THE EFFECTS OF PROCESSED SEAWEED SUPPLEMENTS ON RUMEN FERMENTATION AND METHANE PRODUCTION *IN VITRO*

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Ashley Driemel

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Thesis Examination Committee:

Sabrina L. Greenwood, Ph.D., Advisor
Eric D. Roy, Ph.D., Chairperson
Sarah Y. Morrison, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Seaweed supplementation in ruminants is not a novel practice but has recently regained popularity due to promising nutritional benefits for the animal as well as the presence of bioactive compounds that reduce methanogenesis within the rumen. It is important to broaden our assessment of different seaweed species and identify key seaweed species that have the most efficient CH_4-reducing potential, do not negatively impact rumen fermentation parameters, and are abundant enough to meet consumer demand. Chapter 1 of this thesis reviews the published literature relevant to seaweed supplementation in ruminants, with a focus on the comparison of seaweed processing methods utilized in these studies and how they impacted ruminal fermentation parameters such as methanogenesis. Seaweed species that can be harvested in the northeastern United States are also highlighted.

The objectives of Experiment 1 (Chapter 2) were to evaluate northeastern US-sourced seaweed supplements from *Saccharina latissima* (*SL*) and *Ascophyllum nodosum* (*AN*) and their processing methods on rumen fermentation and methanogenesis using continuous culture fermentation. Experiment 1 utilized a 4x4 Latin Square design consisting of four, 10-d periods. The four experimental treatments in addition to a TMR diet were: 1) unwashed, coarsely milled *SL* at 4.6% dry matter (DM; **UNW**), 2) 3-min rinsed *SL* at 4.5% DM (**3MR**), 3) 20-sec blanched *SL* at 3.8% DM (**20SB**), and 4) a crude phlorotannin extract from *AN* at 4.7% DM (**PHLT**). Treatment did not impact pH, DM, organic matter (OM), or the apparent degradabilities of neutral detergent fiber in amylase, organic matter (aNDFom) or acid detergent fiber (ADF). The processed *SL* treatment 3MR produced less CH_4 *in vitro* than the unprocessed *SL* treatment (UNW). These findings serve as an indication of the importance of washing seaweed supplements and its possible effects on fermentation.

The objective of Experiment 2 (Chapter 3) was to evaluate the dietary inclusion rate of *Chondrus crispus* (*CC*) on these rumen fermentation parameters relative to negative and positive controls *in vitro*. Experiment 2 utilized a 5x5 Latin Square design, consisting of five, 10-d periods. The five experimental treatments were: 1) negative control (**CON**) of TMR only, 2) TMR + 6% DM inclusion of *CC* (**CL**), 3) TMR + 10% DM inclusion of *CC* (**CH**), 4) positive anti-methanogenic control containing TMR + 2.5 mL of 0.6 mM bromoform (**BL**), and 5) positive anti-methanogenic control containing TMR + 5 mL of 0.6 mM bromoform (**BH**). No effects of treatment on pH, DM, OM, aNDFom or ADF apparent degradabilities were observed. Both inclusions of *CC* (treatments CL and CH) produced more CH_4 than the controls (CON, BL and BH). Despite similarities in processing of CL, CH and UNW, as *CC* treatments were also unwashed, further investigation into washing methods as well as dietary cation: anion difference (DCAD) in *CC* are warranted.

Further studies are necessary to determine the feasibility and practicality of implementing *SL* and *CC* in ruminant diets. Our findings only reaffirm the importance of effective seaweed processing methods and dietary inclusion rates, and that these factors affect methanogenesis, even *in vitro*.

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADWG	Average daily weight gain
AN	*Ascophyllum nodosum*
aNDFom	Neutral detergent fiber in amylase, organic matter
A:P	Acetate-to-propionate ratio
BH	Bromoform High inclusion (5 mL of 0.6 mM)
BL	Bromoform Low inclusion (2.5 mL of 0.6 mM)
CC	*Chondrus crispus*
CH	*C. crispus* High inclusion (10% DM)
CL	*C. crispus* Low inclusion (6% DM)
CP	Crude protein
DCAD	Dietary cation: anion difference
DFCCS	Dual-flow continuous culture system
DHA	Docosahexaenoic acid
DM	Dry matter
DMI	Dry matter intake
DMSO	Dimethylsulfoxide
EPA	Eicosapentaenoic acid; Environmental Protection Agency
F:C	Forage:concentrate ratio
FW	Freshwater
GE	Gross energy
KM	Kelp meal
NAN	Non-ammonia nitrogen
NFC	Non-fiber carbohydrate
NH_3-N	Ammonia nitrogen
NR	Not reported
OM	Organic matter
PHLT	Phlorotannin extract derived from *A. nodosum* treatment
PT	Phlorotannin
RUP	Rumen undegraded protein
SL	*Saccharina latissima*
SW	Saltwater
TMR	Total mixed ration
UNW	Unwashed *S. latissima* treatment
VFA	Volatile fatty acid
20SB	20-sec blanched *S. latissima* treatment
3MR	3-min rinsed *S. latissima* treatment

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Dating as far back as 100 BC, macroalgae (commonly known as seaweed) has been intentionally fed to livestock when pasture was scarce. Seaweed meals processed from harvested *Ascophyllum nodosum* gained popularity in European countries in the late 1960s and have since made a comeback in the animal agriculture industry (McHugh, 2003). Recent research is heavily focused on the identification of ideal seaweed candidates for use in ruminant diets, with particular emphasis on their nutrient profile and potential to reduce enteric methane (CH_4) production. Successful and ubiquitous incorporation of seaweed in animal agriculture will rely on the following: 1) a seaweed species and processed form that can supply concentrated bioactive compounds with sustained activity, 2) consistent nutrient profiles within seaweed species that lack contaminants, 3) a palatable and digestible seaweed, and 4) feasible, scalable, and sustainable sourcing, transportation, and processing of these seaweeds (Vijn et al., 2020). The aims of this literature review were to delineate the current knowledge of seaweed supplementation in ruminant diets, describe how different seaweed species impact ruminal performance through a comparative discussion, and identify knowledge gaps, particularly pertaining to how processing method affects enteric CH_4 production by ruminants.

1.2. Methanogenesis and the Rumen

1.2.1. Environmental Implications of Methane

Methane (CH_4) is a major contributor to climate change because of its ability to absorb more energy per unit mass compared with CO_2, according to the National Academies of Sciences, Engineering, and Medicine (NASEM, 2018). Due to its disproportionately large effect on radiative forcing and relatively short atmospheric lifetime when compared with CO_2, CH_4 is the proposed target for emission reduction in hopes of preventing the worst effects of climate change (NASEM, 2018).

The US Greenhouse Gas Inventory (GHGI; EPA, 2017) reported that enteric fermentation of livestock comprised 25% of anthropogenic CH_4 emissions in the US in 2015. Emissions from lactating dairy cows typically range from 200-500 g CH_4/cow/d, whereas beef cattle produce about 50-300 g CH_4/cow/d (Beauchemin et al., 2009; Johnson and Johnson, 1995). With more recent data reporting an estimated 93.6 million cows in the US (United States Department of Agriculture, 2021), this equates to an estimated 194.7 million metric tons of CO_2 equivalent ($MMTCO_2e$) of CH_4 annually from enteric fermentation, representing 27% of total US CH_4 emissions. For comparison, according to the US Environmental Protection Agency (EPA), US landfills released about 122.6 $MMTCO_2e$ of CH_4 in 2021, representing 17% of total US CH_4 emissions. Despite this considerable difference between anthropogenic CH_4 sources, non-animal related emission sources such as coal mining and natural gas systems contributed to an additional 46% of total US CH_4 emissions in 2021. However, it is evident that a reduction in emissions from the livestock sector will still provide considerable environmental benefit. It is projected that global livestock emissions from enteric fermentation and manure

management will increase by 10% between 2015 and 2030, accounting for 24% of *global* non-CO_2 emissions in 2030 (EPA, 2019). Therefore, reduction of these emissions from livestock can benefit not only the producer, but also the greater community by lessening the environmental impacts of agriculture.

1.2.2. Methanogenesis and Rumen Energetics

Through the process of methanogenesis, ruminants lose 2-12% of gross energy (GE) derived from feed (Johnson et al., 1993). For dairy cows specifically, the energy loss is estimated to be 5.5-9% of GE ingested (Johnson et al., 1993). Variation in the proportional loss per ruminant animal is due to physiological and chemical factors of the rumen environment. For example, total gas production is closely associated with volatile fatty acid (VFA) production, substrate digestion and microbial growth (Choi et al., 2021). While an increase in VFA concentrations increases total gas production, pH values outside the range of 6 to 7 will alter the methanogen population and thus further affect gas production (Van Kessel and Russell, 1996). Diets high in fermentable carbohydrates, such as barley, oats, or finely ground corn, that increase ruminal propionate production and decrease acetate formation are associated with less enteric CH_4 production (Moe and Tyrrell, 1979).

Microorganisms in the rumen hydrolyze proteins, starches and plant cell wall polymers into amino acids and sugars, the latter of which are then fermented to VFAs, H_2 and CO_2 (Church, 1988). While most methanogenic *Archaea* convert these fermentation byproducts into CH_4, some can use formate among other substrates (Jones et al., 1991). The molar percentage of VFAs influences the production of CH_4; acetate and butyrate

production liberate H_2 and a net C loss results (Beauchemin et al., 2009). Propionate production serves as a competitive pathway for H_2 as it acts as a H_2 sink, therefore, this synthetic pathway does not contribute excess C to methanogenesis (Demeyer and Van Nevel, 1975; Tedeschi et al., 2022; Ungerfeld, 2020). From a fermentation perspective, methanogenesis is desirable biologically as the formation of CH_4 is a result of microbes removing excess H_2 from the environment. This is especially paramount in the rumen, as this H_2 removal supports ruminal homeostasis (Beauchemin et al., 2009). Ultimately, CH_4 formed in the rumen comprises nearly 87% of the total CH_4 generated by ruminants (Murray et al., 1976).

In the rumen, an interspecies transfer of H_2 occurs between methanogens and protozoa. This transfer aids in the prevention of H_2 accumulation in the rumen while simultaneously supporting the growth of H_2 producing bacteria by promoting further degradation of fibrous feed material (Hegarty and Gerdes, 1999). Due to their hydrophobicity, methanogens attach to feed particles as well as to the surface of protozoa (Krumholz et al., 1983). Protozoa are also directly associated with H_2 production because of their ability to make H_2 via organelles known as hydrogenosomes (Yarlett et al., 1981). Methanogens most often associated with rumen protozoa are from orders *methanobacteriales* and *methanomicrobiales* (Sharp et al., 1998). *Methanobrevibacter* is the most dominant genus of methanogens in the rumen, with the largest group of methanogens in lactating dairy cattle fed a total mixed ration (TMR) being *methanobrevibacter ruminantium* (Whitford et al., 2001). McAllister (1996) reported that *Methanosarcina barkeri, Methanosarcina mazei, Methanobacterium formicicum* and

Methanomicrobium mobile are another four species of methanogenic archaea that are commonly found in the rumen.

1.3. Livestock Feeds and Methane

1.3.1. Impact of Diet on the Rumen Environment

The diet of a ruminant greatly influences the rumen microbiome and thus impacts enteric CH_4 production. Diet affects CH_4 production through its: 1) fiber content, 2) degradability, and 3) fermentability. Increased intake and fermentation of structural carbohydrates, which are particularly rich in forages such as legumes and grass silage, leads to a greater loss of energy as CH_4 due to a decrease in overall rumen capacity, fermentation rate, and passage rate (Hegarty and Gerdes, 1998). By contrast, starch-rich diets favor propionate production due to their fermentability and rapid passage rate. Propionate formation yields less H_2 as a by-product and, therefore, creates less CH_4. Indeed, when compared with forage diets, feeding ruminants a grain-based diet typically yields less enteric CH_4 (Johnson and Johnson, 1995).

1.3.2. Strategies to Manipulate Rumen Environment and Rumen Methane Production

The process of methanogenesis is often the target for enteric CH_4 reduction strategies. Methane emission reductions have been achieved through defaunation techniques within the rumen (Kreuzer et al., 1986), diet manipulation (Holter and Young, 1992), and even the use of vaccines that stimulate antibody production against methanogens (Wedlock et al., 2010; Wright et al., 2004). Strategic use of dietary fats (Dong et al.1997), dietary inclusion of highly fermentable forages (Boadi et al., 2002)

and increased levels of grains in the diet (Hegarty, 1999) have all been applied to manipulate fermentation and directly inhibit methanogens. While all these dietary strategies alter the ruminal environment and substrate availability, ultimately targeting methanogens directly has been proposed to result in a greater reduction in CH_4 production with longer lasting effects (Hook et al., 2010). This is due to the adaptation of rumen microbiota to changes in substrate availability, occurring in strategies like defaunation, thus decreasing the anti-methanogenic effects observed over time when utilizing these strategies (Guan et al., 2006). Despite this, feed additives and supplements have been the most prominent, promising strategies thus far and include: 1) ionophores, 2) tannins, 3) 3-nitrooxypropanol (3-NOP), 4) essential oils, and 5) seaweeds, among others.

1.4. Seaweeds as Feed Additives in Ruminant Diets

The use of seaweeds in ruminant diets is not new; seaweeds were and still are commonly fed in coastal areas during seasons of sparce crop yield (Newton, 1951). However, the US Food and Drug Administration (FDA) limits the use of seaweeds in ruminant diets to only specific species that have been extensively researched, further detailed by the Association of American Feed Control Officials (AAFCO, 2021). Additionally, supplementation is not widespread due to the need for regulatory approval, lack of incentives to farmers who implement seaweeds into diets, and the lack of legislative mandates regarding agricultural CH_4 reductions (Vijn et al., 2020). Changes in fermentation, rumen health and digestibility have been observed in studies supplementing seaweeds in cattle diets but large variabilities in response to different seaweeds have been

reported (reviewed by Abbott et al., 2020). This variability is most likely due to the exposure of seaweed biomass to environmental factors such as water temperature, light and nutrient availability (Dawes, 1998), as well as seaweed species, sampling site (Billot et al., 2003; Young et al., 2013), and processing after harvest (Sappati et al., 2019; Vucko et al., 2017). Seaweed species that are the most prominent in currently available published literature as potential ruminant feed additives are presented in **Table 1.1**.

1.4.1. Sustainability of Seaweed Utilization as Feed Supplements

Incorporation of feed additives that are 1) sustainable and 2) do not heavily compete with human commodity markets are important considerations that must be addressed for us to meet the food systems mission of reducing environmental impact of production animals. Seaweeds appear to be a perfect fit to this model as they do not compete for arable land used for human food chains or potable water and can be grown year-round, dependent on location, in aquatic environments unlike terrestrial crops (Forster and Radulovich, 2015). For example, seaweeds such as *Laminaria japonica*, currently used in human food chains, yielded 20 t/ha/yr dry weight in China, compared with common feedstuffs such as corn (10 t/ha/yr) and soybean (3 t/ha/yr) yields in the US (Forster and Radulovich, 2015). Additionally, a recent study by Spillias et al. (2023) concluded that substituting 10% of human food with seaweed by 2050 could spare 110 million ha of land, not including substitutions from animal diets that would allow for even more land to be used for purposes other than for agriculture.

Despite this abundant seaweed yield reported, it must be considered whether these yields would theoretically be enough to supply all the dairy cows within a certain

region of the world, and if the yield is high enough quality to be incorporated into ruminant diets. The state of Maine produced 800,000 pounds of seaweed in 2021 (Castrodale, 2021), which was enough to feed 0.02% of the cows in the New England region of the US at a 1% DM inclusion rate (estimated 3.5 million cows; USDA, 2021). Although it is projected that Maine will be capable of producing 3 million pounds of seaweed annually by 2035 (Castrodale, 2021), that would still only supplement about 0.08% of cows in New England at a 1% DM inclusion rate. However, the seaweed industry has consistently grown in the last decade, where production in the US has increased from 18 tons in 2017 to 440 tons in 2021 (Elliott, n.d.). With this rapid expansion of infrastructure, there is optimism that the US will have the capacity to cultivate sufficient seaweed for cattle by maximizing the efficiency of aquaculture-compatible zones along coastlines. This development is crucial to meet the growing demand for locally sourced seaweed.

1.4.2. Considerations for Success in the Animal Industry

As stated above, the primary considerations for seaweed inclusion in livestock diets as outlined by Vijn et al. (2020) include a seaweed species and processed form that can supply concentrated bioactive compounds, consistent nutrient profiles within seaweed species, a palatable and digestible seaweed, and feasible, scalable, and sustainable sourcing, transportation, and processing. Herein we will discuss these considerations.

1.4.2.1. Bioactive Compounds

Despite efforts to determine optimal inclusion rates of different seaweed species, the variable concentrations of bioactive compounds within seaweeds present a challenge and potential obstacle to this pursuit. Given the effectiveness of seaweed supplementation is largely reliant on both its bioactive compound profile and dietary inclusion rate (Kinley et al., 2016; Li et al., 2016; Machado et al., 2016), it is essential that this issue of bioactive consistency be further investigated. To do this, we must first understand what bioactive compounds are present within seaweed.

One of the primary bioactive compounds of focus within seaweed to-date is bromoform. Containing nearly 70% of the world's bromoform, marine red macroalgae serve as an important source of halocarbons (Carpenter and Liss, 2000). Seaweed species within the genus *Asparagopsis* accumulate bromoform naturally from their environment, causing variability in bromoform content among species and seaweed growth locations (Young et al., 2013). This bioactive compound has only recently been associated as the cause of anti-methanogenic activity from red seaweed supplementation (Machado et al., 2016). Bromoform inhibits the cobamide-dependent methyl transferase at the terminal step of the methanogenic pathway (Wood et al., 1968), and thus can reduce the amount of CH_4 produced by ruminants. However, the implications of bromoform supplementation to cattle and how their productivity may be affected are still being investigated. The US EPA has established a 0.08 mg/L limit for trihalomethanes in drinking water for humans, meaning that levels above this in milk would present a safety concern for human consumption (EPA, 2018).

Tannins are another naturally occurring bioactive compound and are widespread in terrestrial (typically as condensed tannins) and marine plants (as phlorotannins) such as brown seaweeds. Tannins can form insoluble complexes with proteins to yield non-digestible rumen undegraded protein (RUP) while others bind temporarily, thus protecting them from rumen degradation but still allowing for digestion (digestible RUP; Mueller-Harvey, 1999). Due to varying astringency among species, some phlorotannin-containing seaweeds have been associated with less efficient use of dietary proteins and can be considered anti-nutritive (Deal et al., 2003; Shahidi and Naczk, 1995). However, the results are not consistent; benefits such as a reduction in pasture-induced bloat from terrestrial tannins (when fed 35% sainfoin in mixed alfalfa pasture; Wang et al., 2006), and improved protein utilization, animal productivity and antimicrobial properties from phlorotannins (Horikawa et al., 1999; Nagayama et al., 2002) have been reported with tannin supplementation. Cassani et al. (2020) provided a comprehensive review of how intrinsic and environmental factors, processing and extraction methods, and storage methods can affect the phlorotannin content in seaweeds. Thus, purified, concentrated bioactive compounds such as bromoform or phlorotannins delivered via capsule or injectable forms may be targeted methods for the future rather than feeding biomass due to these aforementioned factors (Cassani et al., 2020; Baumont, 1996).

1.4.2.2. Nutrient Profiles

Currently, one of the most considerable challenges to address has been the need for consistent nutrient profiles across seaweeds of the same species. Ruminant production, including growth, pregnancy, and lactation, all require a consistent plane of nutrition, hence consistent dietary nutrient intake is critical to animal health and

performance. Seaweed is widely variable in protein (Mwalugha et al., 2015; Tayyab et al., 2016), mineral (El-Said and El-Sikaily, 2012), lipid (Bikker et al., 2020), and fiber (Dubois et al., 2013) composition between species as well as within species because of factors such as growth location, harvest seasonality, light availability, and environmental nutrient fluxes.

Compared with other common protein sources included in ruminant diets, such as cereal and soy flours, many seaweeds contain a similar high-quality, but variable, crude protein (CP) content (Morais et al., 2020). Green seaweed of the genus *Ulva* had the highest reported CP content at 382 g/kg DM (Dubois et al., 2013), comparable to grass-clover pasture (Tayyab et al., 2016). All 15 seaweed species studied by Dubois et al. (2013), especially the green seaweeds, were high in CP when compared with Rhodes grass. Green genus *Acrosiphonia* contained 310 g/kg DM CP (Tayyab et al., 2016). Red seaweeds of genus *Porphyra* and *Pyropia* can range from 180-500 g CP/kg DM, according to Marsham et al. (2007) and Rupérez et al. (2001). Gaillard et al. (2018) and Tayyab et al. (2016) reported *Porphyra sp.* containing an average CP of 334 g/kg DM and 347 g/kg DM, respectively. These values are comparable to soybeans which contain an average CP of 350 g/kg DM (Randoin et al., 1987) and oil seed by-products such as sunflower and rapeseed meal (Gaillard et al., 2018). *Palmaria palmata* was reported to contain up to 290 g CP/kg DM (Makkar et al., 2016), with an average CP content of 223 g/kg DM (Tayyab et al., 2016). Due to their richness in CP, red and green seaweeds are recommended as protein source additives in ruminant diets as opposed to brown seaweeds (Gaillard et al., 2018). However, variation across species is still a critical consideration. For example, Roque et al. (2019a) reported that *A. taxiformis*, the most

widely studied red seaweed species, contained 178 g CP/kg DM. This is a considerably lower CP content than *P. palmata*, yet *P. palmata* has not received much attention as an animal feed prospect. Brown seaweeds are recognized as having the lowest CP contents, ranging from 50-150 g CP/kg DM (Dawczynski et al., 2007; Mišurcová, 2011). *Pelvetia canaliculata, Cystoseira trinodis* and *Saccharina latissima* contained less CP than red and green seaweeds (90, 112 and 145 g CP/kg DM, respectively), despite being amino-acid rich (Gaillard et al., 2018; Tayyab et al., 2016; Dubois et al., 2013).

More than 75% of seaweeds assessed by Maehre et al. (2014; 2015) had higher proportions of total essential amino acids compared with wheat flour and were 50% higher than soy flour. Makkar et al. (2016) presented a comparison of the amino acid profiles of brown seaweeds *A. nodosum* (AN*)*, *Undaria pinnatifida* (UP), *Saccharina japonica* (SJ), and *Macrocystis pyrifera* (MP) relative to that of soymeal reported in previous literature. Compared with soymeal, these seaweeds had, based on their average concentration, equivalent or higher concentrations of the following essential amino acids: methionine (all), cysteine (SJ, MP), valine (UP, SJ, MP), isoleucine (UP), leucine (UP), histidine (SJ), lysine (UP), and threonine (UP, SJ, MP; Makkar et al., 2016). Min et al. (2021) reported (on g/100 g protein basis) that *M. pyrifera* and *Laminaria digitata* were both higher in cysteine composition compared with soymeal, while *L. digitata* was higher in valine and *M. pyrifera* was higher in threonine. Smith and Young (1955) reported that *C. crispus* had the highest arginine content and was the only species they assessed that contained citrulline and ornithine. Norziah and Ching (2000) reported that *Gracilaria changgi* was rich in glycine, arginine, alanine, glutamate, proline, and aspartate, but had low concentrations of tyrosine and cysteine.

