armata* inclusion, respectively (P = 0.0001). Figure 3-2 shows the CH_4, hydrogen and carbon dioxide intensity in cows fed seaweed compared to control. When standardized by level of milk yield (MY), CH_4 intensity (g/kg MY) decreased by 18.2 and 60.1%, at low and high levels of *A. armata* inclusion, respectively (P = 0.0001). Average hydrogen intensity increased by 33.3% for cows consuming diet with 0.5% *A. armata*, and 61.7% for cows consuming diet with 1% *A. armata*. Carbon dioxide intensity between control and 1% *A. armata* inclusion remained relatively similar; however, the low level inclusion showed a reduction in carbon dioxide intensity of about 3.5% (P = 0.02). Bromoform concentrations in milk were numerically greater in cows fed diets with *A. armata* at both 0.5 and 1% levels. However, the differences were not significant compared to control (Table 3-2).

3.4.2 Animal and production parameters

Animal characteristics, milk yield, and milk composition responses to *A. armata* inclusion in the TMR are given in Table 3-2. Dry matter intake decreased significantly by 2.98 kg/d (P < 0.001) at low level of inclusion and further went down by 10.6 kg/d at the highest level compared to control, representing a 10.8 and 38.0% decrease, respectively. There was no significant body weight change between cows receiving *A. armata* at low inclusion compared to control; however, cows receiving the 1% level gained 9.72 kg less than control cows. Adjusted feed conversion efficiency was numerically greater in cows consuming *A. armata* at 0.5% level compared to control. However, it was significantly

greater (by 0.95 kg milk/kg intake) in cows fed diet with *A. armata* at 1% level compared to control. Milk yield did not differ significantly between cows in the control group and those at low level of *A. armata* inclusion. However, cows fed at the higher level of *A. armata* inclusion produced 11.6% less milk compared to control ($P < 0.001$). Milk protein content decreased as *A. armata* inclusion increased; however, significant differences were only observed in cows fed diets with 1% *A. armata* ($P < 0.0001$). No significant differences were found in milk fat, lactose, solids non-fat, milk urea nitrogen, or somatic cell count with both levels of macroalgae inclusion.

3.5 Discussion

Livestock systems, particularly ruminants, contribute to greenhouse gas emissions, and particularly in the form of enteric CH_4. A review of mitigation options for enteric CH_4 from ruminants showed that some of the effective strategies include increasing forage digestibility, replacing grass silage with corn silage, feeding legumes, adding dietary lipids and concentrates (Hristov et al., 2013b). Although effective, these types of system management options may not offer the scale of reduction required to dramatically change the agriculture contribution to the global GHG inventory and subsequent negative effects on climate change. However, the results of the present study and others suggest that feed additives may provide potent emissions reduction methodology.

Feed additives have been tested to reduce CH_4 emissions with mixed results (Table 3-3). For example, Appuhamy et al. (2013) showed about a 10% reduction using ionophores, specifically monensin in dairy and beef diets. Nitrates have also shown a

potential to reduce emissions by 16% (van Zijderveld et al., 2011b). Dijkstra et al. (2018) conducted a meta-analysis on the effect of 3-nitrooxypropanol to reduce CH_4 emissions and reported that it is effective in reducing enteric CH_4 by 39% in dairy and 22% in beef. The seaweed tested in this study was reported to have anti-methanogenic effect that reduces CH_4 yield during in vitro fermentation (Kinley et al., 2016a), which was confirmed in in vivo using sheep (Li et al., 2018).

3.5.1 Enteric CH_4 emissions

The CH_4 production reductions demonstrated in this study are among the highest ever reported. Adjusting for differences in intake, CH_4 yield in cows fed diets with *A. armata* showed a sharp decline for low and high levels of inclusion compared to the control group (Figure 3-1). Using a closely-related species of *Asparagopsis (A. taxiformis)* Li et al. (2018) reported a reduction of 50 - 80% in enteric CH_4 emissions over a 72-day period at inclusion levels between 1 and 3% of OM in sheep. The difference in effectiveness reported in the in vivo trials may be related to the concentration of the active compounds responsible for anti-methanogenic activity. The bioactive compounds are halogenated compounds including primarily bromoform, but also to lesser degree dibromochloromethane, bromochloroacetic acid, and dibromoacetic acid (Machado et al., 2016a). *Asparagopsis* accumulate bromoform (Paul et al., 2006a), and in all studies to date, the seaweed has been harvested in the wild where there is large variation between collections. The mode of action resulting in CH_4 reduction by rumen methanogens due to dietary *Asparagopsis* containing the halocarbon bromoform has been previously described using BCM as a model for bromoform (Denman et al., 2007; Kinley et al.,

2016a). This mode of action is a result of inhibition of the methanogenic pathway by inhibition of the cobamide-dependent methyl transferase at the terminal step of the pathway. Prior to this project this theory has been the accepted mode of action however further investigation into any distinctions or deviations is ongoing similar to Duin et al. (2016) who characterized the mode of action for 3-nitrooxyproponal which also has the enzyme inhibition mode of action. Some haloalkanes are structural analogs of methyl coenzyme-M reductase, the enzyme that catalyzes the last step of methanogenesis and inhibit the methyl transfer reactions that are necessary in CH_4 biosynthesis (Ermler et al., 1997; Liu et al., 2011).

The differences in CH_4 reduction when accounting for milk production for cows fed diets with the low and high level of *A. armata* inclusion were similar to the reductions in overall CH_4 production but greater compared to CH_4 yield (Figures 3-1 and 3-2). This is mainly due to milk production being not as greatly affected as would be expected with reductions in DMI for the high level of inclusion. During lactation, milk production is given a higher priority and body reserves are used to compensate if the cow is not consuming enough feed intake to support a given level of milk production (NRC, 2001).

Concentrations of volatile fatty acids in ruminal contents were not analyzed in the current experiment because the cows were not rumen cannulated. In vitro studies showed that volatile fatty acid production decreased with inclusion of *Asparagopsis* (Machado et al., 2016b). However, the inclusion level used was twice as much compared to this study. The authors also reported that the proportion of propionate, butyrate, valerate and isovalerate increased in samples with *Asparagopsis* level of 2% OM, which suggests that rumen fermentation favors propionate producing bacteria when methanogenesis is

inhibited. In vivo experiments of Li et al. (2018) also demonstrated that propionate production (which is a hydrogen sink) increased as the levels of *Asparagopsis* increased. Indeed, virtually all feed additives that reduce CH_4 production demonstrate a concomitant reduction in the acetate to propionate ratio (e.g. Hristov et al. (2015)). Hydrogen is a key product of rumen fermentation and has been suggested to thermodynamically control the production of the various volatile fatty acids, which may shift based on hydrogen partial pressure in the rumen (van Lingen et al., 2016).

3.5.2 Hydrogen and carbon dioxide emissions

The 55- and 79-fold increases in hydrogen emission in low and high macroalgae inclusion groups, respectively, compared to control is remarkable but not surprising as hydrogen would normally be converted to CH_4 and eructated out if methanogenesis were not inhibited. Mitsumori et al. (2012), using bromochlormethane, reported similar effects in hydrogen emissions, where they observed the majority of the metabolic hydrogen to be expelled as hydrogen gas. Hristov et al. (2015) also reported a 64-fold increase in hydrogen emissions in dairy cows supplemented with 3NOP, a compound that inhibits methanogenesis in a similar way as *Asparagopsis*. Metagenomic analysis of the rumen microbial community following inhibition of CH_4 formation by a halogenated CH_4 analog showed that CH_4-inhibited rumen appeared to adapt to the higher hydrogen levels by shifting fermentation to propionate which was mediated by an increase in the population of hydrogen consuming Prevotella and Selenomonas spp. (Denman et al., 2015).

Carbon dioxide production in livestock fed *Asparagopsis* has not been reported. However, Hristov et al. (2015), using the anti-methanogenic compound 3NOP, reported

that there was no significant difference compared to control. In our study the significant increases of carbon dioxide in cows fed both levels of *Asparagopsis* suggest that eructation is one of the ways to expel metabolic carbon dioxide. When adjusted for feed intake or milk yield, carbon dioxide emissions showed small differences.

3.5.3 Effect of macroalgae on dry matter intake

Some differences in DMI were observed and this could be due to the greater mineral concentration in *Asparagopsis*, which may be less palatable, especially at higher level of inclusion in the TMR. As a direct consequence of reduced DMI, high inclusion level cows were found to have a lower milk yield and milk protein percentage when compared to the control group (Table 3-2). Optimum inclusion levels are likely to change with the concentration of active compounds in the macroalgae used and method of incorporation in the diet. Further research is needed to determine how best *A. armata* could be delivered to the cows in order to maintain DMI more effectively than the molasses mix used in this study. In addition, studies that used CH_4 analogues as feed additives, specifically BCM, mixed with cottonseed meal found similar results in cattle and lambs (Sawyer et al., 1974; Tomkins et al., 2009). However, decreased DMI when dosing ruminants with BCM have been reported. For example, McCrabb et al. (1997) reported 8% DMI reduction in growing steers when BCM was mixed into cottonseed meal. Tomkins et al. (2009) found that 0.60 g/100 kg live weight of BCM inclusion was sufficient for a 94% reduction in CH_4 with no change in feed intake whereas McCrabb et al. (1997) used 2.4 g/100 kg live weight to obtain a 100% reduction in CH_4. Chalupa (1977), who observed reductions in DMI while feeding BCM, hypothesized that these changes may be due to

increased hydrogen concentrations in the rumen, impaired B12 production, or even taste aversion to the additive. Adjusted feed conversion efficiency increased in cows fed diets with the higher level of *A. armata* compared to control (Table 3-2) mainly due to the combination of reduced DMI while holding milk production above what is expected for the level of feed intake. Further work needs to be conducted to investigate if increased efficiency will be maintained over a full lactation.

3.5.4 Bromoform in algae and animal product

The main anti-methanogenic compound in *Asparagopsis*, bromoform, is naturally produced by phytoplankton and seaweeds in the ocean (Wever and van der Horst, 2013). Human consumption of high levels of bromoform could be hazardous, so the US EPA (2008) has set drinking water regulations on bromoform consumption to 80 mg/L. Milk produced by cows fed the low and high *Asparagopsis* additive were in the range of 0.11e0.15mg/L, which is over 500 times lower than the maximum standard. However, other minerals, iodine in particular, could be present in greater quantities in milk from cows fed macroalgae additive. Processing of the macroalgae to remove minerals may be necessary to support the attainment of the U.S. Food and Nutrition Board's recommended daily allowances without exceeding tolerable upper limits.

3.6 Conclusions

The potential of the macroalgae *Asparagopsis* to reduce CH_4 emissions shown in in vitro studies was investigated in vivo using dairy cattle. Adding *Asparagopsis* at 0.5% of diet OM resulted in reductions of 26.4% in CH_4 production, 20.5% in CH_4 yield (adjusted

for feed intake) and 26.8% in CH_4 intensity (adjusted for milk production) without compromising milk yield or intake. Increasing the inclusion level to 1% resulted in reductions of 67.2% CH_4 production, 42.6% CH_4 yield, and 60.0% CH_4 intensity. However, feed intake and milk yield were also reduced. Bromoform concentration in milk was not significantly different in cows that consumed macroalgae compared to control. Other mineral concentrations in milk may be increased so some processing may be necessary for *Asparagopsis* to be used as a feed additive effectively. The implication of this study is that enteric CH_4 emissions could potentially be halved by using seaweed as a feed additive to dairy cattle.

3.8 Tables and Figures

Table 3-1. **Ingredients and chemical composition of the basal experimental diet (% of DM)**

Ingredients	Basal Diet	*Asparagopsis armata*
% Dry matter		
Organic matter	91.3	49.6
Crude protein	17.6	18.3
Neutral detergent fiber	30.1	27.2
Acid detergent fiber	21.2	10.9
Fat	4.65	0.32
Total digestible nutrients	70.9	30.5
Lignin	5.04	2.83
Calcium	1.14	4.47
Phosphorus	0.44	0.27
Sodium	0.24	9.36
Magnesium	0.33	1.38
Parts per million		
Iron	415	1188
Manganese	60.0	62.3
Zinc	68.0	66.3
Copper	13.0	13.3

Table 3-2. Effect of *Asparagopsis armata* inclusion at 0.5% (LS) and 1% (HS) on dry matter intake (DMI), body weight, milk production, and milk composition of dairy cows.

	Treatment Groups			
Item	**Control**	**LS**	**HS**	**SEM**
Animal (kg)				
DMI	27.9[a]	24.9[b]	17.3[c]	1.29
Initial body weight	720	732	737	24.9
Body weight change	31.0[a]	32.7[a]	21.3[b]	3.23
adj.FCE[1]	1.29[a]	1.55[a]	2.24[b]	0.10
Milk production				
Milk yield (kg)	36.2[a]	37.2[a]	32.0[b]	2.20
Fat (%)	3.98	3.84	3.71	0.13
Protein (%)	3.12[a]	3.01[ab]	2.93[b]	0.06
Lactose (%)	4.74	4.75	4.69	0.04
Solids non-fat (%)	8.65	8.55	8.40	0.08
MUN (mg/dl)	16.7	15.1	15.2	1.79
SCC (x 103/ml)	126	100	129	30.9
Bromoform µg/L	0.11	0.15	0.15	0.03

[1]adj.FCE accounts for average body weight changes = (Milk yield (kg) / DMI (kg))*(Final body weight (kg) /Initial body weight (kg))

[a,b,c]Means within row with different superscripts differ ($P < 0.05$)

Table 3-3 Estimates of methane reduction through the use of feed additives.

