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Effects of Biosynthesized Bromoform on Enteric Methane Production, Animal Performance and Tissue Residues in Cattle

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EFFECTS OF BIOSYNTHESIZED BROMOFORM ON ENTERIC METHANE PRODUCTION, ANIMAL PERFORMANCE AND TISSUE RESIDUES IN CATTLE

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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ABSTRACT OF THESIS

EFFECTS OF BIOSYNTHESIZED BROMOFORM ON ENTERIC METHANE PRODUCTION, ANIMAL PERFORMANCE AND TISSUE RESIDUES IN CATTLE

Greenhouse gas (GHG) emissions have been implicated in global warming and extreme climate change conditions. The Environmental Protection Agency (EPA) has identified agriculture, more specifically cattle production, as a major contributor to global GHG production. When expressed as liters of gas, CO_2 is considered the primary GHG produced, however, in terms of global warming potential (GWP), CH_4 has 25-times greater potential than that of CO_2. Due to the elevated GWP of CH_4, we have constructed a series of experiments to investigate the use of a halogenated CH_4 analogue derived from kelp (i.e., bromoform) as an inhibitor of enteric CH_4 production in cattle. In experiment 1, 12 Holstein steers were used in a randomized complete block design to test the hypothesis that kelp reduces enteric methane production. Dietary treatments included a corn-silage basal diet fed at 1.5x NE_m, containing either no kelp (ground corn carrier only) or ground corn carrier plus kelp at 10% of carrier. Treatments were administered at 0.5% of total ration dry matter (bromoform content= 10 mg/g product). Steers were adapted to their respective diets for 14-d prior to placement into metabolism stalls fitted with stainless steel headboxes for continuous measurements of CH_4 and CO_2 production and O_2 consumption over a 3-d period. Treatment had no effect ($P>0.11$) on DMI (g/kg BW) during the adaptation or respiratory gas collection period. In contrast, steers receiving kelp had lower ($P<0.001$) CH_4 production (<2.2L/d) compared to the control steers (88.7 L/d). Kelp substantially reduced methane production without altering DMI or oxidative metabolism. In experiment 2, 6 Holstein steers were used in a 17-d respiratory gas collection experiment to test the hypothesis that suppression of enteric CH_4 would persist after removal of kelp from the ration. Dietary treatments contained either 0, 0.5 or 1% of diet DM as kelp (bromoform content= 2 mg/g product) top dressed to a corn silage-based diet provided at 1.5x NE_m. All feed was consumed on all treatments throughout the adaptation, treatment, and recovery periods. Kelp was fed from d 1 through d 11 such that days 1 and 2 of the respiratory gas collection period represent the effects of bromoform on enteric CH_4 production, and responses on subsequent days represent residual effects. On d 1, the 1% inclusion decreased production below the limits of detection, however, 0.5% was not different from the control. A treatment x day interaction ($P\leq0.0003$) was observed for enteric CH_4 production (L/d) and yield (g/g DMI). Steers consuming 0.5% of DM did not differ from the control animals ($P\geq0.3$). Methane production and yield were not affected by day in control animals; however, a quadratic response was observed ($P<0.0001$) for the 1% treatment and a cubic response was observed for the 0.5% treatment. In experiment 3, 12 Angus steers were used in a 30-d feeding trial to test the hypothesis that kelp would not contribute to differences in intake or measurable accumulations of bromoform residues in tissue. Dietary treatments included a corn-silage basal diet fed ad libitum, adjusted twice weekly for intake, and a top-dress composed of ground corn and distillers dried grain (DDG) and either no kelp (ground corn and DDG carrier), or carrier plus kelp (bromoform content= 2 mg/g

product) at 10, or 20% of carrier. Treatments were administered at 0.5% of total ration dry matter. Steers were adapted to ad libitum intake of an 80% concentrate basal diet for a minimum of 14-d prior to introduction of the treatment. Treatment had no effect (P=0.53) on DMI (kg/kg BW) following 30-d of kelp supplementation. Concurrently, treatment had no effect (P=0.55) on ADG (kg/d) or growth efficiency (g/kg) (P=0.82). In support of our stated hypothesis, kelp supplementation did not result in detectable bromoform residues in liver, kidney, adipose or muscle samples collected at harvest following the 30-d supplementation period.

KEYWORDS: Cattle, Methane Mitigation, Bromoform, Residues, Growth Performance

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12/7/2023

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DEDICATION

To my family, for your constant support throughout this adventure.

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LIST OF ABBREVIATIONS

BW= body weight

ADG= average daily gain

G: F= gain: feed ratio

CO_2= carbon dioxide

CH_4= methane

DMI= dry matter intake

3-NOP= 3-nitrooxypropanol

F:C= forage: concentrate ratio

VFA= volatile fatty acids

MCR= methyl coenzyme M reductase

CHAPTER 1. INTRODUCTION

In the last decade, climate research has focused on the impact of ruminant livestock greenhouse gas emissions, particularly the production of carbon dioxide (CO_2) and methane (CH_4). Although greenhouse gases (GHG) are naturally occurring and can be found dispersed throughout the atmosphere, anthropogenic activities have contributed to increased atmospheric concentrations. Substantial evidence suggests that increased atmospheric greenhouse gas concentrations have caused a gradual warming of the earth. Greenhouse gases naturally trap infrared heat that would have otherwise dissipated into space under normal atmospheric conditions, contributing to increased surface temperatures. Livestock account for approximately 12% of global GHG emissions, with a major contribution coming from ruminant animal production and fermentative processes.

Ruminant animals rely on a diverse ecosystem within their rumen which ferments feedstuffs, however, enteric fermentation is responsible for the production of enteric gases, importantly CH_4. Methane is generated by a group of microorganisms known as methanogens which belong to the domain *Archaea* and the phylum *Euryarchaeota* (Hook et al., 2010). While many GHG are important in climate change, much recent research has been aimed at reducing CH_4 production due to its enhanced ability to trap heat compared to its gaseous counterpart CO_2. Ruminant GHG emissions will become particularly important as the United Nations predicts global populations to increase to 9.4-10.2 billion by 2050, generating increased livestock production to meet the increased demand for meat and milk products (United Nations, 2017). Current research targeting

enteric CH_4 is focused on the development of strategies including microbial manipulation and inclusion of dietary additives which alter fermentative end products.

CHAPTER 2. LITERATURE REVIEW

2.1 Recent Trends in Greenhouse Gas Emissions

Greenhouse gases serve a vital role in maintaining life on earth. Without GHG earth's surface temperatures would be 60 ºF (33.3 ºC) colder, which would lead to catastrophic changes to the environment (EPA, 2022). But there are upper limits; beyond which point atmospheric GHG can have an equally catastrophic effect on the environment. A significant increase in GHG emissions occurred following the industrial era, since then CO_2 and CH_4 concentrations have increased 40 and 150%, respectively, far surpassing levels of atmospheric GHG needed to maintain habitable environments (IPCC, 2014). Recent trends indicate total GHG emissions have decreased 7% during the COVID-19 pandemic due to the rapid decline in fossil fuel use (IPCC, 2023). While this trend is promising, the impact of elevated concentrations contributes to climate change events such as increased surface temperatures, storm frequency and extreme weather events (EPA, 2022).

2.2 Effects of Greenhouse Gases

The warming of the planet due to the trapping of infrared radiation by GHG, global warming, is primarily a function of the concentrations of water vapor, CO_2, CH_4, and N_2O (IPCC, 2014). Although CH_4 represents a much smaller fraction of total GHG emissions, compared to CO_2, CH_4 has a much higher GWP, and approximately 43x the radiative effect compared to CO_2 (IPCC, 2023). The 2023 IPCC report shows that

although the contribution of agricultural to CH_4 emissions is significant, non-ag and food system emissions are greater than that from agriculture (IPCC, 2020). Enteric CH_4 accounts for approximately 30% of global CH_4 emissions, much of which is attributable to cattle which have the ability to generate up to 500 L of CH_4 per day (Johnson and Johnson, 1995). While ruminants are significant producers of CH_4, manipulation and complete elimination of CH_4 can come with significant productivity losses and health implications due to the physiological importance of CH_4.

2.3 Importance of Methane Production in Ruminants

During rumination, cattle can generate and eructate nearly 500 liters of gases per day (Sherwood et al., 2012). Ruminants depend on a variety of bacteria, archaea, protozoa and fungi to increase the availability of nutrients in plant material via fermentation to generate substrates that can be utilized for energy by the animal. The primary products of fermentation are short chain fatty acids (SCFA), or volatile fatty acids (VFA), that account for 50-70% of the energy ruminants obtain (Nagaraja, 2016). Remaining products that cannot be directly used by the animal, including H_2 and CO_2, require existing interrelationships between microorganisms which allow these products to be utilized by another class of microbiota. For example, methanogens, which constitute approximately 2-4% of the ruminal population, utilize H_2 and formate to reduce CO_2 to generate CH_4 (Nagaraja, 2016). During this process, methanogens also provide a hydrogen sink, required for the regeneration of NAD^+, an essential molecule of energy metabolism. Although the generation of CH_4 is beneficial for the regeneration of

substrates, it can account for 2-12% loss of gross energy intake (Nagaraja, 2016; Abbott et al., 2020). Due to the loss of productivity, and the impact of CH_4 generation on the atmosphere, research has focused on methods to mitigate enteric CH_4 emissions from cattle.

2.4 Classification of Methanogenic Microflora

The diverse environment of the rumen enables ruminant animals to obtain nutrients from plant matter such as cellulose, which is not immediately soluble in the rumen. Each class of organism in the rumen has a specific function and generates its own products. However, the efficiency at which each species converts feed consumed by the animal into VFA's which are in turn metabolizable by the animal is highly variable. For example, methanogens are responsible for generating 40% of global CH_4 emissions, and the inefficiency of the process of methanogenesis accounts for a 6-12% loss of gross energy (GE) intake (Smith et al., 2019). Methanogens are classified as those belonging to the domain *Archaea* and the phylum *Euryarchaeota* (Hook et al., 2010). Methanogens lack peptidoglycan in their cell walls, distinguishing them from other bacterial species (Hook et al., 2010). The characteristics of methanogens vary regarding physical characteristics, substrate preferences, environmental optima, etc. All methanogens have coenzyme F_{420}, which is a cofactor which is essential for the use of formate to generate CH_4 during metabolism (Hook et al., 2010). Hydrogen ions do not accumulate in the rumen, rather, hydrogenases transfer electrons to H^+ to form H_2 which then can be utilized by methanogens. When methanogenesis is inhibited, there must be a redirection of H_2 to

other pathways, including propionate production which requires hydrogen during reduction reactions leading to formation of propionate (Ungerfeld, 2020).