Despite an abundance of amino acids, the degradability of seaweed protein profiles when ingested must also be considered. Gaillard et al. (2018) reported that *Porphyra sp., P. palmata, Ulva sp., and Cladophora rupestris* are feasible protein sources due to their degradability and richness in: threonine, serine, glycine, valine, leucine, lysine, and arginine. In this study, total tract degradability *in situ* of cysteine, aspartate and threonine differed between seaweed species but not seasonally (Gaillard et al., 2018). While *Porphyra sp.* demonstrated the highest amino acid degradability *in situ* in the research by Gaillard et al. (2018), *L. digitata* had the lowest. Although the red seaweed, *Mastocarpus stellatus,* was found to have CP content comparable to grass-clover pasture (Tayyab et al., 2016), *M. stellatus* was not considered a good feed additive candidate because of its low amino acid degradability *in situ* (Gaillard et al., 2018).

It is important to note that both CP content and individual amino acid profiles from seaweeds can be variable, and are affected by harvest season, harvest location, and potentially processing techniques. Spring-collected samples of *L. digitata*, *Alaria esculenta*, *P. palmata*, *P. canaliculata*, and *Porphyra sp*. characterized in the research by Tayyab et al. (2016) and Gaillard et al. (2018) had higher CP content (g/kg DM) compared with autumn-collected samples of the same species. These findings were consistent within *S. latissima* and *M. stellatus* when investigated by Gaillard et al. (2018), while Tayyab et al. (2016) did not observe a seasonal effect on CP content of *M. stellatus*. Galland-Irmouli et al. (1999) noted that *P. palmata* powder samples were absent of lysine, histidine, arginine, and proline during spring months, where Mathieson and Tveter (1975) suggested this was most likely due to the availability of nitrogenous compounds in the water. Cultivation latitude and seasonality of harvest were found by

Forbord et al. (2020) to affect *S. latissima* CP content (mg/g DM) among samples collected from three different sites along the Norwegian coast. Generally, CP content increased from the southern (~30 mg protein/g DW) to northern (~100 mg/g DW) growth sites in samples taken at a depth of 1-2 m, most likely due to differing sun exposure (Forbord et al., 2020). Protein variability between other studies was thought to be a result of seaweed washing and processing techniques (Gaillard et al., 2018) but Sappati et al. (2019) found that exposure to different drying conditions (oven drying at 30°C vs sun drying vs freeze drying), regardless of set humidity (25% or 50%), did not affect CP content in *S. latissima*. However, Sappati et al. (2019) did indicate that higher oven drying temperatures (50°C and 70°C) may affect CP content compared with sun dried and freeze dried samples. Gaillard et al. (2018) and Tayyab et al. (2016) utilized seaweeds from the same harvest location, same harvest years and with the same processing methods (rinsed in 30% seawater, then freshwater, frozen at -20°C, freeze dried, milled to 1.5 mm), which is what likely led to similarities in CP contents across the two articles. Studies have attributed intrinsic protein variability to high light intensity stimulating N metabolism and thus reducing CP content (Sappati et al., 2019), or to the presence of protein-rich epibionts on fouled seaweed biomass that falsely elevate CP concentrations (Forbord et al., 2020). Additionally, the effects of washing technique on protein concentrations in seaweeds have not been extensively studied to our knowledge and therefore, could potentially still prove to have a pronounced effect on CP content.

Apart from the CP and amino acid profile, the carbohydrate profile of seaweed is also of particular interest due to its impact on rumen function if incorporated into ruminant diets. The fiber content of various seaweed species is comparable to other

common ruminant feedstuffs. The neutral detergent fiber (NDF) determined on an organic matter (OM) basis with amylase (aNDFom) of *Acrosphonia sp., P. palmata and Porphyra sp.* were in range of typical ryegrass aNDFom content (430g/kg DM; Tayyab et al., 2016). Tayyab et al. (2016) also reported that *M. stellatus, P. canaliculata,* and *Ulva sp.* were similar in aNDFom content to white clover (280g/kg DM; Moharrery et al., 2009), with the lowest NDF value for *Ulva sp.* being approximately 219 g/kg DM (Dubois et al., 2013). The NDF content of *C. trinodis* was also within this range, with an NDF content of 311 g/kg DM (Dubois et al., 2013). According to Maia et al. (2019), *S. latissima* had the lowest NDF content of all species measured in that study (71 g/kg DM) while *A. esculenta* harvested in autumn by Tayyab et al. (2016) was the lowest in aNDFom (90 g/kg DM). The red seaweed, *A. taxiformis*, had a reported NDF of 369 g/kg DM (Roque et al., 2019a). Variability in NDF and aNDFom across studies could be a result of harvesting season, as Tayyab et al. (2016) found aNDFom concentrations in all species to be higher in autumn samples than in spring samples. Autumn-harvested *P. palmata* had the highest aNDFom among species and samples (501 g/kg DM; Tayyab et al., 2016). This wide range in NDF and aNDFom is an important consideration for ruminant systems due to its impact on digestibility.

Lipids typically constitute approximately 10-50 g/kg DM (1-5% of DM) of seaweed. Large variation in lipid content was reported among brown seaweed species; *C. crispus* had the lowest crude fat content (7 g/kg DM in Irish samples) and *A. nodosum* had the highest reported value at 38 g/kg DM in Scottish samples (Bikker et al., 2020). Seaweeds are rich in omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), especially brown macroalgae, which are abundant in the essential fatty acids linoleic and

alpha-linolenic acid, and oleic acid (Dawczynski et al., 2007; Fleurence et al., 1994). The ether extract profile of red seaweed, *Gracilaria changgi*, was reported to be 75% unsaturated fatty acids, which were mainly eicosapentaenoic acid (EPA; 33% of total free fatty acids) and docosahexaenoic acid (DHA; 13% of free fatty acids; Norziah and Ching, 2000). *P. palmata* was also rich in the omega-3 EPA (Ackman and McLachlan, 1977; Morgan et al., 1980). In the rumen, a high percentage of dietary PUFAs are converted to saturated fatty acids (SFAs) through rumen biohydrogenation, thus acting as H_2 sinks so less H_2 is directed to methanogenesis (Czerkawski et al., 1966; Johnson and Johnson, 1995). Although added fats in the diet from the supplementation of seaweeds can decrease CH_4 in this way as well as by inhibiting protozoa, caution must be used when supplementing unsaturated fatty acids because high levels of specific isomers can reduce diet digestibility, DM intake (DMI; Eugene et al., 2008; Beauchemin et al., 2009; National Research Council, 2001) and milk production (Martin et al., 2008).

Mineral content of seaweed, measured as ash, is simultaneously a major concern and advantage when being considered as a ruminant feed additive. *S. latissima* and *Porphyra sp.* had the lowest reported ash contents (171 g/kg DM and 128 g/kg DM, respectively; Maia et al., 2019; Tayyab et al., 2016). Similar to its protein content, *Ulva* sp. had the highest ash (438 g/kg DM; Tayyab et al., 2016). *P. palmata, L. digitata* and *A. nodosum* are rich sources of calcium (Ca) and magnesium (Mg), as demonstrated by Lorenzo et al. (2017) who reported average Ca and Mg contents of 10 g Ca/ kg DM and 9 g Mg/ kg DM measured in *A. nodosum* samples. Makkar et al. (2016) summarized mean mineral values for *A. nodosum* outlined in previous literature and calculated the mean as 20 g Ca/kg DM and 8 g Mg/kg DM. Interestingly, the values determined by Lorenzo et

al. (2017) and Makkar et al. (2016) are similar for Mg content, but a large disparity (10 vs 20 g/kg DM) was reported in Ca content, highlighting the large variability across samples. Green seaweeds *Ulva lactuca* and *Codium tomentosum*, harvested from the Egyptian Mediterranean Sea coast, contained an average of nearly double the Ca:Mg, compared with the red and brown seaweeds studied by El-Said and El Sikaily (2013). These authors also reported that red and brown species contained more sodium (Na; 23 g/kg) and potassium (K; 6 g/kg and 10 g/kg, respectively) than the green species (11 g/kg Na; 5 g/kg K). High Na content in seaweeds may make them less desirable for use in livestock rations depending on the base diet, due to the impact of Na on metabolism, intake, and palatability (Rogers et al., 1982). However, NaCl at an inclusion of 2% DM increased water consumption of lactating Holsteins with no effect on DMI when fed a 3:1 forage:concentrate (F:C) diet in Rogers at al. (1982). Ash content of seaweeds has been reviewed extensively; in-depth information regarding individual mineral contents of seaweed species is outlined by Min et al. (2021) and Morais et al. (2020).

While certain minerals in seaweed supplements could serve as beneficial contributions to ruminant diets, the presence of excessive minerals and heavy metals must be closely examined. Wild harvested seaweed is not consistently assessed for quality, leading to large variations in the content of heavy metals, as well as other minerals, such as I and Na, in biomass (Holdt and Kraan, 2011). According to Tayyab et al. (2016), differences in ash and metal content observed between seaweed species could be due to the size and shape of plant stem morphology, as well as light availability, location of growth and other environmental factors (Jørgensen and Richardson, 1996). Rey-Crespo et al. (2014) reported that feeding a seaweed supplement (*Ulva rigida,*

Sargasum muticum, and Saccorhiza polyschides) improved mineral status of lactating Holstein Friesian cows, having a significant effect on iodine (I) milk and blood plasma concentrations. These concentrations were still within a safe consumer range according to the European Commission (EC; 2002). *A. nodosum* increased milk I by approximately 309% (average concentration of 480 µg/L milk; Antaya et al., 2019) and 1015 µg/L milk (Antaya et al., 2015) when Jersey cows were offered 113 g seaweed/d. Antaya et al. (2019) further analyzed these findings and their consequences in both animal and human health regarding I consumption. Concentrations of toxic metals such as arsenic (As), cadmium (Cd) and lead (Pb) were found to be 1-3x higher in the seaweed supplement (*U. rigida, S. muticum, and S. polyschides*) than the base diet in Rey-Crespo et al. (2014). Brown seaweeds have been found to contain higher levels of As when compared with red and green species (Sá, 2019). However, the seaweed inclusion rates are typically so low that the metal contents of the total diet are still within a safe feeding range. Similar to how excess Na concentrations can affect feed intake, higher metal content may as well, as demonstrated by Gawęda (2007) when vegetables exposed to Pb contained less sucrose content.

1.4.2.3. Palatability

A further concern regarding the success of feeding seaweed to animals is intake, which is influenced by its palatability. Mineral content and source influence the palatability of pig and cow diets (Caramalac et al., 2017). Hydroxy-based mineral sources have been found to increase DMI and be more palatable to beef calves (Caramalac et al., 2017) and Brahman-British cross cows and calves (Moriel and Arthington, 2013) compared with sulfate-based minerals, as sulfate sources contain weaker metallic bonds

that can interact with other nutrients and change flavors (Villagómez-Estrada et al., 2020). Flavors such as bitter, salty, and sour predominantly result from plant-derived compounds and in combination with olfactory senses, can trigger chemosensory-related appetite reduction, reducing animal feed intake (Roura et al., 2008, 2019). Since seaweed cell walls are rich in sulfated polysaccharides (Cunha and Grenha, 2016), these sulfate-based mineral sources will likely negatively affect intake in ruminants. The presence of phlorotannins in brown seaweeds may further affect palatability and feed intake, as phenolic compounds correlate to a bitter taste profile (Roura et al., 2008).

Depending on processing method, seaweeds may not only have a higher mineral content that affects palatability, but they can often have a marine odor. Roque et al. (2019b) combined *Asparagopsis armata* with 400 mL molasses and water for palatability, which was then hand mixed into the TMR. Despite this mixture, DMI decreased by 11 and 38% compared with a control diet when seaweed was included at 0.5 and 1% (on OM basis), respectively. Kinley et al. (2020) reported that feeding *A. taxiformis* (biomass stored at -15°C, freeze dried, ground to 2-3 mm, stored at -15°C) at 0.1% and 0.2% of OM intake increased average daily weight gain (ADWG) of beef cattle after a 60-d treatment period by 51% and 42%, respectively, with no effect on DMI when compared with the control. Conversely, Stefenoni et al. (2021) reported a decreased DMI of 4-7% in dairy cows also fed *A. taxiformis* at 0.5% diet DM (Ex 3) when compared with a 0.25% *A taxiformis* inclusion and the control, likely due to palatability issues. Antaya et al. (2019) reported an increase in DMI of Jersey cows when fed *A. nodosum* at a 0.8% DM inclusion in combination with a 70:30 F:C diet (cool-season perennial herbage and partial TMR containing mixed grass baleage and ground corn-barley-based

concentrate blend). These findings from Antaya et al. (2019) could therefore indicate 1) that *A. nodosum* does not negatively impact palatability as severely as other seaweed species, or 2) the profile of the base diet may interplay with the herd's acceptance of seaweed in the diet. As an example of the suggestion that other nutrient factors may play an important role in seaweed palatability, the presence of amino acids glycine and alanine in high concentrations provide a sweet flavor (glycine = 55.7 μM/g DM and alanine = 412 μM/g DM in *Porphyra linearis*; McLachlan, 1972). Therefore, cows may find seaweeds such as *P. linearis* and *G. changgi,* which is rich in both (Norziah and Ching, 2000), more palatable due to the sweetness.

1.4.2.4. Sourcing and Transportation

As the goal for more widespread inclusion of seaweed in ruminant diets has intensified, so have concerns relating climate change's impact on global seaweed abundance, distribution, accessibility, and quality (Buschmann et al., 2017). Variations in seaweed quality and nutrient profile are also affected by the location of growth (Bikker et al., 2020; Billot et al., 2003; Young et al., 2013), harvest season (Craigie et al., 2008; Manns et al., 2017; Pandey et al., 2022; Sappati et al., 2019; Tayyab et al., 2016), and processing techniques used (Craigie et al., 2008; Sappati et al., 2019; Vucko et al., 2017). In comparing samples of *Ulva lactuca*, Bikker et al. (2020) observed that CP content ranged from 70 g/kg DM when harvested in Scotland to 248 g/kg DM when harvested in Ireland. Additional distinctions in crude fat, crude fiber, and ash contents between and within species from different locations are reported by Bikker et al. (2020). Young et al. (2013) collected samples of *Plocamium cartilagineum* at different locations along the coast of Antarctica and reported site-specific differences in phylogeny and secondary

metabolism between closely spaced (125 m apart) sites. While Young et al. (2013) also identified genetic and chemical similarities between samples from sites further apart (4 km), Billot et al. (2003) reported genetic differences in *L. digitata* samples from sites greater than 10 km apart in the English Channel. While these geographically-driven differences lead to variation in nutrient profile, and thus create challenges for sourcing of a consistent seaweed product for livestock diets, Tayyab et al. (2016) and Gaillard et al. (2018) did establish that consistent CP profiles can be achieved in seaweed samples from the same harvest location, year, and when exposed to the same processing methods (rinsed in 30% seawater, then freshwater, frozen at -20°C, freeze dried, milled to 1.5 mm).

Biomass availability also varies greatly based on location; therefore, depending on the species that are deemed ideal candidates, there will be a need to strategically envision how to efficiently and sustainably produce enough seaweed to supply the beef and dairy industries (Vijn et al., 2020). Just like variation in terrestrial annual crop yield, growth of marine biomass fluctuates as demonstrated by Bell et al. (2015). Giant kelp forests along coastal California ranged in biomass from 4.74×10^6 kg (Spring 1998) to 4.14×10^8 kg (Autumn 2005), over a study period of 25 years, fluctuating with wave disturbance and nitrate availability (Bell et al., 2015). While species such as *Pyropia* may seem like appropriate candidates for ruminant diets nutritionally, it is already in high demand in the human food chain, which could lead to sourcing difficulties and competition (Buschmann et al., 2017; FAO, 2016).

Transportation of seaweeds for use on farms is another critical aspect to consider. While the supplementation of the seaweed being transported may reduce CH_4

emissions by the animals it is fed to, trans-oceanic or global transportation may counteract the environmental benefit of feeding the seaweed by creating more transportation emissions (Gray et al., 2021). Although not specifically for seaweed transportation, nearly 99% of total shipping emissions of CH_4 are generated by international ships (Smith et al., 2015). By locally sourcing seaweeds rather than requiring trans-oceanic shipping, some of these emissions could potentially be reduced. Therefore, the transportation method and distance must be factored into overall impact to ensure a net emission reduction. This issue can be circumnavigated by implementing specific seaweed species that are grown for more localized use, allowing for minimal transportation. *L. digitata and A. nodosum* are readily available in Europe, Scandinavia, and eastern North America (McHugh, 2003), which may make them more environmentally beneficial supplement options in those regions. Similarly, *S. latissima* is readily available along North American shores (McHugh, 2003), making it another candidate for supplementation in North American coastal regions.

1.4.2.5. Processing Methods

It is well documented that CH_4 production by animals is dependent on the method of plant preservation (Sundstol, 1981), including any chemical and physical processing of forages and other crops such as soybeans and corn silages, prior to feeding (Johnson et al., 1996; LeLiboux and Peyraud, 1999; McAllister et al., 1996). Recent findings suggest that seaweed is no exception, and its impact on CH_4 emissions after ingestion are also processing-dependent (Vucko et al., 2017). Processing of seaweed, such as washing procedures, may be necessary to remove sand, residing organisms, epiphytes, and excess minerals such as Na and I to satisfy daily mineral allowance

standards for cow consumption set by the National Research Council (NASEM, 2021). However, this would affect ash concentration, and could increase processing-driven variability in product profile. Kolawole et al. (2014) demonstrated that when leafy green vegetables indigenous to Nigeria were subjected to blanching and squeeze washing, respectively, they contained less iron (114-150% less; 116-193% less), Ca (103-578% less; 112-651% less), Mg (104-502%; 113-1243% less), K (106-283% less; 115-323% less) and Na (104-348% less; 115-439% less) compared with unprocessed, fresh samples. Therefore, processing methods such as blanching may have similar effects on the mineral content of seaweeds due to mineral leaching during washing procedures. While no studies directly address these effects in seaweeds, Abbott et al. (2020) highlighted the gap in knowledge of seaweed harvesting, processing, and storage methods.

It is evident that currently utilized seaweed preservation and processing methods vary greatly between studies, and a summary of available processing methods of various studies are presented in **Table 1.1.** Vucko et al. (2017) reported that the most effective methods in preserving anti-methanogenic activity in *A. taxiformis* were the following: 1) unrinsed biomass, frozen then freeze dried, 2) dip-rinsed biomass, frozen then freeze dried, 3) 1 min-submerged biomass, frozen then freeze dried, 4) 3 min-submerged biomass, frozen then freeze dried, 5) 6 min-submerged biomass, frozen then freeze dried 6) unrinsed biomass, freshly kiln-dried without freezing, and 7) unrinsed biomass, freshly dehydrated without freezing. These processes completely inhibited CH_4 production *in vitro* with a 2% OM inclusion, when compared with the control. Despite similar findings among these treatments, the unrinsed frozen and then freeze-dried samples contained the highest concentrations of bromoform (4 mg/g DM; Vucko et al., 2017), which may

indicate an upper threshold for bromoform dosage efficiency. Dubois et al. (2013), Gaillard et al. (2018) and Tayyab et al. (2016) washed seaweed samples in seawater followed by freshwater prior to freeze drying. Liot et al. (1993) reported that seawater was preferred over freshwater when rinsing seaweed, as freshwater rinsing created high osmotic pressure and caused cell rupture, allowing for contaminant microbes to degrade *P. palmaria* and *U. rigida* samples within 14 d of storage. Various studies (Gaillard et al., 2018; Kinley et al., 2020; Roque et al., 2019a, 2019b) froze and stored samples at 25 to -20°C before freeze drying. Kinley et al. (2020), Roque et al. (2019a), and Tayyab et al. (2016) all milled samples after freeze drying, while Machado et al. (2014) rinsed, centrifuged to remove excess water, freeze dried and then ground seaweed samples. Several studies did not outline any use of washing methods performed (Antaya et al., 2015, 2019; Belanche et al., 2016; Bikker et al., 2020; Choi et al., 2021; Kinley and Fredeen, 2015; Kinley et al., 2020; Mihaila et al., 2022; Rey-Crespo et al., 2014; Roque et al., 2019a, 2019b; Wang et al., 2008) and a few did not specify harvesting details (Antaya et al., 2015, 2019; Choi et al., 2021; Kinley and Fredeen, 2015; Rey-Crespo et al., 2014).

Apart from research-specific uses, numerous commercially produced seaweed supplements are currently available to researchers and producers, most of which use physical processing techniques or basic drying or rinsing protocols. Rey-Crespo et al. (2014) and Antaya et al. (2019) used powdered supplements (*U. rigida, S. muticum and S. polyschides* at 100 g DM/animal/d; and kelp meal from *A. nodosum* at 115 g DM/animal/d, respectively) mixed into concentrate portions of diets *in vivo*. The kelp meal fed by Antaya et al. (2019) was geothermally dried at a maximum temperature of

85°C according to the manufacturer (Thorvin Inc., New Castle, VA, USA), which is an important clarifying aspect of the methodology as heat drying is known to reduce the bioactive content of seaweeds (Vucko et al., 2017). Maia et al. (2019) also heat dried samples at 65°C after rinsing. Yen et al. (2021) subjected *S. latissima* and *A. esculenta* to different ensiling conditions and found that seaweeds ensiled for 12 months had a significant reduction in antioxidant activity. Craigie et al. (2008) was able to distinguish among commercial *A. nodosum* extracts produced by different manufacturers based on molecular structure, most likely due to differences in seaweed processing methods. These findings indicate the need for careful consideration and explicit descriptions of seaweed processing methods in literature.

Although less common, some studies have chemically processed seaweeds for extractions prior to utilization in ruminant feed research. Choi et al. (2021) cut or crushed, freeze dried and then ground brown seaweeds (*Undaria pinnatifida, Sargassum fusiforme, and Sargassum fulvellum*) into a fine powder. The product was further treated with 80% (v/v) ethanol and later dissolved in the solvent dimethylsulfoxide (DMSO). In Wang et al. (2008), whole plant material from *A. nodosum* was ground to pass through a 500 μm screen and 100 g was mixed with 2500 mL of 80% (v/v) methanol, where the aqueous fraction was then freeze dried. Although the reasoning for chemical processing is not explicitly stated in these studies, Cassani et al. (2020) reviewed the role of solvent polarity and compound solubility on phlorotannin yield. Koivikko et al. (2005) found that the effectiveness of phlorotannin extractants for *F. vesiculosus* were the following (in order from most to least effective): 70% aqueous acetone, water, 80% aqueous methanol, 80% aqueous ethanol, 100% ethanol and 100% methanol performed equally, and lastly,

100% acetone. These findings reaffirm the notion that a mixture of water and an organic solvent have a greater extraction potential than a single component solvent system, with the exception of water (Waterman and Mole, 1994). However, further investigation of how chemical treatment affects seaweed nutrient profiles, digestibility, and palatability, and how the seaweeds will affect rumen fermentation is necessary to compare these processes with other common processing methods.