Feed additive	N	Methane Reduction (%)	P value	Reference
3NOP	11	-39.0	0.002	Dijkstra et al. (2018)
Essential Oils	4	-14.8	0.172	Beauchemin et al. (2006)
Fibrolytic enzymes	4	+27.0	0.223	Chung et al. (2012)
Tannins	4	-3.7	0.018	Staerfl et al. (2012)
Grape pomace	2	-18.6	0.051	Moate et al. (2014)
Monensin	11	-6	0.065	Appuhamy et al. (2013)
Nitrate	43	-39.5	0.001	van Zijderveld et al. (2011)
Saponins	3	-16.6	0.018	Mao et al. (2010)
Yeast	9	-9.9	0.015	Chung et al. (2011)
Yucca	12	-2.0	0.172	(van Zijderveld et al., 2011a)
A. armata	1	-46.7 (average)	<0.0001	This study

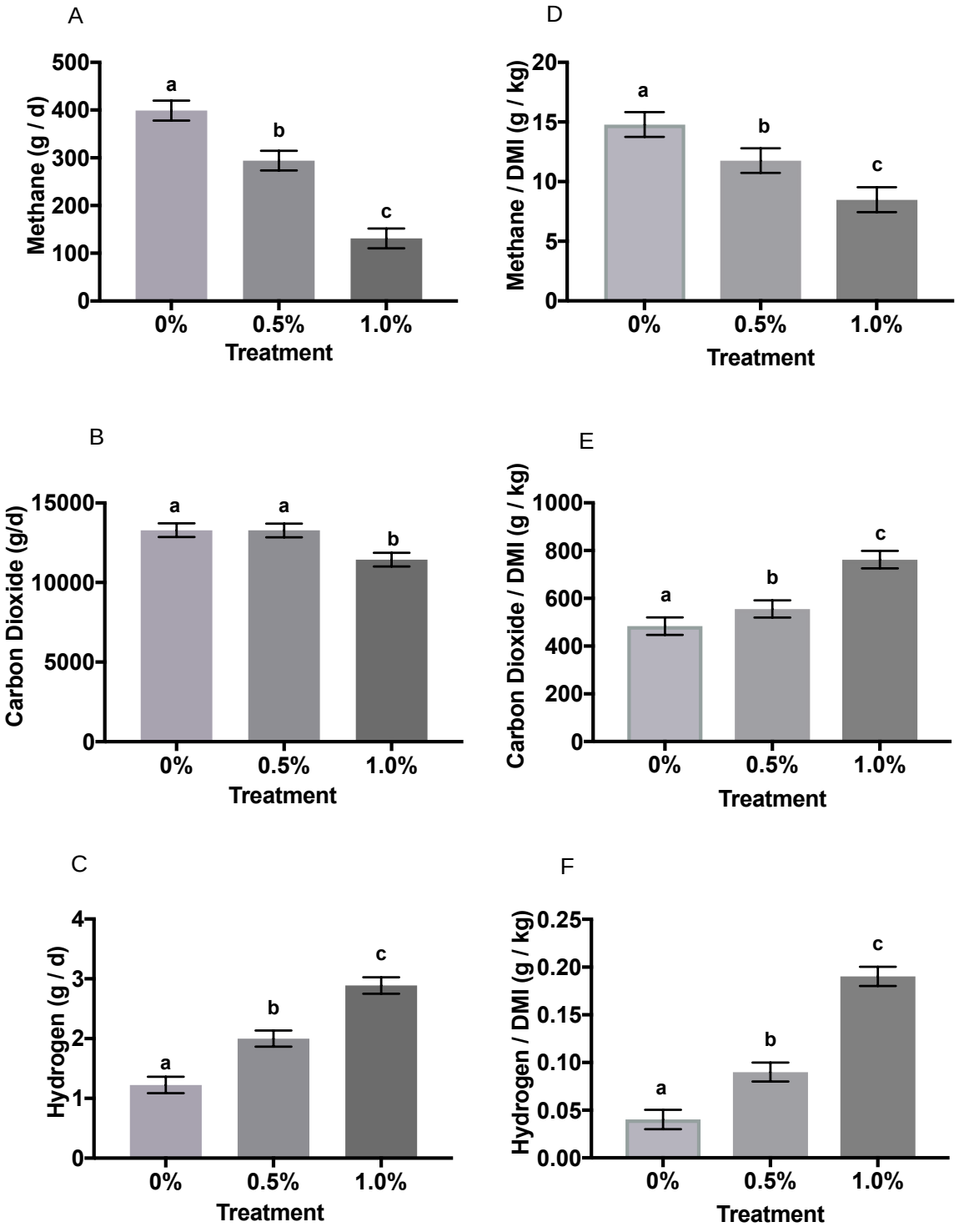


Figure 3-1. Means, standard deviations, and statistical differences of methane, hydrogen, and carbon dioxide production, g/d, (A,B,C) and yield, g/kg dry matter intake (DMI), (D,E,F) for 0%, 0.5%, and 1% *A. armata* inclusion. Means within a graph with different alphabets differ ($P < 0.05$)

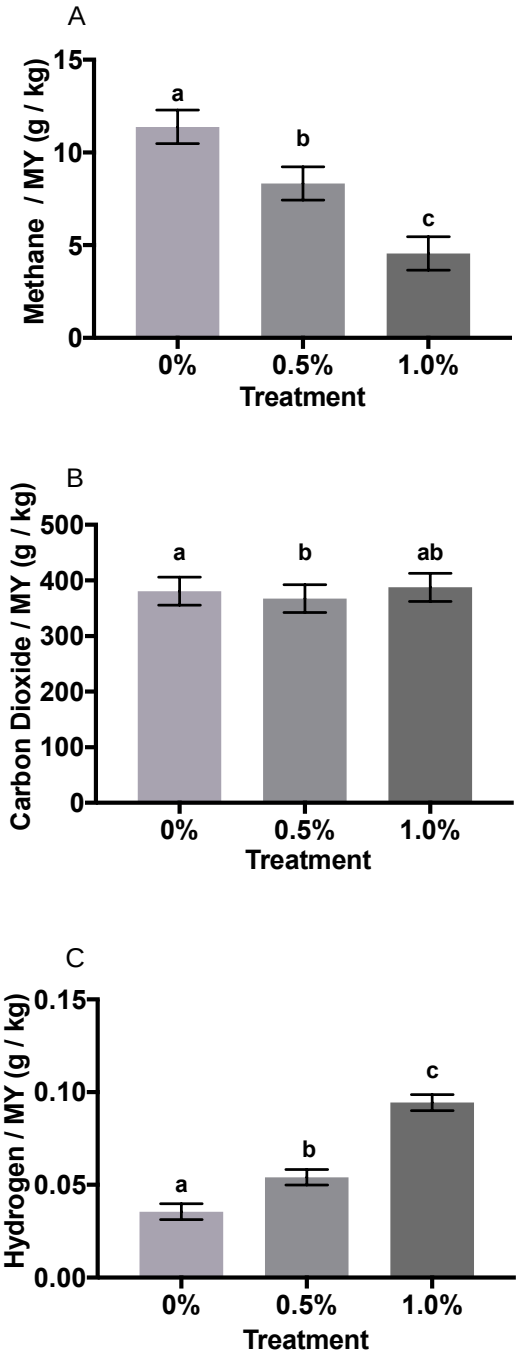


Figure 3-2. Means and standard deviations of methane (A) , hydrogen (B), and carbon dioxide (C) intensity (g/kg milk yield (MY)), for cows supplemented with 0, 0.5, and 1% *A. armata*. Means within a graph with different alphabets differ ($P < 0.05$)

Chapter 4: Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers.

Roque et al. (2021) *Plos One*

4.1 Abstract

The red macroalgae (seaweed) *Asparagopsis spp*. has shown to reduce ruminant enteric CH_4 production up to 99% *in vitro.* The objective of this study was to determine the effect of *Asparagopsis taxiformis* on CH_4 production (g/day per animal), yield (g CH_4/kg dry matter intake (DMI)), and intensity (g CH_4/kg ADG); average daily gain (ADG; kg gain/day), feed conversion efficiency (FCE; kg ADG/kg DMI), and carcass and meat quality in growing beef steers. Twenty-one Angus-Hereford beef steers were randomly allocated to one of three treatment groups: 0% (Control), 0.25% (Low), and 0.5% (High) *A. taxiformis* inclusion based on organic matter intake. Steers were fed 3 diets: high, medium, and low forage total mixed ration (TMR) representing life-stage diets of growing beef steers. The Low and High treatments over 147 days reduced enteric CH_4 yield 45 and 68%, respectively. However, there was an interaction between TMR type and the magnitude of CH_4 yield reduction. Supplementing low forage TMR reduced CH_4 yield 69.8% (P <0.01) for Low and 80% (P <0.01) for High treatments. Hydrogen (H_2) yield (g H_2/DMI) increased (P <0.01) 336 and 590% compared to Control for the Low and High treatments, respectively. Carbon dioxide (CO_2) yield (g CO_2/DMI) increased 13.7% between Control and High treatments (P =0.03). No differences were found in ADG, carcass quality, strip loin proximate analysis and shear force, or consumer taste preferences. DMI tended to decrease 8% (P =0.08) in the Low treatment and DMI decreased 14% (P <0.01) in the High treatment. Conversely, FCE tended to increase 7%

in Low (P =0.06) and increased 14% in High (P <0.01) treatment compared to Control. The persistent reduction of CH_4 by *A. taxiformis* supplementation suggests that this is a viable feed additive to significantly decrease the carbon footprint of ruminant livestock and potentially increase production efficiency.

4.2 Introduction

Livestock production, particularly ruminants, contributes to anthropogenic greenhouse gas (GHG) emissions globally. These emissions are estimated to be 7.1 Gt carbon dioxide (CO_2) equivalents annually which accounts for approximately 14.5% of the global anthropogenic GHG emissions (Gerber et al., 2013). The majority of GHG emissions from livestock production is in the form of CH_4, which is produced largely through enteric fermentation and to a lesser extent manure decomposition. Enteric CH_4 emissions not only contribute to total agricultural GHG emissions but also represent an energy loss amounting up to 11% of dietary energy consumption (Moraes et al., 2014). Therefore, reducing enteric CH_4 emissions decreases the total agricultural contribution to climate change and can improve productivity through conservation of feed energy. There is potential for mitigation of enteric CH_4 emissions through a variety of approaches with a focus on the use of feed additives, dietary manipulation and forage quality (Hristov et al., 2013b).

Feed additives used in CH_4 mitigation can either modify the rumen environment or directly inhibit methanogenesis resulting in lower enteric CH_4 production (g/day per animal) and yield (g/kg dry matter intake [DMI]). Reductions in CH_4 production of beef cattle, through the direct inhibition of methanogenesis, have been reported for feed

additives at 22, 93, and 98% for short-chain nitro-compounds (3-nitrooxypropanol; 3-NOP, (Dijkstra et al., 2018)), synthetic halogenated compounds (Tomkins et al., 2009), and naturally synthesized halogenated compounds in seaweed (Kinley et al., 2020), respectively. The compound 3-NOP inhibits the enzyme methyl-coenzyme M reductase (MCR) which catalyzes the final step in methanogenesis in rumen archaea (Duin et al., 2016). Halogenated CH_4 analogs, such as bromoform, act on the same methanogenesis pathway by binding and sequestering the prosthetic group required by MCR in order to form CH_4 (Smith et al., 1962; Wood et al., 1968; Johnson et al., 1972). Some haloalkanes are structural analogs of CH_4, and therefore competitively inhibit the methyl transfer reactions that are necessary in CH_4 biosynthesis (Ermler et al., 1997; Liu et al., 2011). These CH_4 analogues include BCM, bromoform, and chloroform and have been proven to be most effective for reducing CH_4 production. A 93% reduction of CH_4 was shown in Brahman cattle with a feed inclusion of BCM at 0.30 g/100 kg LW twice daily for 28 days, however feed intake, weight gain, carcass quality or feed efficiency were not statistically different (Tomkins et al., 2009). Conversely, Abecia et al. (2012) reported that the inclusion of BCM at 0.30 g/100 kg once per day decreased CH_4 production 33% and increased milk production 36%. The authors speculated that increased milk production in BCM treated cows could be attributed to a shift to more propionate production in the rumen, which is a hydrogen (H_2) sink and provides more energy compared to other volatile fatty acids. However, long-term efficacy of CH_4 analogues in the rumen remains to be confirmed. For example, Tomkins et al. (2009) reported a second experiment resulting in a 57.6% CH_4 reduction after 30 days of treatment which is far less than the reductions found during the first 28 days. Additionally, chloroform fed to fistulated dairy

cows was effective at reducing enteric CH_4 production through reduced abundance and activity of methanogenic archaea, but only over a 42-day period (Knight et al., 2011).

Types of feedstuffs can also impact CH_4 production by providing different substrates to microbial populations which are the drivers of volatile fatty acid (VFA) production in the rumen. There are ways to influence the types of VFA produced in the rumen by changing the types of feed in the diet (Van Soest, 1994; Russell, 1997). This is important for two reasons; first VFAs are utilized as an energy source for animal productivity and second VFA pathways, such as the production of propionate, are able to utilize reducing equivalents that normally would be shifted to methanogenesis (Blaxter and Clapperton, 1965; Johnson and Johnson, 1995). Concentrates contain non-structural carbohydrates, such as starch and sugar, that are rapidly fermented which drives pH down, negatively impacting methanogenic populations, and are an effective way to increase propionate production (Bannink et al., 2006; Bannink et al., 2008). Forages contain structural carbohydrates, such as neutral detergent fiber (NDF), and have been linked to increased CH_4 production (Niu et al., 2018). As dietary NDF increases, rumen pH also increases resulting in preferential production of acetate over propionate, which generates reducing equivalents that are then used in the methanogenesis pathway (Hungate, 1966; Janssen, 2010). Fiber content in feeds play a significant role in CH_4 production, including impacting the efficacy of anti-methanogenic compounds, such as 3-NOP and bromoform that specifically target MCR (Dijkstra et al., 2018). This hypothesis is based on the assumption that when high grain diets are fed, NDF decreases and ruminal MCR concentration is likely lowered thus granting greater efficacy for anti-

methanogenic compounds to target a greater proportion of MCR which results in greater CH_4 reductions (Vyas et al., 2018).

Some red seaweeds are anti-methanogenic, particularly the genus *Asparagopsis*, due to their capacity to synthesize and encapsulate halogenated CH_4 analogues, such as bromoform and dibromochloromethane, within specialized gland cells as a natural defense mechanism (Paul et al., 2006a). In a screening process to identify CH_4 reduction potential of select macroalgae in Australia, *Asparagopsis taxiformis* was demonstrated to be the most promising species with a 98.9% reduction of CH_4 when applied at 17% OM in vitro (Machado et al., 2014). Although that level of inclusion of seaweeds is not practical for livestock production, subsequent studies demonstrated effective inclusion levels below 2.0% OM for *Asparagopsis* in vitro (Kinley et al., 2016b; Kinley et al., 2016a) without affecting total VFA concentrations or substrate digestibility. There are only two published studies that measured CH_4 reduction by supplementing *Asparagopsis* in cattle diets. Reductions in CH_4 as high as 98% were reported when *A. taxiformis* (containing 6.6 mg bromoform/g DMI) was supplemented at 0.2% OM in a high concentrate feedlot TMR (Kinley et al., 2020). In dairy, a 67% CH_4 reduction was observed when *Asparagopsis armata* (at 1.3 mg bromoform/g DMI) was supplemented at 1% OM over a two-week feeding period (Roque et al., 2019b). The differences in efficacy between the two studies were the concentration of bromoform in the naturally variable wild harvested seaweed and diet formulation (high grain versus low grain) (Kinley et al., 2020). *A. taxiformis* reduces CH_4 more effectively compared to similar inclusions of pure bromoform in vitro probably be due to multiple anti-methanogenic CH_4 analogues working synergistically in the macroalgae (Machado et al., 2018). Furthermore, *A. taxiformis* synthesizes multiple

anti-methanogenic CH_4 analogues such as bromo- and iodo- methanes and ethanes (Lanigan, 1972) and that methanogen species are differentially sensitive to CH_4 inhibitors (Ungerfeld et al., 2004).

For adoption of the seaweed by industry it is crucial that meat quality be maintained or improved. As with any feed additive, feeding *A. taxiformis* to livestock has the potential to alter meat quality, tenderness, taste, and consumer acceptability. Marbling, for instance, directly impacts flavor and juiciness and it has been shown that marbling can directly influence consumer preference with some willing to pay a premium (Killinger et al., 2004).

We hypothesize that a significant anti-methanogenic effect of *A. taxiformis* would 1.) persist throughout introduction, transition, and finishing periods in a typical beef feedlot scenario, 2.) have no detrimental effects on animal productivity or meat quality and 3.) not contain bromoform residues within the meat and liver would be present.

4.3 Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Protocol No. 20803).

4.3.1 Study design, animals, and diets

Twenty-one Angus-Hereford cross beef steers, blocked by weight, were randomly allocated to one of three treatment groups: 0% (Control, n = 7), 0.25% (Low, n = 7), and 0.5% (High, n = 6) inclusion rates of *A. taxiformis* based on OM intake. The unbalanced number of steers between treatment groups was due to an unexpected animal injury

during the last three weeks of the trial to which all data from this steer was removed from statistical analysis. The steers used in this study were obtained from the Shasta Livestock Auction Yard (Cottonwood, CA), all of which were sourced from the same ranch, and were approximately 8 months of age weighing approximately 352 ± 9 kg at the start of the trial. Each steer was randomly assigned to an individual pen, fitted with its own feed bunk, and were fed twice per day at 0600 and 1800 hours at 105% of the previous day's intake.