16S rRNA gene sequence analysis is a preferred method of identifying and comparing bacterial alpha and beta diversity and can categorize bacteria down to the genus or species level. The 16S gene is highly conserved across bacterial species, making it an appropriate candidate for comparison of groups within the microbiome. Whitford et al. (2001) identified *Methanobrevibacter* spp. as the primary species of methanogens in the bovine rumen, followed by eleven species belonging to the *Methanobacteriaceae* family (Whitford et al., 2001). Similar to the findings of Whitford et al. (2001), Tymensen et al. (2012) identified *Methanbrevibacter* species as having the highest sequence identity to the selected primers, followed by *Methanomicrobium* spp. (Tymensen et al., 2012). Across literature it is understood that bacterial populations vary based on the composition of the diet being consumed by the animal from which the rumen fluid was harvested, generating significant variation across studies. For example, feedlot cattle being fed a diet composted of predominantly corn were found to have 95% sequence similarity with *Methanobrevibacter ruminantium*, *Methanbrevibacter thaueri, Methanobrevibacter smithii, and Methanosphera stadtmanae,* (Wright et al., 2007). However, in the same study cattle that were fed a diet composed of primarily potato by-products were found to have 95% sequence similarity with *Methanobrevibacter smithii, Methanobrevibacter ruminantium and Methanobrevibacter thaueri*, further supporting the belief that diet influences aspects of microbiome prevalence (Wright et al., 2007). The sensitivity of the microbiome to changes allows for identification of mechanisms that affect CH_4 production.

2.5 Factors Affecting Methane Production

The rapidly adapting environment of the rumen is responsive to minor changes to inputs or conditions, which can result in substantial changes in the rumen flora and fermentative end products. Changes in the rate of methanogenesis result in altered efficiency, productivity, and energy utilization, thus it is important to minimize CH_4 and maximize feed utilization and animal efficiency (Ungerfeld, 2020). Methane losses are variable and influenced by a variety of factors including feed quality and composition, intake, feed processing, and geographical location (Hook et al., 2010).

2.5.1 Influence of Dietary Composition on Methane Production

The dynamic environment of the rumen responds and adapts to changes in diet quality. The primary VFA generated in the rumen are acetate, propionate, and butyrate, which account for 95% of total VFA production (Ungerfeld, 2020). VFA's are the predominant source of energy for ruminants, accounting for approximately 75% of metabolizable energy (ME) (Ungerfeld, 2020). Diet composition is highly influential on VFA proportions, particularly the proportions of acetate and propionate. Dietary carbohydrate fractions impact the amount of CH_4 generated via influence on pH and rumen microbial populations (Sherwood et al., 2012). The fermentation of cell wall fiber components (i.e. cellulose), is associated with higher CH_4 production via increased acetate production, whereas high soluble carbohydrate fractions are associated with lower CH_4 production through increased propionate production (Sherwood et al., 2012). Altering the forage-to-concentrate ratio is the primary way to alter the VFA composition

of the rumen, thus, influencing the production of CH_4 via alternative hydrogen sinks (i.e., propionate).

Typical VFA proportions are 60-70% acetate, 14-20% propionate, and 10-14% butyrate, however, propionate is the only glucogenic VFA (Sherwood et al., 2012). Compared to non-ruminant animals, ruminants require much higher levels of gluconeogenesis, where propionate is the primary substrate for the generation of glucose. Approximately 70% of the animals glucose requirement is fulfilled by converting propionate to glucose (Sherwood et al., 2012). The other VFA's (i.e., acetate and butyrate) enter the citric acid cycle as acetyl-CoA where a series of reactions generate 2 ATP as well as 6 NADH and 2 $FADH_2$. The profile of VFA absorbed from the rumen has important implications to metabolism post-absorption. For example, due to the importance of propionate in gluconeogenesis, a shift towards higher propionate production can be beneficial when glucose requirements are elevated. However, propionate can also act as a satiety signal, causing decreased feed intake and milk fat, whereas acetate does not cause milk fat suppression, an important consideration when manipulating VFA profile (Ungerfeld, 2020).

Due to shifting molar proportions of VFA's having an effect on CH_4 production studies have observed the effects high starch diets on fermentative end products, finding that these diets are associated with decreased CH_4 production. Hatew et al. (2015) observed the effects of diets differing in starch rate of fermentation and level of inclusion in the concentrate in lactating dairy cattle. CH_4 emissions were not affected by increasing concentrate proportion or rate of fermentation ($P=0.14$) or source ($P=0.67$) (Hatew et al., 2015). However, Hassanat et al. (2013) examined the effects of replacing alfalfa silage

(AS) with corn silage (CS) in dairy cow diets, suggesting that the shift from AS to CS would increase dietary starch supply and decrease CH_4 production (Hassanat et al., 2013). Corn silage was included at 0, 50 or 100% of the diet, CH_4 production (g/d) quadratically increased (440, 483, 434 g/d, respectively; $P<0.01$) in response to increasing corn silage proportion, finding that 50% inclusion resulted in the highest production of CH_4 (Hassanat et al., 2013). Similarly, a quadratic response was observed when CH_4 was presented as g/kg DMI, % of GE intake, and in proportion to milk components (Hassanat et al., 2013). The findings of this study suggest that there is a minimum level of inclusion for starch required to observe significant effects on CH_4 production. However, a concern expressed by Dijkstra et al. is that increasing starch concentrations of the diet may reduce CH_4 emissions, but increase N excretion, which can incorporate with oxygen to form N_2O, another GHG (Dijkstra et al., 2011). Another proposed, potentially low cost method of reducing CH_4 production in ruminants, is increasing the digestibility of nutrients in pasture (Hart et al., 2009).

2.5.2 Influence of Forage Digestibility on Methane Production

Forage digestibility is determined by a variety of anti-quality factors which decrease the bioavailability of nutrients in forages. One anti-quality factor responsible for decreasing forage digestibility is lignin, an indigestible component of the plant cell wall associated with increasing plant maturity (Waldo, 1986). As plants mature, the concentration of dietary neutral detergent fiber (dNDF) increases, also causing a linear increase in CH_4 production (Pinares-Patino, 2003). This finding is in agreement with an earlier study from Moe and Tyrell which concluded that soluble residue and digestible

hemicellulose and cellulose fractions were the most significant components in CH_4 production (Moe and Tyrrell, 1979). The relationship between NDF and CH_4 suggests that CH_4 production in the rumen is primarily a function of the concentration of cell wall components (Pinares-Patino, 2003). Across six studies, decreasing plant maturity, and thus NDF, generated a mean decrease in CH_4 of 13% (per unit of milk), and a 9% increase in milk yield (Arndt et al., 2022). Thus, decreasing forage maturity decreases NDF content, increases the proportion of digestible energy intake, and decreases CH_4 per unit of production (i.e., milk or meat). These changes are likely due to increased energy and digestible protein intake which contributes to higher milk production or tissue accretion (Arndt et al., 2022). However, increased protein consumption can lead to increased nitrogen excretion which is also a concern around the agricultural industry as it has been implicated in other climate concerns. Although anti-quality factors have a significant impact on forage quality, it is important to consider all factors when evaluating CH_4 production and mitigation potential.

VFA proportions are highly influential on CH_4 production due to the availability of H_2. Bacteria that favor propionate production will reduce H_2 availability for methanogenesis (Russell, 1998). Russel (1998) concluded that 25% of the difference in VFA proportions could be explained by the effects of manipulating pH (Russell, 1998). The production of CH_4 is thus influenced by a combination of the substrate availability and VFA production (Dijkstra et al., 2008). However, many models including that of Mills et al. suggest that the addition of pH to mechanistic models aiming to predict CH_4 production, did not contribute to significant increases in the model's ability to predict CH_4 production (Mills et al., 2001). Instead, many models focus on the inclusion of NDF

as a determinant of CH_4 production, as it contributes to VFA production, and increases the reliability and accuracy of the model to a greater extent than pH (Mills et al., 2001).

2.5.3 Protozoa and Methanogens, the Effects of Defaunation on Methane Emissions

While much of the discussion up to this point has been about methanogens, it is important to note that some methanogen communities require the presence of protozoa to survive. Protozoa-associated methanogens (PAM), are methanogens which act as ectosymbionts, meaning they dwell on the outside of the protozoa. Due to the close relationship of methanogens and protozoa, defaunation of the rumen has been discussed as a possible mechanism for the reduction of CH_4 production in the rumen. Defaunation removes protozoa, which constitute approximately 50% of the rumen biomass, and also cause a decrease in the number of PAM, however, when these PAM's are removed there is an increase in the amount of free hydrogen in the rumen (Santra and Jakhmola, 1998; Newbold et al., 2015). Increased hydrogen availability in the rumen contributes to decreased pH which can interfere with the ability of other microorganisms to function. Defaunation of the rumen by Newbold et al. (2015) resulted in an 11% reduction in CH_4 production, accompanied by a 30% increase in microbial protein supply (Newbold et al., 2015). The associated increase in microbial protein is due to an increase in microbial presence in response to decreased protozoal predation (Newbold et al., 2015). However, in lambs, there was no significant changes in CH_4 production (L/d) when lambs were defaunated (Hegarty et al., 2008). Which opposes the conclusion made by Nguyen et al. who observed a tendency for reduced CH_4 yield (g CH_4/ kg DMI; $P=0.07$) when

defaunated and refaunated grazing sheep were observed via GreenFeed system (Nguyen et al., 2018).

2.5.4 Influence of Particle Size and Passage Rate on Methane Production

Passage of digesta is a dynamic process which is influenced by a variety of factors that alter rumen kinetics. For example, it has been suggested that particles must reach a size at which they are able to pass through the reticulo-omasal orifice. It is understood that by decreasing particle size through either external processing of feeds or through mastication and digestion, passage rate will increase. Rumen motility patterns stratify particulate matter into layers, larger particles remain at the top of the rumen layers, known as the hay mat. It was believed that once those particles reached a certain size that they would pass through the reticulo-omasal orifice, however, Kammes and Allen suggested that a large portion of the particles retained in the rumen were smaller than the necessary size to pass through the orifice, thus it was concluded that particle size is not solely responsible for passage rate (Kammes and Allen, 2012). While not the sole factor in determining passage rate, decreasing particle size decreases CH_4 production, likely due to increased rate of digestion and passage (Hook et al., 2010). Ruminal passage rate has also been directly associated with methanogen growth rate and decreased CH_4 production per unit of feed digested, and Pinares-Patiño reported a negative correlation between CH_4 production and rate of passage (Pinares-Patino, 2003). Kennedy and Milligan also reported decreased CH_4 production when the rate of liquid and solid passage from the rumen increased (Kennedy and Milligan, 1978). Both groups suggest that passage can

influence CH_4 production, however, it is also important to consider the influence of intake level as it contributes to passage rate and CH_4 production.