1.4.3. Impact of Seaweed and Seaweed-derived Products on Rumen Environment

Due to their complex nutrient and bioactive profiles, seaweeds impact different aspects of rumen fermentation, such as reducing N emissions from ruminants and affecting VFA proportions. Wang et al. (2008) reported that supplementation of kelp meal (whole *A. nodosum* ground to pass through a 500 μm screen, mixed with methanol, stirred at 22°C, evaporated at 40°C, freeze dried) *in vitro* improved N utilization by reducing ammonia-N (NH_3-N) concentrations, thus reducing N output into the environment. Up to 92.5% (Exp 1) and 38.2% (Exp 2) reductions in NH_3-N concentrations were observed by Wang et al. (2008), in batch culture given a forage substrate (Exp 1) or concentrate substrate (Exp 2) with 500 μg/mL phlorotannin without polyethylene glycol, respectively. These reductions were attributed to reduced proteolysis by rumen microbes as proteins were protected by the phlorotannins present in *A. nodosum*. A reduction of NH_3-N output, in tandem with an increase in VFA production and no concurrent change in digestibility, was also observed by Choi et al. (2021) when supplementing brown algae extracts (plant matter cut or crushed, freeze-dried, ground to fine powder, extracted with ethanol solvent, and infused with DMSO) *in vitro*. Bendary et

al. (2013) found that a seaweed supplement sourced from Crossgates Bioenergetics-Seaweeds Company (Skipton, UK; species and processing not reported) resulted in a higher total VFA production, a lower NH_3-N concentration and higher digestibility when compared with a negative control, suggesting that the high content of amino acids, fatty acids and ash stimulated fermentation activity. Conversely*, in vitro* supplementation of *A. taxiformis* (biomass rinsed in freshwater, centrifuged, freeze dried at -55°C, ground to 1 mm, stored at -20°C) reportedly had lower acetate to propionate ratio (A:P) and lower NH_3-N concentration compared with a negative control (Machado et al., 2014).

Shifts in VFA proportions with the addition of seaweeds have been highly variable across studies. Machado et al. (2016; 4 different solvents; biomass rinsed, freeze-dried, ground to 1 mm, mixed with solvent and agitated, dissolved in DMSO) observed a decrease in total VFA production which is an undesirable effect as it decreases diet-derived energy efficiency. In supplementing *A*. *taxiformis*, both Machado et al. (2014; inclusion of 20% OM intake; biomass rinsed, freeze-dried, ground to 1 mm, mixed with solvent and agitated, dissolved in DMSO) and Li et al. (2016; inclusion of 0.5, 1, 2 or 3% OM intake; biomass rinsed in clean seawater, dried in kiln at 45°C, ground to 3 mm) observed increased ruminal concentrations of propionate *in vitro* and in sheep, respectively. Conversely, Roque et al. (2019a) did not observe changes to VFA concentrations *in vitro* as a result of supplementing *A. taxiformis* at 5% OM inclusion (biomass frozen, stored at -15°C, freeze dried, ground to 2-3 mm). Danielsson et al. (2017) found that low CH_4 emitting dairy cows (producing 291 ± 7.7 g CH_4/d) produced less butyrate when compared with high CH_4 emitters (producing 345 ± 8.1 g CH_4/d). While Kinley et al. (2020; biomass stored at -15°C, freeze dried, ground to 2-3 mm,

stored at -15°C) reported no changes in total VFAs between the control and treatments in Brahman-Angus cross steers, Machado et al. (2014) found that *Dictyota bartayresii* and *A. taxiformis* (biomass of both species rinsed in freshwater, centrifuged, freeze dried at -55°C, ground to 1 mm, stored at -20°C) supplementation yielded the lowest total VFA concentrations among the twenty species studied *in vitro*.

Tangential to VFAs, rumen pH may also be impacted by seaweed intake. For instance, Bendary et al. (2013) reported that seaweed meal sourced from Crossgates Bioenergetics-Seaweeds Company (Skipton, UK; species and processing undeclared) raised rumen pH in lactating Friesian cows compared with the negative control group. Montañez-Valdez et al. (2012) reported that a calcified seaweed extract from *Lithothamnium calcareum* (processing undeclared) successfully increased rumen pH at 0.5 g/kg DM in Holstein steers without modifying VFA concentrations or ruminal disappearance kinetics. Another study supplemented *L. calcareum* at 0.4% of dietary DM in Holstein cows, where the algae treatment cows spent 4 h/d with a rumen pH below 5.5, in comparison with sodium bicarbonate-treated (7.5 h/d) and control cows (13.8 h/d; Cruywagen et al., 2015). However, despite a high concentrate-based diet, treatment cows in Cruywagen et al. (2015) produced more acetate and less propionate than control cows.

Positive or minimal effects on animal health (Antaya et al., 2019; Karatzia et al., 2012), energy utilization (Karatzia et al., 2012), N metabolism (Wang et al., 2008), and milk quality and safety (Karatzia et al., 2012; Newton et al., 2021) have been demonstrated as a result of *A. nodosum* supplementation in dairy cows. These null results have been reported both *in vivo* (Antaya et al., 2019; Karatzia et al., 2012; Newton et al. 2021) and *in vitro* (Wang et al., 2008). Another *in vitro* study by Belanche et al. (2016)

observed a 24% decrease in N degradability compared with the control when supplementing *A. nodosum* at 5% DM, but also found no impact of *L. digitata* on N degradability at the same dietary inclusion, most likely due to the higher phlorotannin content of *A. nodosum* compared with *L. digitata*. Newton et al. (2021) reported that Icelandic cows fed a seaweed mixture (91% DM *A. nodosum* and 9% DM *L. digitata*) at 1.5% of concentrate DM produced milk with less protein, copper, and selenium than the control cows but with more I and As. Despite these significant changes seen in milk nutrient profile, Newton et al. (2021) reported that these changes do not impact milk safety for human consumption. Karatzia et al. (2012) found no effects of seaweed on average daily milk production, milk fat or protein when supplementing 80 g *A. nodosum*/cow/d to Holsteins. Additionally, *A. nodosum* increased blood glucose by 13% in 7 weeks compared with the control, without effects on red or white blood cells (Karatzia et al., 2012). Since blood glucose serves as a main indicator of energy status in ruminants, this increase could help provide enough glucose to promote more lactose synthesis in high-producing animals (Schultz, 1968).

The most alluring aspect of utilizing seaweeds as ruminant feed additives is the potential for enteric CH_4 reductions; however, results among studies are highly variable. While the first *in vitro* study to report lowered gas production from supplementation of seaweed extracts was Wang et al. (2008), Dubois et al. (2013) was the first to report reductions from the use of whole seaweed material. Machado et al. (2014) reported similar findings shortly after, where all seaweed species tested had similar or lower total gas production (TGP) and CH_4 when compared with the negative control. In this study, twenty different seaweeds were supplemented via batch culture at 0.2 g OM in 125 mL of

rumen fluid with 0.1 g OM Flinders grass (Machado et al., 2014). Abbott et al. (2020) later summarized more than 21 seaweed species that have been shown to reduce CH_4 emissions *in vitro*. Percentages of CH_4 reductions reported from supplementation with various seaweed species among key studies are represented in **Figure 1.1.** To better compare these studies, information regarding animals used, the base diet fed, inclusion rates and processing methods of seaweed supplemented are presented in **Table 1.1.**

Among the species evaluated and listed in **Figure 1.1.**, *A. taxiformis* seems to be the most promising seaweed from a CH_4 emission standpoint, as supplementation decreased CH_4 production up to 98.9% *in vitro* (Machado et al., 2014; Roque et al., 2019a) and 98% *in vivo* (Kinley et al., 2020). Both TGP and CH_4 production were reduced when *A. taxiformis* was supplemented in Machado et al. (2014; biomass rinsed in freshwater, centrifuged, freeze dried at -55°C, ground to 1 mm, stored at -20°C) and Roque et al. (2019a; biomass frozen, stored at -15°C, freeze dried, ground to 2-3 mm). While a 98.9% CH_4 reduction after 72 h *in vitro* was reported by Machado et al. (2014) when *A. taxiformis* was added at an inclusion rate of 0.2 g OM, Kinley et al. (2020; biomass stored at -15°C, freeze dried, ground to 2-3 mm, stored at -15°C) reported a similar reduction of up to 98% *in vivo*, when beef cattle were fed an inclusion rate of 0.2% of TMR (OM basis). Kinley et al. (2020) also noted a significant but less impressive CH_4 reduction of 38% when included at 0.1% of TMR (on OM basis). Despite differences in processing, Machado et al. (2014) and Kinley et al. (2020) still yielded very comparable CH_4 reduction results, which could indicate that freezing prior to freeze drying and milling differences may not directly affect CH_4 production. It is important to note that the *A. taxiformis* used in both studies was harvested in the coastal area near

Queensland, Australia, which could also contribute to the consistency of reduction findings.

Feeding other red seaweed species, such as *A. armata*, has yielded CH_4 reductions as well. Roque et al. (2019b) reported that CH_4 yield (g/kg DMI) decreased up to 42.7% *in vivo* with a 1% OM inclusion of *A. armata* (whole plant; blast frozen at -25°C*,* vacuum freeze dried) when normalized for amount of feed intake. *Gracilaria vermiculophylla* was supplemented *in vitro* at 25% (DM basis), yet no effect was observed on daily CH_4 production (Maia et al., 2019). However, *G. vermiculophylla* was oven dried prior to supplementation in the research reported by Maia et al. (2019), which could have affected the retention of bioactive compounds, such as bromoform, that would impact methanogenesis. Additionally, Maia et al. (2019) reported that *G. vermiculophylla* had the highest ash content when compared with *S. latissima* and *U. rigida* (all rinsed in freshwater, oven dried at 65°C, ground to 4 mm), which could indicate that *G. vermiculophylla* simply contained less bioactive compounds. Despite the lack of *C. crispus* studies *in vivo*, Kinley and Fredeen (2015) examined CH_4 reductions of a seaweed mixture containing equal proportions of *C. crispus*, *Laminaria longicruris*, *Furcellaria sp.*, and *Fucus vesiculosus in vitro*. It was found that all seaweed treatments, including individual supplementation of *C. crispus* and *Furcellaria sp.*, reduced CH_4 production compared with the control *in vitro*. While a 16% maximum reduction of CH_4 production (g/d) resulted from a 0.28 g/DM/d inclusion of the seaweed mixture, there was no difference found in CH_4 production when *C. crispus* and *Furcellaria sp.* were supplemented individually at 0.14 g/DM/d (Kinley and Fredeen, 2015).

Supplementation with brown seaweeds creates quantifiable but less significant impacts on enteric CH_4 (Antaya et al., 2019; Choi et al., 2021; Wang et al., 2008) when compared with other phyla. However, brown algae are still of interest as they are the only seaweed type known to contain the bioactive compounds phlorotannins, which inhibit ruminal methanogenic archaea (Antaya et al., 2019) and thus impact methanogenesis through altered microbial diversity. This is possibly due to phlorotannin's protein-binding activity (Wang et al., 2008) which decreases digestibility and consequently decreases CH_4 production (Jayanegara et al., 2011). While Patra et al. (2011) noted that differences in the magnitude of methanogenic response to phlorotannin could be observed due to the source, type, and concentration of phlorotannin supplemented, several studies have reported benefits. When supplementing a phlorotannin extract derived from *A. nodosum* (whole plant; ground to pass through a 500 µm screen, mixed with methanol, stirred at 22°C, evaporated at 40°C, freeze dried) at 500 µg/mL, Wang et al. (2008) found that gas production *in vitro* was reduced up to 44.2% (Exp 1) and 7.7% (Exp 2) at 24h. The impact of *A. nodosum* reported by Belanche et al. (2016) were dissimilar, as no effects of *A. nodosum* (frozen at -80°C, freeze dried, ground to 1 mm, stored at -20°C) on CH_4 production, feed degradability or rumen fermentation were reported but a 24% decrease in N degradability was observed.

While Dubois et al. (2013) also reported that *C. trinodis* (fed as whole plant; rinsed in seawater and dechlorinated freshwater, water removed via centrifugation, frozen at -10°C, freeze-dried) reduced more CH_4 *in vitro* as dosage increased from 0 to 80 mg/g substrate OM, these authors suggested that the anti-methanogenic properties were due to terpenes, another group of phenolic compounds. Methane production declined over time

when *U. pinnatifida*, *S. fusiforme*, and *S. fulvellum* extracts were supplemented at 0.25 mg/mL (plant matter cut or crushed, freeze-dried, ground to fine powder, extracted with ethanol solvent, DMSO infusion) *in vitro* (Choi et al., 2021). Unlike the work reported by Machado et al. (2016), where 20 µL of *A. taxiformis* extracts (4 different solvents; biomass rinsed, freeze-dried, ground to 1 mm, mixed with solvent and agitated, dissolved in DMSO) did not affect TGP, TGP increased in response to seaweed supplementation in the *in vitro* trial by Choi et al. (2021). This disparity could again be due to the different seaweed species and substrates studied. Maia et al. (2019) supplemented *S. latissima* (rinsed in freshwater, oven dried at 65°C, ground to 4 mm) at an inclusion rate of 25% DM *in vitro* and observed no effects on CH_4 production. However, samples were oven dried rather than freeze dried in the study outlined by Maia et al. (2019), which could have affected the retention of bioactive compounds in *S. latissima* that would result in CH_4 reductions.

Apart from differences in immediate CH_4 outputs, it is unknown if seaweed-induced inhibition of methanogens is temporary and little research has characterized how long CH_4 emission reductions last after or during supplementation. Few studies have investigated the long-term effects of seaweed supplementation on CH_4 reductions. To our knowledge, the longest study to date was performed by Kinley et al. (2020), where beef cattle had a 90-d adaptation period of *A.taxiformis* followed by a 60-d period at complete inclusion levels up to 0.2% of the TMR on an OM basis. Kinley et al. (2020) reported CH_4 reductions up to 98% for the highest inclusion rate, making it the highest reported CH_4 reduction *in vivo* among any seaweed supplementation study in ruminants. However, it is still unclear if these CH_4 reductions would be sustained longer-term. Temporary CH_4

reductions from lactating Jersey cows fed kelp meal (*A. nodosum*) were reported by Antaya et al. (2019), but only during the first 28-d experimental period. Since subsequent periods showed no changes in CH_4 production, it was uncertain whether the methanogenic communities of the cows adapted to the kelp meal over time (Antaya et al., 2019). Ultimately, whether processing interplays with the longevity of response, in addition to the magnitude, is another area requiring further study.

1.5. CONCLUSION

Identification and characterization of plant-based supplements that can support improved sustainability through decreased environmental outputs, is an exciting prospect. Seaweeds appear to be feasible candidates that can provide such benefit. While it is evident that more research is required both *in vitro* and *in vivo* before pursuing widespread, long-term inclusion of seaweed in ruminant diets, some preliminary reports are promising. However, more research is needed to better understand the impact of seaweed supplementation in ruminant diets on animal health, with a more in depth and complete characterization of seaweed processing, including how washing or drying techniques may affect rumen fermentation and overall effectiveness in mitigating CH_4 emissions. Additionally, there is a need to consider both scalability and financial feasibility of candidate seaweed species prior to widespread supplementation trials *in vivo*.

Based on our current knowledge of seaweed species and their dietary potential, the following hypotheses and objectives were developed in this thesis; it was hypothesized that there would be differences in CH_4 production of *S. latissima* due to

processing method, with less processing correlating to less CH_4 production *in vitro* in experiment 1. For experiment 2, it was hypothesized that fermenters supplemented with *C. crispus* would produce less CH_4 as inclusion rate increased and that reductions would be comparable to that seen in fermenters given bromoform dosages. The objectives were to evaluate the effects of dietary supplements derived from *S. latissima* and *A. nodosum* subject to different processing methods, as well as *C. crispus* at different inclusion rates, on microbial CH_4 concentration and fermentation profile through continuous culture fermentation.

1.6. ACKNOWLEDGEMENTS

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Table 1.1. Summary of rumen fermentation and dairy cow performance (where applicable) in response to seaweed supplementation and processing methods. Specific differential responses are separated by dose or treatment if more than one is being compared.

Seaweed Species	Reference	Phyla	Seaweed Washing/Processing	Animal	Diet/Inclusion	Response
A. esculenta	[5]Pandey et al., 2022	Brown	Whole plant washed in SW[1], then 30 SW:70 FW[1], then FW Samples frozen (-40°C), freeze-dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM[2] inclusion	↓[3] OMdeg[4] (autumn); butyrate production (autumn) ↔[3] TGP[4]; CH_4 (mL/g OM); tVFA[4]; propionate production; Euryarchaeota relative abundance; *Methanobrevibacter spp*.
A. nodosum	Antaya et al., 2019	Brown	Washing NR[1] Geothermally dried at ≤ 85°C	*in vivo*; lactating Jersey cows	70:30 (F:C[2]); 48% DM cool-season perennial herbage, 52% DM partial TMR[2] 113 g KM[2]/d inclusion	↑[3] DMI[4]; milk I ↔ CO_2; CH_4 (g/kg DMI); milk yield; FE[4]; DMdig[4]
A. nodosum	Belanche et al., 2016	Brown	Washing NR Frozen (−80°C within 1 h of harvesting), freeze-	*in vitro*; inoculum collected from open Holstein-	50:50 (F:C) 5% DM inclusion	↓ Valerate production ↔ OMdeg; TGP; CH_4 (mmol/g DOM[4]); rumen pH; tVFA; NH_3-N[4]; NAN[4]

			dried, ground (1 mm^2), stored (–20°C)	Friesian cows		
A. nodosum	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW: 70 FW, then FW Samples frozen (-40 °C), freeze-dried (72 h; -50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ OMdeg; tVFA (autumn); TGP; CH_4 (mL/g OM; autumn); butyrate production (autumn); Euryarchaeota relative abundance ↔ Propionate production; *Methanobrevibacter spp.*
A. nodosum	Wang et al., 2008	Brown	Washing NR Whole plant material ground (500 µm), 100 g was mixed with 2500 mL of 80% (v/v) methanol, stirred (22°C, 2 h), filtered, aq fraction freeze-dried	*in vitro*; Jersey steers	Barley silage/alfalfa (Ex 1) Ground barley grain (Ex 2) 125, 250 or 500 g PT^2/mL of medium (40 mL)	<u>Ex 1 (all doses, 48 h)</u> ↑ A:P[4] ↓ TGP; aNDFd[4]; NH_3-N; tVFA; BVFA[4] <u>Ex 2 (all doses, 24 h)</u> ↓ TGP; starch disappearance; NH_3-N; BVFA; A:P ↔ tVFA
A. armata	Roque et al., 2019b	Red	Washing NR Blast frozen (-25°C; 6 h), freeze-dried (30 h)	*in vivo*; lactating Holstein cows	TMR 0.5% or 1% OM inclusion	<u>0.5% OM</u> ↓ CH_4 yield (g/kg DMI) ↔ Milk yield; fat %; protein %; lactose %; MUN[4]; SCC[4]; milk $CHBr_3$[4] <u>1% OM</u>

						↓ CH_4 yield (g/kg DMI); DMI; milk yield; protein % ↔ Fat %; lactose %; MUN; SCC; milk $CHBr_3$
A. taxiformis	Kinley et al., 2016	Red	Biomass rinsed in SW (2 min), dipped in FW Centrifuged (1000 x g, 6 min), stored (-10°C), freeze-dried, ground (1 mm), stored (-10°C)	*in vitro*; Brahman steers	Rhodes grass 0.5%, 1%, 2%, 5% or 10% OM inclusion	<u>0.5% OM</u> ↔ OMdeg; tVFA; TGP; CH_4 (mL/g OM) <u>1% OM</u> ↔ OMdeg; tVFA; TGP; CH_4 (mL/g OM) <u>2% OM</u> ↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; tVFA <u>5% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM) ↔ OMdeg <u>10% OM</u> ↓ OMdeg; tVFA; TGP; CH_4 (mL/g OM)
A. taxiformis	Kinley et al., 2020	Red	Washing NR Biomass stored (-15°C), freeze-dried, ground (2-3 mm), stored (-15°C)	*in vivo*; Brahman-Angus cross steers	High grain TMR; 80% DM Rhodes grass hay, steam rolled barley	<u>0.05% OM</u> ↓ A:P ↔ CH_4 (g/kg DMI); H_2; DMI; tVFA; meat eating quality; butyrate production; ADWG[4]

					0.05%, 0.1% or 0.2% OM inclusion	<u>0.1% OM</u> ↑ H_2; ADWG ↓ CH_4 (g/kg DMI); A:P ↔ DMI; tVFA; meat eating quality; butyrate production <u>0.2% OM</u> ↑ H_2; ADWG ↓ CH_4 (g/kg DMI); A:P ↔ DMI; tVFA; meat eating quality; butyrate production
A. taxiformis	Li et al., 2016	Red	Biomass rinsed in SW Dried in a forced-air solar kiln (45°C, 72 h), ground (3 mm)	*in vivo*; Merino-cross wethers	High fiber pellet 0.5%, 1%, 2%, or 3% OM inclusion	<u>0.5% OM</u> ↔ CH_4 yield (g/kg DMI); LW^4; DMI <u>1% OM</u> ↓ CH_4 yield (g/kg DMI); tVFA ↔ LW; DMI <u>2% OM</u> ↓ CH_4 yield (g/kg DMI); tVFA ↔ LW; DMI <u>3% OM</u> ↓ CH_4 yield (g/kg DMI); tVFA ↔ LW; DMI

A. taxiformis	Machado et al., 2014	Red	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
A. taxiformis	Roque et al., 2019a	Red	Biomass was frozen, stored (− 15°C), freeze-dried, ground (2–3 mm)	*in vitro*; non-lactating Jersey and Holsteins	70:30 (F:C); alfalfa, dried distillers grain, rolled corn 5% OM inclusion	↑ A:P ↓ TGP; CH_4 (mL/g OM; 96 h); Euryarchaeota relative abundance ↔ CO_2 (96 h); tVFA; rumen pH
A. taxiformis	Stefenoni et al., 2021	Red	Biomass rinsed Frozen (−40°C), stored (-20°C to −25°C), freeze-dried, ground (1 mm), stored in airtight locking barrels (4°C)	*in vitro*; lactating dairy cows (Ex 1) *in vivo;* lactating Holsteins (Ex 3)	30:70 (F:C; Ex 1, 3); 43% corn silage, 14% haylage 1% DM inclusion (Ex 1) 0.25% or 0.50% DM (Ex 3)	<u>Ex 1</u> ↑ H_2 ↓ TGP; CH_4/g of TMR <u>Ex 3 (0.25% DM)</u> ↑ H_2 ↓ lactose % ↔ CH_4 (g/kg of DMI); DMI; milk yield; milk TP[4]; milk FE; ECM[4]; milk fat; TP; SNF[4]; MUN; SCC; LW; milk $CHBr_3$; CO_2; tVFA; A:P; insulin; BUN:creatinine; TP; albumin:globulin