The experiment followed a completely randomized design, with a 2-week covariate period, used as a baseline period, before treatment began followed by 3-week data collection intervals for 21-weeks; a total of 147 days (Figure 4-1). During data collection intervals, alfalfa pellets offered through the gas measuring device (GreenFeed system, C-Lock, Inc., Rapid City, SD) were included as part of daily feed intake. Steers were fed 3 diets during the study; high (starter diet; 63 days), medium (transition diet; 21 days), and low (finisher diet; 63 days) forage TMRs, which are typical life-stage TMRs of growing beef steers (Table 4-1). Samples from the three diets and alfalfa pellets were collected once per week and bags of *A. taxiformis* were randomly sampled and analyzed (Table 4-2) for dry matter, acid detergent fiber, NDF, lignin, starch, crude fat, total digestible nutrient and mineral content (Cumberland Valley Analytical Services, Waynesboro, PA). Steers were offered water ad libitum.

The *A. taxiformis* used as a feed additive was provided by Commonwealth Scientific and Industrial Research Organization (CSIRO) Australia. The seaweed was collected during the gametophyte phase from Humpy Island, Keppel Bay, QLD (23o13'01"S, 150o54'01"E) by Center for Macroalgal Resources and Biotechnology of James Cook University, Townsville, Queensland, Australia. Once collected, the *A.*

taxiformis was frozen, stored at −15 °C, then freeze dried at Forager Food Co., Red Hills, Tasmania, Australia, and later ground using a Hobart D340 mixer (Troy, OH, USA) and 3mm sieve. Total seaweed inclusion ranged from 46.7 to 55.7 g/day for Low and 76.1 to 99.4 g/day for High treatment. The seaweed used in the study contained bromoform at a concentration of 7.8 mg/g dry weight as determined by Bigelow Analytical Services (East Boothbay, ME, USA). To increase palatability and adhesion to feed, 200 ml of molasses and 200 ml of water was mixed with the *A. taxiformis* supplement, then the molasses-water-*A. taxiformis* mixture was homogenously incorporated into the TMR, by hand mixing, for each treatment animal. The Control group also received 200 ml of both molasses and water with their daily feed to ensure *A. taxiformis* was the only difference between the three treatments.

4.3.2 Sample collection and analysis

CH_4, CO_2, and H_2 gas emissions from steers were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD, USA). Gas emissions were measured during the covariate (baseline) period and experimental period during weeks 3, 6, 9, 12, 15, 18, and 21. In each measurement period, gas emission data were collected during 3 consecutive days as follows: starting at 0700, 1300, and 1900 hours (sampling day 1); 0100, 1000, and 1600 hours (sampling day 2); and 2200 and 0400 hours (sampling day 3). Eructated gas samples from each steer were taken at random across each treatment group. The GreenFeed machine was manually moved to each steer pen where the steer was allowed to enter the machine by choice and induced to eat from the machine for 3 to 5 minutes, followed by a 2-minute background gas sample collection. One GreenFeed unit was used

for all gas emissions samples and took approximately 140 minutes to complete each timepoint. The GreenFeed system was calibrated before each measurement period with a standard gas mixture containing (mol %): 5000 ppm CO_2, 500 ppm CH_4, 10 ppm H_2, 21% O_2 and nitrogen as a balance (Air Liquide America Specialty Gases, Rancho Cucamonga, CA). Recovery rates for CO_2, CH_4, and H_2 observed in this study were within +/− 3% of the known quantities of gas released. Alfalfa pellets were offered at each sampling event as bait feed and was kept below 10% of the total DMI during each 3-day measurement period. The composition of alfalfa pellets is shown in Table 4-2. Feed residuals were collected daily before the morning feeding to determine the previous day's intake. Feed intake and feed costs were recorded daily and bodyweight (BW) was measured once weekly, using a Silencer Ranch Model hydraulic squeeze chute (Dubas Equipment Stapleton, NE) equipped with a scale, at 0500 before morning feeding to reduce variability due to gut fill.

After the feeding trial was completed, all 20 steers were sent to a USDA-inspected commercial packing plant (Cargill Meat Solutions, Fresno, California) for slaughter. On the day of slaughter, steers were marked and followed throughout the process. On the first day, livers were collected, placed in individually labelled freezer bags and stored on dry ice until placed in a −20°C freezer. Carcasses were aged for 48 hours in a large cooler and then graded by a certified USDA grader. Directly after grading, carcasses were sent to fabrication where the strip loin from the left side of each carcass was cut and saved for further analysis. All 20 strip loins were vacuum packed then stored on ice and transported back to the University of California, Davis where they were cryovac packaged and stored at 4°C in dark for 14 days. After 14-day of aging, strip loins were cut into steaks (2.45 cm

thickness) and individually vacuum packaged and stored at −20°C. Samples of steaks and livers were analyzed by Bigelow Analytical Services (East Boothbay, ME, USA) for bromoform concentrations following a modified protocol described by Paul et al. (2009) [25]. The limits of bromoform detection and quantification were 0.06 mg/kg and 0.20 mg/kg, respectively. Steaks were also analyzed for proximate analysis by Midwest Labs (Omeha, Nebraska) for moisture (AOAC 950.46), protein (AOAC 992.15), fat (AOAC 991.36), ash (AOAC 900.02, 920.155, 920.153), calories (21 CFR P101.9), carbohydrates (100 – Moisture – Protein – Fat - Ash), and iodine (USP 233) concentration.

To test for objective tenderness, slice shear force (SSF) and Warner-Brazler shear force (WBSF) were measured following the protocol described by (AMSA, 2016). One steak from each animal was thawed overnight and cooked to an internal temperature of 71°C. Within 1 to 2 minutes after cooking, the SSF were measured using machine texture analyzer (TMS Pro Texture Analyzer, Food Technology corporation, Sterling, VA, USA) with a crosshead at the speed of 500 mm/minute. To test WBSF, cooked steaks were cooled at 4°C overnight, and then four cores were cut using WEN 8-inch 5 Speed Drill Press from one steak from each animal parallel to the muscle fiber orientation. The WBSF was measured using the TMS Pro texture analyzer with a Warner Bratzler blade (2.8 mm wide) and a crosshead at speed of 250 mm/minute. The average peak forces for all four cores were recorded.

A consumer sensory panel was conducted at UC-Davis. Strip steaks were thawed at 4°C for 24 hours then cooked to an internal temperature of 71°C using a George Foreman clamshell (Spectrum Brands, Middleton, WS, USA). Internal temperature was

taken from the geometric center of each steak using a K thermocouple thermometer (AccuTuff 351, model 35100, Cooper-Atkins Corporation, Middlefield, CT, USA). Following cooking, steaks were rested for 3 minutes then cut into 1.5 cm3 pieces. Each steak was randomly assigned a unique three digit number, placed into glass bowls covered in tin foil then stored in an insulated food warmer (Carlisle model PC300N03, Oklahoma, OK, USA) for longer than 30 minutes prior to the start of each sensory evaluation session. A total of 112 participants evaluated steak samples during one of the 5 sessions held over a 4-day period. Each participant evaluated a total of three steak samples, one from each treatment group, with a minimum of two 1.5 cm3 pieces per steak. Each participant was asked to evaluate tenderness, flavor, juiciness, and overall acceptance using a 9-point hedonic scale (1 = Dislike extremely and 9 = Like extremely).

4.3.3 Statistical analysis

Statistical analysis was performed using R statistical software (version 3.6.1; The R Foundation for Statistical Computing, Vienna, Austria). The linear mixed-effects models (lme) procedure was used with the steer as the experimental unit. GreenFeed emission data were averaged per steer and gas measurement period, which was then used in the statistical analysis. The statistical model included treatment, diet, treatment × diet interactions, and the covariate term, with the error term assumed to be normally distributed with mean = 0 and constant variance. Individual animal was used as random effect, whereas all other factors were considered fixed. Data was analyzed as repeated measures with an autoregressive 1 correlation structure. Statistical significance was established when $P \leq 0.05$ and a trend at $0.05 > P \leq 0.10$. The consumer sensory

evaluation data were analyzed using the Kruskal-Wallis test. The Dunn's test with P-value adjustment following Bonferroni methods was used for post-hoc pair-wise comparisons. Dry matter intake (DMI) and cost per kg of gain (CPG) data was averaged by week and used in the statistical analysis. Average daily gain (ADG) was calculated by subtracting initial BW from final BW then dividing by the number of experimental days for each diet regimen and the duration of the study (i.e. 63 days on high forage (starter) TMR, 21 days on medium forage (transition) TMR, then 63 days on low forage (finisher) TMR with total study duration of 147 days). Feed conversion efficiency (FCE) was calculated by dividing ADG by DMI for each diet regimen and the duration of the study. Carbon Dioxide (CO_2), CH_4, and H_2 emissions are reported as production (g / day), yield (g / kg DMI), and intensity (g / kg ADG).

4.4 Results

4.4.1 Gas parameters

The emissions as production (g/day), yield (g/kg DMI), and intensity (g/kg ADG) of CH_4, H_2, and CO_2 gases from the steers in the three treatment groups (Control, Low, and High) are presented in Figure 4-2 (for the duration of the trial) and Table 4-3 (divided by the three diet regimes). Inclusion of *A. taxiformis* in the TMR had a significant linear reduction in enteric CH_4 production, yield, and intensity. For the duration of the experimental period, CH_4 production, yield and intensity declined by 50.6 and 74.9%, 45 and 68%, and 50.9 and 73.1% for Low and High treatments, respectively, compared to Control. Hydrogen production, yield, and intensity significantly increased by 318 and 497%, 336 and 590%, and 380 and 578% in the Low and High treatments, respectively,

for the duration of the experiment. Carbon dioxide (CO_2) production and intensity factors were not affected by either Low or High treatments, however, CO_2 yield was significantly greater in High treatment compared to Control (P = 0.03).

An interaction was observed between diet formulation and magnitude of CH_4 reduction and H_2 formation for production, yield, and intensity factors (Table 4-3). CH_4 production, yield, and intensity in steers on the high forage TMR and supplemented with *A. taxiformis* reduced by 36.4 and 58.7%, 32.7 and 51.9%, and 36.9 and 56.4% for Low and High treatments, respectively. Hydrogen production, yield, and intensity increased by 177 and 360%, 198 and 478%, and 256 and 524% for the Low and High treatments, respectively. CH_4 production, yield, and intensity in steers fed the medium forage TMR and supplemented with *A. taxiformis* was reduced by 51.8 and 86.8%, 44.6 and 79.7%, and 54.4 % and 82.4%% for the Low and High treatments, respectively. Furthermore, H_2 production, yield and intensity significantly increased by 326 and 535%, 404 and 753%, and 341 and 626% for the Low and High treatments, respectively. Steers fed low forage TMR and supplemented with *A. taxiformis* reduced CH_4 production, yield, and intensity by 72.4 and 81.9%, 69.8 and 80.0%, and 67.5 and 82.6% for Low and High treatments, respectively. Additionally, H_2 production, yield, and intensity increased by 419 and 618%, 503 and 649%, and 566 and 559% for the Low and High treatments, respectively. No significant differences were found in CO_2 production, yield, or intensity in any of the three diets.

4.4.2 Animal production parameters

Dry matter intake (DMI), average daily gain (ADG), feed conversion efficiency (ADG/DMI; FCE) and cost per gain ($USD/kg weight gain; CPG) as impacted by treatment groups (Control, Low, and High) for the entire experimental period is presented in Table 4-4 and for the individual TMRs in Table 4-5. Initial BW, final BW, carcass weight and total weight gained are shown in Table 4-4. During the entire experiment (Table 4-4), DMI in Low treatment tended ($P = 0.08$) to decrease by 8% and High treatment DMI significantly reduced by 14% ($P < 0.01$) whereas no significant effects were observed in ADG by either Low or High treatment groups when compared to Control. With the reduction of DMI in Low and High treatments and similar ADG among all 3 treatments, FCE tended to increase 7% ($P = 0.06$) in Low treatment and increased 14% ($P < 0.01$) in High treatment. No significant differences between initial BW, final BW, total gains, CPG or carcass weight were found between Control and treatment groups. While no significant differences were found in CPG, there was a $0.37 USD/kg gain differential between High and Control and $0.18 USD/kg gain differential between Low and Control.

Decreases in DMI were also found over the three different TMR diets (Table 4-5) where steers fed the high and medium forage TMR and the High treatment decreased their DMI 18.5 ($P = 0.01$) and 18.0% ($P < 0.01$), respectively. No significant effects were observed in ADG, CPG, or FCE by the Low or High treatment groups during the individual TMR diets. Additionally, cost differentials for High treatment were $0.29, $0.40, and $0.34 USD/kg gain and for Low treatment were $0.15, $0.49, and $0.34 USD/kg gain for the high, medium, and low forage TMRs, respectively.

4.4.3 Carcass and meat quality parameters

There was no statistical difference between treatment groups for rib eye area (Table 4-6). No effects were found between Control, Low, and High treatments in moisture, protein, fat, ash, carbohydrates, or calorie content of strip loins (Table 4-6). The average WBSF values for the Control, Low and High groups were 2.81, 2.66 and 2.61 kg, respectively. Additionally, the SSF averages were measured as 17.1 for Control, 16.75 for Low and 17.4 kg for High treatments. No significant differences ($P > 0.05$) were found in shear force resistance among treatment groups. Mean scores of all sensory attributes (tenderness, juiciness, and flavor) by consumer panels were not significantly different ($P > 0.05$) among treatment groups (Table 4-6). The taste panel considered all steaks, regardless of treatment group, to be moderately tender and juicy. This was consistent with the taste panel stating that they moderately liked the flavor of all steaks regardless of treatment group. There was no difference ($P > 0.05$) in overall acceptability among treatment groups. There was a linear increase in iodine concentrations in both Low ($P < 0.01$) and High ($P < 0.01$) compared to Control. Iodine concentrations for the Control treatment group were below detection levels, which was set at 0.10 mg/g (Table 4-6). However, 5 out of 7 steers in Low treatment group had iodine levels above the detection level with a treatment average of 0.08 mg/g ($P < 0.01$). All 6 steers in the High treatment group were found to contain iodine levels above the detection level with concentration levels ranging between 0.14 – 0.17 mg/g with a mean of 0.15 mg/g ($P < 0.01$). Bromoform concentrations for all treatment groups were below detection levels, which were 0.06 mg/kg.

4.5 Discussion

4.5.1 Enteric CH_4 production, yield, and intensity

This study demonstrated that dietary inclusion of *A. taxiformis* induces a consistent and considerable reduction in enteric CH_4 production from steers on a typical feedlot style diet. Enteric CH_4 is the largest contributor to GHG emissions from livestock production systems. Significant reductions in CH_4 yield, which is standardized by DMI, when *Asparagopsis* is supplemented to beef cattle diets has been established in this study and are similar to the reductions found in previous studies (Li et al., 2018; Roque et al., 2019b; Kinley et al., 2020). While CH_4 intensities have been previously reported for dairy cows fed *A. armata* (Roque et al., 2019b), this is the first study to measure CH_4 intensity differences in beef cattle fed *A. taxiformis*. Intensity reports are important to determine the amount of CH_4 being produced per unit of output for ruminant livestock systems. There is a concern that feed additives and other CH_4 reducing agents decrease in efficacy over time (Knight et al., 2011). This study provided evidence that the seaweed inclusion was effective in reducing CH_4 emissions, which persisted for the duration of the study of 147 days (Figure 4-3). Notably, until this study the longest exposure to *A. taxiformis* had been demonstrated for steers in a study ending after a 90-d finishing period (Kinley et al., 2020). To date, only three in vivo studies have been published using *Asparagopsis spp*. to reduce enteric CH_4 emissions in feedlot Brangus steers (Kinley et al., 2020), lactating dairy cattle (Roque et al., 2019b), and sheep (Li et al., 2018). All studies show considerable yet variable reductions in enteric CH_4 emissions. The differences in efficacy are likely due to levels of seaweed inclusion, formulation of the diets, and differences in seaweed quality based on bromoform concentrations.