2.5.5 Influence of Level of Intake on Methane Production

Intake has also been identified as a factor which indirectly influences ruminal CH_4 production. It is understood that as intake increases, the amount of substrate available for the generation of CH_4 would also increase. Cattle being limit fed (75% of ad libitum) had lower CH_4 production than cattle fed ad libitum (126 g CH_4/d vs 156 g CH_4/d, respectively), however there were no differences in CH_4 yield (CH_4/kg DMI) (Winders et al., 2018). However, dairy cow herds consuming ad libitum intake had lower CH_4 production on a herd basis compared to those consuming a restricted intake (1,310 vs 1,583 MJ/herd/d) (Mills et al., 2001). Johnson and Johnson reported that as DMI increased, the percentage of dietary GE lost as CH_4 decreases by an average of 1.6% per level of maintenance intake (Johnson and Johnson, 1995). However, Blaxter and Clapperton observed the effects of amount and apparent digestibility of feed (roughage, pelleted or milled feeds, or mixed diets) on CH_4 production, where intake was set to maintenance, 2 times maintenance or 3 times maintenance. From these trials it was concluded that the CH_4 intensity (kcal/100 kcal feed) increased as apparent digestibility increased at maintenance level feeding, but when fed at 2x maintenance requirements, the increasing digestibility caused less of an impact on CH_4 production compared to maintenance intake (Blaxter and Clapperton, 1965). At 3 times maintenance intake the resulting effects of increasing digestibility were unlike maintenance and two times

maintenance intake, there was a decrease in CH_4 production (kcal/100 kcal feed) as apparent digestibility of the diet increased (Blaxter and Clapperton, 1965). Decreases in CH_4 emissions (CH_4 g/ kg DMI) in response to increasing intake has been well documented across literature since Blaxter and Clapperton. Winders et al. observed a tendency for increased CH_4 intensity, 18.7 and 20.3 g/kg DMI (P=0.07) for cattle fed 75% of ad libitum intake compared to ad libitum intake of a diet composed of 45% alfalfa, 30% sorghum silage and 22% modified distillers grains plus soluble, respectively (Winders et al., 2018). Increasing level of intake causes reductions in CH_4 intensity (g/ kg DMI) due to decreasing digestibility and increasing passage as intake increases (Blaxter and Clapperton, 1965; Knapp et al., 2014).

2.6 Heritability and Genetic Selection for Sustained Methane Mitigation

While dietary manipulation and supplementation has been a focus of research for many years, recent genetics research has focused on determining the influence of animal genetics on CH_4 emissions. Selection for genetic traits which produces long term, sustained mitigation suggest that both phenotypic and genetic variation contribute to CH_4 emissions in cattle (Haas et al., 2019). Methane production has been reported as a heritable trait (MET) with a heritability estimate of 0.1 and 0.4 (De Haas et al., 2011; Lassen and Løvendahl, 2016). The MET selection criteria is based on the sum of CH_4 production (kg) of a large population of cows across a single lactation, giving MET a phenotypic value (kg/yr) (González-Recio et al., 2020). González-Recio et al. estimated that if MET is included in breeding criteria CH_4 emissions could be expected to decrease

4-6% in 10 years due to the positive correlation between production traits and MET (González-Recio et al., 2020). Thus, many researchers have been searching for criteria which would directly or indirectly reduce CH_4 production.

Basarab et al. examined residual feed intake (RFI) as a potential selection criterion for reducing CH_4 emissions based on the theory that cattle selected for lower RFI would consume less dry matter, have higher feed conversion efficiency, and produce less CH_4 (Basarab et al., 2013). Basarab et al. concluded that while selecting for RFI on a single occasion does not contribute to rapid change, repeated selection overtime would generate an improvement to feed efficiency and CH_4 intensity (CH_4/kg DMI) (Basarab et al., 2013). Breider et al. determined that the heritability of CH_4 ranged from 0.12 to 0.45 and had a positive genetic correlation with milk yield (MY) of 0.49-0.54 when evaluated across 184 Holstein-Friesian dairy cows (Breider et al., 2019). These findings were similar to López-Paredes et al. (2020), which found that CH_4 concentration (ppm) CH_4 production (g/d) had a heritability of 0.12 (López-Paredes et al., 2020). Carberry et al. (2014) aimed to quantify microbial methanogenic populations in cattle selected for RFI while being offered high or low forage diets, concluding that the abundance of a specific methanogen did not contribute to the variation in CH_4 production between efficient and inefficient animals, however, dietary manipulation did alter the total abundance of methanogens which then causes the differences in observed efficiency (Carberry et al., 2014). Genetic control of CH_4 production is a rapidly developing area of animal science research; however, it is quickly becoming a major focus in the conversation of CH_4 mitigation.

2.7 Methane Mitigation Strategies Utilized in Ruminant Production

2.7.1 Ionophores

Ionophores, such as monensin and lasalocid, are polyether antibiotics that have long been used to improve feed efficiency and gain in cattle. Ionophores have been shown to be effective at combating CH_4 emissions via suppression of bacterial metabolism (Marques and Cooke, 2021). By adhering to the bacterial membrane, particularly of gram-positive bacteria, which lack the protective membrane present in gram-negative bacteria, ionophores cause a disruption in the ion gradient that is required for cell survival. Following a loss of ion balance, cell sodium/potassium pumps and hydrogen ATPase systems activate, to pump H^+ out of the cell to attempt to regain ion balance. However, with the ionophores adhered, ion balance cannot be regained, and ATP depletion causes decreased gram-positive bacteria proportion in the rumen (Marques and Cooke, 2021).

Monensin supplementation to dairy cattle consuming a 60:40 forage to concentrate TMR, reduced ($P< 0.01$) CH_4 production (g/d) by 7% and persisted through six months of use (Odongo et al., 2007). However, studies suggest a loss of efficacy occurs during long-term ionophore use, Guan et al. reported that CH_4 suppression due to the inclusion of ionophores only persisted through four-weeks in low concentrate diets, and for two-weeks in high concentrate diets (Guan et al., 2006). While the use of ionophores mitigates CH_4 emissions, the loss of efficacy coupled with a negative public perception of ionophores has motivated researchers to develop other methods for reducing CH_4 emissions.

2.7.2 Dietary Lipids

Dietary lipids negatively impact CH_4 production, however, DMI and digestibility is impeded when lipid inclusion exceeds 6% of DM (Beauchemin et al., 2007). Inclusion levels less than 6% can have additional benefits such as altering the fatty acid (FA) composition of animal products and increasing the absorption of fat soluble nutrients (Beauchemin et al., 2007). Lipid sources containing long-chain FA inhibit microbes, particularly fiber digesting bacteria contributing to observed decreases in digestibility at high inclusion levels (Beauchemin et al., 2007). When tallow, sunflower oil and sunflower seeds were supplied to provide an additional 33 g/kg DM of lipids, CH_4 production (% GE intake) decreased ($P< 0.001$) 15% (Beauchemin et al., 2007). In a later review published by Beauchemin et al, over a range of conditions, each 1% inclusion of fat in the diet was associated with a 5.6% decrease in CH_4 yield (g/kg DMI) (Beauchemin et al., 2008). These results were much higher than that shown by Grainger et al. (2010) who observed a 2.9% decrease ($P= 0.01$) in CH_4 production (g/d) over 12 weeks of supplementation of lipids (52.6 g/kg DM fat). Concurrently, a 10% increase ($P< 0.001$) in milk yield, 11% increase ($P<0.001$) in milk fat yield, and 31% increase ($P= 0.01$) in body weight gain were observed when lipids were supplemented to lactating dairy cows (Grainger et al., 2010). Persistence of CH_4 mitigation shown in this study suggests lipids could be a promising option for long-term use in cattle production (Grainger et al., 2010). Although these three sources of lipids produced approximately the same mitigation potential, the source of the lipid can also influence its CH_4 mitigation potential.

Dietary lipid source is influential on its mitigation potential, for example, when supplementing tallow, sunflower oil or whole sunflower seeds to supply an additional 3.3% added fat, tallow and sunflower oil reduced ($P< 0.001$) CH_4 production (% GE intake) by 15%, while sunflower seeds reduced ($P< 0.001$) CH_4 production by 25% (Beauchemin et al., 2007). However, when adjusted for DE intake all three sources reduced ($P=0.01$) CH_4 emissions by 15% (Beauchemin et al., 2007). Meanwhile, Eugéne et al. (2008) reported that regardless of the nature of the fatty acid, a mean decrease ($P=0.03$) in CH_4 emissions (MJ/d; 19.6 vs 17.9 MJ/d) of 9% was observed when expressed as MJ/d when supplemented to lactating dairy cows. However, regardless of source, lipid supplementation effects appear to lack any carryover following the cessation of treatment, suggesting that continued supplementation is required to maintain reductions (Muñoz et al., 2021). Another key consideration when feeding dietary lipids for CH_4 mitigation is the influence of lipid source and degree of hydrogenation.

Biohydrogenation is a process in which rumen microorganisms saturate dietary unsaturated fatty acids. The biohydrogenation pathway of unsaturated fatty acids competes with CH_4 and propionate production pathways for H^+, supplementing dietary unsaturated fatty acids is reported to be a viable option for decreasing H^+ availability to methanogens (Sun et al., 2022). Sun et al. reported that supplementing 3.04:1 ratio of n-6 to n-3 fatty acids significantly reduced CH_4 and propionate ($P<0.05$ and $P<0.01$) *in vitro* (Sun et al., 2022). While unsaturated fatty acids reduce CH_4 production, it is also important to note that unsaturated fatty acids are toxic to fibrolytic bacteria, protozoa and methanogens, and thus cause a reduction in organic matter fermentation (Beauchemin et al., 2008). While dietary manipulation is potentially useful for mitigating CH_4 emissions,

supplementation with other feed additives has been shown to produce higher mitigation potential.

2.7.3 Nitro Compounds

The reduction of nitrate (NO_3^-) to ammonium (NH_4^+) is thermodynamically favored over the generation of CH_4, shown by the differences in Gibbs free energy between the reactions. NO_3^- is reduced to nitrite (NO_2^-) in a reaction with a Gibbs free energy (ΔG) of -130 kJ. Next, NO_2^- is further reduced to form ammonium (NH_4^+) in a reaction with a ΔG of -371 kJ (Olijhoek et al., 2016). The reduction of NO_3^- to NH_4^+ has a lower ΔG compared to the reaction of CO_2 and H_2 to form CH_4, which has a ΔG of -67 kJ. The formation of NH_4^+ thus is energetically favorable for the available hydrogen, causing reduced CH_4 production in the rumen. An added benefit of nitro compound supplementation is that it is a useful source of non-protein nitrogen, replacing urea, and increasing the synthesis of microbial protein (Lee and Beauchemin, 2014).