						Ex 3 (0.50% DM) ↑ H_2 ↓ CH_4 (g/kg of DMI); DMI; milk yield; milk fat; milk TP; lactose %; ECM; tVFA; A:P; BUN:creatinine ↔ Milk FE; TP; SNF; MUN; SCC; LW; milk $CHBr_3$; CO_2; insulin; TP; albumin:globulin
A. taxiformis	Vucko et al., 2017	Red	Washed with SW, DR[1], Sub1[1], Sub3[1] or Sub6[1] [6,7] See footnotes for specific processing All milled (1 mm), stored (-15°C) with desiccant	*in vitro*; Brahman steers	Rhodes grass 2% OM inclusion	Treatments ([6,7]ALL Froz FD; ALL Fres Deh; ALL Fres Kiln) ↓ TGP; CH_4 (mL/g OM) Treatments ([6,7]ALL Froz Deh; ALL Froz Kiln) ↔ TGP; CH_4 (mL/g OM)
B. hamifera	Mihaila et al., 2022	Red	Washing NR Frozen, stored (–20°C), freeze-dried (48 h, 50 mbar, milled to powder with blender, stored (–80°C)	*in vitro*; non-lactating Friesian x Jersey cows	Perennial ryegrass 2%, 6%, or 10% OM	2% OM ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg 6% OM ↑ H_2 ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg 10% OM ↑ H_2

						↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg
C. taxifolia	Dubois et al., 2013	Green	Washed in SW (2 min); rinsed in dechlorinated FW (1 min) Centrifuged (100 µm mesh; 1000 rpm, 6 min), stored (-10°C), freeze-dried, stored (-10°C)	*in vitro*; Brahman steers	Rhodes grass 2%, 4%, 8% or 16% OM (Ex 2)	<u>2% OM</u> ↔ CH_4 (mmol/mL); TGP; rumen pH <u>4% OM</u> ↔ TGP; rumen pH CH_4 NR <u>8% OM</u> ↓ TGP ↔ Rumen pH CH_4 NR <u>16% OM</u> ↔ CH_4 (mmol/mL); TGP; rumen pH
C. taxifolia	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ CH_4 (mL/g OM) ↔ TGP; OMdeg; rumen pH
C. linum	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher &	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH

			Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)			
C. crispus	Kinley and Fredeen, 2015	Red	Washing NR Ground (2 mm)	*in vitro;* lactating Holstein cows	60:40 (F:C); timothy/alfalfa 0.14 g DM/d	↓ NH_3-N; CH_4 (g/d) ↔ Rumen pH; OMdeg; tVFA; A:P
C. crispus	[5]Pandey et al., 2022	Red	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze-dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ OMdeg; CH_4 (mL/g OM; spring) ↔ tVFA; TGP; propionate production; butyrate production; Euryarchaeota relative abundance; *Methanobrevibacter spp.*
C. coelothrix	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↔ TGP; CH_4 (mL/g OM); OMdeg; rumen pH
C. patentiramea	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH

			dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)			
C. sinuosa	Machado et al., 2014	Brown	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
C. trinodis	Dubois et al., 2013	Brown	Washed in SW (2 min); rinsed in dechlorinated FW (1 min) Centrifuged (100 µm mesh; 1000 rpm, 6 min), stored (-10°C), freeze-dried, stored (-10°C)	*in vitro*; Brahman steers	Rhodes grass 2%, 4%, 8% or 16% OM (Ex 2)	<u>2% OM</u> ↑ Rumen pH ↔ CH_4 (mmol/mL); TGP CH_4 NR[4] <u>4% OM</u> ↓ CH_4 (mmol/mL) ↔ TGP; rumen pH <u>8% OM</u> ↓ CH_4 (mmol/mL); TGP ↔ Rumen pH <u>16% OM</u> ↔ TGP; rumen pH CH_4 NR
D. tenuissima	Machado et al., 2014	Green	Biomass rinsed in FW	*in vitro*; Bos	Flinders grass 0.2 g OM	↔ TGP; CH_4 (mL/g OM); OMdeg; rumen pH

			Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	indicus steers		
D. bartayresii	Machado et al., 2014	Brown	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
E. radiata	Mihaila et al., 2022	Brown	Washing NR Frozen, stored (–20°C), freeze dried (48 h, 50 mbar, milled to powder with blender, stored (–80°C)	*in vitro*; non-lactating Friesian x Jersey cows	Perennial ryegrass 2%, 6%, or 10% OM	<u>2% OM</u> ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg <u>6% OM</u> ↓ TGP; CH_4 (mL/g OM); OMdeg ↔ tVFA; H_2 <u>10% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2
E. formisissima	Mihaila et al., 2022	Red	Washing NR Frozen, stored (–20°C), freeze dried (48 h, 50	*in vitro*; non-lactating Friesian x	Perennial ryegrass	<u>2% OM</u> ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg

			mbar, milled to powder with blender, stored (– 80°C)	Jersey cows	2%, 6%, or 10% OM	<u>6% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2 <u>10% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2
F. serratus	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (– 40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ OMdeg; TGP (spring); butyrate production (autumn) ↔ tVFA; propionate production; CH_4 (mL/g OM); Euryarchaeota relative abundance; *Methanobrevibacter spp.*
F. vesiculosus	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (– 40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ OMdeg; tVFA; butyrate production; TGP; CH_4 (mL/g OM; autumn); Euryarchaeota relative abundance; *Methanobrevibacter spp.* ↔ Propionate production
Furcellaria sp.	Kinley and Fredeen, 2015	Brown	Washing NR Ground (2 mm)	*in vitro;* lactating Holstein cows	60:40 (F:C); timothy/alfalfa 0.14 g DM/d	↓ NH_3-N; CH_4 (g/d) ↔ Rumen pH; OMdeg; tVFA; A:P

G. vermiculophylla	Maia et al., 2019	Red	Rinsed in FW Oven dried (65°C), ground (4 mm)	*in vitro*; inoculum sampled from non-lactating Holsteins	TMR diet (mostly corn silage and haylage) 25% DM inclusion	↑ A:P; DMdig ↔ Rumen pH; NH_3-N; tVFA; TGP; CH_4 (mL/g DM); H_2; OMdig[4]
H. floresii	Machado et al., 2014	Red	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↔ TGP; CH_4 (mL/g OM); OMdeg; rumen pH
H. elongata	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↔ tVFA; propionate production; butyrate production; OMdeg; TGP; CH_4 (mL/g OM); Euryarchaeota relative abundance; *Methanobrevibacter spp.*
H. triquetra	Machado et al., 2014	Brown	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ CH_4 (mL/g OM) ↔ TGP; OMdeg; rumen pH

H. pannosa	Machado et al., 2014	Red	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ CH_4 (mL/g OM) ↔ TGP; OMdeg; rumen pH
L. digitata	Belanche et al., 2016	Brown	Washing NR Frozen (−80°C within 1 h of harvesting), freeze-dried, ground (1 mm^2), stored (–20°C)	*in vitro*; open Holstein-Friesian cows	50:50 (F:C) 5% DM inclusion	↔ OMdeg; TGP; CH_4 (mmol/g DOM); rumen pH; tVFA; NH_3-N; NAN; valerate production
L. digitata	[5]Pandey et al., 2022	Brown	Blades and stipes washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↔ tVFA; propionate production; OMdeg; TGP; CH_4 (mL/g OM); butyrate production; Euryarchaeota relative abundance; *Methanobrevibacter spp.*
L. filiformis	Machado et al., 2014	Red	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH

P. australis	Machado et al., 2014	Brown	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
P. palmata	[5]Pandey et al., 2022	Red	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↔ tVFA; propionate production; OMdeg; butyrate production; TGP; CH_4 (mL/g OM); Euryarchaeota relative abundance; *Methanobrevibacter spp.*
P. canaliculata	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ OMdeg ↔ tVFA; propionate production; butyrate production; TGP; CH_4 (mL/g OM); Euryarchaeota relative abundance; *Methanobrevibacter spp.*
P. cirrhosum	Mihaila et al., 2022	Red	Washing NR Frozen, stored (–20°C), freeze dried (48 h, 50 mbar, milled to powder	*in vitro*; non-lactating Friesian x Jersey cows	Perennial ryegrass 2%, 6%, or 10% OM	<u>2% OM</u> ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg <u>6% OM</u>

			with blender, stored (–80°C)			↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2 <u>10% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2
P. umbilicalis	[5]Pandey et al., 2022	Red	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↔ tVFA; propionate production; butyrate production; OMdeg; TGP; CH_4 (mL/g OM); Euryarchaeota relative abundance; *Methanobrevibacter spp.*
S. flavicans	Machado et al., 2014	Brown	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ CH_4 (mL/g OM) ↔ TGP; OMdeg; rumen pH
S. fulvellum	Choi et al., 2021	Brown	Washing NR Plant matter cut or crushed, freeze-dried, ground to fine powder, extracted with 80 (v/v) ethanol solvent, DMSO	*in vitro*; non-lactating Hanwoo cows	60:40 (F:C); timothy hay, corn grain 5% substrate (0.25 mg/mL)	↑ tVFA ↓ NH_3-N (48 h); A:P (24 h); CH_4 (mL/g DM) ↔ Rumen pH (48 h); DMdig; methanogen abundance

			infusion (50 mg/mL), diluted with culture media			
S. fusiforme	Choi et al., 2021	Brown	Washing NR Plant matter cut or crushed, freeze-dried, ground to fine powder, extracted with 80 (v/v) ethanol solvent, DMSO infusion (50 mg/ml), diluted with culture media	*in vitro*; non-lactating Hanwoo cows	60:40 (F:C); timothy hay, corn grain 5% substrate (0.25 mg/mL)	↑ tVFA; methanogen abundance ↓ NH_3-N (48 h); A:P (24 h); CH_4 (mL/g DM) ↔ Rumen pH (48 h); DMdig
S. latissima	Maia et al., 2019	Brown	Rinsed in FW Oven dried (65°C), ground (4 mm)	*in vitro*; non-lactating Holsteins	TMR diet (mostly corn silage and haylage) 25% DM inclusion	↑ DMdig; OMdig ↔ Rumen pH; NH_3-N; tVFA; TGP; CH_4 (mL/g DM); H_2
S. latissima	[5]Pandey et al., 2022	Brown	Whole plant washed in SW, then 30 SW:70 FW, then FW Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)	*in vitro*; Jersey heifers	Corn silage 20% DM inclusion	↓ Euryarchaeota relative abundance ↔ tVFA; propionate production; OMdeg; butyrate production; TGP; CH_4 (mL/g OM); *Methanobrevibacter spp.*
U. lactuca	[5]Pandey et al., 2022	Green	Whole plant washed in SW, then 30 SW:70 FW, then FW	*in vitro*; Jersey heifers	Corn silage	↓ Euryarchaeota relative abundance

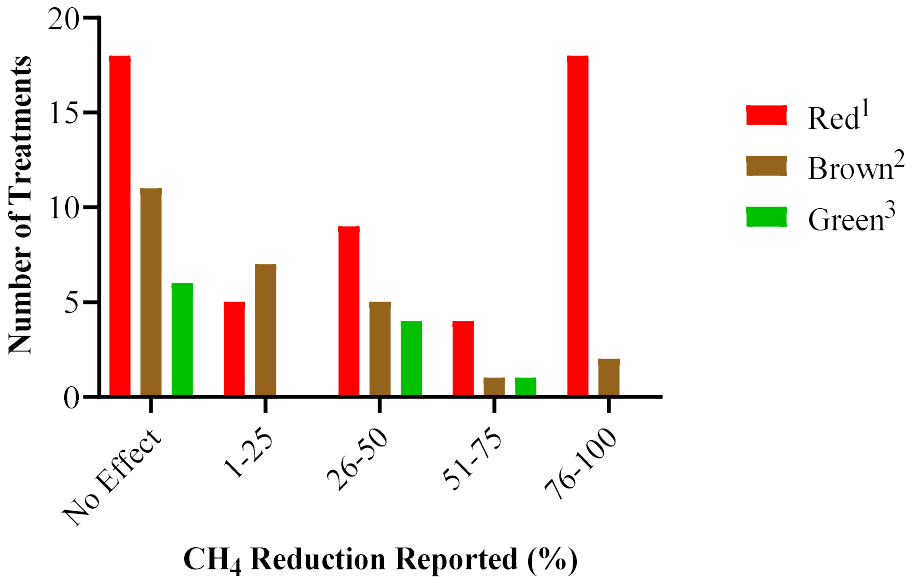
			Samples frozen (–40°C), freeze dried (72 h; – 50°C; < 0.1 mbar), ground (2 mm)		20% DM inclusion	↔ tVFA; propionate production; OMdeg; butyrate production; TGP; CH_4 (mL/g OM); *Methanobrevibacter spp.*
U. ohnoi	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
U. pinnatifida	Choi et al., 2021	Brown	Washing NR Plant matter cut or crushed, freeze-dried, ground to fine powder, extracted with 80 (v/v) ethanol solvent, DMSO infusion (50 mg/mL), diluted with culture media	*in vitro*; non-lactating Hanwoo cows	60:40 (F:C); timothy hay, corn grain 5% substrate (0.25 mg/mL)	↓ CH_4 (mL/g DM) ↔ Rumen pH (48 h); DMdig; NH_3-N (48 h); tVFA; A:P (24 h); methanogen abundance
U. rigida	Maia et al., 2019	Green	Rinsed in FW Oven dried (65°C), ground (4 mm)	*in vitro*; non-lactating Holsteins	TMR diet (mostly corn silage and haylage) 25% DM inclusion	↑ DMdig ↔ A:P; rumen pH; NH_3-N; tVFA; TGP; CH_4 (mL/g DM); H_2; OMdig

Ulva sp.	Machado et al., 2014	Green	Biomass rinsed in FW Centrifuged (1000 rpm [MW512; Fisher & Paykel], 5 min), freeze-dried (-55°C, 120 µbar, 48 h), ground (1 mm), stored (-20°C)	*in vitro*; Bos indicus steers	Flinders grass 0.2 g OM	↓ TGP; CH_4 (mL/g OM) ↔ OMdeg; rumen pH
Ulva sp.	Mihaila et al., 2022	Green	Washing NR Frozen, stored (–20°C), freeze dried (48 h, 50 mbar, milled to powder with blender, stored (–80°C)	*in vitro*; non-lactating Friesian x Jersey cows	Perennial ryegrass 2%, 6%, or 10% OM	<u>2% OM</u> ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg <u>6% OM</u> ↓ OMdeg ↔ tVFA; TGP; CH_4 (mL/g OM); H_2 <u>10% OM</u> ↓ tVFA; TGP; OMdeg ↔ CH_4 (mL/g OM); H_2
V. colensoi	Mihaila et al., 2022		Washing NR Frozen, stored (–20°C), freeze dried (48 h, 50 mbar, milled to powder with blender, stored (–80°C)	*in vitro*; non-lactating Friesian x Jersey cows	Perennial ryegrass 2%, 6%, or 10% OM	<u>2% OM</u> ↔ tVFA; TGP; CH_4 (mL/g OM); H_2; OMdeg <u>6% OM</u> ↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2 <u>10% OM</u>

↓ tVFA; TGP; CH_4 (mL/g OM); OMdeg ↔ H_2

[1]SW = saltwater; FW = freshwater; 30 SW:70 FW = 30% saltwater, 70% freshwater mixture; NR = not reported; DR = dip-rinsed (in dechlorinated freshwater); Sub1 = submerged for 1 min (in dechlorinated freshwater); Sub3 = submerged for 3 min (in dechlorinated freshwater); Sub6 = submerged for 6 min (in dechlorinated freshwater).
[2]DM = dry matter; F:C = forage:concentrate ratio; KM = kelp meal; TMR = total mixed ration; PT = phlorotannin.
[3]↑ = increase; ↓ = decrease; ↔ = no change.
[4]OMdeg = organic matter degradability; TGP = total gas production; tVFA = total volatile fatty acids; DMI = dry matter intake; FE = feed efficiency; DMdig = dry matter digestibility; DOM = dissolved organic matter; NH_3-N = Ammonia-N; NAN = Non-ammonia N; A:P = acetate:propionate ratio; aNDFd = disappearance of neutral detergent fiber with amylase; BVFA= branched-chain volatile fatty acid; MUN = milk urea nitrogen; SCC = somatic cell count; milk $CHBr_3$ = bromoform in milk; ADWG = average daily weight gain; LW = live weight; TP = true protein; ECM = energy corrected milk; SNF = solids-not-fat
[5]If season is not specified under responses from Pandey et al., 2022, responses were not affected by seaweed harvest seasonality.
[6]Treatments from Vucko et al., 2017: SW Froz FD; SW Fres Deh; SW Fres Kiln; SW Froz Deh; SW Froz Kiln; DR Froz FD; DR Fres Deh; Dr Fres Kiln; DR Froz Deh; DR Froz Kiln; Sub1 Froz FD; Sub1 Fres Deh; Sub1 Fres Kiln; Sub1 Froz Deh; Sub1 Froz Kiln; Sub3 Froz FD; Sub3 Fres Deh; Sub3 Fres Kiln; Sub3 Froz Deh; Sub3 Froz Kiln; Sub6 Froz FD; Sub6 Fres Deh; Sub6 Fres Kiln; Sub6 Froz Deh; Sub6 Froz Kiln.
[7]Froz = frozen (5 d, −80°C prior to freeze-drying; 5 d, −15°C prior to dehydration or kiln-drying); FD = freeze-dried (−55°C, 120 μbar, 48 h); Fres = fresh (not frozen); Deh = dehydrated (45°C, 48 h); Kiln = kiln-dried (45°C, 48 h); OMdig = organic matter digestibility.

Figure 1.1. Effect of 40 seaweed species, based on phyla, on methane reduction when supplemented *in vitro* and *in vivo* to ruminants. Treatments reported as "no effect" were not significant compared to the control of the corresponding study.



[1]***A. armata*** (Roque et al., 2019); ***A. taxiformis*** (Kinley et al., 2016; Kinley et al., 2020; Li et al., 2016; Machado et al., 2014; Roque et al., 2019; Stefenoni et al., 2021; Vucko et al., 2017); ***B. hamifera*** (Mihaila et al., 2022); ***C. crispus*** (Kinley and Fredeen, 2015); ***E. formisissima*** (Mihaila et al., 2022); ***G. vermiculophylla*** (Maia et al., 2019); ***H. floresii*** (Machado et al., 2014); ***H. pannosa*** (Machado et al., 2014); ***L. filiformis*** (Machado et al., 2014); ***P. palmata*** (Pandey et al., 2022); ***P. cirrhosum*** (Mihaila et al., 2022); ***P. umbilicalis*** (Pandey et al., 2022).
[2]***A. esculenta*** (Pandey et al., 2022); ***A. nodosum*** (Antaya et al., 2019; Belanche et al., 2016; Pandey et al., 2022; Wang et al., 2008); ***C. sinuosa*** (Machado et al., 2014); ***C. trinodis*** (Dubois et al., 2013; Machado et al., 2014); ***D. bartayresii*** (Machado et al., 2014); ***E. radiata*** (Mihaila et al., 2022); ***F. serratus*** (Pandey et al., 2022); ***F. vesiculosus*** (Pandey et al., 2022); ***Furcellaria spp.*** (Kinley and Fredeen, 2015); ***H. elongata*** (Pandey et al., 2022); ***H. triquetra*** (Machado et al., 2014); ***L. digitata*** (Belanche et al., 2016; Pandey et al., 2022); ***P. australis*** (Machado et al., 2014); ***P. canaliculata*** (Pandey et al., 2022); ***S. flavicans*** (Machado et al., 2014); ***S. fulvellum*** (Choi et al., 2021); ***S. fusiforme*** (Choi et al., 2021); ***S. latissima*** (Maia et al., 2019; Pandey et al., 2022); ***U. pinnatifida*** (Choi et al., 2021).
[3]***C. taxifolia*** (Machado et al., 2014); ***C. linum*** (Machado et al., 2014); ***C. coelothrix*** (Machado et al., 2014); ***C. patentiramea*** (Machado et al., 2014); ***D. tenuissima*** (Machado et al., 2014); ***U. lactuca*** (Pandey et al., 2022); ***U. ohnoi*** (Machado et al., 2014); ***U. rigida*** (Maia et al., 2019); ***Ulva sp.*** (Machado et al., 2014; Mihaila et al., 2022).

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CHAPTER 2: EFFECTS OF PROCESSED, DIETARY *SACCHARINA LATISSIMA* AND *ASCOPHYLLUM NODOSUM* ON FERMENTATION AND METHANE PRODUCTION IN CONTINUOUS CULTURE

2.1. ABSTRACT

Dietary supplements derived from *Saccharina latissima* (*SL*) and *Ascophyllum nodosum* (*AN*) were utilized to evaluate the effects of these processed seaweeds on microbial methane (CH_4) production and fermentation profile. The following supplements were used in a 4x4 Latin Square design with 4 continuous culture fermenters: unwashed, coarsely milled *SL* (UNW), 3-min rinsed *SL* (3MR), 20-sec blanched *SL* (20SB), and a crude phlorotannin extract from *AN* (PHLT). Treatments were randomly assigned to fermenters and were topdressed on the total mixed ration (TMR) at inclusion rates of 3.8-4.7% of total dry matter (DM). Each experimental period (n = 4) was 10 d, with the final 3 d for sampling. Vessel pH was continually measured but not adjusted. Effluent samples were collected for volatile fatty acid (VFA) analysis, N flows and calculation of DM, organic matter (OM), neutral detergent fiber in amylase, organic matter (aNDFom) and acid detergent fiber (ADF) degradabilities. Methane was measured in triplicate prior to the first AM and PM feedings during the last 3 d of each period. Data were analyzed using the PROC MIXED procedure of SAS (9.4) with fermenter as the random effect and period and treatment as fixed effects. The unprocessed *SL* treatment (UNW= 22.9 mg/dL) yielded more CH_4 (P = 0.009) than the processed, rinsed treatment (3MR= 1.02 mg/dL). When comparing 20SB with 3MR, the washing method did not affect CH_4 concentration. Apparent degradabilities of DM, OM, aNDFom and ADF, as

well as hours spent below pH 5.6 were not affected by treatment. The *AN* treatment (PHLT) had the greatest acetate: propionate ratio ($P < 0.0002$) among treatments (5.62 vs 1.28-1.55, respectively). These results indicate that seaweed processing method is an important factor to consider for seaweed implementation in ruminant diets, as these processes impact methanogenesis *in vitro*.