It has been previously hypothesized that NDF levels can also influence the rate at which CH_4 is reduced with the inclusion of inhibitors (Dijkstra et al., 2018; Vyas et al., 2018). In the current study, the magnitude of reductions in CH_4 production were negatively correlated (r2 = 0.89) with NDF levels in the 3 diet regimens that contained 33.1% (high forage), 25.8% (medium forage), and 18.6% (low forage) NDF levels. Enteric CH_4 production was reduced 32.7, 44.6 and 69.8% in steers on the Low treatment and 51.9, 79.7, and 80.0% on High treatment with high, medium and low forage TMRs, respectively. The low forage TMR, containing the lowest NDF levels, was the most sensitive to the inclusion of *A. taxiformis* with CH_4 reductions above 70% at equivalent inclusion levels compared to the higher forage TMRs. Vyas et al. (2018) showed similar trends of greater CH_4 reduction potential in high grain, low NDF, diets in combination with the anti-methanogenic compound 3-NOP. It has been hypothesized to increase efficacy by a reduction in rumen MCR concentration when low NDF is fed, thus increasing the MCR targeting capability of the anti-methanogenic feed additive. An 80.6% reduction of CH_4 yield in sheep fed diets containing 55.6% NDF, however, the level of *A. taxiformis* intake by the sheep was unclear but was offered at 6 times the High treatment in our study (Li et al., 2018). A 42.7% reduction in CH_4 yield was observed in lactating dairy cattle fed a diet containing 30.1% NDF at 1% inclusion rate of *A. armata* (Roque et al., 2019b). The high forage TMR in our study had a similar NDF level to the dairy study, however, had approximately double the reduction of CH_4, even when consuming 50% less seaweed. These differences relate to a large degree to the quality of seaweed in terms of the concentration of bromoform, which was 1.32 mg/g in the dairy study (Roque et al., 2019b)

compared to 7.82 mg/g in the current study. The same collection of *A. taxiformis* was used in a previously published in vivo study focused on Brangus feedlot steers for a duration of 90 days (Kinley et al., 2020). This seaweed had bromoform concentration of 6.55 mg/g, which was marginally lower than our study and may be due to variation in the collection, sampling, analysis techniques, or storage conditions. Despite the marginally lower bromoform concentration in the seaweed and using 0.20% inclusion rate of *A. taxiformis* on OM basis, the CH_4 yield was reduced by up to 98% in Brangus feedlot steers. The diet used by Kinley et al. (2020) included 30.6% NDF, which was similar to our high fiber diet. The greater efficacy of *A. taxiformis* in that study could be due to collective feed formulation differences such as the energy dense component of barley versus corn, which is typical of Australian and American feedlots, respectively. Additionally, it could be due to beneficial interaction with the ionophore, monensin, that was used in the Australian study. Monensin has not been used in any other feed formulation in other in vivo studies with the inclusion of *Asparagopsis* species. Use of monensin in diets has shown to decrease CH_4 yields by up to 6% in feedlot steers while also having an enhanced effect in diets containing greater NDF levels (Appuhamy et al., 2013). A potential enhancing interaction of the seaweed with monensin is of great interest and further investigation will elucidate this potential that could have significant beneficial economic and environmental impact for formulated feeding systems that use monensin.

4.5.2 Enteric hydrogen and carbon dioxide emissions

Increases in H_2 yield have typically been recorded when anti-methanogenic feed additives are used, and with the addition of *Asparagopsis* species in dairy cattle (1.25-

3.75 fold) (Roque et al., 2019b) and Brangus feedlot steers (3.8-17.0 fold) (Kinley et al., 2020). Similar increases in H_2 yield have been reported in feed additives that reduce enteric CH_4 emissions targeting methanogens. For example, in lactating dairy cows supplemented with 3-NOP, H_2 yield increased 23-71 fold (Hristov et al., 2015). BCM fed to goats increased H_2 (mmol/head per day) 5-35 fold, while chloroform fed to Brahman steers increased H_2 yield 316 fold (Mitsumori et al., 2012; Martinez-Fernandez et al., 2016). Although feeding *Asparagopsis spp*. increased overall H_2 yield (Figure 4-4), the magnitude was considerably lower (1.25 to 17-fold) compared to alternative CH_4 reducing feed additives (5-316 fold), with similar levels of reductions in CH_4. This indicates that there may be a redirection of H_2 molecules that would otherwise be utilized through the formation of CH_4 and redirected into different pathways that could be beneficial to the animal. For example, increased propionate to acetate concentrations have been recorded *in vitro* (Machado et al., 2016b; Roque et al., 2019a) and *in vivo* (Kinley et al., 2020) using *A. taxiformis* and BCM (Denman et al., 2015) for CH_4 mitigation which may indicate that some of the excess H_2 is being utilized for propionate production.

Similar to the lactating dairy cattle study with 1% *A. armata* supplementation (Roque et al., 2019b), the CO_2 yield in the current study also increased in the High group (Figure 4-2). However, in the current study no differences in CO_2 production were seen. Typically, CO_2 and H_2 are used in the methanogenesis pathway to form CH_4 thus increases in exhaled CO_2 is expected with the addition of anti-methanogenic compounds. The fact that only CO_2 yield increased may be due to decreases in DMI, which could have reduced overall CO_2 generation thus resulting in no increases seen in CO_2 production factors.

4.5.3 Animal production parameters

Dry matter intake reductions observed in this study were consistent with previous studies in lactating dairy cows where decreases in DMI were found to be 10.7 and 37.9% at 0.50 and 1.0% inclusion rate of *A. armata* (Roque et al., 2019b), respectively. Decreases in DMI have also been reported in cattle fed other anti-methanogenic feed additives in a linear dose-response fashion. For example, Tomkins et al. (2009) reported 3 to 19% reductions in DMI in steers supplemented with BCM at dosages between 0.15 and 0.60 g/100 kg live weight. Additionally, Martinez-Fernandez et al. (2016) found 1.7 to 15% reductions in DMI when chloroform was directly applied to the rumen, through a rumen fistula, at dosages between 1 to 2.6 g/100g liveweight. In contrast, Kinley et al. (2020) reported no significant differences in DMI at the highest *A. taxiformis* level of 0.20%. However, the inclusion level was less than our study's lowest inclusion rate, so based on previous experiment's observation of reduced DMI in a dose-response manner (Roque et al., 2019b), it was expected to have lower effect on DMI. Decreases in DMI are normally associated with lower productivity due to lower levels of nutrients and dietary energy consumed. However, there was no significant difference in ADG between steers in the High treatment and Control (average 1.56 kg/day) groups despite consuming 14% less feed. The results were in agreement with a previous study (Roque et al., 2019b), in which milk production was not compromised at a 0.5% OM inclusion level despite reductions in DMI. The FCE (ADG/DMI) increased significantly in High treatment group, suggesting that inclusion rates of *A. taxiformis* at 0.5% improves overall feed efficiency in growing beef steers. Since a large proportion of on farm costs is the purchase of feed, an

improved feed efficiency is particularly exciting for producers to decrease feed costs while also producing the same amount of total weight gains. Total gains were between 224 kg (Low) to 236 kg (High) combined with an average cost differential of ~$0.18 USD/kg gain (Low) and ~$0.37 USD/kg gain (High). A producer finishing 1000 head of beef cattle has the potential to reduce feed costs by $40,320 (Low) to $87,320 (High) depending on seaweed dosage. While the CPG in this study were not statistically significant, this may be due to low animal numbers in each treatment and warrants further investigation on a larger feedlot setting to reduce animal variability.

4.5.4 Bromoform and iodine residues

Bromoform is the major active ingredient responsible for CH_4 reduction when fed to cattle (Machado et al., 2016a). However, high levels of bromoform are suspected to be hazardous for humans and mice. While bromoform intake limits are yet to be defined for cattle specifically, the US EPA (2017) has suggested a reference dose for bromoform, an estimated level of daily oral exposure without negative effects, to be 0.02 mg/kg BW/day for human consumption. It is essential that food products from livestock consuming the seaweed are confirmed as safe for consumption and that bromoform residues are not transferred to the edible tissues and offal of bovines at levels detrimental to food safety. Previous studies have demonstrated that bromoform was not detectable in the kidney, muscle, fat deposits, blood, feces, and milk in either Brangus feedlot steers (Kinley et al., 2020), dairy cows (Roque et al., 2019b), or sheep (Li et al., 2018). Strip loin and liver samples from steers were collected and in agreement with previous studies, no bromoform was detected in this study.

The National Academies of Sciences, Engineering, and Medicine recommendations for daily iodine intake in growing beef cattle is 0.5 mg/kg DMI and maximum tolerable limit is 50 mg/kg DMI (NASEM, 2016). Based on DMI intake from steers in this study, recommended daily iodine intake levels were 5.2 mg/day and 4.85 mg/day and maximum limits are 521 mg/day and 485 mg/day for Low and High treatment groups, respectively. The iodine level in the *A. taxiformis* fed in the current study contained 2.27 mg/g, therefore, maximum daily intake of seaweed iodine was 106 – 127 mg/day and 173 – 225 mg/day for the Low and High treatment groups, respectively. While these levels do not exceed maximum tolerable limits, they exceed daily iodine intake recommendations for cattle, therefore it was appropriate to test for iodine residue levels in meat used for human consumption. The US Food and Nutrition Board of the National Academy of Sciences has set a tolerable upper intake level (UL) for human consumption of foods, which is defined as the highest level of daily intake that poses no adverse health effects (Trumbo et al., 2001). The iodine UL ranges between 200 ug/day to 1,100 ug/day depending on age, gender, and lactation demographics. Strip loins tested for iodine residues had levels of 0.08 and 0.15 ug/g from steers in treatments Low and High, respectively. These iodine residues are far under the UL limits for human consumption. For example, UL for a person under 3 years of age is 200 ug/day meaning that this person would have to consume more than 2.5 kg/day and 1.3 kg/day of meat from a Low and High steers, respectively, to reach the UL. An adult over the age of 18 has an UL of 1,100 ug/day and would have to consume more than 13.8 kg/day and 7.3 kg/day of meat from a Low and High steers, respectively, to reach their UL of iodine intake. At the inclusion levels and iodine concentration of *A. taxiformis* used in this study the margin of safety is

extremely high and the likelihood of iodine toxicity from consuming the meat is extremely low. The health hazards of consistently consuming any meat at such levels is much higher than the iodine toxicity risks. Low level iodine in meat may provide for provision of iodine to populations that suffer from natural iodine deficiency, a common issue in populations with low intake of marine food products (Pearce, 2017).

4.5.5 Carcass and meat quality parameters

Marbling scores ranged from 410 – 810 while all carcasses, regardless of treatment, graded as either choice or prime. The value placed on tenderness in the marketplace is high and has even been found that consumers are likely to pay premiums for more tender beef (Miller et al., 2001). Many factors can greatly affect meat tenderness, such as animals' age at slaughter, breed, marbling, and diet (Warner et al., 2010; Corbin et al., 2014; Blank et al., 2017). All animals used in the current study were of similar age and breed. Additionally, no significant difference in average marbling scores was observed. The lack of significant differences seen in these factors further supports that the supplementation of *A. taxiformis* at the current dosage did not impact the tenderness of meat. This is in agreement with Kinley et al. (2020)'s meat taste assessment where no differences between Control and *A. taxiformis* supplemented beef cattle were found. The combination of both the current study as well as the Kinley et al. (2020) study indicates that the supplementation of *A. taxiformis* at or below 0.5% to cattle does not significantly impact overall meat quality nor alter the sensory properties of the steaks.

4.6 Conclusions

This study demonstrated that the use of *A. taxiformis* supplemented to beef cattle diets reduced enteric CH_4 emissions for a duration of 21 weeks without any loss in efficacy. The efficacy was highly correlated with the proportion of NDF in the diet as demonstrated through the typical stepwise transition to a feedlot finishing diet formulation. Additionally, supplementing *A. taxiformis* had no measurable bromoform residues, no detrimental iodine residual effects in the product, and did not alter meat quality or sensory properties. Importantly, the use of *A. taxiformis* impacts DMI and not ADG, therefore increasing overall feed efficiency (FCE) in growing beef steers. This study also demonstrated a potential to reduce the cost of production per kg of weight gain. These feed cost reductions in combination with significantly reduced CH_4 emissions have a potential to transform beef production into a more economically and environmentally sustainable red meat industry.

Next steps for the use of *Asparagopsis* as a feed-additive would be to develop aquaculture techniques in ocean and land-based systems globally, each addressing local challenges to produce a consistent and high-quality product. Processing techniques are evolving with the aim of stabilizing as feed supplement and the economics of the supply chain. The techniques include utilization of already fed components as carriers and formats such as suspensions in oil which may be done using fresh or dried seaweed, and options in typical feed formulations such as mixtures are being explored (Magnusson et al., 2020). Transportation of the processed or unprocessed seaweed should be kept to a minimum, so cultivation in the region of use is recommended specially to avoid long-haul shipping.

4.8 Tables and Figures

Table 4-1. Ingredients of the experimental diet containing high, medium, and low forage concentrations (% of DM)

Ingredients	High forage	Medium forage	Low forage
Forage			
Alfalfa hay	35.0	25.0	5.00
Wheat hay	25.0	15.0	6.00
Dry distiller grain	12.0	14.0	6.00
Concentrate			
Rolled corn	20.0	37.0	72.0
Molasses	5.0	5.00	3.00
Fat	1.5	2.00	3.00
Urea	0.35	0.40	1.80
Beef trace salt[1]	0.32	0.32	1.00
Calcium carbonate	0.82	1.15	1.80
Magnesium oxide			0.20
Potassium chloride			0.50

[1]Beef Trace Salt sourced from A.L Gilbert (Oakdale, California) contains; salt manganous oxide, vegetable oil, zinc oxide, copper sulfate, ethylene, diamine dihydriodide, sodium selenite.