When dietary nitrates were fed to beef steers and heifers, a linear decrease ($P< 0.001$) in CH_4 production from 491 L/d in control animals to 468, 424, and 396 L/d in animals receiving 5.3, 13.6, and 21.1 g NO_3^-/kg DM, respectively (Olijhoek et al., 2016). Nitrate toxicity is a concern of utilization of nitrates to reduce the production of CH_4 in ruminants. High nitrate supplementation has been associated with an increase in nitrate bound red blood cells and changes the Fe^{2+} that is normally associated with hemoglobin to Fe^{3+} to form methemoglobin (Lee and Beauchemin, 2014). Crawford et al. clearly represented the sigmoidal relationship between blood methemoglobin levels and nitrate

consumption by ruminants where a rapid increase in methemoglobin was observed when nitrate supplementation increased beyond 5 g/100 lbs BW (Lee and Beauchemin, 2014). Each 1% inclusion, nitrates decreased CH_4 yield (L/kg DMI) by 10.9%, in agreement with Van Zijderveld et al. (2011) which showed a 16% decrease in CH_4 production. Nitrates persistently decreased CH_4 production in lactating dairy cows over four consecutive 24-d periods (Van Zijderveld et al., 2011; Olijhoek et al., 2016). Nitrate supplementation decreased ($P< 0.001$) CH_4 production (L/kg DMI) by 10.9% per 1% nitrate inclusion by for 5-h after feeding, however, this period is associated with a quadratic increase ($P= 0.02$) in hydrogen emissions (L/kg DMI) (Van Zijderveld et al., 2011; Olijhoek et al., 2016). An increase in hydrogen emissions is commonly seen throughout CH_4 mitigation research due to the loss of CH_4 as a hydrogen sink, which causes a redirection of H_2 to other sources. Although NO_2 is typically reduced with hydrogen to form NH_4, the utilization of nitrates at high levels can exceed the capacity of rumen microbial NH_4 synthesis in the rumen (Lee and Beauchemin, 2014). Similar to Olijhoek et al., Van Zijderveld et al. did not observe any changes to animal health throughout the nitrate supplementation period. Although nitrates did suppress CH_4 production, the spared GE losses did not contribute to any significant increases in milk production or energy balance (Van Zijderveld et al., 2011).

2.7.4 3-Nitrooxypropanol

3-nitrooxypropanol (3-NOP) is a synthetic inhibitor of methanogenesis which binds the active form of methyl coenzyme M reductase (MCR), inhibiting methanogen

metabolism (Romero-Perez et al., 2015). 3-NOP supplementation at 150, 175, or 200 mg/kg DM to beef cattle rations containing 70% corn or barley silage and 30% steam-flaked barley grain silage diets decreased CH_4 production (g/d) by 20.1, 25.5. and 21.1%, respectively (Alemu et al., 2021). Similar to other methods of mitigation, and the inverse relationship between CH_4 production and NDF concentration, suggest that the effectiveness of 3-NOP is highly dependent on the neutral detergent fiber (NDF) content of the diet (Alemu et al., 2021). 3-NOP appears to be more effective in high-grain diets than high-forage diets due to the effects of starch content on pH (van Gastelen et al., 2022). Decreasing ruminal pH has an inhibitory effect on methanogens, in turn decreasing methyl coenzyme M reductase in methanogens (van Gastelen et al., 2022). Although DMI had a tendency to decrease (P=0.06; 8.07 kg/d vs 7.86), gain:feed tended to be improved (P=0.06) 2.5% with no negative impacts on animal health when 3-NOP was supplemented to beef cattle (Alemu et al., 2021). Methane yield decreased with increasing 3-NOP inclusion, and long-term studies suggest that extended use of 3-NOP may be a viable option, without a loss of efficacy (Hristov et al., 2015; Romero-Perez et al., 2015; Vyas et al., 2016; Vyas et al., 2018; Winders et al., 2018). Romero-Perez et al. (2015) concluded that 3-NOP decreased 16S rRNA of methanogens in the solid phase, however, it was less effective on methanogens in the liquid phase compared to those in the solid phase (P=0.98 vs P<0.01), suggesting that the effects of 3-NOP are phase dependent. Meale et al. (2021) observed 18 female Holstein x Montbéliarde cows receiving 10% 3-NOP along with their daily milk allowance through 14 weeks of age. When reported one-year post-weaning CH_4 emissions were 17.5% lower (P=0.002) in heifers that received 3-NOP. Concurrently, ruminal and fecal methanogenic archaea were

also reduced in response to 3-NOP, suggesting that methanogenesis can be influenced during early development using 3-NOP (Meale et al., 2021). Van Gastelen et al. (2022) observed the effects of 3-NOP included in a 70:30 (forage: concentrate; DM basis) TMR containing 59 mg 3-NOP for one year, including a lactation, 3-NOP did not significantly affect daily milk yield (26.4kg/d vs 27.5 kg/d) or total DMI (21.0 kg/d vs 20.8 kg/d). Pitta et al. (2022) reported a 30% reduction in CH_4 which was sustained for 15-weeks when supplemented at 60 mg/kg DM (0.006%) to lactating dairy cows. Concurrently, methyl coenzyme M reductase enzyme transcripts in rumen fluid samples was reduced 4, 30 and 14% during weeks 4, 8, and 12, respectively (Pitta et al., 2022). The ability of 3-NOP to reduce CH_4 emissions by 26-30%, while not impacting animal performance or health makes it a viable option for implementation into ruminant production systems (Pitta et al., 2022).

2.7.5 Tannins

Tannins are a plant based compound composed of repeating phenolic groups, which have the ability to form complexes with other nutrients including protein, starch, cellulose and minerals (Kelln et al., 2021). Tannins can be further classified based on structure and reactivity as either hydrolysable or condensed tannins. Hydrolysable tannins have a lower molecular weight making them more absorbable, however, this gives hydrolysable tannins a higher potential for toxic effects (Zhang et al., 2019). Condensed tannins have a higher molecular weight, and have been shown to have negative impacts on fiber and protein digestion due to the formation of complexes (Zhang et al., 2019).

Anti-methanogenic properties of tannins have been shown across many studies, however, the mechanism is not well understood.

One suggested mechanism is that tannins act directly on methanogens to reduce CH_4 production. However, it has also been suggested that tannins may act indirectly as an alternative hydrogen sink, reducing hydrogen availability to methanogens, thus decreasing CH_4 production. A third possible mechanism is that tannins decrease the nutrient availability to other microorganisms that produce the H_2 and CO_2 utilized as substrates for methanogenesis (Kelln et al., 2021). Regardless of the mechanism, a 2% inclusion of condensed tannins reduced ($P<0.01$) *in vitro* CH_4 emissions (14.9 mL/g DM to 10.0 mL/g DM) by 39.36% (Tan et al., 2011). *In vitro* tannin inclusion caused nearly complete elimination of 16S rRNA of methanogens in rumen fluid, suggesting that tannins may decrease CH_4 production via direct action on methanogens (Tan et al., 2011). In beef cattle consuming an alfalfa silage diet, Aboagye et al. reported a 9% reduction ($P= 0.07$) in CH_4 yield (g/kg DMI when gallic acid, a hydrolysable tannin, was included at 1.5% of diet DM to a high-forage alfalfa-based diet (Aboagye et al., 2019). However, two other hydrolysable tannins, tannic acid and chestnut did not significantly reduce CH_4 production (g/d) compared to the control, which suggests that the source of the tannin may influence its mitigation potential (Aboagye et al., 2019).

While tannins have the ability to form complexes that decrease the digestibility of nutrients, this influence appears to be related to the source of the tannin (Zhang et al., 2019). For example, A. *mangium*, a condensed tannin, decreased dry matter, neutral detergent fiber, acid detergent fiber and crude protein digestibility at higher rates than bayberry and valonia, two other tannin sources when supplemented at 3% DM (Zhang et

al., 2019). Concurrently, Valonia, a source of hydrolysable tannins, decreased dry matter, neutral detergent fiber and crude protein digestibility, however, only crude protein was significantly reduced ($P<0.01$) (Zhang et al., 2019). Significant decreases in crude protein digestibility ($P<0.001$) were also observed by Aboagye et al. when hydrolysable tannins of gallic acid, tannic acid and chestnut were supplemented to beef cattle consuming alfalfa silage (Aboagye et al., 2019). Concurrently, nitrogen excretion (% of total N excretion) in feces and urine increased (38.3% and 61.7% vs 31.7% and 53.5%), suggesting that the inclusion of tannins decreased protein degradation due to the formation of nutrient complexes causing increased excretion of nitrogenous products (Aboagye et al., 2019). *In vivo* use of tannins, regardless of source, did not impact milk production (kg/d), fat corrected milk FCM), energy corrected milk (ECM), or milk composition measures of fat, protein or lactose in multiparous Holstein cows (Zhang et al., 2019). Milk urea nitrogen (MUN) was significantly decreased ($P<0.01$) in all tannin treatments, suggesting a decrease in ruminal protein degradation, likely due to the formation of protein-tannin complexes (Zhang et al., 2019). Although many *in vitro* studies suggest that tannins are an option for future CH_4 mitigation, there is a lack of *in vivo* research, thus future research is required to evaluate health effects, and long-term efficacy of tannin use.

2.7.6 Saponins

Saponins are naturally occurring compounds found in legumes which are composed of a skeleton of at least four hydrocarbons with sugars attached. Depending on

their structure, saponins are either classified as steroidal or triterpenoid (Kregiel et al., 2017). The hydrophobic nature of saponins gives them the ability to form complexes with the hydrophilic lipid membrane of bacteria, thus, increasing the permeability of the cell (Ku-Vera et al., 2020). Following the formation of the membrane-saponin complex, cell ion balance is interrupted. Prolonged imbalance will eventually cause death of the microorganism and reduced microbial presence in the rumen (Ku-Vera et al., 2020). It is also suggested that saponins can form complexes with the sterols on the protozoal membrane surface, which causes the breakdown of the membrane surface and defaunation of the rumen environment (Ku-Vera et al., 2020). Due to the ecto-symbiotic relationship of protozoa and methanogenic archaea, meaning methanogens adhere to the surface of protozoa, defaunation contributes to reductions in enteric methanogenesis (Tan et al., 2020). The ecto-symbiotic relationship between protozoa and methanogens means both benefit from the relationship. As protozoa generate H_2 it is utilized by methanogens to generate CH_4, which in turn is beneficial to protozoa (Patra et al., 2016). Protozoa are sensitive to changes in pH, when pH is above 7.8 or below 5.0 protozoa are not able to survive, and Li et al. showed that the effects of saponins on protozoa are greater when pH of the rumen is lower (Clarke and Bauchopp, 1977; Li et al., 2009). A strong positive interaction was observed between protozoal numbers and CH_4 production, which suggests that methanogens depend on the ecto-symbiotic relationship to survive (Morgavi et al., 2010). Saponins have established ion gradient preference, selectively interrupting Ca^{2+} channels and Na^{+}-K^{+} ATPases which causes defaunation of the rumen (Ku-Vera et al., 2020). Although the mechanism of saponins appears to be selective inhibition of ion channels, it is important to consider the variability of saponin concentrations.

Concentrations of saponins in plants appears to be impacted by classification of saponin, plant type, stage of maturity and environment (Kregiel et al., 2017).