2.2. INTRODUCTION

Global livestock methane (CH_4) emissions from enteric fermentation and manure management are expected to account for 24% of global non-CO_2 emissions by 2030, which is a projected 10% increase from 2015 (EPA, 2019). Ruminants produce CH_4 as a by-product of the natural process of fermentation, where ingested feed is converted to energy and products via microbes. While this process of methanogenesis serves to rid the rumen of excess hydrogen (H_2), CH_4 losses translate to a 2-12% loss of feed-derived energy (Johnson et al., 1993). This loss of energy represents production and economic inefficiency. Although CH_4 has a relatively short atmospheric lifetime when compared with CO_2, its disproportionately large effect on radiative forcing contributes to climate change (NASEM, 2018). If a redirection of energy to production parameters instead of other detrimental H_2 sinks can be achieved, reductions in these energy losses could not only increase net revenue for producers but could also benefit the greater community by lessening the impact of agricultural CH_4 on the environment.

Various CH_4 reduction strategies have been assessed and implemented in ruminants to address these emissions, as outlined in Boadi et al. (2004) and Hook et al. (2010). Of these strategies, most are diet manipulations or feed additives. Dietary

inclusion of essential oils (Evans and Martin, 2000; Oh et al., 1967), defaunation of the rumen (Kreuzer et al., 1986; Morgavi et al., 2008) and supplementation with ionophores (Guan et al., 2006; Odongo et al., 2007), are all approaches that limit substrate availability or methanogens, ultimately reducing methanogenesis. However, these strategies have yielded mixed results (Beauchemin and McGinn, 2006; Hook et al., 2009; Karnati et al., 2009) or only temporary reductions in emissions (Sauer et al., 1998; Guan et al., 2006). Hook et al. (2010) noted that targeting methanogens directly could yield greater and more sustainable reductions in CH_4 output.

Feed additives such as seaweeds have moved to the forefront of nutrition and CH_4 mitigation research due to the unique bioactive profiles of seaweed species. The use of brown seaweeds in ruminant diets is not new; farmers in coastal areas have fed seaweed species such as *Ascophyllum nodosum* (*AN*) to their herds for decades during lean feed seasons (McHugh, 2003). Despite this, little is known about the nutritional profiles of numerous brown seaweeds (Gaillard et al., 2018; Maia et al., 2019; Tayyab et al., 2016) or their effects on the rumen environment (Dubois et al., 2013; Wang et al., 2008). Brown seaweeds belong to the phylum phaeophyta and attain their brown color from the phenolic-rich pigment fucoxanthin (Nomura et al., 1997). Additionally, brown seaweed contains bioactive phlorotannins, which are naturally occurring phenolic compounds that have been shown to have antimicrobial activity (Bach et al., 2008; Braden et al., 2004; Nagayama et al., 2002).

Multiple variables such as the sourcing, harvesting, and processing of seaweeds fed are important to consider when examining CH_4 reductions in ruminants. Since transportation methods also produce greenhouse gas emissions, it is vital to consider local

sourcing of seaweeds to minimize shipment lengths and ensure a net emission reduction. The brown seaweed AN is widespread along the northern Atlantic, spanning from Arctic shores to as far south as New Jersey and Portugal (Makkar et al., 2016). Since *AN* resides in the eulittoral zone, it is exposed at low tides which makes it easy to harvest (McHugh, 2003). *Saccharina latissima* (*SL*), commonly known as sugar kelp, is another brown seaweed species that grows in cold water temperatures of the Atlantic and North Pacific oceans (McHugh, 2003). It grows on rock shores visible at low tide and in the subtidal zone to depths of 8–30 m (Makkar et al., 2016). Therefore, these two brown seaweed species are appropriate candidates to evaluate for implementation in ruminant diets in northeastern US due to their location and ease of harvest.

Similar to the impact of terrestrial forage processing on CH_4 emission outcomes (Johnson et al., 1996; LeLiboux and Peyraud, 1999), seaweed processing methods such as rinsing, blanching, grinding, heating and freeze drying may impact rumen fermentation and methanogenesis (Vucko et al., 2017). Seaweed meals processed from harvested *AN* gained popularity in European countries in the late 1960s and have since made a comeback in the animal agriculture industry (McHugh, 2003). Commercialized products containing phlorotannin extracts derived from seaweeds such as *AN* have emerged as well, marketed for ease of supplementation and greater efficiency compared with feeding seaweed biomass. Additionally, ensiling has also recently been identified as a viable processing option for the brown seaweeds *SL and Alaria esculenta* (Yen et al., 2021).

Washing procedure is thought to reduce both the bioactive compound concentration (Vucko et al., 2017) and ash concentration (Tayyab et al., 2016) in seaweed biomass. Vucko et al. (2017) reported that both dip-rinsing and submerging of

Asparagopsis taxiformis samples decreased their bromoform content when compared with unwashed samples. Loss of bromoform and mineral concentrations is due to osmotic lysis and cell damage from rinsing of the biomass (Benjamin et al., 1999). However, it is important to note that processing of seaweeds may still be necessary prior to supplementation to remove excess minerals, such as iodine and metals. To highlight this potential need, the control and kelp meal (KM) diets fed to lactating Jersey cows in Antaya et al. (2019) exceeded the dietary I intake requirement of 6.23 mg/d by 184 and 1404%, respectively, but did not exceed the maximum tolerable dietary I concentration of 50 mg/kg DM (NRC, 2005). When fed at 113 g KM/d, the KM diet composed of *AN* resulted in a milk I concentration of 480 µg/L, which is just below the recommended 500 µg/L allowance for human I consumption (Antaya et al., 2019; EFSA, 2013). However, when supplementing the same KM dosage, Antaya et al. (2015) reported milk I concentrations of 1015 µg/L. Therefore, studying the effects of processing techniques of seaweeds is an important next step that may be crucial in achieving safe mineral concentrations in both animal diets and animal by-products consumed by humans.

Few studies have acknowledged the potential effect of seaweed processing method on rumen fermentation parameters. Vucko et al. (2017) investigated the effect of processing method on the CH_4 reduction potential of *A. taxiformis* in ruminant diets. It was hypothesized that there would be differences in CH_4 concentration of *SL* due to processing method, with less processing correlating to less CH_4 concentration *in vitro*. The objectives of this experiment were to evaluate the effects of dietary supplements derived from unwashed, rinsed, or blanched *SL* as well as an *AN* extract at inclusion rates

of 3.7-4.6% DM, on microbial CH_4 concentration and fermentation profile through *in vitro* continuous culture fermentation.

2.3. MATERIALS AND METHODS

This experiment was conducted at the University of Vermont (Burlington, VT, USA). Handling and use of animals outlined in this experiment were approved by the Institutional Animal Care and Use Committee of the University of Vermont (Protocol # PROTO201900019) in accordance with the requirements of the Office of Laboratory Animal Welfare.

2.3.1. Experimental Design and Diet

The experiment was conducted as a 4 x 4 Latin square design using dual-flow continuous culture fermenters. Fermenters were given a total mixed ration (**TMR**) mixture (**Table 2.1.**) 4 times daily (105 g of dry matter (**DM**)/fermenter/d) as adapted from Gregorini et al. (2008); 30% (on DM basis) of the base diet was added at 0700h, 20% was added at 0820h followed by the addition of the final 30% and 20% of DM at 1600h and 1720h, respectively. Feed was stored at -20°C, ground to a 2 mm particle size (Wiley Mill; Thomas Scientific, Swedesboro, NJ, USA), frozen again at -20°C, then thawed prior to addition. A composite sample of the ground TMR was submitted for commercial wet chemistry analysis (DairyOne Cooperative, Inc., Ithaca, NY, USA). Treatments were comprised of the inclusion of the following seaweeds processed as described: unwashed, coarsely milled *SL* (**UNW**; 4.6% of diet DM), 3-min rinsed, coarsely milled *SL* (**3MR**; 4.5% of diet DM), 20-sec blanched, coarsely milled *SL* (**20SB**; 3.8% of diet DM), and a crude phlorotannin extract from AN (**PHLT**; 4.7% of diet DM).

All *SL* samples were harvested in the sporophyte stage in summer 2016 from the coast of Maine (Maine Coast Sea Vegetables, Hancock, ME, USA) where they were hung on clothes lines to dry after harvest. Seaweed processed as 3MR was rinsed in deionized water for 3 min while 20SB was blanched in boiling DI water for 20 s. A clean 3 L of DI water was used per 200 g of dried *SL* rinsed or blanched. All *SL* samples were then dried at 60°C for 48 h and later stored at -20°C. The PHLT supplement was fed as the dried, ground commercial product, InSea^2 (InnoVactiv, Quebec, Canada). The TMR and seaweed supplements were stored separately, and the seaweeds were proportionally topdressed (20% of daily supplement DM at 0820 h and 1720 h; 30% at 0700 h and 1600 h) before feeding by manually mixing the seaweed directly into the TMR. Chemical profiles of seaweed supplements were determined by commercial wet chemistry analysis (DairyOne Cooperative, Inc., Ithaca, NY, USA) and are listed in **Table 2.2.**, along with total diet chemical profiles.

2.3.2. Continuous Culture Operation

Rumen contents were collected from 2 ruminally fistulated, lactating Holstein cows housed at the Paul R. Miller Research and Educational Center (University of Vermont, Burlington, VT, USA). The cows were fed a TMR diet similar to that which the fermenters received, listed in **Table 2.1.** Rumen digesta collected from the dorsal, ventral, caudodorsal and caudoventral rumen sacs via cannula was manually strained, and liquid samples were pooled then immediately transported to the lab in an electric heated container maintained at 39° C. Modified FerMac 320 modular bioreactors (Electrolab Biotech Ltd., Tewkesbury, UK) were each inoculated with approximately 3 L of rumen

fluid within 30 min of the start of collection, leaving approximately 3 L of gas headspace. Fermenter modification included the addition of a gravity-fed outflow spout to the front of each vessel, enabling a dual-flow system and future sample collection. Filters fitted for protozoa retention were adapted from Karnati et al. (2009). Briefly, 3-D printed cages (Baker, 2021) were fitted inside Ankom bags (50 ± 10 µm porosity; Ankom Technology, Macedon, NY, USA) and attached to the collection end of the peristaltic pump-regulated liquid outflow tubing. Filters were replaced on d 5 of each period with a second filter that was pre-installed to the anterior of the fermenter lid prior to inoculation.

The fermenter systems used in this experiment were maintained under similar conditions to that described by Hoover et al. (1976). Fermenters were maintained at 39°C with continuous agitation for a 10 min cycle including agitation at 70 rpm for 9 min, followed by 1 min at 200 rpm. Temperature and pH were recorded every min. Fermenter pH was monitored but not adjusted. The pH probe was calibrated prior to d 0 inoculation. Prior to inoculation, fermenters were pre-warmed to 39°C and a continuous flow of CO_2 into the buffer solution.

On the day of inoculation, clarified rumen fluid (previously described by Wenner et al., 2017) was included in the mineral buffer solution (Weller and Pilgrim, 1974) pumped to each fermenter. Briefly, rumen fluid was collected as described above, centrifuged (100 x g, 4°C, 15 min), diluted to a 1:20 rumen fluid: mineral buffer ratio and autoclaved (250°C, 75 min). Clarified rumen fluid was supplied to the fermenters via peristaltic pump for the first 24 h of each period to aid in protozoal adaptation to the system. From d 1 to 5 of each period, sterile mineral buffer was then used with a urea inclusion of 0.4 g/L as previously described (Weller and Pilgrim, 1974). On d 5 of each

period, a priming dose of 100 mL [70 mg $(^{15}NH_4)_2SO_4$ 10% atom excess (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 600 mL buffer without urea] was injected into each fermenter. Urea from the buffer was then continually substituted with an addition of 0.084 g $(^{15}NH_4)_2SO_4$ 10% atom excess/4L buffer/d until the end of the period. Data from this ^{15}N enrichment is not reported here.

2.3.3. Sampling and Analysis

Each 10-d period consisted of 7 d of equilibration followed by 3 d of sample collection. Samples of inoculum collected on d 0 served as a control for determination of DM, volatile fatty acid (VFA) concentration and ammonia-N (NH_3-N) concentration.

During sample collection on d 8-10, effluent was collected in 19 L carboys attached to the gravity-fed outflow spout and was chilled on ice. Solid and liquid effluent weights were recorded on d 1-7 at 0830 h and effluent was subsequently discarded as previously described (Bargo et al., 2003). On d 8-10, representative 1.5 L liquid outflow subsamples from the carboys were collected every 24 h for VFA and NH_3-N concentration determination, N flow calculation and DM determination. For NH_3-N analysis, samples collected from carboys were strained through 4 layers of cheesecloth and acidified with 2 mL of 25% meta-phosphoric acid (per 8 mL rumen fluid) before being frozen and stored at -20°C. As per methods outlined by Chaney and Marbach (1962) and Weatherburn (1967), samples collected for NH_3-N concentration determination were later thawed and analyzed using a Berthelot reaction to form indophenol. Additional effluent samples collected on d 8-10 were dried at 65°C, pooled within fermenter across d and submitted to DairyOne Cooperative, Inc. (Ithaca, NY,

USA) for wet chemistry analysis and nutrient profile determination. Samples collected for VFA analysis were strained through 4 layers of cheesecloth and stored at -20°C until they were analyzed via gas chromatography (Bulletin 856B, Supelco Inc., Bellefonte, PA) at the William H. Miner Agricultural Research Institute (Chazy, NY, USA). Briefly, a Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a flame-ionization detector and an 80/120 Carbopack B-DA/4% Carbowax 20M column (Supelco Inc.) was used to determine VFA concentrations. A Sewerin Multitech 545 gas measuring device (Gütersloh, Germany) was used to measure CH_4 twice daily (at 0630 h and 1530 h) prior to feedings on d 8-10. Gas readings were recorded in triplicate.

2.3.4. Statistical Analysis and Calculations

Data was analyzed using the PROC MIXED procedure of SAS (9.4; SAS Institute Inc., Cary, NC, USA) with fermenter as the random effect and period and treatment as fixed effects. All data were presented as least square means (LSM) ± standard error of means (SEM). Multiple comparison Tukey adjustments were used to compare treatment means over d 8-10 of all periods. Treatment effects were considered significant if $P \leq 0.05$ and tendencies were reported if $0.05 < P \leq 0.10$.

Apparent (DM, OM, aNDFom and ADF) degradabilities were calculated using equations outlined by Soder et al. (2013). Fermentation times spent below pH 5.8 and 5.6 were calculated by using the mean of the sums of minutes under the specified threshold. These rumen pH have previously been identified as thresholds that, below which, pH negatively impacts the microbial population, particularly protozoa (Dehority, 2010;

Mertens, 1997). Calculations of N flows were based on Calsamiglia et al. (1996) and Soder et al. (2013), using equations from Bennett et al. (2021).

2.4. RESULTS

2.4.1. Nutrient Degradability

Apparent degradabilities of DM, OM, aNDFom and ADF were not affected by treatment (**Table 2.2.**).

2.4.2. Fermenter pH, VFA, and CH_4 Concentration

Time spent below pH 5.8 and 5.6 during continuous culture fermentation was not affected by treatment (**Table 2.3.**). Mean time spent below pH 5.6 ranged from 28.7 – 37.4 h, representing 60-78% of the d 8-9 (48 h) fermentation time.

Total and individual VFA concentrations as well as the acetate: propionate ratio (A:P) and the acetate + butyrate: propionate ratio (A+B:P) are listed in **Table 2.3.** Total VFA (mM) tended to be higher ($P = 0.09$) in 3MR- than PHLT-treated systems. The A:P ratio ($P = 0.0002$) and A+B:P ratio ($P = 0.0003$) were higher due to the PHLT (*AN*) treatment compared with the 20SB, 3MR and UNW treatments. The propionate concentration (mol/100mol) in PHLT-treated systems was lower ($P < 0.0001$) than the other treatments, while the acetate concentration in PHLT- treated systems was higher ($P < 0.0001$) compared with the *SL* treatments. Butyrate concentrations due to PHLT treatment were higher than 3MR ($P = 0.03$) and UNW ($P = 0.001$) treatments, and tended to be higher than 20SB- treated fermenters ($P = 0.06$). The valerate concentration (mol/100 mol) in UNW-treated systems was higher than the 20SB and PHLT treatments ($P = 0.05$, $P = 0.0002$, respectively) but valerate concentration in 3MR-treated systems

tended to be higher ($P = 0.07$) than PHLT-treated systems. Individual concentrations (mol/100mol) of isobutyrate and isovalerate did not differ between treatments.

Treatment 3MR yielded less CH_4 ($P = 0.0089$) than the unprocessed treatment, UNW. Mean CH_4 concentration per treatment is shown in **Figure 2.1.** When comparing processed treatments, fermenters supplemented with 20SB and 3MR did not differ in CH_4 concentration, and neither did treatments 20SB nor UNW differ from PHLT in CH_4 concentration. However, the UNW treatment did tend to yield more CH_4 than 20SB ($P = 0.06$).

2.4.3. N metabolism

Concentrations of NH_3-N in effluent, total N flow, NH_3-N flow, and NAN flow did not differ between treatments (**Table 2.4.**).

2.5. DISCUSSION

Seaweed species and processing method did not impact overall nutrient degradability, N flows, or fermenter pH, cumulatively supporting the suggestion that the fermenter environment was not altered. Interestingly, despite the lack of shifts in these parameters, shifts in VFA and CH_4 concentration were observed, suggesting the functionality of microbes within the system were impacted by treatment.

In summary, the key findings of this study were that: 1) *SL*-based treatments, regardless of processing method, caused rumen microbial shifts, 2) consistent disparities in VFA profile were observed between the *SL*- and *AN*- based treatments most likely due to dietary fiber profiles, 3) washing method did not affect CH_4 concentration, and 4)

phlorotannins from the seaweed treatments and dietary carbohydrates may be acting together to drive the microbial changes observed.

In the current trial, VFA concentrations produced in the fermenters were affected by seaweed supplements, and a consistent disparity between the *SL*-based treatments (UNW, 3MR and 20SB) and the *AN*-based phlorotannin extract (PHLT) was observed. The supplement derived from *AN* (PHLT) yielded the lowest propionate (31-36% lower than average of other treatments) and highest acetate (14-23% higher than average of other treatments) concentrations compared with the 3MR, 20SB and UNW treatments. As a result, the A:P ratio (5.62) calculated in response to the PHLT treatment is higher than the typical targeted range of 2-3:1 (Van Soest, 1994). While this unique VFA profile is not in line with the degradability profiles, the estimated hemicellulose content (calculated as aNDFom-ADF) of the extract used in the PHLT treatment is 5-20x greater than the hemicellulose content of the other supplements. The increased acetate production in this study could be due to greater hemicellulose content within the PHLT diet and could indicate the presence of fibrolytic bacteria such as *Prevotella sp.* and *Butyrivibrio fibrisolvens* that digest hemicellulose, yielding acetate at increased levels (Russell, 2002). Additionally, the hemicellulose content could be an indication of the harvest season of *AN*, as Solden et at. (2017) reported that winter plants contained higher lignin, cellulose, and hemicellulose (as % of DM) compared with spring and summer plants. While it is unknown what season the *AN* used for the PHLT extract treatment was harvested, the *SL* utilized in this study was harvested in summer 2016, and all *SL*-based treatments contained less hemicellulose than PHLT. Hemicellulose properties also differ among

plant species (Van Soest, 1994), leading to inherent variability even between seaweed species *AN* and *SL*.

The different VFA profiles resulting from the treatments suggest that perhaps the carbohydrate profile yielded some differences in microbial activity. For instance, valerate was higher in samples from unwashed *SL*- treated (UNW) vessels compared with 20SB and PHLT, but not in 3MR. This could be due to higher amounts of lactic acid being produced by microbes in response to the UNW and 3MR *SL* treatments, as the conversion of lactate to valerate and propionate serves as a method of sequestering H_2 to maintain ruminal pH (Bramley et al., 2008; Ladd, 1957). Elevated concentrations of lactate could indirectly result from the type of starch present in UNW and 3MR, where readily fermentable carbohydrates increase VFA production, drive rumen pH down and thus promote the growth of lactate-producing bacteria (reviewed by Owens et al., 1998). However, starch content did not differ among treatments, indicating that another diet component must be affecting valerate concentrations.

While increased valerate production is noteworthy as it has been associated with decreased rumen pH and incidence of subacute ruminal acidosis (SARA; Bramley et al., 2008; Morgante et al., 2007), our findings indicated no differences in the number of hours spent below pH of 5.8 among treatments. However, valerate was identified as a key indicator of ruminal change by Lean et al. (2013) when these researchers fed 5 different grain types *in vivo* to assess acidosis parameters. These findings thus support our observation that the *SL*-based treatments, regardless of processing method, caused rumen microbial shifts.

Moreover, secondary compounds within the seaweed treatments, namely phlorotannins, may be working in concert with the carbohydrate profile of the diet to amplify microbial activity changes in the vessels. While tannins mostly affect proteins, they have also been shown to affect carbohydrates, where fiber degradability is drastically inhibited by tannin-rich feeds (Barry and McNabb, 1999; McSweeney et al., 2001). Tannins are thought to inhibit fiber degradability by complexing with lignocellulose which decreases microbial digestion, or via impeding fibrolytic enzymes (Bae et al., 1993). This relationship between fiber degradation and digestibility as caused by dietary tannins has been extensively reviewed in ruminants (Cardoso-Gutierrez et al., 2021; Frutos et al., 2004; Patra and Saxena, 2011). Degradability of both *SL*- and *AN*-treated fermenters was lower than expected and previously reported in Brandao and Faciola (2019), which therefore, could have been a result of phlorotannins present in all diets supplemented in this study.

One additional fiber-associated aspect of our trial that could have impacted degradability, and hence total VFA concentrations, of all treatments is that seaweed supplements were high in ADF (as a % of DM). More specifically, supplements were particularly high in lignin (supplements ranged from 20.8-33.3% lignin as a % of DM), which resulted in a higher lignin content of the total diet (5.12-5.71 as % of DM). High ADF is known to provide less energy to ruminants as ADF contains the least digestible fractions of the diet, lignin and cellulose. Since microbes in the rumen utilize dietary carbohydrates to create VFAs which serve as major energy source for ruminants, higher concentrations of dietary lignin reduce VFA production and disrupt overall energy derivation from the diet. While the seaweed supplements had an estimated 8.4-20.6%

cellulose (as a % of DM; calculated as ADF- lignin), supplements ranged from 20.8-33.3% lignin (as a % of DM). Therefore, the high lignin values of the seaweeds supplemented could have affected VFA production in the fermenters.