Table 4-2. Nutritional composition of experimental diets, *Asparagopsis taxiformis*, and alfalfa pellets

	High Forage	Medium Forage	Low Forage	Pellets	*A. taxiformis*
% Dry matter					
Organic matter	92.1	93.1	94.8	88.6	50.9
Crude protein	17.2	17.4	13.2	17.1	16.8
ADF	22.6	16.7	10.5	28.1	11.5
NDF	33.1	25.8	18.6	35.9	33.7
Lignin	4.08	3.05	1.73	6.16	4.08
Starch	16.9	25.0	46.7	0.90	0.35
Crude fat	4.92	6.04	6.77	3.02	0.63
Calcium	0.77	1.00	0.50	2.06	5.29
Phosphorus	0.33	0.38	0.28	0.24	0.18
Magnesium	0.38	0.38	0.23	0.37	0.81
Potassium	1.74	1.42	0.94	2.10	2.02
Sodium	0.18	0.25	0.30	0.20	6.34
Parts per million					
Iron	438	335	127	1508	8494
Manganese	61.7	56.0	64.0	88.0	142.5
Zinc	43.2	51.50	58.0	19.0	53.5
Copper	8.67	8.00	7.00	10.0	22.5

Chemical analysis was performed by Cumberland Valley Analytical Services, Waynesboro, PA

Table 4-3. Effect of *Asparagopsis taxiformis* inclusion at 0.25% (Low) and 0.5% (High) feed organic matter on enteric gas emissions using high-, medium-, and low- forage diets

		High Forage					Medium Forage					Low Forage				
Gas Emission Data		Control†	Low†	High‡	SEM*	P**	Control†	Low†	High‡	SEM*	P**	Control†	Low†	High‡	SEM*	P**
Methane																
Production	(g/day)	237[a]	151[b]	98.0[c]	11.4	<0.01	241[a]	116[b]	31.9[c]	15.3	<0.01	139[a]	38.4[b]	25.2[b]	11.4	<0.01
Yield	(g/kg DMI)	22.1[a]	14.9[b]	10.6[c]	1.02	<0.01	19.2[a]	10.6[b]	3.92[c]	1.36	<0.01	12.4[a]	3.75[b]	2.50[b]	1.02	<0.01
Intensity	(g/kg ADG)	150[a]	94.6[b]	65.3[c]	7.92	<0.01	176[a]	80.3[b]	31.0[c]	12.4	<0.01	88.6[a]	28.8[b]	15.4[b]	7.93	<0.01
Hydrogen																
Production	(g/day)	1.25[a]	3.48[b]	5.77[c]	0.44	0.01	1.38[a]	5.88[b]	8.76[c]	0.56	<0.01	0.97[a]	5.71[b]	6.94[b]	0.44	<0.01
Yield	(g/kg DMI)	0.12[a]	0.35[b]	0.68[c]	0.05	0.02	0.11[a]	0.55[b]	0.93[c]	0.06	<0.01	0.09[a]	0.53[b]	0.66[b]	0.05	<0.01
Intensity	(g/kg ADG)	0.60[a]	2.15[b]	3.77[c]	0.29	0.01	0.93[a]	4.11[b]	6.77[c]	0.42	<0.01	0.60[a]	4.00[b]	3.96[b]	0.29	<0.01
Carbon dioxide																
Production	(g/day)	7422	7399	7035	324	1.00	8393	8335	7185	365	0.67	7577	7770	7795	324	1.00
Yield	(g/kg DMI)	706	742	815	27.3	0.57	694	779	806	32.9	0.43	678	731	744	27.3	0.78
Intensity	(g/kg ADG)	4884	4658	4689	272	1.00	6460	5932	5874	442	1.00	4784	5507	4532	272	0.62

† Control and Low; n = 7 steers per treatment; ‡High; n = 6 steers

*Standard Error of the mean; standard error pooled across treatments.

**P values are pooled across treatments

[a,b,c] superscripts = note significant differences (P= <0.05) between treatment groups

Table 4-4. Effect of Asparagopsis taxiformis inclusion at 0.25% (Low), and 0.5% (High) feed organic matter on beef animal parameters over 21 weeks.

Animal Parameters		Control†	Low†	High‡	SEM*	P**
Initial BW	(kg)	357	348	350	9.21	0.78
Final BW	(kg)	589	572	587	11.1	0.73
Total gain	(kg)	232	224	236	6.09	0.72
ADG	(kg/day)	1.6	1.52	1.56	0.06	0.72
DMI	(kg/day)	11.3[a]	10.4[ab]	9.69[b]	0.29	0.04
FCE	(ADG/DMI)	0.14[a]	0.15[a]	0.16[b]	0.01	0.04
CPG	($/kg gain)	2.25	2.07	1.88	0.18	0.53
Carcass weight	(kg)	370	361	350	13.4	0.61

BW, body weight; ADG, average daily gain; DMI, dry matter intake; FCE, feed conversion efficiency; CPG, cost per gain

† Control and Low; n = 7 steers per treatment; ‡High; n = 6 steers

*Standard Error of the mean; standard error pooled across treatments.

**P values are pooled across treatments

[a,b,c] superscripts = note significant differences (P = <0.05) between treatment groups

Table 4-5. Effect of *Asparagopsis taxiformis* inclusion at 0.25% (Low) and 0.5% (High) feed organic matter on beef animal parameters using high, medium-, and low- forage diets.

	High Forage					Medium Forage					Low Forage				
Animal Parameters[1]	Control†	Low†	High‡	SEM*	P**	Control†	Low†	High‡	SEM*	P**	Control†	Low†	High‡	SEM*	P**
DMI (kg/day)	10.3[a]	9.69[ab]	8.40[b]	0.33	0.34	12.2[a]	10.8[ab]	9.99[b]	0.33	0.05	11.5[a]	10.8[ab]	10.7[b]	0.33	0.34
ADG (kg/day)	1.58	1.61	1.53	0.11	1.00	1.62	1.50	1.38	0.11	0.94	1.60	1.44	1.75	0.11	0.82
FCE (ADG/DMI)	0.15	0.17	0.18	0.01	0.78	0.13	0.14	0.14	0.01	1.00	0.14	0.13	0.17	0.01	0.63
CPG ($/kg ADG)	1.83	1.68	1.54	0.25	0.97	2.67	2.18	2.20	0.26	1.00	2.24	2.35	1.90	0.27	0.95

[1]DMI, dry matter intake; ADG, average daily gain, FCE, feed conversion efficiency; CPG, cost per gain

† Control and Low; n = 7 steers per treatment; ‡High; n = 6 steers

*Standard Error of the mean; standard error pooled across treatments.

**P values are pooled across treatments

[a,b,c] superscripts = note significant differences (P= <0.05) between treatment groups

Table 4-6 Effect of *Asparagopsis taxiformis* inclusion at 0.25% (Low) and 0.5% (High) on carcass quality, proximate analysis, shear force, and consumer panel preference.

Carcass Quality		Control†	Low†	High‡	SEM*	P**
Rib eye area	(cm)	28.7	28.4	26.9	0.37	0.65
Proximate Analysis						
Moisture	(g/100g)	53.9	55.4	55.3	1.70	0.86
Protein	(g/100g)	16.1	17.1	17.2	0.70	0.68
Fat	(g/100g)	29.1	26.1	26.3	2.20	0.73
Ash	(g/100g)	0.73	0.86	0.88	0.05	0.92
Carbohydrates	(g/100g)	0.24	1.01	0.42	0.38	0.57
Calories		327	307	307	17.7	0.79
Iodine	(PPM)	0[a]	0.08[b]	0.15[c]	0.02	<0.01
Shear Force						
Warner-Bratzler	(kgf)	2.81	2.66	2.61	0.24	0.82
Slice Shear Force	(kgf)	17.1	16.8	17.4	1.87	0.98
Consumer Panel [1]						
Tenderness		6.72	6.68	6.45	0.17	0.37
Juiciness		6.35	6.33	6.07	0.17	0.40
Flavor		6.63	6.34	6.24	0.15	0.10
Overall		6.66	6.36	6.46	0.16	0.26

† Control and Low; n = 7 steers per treatment; ‡High; n = 6 steers

*Standard Error of the mean; standard error pooled across treatments.

**P values are pooled across treatments

[a,b,c] superscripts = note significant differences (P = <0.05) between treatment groups

[1] A 9-point hedonic scale was used (1 = Dislike extremely and 9 = like extremely)

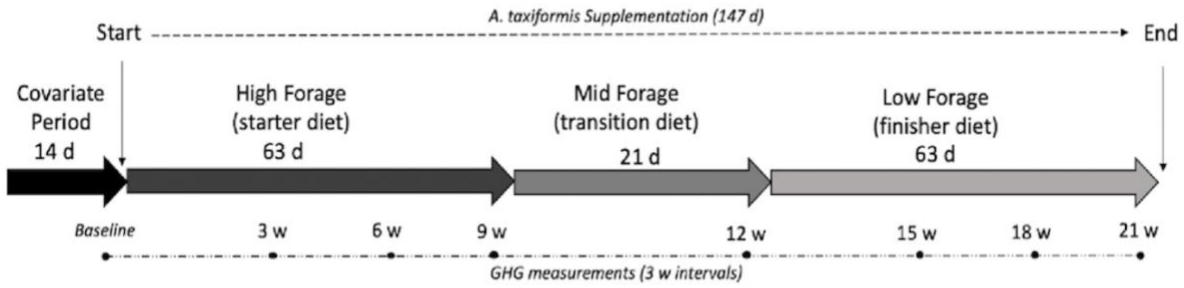


Figure 4-1. Experimental timeline including covariate period, *Asparagopsis taxiformis* implementation, dietary regime, and greenhouse gas measurement intervals.

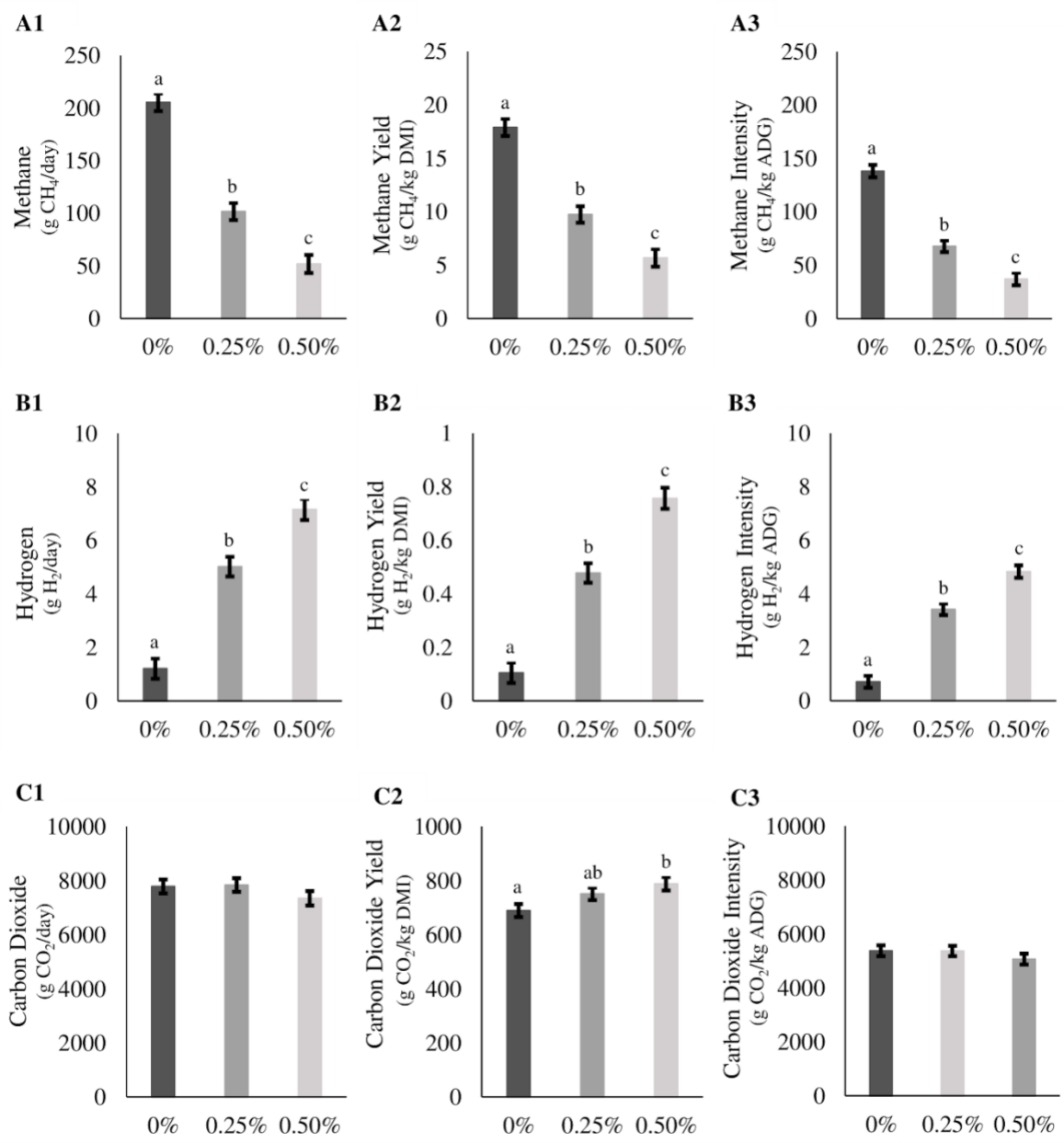


Figure 4-2. *Asparagopsis taxiformis* inclusion effects on methane, hydrogen and carbon dioxide emissions over a 147-day period. Means, standard deviations, and statistical differences of methane, hydrogen, and carbon dioxide production (g/d) (A1,B1,C1), yield (g/kg dry matter intake (DMI)) (A2,B2,C2), and intensity (g/kg average daily gain) (A3,B3,C3) for 0%, 0.25% (Low), and 0.50% (High) *Asparagopsis taxiformis* inclusion. Means within a graph with different alphabets differ ($P < 0.05$).

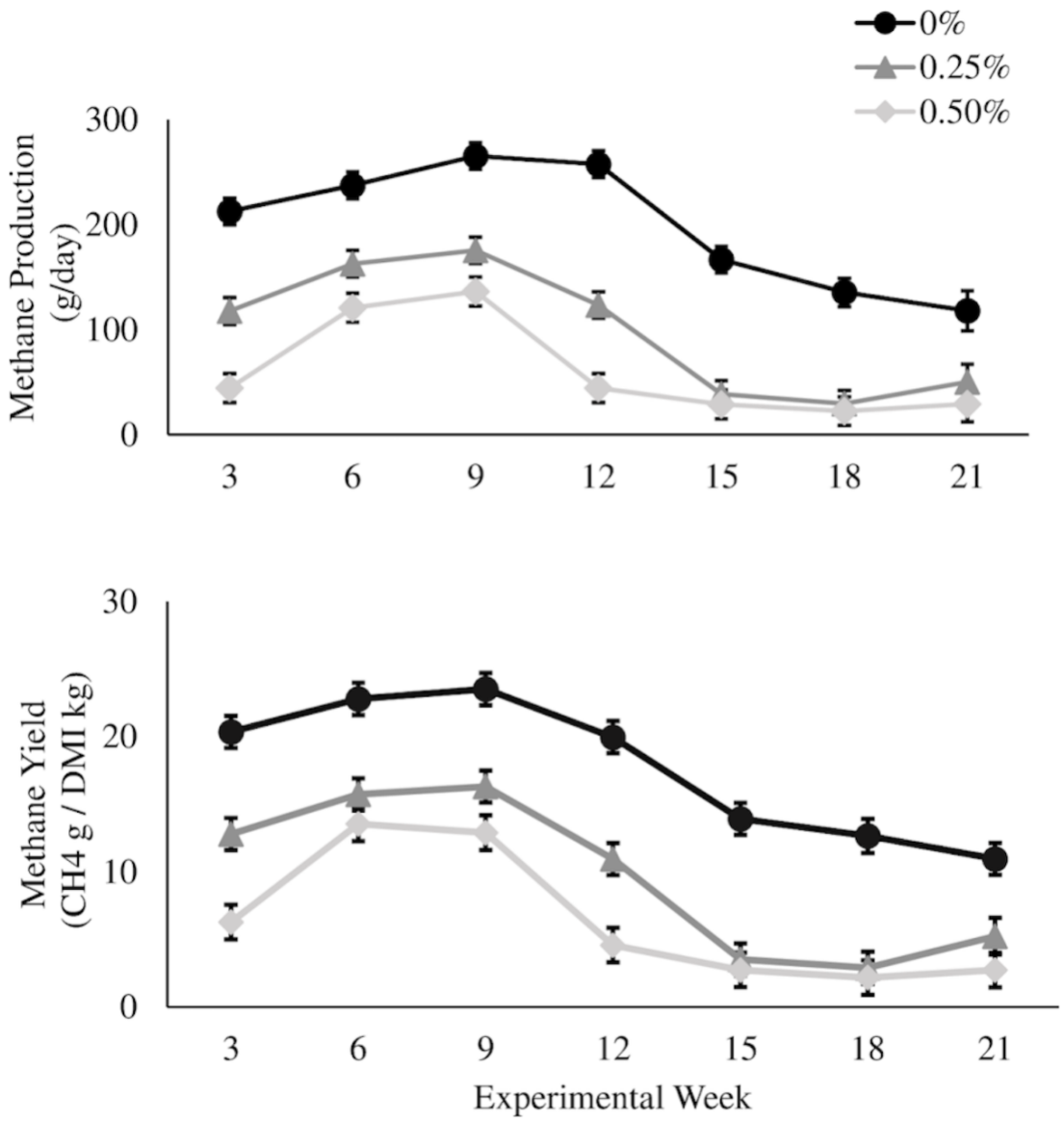


Figure 4-3. *Asparagopsis taxiformis* inclusion effects on methane emissions during the 21 week experimental period. Methane production [g CH_4/day] (A) and methane yield [g CH_4/kg DMI] (B) from beef steers supplemented with *Asparagopsis taxiformis* at 0%, 0.25%, and 0.5% of basal total mixed ration on an organic matter basis during the 21 week experimental period. Data points are treatment means for each gas collection timepoint and error bars represent standard errors.