Triterpene saponins are typically found in dicotyledonous plants, such as beans and peas, whereas steroidal saponins are found in crops such as asparagus, yams and yucca (Kregiel et al., 2017). Grasses and cereal grains contain minimal saponins, however, there are some exceptions such as oats, which have the ability to be a source of both triterpenoid and steroidal saponins (Kregiel et al., 2017). Saponins cannot be found in all legumes, however, notable sources which generate CH_4 mitigation include tea, quillaja, and yucca. Yuan et al. reported an 8.5% decrease in CH_4 emissions when tea saponin was supplemented at 5 g/kg DM (Yuan et al., 2007). However, much higher CH_4 reductions ($P< 0.05$) were observed by Hu et al. which included 2, 4, 5, and 8 mg of tea saponin to 200 mg of corn and grass meal and observed a 13, 22, 25, and 26% decrease in *in vitro* CH_4 concentration, respectively (Hu et al., 2005). *In vitro* inclusion of Quillaja saponins at 30 and 45 g/kg of substrate DM reduced ($P< 0.01$) CH_4 production by 11.4 and 12.2%, respectively (Holtshausen et al., 2009). Concurrently, total gas production (mL/g of DM) and *in vitro* dry matter digestibility (IVDMD, %) was also reduced ($P< 0.01$) by both inclusions, suggesting a depression in digestibility due to the inclusion of Quillaja saponins (Holtshausen et al., 2009). To reduce the effects of Quillaja on digestibility, a subsequent study supplied the Quillaja saponin to a dairy cow TMR at 10 g/kg of DM, however, at this inclusion there was no reduction ($P= 0.42$) in CH_4 production (g/d) (Holtshausen et al., 2009). *Yucca schidigera* contains steroidal saponins which have been shown to reduce CH_4 production *in vitro* across a variety of diets (Xu et al., 2010). *In vitro* supplementation of *Yucca schidigera* yielded a dose response when

provided at 0, 2, 4, and 6 mL/L to rumen fluid from non-lactating Holstein cows (Pen et al., 2006). Inclusion of *Yucca schidigera* at 0, 2, 4, and 6 mL/L reduced ($P< 0.001$) *in vitro* methane production by 0.24, 0.20, 0.17, 0.14 mL/min (Pen et al., 2006). However, with Holtshausen et al. did not observe differences in CH_4 production (g/d), yield (g/kg DMI), or intensity (% of GE intake) in response to the inclusion of *Yucca schidigera* when supplemented at 10 g/kg DM (Holtshausen et al., 2009). Trotta et al. (2023) observed no changes in in-situ passage kinetics in response to the inclusion of Yucca or Quillaja saponins, which suggests that the influence of saponins is not due to changes in digestibility of nutrients. Researchers suggest that the mechanism of saponins is likely due to its effects on decreasing the presence of bacteria, methanogenic archaea or protozoa (Hristov et al., 1999; Pen et al., 2006; Trotta et al., 2023).

2.7.7 Bromochloromethane

Bromochloromethane (BCM), a halogenated CH_4 analogue, acts as an inhibitor of methyl coenzyme M reductase, a major step of methanogenesis, effectively reducing CH_4 production when fed to cattle. Bromochloromethane diffuses through cell membranes and targets the binding site of cofactor$_{430}$, causing inhibition of the enzyme (Duin et al., 2016). Cattle consuming ad libitum Rhodes grass and proprietary BCM containing pellets (0.3 g/100 kg live weight) twice daily had reduced ($P<0.05$) CH_4 emissions (mL/min) by 30% on average over 12-hr compared to control animals consuming the same diet (Denman et al., 2007). However, Tompkins et al. (2009) reported a 60% reduction ($P<0.05$) of CH_4 production (L/h) when cattle were supplemented to a grain-based diet at

the same rate for 90-d. Following 80-d of supplementation, no changes were observed in DMI or live weight gain (Denman et al., 2007; Tomkins et al., 2009). While BCM appears to be an effective CH_4 inhibitor, bromoform, another halogenated methane analogue, has caused almost complete mitigation of CH_4.

2.7.8 Bromoform Containing Macroalgae

Numerous approaches for mitigation of enteric CH_4 emissions have been evaluated, however, few have been as effective as macroalgae. Feeding ruminants brown, green, and red macroalgae has been shown to reduce emissions, although the resulting mitigation potentials have been variable. Brown macroalgae species have been shown to contain nearly 1,100 secondary metabolites (Wang et al., 2008).

While many species have been shown to have anti-microbial activity, brown species have been shown to be more effective against cellulolytic bacteria. When supplied at 20% of DMI, the brown macroalgae species E. *maxima* and L. *japonica*, reduced ($P= 0.017$) CH_4 yield (mL CH_4/ g DDM) by 18 and 21%, respectively (Ahmed et al., 2022). While brown macroalgae has been shown to be effective at mitigating CH_4 emissions, green macroalgae have been minimally investigated. In one of the few studies performed, *in vitro* testing of 0.2 g OM inclusion of the green macroalgae C. patentiramea reduced ($P< 0.0001$) CH_4 production (mL CH_4/ g OM) by 66.3% compared to the control diet (Machado et al., 2014). Differences in observed CH_4 reduction potentials between red, brown, and green are due to differences in the active compounds within each species, one of particular importance is bromoform. Red macroalgae species

Asparagopsis taxiformis and *Asparagopsis armata* have 1723 and 1320 mg bromoform/kg DM, higher than both brown and green species (Min et al., 2021). Although each of the species of macroalgae has been shown to have mitigation potential, red macroalgae has consistently generated the highest mitigation potential when supplemented to cattle.

Red macroalgae generates the largest reduction in CH_4 emissions, for example, Roque et al. reported a 99% reduction ($P< 0.05$) in CH_4 emissions *in vitro* (Roque et al., 2021). Red seaweed species, particularly those belonging to the *Asparagopsis* family have been the subject of recent research due to their enhanced ability to reduce CH_4 emissions *in vivo*. Red macroalgae have been shown to generate halogenated compounds, particularly bromoform (Roque et al., 2021). Similar to bromochloromethane, bromoform binds methyl coenzyme M reductase, inhibiting methanogenesis. While the *Asparagopsis* family of macroalgae contains many species, two species have been selected for their enhanced ability to reduce CH_4 emissions, *Asparagopsis armata* and *Asparagopsis taxiformis.*

When supplemented to dairy cows at 0.5 or 1.0% inclusion of A. *armata* (bromoform content not specified) Roque et al. (2019) reported a 26.4 and 67.2% reduction ($P<0.05$) in CH_4 production (g/d), respectively. Concurrently, the inclusion of the macroalgae was associated with a decrease in DMI at both levels, contributing to an 11.6% decrease ($P<0.05$) in milk yield and 6% decrease ($P<0.05$) in milk protein at 1% inclusion (Roque et al., 2019). However, another species of *Asparagopsis* has been shown to cause nearly complete elimination of CH_4 production (g/d) when included at 1% of ration DM, *Asparagopsis taxiformis*. Beef cattle consuming a TMR containing *A.*

taxiformis at 0.25 or 0.5% of ration DM had 50.6% and 74.9% lower ($P<0.05$) CH_4 production (g/d) when compared to beef cattle receiving no macroalgae (Roque et al., 2021). Concurrently, reduced CH_4 production was associated with a 14% reduction in DMI and a 15% increase ($P= 0.04$) in feed conversion efficiency (ADG/DMI) at 0.5% inclusion, suggesting a recapturing of the energy loss associated with CH_4 production (Roque et al., 2021). Dairy cattle consuming 0.5% DM A. *taxiformis* produced 34% less ($P< 0.001$) CH_4 (g/d), however, DMI decreased ($P= 0.006$) by 7% and milk yield decreased ($P=0.006$) 6.5% (Stefenoni et al., 2021). The reduction in DMI is likely the cause of decreased milk yield in dairy cattle consuming macroalgae. Concurrently, decreased CH_4 production was also associated with a 527% increase ($P< 0.001$) in hydrogen emissions (Stefenoni et al., 2021). Observed increases in hydrogen emissions are commonly seen when methanogenesis is inhibited, likely due to the shift away from CH_4 as a hydrogen-sink. Inclusion rates are variable among studies, ranging from 0.1-10% inclusion, however, typical inclusion rates are between 0.25 and 1% inclusion due to the observed impacts on digestibility and intake at higher inclusion rates. *In vitro* inclusion rates greater than 2% DM decreased total gas production, suggesting that digestibility was impeded (Kinley et al., 2016). *In vitro* VFA production also suggests that there is a shift in VFA proportions, resulting in higher propionate production, attributable to increased hydrogen pressure causing propionate to become an alternative hydrogen sink in the absence of adequate CH_4 production (Kinley et al., 2016).

Binding of the active site of MCR by bromoform, the active compound of A. *taxiformis,* also contributes to significant changes to the rumen microbiome. 16S rRNA gene sequencing showed a shift in archaeal populations from *Methanobrevibacter* to

Methanomethylophilaceae and a decreased abundance of *Prevotella* in the bacterial population when A. *taxiformis* was supplemented at 0.5% OM (Krizsan et al., 2023). Decreased *Prevotella* abundance observed by Krizsan et al. disagrees with other studies such as O'Hara et al. which observe increased *Prevotella* abundance when CH_4 emissions are reduced (Krizsan et al., 2023; O'Hara et al., 2023). *Prevotella* are a prominent gram-negative anaerobe which are believed to redirect H_2 to propionate during periods of CH_4 inhibition (Aguilar-Marin et al., 2020). However, Krizsan et al. observed decreased propionate concurrently with decreased Prevotella abundance, suggesting that this mechanism may not be correct (Krizsan et al., 2023).

Unlike ionophores, it appears that the effects of macroalgae species, such as *A. taxiformis*, persist when supplemented over an extended period of time. Roque et al. (2021) reported that 0.25 and 0.5% inclusion reduced ($P< 0.05$) CH_4 yield (g/kg DM) by 45 and 68%, respectively, and these reductions persisted for 147-d in beef cattle. While the use of macroalgae appears to be an effective strategy to reduce the impact of cattle on climate change, the persistence of the effects following cessation of supplementation remains to be determined.

The substantial reduction in CH_4 emissions observed at low inclusion rates makes macroalgae a promising area for future research, however, concerns have been raised regarding bromoform, the active compound found in the algae. When consumed in moderate amounts over an extended period of time bromoform can have mild to deadly effects on humans and animals (ATSDR, 2005). Individuals are most likely to encounter these compounds in drinking water, however, the concentration is typically between 1 and 10 ppb, which are not associated with health implications (Risher et al., 2005). The

effects of exposure to bromoform are based on the concentration and length of exposure, although substantial exposures are unlikely to occur. The main symptoms of bromoform exposure include temporary decreases in brain activity leading to a sedated sensation (Risher et al., 2005). The effects of bromoform exposure only persist for a brief time, however, repeated exposure can extend and worsen the effects. Residues from animals receiving bromoform have not been detected in tissues or milk at this time. With detection levels of 0.06 mg/kg, Roque et al. (2021) did not observe residues in longissimus dorsi muscle samples from cattle consuming *A. taxiformis* for 21 weeks. A similar study in dairy cows also concluded that macroalgae supplementation did not cause significant changes in bromoform concentrations in milk (Stefenoni et al., 2021).

In a recent study examining the effects of supplementing 1.26 mg bromoform/ kg DM to lactating dairy cows, Muizelaar et al. (2021) observed a visual absence of rumen papillae on a large portions of the rumen, and a thickening of the rumen wall. Histological examination showed reduced presence of ruminal papillae, the formation of blisters in the epithelial layer and the invasion of inflammatory cells in areas of micro-abscesses (Muizelaar et al., 2021). While bromform residues have not been detected in these tissues, it is important to consider other modes of storage and excretion including the liver, kidney, and adipose tissue. While both bromochloromethane and bromoform do not appear to be retained in tissues, volatilization of these compounds is associated with ozone depletion, leading many governments to limit or prohibit their use in animal feeds (Tomkins et al., 2009).