In the current study, the *SL* treatment processed using a rinsing technique (3MR) produced less CH_4 than unprocessed *SL* (UNW). This was a surprising result, as Vucko et al. (2017) found that unrinsed seaweed treatments resulted in the highest CH_4 reductions compared with samples that were dip rinsed or submerged in water. Additionally, Chowdhury et al. (2011) reported that unwashed *Ecklonia cava* tissues had higher crude phlorotannin content than tap water rinsed tissues, which should also translate to greater methanogenesis inhibition from unwashed samples. In Sappati et al. (2019), drying temperature and humidity affected the total phenolic content of *SL*. Drying at 70°C, regardless of humidity, decreased the total phenolic content of *SL* compared with fresh *SL* in that study (Sappati et al., 2019). Since phenolic content affects methanogenesis, it can be inferred that less phenolic content in *SL* due to drying at temperatures above 50°C may decrease the methanogenesis reduction potential of *SL*.

The findings from the current study could be a result of the rinsing protocols resulting in the removal of water-soluble fractions, including some carbohydrate, proteins, and organic acids, thus increasing the proportional content of secondary metabolites/g DM of 3MR. This was demonstrated by Chirinos et al. (2006), when the use of water as a solvent for polyphenol extraction from mashua tubers resulted in extracts with many impurities and 13-30% less total phenolic content than derived when using other solvents. While Chowdhury et al. (2011) found that phlorotannins were also rinsed away from samples with other water-soluble components, Koivikko et al. (2005)

and Wang et al. (2012) confirmed that polyphenolic compounds are less soluble in water than in a polar organic solvent like acetone. Therefore, certain phlorotannins in *SL* may not be soluble in water compared with phlorotannins found in other species, resulting in retention of these bioactive compounds despite washing.

Additionally, while the majority of phlorotannins in seaweeds are soluble and present in organelles in the cytoplasm, cell wall-bound phlorotannins are also present (Koivikko et al., 2005). Rinsing or boiling is known to modify plant tissues (Ologunde et al., 2005) and can cause cell wall rupture, which may result in the loss of soluble phlorotannins at a greater rate compared with cell wall-bound phlorotannins. This in turn could make cell wall-bound phlorotannins more resistant to processing methods as they may be less likely to be rinsed out when the cell wall ruptures from rinsing or boiling, as seen by 3MR and 20SB. Furthermore, the tendency of 20SB to yield less CH_4 compared with UNW indicated that washing processes in either form (3MR or 20SB) affected overall CH_4 reduction potential compared with the unwashed *SL* treatment. However, differences in washing process (blanching vs rinsing) did not seem to affect overall CH_4 reduction as 20SB and 3MR did not differ.

The PHLT-treated fermenter (*AN*) did not differ in CH_4 concentration from any of the *SL*-treated fermenters (3MR, 20SB, and UNW), regardless of processing methods. Despite the lack of differences in CH_4 reducing potential in *SL*- and *AN*- treated vessels found in this study, variation was expected due to processing differences as well as inherent species differences. Numerical variation, although not significant, found between *SL* and *AN* could be a result of the methods in which *AN* was processed by the manufacturer and supplemented in powder-form, which could have affected its

phlorotannin content. Phlorotannins are structurally complex polyphenolic compounds synthesized in brown seaweeds and up to 150 different forms of phlorotannins (Martínez et al., 2013) have been isolated from 33 species (Khan et al., 2022). This structural diversity in phlorotannins as well as intrinsic and environmental factors that affect phlorotannin content in seaweeds (reviewed by Cassani et al., 2020) also contributes to inherent variation among different species.

There are significant gaps in knowledge regarding the mechanism in which phlorotannins from seaweeds impact methanogens in the rumen. Alterations in microbial diversity from phlorotannins are often attributed to phlorotannin protein-binding activity (Wang et al., 2008) which decreases digestibility and consequently decreases CH_4 production (Jayanegara et al., 2011). However, little is known about the specific mechanisms that disrupt these methanogen populations in the rumen when phlorotannins are present. Kim et al. (2022) found that the number of hydroxyl groups and ether linkages of phlorotannin derivatives, phlorofucofuroeckol-A (PFFA), dieckol (DE), and 8,8′-bieckol (BE), were directly associated with CH_4 reductions observed *in vitro*. A similar observation was also documented in Yotsu-Yamashita et al. (2013), where the number of hydroxyl groups were linked to the degree of antioxidant activity. Phlorotannins can have up to eight phenolic rings, which means they have more hydroxyl groups than other tannin forms (Shannon and Ghannam, 2016). More hydroxyl groups allow for the formation of more hydrogen bonds between phlorotannin hydroxyl groups and bacterial surface proteins (Wang et al., 2009). This led to cell denaturation, exhibited by *AN* in the research reported by Wang et al. (2009). Although this reaction has been documented mostly in bacterial cells, archaea also have cell surface proteins (Varki et al.,

2009), which could be affected similarly to bacterial cell surface proteins. Additionally, phlorotannin derivatives BE and DE were reported to disrupt proteolysis via protease inhibition (Khan et al., 2022), further interrupting bacterial cell defense mechanisms to break newly formed bonds to phlorotannins.

2.6. CONCLUSION

Rinsed (3MR) *SL*-derived treatment reduced CH_4 concentration compared with the unwashed (UNW) *SL*-derived treatment. However, the washing method (rinsing vs blanching) did not result in CH_4 concentration differences across washing method (3MR and 20SB). There was no difference in comparing the *in vitro* CH_4 concentration results between *SL* and *AN* in this study. Despite this lack of difference, numerous intrinsic (environment, harvest location, harvest season) and external factors (washing, drying, grinding, solvents used for extraction) are known to impact the phlorotannin content of seaweeds, making it imperative that a more structured approach to testing these seaweed-derived supplements be established. Additionally, the mechanism in which phlorotannins impact methanogenesis is still unclear but is suspected to be due to protein binding and the difficulty of breaking these bonds between phlorotannins and bacterial cell surface proteins, often affected by the number of hydroxy groups present within the phlorotannin compound. Therefore, further investigation into the types of phlorotannins present in *SL* and *AN*, their mechanisms, and how specific processing techniques impact their efficacy and extraction potential is warranted.

2.7. ACKNOWLEDGEMENTS

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Table 2.1. Chemical composition (% DM unless specified) of unwashed *S. latissima* (UNW), rinsed *S. latissima* (3MR), blanched *S. latissima* (20SB), or *A. nodosum* phlorotannin extract (PHLT) and TMR base diet and mixed diets.

Item (% DM)	Base diet TMR[1]	Treatments[2]				Total Diet[3]			
		UNW	3MR	20SB	PHLT	UNW	3MR	20SB	PHLT
DM[4], %	44.3	81.1	24.8	20.5	89.6	45.9	43.4	43.4	46.3
Ash	6.78	33.9	19.8	22.2	17.0	7.98	7.34	7.35	7.24
CP[4]	15.7	11.5	13.5	14.4	3.4	15.5	15.6	15.6	15.1
aNDFom[4]	29.3	29.2	32.5	34.6	13.7	29.2	29.4	29.4	28.5
ADF[4]	20.5	36.5	34.8	37.4	53.9	21.2	21.1	21.1	22.0
Crude Fat	5.61	1.07	1.04	1.56	0.47	5.41	5.41	5.46	5.38
TDN[4]	74.0	32.0	44.0	41.0	62.0	72.0	73.0	73.0	74.0
Ca	0.71	1.19	1.57	1.78	0.79	0.73	0.75	0.75	0.71
P	0.39	0.38	0.30	0.34	0.05	0.39	0.39	0.39	0.37
Mg	0.33	0.79	0.78	0.78	0.90	0.35	0.35	0.35	0.36
K	1.24	7.18	4.13	4.58	1.78	1.50	1.36	1.36	1.26
Na	0.62	3.71	1.83	2.01	2.46	0.76	0.68	0.68	0.71
Cl	0.48	10.30	2.34	3.17	0.08	0.92	0.56	0.58	0.46
S	0.28	1.54	1.44	1.57	4.13	0.34	0.33	0.33	0.46
Starch	25.2	0.50	0.50	0.80	0.30	24.10	24.13	24.29	24.07
NFC[4]	42.6	24.3	33.1	27.2	65.4	41.8	42.2	42.0	43.6

ESC[4]	5.60	1.50	1.90	1.10	0.40	5.41	5.44	5.43	5.36
Lignin	4.40	20.8	26.4	24.8	33.3	5.12	5.35	5.15	5.71
DCAD[5], mEq/100g	+28	-41	+30	+17	-107	+24	+28	+27	+22

[1]Base diet TMR (DM basis) included 52% corn silage, 9% haylage, 39% custom pellet formulated by Poulin Grain Inc. (Newport, VT, USA). Pellet formula was proprietary and is not reported here.
[2]Treatments: UNW= unwashed *S. latissima* (*SL*); 3MR= 3-min rinsed *SL*; 20SB= 20-sec blanched *SL*; PHLT= *A. nodosum* (AN) phlorotannin extract.
[3]Total diet (DM basis) consisted of 95% TMR, 5% seaweed inclusion and 0.6 g of $NaHCO_3$ (Fisher Scientific International INC., Pittsburgh, PA, USA)/d/fermenter.
[4]DM= dry matter; CP= crude protein; aNDFom= neutral detergent fiber in amylase, organic matter; ADF= acid detergent fiber; TDN= total digestible nutrient; NFC= non-fiber carbohydrate; ESC= ethanol soluble carbohydrate.
[5]DCAD calculated as mEq [(Na + K) – (S + Cl)]/100 g DM.

Table 2.2. Effect of inclusion of unwashed *S. latissima* (UNW), rinsed *S. latissima* (3MR), blanched *S. latissima* (20SB) or *A. nodosum* phlorotannin extract (PHLT) on *in vitro* nutrient degradability with TMR during fermentation with continuous culture fermenters.

	Treatments[1]					*P*-value
Item (%)	UNW	3MR	20SB	PHLT	SEM	Treatment
Apparent degradability, %						
DM[2]	69.5	62.9	71.2	61.4	3.31	0.128
OM[2]	66.6	59.6	67.3	58.8	3.18	0.166
aNDFom[2]	64.3	49.3	64.3	46.9	7.01	0.219
ADF[2]	65.5	56.8	67.4	46.7	6.39	0.168

[a-c]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: UNW= unwashed *S. latissima* (*SL*); 3MR= 3-min rinsed *SL*; 20SB= 20-sec blanched *SL*; PHLT= *A. nodosum* (AN) phlorotannin extract.
[2]DM= dry matter; OM= organic matter; aNDFom= neutral detergent fiber in amylase, organic matter; ADF= acid detergent fiber.

Table 2.3. Effect of inclusion of unwashed *S. latissima* (UNW), rinsed *S. latissim*a (3MR), blanched *S. latissima* (20SB) or *A. nodosum* phlorotannin extract (PHLT) on *in vitro* pH, VFA concentration and molar proportion, and CH_4 concentration during fermentation with continuous culture fermenters.

	Treatments[1]					*P*-value
Item	UNW	3MR	20SB	PHLT	SEM	Treatment
pH[2]						
Hrs below 5.8	40.3	37.3	35.4	34.4	379	0.899
Hrs below 5.6	37.4	29.8	31.7	28.7	417	0.811
Mean pH	5.42	5.47	5.44	5.52	0.15	0.970
Total VFA, mM	82.8	87.6	86.0	79.0	2.60	0.094
Individual VFA, mol/100mol						
Acetate (A)	48.1^{b}	44.7^{b}	47.0^{b}	54.8^{a}	1.23	< .0001
Propionate (P)	32.8^{a}	35.6^{a}	33.0^{a}	22.7^{b}	1.77	< .0001
Butyrate (B)	13.1^{b}	14.8^{b}	15.2^{ab}	18.5^{a}	1.08	0.0017
Isobutyrate	0.53	0.50	0.53	0.52	0.05	0.844
Valerate	4.65^{a}	3.79^{ab}	3.53^{b}	2.74^{b}	0.33	0.0005
Isovalerate	0.79	0.70	0.78	0.77	0.06	0.333
A:P	1.55^{a}	1.28^{a}	1.47^{a}	5.62^{b}	1.10	0.0002
A+B:P	1.97^{b}	1.70^{b}	1.96^{b}	7.95^{a}	1.63	0.0003

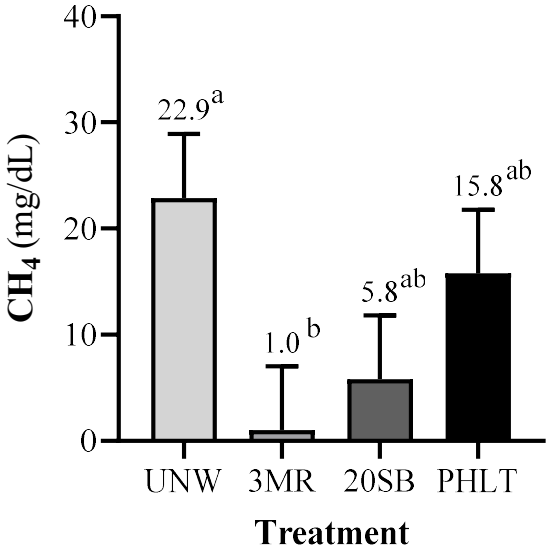
[a-c]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: UNW= unwashed *S. latissima* (*SL*); 3MR= 3-min rinsed *SL*; 20SB= 20-sec blanched *SL*; PHLT= *A. nodosum* (AN) phlorotannin extract.
[2]Time continuous culture fermentation spent below pH of 5.8 and 5.6, and mean pH over 48 h.

Table 2.4. Effect of inclusion of unwashed *S. latissima* (UNW), rinsed *S. latissima (*3MR), blanched *S. latissima* (20SB) or *A. nodosum* phlorotannin extract (PHLT) on ruminal N flows and NH_3-N concentration during fermentation with continuous culture fermenters.

	Treatments[1]					*P*-value
Item	UNW	3MR	20SB	PHLT	SEM	Treatment
N-NH_3[2], mg/dL	3.92	3.76	3.87	3.87	0.12	0.809
N flows, g/d						
Total N	1.49	1.76	1.52	1.70	0.13	0.238
NH_3-N	0.11	0.11	0.11	0.11	0.01	0.905
NAN[2]	1.38	1.66	1.41	1.60	0.13	0.250
NAN flow, % of N intake	53.2	63.8	54.6	63.2	5.15	0.228
N intake, g/d	2.58	2.60	2.59	2.52		

[a-c]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: UNW= unwashed *S. latissima* (*SL*); 3MR= 3-min rinsed *SL*; 20SB= 20-sec blanched *SL*; PHLT= *A. nodosum* (AN) phlorotannin extract.
[2]N-NH_3= ammonia nitrogen; NAN= non-ammonia nitrogen.

Figure 2.1. Mean CH_4 concentration (mg/dL) per treatment during continuous culture fermentation on d 8-10. UNW= unwashed *SL*; 3MR= 3-min rinsed *SL*; 20SB= 20-sec blanched *SL*; PHLT= AN extract. [a-c]Least square means with different superscripts are different ($P < 0.05$). Error bars represent SEM.



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CHAPTER 3: EFFECTS OF DIETARY *CHONDRUS CRISPUS* INCLUSION ON FERMENTATION AND METHANE PRODUCTION IN CONTINUOUS CULTURE

3.1. ABSTRACT

Red seaweeds are being investigated as a ruminant feed material, due to their advantageous nutrient profiles and potential to reduce enteric methane (CH_4) production. Five dual-flow continuous culture fermenters were utilized to assess the impact of two inclusion rates of dietary *Chondrus crispus* (*CC*) addition on fermentation profile and microbial CH_4 concentration. The 5x5 Latin Square design experiment consisted of these treatments: 1) negative control (TMR only; **CON**), 2) 6% dry matter (DM) inclusion of CC (**CL**), 3) 10% DM inclusion of CC (**CH**), 4) positive anti-methanogenic control containing TMR + 2.5 mL of 0.6 mM bromoform (**BL**), and 5) positive anti-methanogenic control containing TMR + 5 mL of 0.6 mM bromoform (**BH**). Treatments were mixed into the total mixed ration (TMR), in equal proportions split between two feedings. Five, 10 d experimental periods were performed, consisting of 7 d of equilibration followed by 3 d of sampling. Effluent samples were collected for volatile fatty acid (VFA) analysis and calculation of DM, organic matter (OM), neutral detergent fiber in amylase, organic matter (aNDFom) and acid detergent fiber (ADF) degradabilities, while vessel pH was continually recorded. Methane (CH_4) was measured prior to feedings during the 3 d sample collection. Data were analyzed using the PROC MIXED procedure of SAS (9.4) with fermenter as the random effect, and period and treatment as fixed effects. *C. crispus* treatments (CL= 10.7 and CH= 11.0 mg/dL) yielded

more CH_4 ($P < 0.0001$) than the controls (CON= 5.98, BL= 3.18 and BH= 3.62 mg/dL). Time spent under a pH of 5.8 during fermentation and apparent degradabilities of DM, OM, aNDFom and ADF were not affected by treatment. The highest *CC* inclusion (CH) produced the lowest valerate concentrations (4.08 mol/100 mol; $P < 0.0001$) compared with the other treatments (CON, CL, BL and BH). Further investigation characterizing the impact of processing method and inclusion of *CC* that could have reduced bioactive content and efficacy is necessary prior to discounting *CC* as a potential feed additive for CH_4 mitigation in ruminants.

3.2. INTRODUCTION

Enteric fermentation by livestock represented 25% of anthropogenic methane (CH_4) emissions in the US in 2015 (US Greenhouse Gas Inventory; EPA, 2017). Fermentation is a natural process, whereby microbes transform ingested feed into energy, releasing CH_4 and volatile fatty acids (VFAs) as byproducts of this fermentation process. Dairy cows lose 5.5-9% of feed-derived energy through the process of methanogenesis (Johnson and Johnson, 1995). This energy loss represents production and economic inefficiency. Methane can absorb more energy as light per unit mass than CO_2 does, making it a major contributor to global warming and a primary industrial target for emission reduction (NASEM, 2018).

While numerous CH_4 reduction strategies have been investigated and applied to ruminant diets, the most abundant are diet manipulations and feed additives, as reviewed by Beauchemin et al. (2008) and Hook et al. (2010). Feed materials derived from seaweeds have gained popularity in ruminant nutrition due to the unique bioactive

profiles within seaweed species and their CH_4 reduction potential. Bromoform, a bioactive compound within some seaweed species, inhibits methanogenesis in the rumen by impeding enzymes that are essential to the terminal step of the pathway (Denman et al., 2007). Machado et al. (2016) reported that bromoform had the highest CH_4 mitigation activity at concentrations ≥ 1 mM *in vitro*. *Asparagopsis taxiformis*, the most widely studied red seaweed to date (Machado et al., 2014; Kinley et al., 2016; Li et al., 2016; Vucko et al., 2017; Roque et al., 2019; Stefenoni et al., 2021), contains a high concentration of bromoform (Machado et al., 2014). *A. taxiformis* has been shown to reduce CH_4 emissions by up to 98% in beef cattle fed a high grain diet (Kinley et al., 2020).

While *A. taxiformis* has been a central focus, it is not the only red seaweed species. Red algae are part of the phylum rhodophyta and encompasses over 7,000 currently recognized species, making it one of the largest algal phyla. Consumed by humans for centuries in Europe and Asia, red seaweeds such as *Palmaria palmata* and *Porphyra sp.* are major constituents of human nutrition, functional food additives and pharmaceuticals (Holdt and Kraan, 2011; McHugh, 2003). Despite this, research evaluating the effects of red seaweeds in ruminant diets has just recently emerged. Kinley and Fredeen (2015) examined CH_4 reductions of a seaweed mixture containing equal part *Chondrus crispus* (*CC*), *Laminaria longicruris*, *Furcellaria sp.*, and *Fucus vesiculosus*, and reported a maximum reduction of 16% CH_4 production (g/d) *in vitro* when supplemented at 0.28 g DM/d. Supplementation of either *CC* or *Furcellaria sp.* at 0.14 g DM/d also reduced CH_4 production compared with the control. These results clearly demonstrate an opportunity for use of multiple seaweed species for CH_4 mitigation;

however, more research is needed to determine how *CC* and other less characterized species impact rumen fermentation and methanogenesis, both when fed individually and in conjunction with other seaweeds.

Regionality of growth and optimization of inclusion rates are also important factors to consider when assessing seaweeds as potential CH_4 reduction candidates for ubiquitous use in animal agriculture. The abundance of wild *CC* along the Northeastern shores of North America makes it a suitable feed candidate to investigate for supplementation in that region. Kinley et al. (2016) demonstrated that when supplemented at ≥ 5% OM, *A. taxiformis* reduced *in vitro* CH_4 production to undetectable levels. However, *A. taxiformis* inclusion at ≥ 10% OM significantly reduced OM degradability *in vitro* (Machado et al., 2014). Therefore, inclusion rates of ≤ 10% OM for other red seaweeds such as *CC* may be a logical starting point for further supplementation trials.

Few studies have investigated the potential effect of *CC* supplementation on rumen fermentation parameters. It was hypothesized that inclusions of 6% and 10% DM of *CC* would produce less CH_4 when compared with a negative control, where increased dosage would result in increased CH_4 reduction. The objective of this experiment was to evaluate two inclusion rates of a dietary supplement derived from *CC* on microbial CH_4 concentration and fermentation profile when compared with bromoform supplementation in continuous culture fermentation.

3.3. MATERIALS AND METHODS

This experiment was conducted at the University of Vermont (Burlington, VT, USA). Handling and use of the animals outlined in this experiment were approved by the Institutional Animal Care and Use Committee of the University of Vermont (Protocol #PROTO201900019) in accordance with the requirements of the Office of Laboratory Animal Welfare.

3.3.1. Experimental Design and Diet

The current experiment was conducted as a 5x5 Latin square design that included the use of dual-flow continuous culture fermenters. Fermenters were given a total mixed ration (TMR, **Table 3.1.**) 2 times daily (105 g of DM total diet/fermenter/d) in equal proportions at 0700 h and 1700 h with the seaweed supplement topdressed in equal proportions during feedings. The TMR was ground to a 2 mm particle size (Wiley Mill; Thomas Scientific, Swedesboro, NJ, USA) frozen at -20°C, and a subsample was submitted for commercial wet chemistry analysis (DairyOne Cooperative, Inc., Ithaca, NY, USA). Treatments included the following: 1) a negative control (TMR only; **CON**), 2) 6% DM inclusion of *CC* (**CL**), 3) 10% DM inclusion of *CC* (**CH**), 4) positive anti-methanogenic control containing TMR + 2.5 mL of 0.6 mM bromoform (**BL**), and 5) positive anti-methanogenic control containing TMR + 5 mL of 0.6 mM bromoform (**BH**). All *CC* was wild harvested off the coast of Maine, USA, commercially air-dried and milled (Vitamin Sea Seaweed, ME, USA). For use in this experiment, seaweed was further ground to 1 mm (Wiley Mill; Thomas Scientific, Swedesboro, NJ, USA) and stored in sealed containers separately from the TMR, at room temperature. A 0.6 mM

bromoform solution (with di H_2O; Sigma-Aldrich, St. Louis, MO, USA) was prepared and maintained under continuous agitation at 350 rpm on a Corning Hot Plate Stirrer PC-351 (temperature setting not utilized; Corning Inc., Corning, NY, US) for the duration of the study. From that bromoform stock solution, 2.5 mL (for BL) or 5.0 mL (for BH) per feeding (2 times daily) was pipetted into the fermenters directly after TMR was given. The *C. crispus* supplement was stored in an airtight container where the chemical profile of a representative sample was determined by commercial wet chemistry analysis (DairyOne Cooperative, Inc., Ithaca, NY, USA).