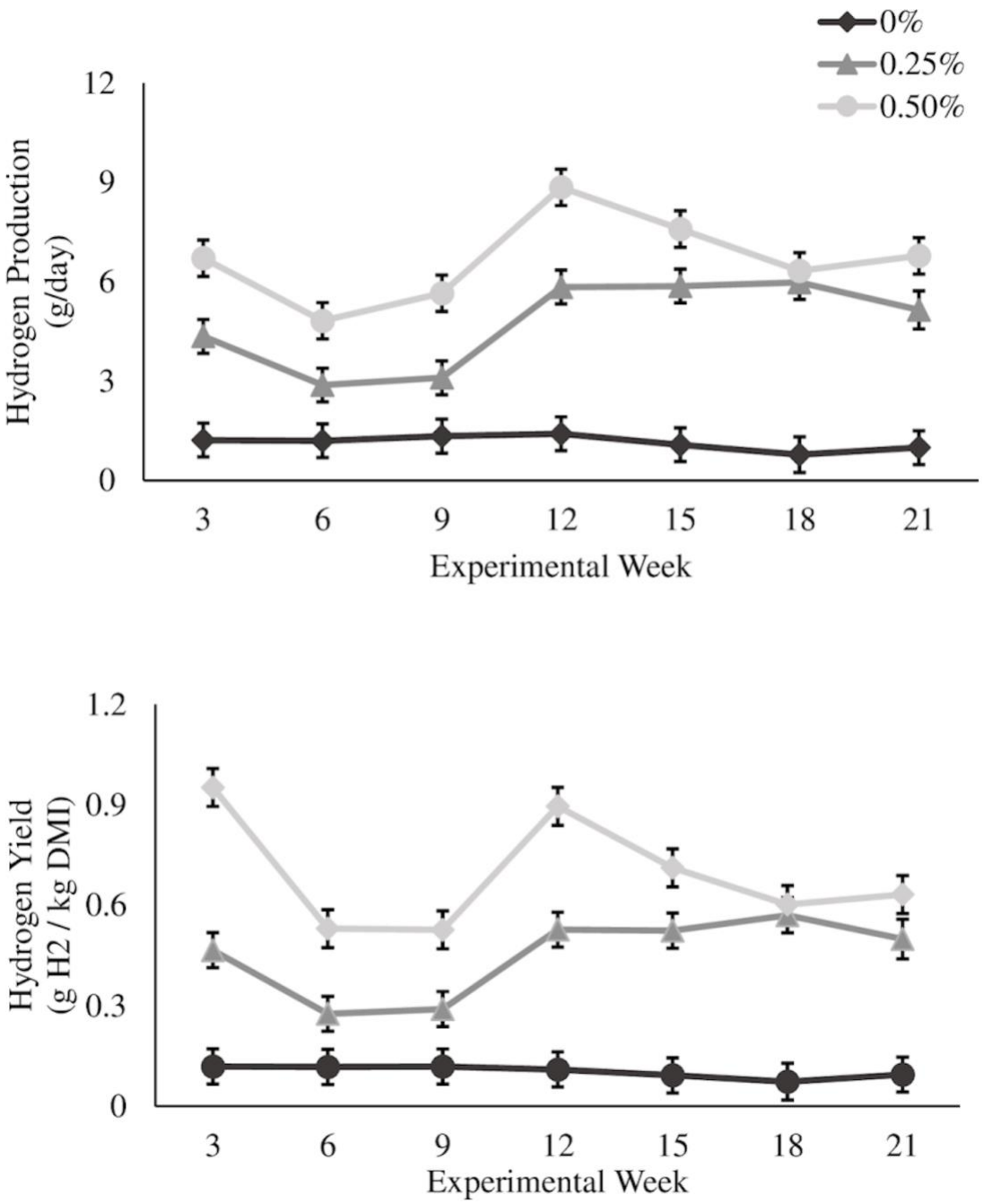


Figure 4-4. *Asparagopsis taxiformis* inclusion effects on hydrogen emissions during the 21-week experimental period. Hydrogen production [g H_2/day] (A) and Hydrogen yield [g H_2/kg DMI] (B) from beef steers supplemented with *Asparagopsis taxiformis* at 0% , 0.25% , and 0.5% of basal total mixed ration on an organic matter basis during the 21 week experimental period. Data points are treatment means for each gas collection timepoint and error bars represent standard errors.

Chapter 5: Insights into rumen microbiome molecular responses to the methane reducing macroalgae *Asparagopsis armata* using comparative omics analyses.

5.1 Abstract

Macroalgae belonging to the genus of *Asparagopsis* has shown to reduce CH_4 production during rumen fermentation when added to cattle diets. Additionally, supplementing cattle feed with *Asparagopsis armata (A. armata)* resulted in increased feed efficiency (milk production/dry matter intake). In this study, we utilized a combination of metagenomic and metatranscriptomic data generated from rumen fluid that was collected from lactating dairy cows fed a diet with (treatment) and without (control) *A. armata* added. Animals supplemented with *A. armata* showed 67% lower CH_4 yield and feed efficiency that was 74% higher than what was measured for the control group [Chapter 3]. Previous research indicates that the inhibition of CH_4 production does not necessarily coincide with a reduced abundance of methanogens *in vitro* [Chapter 3]. In this study, we performed comparative omics analyses to determine if the reduction of rumen CH_4 was caused primarily by a reduced abundance of methanogens or rather by the downregulation of methanogenic genes and pathways. Results suggest that the abundance of the methanogenic population remains stable and reduction of methanogenesis in the presence of *A. armata* is triggered by the promiscuous downregulation of several genes of the methanogenesis pathway.

5.2 Introduction

Ruminant livestock represents a significant source of anthropogenic CH_4, accounting for almost 30% of the CH_4 emissions, both within the United States and globally (Gerber, 2014). CH_4 is a greenhouse gas that is 28-fold more potent than carbon dioxide (CO_2) on a 100-year scale and, with increasing demand for products from ruminant animals, potent and sustainable CH_4 mitigation strategies are needed to reverse the trend of increased CH_4 emission from the livestock industry. Such successful strategies can only be developed when we have a mechanistic understanding of the molecular processes that build the foundation for these intervention strategies. Primary production of CH_4 from ruminant livestock occurs during the anaerobic fermentation process of the ruminant's feed. This process, which enables the removal of excess reducing equivalents such as hydrogen (H_2) (Janssen, 2010), is driven by the symbiotic activity of microbes, especially between the feed-degrading bacteria, protozoa, and fungi and the methanogenic archaea that inhabit the ruminants' foregut (i.e., the rumen). The microbial methanogenesis has been previously described in detail and three main pathways encoded by a set distinct genes were identified: hydrogenotrophic (carbon dioxide reduction), acetoclastic (acetate reduction), and methylotrophic (methanol and methylated amine reduction) methanogenesis (Costa and Leigh, 2014; Buan, 2018). On top of this fundamental metabolic understanding, a core methanogenic population has been identified for the rumen ecosystem with hydrogenotrophic methanogens, such as *Methanobrevibacter ruminantium* and *Methanobrevibacter gotschalkii,* making up approximately 74% of the methanogenic population (Henderson et al., 2015). Despite differences in substrate utilization, all methanogens utilize the methyl coenzyme M

reductase (MCR) enzyme to promote the formation of CH_4 during the last step in methanogenesis (Thauer, 2019), thus making this enzyme a particularly promising target for natural and engineered CH_4 mitigation strategies (Leahy et al., 2010).

Halogenated CH_4 analogues, such as bromoform (Lanigan, 1972), BCM (Tomkins et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012), chloroform (Knight et al., 2011; Martinez-Fernandez et al., 2016), and dichloromethane (Lanigan, 1972), have shown significant reductions of enteric methanogenesis, and these brominated and chlorinated CH_4 analogues are thought to inhibit CH_4 formation by binding and sequestering the prosthetic group of MCR (Smith et al., 1962; Wood et al., 1968; Johnson et al., 1972). While halogenated CH_4 analogues seem to be quite effective at reducing the production of enteric CH_4, it appears that the extent of reduction is specific to the type of inhibitor. It has been hypothesized that different methanogenic species may be differentially sensitive to these halogenated CH_4 analogues (Ungerfeld et al., 2004) and thus might not only suppress MCR but might also target other enzymes/genes within the methanogenesis pathway (Bauchop, 1967).

Interestingly, members of the genus *Asparagopsis*, a red seaweed that owes its red color to the presence of the pigment phycoerythrin (Dumay et al., 2015), synthesize and store multiple halogenated CH_4 analogues in vacuoles within the cell wall (Paul et al., 2006b; Machado et al., 2016a). Among these, macroalgae *A. armata* and *A. taxiformis* have been identified as particularly efficient in reducing enteric methanogenesis, with CH_4 reduction as high as ~70% in dairy cows (Roque et al., 2019a) and ~98% in beef steers

(Kinley et al., 2020), respectively. Moreover, the CH_4-mitigating effect of *Asparagopsis* was sustained in an extended animal trial over 21 weeks, while also increasing the feed efficiency of the animals, suggesting that the rumen microbiome may experiencing a metabolic shift that enables the animal to obtain energetic precursors without the production and release of CH_4 (Roque et al., 2021). *Asparagopsis taxiformis,* one of the most promising species, reduces *in vitro* methanogenesis at a greater efficacy compared to halogenated CH_4 analogues such as bromoform (Machado et al., 2018) and it has been hypothesized that this increased efficacy of the red seaweed may be due to multiple halogenated CH_4 analogues working synergistically, affecting multiple reactions within the methanogenesis pathway (Machado et al., 2018), whereas other intervention strategies are targeting primarily MCR. To elucidate if and which reactions that are part of the methanogenesis pathway may be downregulated, we performed a comparative analysis of the gene expression profile of the rumen microbiome of lactating dairy cows fed a control diet and the base diet with *A. armata* at an inclusion rate of 1% organic matter (OM) (Roque et al., 2019b). The obtained results provide strong evidence that *A. taxiformis* indeed targets numerous reactions in methanogenesis by downregulating several genes in the three major methanogenesis pathways. Furthermore, our data suggests that there is almost a complete shutdown of the methanotrophic CH_4 synthesis by *A. taxiformis* almost fully silencing gene expression of several key enzymes of this pathway, which is the dominant CH_4 production mechanisms in the rumen ecosystem.

5.3 Materials and Methods

All procedures were reviewed and approved through the University of California, Davis Institution for Animal Care and Use Committee (IACUC) under protocol number 20398. Details of the animal trial have been described previously by (Roque et al., 2019b). In brief, three sets of four cows (total n=12) were randomly assigned to one of three treatment groups (Control group: basal diet; Low Dose group: basal diet + 0.5% OM *Asparagopsis armata*; High Dose group: basal diet + 1% OM *A. armata*), then fed the allocated diet for 14 days during which milk production and components, dry matter intake (DMI), body weight (BW), feed conversion efficiency (FCE), CH_4, carbon dioxide (CO_2), and hydrogen (H_2) production were recorded. After the two-week feeding period ended, cows were fed the basal diet for 7 days (washout period), before treatments were randomly reassigned to a different set of cows. This ensured all 12 cows experienced each of the three treatment groups for a 14-day period. For the work described here, rumen fluid was collected from the Control and High Dose groups during the last day of the last period of experiment. The experimental design is outlined in Figure 5-1.

5.3.1 Sample Collection and Preparation

Two hours after feeding, cows were moved to a head gate where rumen fluid was collected using an oral stomach tube technique (Geishauser, 2019). An oral-ruminal stomach tube fitted with a perforated brass probe head (Anscitech Co Ltd. Wuhan, China) was inserted through the mouth and into the rumen at an insertion depth of 200 cm to ensure that samples were collected from the central portion of the rumen, providing for less variability between samples (Shen et al., 2012). During collection, the first 500 mL of sample was discarded to limit saliva contamination (van Gastelen et al., 2017). Approximately 200 mL of rumen fluid was captured in a 1 gallon prewarmed, insulated

cannister (Thermos, Schaumburg, IL), strained through four layers of cheese cloth into 100 mL centrifuge tubes, flash frozen in liquid nitrogen and then stored at -80°C to prevent sample degradation.

5.3.2 RNA extraction and sequencing

To obtain RNA, approximately 1 mL of frozen rumen fluid was thawed and homogenized using an 18-gauge needle and syringe, RNA was extracted and purified using the PureLink RNA Mini Kit (Invitrogen, San Diego, CA). Samples were then treated with DNA-Free Kit DNase treatment and removal (Invitrogen, Waltham, Massachusetts, USA) at room temperature for 15 minutes. RNA was quantified using a Bioanalyzer (Agilent, Santa Clara, CA) and RNA quality was evaluated using a sodium hypochlorite (6%) agarose gel as described previously (Aranda et al., 2012). All RNA samples were equally split in half then subjected to two different ribosomal RNA removal techniques [i.e., Ribo-Zero(TM) rRNA Removal Kit (Illumina, San Diego, CA, USA) and QIAseq FastSelect rRNA Removal Kit (Qiagen, Germantown, MD]. Both RNA sample quantities were determined using Qubit 3.0 Fluorometer (Invitrogen, Waltham, Massachusetts, USA)). JGI, RiboZero: rRNA was removed from 10 ng of total RNA using Ribo-Zero(TM) rRNA Removal Kit (Illumina, San Diego, CA, USA). Stranded cDNA libraries were generated using the Illumina Truseq Stranded mRNA Library Prep kit. The rRNA depleted RNA was fragmented and reversed transcribed using random hexamers and SSII (Invitrogen, Waltham, MA, USA) followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles of PCR. The prepared libraries were quantified using KAPA Biosystems' next-generation sequencing

library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flowcell, following a 2x151 indexed run recipe. JGI, FastSelect: The prepared libraries were quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flowcell, following a 2x151 indexed run recipe.

5.3.3 Statistical Analysis

Differential expression of gene/transcript data was analyzed using both the Fisher's Exact Test as well as using Bioconductor (Gentleman et al., 2004) software package EdgeR (Robinson et al., 2010). The Benjamini Hochberg Method (1995) was used to control for false positives during multiple comparisons. Raw gene counts were normalized by counts per million (CPM) for both tests. Additionally, EdgeR was used to automatically fit the data to a negative binomial generalized linear model (glm) for use in differential expression analysis between treatment and control (Robinson et al., 2010). No outliers were found in either technique or treatment group. To correct for multiple comparisons, statistical significance was determined at a P-value < 0.1.