2.7.9 Stability of Halogenated Compounds

Delivery of bromoform in Asparagopsis has become a widely studied area for its potent ability to mitigate CH_4 emissions in cattle. However, stability of these compounds is vital to understanding their ability to be fed over time. Stefenoni et al. (2021) examined the concentration of $CHBr_3$ over four months when exposed to light and dark conditions as well as a range of temperatures and observed a 75 and 84% reduction ($P< 0.05$) in $CHBr_3$ concentration, in samples stored in dark and luminescent light conditions, respectively, regardless of temperature. However, Tan et al. (2023) observed a significant decrease in $CHBr_3$ concentrations when freeze-dried A. *taxiformis* samples were stored above 4 ºC during a 24-week period. When stored in open air (40 ºC), incubated at 40 ºC or 25 ºC, or refrigerated at 4 ºC, $CHBr_3$ concentration decreased ($P< 0.05$) 71, 74, 53, and 5.5%, respectively (Tan et al., 2023). Concurrently, samples stored at -20 ºC resulted in no notable changes in $CHBr_3$ concentration (Tan et al., 2023). Suggesting that samples should be stored at -20 ºC and exposed to minimal light in order to preserve maximum $CHBr_3$ concentration.

2.8 Mitigation Strategies Across Species

While cattle generate the largest proportion of CH_4, dietary strategies which aim to reduce CH_4 emissions generate variable results between species. Physiological and microbiological differences exist between ruminant species which contribute to the

variability in responses observed across literature. Thus, application of mitigation strategies across species without proper research should be exercised with caution.

2.8.1 Influence of Rumen Physiology, Level of Intake and Rumen Microbial Community

Daily CH_4 production from sheep is approximately 10% of that generated by a dairy cow, causes of these differences can be attributed to a variety of factors including level of intake, and rumen microbial community (Ulyatt et al., 2002). It is obvious that cattle consume more dry matter daily compared to sheep due to their larger body size and increased nutritional requirements. However, it has also been suggested that differences in rumen kinetics can also influence intake between sheep and cattle. For example, Colucci et al. (1989) consistently observed higher organic matter digestibility in sheep regardless of intake level or concentrate proportion of the diet compared to cattle, suggesting that digestive physiology between species is sufficiently different to cause observed differences in mitigation strategy effectiveness.

Rumen microbial populations have also been implicated for variation in CH_4 emissions, and because many mitigation strategies target the microbiome, understanding differences in the microbiome between cattle and sheep may also clarify the observed variation in mitigation potential. Henderson et al. (2015) suggest that diet and host are both influential on microbial structure of the rumen. When animals were separated by diet type (forage vs concentrate) and by species, Henderson et al. found similar abundance of *Ruminococcus*, however cattle had significantly lower relative abundance

of *Veillonellaceae*, and higher abundance of *Fibrobacter* compared to sheep, suggesting that species as well as diet contribute to differences in the microbiome (Henderson et al., 2015).

2.9 Techniques Utilized to Measure Gas Production

2.9.1 *In vitro* Incubation

In vitro rumen fermentation techniques have been used extensively to achieve simulated rumen conditions. There are two methods utilized when preparing *in vitro* vessels, batch culture *in vitro* techniques are prepared with fresh rumen fluid, and vessels remain sealed until termination of the experiment, while continuous culture *in vitro* involves maintaining fresh rumen fluid supplies over a longer period of time (Vinyard and Faciola, 2022). Batch culture methods are best to quickly estimate nutrient degradation and nutritional quality, while continuous culture systems allow for both long and short-term simulation of the rumen environment. Measurements of total gas production during incubation allow for the analysis of *in vitro* CH_4 production (Patra et al., 2016). The principle of *in vitro* measurement of gas production is that it relies on proper incubation of rumen inoculum with substrate while maintaining an anaerobic environment (Goopy et al., 2016). Bhatta et al. (2006; 2008) compared *in vitro* gas production values to values obtained using the sulfur hexafluoride (SF_6) and respiratory chamber technique and concluded that all three were comparable. While both batch and continuous culture *in vitro* fermentation methods are beneficial, each has its own

shortcomings. Batch cultures allow for the collection and measurement of fermentation end production, however, the accumulation of these products requires venting of vessels and additional buffer inclusion to limit the impact of pH on fermentation (Vinyard and Faciola, 2022). Due to the inclusion of buffers, batch culture methods may not be as applicable *in vivo* (Vinyard and Faciola, 2022). Continuous culture allows for steady state measurements of fermentation, however, it is difficult to measure protozoal counts and has the same limitations as batch culture when applying results *in vivo* (Vinyard and Faciola, 2022). Although there is disagreement about the applicability of results obtained *in vitro* to *in vivo* applications, this method provides rapid results and does not require the care and keeping of animals.

2.9.2 Estimation of Methane Production (Modeling)

Direct measurement of CH_4 emissions requires expensive equipment and significant labor input, however, modeling provides a method of estimating a predicted CH_4 output given intake and digestibility of the feedstuff(s) of interest. A number of models have been generated to predict gas production; however, emissions can be variable, meaning that model generated values are not always accurate. Tompkins et al. (2011) reported that modeling algorithms generated a 36% range in predicted CH_4 values. Models are typically classified as either statistical (static), which estimate CH_4 production from intake, or mechanistic, which predict CH_4 emissions using mathematical equations which aim to describe fermentation (Patra, 2016). While mechanistic models provide more accurate values of CH_4 emissions, they are more complex than static models, and

can only provide information for the population of animals in which they were originally designed in (Patra, 2016). Multiple equations have been published which attempt to predict CH_4 production in beef and dairy cattle, including the model published by Moe and Tyrrell, which states that CH_4 production (Mcal/d) is related to the amount of each carbohydrate fraction that is digested through the equation;

CH_4,Mcal/d= .439+.273 (kg digested NSC)+.512(kg digested H)+1.393(kg digested C); where NSC is the soluble fraction, H is hemicellulose, and C is cellulose (Moe and Tyrrell, 1979). This equation was established by reviewing 404 total energy balance trials of Holstein cows, where CH_4 production was measured during three or four consequtive 24-hr periods (Moe and Tyrrell, 1979). This equation has however been adapted for more accurate predictions which predict CH_4 production per unit of feed intake (gross energy; GE or DM), and appear to be more applicatble with less variability in input values (Ramin and Huhtanen, 2013). Ellis et al. (2007) evaluated a series of equations related to the prediction of CH_4 production in dairy cows and concluded that models that include dietary variables generate values with lower systematic error. In agreement with Niu et al, which reviewed an international database of models, concluding that those which included variables for DMI, NDF, EE, MF, and BW generated the most accurate estimates of CH_4 production (Niu et al., 2018). A mechanistic model generated by Mills et al. (2001) evaluates fermentation parameters and postruminal digestion to more accurately predict CH_4 production in dairy cows. Application of the model to experimental data collected from 67 calorimetry observations resulted in an root mean square prediction error of 12.4% and r^2=0.46 for the prediction of CH_4, suggesting that the model underpredicts CH_4 emissions (Mills et al., 2001). Modeling is associated with

errors, however, they are useful in determining predicted values. As research continues to advance our understanding of factors influencing CH_4 production, more complex models are being developed which increase reliability and decrease errors assocated with prediction of CH_4 production.

2.9.3 Portable Accumulation Chambers

Portable accumulation chambers (PAC) capture and measure exhaled CH_4 and CO_2, however, portable accumulation chambers lack airflow. The short-term use of PAC allows for the measurement of CH_4 at the end of a 1–2-hour collection period. Following collection, calculations yield a short-term, repeatable measurement of CH_4 emissions. The chamber material varies, however, regardless of design, the chamber will be airtight and equip with a portable gas analyzer probe which captures and transports gasses to a gas meter (Jonker et al., 2020). O'Connor et al. (2021) utilized a PAC for gas measurements in sheep over 17-d, consisting of 50 min gas collection periods for 48 ewes, and concluded that the measurements were moderately repeatable and had high precision when measurements were taken over three days. Inter-day variation between animals was 23% and between day was 39% (O'Connor et al., 2021). Following gas collection measurements, CH_4 concentration (ppm) within the chamber is converted to CH_4 production (L/d) using the following equation (Jonker et al., 2020):

CH_4 (L/d)=[((CH_4, ppm–background, ppm)/ 100,000) x (air vol. in PAC,L//min in PAC)]

Variation, repeatability, precision and accuracy were moderately repeatable outside of a research setting, suggesting that this method could be a viable option in some GHG research settings (Goopy et al., 2016).

2.9.4 Greenfeed System (C-Lock Inc.)

GreenFeed System, a product of C-Lock Inc. (Rapid City, SD, USA), is designed to measure flux of CH_4, CO_2, O_2, and H_2 from individual animals. This system provides bait, typically pelleted feed, to attract animals to the system. Each animal is equip with an RFID tag which is used to track the identity of the animal visiting the system. When the RFID is detected at the feeder, a predetermined amount of pelleted bait is dispensed, and gas collection begins. Following gas collection, the gases pass through a series of analyzers and measurements become available on an online computer interface. The objective of GreenFeed is to use multiple short-term measurements throughout the day to estimate, via calculation, average daily CH_4 emissions.

$$CH_4, L/min = C_{p(i)} \times ([CH_4]_{c(i)} - [CH_4]_{b(i)}) \times F_{air(i)} / 10$$

Where $C_{p(i)}$ is the fractional capture rate of air at time I, $[CH_4]_{c(i)}$ and $[CH_4]_{b(i)}$ are the concentrations (ppm) of capture gas and background gas, respectively, time I; and $F_{air(i)}$ is the volumetric air flow rate (L/min) at time i (Patra, 2016).

The GreenFeed system provides a method of measuring gaseous emissions in a variety of locations that would otherwise prohibit such activities, and minimizes errors associated with attempted replication of normal pasture feeding patterns. While utilizing

the GreenFeed system, proper training and adaptation may not be sufficient to ensure all animals visit the system. Also, compared to other methods including respiratory calorimeters and sulfur hexafluoride tracers, the GreenFeed has been shown to cause higher variance (Patra et al., 2016). McGinn et al. reported that CH_4 and CO_2 measurements made by the GreenFeed system differed by 1% and 2%, respectively, from those taken with respiratory calorimeters (McGinn et al., 2021). Respiratory calorimeters are considered the 'gold standard,' however, GreenFeed allows for remote measurement across a wider range of environments and generates comparable results compared to respiratory chambers, suggesting that the GreenFeed could be a viable alternative for situations that do not allow for the use of respiratory chambers.