3.3.2. Continuous Culture Operation

Rumen contents were collected from 3 fistulated, mid-lactation Holstein cows housed at the Paul R. Miller Research and Educational Center (The University of Vermont, Burlington, VT, USA). The rumen fluid donors were fed a TMR diet similar to that which the fermenters received (**Table 3.1.**). Rumen digesta collected from the dorsal, ventral, caudodorsal and caudoventral rumen sacs via cannula was manually strained and liquid samples were pooled in a pre-warmed, insulated container. Rumen fluid was transported to the laboratory, manually mixed. FerMac 320 modular fermenters (Electrolab Biotech Ltd., Tewkesbury, UK) were each inoculated with approximately 3 L of rumen fluid. Fermenters were inoculated within 30 min of the start of rumen fluid collection. To enable a dual-flow system, fermenter vessels were modified to include a gravity-fed outflow spout and liquid outflow tubing with peristaltic pump. Liquid outflow included filters fitted for protozoa retention. Filters were adapted from Karnati et al. (2009) and consisted of 3-D printed cages inside Ankom bags (50 ± 10 µm porosity;

Ankom Technology, Macedon, NY, USA) that were placed in the fermenter vessel prior to inoculation. One active filter per fermenter was switched on d 5 of each period with a second filter that was pre-installed to the fermenter interior prior to inoculation to avoid disruption to the system.

Prior to inoculation, fermenters were pre-warmed to 39°C, pH probes were calibrated and a continuous flow of CO_2 into the system was established. Similar to the system described Hoover et al. (1976), fermenters were maintained at 39°C. Temperature and pH were recorded every min, and fermenter pH was monitored but not adjusted Fermenters were equipped with internal agitation systems and programmed for continuous agitation for 9 min at 70 rpm, followed by 1 min at 200 rpm, as part of a 10 min cycle.

On the day of inoculation, each fermenter received a mineral buffer (Weller and Pilgrim, 1974) with the addition of clarified rumen fluid (previously described by Wenner et al., 2017). Briefly, rumen fluid was collected as described above, centrifuged (100 x g, 4°C, 15 min), diluted to a 1:20 rumen fluid: mineral buffer ratio and autoclaved (250°C, 75 min). The clarified rumen fluid-buffer solution was added to the fermenters via peristaltic pump for the first 24 h of each period to aid in protozoal adaptation to the system. Fermenters were then given only the sterile mineral buffer with a urea inclusion of 0.4 g/L as previously described (Weller and Pilgrim, 1974) until d 5 of each period. On d 5 of each period, a priming dose of 100 mL was injected into each fermenter and contained 70 mg $(^{15}NH_4)_2SO_4$ 10% atom excess (Sigma-Aldrich, St. Louis, MO, USA) dissolved in the buffer (600 mL) without urea. From d 5 on, 0.084 g $(^{15}NH_4)_2SO_4$ 10%

atom excess per day was added to the buffer and excluded urea until the end of the period as part of a larger experiment (15N data is not presented herein).

3.3.3. Sampling and Analysis

Each period ran for 10 d, consisting of 7 d of equilibration followed by 3 d of sample collection.

During sample collection on d 8-10, effluent was continuously collected in 19 L carboys attached to the gravity-fed outflow spout and was chilled on ice. Effluent weights from the carboys were recorded on d 8-10 every 24 h at 0800h, where a representative 1.5 L subsample of effluent was collected and stored at -20°C for VFA and ammonia-N (NH_3-N) concentration determination, N flows and DM determination. Subsamples for effluent were dried at 65°C, pooled within fermenter across d and submitted to DairyOne Cooperative, Inc. (Ithaca, NY, USA) for wet chemistry analysis. For NH_3-N analysis, samples taken from carboys were strained through 4 layers of cheesecloth and acidified with 2 mL of 25% meta-phosphoric acid (per 8 mL rumen fluid) before freezing at -20°C. Samples collected for VFA analysis were strained through 4 layers of cheesecloth and analyzed with a Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA;) equipped with a flame-ionization detector and an 80/120 Carbopack B-DA/4% Carbowax 20M column (Supelco Inc.). Analysis of VFA concentrations followed the protocol outlined by Supelco (Bulletin 856B, Supelco Inc., Bellefonte, PA, USA) and was performed by William H. Miner Agricultural Research Institute (Chazy, NY, USA). To determine NH3-N concentrations, samples were analyzed using a Berthelot reaction to form indophenol (Chaney and Marbach 1962; Weatherburn 1967). Methane

measurements were performed in triplicate using a Sewerin Multitech 545 gas measuring device (Gütersloh, Germany), twice daily (at 0630 h and 1630 h) prior to feedings on d 8-10.

3.3.4. Statistical Analysis and Calculations

The PROC MIXED procedure of SAS (9.4; SAS Institute Inc., Cary, NC, USA) was used for data analysis, with fermenter as the random effect, and period and treatment as fixed effects. All data were presented as least square means (LSM) ± standard error of means (SEM). Multiple comparison Tukey adjustments were used to compare treatment means over d 8-10 of all periods. P-values were significant if $P \leq 0.05$ and tendencies were reported if $0.05 < P \leq 0.10$.

Apparent (DM, OM, aNDFom and ADF) degradabilities were calculated using equations from Soder et al. (2013). Fermentation times spent below pH 5.8 and 5.6 were calculated by using the mean of the sums of minutes under the specified threshold, which were based off known lower limits of rumen pH values that negatively impact the microbial population, especially protozoa, from animals fed a concentrate diet (Dehority, 2010; Mertens, 1997). Calculations of N flows were based on Calsamiglia et al. (1996) and Soder et al. (2013), using equations from Bennett et al. (2021).

3.4. RESULTS

3.4.1. Nutrient Degradability

Seaweed treatments did not affect apparent DM, OM, aNDFom and ADF degradabilities, as reported in **Table 3.2.**

3.4.2. Fermenter pH, VFA, and CH_4 Concentration

During continuous culture fermentation, time spent under a pH of 5.8 and 5.6 were not affected by treatment (**Table 3.3.**). Mean time spent below pH 5.6 ranged from 2.8- 18.3 h (**Table 3.3.**), representing 6-38% of the d 8-9 (48 h) fermentation time.

The total VFA concentration (mM) was lower in samples collected from the CH-treated fermenters (87.3 mM; $P = 0.0007$) when compared with CON- and BL- treated fermenters (94.2 and 95.8 mM, respectively) and the other *CC* treatment (*CC* at 6% DM inclusion, CL = 95.9 mM). While the CON- treated fermenter tended to have a higher concentration of propionate ($P = 0.0809$) compared with the CH (*CC* at 10% DM inclusion) treatment, CH yielded the highest acetate concentration ($P < 0.0001$) among all treatments. However, it was the acetate: propionate (A:P) ratio of the CL- treated (*CC* at 6% DM inclusion) fermenter that tended to be higher compared with the BH- treated fermenter ($P = 0.0775$). The CH treatment resulted in a lower valerate concentration ($P < 0.0001$) when compared with the other treatments (CON, CL, BL and BH). Both *CC*-derived treatments (CL and CH) yielded higher concentrations of butyrate compared with the positive anti-methanogenic controls (CL vs BL, $P = 0.0011$; CL vs BH, $P < 0.0001$; CH vs BL and BH, $P < 0.0001$). The CH treatment tended to have a higher concentration of butyrate compared with CON (TMR only; $P = 0.0867$). No differences in isovalerate or isobutyrate concentrations (mM) were reported across treatments ($P = 0.959$ and $P = 0.923$, respectively).

The *CC* treatments (CL= 10.7 and CH= 11.0 mg/dL) yielded higher CH_4 concentrations ($P < 0.0001$) compared with the negative and bromoform controls (CON= 5.98, BL= 3.18 and BH= 3.62 mg/dL). No difference in CH_4 concentration across *CC*

treatments was observed. Mean CH_4 concentration per treatment is graphically displayed in **Figure 3.1.**

3.4.3. N metabolism

Concentrations of NH_3-N in effluent, total N flow, NH_3-N flow, and NAN flow did not differ between treatments (**Table 3.4.**).

3.5. DISCUSSION

Supplementation of *CC* at dietary inclusion rates of 6% and 10% DM had no effect on apparent DM, OM, aNDFom and ADF degradabilities, N flows, or fermenter pH between treatments, indicating the fermenter environment was not significantly altered by the addition of *CC*. Interestingly, despite the lack of shifts in these parameters, shifts in VFA and CH_4 production were observed, suggesting the functionality of microbes within the system were impacted by *CC* treatment or inclusion.

In summary, the key findings of this study were that: 1) inclusion rate of *CC* did not affect CH_4 concentrations, 2) *CC*-based treatments, regardless of inclusion rate, caused VFA profile shifts that could be due to DCAD, and 3) reduced CH_4 reduction potential of *CC*-based treatments was most likely a result of seaweed preservation method that decreased bromoform concentrations.

In the current trial, VFA concentrations produced in the fermenters were affected by the seaweed supplement and their inclusion rates, especially at 10% DM inclusion, compared with the controls. The 10% DM inclusion of *CC* (the CH treatment) produced the lowest total VFA concentration (7-9% lower than average of other treatments) when compared with CON, CL and BL. Whereas CL differed from BL and BH by yielding less

propionate (9-13% less, respectively) and valerate (12-25% less, respectively) but produced more butyrate than both of the positive anti-methanogenic controls (BL and BH; 17-21% more, respectively). Cumulatively, the shifts in VFA concentrations and profile could be a result of the dietary cation anion difference (DCAD) of the *CC*-containing diets compared with the negative control (CON) and bromoform-containing (BL and BH) diets. The minerals S, Cl, K and Na in DCAD play essential roles not only post-absorptively in the ruminant (Block, 1994) but also affect rumen function by altering VFA patterns and pH as reported in the meta-analysis by Iwaniuk and Erdman (2015). However, differences in pH values between negative DCAD-yielding treatments (CL and CH) and the other positive DCAD-yielding treatments (CON, BL and BH) were not observed in this study, potentially indicating that DCAD values were not low enough or VFA profile changes were not substantial enough in our systems to alter rumen pH. The negative DCAD values may be an indication that *CC*-derived supplements such as CL and CH are better suited for inclusion in dry cow diets though, as higher DCAD values are considered beneficial for lactating cows (Apper-Bossard et al., 2006; Beede, 2005).

The lignin content of *CC*-derived treatments could be another dietary component responsible for the VFA profile shifts observed, as *CC* contained 65% more lignin compared with the negative control and bromoform-containing treatments. Higher lignin content results in less dietary energy capture as lignin is indigestible and lowers diet digestibility (Van Soest, 1967), and thus would support findings of reduced total VFA concentration. However, CH and BH did not differ in total VFA production. Therefore, a 2% and 6% DM difference in lignin content between CH vs CL, and CH vs CON, BL, and BH, respectively, likely did not cause this total VFA profile shift (Broderick et al.,

2008). Since dietary NDF content was clearly not the driving force in this study, it is possible that ash from the seaweed supplements had a larger impact than expected, as previously mentioned when evaluating DCAD values.

While the NFC content of the different diets was similar, the reduced valerate production by CH-treated vessels could be attributed, at least in part, to having the lowest starch and non-fiber carbohydrate (NFC) load among treatments. Lean et al. (2013) demonstrated that increased grain in the diet (as % of body weight) increased valerate production, causing changes in the rumen environment due to dietary NFC content. Therefore, shifts in valerate concentration in the current study could indicate that valerate was once again reactive to rumen environment changes related to dietary inclusion of fiber. In terms of biological significance, valerate is a lesser studied VFA but has been shown to be an effective method of sequestering H_2 in the rumen (Bramley et al., 2008), serving as a critical component of intestinal health and in the regulation of intestinal homeostasis (Gao et al., 2022). Moreover, valeric acid addition to rumen fluid *in vitro* by Bentley et al. (1955) resulted in 56% more cellulose digestion than the control while also exhibiting an increase in cellulose digestion in Cline et al. (1958). This relationship between valerate production and fiber content sheds light on necessary considerations for more efficient utilization of dietary forage components. Therefore, less valerate production as a result of *CC* supplementation at 10% DM warrants further investigation *in vivo* to ensure that ruminal and post-ruminal digestion efficiency is not detrimentally decreased due to ADF.

As expected, the positive controls (BL and BH) had the lowest CH_4 concentration among treatments, yet surprisingly, CH had the highest (over 3x greater CH_4

concentration compared with BH). Despite the lack of *CC* studies, these CH_4 concentration findings were unexpected as *CC* has been shown to reduce CH_4 production *in vitro* (Kinley and Fredeen, 2015) and *in vivo* (Brito, University of New Hampshire, Durham, NH, personal communication). Kinley and Fredeen (2015) found that *CC* reduced CH_4 production by 12-16% compared with the negative control when supplemented individually at 0.14 g/DM/d and in a seaweed mixture (*CC*, *Laminaria longicruris*, *Furcellaria sp.*, and *Fucus vesiculosus*). While Kinley and Fredeen (2015) attributed the reduction in CH_4 production with seaweed supplementation to an interaction between the protein in dietary seaweed treatments and *in vitro* gas production as previously postulated (Cone and van Gelder, 1999), supported by reduced NH_3-N concentrations, the current study did not observe a difference in NH_3-N between seaweed-supplemented fermenters and non-seaweed-supplemented fermenters. The *CC* diet utilized by Kinley and Fredeen (2015) contained 77 g CP/kg DM and was in range of the *CC* diets fed in the current study (64 – 107 g CP/kg DM), serving as a possible indication that factors other than protein interactions affected CH_4 concentration. While the fiber and individual diet lignin values were not reported by Kinley and Fredeen (2015), perhaps the results from the current trial provide some support for future consideration of these dietary constituents.

When considering the gross diet, it is important to highlight that the lack of CH_4 reduction demonstrated by the *CC*-supplemented fermenters could also be a result of *CC* processing method. Vucko et al. (2017) analyzed the effects of different processing and preservation methods of *A. taxiformis*, another red seaweed that is known for its bromoform content. Although the method that resulted in *A. taxiformis* with the highest

bromoform content was unrinsed biomass which was frozen and then freeze dried, unrinsed biomass which was kiln-dried or dehydrated without freezing still completely inhibited CH_4 production *in vitro* (Vucko et al. 2017). The *CC* supplemented in the current study was unrinsed and air dried without freezing prior to supplementation, which is similar to methods assessed by Vucko et al. (2017). Since Vucko et al. (2017) described that freeze drying was the best method to preserve bioactive compound content, the lack of methanogenesis inhibition observed in the current trial could be due to reduced bromoform content from not freeze-drying *CC* samples, or from milling samples twice. Additionally, it is unknown how *CC* samples compare with other red seaweeds regarding average bromoform content after processing, which is another consideration that should be researched further.

3.6. CONCLUSION

The objective of this experiment was to investigate applicable inclusion rates of a dietary supplement derived from *CC* on microbial CH_4 concentration and fermentation profile in continuous culture fermentation and how these supplements compared with positive anti-methanogenic controls. Inclusions of 6% (CL) and 10% DM (CH) *CC* treatments produced more CH_4 than both the positive (BL and BH) and negative (CON) controls. However, CH_4 concentration did not differ between the different inclusion levels of *CC* (CL and CH). Investigation into how processing methods could have affected bioactive compound efficacy in *CC*, and thus CH_4 concentration, should be considered succeeding this study but prior to implementation *in vivo*. While the mechanism in which bromoform inhibits methanogenesis is well established, the abundance of bromoform in

different red seaweed species is still unknown, therefore indicating potential for further exploration. With CH producing the least amount of valerate among treatments, further evaluation *in vivo* would then be necessary to ensure that *CC* supplementation at inclusions of 10% DM or above will not negatively impact feed utilization. Additionally, the inclusion of *CC* should be further evaluated for use in non-lactating cow diets, due to DCAD.

3.7. ACKNOWLEDGEMENTS

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Table 3.1. Chemical composition (% DM unless specified) of 6% DM inclusion of *C. crispus*, 10% DM inclusion of *C. crispus*, TMR + 2.5 mL of 0.6 mM bromoform, or TMR + 5 mL of 0.6 mM bromoform, total mixed ration base diet and mixed diets.

		Seaweed[2]	Total Diet[3]				
Item (% DM)	Base Diet TMR[1]	CC	CON	CL	CH	BL	BH
DM[4], %	42.3	78.5	42.3	44.6	46.1	42.3	42.3
Ash	7.69	38.3	7.69	9.64	10.9	7.69	7.69
CP[4]	17.9	14.1	17.9	17.7	17.5	17.9	17.9
aNDFom[4]	38.8	22.6	38.8	37.8	37.1	38.8	38.8
ADF[4]	20.9	8.30	20.9	20.1	19.6	20.9	20.9
Crude Fat	6.97	0.58	6.97	6.56	6.30	6.97	6.97
TDN[4]	72.0	39.0	72.0	69.9	68.5	72.0	72.0
Ca	1.07	1.06	1.07	1.07	1.07	1.07	1.07
P	0.45	0.32	0.45	0.44	0.44	0.45	0.45
Mg	0.46	1.00	0.46	0.49	0.52	0.46	0.46
K	1.20	4.89	1.20	1.44	1.60	1.20	1.20
Na	0.42	5.85	0.42	0.77	1.00	0.42	0.42
Cl	0.51	9.43	0.51	1.08	1.45	0.51	0.51
S	0.30	6.05	0.30	0.67	0.91	0.30	0.30

Starch	22.8	1.40	22.8	21.4	20.5	22.8	22.8
NFC[4]	28.6	24.3	28.6	28.3	28.1	28.6	28.6
ESC[4]	1.00	2.00	1.00	1.06	1.11	1.00	1.00
Lignin	4.00	6.20	4.00	4.14	4.23	4.00	4.00
DCAD[5], mEq/100g	+16	-260	+16	-2	-13	+16	+16

[1]Base diet TMR (DM basis) included 67% corn silage and haylage, 33% custom mash formulated by Poulin Grain Inc (Newport, VT, USA). Mash (DM basis) included 22% fine corn meal, 21% canola meal, 9% soybean meal, 7% soy hulls, 41% minerals, vitamins, and additives.
[2]Treatments: CON= Control, TMR only; CL= 6% DM inclusion of *C. crispus* (*CC*); CH= 10% DM inclusion *CC*; BL= TMR + 2.5 mL of 0.6 mM bromoform; BH= TMR + 5 mL of 0.6 mM bromoform.
[3]Total diet consisted of 100% TMR (DM basis) for CON; 94% TMR + 6% *CC* (DM basis) for CL; 90% TMR + 10% *CC* (DM basis) for CH; 100% TMR (DM basis) + bromoform dosage for BL and BH.
[4]DM= dry matter; CP= crude protein; aNDFom= neutral detergent fiber in amylase, organic matter; ADF= acid detergent fiber; TDN= total digestible nutrient; NFC= non-fiber carbohydrate; ESC= ethanol soluble carbohydrate.
[5]DCAD calculated as mEq [(Na + K) – (S + Cl)]/100 g DM.

Table 3.2. Effect of 6% DM inclusion of *C. crispus*, 10% DM inclusion of *C. crispus*, TMR + 2.5 mL of 0.6 mM bromoform, or TMR + 5 mL of 0.6 mM bromoform on *in vitro* nutrient degradability with TMR during fermentation with continuous culture fermenters.

	Treatment[1]						*P*-value
Item (%)	CON	CL	CH	BL	BH	SEM	Treatment
Apparent degradability, %							
DM[2]	37.5	36.0	33.7	40.1	41.3	2.64	0.307
OM[2]	39.4	38.6	36.3	41.0	41.0	2.17	0.535
aNDFom[2]	53.0	52.5	50.9	52.0	50.8	3.24	0.977
ADF[2]	52.5	51.3	51.3	54.8	50.0	3.59	0.869

[a-d]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= Control, TMR only; CL= 6% DM inclusion of *C. crispus* (*CC*); CH= 10% DM inclusion *CC*; BL= TMR + 2.5 mL of 0.6 mM bromoform; BH= TMR + 5 mL of 0.6 mM bromoform.
[2]DM= dry matter; OM= organic matter; aNDFom= neutral detergent fiber in amylase, organic matter; ADF= acid detergent fiber.

Table 3.3. Effect of 6% DM inclusion of *C. crispus*, 10% DM inclusion of *C. crispus*, TMR + 2.5 mL of 0.6 mM bromoform, or TMR + 5 mL of 0.6 mM bromoform on *in vitro* pH, VFA concentration and molar proportion during fermentation.

	Treatment[1]						*P*-value
Item	CON	CL	CH	BL	BH	SEM	Treatment
pH[2]							
Hrs below 5.8	38.1	30.6	23.4	30.5	29.1	324	0.468
Hrs below 5.6	18.3	11.7	2.8	10.8	12.8	315	0.389
Mean pH	5.63	5.73	5.82	5.74	5.81	0.08	0.511
Total VFA, mM	94.2^{a}	95.9^{a}	87.3^{b}	95.8^{a}	91.9^{ab}	1.69	0.0007
Individual VFA, mol/100mol							
Acetate (A)	44.9^{b}	45.3^{b}	48.2^{a}	43.3^{b}	44.2^{b}	0.68	< .0001
Propionate (P)	29.7^{bc}	28.3^{b}	27.6^{b}	32.4^{a}	31.0^{ac}	0.60	< .0001
Butyrate (B)	16.4^{ac}	17.6^{a}	18.3^{a}	14.6^{bc}	13.9^{b}	0.60	< .0001
Isobutyrate	0.86	0.88	0.85	0.90	0.80	0.08	0.923
Valerate	7.19^{b}	6.89^{b}	4.08^{c}	7.81^{a}	9.14^{a}	0.59	< .0001
Isovalerate	0.98	0.95	0.94	0.97	0.91	0.06	0.959
A:P	1.52^{c}	1.62^{ac}	1.76^{a}	1.34^{b}	1.45^{bc}	0.05	< .0001
A+B:P	2.07^{cd}	2.25^{ad}	2.43^{a}	1.79^{b}	1.90^{bc}	0.07	< .0001

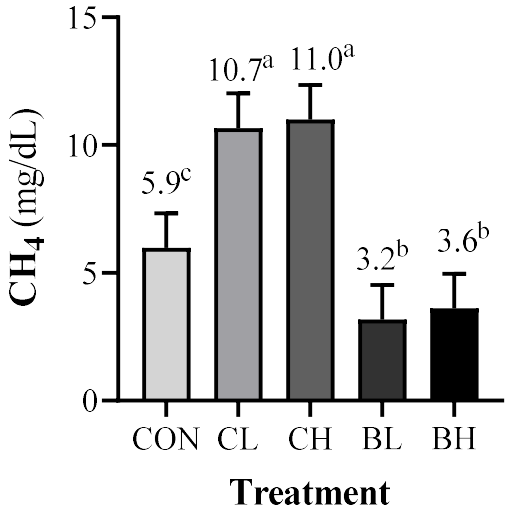
[a-d]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= Control, TMR only; CL= 6% DM inclusion of *C. crispus* (*CC*); CH= 10% DM inclusion *CC*; BL= TMR + 2.5 mL of 0.6 mM bromoform; BH= TMR + 5 mL of 0.6 mM bromoform.
[2]Time continuous culture fermentation spent below pH of 5.8 and 5.6, and mean pH over 48 h.