5.4 Preliminary Results

5.4.1 Taxonomic variation of gene expression in response to Asparagopsis taxiformis

Metatranscriptomic sequencing summary for each cow, and for both Ribozero and FastSelect techniques, are described in Table 5-1. Distribution of gene expression across bacterial and archaea presented as percent proportion of bacterial and archaeal activity on the phylum level are shown in Figure 5-2. Gene expression of *Bacteroidetes, Firmicutes, Proteobacteria,* and *Fibrobacteres*, the four dominant rumen bacterial phyla, appears rather stable between control and treatment group with minor upregulation patterns in *Bacteroidetes* (67.9% vs 78.2%) and *Fibrobacteres* (5.9% vs 6.4%) and minor downregulation patterns in *Firmicutes* (10.3% vs 8.4%) and *Proteobacteria* (6.6% vs 4.4%). However, gene expression for the *Spirochaetes* phyla went from 4.6% in the control group to 1.96% in the treatment group. As expected, gene expression greatly decreased in the two main rumen methanogenic phyla of *Candidatus thermoplasmata* (3.04% vs 0.10%) and *Euryarchaeota* (0.67% vs 0.12%). Differential expression of gene transcripts can be visualized in Figure 5-3. In total, 38 genes were significantly upregulated and 409 genes were significantly downregulated in the treatment group compared to control.

5.4.2 Hydrogenotrophic methanogenesis

Comparative heatmaps were created based on differential expression analysis of the three methanogenesis pathways; hydrogenotrophic pathway, methylotrophic pathway, and acetogenic pathway (Figure 5-4). The hydrogenotrophic pathway utilizes hydrogen (H_2) and carbon dioxide (CO_2) to form CH_4 in a stepwise progression and utilizes enzyme complexes such as formylmethanofuran dehydrogenase (Fwd), formylmethanofuran-tetrahydromethanopterin (H_4MPT) N-formyltransferase (Ftr),

methenyl-H_4MPT cyclohyrdogenase (Mch), methylene-H_4MPT dehydrogenase (Mtd), 5,10-methenyl-H_4MPT hydrogenase (Hmd), methylene-H_4MPT reductase (Mer), and F_{420}-reducing hydrogenase (Frh). The first step includes the reduction of CO_2 to formylmethanofuran and utilizes genes that code for Fwd subunits. Four of the 8 genes coding for Fwd (*fwdABDF*) were found to be significantly downregulated ($P<0.1$), however all 8 Fwd coding genes in the treatment group were recorded at 0 read counts indicating a complete suppression of the first step in methanogenesis. Lack of statistical significance for the latter 4 genes (*fwdCHGE*) is likely due to low concentrations in control group gene expression which may indicate low importance of these genes for this step. The next two steps outlined for hydrogenotrophic methanogenesis is the conversion of formyl-methanofuran (Mfr) to formyl-H_4MPT, catalyzed by the Ftr enzyme, then from formyl-H_4MPT to Methenyl-H_4MPT, catalyzed by the Mch enzyme. The ftr and *mch* genes that encode for these two enzymes were not found in high concentrations in either the treatment or control groups, thus no significant difference was detected. The fourth step in methanogenesis is the reduction of methenyl-H_4MPT to methylene-H_4MPT catalyzed by either Mtd or Hmd enzymes, using reduced F_{420} as the electron donor. While either Mtd or Hmd enzymes can be used for this step, Mtd has been previously shown to be more active in hydrogen limited conditions (Goldman et al., 2009). The results of this study are in agreement with this previous finding in that *mtd* gene expression is far greater that *hmd* gene expression in the control group. When comparing control and treatment groups, *mtd* gene expression is completely downregulated with zero read counts found in the treatment group ($P = 0.05$). Gene expression for *mer,* which codes for the Mer enzyme responsible for the fifth step of hydrogenotrophic methanogenesis, also shows

complete suppression in the treatment group with zero read counts (P = 0.02). The last two steps in hydrogenotrophic methanogenesis are also shared by all three pathways and will be discussed in the universal gene subsection.

5.4.3 Methylotrophic methanogenesis

The methylotrophic pathway utilizes substrates such as trimethylamine, dimethylamine, monomethylamine, and methanol to form CH_4. All gene complexes associated with methylotrophic methanogenesis were significantly downregulated in the treatment group compared to control except for two genes *mtbA* and *mtmC*. More specifically, genes associated with trimethylamine metabolism, *mttB* and *mttC,* were downregulated 3.7- and 5.3-fold, respectively. Dimethylamine metabolism genes *mtbB,* and *mtbC* were downregulated 4.2-fold and 8.7-fold, respectively. Monomethylamine metabolism gene *mtmB* was downregulated by 9.3-fold. All genes associated with Methanol reduction *mtaA*, *mtaB*, *mtaC* were downregulated by 6.4-, 5.9-, and 7.2- fold, respectively.

5.4.4 Acetoclastic methanogenesis

The acetoclastic pathway utilizes acetate as a substrate to form CH_4. The first step in acetoclastic methanogenesis involves the assimilation of Acteyl-CoA synthesis from a core set of enzymes including Acs, Pta, and Acs and is commonly referred to as the “acetate switch” (Wolfe, 2005). The acetate switch is utilized in many metabolic pathways such as pyruvate metabolism, glucogenesis, VFA production, and CH_4 synthesis. Gene expression of *ackA, ACSS,* and *pta* was less affected by the supplementation of *A. armata*

with no significant downregulation observed. This is likely due to the transient nature of these genes expressed for metabolic pathways other than methanogenesis. However, one gene coding for a Cdh subunit, *cdhE,* is most often utilized only in the acetoclastic methanogenesis pathway was significantly downregulated by 8.6-fold in the treatment group when compared to control.

5.4.5 Universal genes associated with methanogenesis

Universal genes associated with the last two steps of methanogenesis are labelled as shared genes in Figure 5-4. Gene expression for all universal genes were significantly downregulated. The second to last step in any CH_4 formation pathway includes the utilization of methyl-H_4MPT transferase (Mtr) and is responsible for cleavage of the methyl group from methyl-H_4MPT in to methyl-2-mercaptoethanesulfonate (CH_3-S) attached to Coezyme M (CoM). Genes known to encode for methyl-H_4MPT transferase (Mtr) subunits were found to be completely suppressed by the inclusion of *A. armata* with zero read counts found in the treatment group. The last step in methanogenesis includes methyl-coenzyme M reductase (Mcr) and responsible for the conversion of methyl-S-CoM and N-7-mercaptoheptanoylthreonine phosphate (CoB-S-H) to CH_4 and mixed disulfide attached to Coenzyme B (CoB) and CoM. (CoB-S-S-CoM). This is the step that has been previously identified as one that halogenated CH_4 analogues are able to directly target. Genes that encode for methyl-Coenzyme M reductase enzyme (Mcr) subunits were significantly downregulated by 5.6-fold for *mcrA*, 17.5-fold for *mcrA2*, 4.3-fold for *mcrB* and *mcrG*. Universal genes also include those that encode for enzyme subunits that are responsible for electron bifurcation, between heterodisulfide reductase (Hdr) and F_{420}

non-reducing hydrogenase, thus enabling the recirculation of CoM and CoB. (Wagner et al., 2017). Transcription levels for universal genes responsible for electron bifurcation such as those that encode for heterodisulfide reductase (Hdr) subunits were downregulated by between 4.1 - 5.1-fold, F_{420} non-reducing hydrogenase (Mvh) subunits were downregulated by between 6.7 – 7.4-fold, and formate dehydrogenase beta subunit (*fdhB)* was downregulated by 6-fold.

5.5 Next Steps

To expand our understanding of the diversity and insights into the role of beneficial bacterial populations and methanogens in the rumen, we plan to assemble metagenome assembled genomes (MAGs) from our representative metagenome sample for both control and treatment. Additionally, we plan to map metatranscriptomic gene expression on to these MAGs to identify their specific responses to the supplementation of *Asparagopsis* macroalgae.

Table 5-1. Metatranscriptome assembly and annotation statistics

	Control							
Raw Data	**Ribozero**				**Fastselect**			
Cow #	**2634**	**2632**	**2555**	**2490**	**2634**	**2632**	**2555**	**2490**
Sequencing Project ID	1248797	1248796	1248795	1248794	1248797	1248796	1248795	1248794
Read Length	151	151	151	151	151	151	151	151
Raw Reads (millions)	60.5	125	46.4	203	153	1,259	63.9	113
Raw Sequence (Gbp)	9.14	18.8	7.01	30.7	23.14	190	9.65	17.0
Assembled Data								
Filtered Reads (millions, % raw)	11.3 (18.7%)	16.9 (13.5%)	7.86 (16.9%)	59.5 (29.3%)	25 (16.3%)	130 (10.3%)	9.61 (15.0%)	19.0 (16.8%)
Filtered sequence (Gbp)	1.71	2.55	1.19	8.98	3.81	19.7	1.45	2.86
% reads over Q30	95.6%	95.4%	95.4%	95.2%	94.7%	95.3%	94.0%	94.2%
Base Quality Score	36.3 +/- 3.69	36.2 +/- 3.72	36.2 +/- 3.73	36.2 +/- 3.79	36.12 +/- 4.01	36.2 +/- 3.75	36.0 +/- 4.30	36.0 +/- 4.21
% read pairs merged	84.5%	80.3%	86.1%	62.8%	75.1%	58.0%	85.5%	86.3%
Read GC	45.9% +/- 10.1	38.7% +/- 11.0	47.1% +/- 10.6	39.3% +/- 12.2	39.8% +/- 11.3	34.5% +/- 10.1	40.1% +/- 11.4	41.3% +/- 10.9
Potential Contaminants								
Adapters	15.2%	6.16%	12.7%	3.78%	8.95%	2.85%	12.7%	14.4%
rRNA	77.1%	80.6%	80.0%	67.2%	76.77%	81.5%	80.8%	77.2%
Mitochondria	0.00%	3.68%	0.00%	4.79%	1.70%	2.72%	1.62%	1.71%
Chloroplast	0.00%	10.2%	0.00%	8.07%	6.18%	3.56%	8.25%	9.97%
Total	77.1%	94.5%	80.0%	80.1%	84.7%	87.7%	90.7%	88.8%
Contig Assembly								
Contigs Assembled (thousands)	118	163	79.4	676	253	605	146	770
Contig Sequence (Mbp)	61.7	89.6	39.7	414	137	346	76.9	473
Contig N50 (thousands)	36.3	47.4	25.5	180	75	166	44.8	205
Max Contig length (bp)	13,409	13,921	11,381	31,727	14,759	15,870	13,021	31,744
Ratio of reads mapped to contigs	67.3%	83.6%	61.6%	83.7%	76.0%	92.3%	70.5%	85.0%

	Treatment							
Raw Data	**Ribozero**				**Fastselect**			
Cow #	**2553**	**2631**	**2623**	**2497**	**2553**	**2631**	**2623**	**2497**
Sequencing Project ID	1248799	1248801	1248800	1248798	1248799	1248801	1248800	1248798
Read Length	151	151	151	151	151	151	151	151
Raw Reads (millions)	171	131	146	96.2	99.5	90.1	106	79.8
Raw Sequence (Gbp)	25.8	19.8	22.0	14.5	15.0	13.6	15.9	12.0
Assembled Data								
Filtered Reads (millions, % raw)	23.7 (13.9%)	24.7 (18.9%)	35.5 (24.3%)	11.1 (11.5%)	10.4 (10.5%)	12.4 (13.8%)	13.9 (13.11%)	8.1 (10.2%)
Filtered sequence (Gbp)	3.58	3.73	5.35	1.67	1.56	1.87	2.10	1.22
% reads over Q30	94.8%	94.7%	94.6%	95.5%	94.12%	94.2%	94.1%	94.0%
Base Quality Score	36.1 +/- 3.96	36.1 +/- 4.02	36.1 +/- 4.07	36.3 +/- 3.65	36.0 +/- 4.23	36.0 +/- 4.20	36.0 +/- 4.27	36.0 +/- 4.33
% read pairs merged	74.4%	71.1%	70.8%	72.3%	84.3%	88.3%	86.4%	88.5%
Read GC	36.5 % +/- 11.0	40.8% +/- 12.4	38.2% +/- 12.1	35.1% +/- 10.6	38.2 +/- 10.3%	45.2% +/- 11.2	41.4% +/- 11.1	39.2% +/- 10.4
Potential Contaminants								
Adapters	3.12%	3.07%	3.73%	3.80%	14.9%	15.8%	17.1%	15.7%
rRNA	81.5%	76.5%	71.0%	83.2%	81.0%	74.7%	77.9%	81.3%
Mitochondria	4.16%	4.88%	3.52%	4.62%	2.13%	2.80%	2.30%	2.30%
Chloroplast	9.43%	9.42%	9.86%	9.94%	10.4%	10.8%	11.7%	10.7%
Total	95.1%	90.8%	84.4%	97.8%	93.5%	88.3%	91.9%	94.3%
Contig Assembly								
Contigs Assembled (thousands)	266	343	397	128	319	406	470	162
Contig Sequence (Mbp)	148	195	230	71.9	181	233	273	90.9
Contig N50 (thousands)	77.4	99.2	111	37.0	91.9	116	131	46.9
Max Contig length (bp)	18,702	21,755	31,272	13,794	18,064	22,373	37,416	18,898
Ratio of reads mapped to contigs	83.1%	78.1%	81.8%	80.4%	85.3%	78.7%	83.1%	82.1%

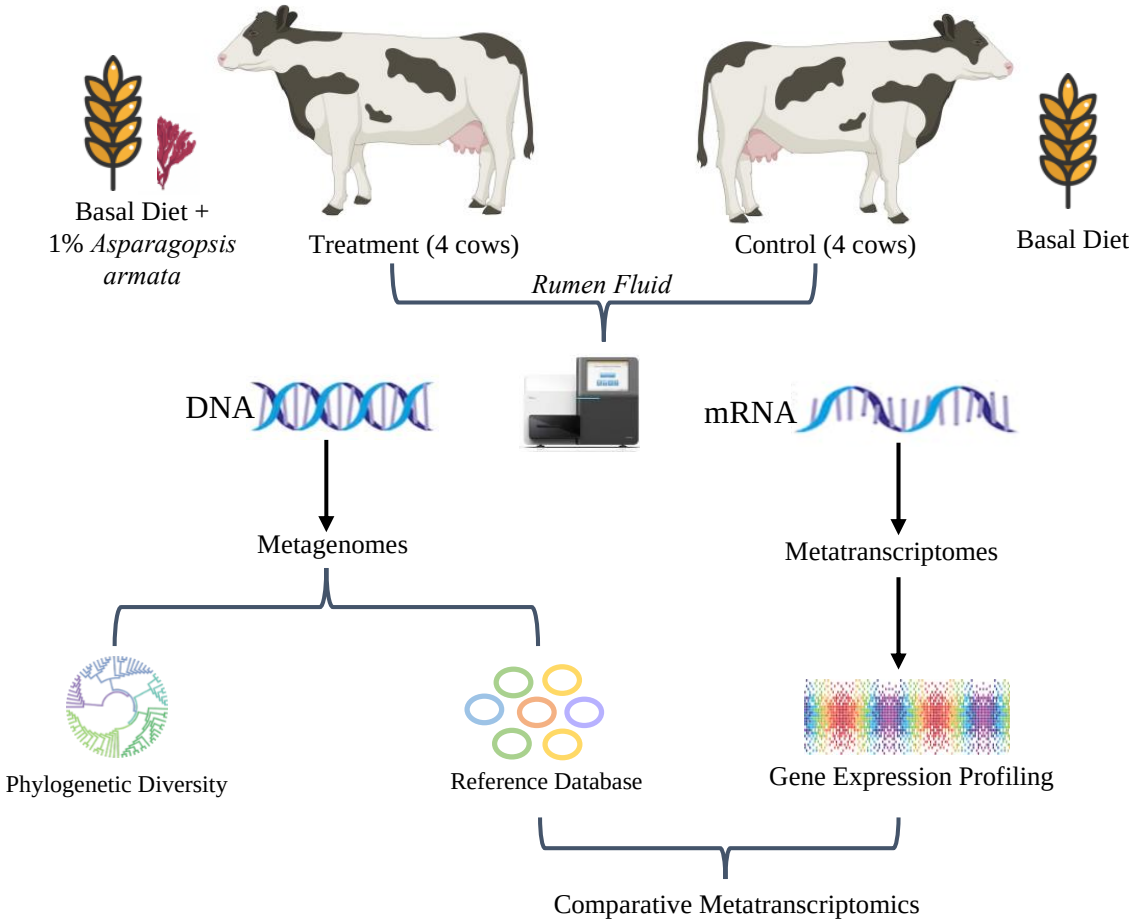


Figure 5-1: Experimental Design. Rumen fluid was collected from animals of the Control group (n=4) and High Dose group (n=4) after 14 days. DNA was extracted for metagenomic sequencing to generate metagenome assembled genomes and to assign phylogeny to genes and scaffolds. RNA was extracted for gene expression profiling.