2.9.5 Respiratory Calorimeters

Calorimetry chambers have been widely used to quantify energy balance and gaseous exchange (Patra et al., 2016). Respiratory chambers measure total emissions from all routes (respiratory and flatus) while many other methods capture only respiratory gas emissions (Garnsworthy et al., 2019). As gas exits the chamber, it is continuously sampled for analysis. Chambers also allow for measurements over several days allowing for capturing of 24-hour CH_4 fluctuations and diurnal variation (National Academies of Science, 2018). Respiratory chambers provide high accuracy and repeatability, however, this method can have potentially negative effects on intake and normal feeding activity, which can be minimized by properly training animals (Patra et al., 2016). Accuracy and

repeatability, as well as low animal-to-animal variation make respiratory chambers the 'gold standard' for measuring CH_4 emissions.

2.9.6 Sulfur Hexafluoride Tracer

The sulfur hexafluoride tracer (SF_6) has been extensively utilized to measure CH_4 emissions from ruminants. A series of assumptions have been validated and are thus key to using this method; 1) the release rate of the tube must be constant and predictable; 2) the tracer cannot have any influence on fermentation; 3) detection of the tracer must be attainable at low concentrations; 4) the tracer must be inert and cannot be toxic (Johnson et al., 2007). Once the SF6 gas production rate of the rumen has been determined, tubes are filled with SF_6 and placed into the rumen of animals. The tubing, which regulates the sampling rate is then connected to an evacuated canister which collects gas samples, typically over a 24-h period (Goopy et al., 2016). The concentration of SF_6 and CH_4 in the collected gas samples are then analyzed by gas chromatography. CH_4 emissions are then calculated using SF_6 release rate and the concentration of each of the gases in the container (Patra et al., 2016).

$$[CH_4], g/d = [SF_6], g/d \times ([CH_4]_c - [CH_4]_{b)}) / ([SF_6]_c - [SF_6]_{b)})$$

Where $[CH_4]_c$ and $[SF_6]_c$ are the concentrations of the gases in the container, while $[CH_4]_b$ and $[SF_6]_b$ are the concentrations of the gases in the background air (Patra, 2016). Unlike many other methods, SF_6 can be used to measure a large number of animals at one time across a variety of feeding scenarios, however, Muñoz et al. (2012) observed an 11% difference in CH_4 production measurements taken by SF_6 and respiratory

calorimetery. Over time this difference leads to an over estimation of CH_4 emissions when using SF_6 technique (Muñoz et al., 2012). The SF_6 technique also had a higher variation coefficient compared to respiratory chambers (16.4 vs 12.8%, respectively) (Johnson et al., 2007). Grainger et al. (2007) observed higher variability between days for the SF_6 tracer (CV=6.1%) than the respiratory chamber (CV=4.3%). Concurrently, animal-to-animal variation was also higher for the SF_6 technique compared to chambers (CV= 19.6 vs 17.8%) (Grainger et al., 2007). The SF_6 technique provides a method of evaluating gas emissions over large populations of animals, however, does have higher variation compared to respiratory chamber measurements.

2.9.7 Ventillated Hood Chambers

The University of Kentucky C. Oran Little Beef Research Facility (Versailles, KY) is equip with four head-box style respiratory chambers, which allow for respiratory gas collection and analysis. The respiratory chambers have been previously described by Koontz et al. (2009). In summary, each headbox is constructed of a stainless steel box with a plexiglass door at the front, and a large opening at the rear where the head of the animal is placed through a canvas shroud (Koontz et al., 2009). The opening is large enough to allow for movement, including lying, and the canvas sheath extends outward to secure comfortably at the base of the neck (Koontz et al., 2009). The secured sheath and negative airflow generated within the headbox prevent the respiratory gases from escaping the chamber (Koontz et al., 2009). Each headbox is also equip with a waterer, feeder, and an air conditioning unit to maintain a consistent temperature and humidity.

Air flow within the headbox is determined by mass flow meters (Columbus Instruments, Columbus, OH) while gas production measures are analyzed using gas meters. The headbox is also equip with software to analyze and supply gas production measures to a computer interface. Headbox systems are limited to the collection of respiratory gases, however, flatus only accounts for approximately 5% of ruminant gas emissions (Goopy et al., 2016; Doyle et al., 2019). Variability was minimal (0.1%) when comparing headbox style respiration chambers and respiratory chambers (Patra, 2016).

2.9.8 Polytunnel

Polytunnels are composed of a series of supports covered in polyethylene with a port for gas measurement, aiming at directly measuring ruminal CH_4 production under normal grazing conditions. The volume of air entering and leaving the tunnel is measured as well as the concentration of CH_4, temperature and humidity (Patra, 2016). Lockyer et al. (1995) measured 104% recovery of CH_4 when pumped into the tunnel. These findings suggest that the polytunnel provides sensitive and accurate measurements of CH_4 emissions (Lockyer and Jarvis, 1995). Similar to other methods there are errors associated with departing from "normal" environmental conditions (Lockyer and Jarvis, 1995). This method also depends on the availability of forages within the tunnel, causing decreased CH_4 production overtime, associated with the decline in forage availability and measurement period (Lockyer and Jarvis, 1995). While the polytunnel allows for recreation of a normal grazing environment, these results can be impacted by the inability to control environmental conditions and forage availability.

2.9.9 Micrometeorological

Micrometeorological methods of CH_4 emissions have been developed to provide emission measures from larger areas (i.e., farms, feedlots, etc.). By measuring the fluxes and concentrations of gases in the atmosphere and relating them to the emissions that are generated by the animals. This method cannot however relate those measures to individual animals which makes it difficult to use in mitigation research (Patra, 2016). CH_4 emissions of animals subjected to respiratory chamber and micrometeorological measurements in response to an antimethanogenic treatment yielded similar reductions (30.1 and 29.7 g/kg DM intake, respectively) (Tomkins et al., 2011). However, a 15% decrease in DMI was observed for animals in respiratory chambers, compared to those who's gaseous emissions were measured by micrometeorological techniques, suggesting a disruption in normal activity occurs when using chambers (McGinn, 2013). Concurrently, McGinn (2013) reported a 7-10% difference in CH_4 emissions between various micrometeorological techniques, suggesting that individual techniques can also influence results. Results obtained using this technique tend to be less precise than direct measurement methods, however, can provide insight into large scale gas production measures without altering feeding conditions.

2.10 Summary

While many methods of mitigation have been shown to generate significant decreases in CH_4 production, yield and intensity, macroalgae is at the forefront of

research due to its ability to nearly eliminate CH_4 production. The mechanism in which macroalgae inhibits the formation of CH_4 is believed to be connected to methanogen metabolism via secondary metabolites such as bromoform (Kinley et al., 2016). Bromoform inhibits the final step of methanogenesis effectively preventing the formation of CH_4, thus mitigating CH_4 emissions from cattle. Additionally, macroalgae has been shown to cause minimal impacts on animal production when included at less than 1% DM (Roque et al., 2021). Although there are concerns over potentially harmful bromoform residues, few studies have found levels above detection, suggesting that residues are not accumulating in tissues or being excreted in milk at harmful levels. Future research is needed to examine the persistency of CH_4 reduction in cattle as well as the accumulation of bromoform in other forms such as bromide (Br^-). Thus, the objectives of the current studies were to 1) examine the effect of kelp supplementation on growth performance, intake, and bromoform accumulation in organ, muscle, and adipose and 2) determine the inhibitory effect of kelp supplementation on CH_4 production and yield in growing steers.

CHAPTER 3. EFFECTS OF KELP CONTAINING BIOSYNTHESIZED BROMOFORM ON ENTERIC METHANE PRODUCTION IN GROWING CATTLE

3.1 Abstract

Twelve Holstein steers (160.5 ± 8.54 kg BW) were used in a randomized complete block design to test the hypothesis that feeding bromoform containing kelp reduces enteric methane production in cattle. Steers were blocked (n=4 per block) by body weight (BW) and randomly assigned to one of two treatments (n=2 per treatment within block). Treatments consisted of a ground corn carrier (control) or a ground corn carrier including kelp (10% of carrier). The biosynthesized bromoform containing kelp product was composed of 10.0 mg bromoform/g product, which was delivered at a rate of 0.5% of the total ration dry matter to the corn-silage based ration which was supplied at 1.5 times maintenance energy requirements. Animals were adapted to 1.5 times maintenance of the corn-silage based diet for 14-days prior to beginning kelp supplementation. Methane production and respiratory gases were measured for 72-hours in headbox-style respiratory chambers following seven days of treatment supplementation. No differences in DMI were observed when intake was expressed on a body weight basis (P=0.11). Methane production was reduced (P<0.001) by kelp treatment. When expressed as L/d methane production of control animals was 88.7 L/d, whereas kelp supplemented steers had a resulting methane concentration which was below the detection limits of the system (<2.2L/d). Oxygen consumption (P≥ 0.20) and CO_2 production (P ≥ 0.28) did not differ between treatments or blocks. The observed decrease in CH_4 production, without observed differences in CO_2 production or O_2 consumption, suggests that kelp

supplementation is effective at reducing CH_4 production without affecting DMI or whole animal oxidative metabolism.

Keywords: bromoform, methane, cattle, greenhouse gases

3.2 Introduction

Livestock production, particularly ruminant fermentation, has been implicated in global warming due to the emission of greenhouse gases (GHG). Carbon dioxide (CO_2) is the primary atmospheric GHG, however, when considering global warming potential, methane (CH_4), has a potential 25-times greater than that of CO_2 per kg. Methane production attributable to cattle is generated by enteric fermentation, which gives them the ability to generate up to 500 L of CH_4 per day (Sherwood et al., 2012). The 2020 EPA report estimates that livestock production accounts for approximately 12% of global GHG emissions (Hook et al., 2010). Methane is generated as a product of enteric fermentation, through the reduction of carbon dioxide. While a variety of mitigation strategies have been tested to reduce methane, few have been as effective as red seaweed (i.e., genus *Asparagopsis*). The addition of *Asparagopsis taxiformis* has been reported to reduce enteric methane production by 50.6-99% when included at 0.25-1.0% of DM to cattle rations (Roque et al., 2021; Stefenoni et al., 2021). The observed reductions in methane emissions observed with the inclusion of red macroalgae has been attributed to their ability to synthesize halogenated methane analogues, such as bromoform. Bromoform inhibits the metabolism of methanogenic archaea inhibiting methyl coenzyme M reductase, effectively reducing enteric methane production. Thus, the

objective of this study was to record the effects of kelp containing biosynthesized bromform on reducing enteric methane production in growing calves.

3.3 Materials and Methods

This experiment was conducted in an environmentally controlled, thermoneutral environment at the University of Kentucky C. Oran Little Beef Research Unit. This experiment was conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

3.3.1 Animal Management and Housing

Twelve Holstein steers (160.5 ± 8.54 kg BW) were used in a 14-d randomized complete block design experiment to test the hypothesis that kelp reduces enteric methane production. Prior to beginning the experiment, steers were vaccinated and backgrounded on a corn-silage based diet for a minimum of 14-d. Steers were housed indoors under thermoneutral conditions (22 °C) individually in 3m x 3m stalls and given ad libitum access to water via continuous water basins. A 14:10 light: dark cycle was established with lights turning on at 0600 h and off at 2000 h each day. During the methane measurement period, steers were moved within the same facility to metabolism stalls (1.25 m x 2 m) fitted with indirect calorimetry headboxes for 72 h of continuous measures of gas production. Animals were fed at 1.5 times the net energy requirements for maintenance of growing steers once daily at 0800.