Table 3.4. Effect of 6% DM inclusion of *C. crispus*, 10% DM inclusion of *C. crispus*, TMR + 2.5 mL of 0.6 mM bromoform, or TMR + 5 mL of 0.6 mM bromoform on *in vitro* N flows and NH3-N concentration during fermentation.

	Treatment[1]						*P*-value
Item	CON	CL	CH	BL	BH	SEM	Treatment
$N\text{-}NH_3$[2], mg/dL	3.84	3.89	4.13	3.99	4.69	0.42	0.621
N flows, g/d							
Total N	2.77	2.72	2.60	2.64	2.56	0.10	0.522
NH_3-N	0.15	0.15	0.16	0.15	0.18	0.02	0.564
NAN[2]	2.62	2.57	2.44	2.50	2.38	0.11	0.510
NAN flow, % of N intake	91.1	90.2	86.1	86.5	82.6	3.69	0.513
N intake, g/d	2.88	2.85	2.83	2.88	2.88		

[a-d]Least square means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= Control, TMR only; CL= 6% DM inclusion of *C. crispus* (*CC*); CH= 10% DM inclusion *CC*; BL= TMR + 2.5 mL of 0.6 mM bromoform; BH= TMR + 5 mL of 0.6 mM bromoform.
[2]$N\text{-}NH_3$= ammonia nitrogen; NAN= non-ammonia nitrogen.

Figure 3.1. Mean CH_4 concentration (mg/dL) per treatment during continuous culture fermentation on d 8-10. CON= Control, TMR only; CL= 6% DM inclusion of *C. crispus* (*CC*); CH= 10% DM inclusion *CC*; BL= TMR + 2.5 mL of 0.6 mM bromoform; BH= TMR + 5 mL of 0.6 mM bromoform. [a-c]Least square means with different superscripts are different ($P < 0.05$). Error bars represent SEM.



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CHAPTER 4: CONCLUSIONS AND IMPLICATIONS

Numerous strategies for CH_4 emission reduction have been investigated to date, but the addition of seaweed in ruminant diets appears promising and appears to also provide other beneficial effects apart from simply reduced methanogenesis. Due to their nutrient density (Dawczynski et al., 2007; Morais et al., 2020), bioactive compound contents (Holdt and Kraan, 2011; Machado et al., 2016; Wang et al., 2008), and sustainability (Forster and Radulovich, 2015), seaweeds may be the next big movement in supplements for production animals. However, published studies have just scratched the surface in identifying suitable seaweed species for ubiquitous, effective, and sustainable implementation of seaweed in ruminant diets. Aspects such as seaweed processing methods, retention of bioactive compounds, seaweed sourcing availability and locations, inclusion rates and sustainable transportation are just a few that need to be addressed.

As discussed in Chapter 1, species such as *A. taxiformis* and *A. nodosum* (AN) have been widely studied in both *in vitro* (Roque et al., 2019; Wang et al., 2008) and *in vivo* (Antaya et al., 2019; Stefenoni et al., 2021) models. While red seaweed species are known for their bromoform content (Machado et al., 2016), few studies have investigated the potential effect of different species, for instance *C. crispus* (*CC*) supplementation, on rumen fermentation parameters (Kinley and Fredeen, 2015). Therefore, it is evident that some species may be overlooked, emphasizing the necessity of thorough evaluation of numerous different species to identify the best candidate for ruminant diets. Another gap in the investigation of seaweed candidates has been determining consistent inclusion rates, which has proven difficult as species, harvest location (Young et al., 2013), and

processing method (Vucko et al., 2017) are just some factors that affect bioactive compound efficacy in seaweeds. Still, few studies have acknowledged how seaweed processing method could affect rumen fermentation parameters. Because of this, Chapter 2 (Experiment 1) focused on the evaluation of processing methods, such as rinsing of *S. latissima* (*SL*), on CH_4 production and other fermentation parameters *in vitro*. Chapter 3 (Experiment 2) considered inclusion rates of 6% and 10% DM of *CC* and evaluated its effects on rumen fermentation *in vitro*.

4.1. Investigation of Bioactive Compounds

Brown seaweeds *SL* and *AN* used in Chapter 2 (Experiment 1) are known to contain phlorotannins. The efficacy of tannin-rich ruminant diets in reducing methanogenesis (Jayanegara et al., 2011; Wang et al., 2008) has been demonstrated throughout the last few decades, and extensively reviewed by Mueller-Harvey (2006) and Waghorn (2008), among others. Phlorotannins function in the rumen by binding with non-enzymatic and enzymatic proteins (Horigome et al., 1988), which decreases protein digestibility and affects enzymes involved in methanogenesis, thus yielding less CH_4.

Tannins are complex and highly variable in chemical structure among plant species, where phlorotannin concentration can vary even within the same algae class due to intrinsic and environmental factors (Cassani et al., 2020). This inherent variability likely contributes to the variability of tannin effects observed on rumen fermentation, depending on tannin source and concentration (Mueller-Harvey, 2006). However, compared with terrestrial tannins, phlorotannins from brown algae are known to be structurally less complex and are produced through the polymerization of phloroglucinol (Ragan and Glombitza, 1986). Despite the breadth of research that has been done on

phlorotannins, long term effects of supplementation in ruminants are still unknown and require further investigation. Namely, the compound phloroglucinol acts on smooth muscle, leading to muscle relaxation via the inhibition of voltage-dependent Ca channels which could influence Ca partitioning within dairy cows, especially transition cows.

While Chapter 2 (Experiment 1) tested phlorotannin-containing *SL* and *AN*, Chapter 3 evaluated bromoform-containing *CC*, comparing this seaweed species with anti-methanogenic bromoform controls. Bromoform reduces CH_4 production in ruminants by inhibiting an essential enzyme at the final step of the methanogenesis pathway (Wood et al., 1968; Denman 2007). Although bromoform is the most abundant and has been identified as likely the major compound that reduces methanogenesis activity, other halogenated compounds present in red seaweed biomass may contribute to a synergistic interaction that yields the most anti-methanogenic activity, as demonstrated in the research done by Machado et al. (2016). The CH_4 production results in this study from supplementing *CC* could therefore indicate that *CC* is not as potent in bromoform as *Asparagopsis* sp., most likely requiring higher dietary inclusion levels.

4.2. Seaweed Species Performance

Rinsing of seaweed supplements did influence CH_4 production *in vitro*, as supplements derived from *S. latissima* produced less CH_4 compared with the unwashed supplement. The processed extract from *A. nodosum* (Chapter 2), however, did not differ in CH_4 reduction *in vitro* compared with the unrinsed *S. latissima* supplement. Contrary to our hypothesis, both inclusion rates of *C. crispus* supplements (Chapter 3) increased CH_4 production *in vitro* compared with the controls. Despite these unexpected results, *CC* still produced more CH_4 at a 10% inclusion rate than the negative control in a subsequent

study (A. W. Driemel, unpublished data). Therefore, more investigation into the bromoform content and potency of *CC* is necessary before transitioning *CC* supplementation *in vivo*.

As discussed previously in Chapter 2, processing method of *AN* could have influenced phlorotannin efficacy in treatment PHLT. While soluble phlorotannins that are stored in organelles in the cell cytoplasm make up to 25% of dried algae, cell wall-bound phlorotannins form complexes with cell wall components (Koivikko et al., 2005). Cell lysis because of processes like washing or boiling may result in the loss of soluble phlorotannins. Therefore, certain processing methods would impact retention of soluble and cell-wall bound phlorotannins differently, thus affecting the rate of methanogenesis when the bioactive is supplemented to ruminants.

Outside of our knowledge, the ionophore monensin (Rumensin; Elanco Animal Health, Greenfield, IN, USA) was included at 0.002% DM in the TMR used in Experiment 2. Rumensin has been shown to have no effect on milk production (Odongo et al., 2007) or increase milk production (Sauer et al., 1998), reduce CH_4 emissions (Sauer et al., 1998; van Vugt et al., 2005; Guan et al., 2006; Odongo et al., 2007), and help control coccidiosis in dairy cows (Fitzgerald and Mansfield, 1973). The unexpected CH_4 results from Experiment 2 (Chapter 3) could have been a result of monensin masking the CH_4 reduction potential of *CC*. Since *CC* was included at 6% and 10% DM in the diet, less monensin would have been present in the seaweed diets compared with the controls, and therefore contributed to the controls yielding less CH_4 than the *CC* treatments. *C. crispus* reduced CH_4 production by 12% compared with the control *in vitro* when supplemented at 0.14 g DM/d in previously published research (Kinley and

Fredeen, 2015). Additionally, monensin was only unknowingly present as part of the TMR used in Experiment 2 (Chapter 3), and not included in the ration from Experiment 1 (Chapter 2).

4.3. Limitations of the Experimental Approach

Dual-flow continuous culture systems (DFCCS) have been demonstrated to be a representative *in vitro* model for the rumen environment. Despite notable differences in comparing *in vivo* and *in vitro* models, there are many benefits to using DFCCS, as described by Brandao and Faciola (2019). Both solid and liquid passage rates, temperature, agitation, artificial saliva additions and DMI are tightly controlled in DFCCS, which allows for a more accurate evaluation of rumen degradation without animal or DMI variabilities. Additionally, VFA production in DFCCS is more indicative of total VFA production as VFAs are not lost due to absorption via the rumen wall (Calsamiglia et al., 2002). Even with the advantages of controlled DFCCS, these systems can never perfectly mimic *in vivo* studies. Therefore, some of the more prominent limitations of the experimental approach included laboratory methodologies regarding sample collection and period effects due to environmental factors during fermenter inoculum collection.

4.3.1. Laboratory Methodology Limitations

In this research (Chapter 2 and 3), CH_4 samples were taken via a Sewerin Multitech 545 gas measuring device (Gütersloh, Germany). To take these samples, a probe attached to the portable analyzer was inserted into a small port located on each fermenter lid. The ports are sealed when not in use but there is likely gas escape during CH_4 sample collection as the port caps must be unscrewed to insert the probe for a

measurement. Additionally, the sensitivity of the analyzer is within ± 1% volume CH_4, thus limiting the precision of our CH_4 data. To combat this, samples collected from fermenter vessels via glass syringe were injected into glass vials and then shipped to Bigelow Laboratory for Ocean Sciences (BLOS; East Boothbay, ME) for analysis via gas chromatography. However, little to no CH_4 was detected from samples using this approach, and therefore their stability during shipment was too questionable to proceed (K. Posman, Bigelow Laboratory for Ocean Sciences, East Boothbay, ME, personal communication).

A more sensitive gas analyzer that is more compatible with our *in vitro* systems would be an important next step moving forward. Units such as an infrared spectrometer that connects directly to the DFCCS would allow for more accurate results without the loss of gas from the fermenter lid. While Tedlar gas collection bags have been previously evaluated in our systems, further investigation into adaptations to the DFCCS may be a more affordable solution compared with new gas analyzers.

For both experiments (Chapter 2 and 3), samples were prepared for proteomic techniques to identify microbial protein pathways in the rumen that were impacted by the seaweeds supplemented. Samples were cleaned and protein was extracted as per methods outlined by Honan et al. (2020). However, multiple extraction and quantification attempts performed by two laboratories yielded too little protein concentration to identify specific microbial proteins present. The presence of mostly degraded proteins was reported, despite the use of carboy samples that were iced during collection and frozen at -80°C prior to analysis. Therefore, the carboys may not have been cold enough for sample

collection to stop further protein degradation from occurring within the collection container.

Regarding different methodological approaches to proteomic procedures, it seems evident that issues are stemming from sample collection methods rather than sample processing. Extraction and labelling procedures utilized in these experiments have been successfully implemented by Honan et al. (2020) and Mulakala et al. (2022) with samples that were not collected from our *in vitro* systems. Rumen fluid sample collection directly from the fermenter vessel that is then immediately put on ice or stored at -80 °C rather than collected from carboys iced over a 24 h collection period may aid in the prevention of protein degradation. Additionally, validating protein concentrations via gel electrophoresis and proceeding with peptide labelling for TMT analysis after in-gel digestion could serve as a useful step to ensure successful results.

One of the largest challenges faced in Experiment 2 (Chapter 3) involved bromoform solubility for addition to the positive control fermenters. A solution containing 2.5 mL bromoform and 2497.5 mL H_2O was made with a targeted concentration of 11.3 mM. However, the bromoform would not solubilize, leaving visible droplets of bromoform in the bottom of the solution. Despite constant agitation, variability in concentrations among the bromoform stock solution led to a targeted 0.6 mM solution during trial periods. Since bromoform samples were shipped and then sample concentrations were analyzed by BLOS, it is unclear if the bromoform solution's immiscibility was affected by temperature during sample shipment or if stirring intensities of samples affected solubilization. Concentration differences were briefly investigated between room temperature and frozen samples, samples shipped in glass and

plastic containers, and samples stirred at different intensities, yet no clear results were observed. Targeted dosages for fermenters were initially 0.1 μM and 0.2 μM but dosages were later increased to 0.5 μM and 1.0 μM as they were effective at reducing CH_4 in a batch culture system by K. Posman (Bigelow Laboratory for Ocean Sciences, East Boothbay, ME, personal communication). For the final stock solution used in Experiment 2, 26.7 mL of bromoform stock solution was taken from the previously made solution (2.5 mL bromoform to 2497.5 mL H_2O; ~5.5 mM) and was diluted with 223.3 mL of di H_2O (0.6 mM). Despite unknown concentrations of bromoform supplemented to the fermenters, the bromoform treated fermenters still successfully served as positive controls and reduced CH_4 production *in vitro* as expected.

Throughout bromoform solubility troubleshooting, different solvents for bromoform other than water were considered. While DMSO was an option as it has been used in seaweed extraction processes by Machado et al. (2014) and Choi et al. (2021), and could be supplemented *in vivo*, it can be challenging to work with. DMSO acts as a permeator and therefore, may carry other dissolved chemicals such as the bromoform into the body if skin contact occurs (SDS). Methanol was also considered for use as a solvent, but some studies suggest that methanol supplementation increased methanogenesis (Czerkawski and Breckenridge, 1972; Neumann et al., 1999) which would then make it a confounding issue in Experiment 2. In proceeding with H_2O as a solvent, concentration analysis performed in-house to avoid shipping would remove one variable. Investigation into how volatile the bromoform and water solution is during shipping and temperature fluctuation as well as without consistent stirring could be another useful step moving forward. To avoid solubility issues, additions of pure bromoform to fermenter vessels

was considered but dosages were so small (0.02- 0.05 μL) which risks doses being administered directly to the rumen fluid, as it could get stuck on the agitation rods or sides of the vessel.

Chapters 2 and 3 also attempted analysis of ^{15}N enriched samples to determine microbial nitrogen utilization during fermentation. Procedures were performed based on Calsamiglia (1996). Liquid effluent taken directly from each fermenter on day 5 was strained through 4 layers of cheesecloth and centrifuged at 1000 x g for 10 min at 4°C to obtain a bacterial pellet (Benedeti et al., 2015). These samples were taken prior to administering the $(^{15}NH_4)_2SO_4$ priming dose to establish background ^{15}N abundances. For d 8-10, effluent was taken directly from the carboys, strained, and centrifuged as described previously to obtain a bacterial pellet. Bacterial pellets were stored at -20°C until freeze dried at -80°C for 72 h. Once freeze dried, bacterial pellet samples were ground using a tissue lyser (TissueLyser II, Qiagen, Hilden, Germany) and 5 mm metal bead at 20 hz for 5 min. Pre-acidified sample was used to determine the ^{15}N NH_3-N enrichment exclusively, following the methods outlined by Hristov et al. (2001). Aliquots of oven dried effluent samples were set aside prior to wet chemistry analysis to determine ^{15}N non-ammonia nitrogen (NAN) enrichment using protocols adapted from Firkins et al. (1992). After drying, samples were also ground using a tissue lyser as described previously. Ground bacterial samples, filter disks obtained from the NH_3-N diffusion and ground NAN samples were then separately encapsulated in tin and sent to the UC Davis Stable Isotope Facility for ^{15}N enrichment analysis via isotope ratio mass spectrometry. Despite similarities in sampling and processing of ^{15}N samples between our studies and others published, values from Experiment 1 and 2 for bacterial atom percent excess

(APE) of N were lower than expected, so bacterial N flows (g/d) and microbial efficiencies could not be determined accurately.

Inadequate ^{15}N concentrations detected in bacterial cells could have been due to bacterial death during fermentation, protein binding in seaweeds (reviewed by Khan et al., 2022), or simply too low of a ^{15}N dosage. Therefore, higher dosages of $(^{15}NH_4)_2SO_4$ may be the next step forward regarding nitrogen utilization analysis. The priming dose administered in Experiments 1 and 2 (0.07 g) was comparable to that given by Calsamiglia et al. (1996; 0.066 g) and Paula et al. (2017; 0.077 g), but less than that added by Bennett et al. (2021; 0.1733 g). However, the ^{15}N enrichment of the buffer was lower in Chapters 2 and 3 (0.021 g/L) compared with dosages administered by Calsamiglia et al. (1996; 0.066 g/L) and Bennett et al. (2021; 0.077 g/L). Thus, a higher concentration of $(^{15}NH_4)_2SO_4$ added to the supplied buffer may increase ^{15}N detected in samples.

While expected DM and OM degradability in DFCCS are 70-80% (Brandao and Faciola, 2019), both Experiments 1 and 2 (Chapters 2 and 3) yielded lower degradability in treatments which could be due to processing methods used for diet components. If seaweed samples were not fully dried, a Maillard reaction could have occurred from the abundance of amino acids in seaweeds, leftover H_2O present and heat applied (Van Soest, 1994). This reaction polymerizes sugars that then have similar properties to lignin, thus decreasing digestibility (Van Soest, 1994). Forage quality in the TMR as well as freezing and thawing cycles in both TMR and seaweed samples could also have contributed to decreased degradability *in vitro*.

4.3.2. Animal-based Experimental Limitations

One of the greatest challenges in using the DFCCS is collecting the nearly 20 L of rumen fluid needed to inoculate up to 6 fermenters in the shortest amount of time possible. Once collected, the rumen fluid is manually strained on farm, and then transported 2.2 miles to the laboratory where each fermenter is inoculated individually. This whole process usually takes up to 30 min and despite all efforts, the rumen fluid is exposed to the aerobic environment, light and temperatures colder than 39°C during that time. Vastly different weather conditions by season in the northeastern US often results in period effects seen in the fermenters as microbes are briefly exposed to temperatures as low as -23 °C during winter. To combat this, heat packs filled with hot water are used, as well as insulated containers equipped with electric heaters to keep the fluid as warm as possible. Additionally, the Latin Square design was chosen specifically to increase statistical power, as it yields up to 6 replicates per treatment in a 6x6 design.

4.4. Future Perspectives

With over 10,000 species of seaweeds that exist today in our oceans, it is difficult to determine whether species that are currently being investigated will be the optimal choice for ruminant diets or if new species deserve evaluation as well.

4.4.1. Current Species Feasibility and New Prospects

Not all seaweeds commonly evaluated in the literature would be appropriate or feasible for supplementation anywhere in the world. While *SL* may be a feasible candidate for supplementation to dairy cows in the Northeast US as it is readily available along Northeastern shores (McHugh, 2003) and therefore, requires less transportation, other seaweed species may be more fitting for different regions.

Some species, such as *Porphyra*, have been investigated little in ruminants and scarcely, if at all, studied in dairy cows. *Porphyra* sp. have been shown to contain the highest CP (on a DM basis) out of species evaluated by Tayyab et al. (2016) and had the highest total tract amino acid digestibility among species reported in Gaillard et al. (2018). Numerous other studies have also investigated or reviewed the nutrient profiles of *Porphyra* (Dawczyski et al., 2007; Holdt and Kraan, 2011; Makkar et al., 2016), as well as storage stability (Harrysson et al., 2021). *Porphyra* sp. are already widely used for humans (detailed in McHugh, 2003) but even if enough of this species cannot be sourced at high enough volume to feed to ruminants, by-products from human consumption may have potential uses in animal feed. For instance, if bioactive compounds like bromoform can be extracted from *Porphyra* either before or after human use, less plant matter would need to be sourced and less waste would result.

4.4.2. Diving Deep into Processing Methods

After our investigation of how the processing method of seaweeds affects *in vitro* fermentation and CH_4 production as well as reviewing previous literature, it is evident that processing method of seaweeds and bioactive compounds should be a focus in future work. Ensiling, as reported in Yen et al. (2021) has shown promise and therefore, warrants further examination as an easily replicable and inexpensive processing and storage method. Establishing universal guidelines for specific seaweed processing techniques for future studies to follow may help ensure more uniform experimental comparisons as vast variability in processing methods between studies makes comparison difficult. The most effective existing processing methods can then be

applied to numerous different species that have shown anti-methanogenic potential, and from there the best seaweed species candidates can be determined.

4.3. Conclusions

The thesis presented herein outlines the impact of different seaweed materials on rumen fermentation via *in vitro* continuous culture fermenters. Contributing to the growing body of knowledge, this research examined feeding seaweed supplements to ruminants by evaluating the impact on pH, methane, and degradability. Chapter 2 studied how processing method of *SL* and *AN* affected rumen fermentation parameters, specifically CH_4 production, *in vitro*. Chapter 3 (Experiment 2) then delved into the investigation of the lesser studied *CC*, and its ability to reduce CH_4 production at inclusion rates of 6% and 10% DM *in vitro*. As this work is just a start in the evaluation of processing methods, different seaweed species, and inclusion rates, it is evident that the implementation of seaweeds in ruminant diets is a multi-faceted and multi-disciplinary venture. Thus, it requires aspects of sustainability, feasibility, economic efficiency, and animal safety to be addressed in studies moving forward prior to successful implementation in ruminant diets.

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