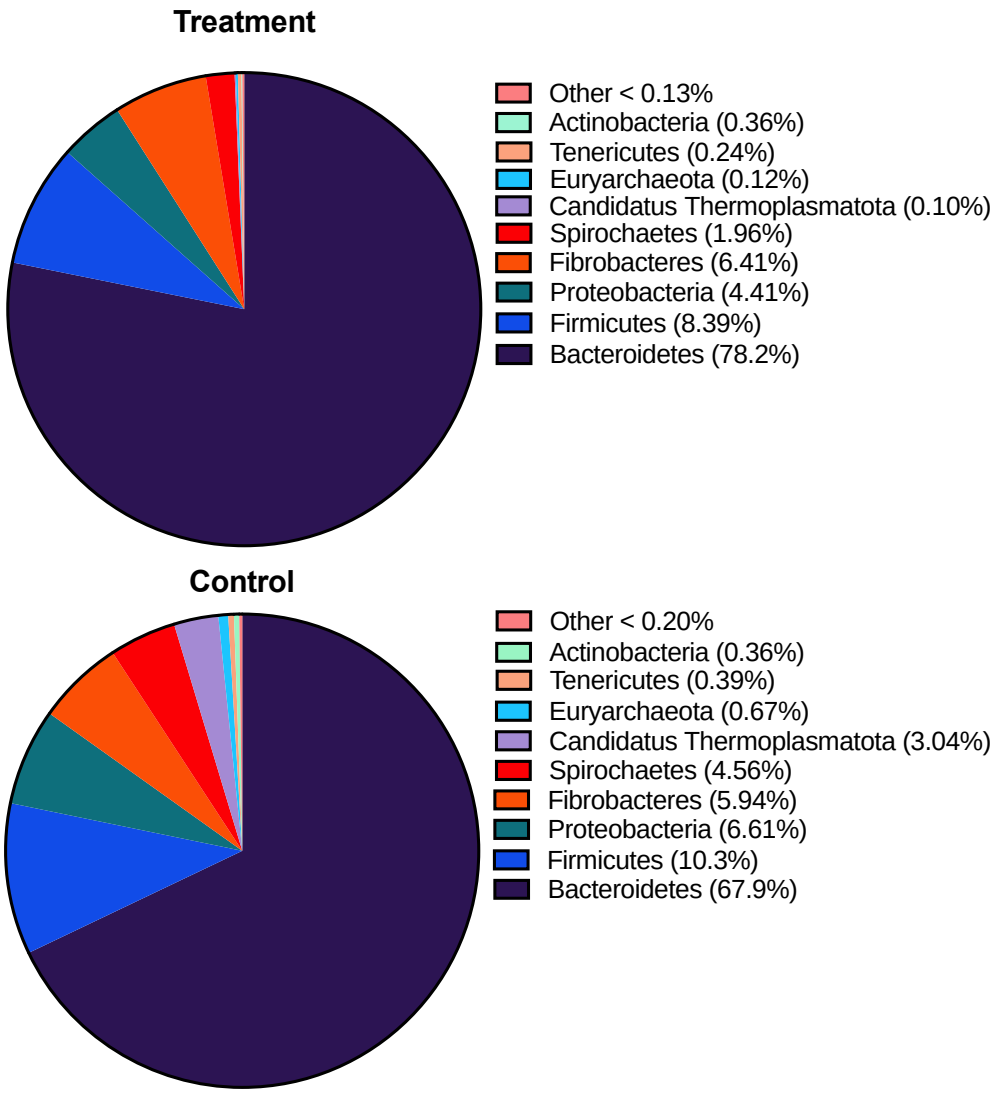


Figure 5-2. Phylogenetic distribution of gene expression. Percent of genes expressed relative to all bacterial and archaeal transcripts for Control (no seaweed) and Treatment (1% seaweed inclusion). Values are averages between the 4 cows per treatment group and do not include transcripts for protists, fungi or viruses.

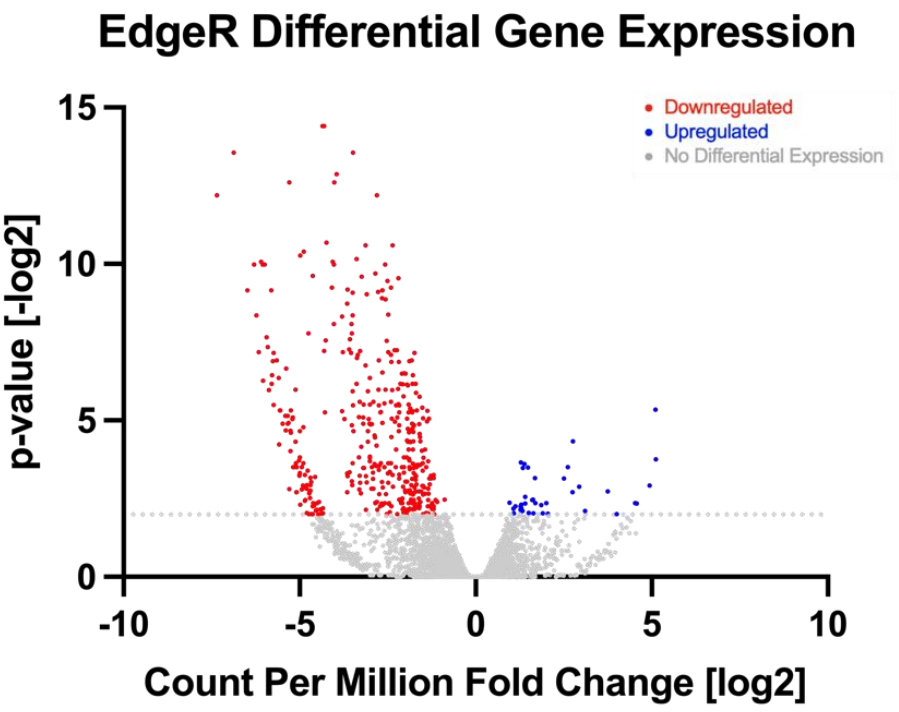


Figure 5-3. Volcano plot of differential gene expression between Control and Treatment. Red dots represent downregulation of genes in treatment group compared to control. Blue dots represent upregulation in treatment group compared to control. Grey dots represent no significant differential expression.

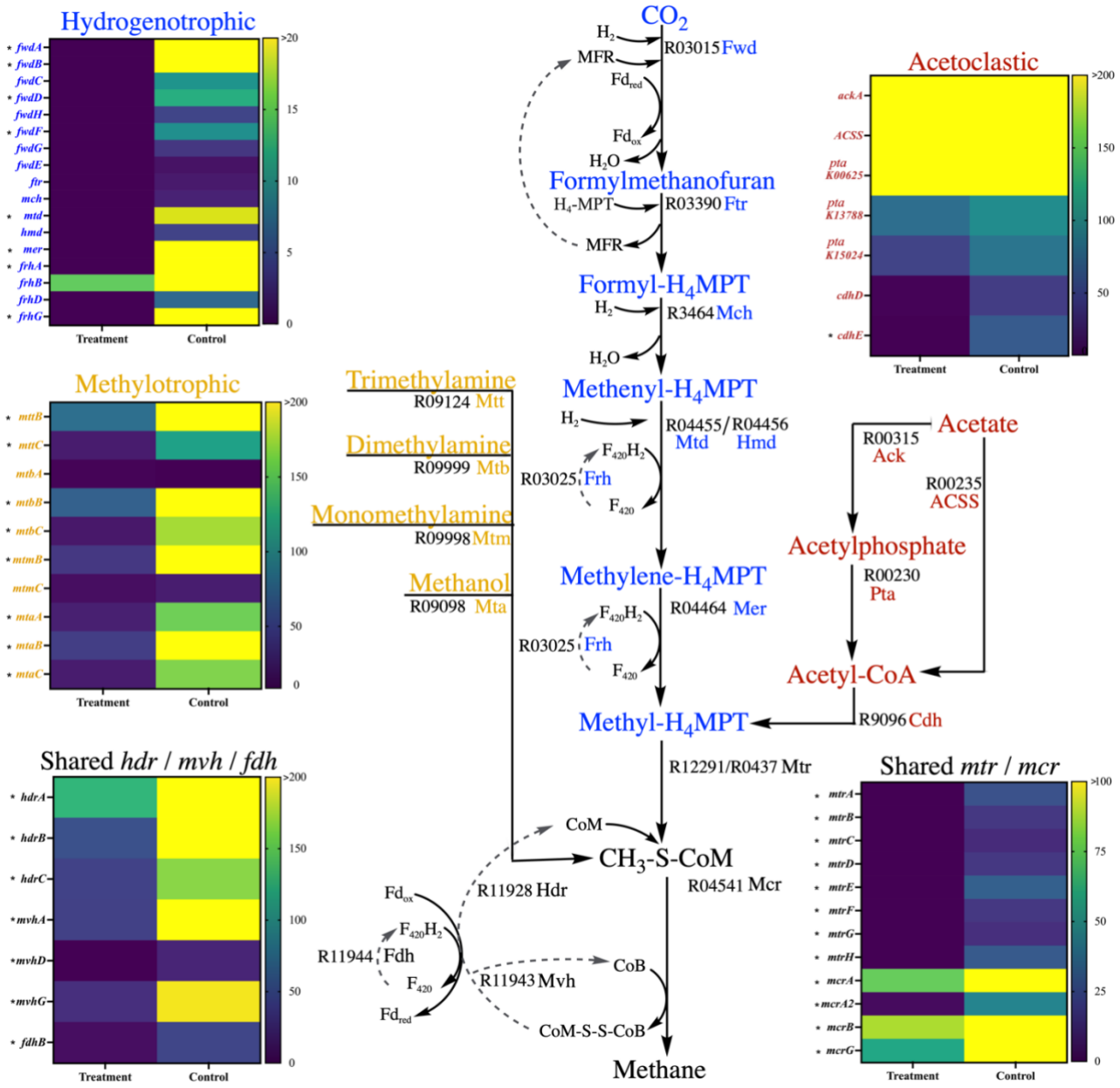


Figure 5-4. Differential gene transcription. Hydrogenotrophic (blue), methylotrophic (yellow), acetoclastic (red), and universal (black) methanogenesis gene expression in the absence (Control) and presence (Treatment) of 1% *Asparagopsis armata* inclusion to dairy cow diets. Genes labelled with an asterisk were statistically different between Control and Treatment groups.

Chapter 6: Concluding Remarks

In summary, there are a variety of promising feed additives to reduce enteric CH_4 production from livestock, some of the most promising candidates being synthetic inhibitors such as 3-NOP and HMAs (bromoform, BCM, and chloroform), dietary lipids sourced from oils and oilseeds, and plant bioactive compounds such as macroalgae, tannins, EOs and EO blends. However, not all products are available on the market for immediate use. For short term CH_4 reductions, dietary lipids, tannins, and EOs may be used to reduce enteric CH_4 as they are currently available for use. However, feed additives with the largest CH_4 reduction capabilities such as 3-NOP (22 – 39% reduction) and macroalgae (67 – 99% reductions) first need regulatory approval to determine the safety of these products not only for the animals but also for animal sourced foods produced for human consumption. While 3-NOP is currently conducting clinical trials across the world and may be available for use in the next few years, macroalgae does not yet have the amount of scientific research to support the need for clinical trials.

The objectives of the work presented here were to evaluate the efficacy and persistence of *Asparagopsis* macroalgae in reducing enteric CH_4 production as well as determine the efficiency of product formation [meat or milk] and overall health of the animal, investigate the safety of animal sources products such as meat and milk, and to observe potential changes in the rumen microbiome when cattle are fed *Asparagopsis*. To summarize the research conducted, *Asparagopsis* macroalgae is extremely effective at reducing enteric CH_4 production *in vitro* and *in vivo*. When *Asparagopsis* was applied to RUSTEC fermentation vessels at an inclusion of 5% OM a 95% reduction in CH_4 was

observed. Additionally, VFA production shifted toward increased propionate to acetate ratios. Methanogen abundance *in vitro* decreased over the 96-hour experimental period, however did not necessarily coincide with CH_4 reduction meaning that *Asparagopsis* likely works as a methanogenesis inhibitor rather than an anti-methanogenic compound. When fed to lactating dairy cows, *A. armata* reduced CH_4 emissions by 26% when fed at a 0.5% OM inclusion level with no decline in milk yield or feed intake. Increasing the inclusion level to 1% resulted in CH_4 reductions of 67.2%, however, feed intake and milk yield were also reduced. Bromoform concentration in milk was not significantly different in cows that consumed macroalgae compared to control, however, other mineral concentrations such as iodine may be increased in milk and need further investigation before determining overall safety for human consumption. When *A. taxiformis* was fed to growing beef steers over a 21-week duration, average CH_4 reductions of 45% and 68% were seen with 0.25% OM and 0.50% OM *Asparagopsis* inclusions and the mitigation effect persistent throughout the course of the experiment. However, it was found that CH_4 reduction efficacy was highly correlated with the proportion of NDF in the diet as demonstrated through the typical stepwise transition to a feedlot finishing diet formulation. For instance, when low forage levels were fed CH_4 mitigation was increased to approximately 70% and 80% using 0.25% and 0.50% *Asparagopsis* inclusions, respectively. Furthermore, the use of *Asparagopsis* impacts DMI and not ADG, therefore increasing overall feed efficiency (FCE) in growing beef steers significantly reducing feed costs per kg of weight gained. Additionally, supplementing *A. taxiformis* had no measurable bromoform residues, no detrimental iodine residual effects in the product, and did not alter meat quality or sensory properties.

The results of these findings conclude that *Asparagopsis* macroalgae shows great promise as a CH_4-mitigating feed additive, however there is little known about the impacts it may have on the rumen microbiome. Preliminary findings suggests that *Asparagopsis* works to suppress most of the necessary enzymes needed for hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis in the rumen. Further investigation is needed to determine the potential benefits or shortfalls *Asparagopsis* may have on host microbiome interactions during dietary *Asparagopsis* inclusion. Additionally, more research is needed to determine the scalability of farming *Asparagopsis* to be applied to ruminant diets globally. Land-based and aquaculture techniques specific to regional areas should be developed in order to produce a consistent and high-quality product.

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