3.3.2 Experimental Design

Steers were blocked (n=4 per block) by weight and randomly assigned to one of two treatments (n=2 per treatment within block). Dietary treatments (Table 3.1) consisted of a corn-silage based diet which was supplemented with either a ground corn carrier (control) or a ground corn carrier including kelp (10% of carrier; bromoform content= 10 mg/g product). Kelp was included in the supplement at a rate which supplied 0.5% of the total ration dry matter.

Kelp supplement was prepared by mixing the biosynthesized bromoform containing kelp with a ground corn carrier in a commercial stainless-steel chopper (Mandeville Company, Inc., Minneapolis, MN, USA) for 10 minutes followed by 10 seconds of pulse-blending (Waring MX1000XTX Extreme, Waring Commercial, McConnellsburg, PA, USA) until observational inspection produced a uniform distribution of kelp throughout the supplement. The control supplement containing only ground corn was subjected to identical procedures to ensure minimal differences in particle size existed between the two supplements. Supplements were prepared prior to each block and stored in sealed containers at 5 °C until fed (Rubbermaid Commercial Products, Sarasota Spring, NY, USA). Each supplement was top-dressed to the corn-silage based ration and hand mixed in the feed bunk. The basal diet was subsampled daily and composited weekly for dry matter analysis (55 °C, forced air oven). Similarly, any feed refusals were weighed, sampled, dried, and included in the calculation of DMI.

3.3.3 Enteric Gas Collection

Enteric methane production, as well as carbon dioxide production and oxygen consumption were measured by confining the animals in metabolism stalls fitted with stainless-steel headbox style indirect respiratory chambers. As previously described Kootz et al. (2009), each headbox style respiration chamber (0.90 m x 1.5 m x 0.60 m) is composed of stainless-steel with three plexiglass windows (.030 m x 0.60 m on sides and 0.90 m x 0.60 m on rear) and is equipped with a shroud to be placed at the base of the animal's neck (Koontz et al., 2009). Each respiration chamber is fitted with a continuous water basin to allow for ad libitum intake of water and a door at the rear of the chamber (0.90 m x 0.60 m) to allow for access to the feeder. Each chamber is built around a stall designed for intensive metabolic studies including an adjustable track to allow for extension of the bedding area (1.84-2.44 m x 1.68 m) which is covered in a rubber mat. Each respiration chamber is also equipped with an air conditioning unit to maintain temperature (21℃) and humidity (35%).

Prior to use, the zero point of O_2, CO_2 and CH_4 gas analyzers was calibrated with pure N_2 gas (American Welding & Gas, Lexington, KY, USA) and the span point of each gas analyzer was calibrated with a custom calibration gas (American Welding & Gas, Lexington, KY, USA) containing 19.90% O_2, 0.70% CO_2 and 0.065% CH_4 that was accurate within 1% tolerance. Each respiration chamber is validated by combusting propane over a 120 min period and determining the mass of propane combusted relative to the amount of CO_2 and O_2 measured within the respiration chamber.

Air flow was maintained at 300 L/min during measurements via flow controller (Flow Max XL; Columbus Instruments, Columbus, OH, USA). Air flow from each respiration chamber was measured by HFM-200 mass flow meters (Teledyne Hastings Instruments, Hampton, VA, USA) with vertically imposed LS-4F laminar flow elements (Teledyne Hastings Instruments, Hampton, VA, USA). Air from the chambers passed through a 10-channel expansion interface, and sample pump (0.5 L/min). Prior to entering analyzers, sample gases passed through sample drying tubes filled with desiccant indicator (W.A. Hammond Drierite Co., LTD, Xenia, OH, USA). Samples were then analyzed for O_2 via paramagnetic sensor (Columbus Instruments, Columbus, OH, USA), while CO_2 (Columbus Instruments, Columbus, OH) and CH_4 (VIA-510; Horiba Ltd., Kyoto, Japan) were measured via infrared sensors. Data was communicated to a computer interface through a CI-Bus Serial Interface (Columbus Instruments, Columbus, OH, USA) at 4-minute intervals each measuring background and chamber gas concentrations. Data are presented via computer interface using Oxymax software (version 4.8.5; Columbus Instruments, Columbus, OH, USA). To ensure animal safety during gas measurement periods, the computer interface and animals were monitored continuously to ensure CO_2 concentrations remained between 0.4-0.7% and power supply remained available. Following collection, O_2 consumption, CO_2 production and CH_4 production were calculated as total volume of gas consumed/produced during 24-hour increments between feedings and were averaged over the 72-h period. All openings of the headboxes were recorded and data measured during this time was excluded from calculations.

3.3.4 Statistical Analysis

Data were analyzed as a randomized complete block design using the GLM procedure of SAS (9.4, Cary, NC, USA). Treatment and block were included as fixed variables. Oxygen consumption and CO_2 and CH_4 production were totaled over each 24-h period and averages for each animal were included as dependent variables. Following collection from all blocks it was determined that CH_4 concentrations in expired air were below detection in the kelp group. To account for this maximal CH_4 production was estimated for this group using the lowest CH_4 concentration (2 ppm) in the exhaust air of control animals. Because of the absence of measurable variance for the kelp treatment group, the variance observed for the control treatment was used across treatments. DMI was analyzed separately within the treatment adaptation period and gas collection period. One steer assigned to the kelp treatment was removed from analyses due to illness and low DMI. Based on the subsequent observation and death two-weeks after the experiment, the illness was not considered related to the experimental treatment. Significance was set at $P \leq 0.05$.

3.4 Results and Discussion

3.4.1 Intake

The amount of feed offered was limited to 1.5 times the net energy required for maintenance of growing steers (Table 3.2). Accordingly, dry matter consumption was

nearly complete and exceeded 2.0% of body weight across treatments. Dry matter intake increased across blocks ($P<0.01$), and a slight reduction in intake was observed for kelp supplemented steers during the gas collection period ($P=0.04$). When DMI is expressed as a function of body weight, there were no significant differences between block ($P\geq0.10$) or treatment ($P\geq0.11$) during either time period. These results disagree with Roque et al. (2021) who observed a significant decrease (9.69 versus 11.3 kg/d; $P=0.04$) in DMI (kg/d) when kelp containing 7.8 mg bromoform/g dry weight (current study: 10.0 mg/g product), was supplemented at 0.5% of feed OM, compared to the control. However, the findings of this study agree with Kinley et al. (2020) who did not observe significant decreases in DMI when beef steers were supplemented kelp at 0, 0.05, 0.10, and 0.20% of OM (bromoform content: 6.55 mg/g product) to cattle consuming a high grain TMR. Differences in DMI between the present study and Roque et al. are not likely due to bromoform concentrations because the levels were higher in this study, thus, there was likely another factor influencing intake in that study.

3.4.2 Enteric Gas Production

Methane production was significantly reduced ($P<0.001$) by kelp treatment (Table 3.3). Steers consuming the control supplement exhibited the expected post-prandial increase in methane production (Figure 3.1), which resulted in a mean production of 88.7 L/d. In contrast, the CH_4 concentrations of exhaust air from steers consuming the kelp treatment were below detectable levels at all measured time points throughout the 72-h measurement period. For the purpose of statistical comparison, the lowest detectable CH_4

concentration was set at 2.2 L/d for the kelp group based on the set flow rate and minimum level of detection for the analyzers. Methane yield of control treatment animals averaged 18.96 g/kg of DMI compared to a maximum of 0.49 g/kg of DMI for kelp treatment animals. Roque et al. (2021) compared varying forage: concentrate ratio with the inclusion of kelp (0.0, 0.25, or 0.5% kelp; bromoform content: 7.35 mg/g product). When cattle were consuming a low forage diet supplemented with kelp, CH_4 yield decreased from 12.4 g/kg DMI in control animals to 3.75 and 2.50 g/kg DMI in 0.25 and 0.5% supplemented animals, respectively (Roque et al., 2021). Roque et al. (2021) observed a 50.6 and 74.9% reduction in methane production for 0.25% and 0.50% of diet inclusion, respectively, which is less than the 97% reduction observed for animals receiving 0.5% kelp in the current study. However, the bromoform concentration of Roque et al was 7.82 mg/g, compared to 10.0 mg/g in the current study, which may have contributed to the reduced effectiveness observed in that study (Roque et al., 2021). Oxygen consumption ($P \geq 0.20$) and carbon dioxide production ($P \geq 0.28$) did not differ between treatments or blocks. Li et al. reported that inclusion of A. *armata* at 3.0% OM (bromoform content= 0.384 mg/g) reduced CH_4 production by 80%. Higher inclusion levels of kelp in Li et al. (2018) are likely included in response to the lower concentration of bromoform in A. *armata* compared to other kelp sources. Beef steers consuming a high grain diet supplemented 0.2% OM A. *taxiformis* (bromoform content= 6.55 mg/g) generated a 98% less CH_4 (Kinley et al., 2020). The supplementation period for the Kinley et al. study was 90-d, which may have allowed for lower bromform inclusion with comparable results to this study (Kinley et al., 2020).

Bromoform, the active component in kelp, binds methyl coenzyme M reductase, which is an essential step of methanogen metabolism (De Bhowmick and Hayes, 2023). By binding the enzyme, catalysis required to form CH_4 cannot be completed, which decreases CH_4 production and methanogen presence in the rumen (De Bhowmick and Hayes, 2023). Alterations in the microbial structure in the rumen can cause detrimental changes to whole animal metabolism. However, due to the observed similarities between treatments for DMI, and thus energy intake between treatments, as well as no differences in CO_2 production and O_2 consumption, oxidative metabolism was unaffected by kelp supplementation.

3.5 Conclusion

Dietary kelp supplementation decreased CH_4 production and yield without affecting DMI or whole animal oxidative metabolism. This suggests that the inclusion of kelp may be a viable option for the mitigation of CH_4 emissions in cattle. Future research should focus on the recovery of CH_4 following the cessation of treatment, to elucidate recovery time of methanogenesis.

Table 3-1 Diet Composition (% DM basis)

	Treatments	
	Control	Kelp
Item	%, DM Basis	
Basal Diet		
Corn Silage	59.8	59.8
Distillers Dried Grain	27.1	27.1
Soybean Meal	5.8	5.8
Calcium Carbonate	1.6	1.6
Trace Mineral Mix	0.6	0.6
ADE Vitamin Premix	<0.1	<0.1
Topdress Supplement		
Ground Corn	5	4.5
Proprietary Kelp	0	0.5

Table 3-2 Effects of dietary treatment on dry matter intake of steers during adaptation and respiratory gas collection periods

	Treatment			P-value	
Item	Control	Kelp	SE[a]	Block	Treatment
Dry matter intake, kg					
Diet Adaptation	3.39	3.34	0.027	<0.01	0.20
Gas Collection	3.35	3.21	0.045	<0.01	0.04
Dry matter intake, kg/kg BW					
Diet Adaptation	0.021	0.021	0.0002	0.1	0.17
Gas Collection	0.021	0.020	0.0021	0.51	0.11

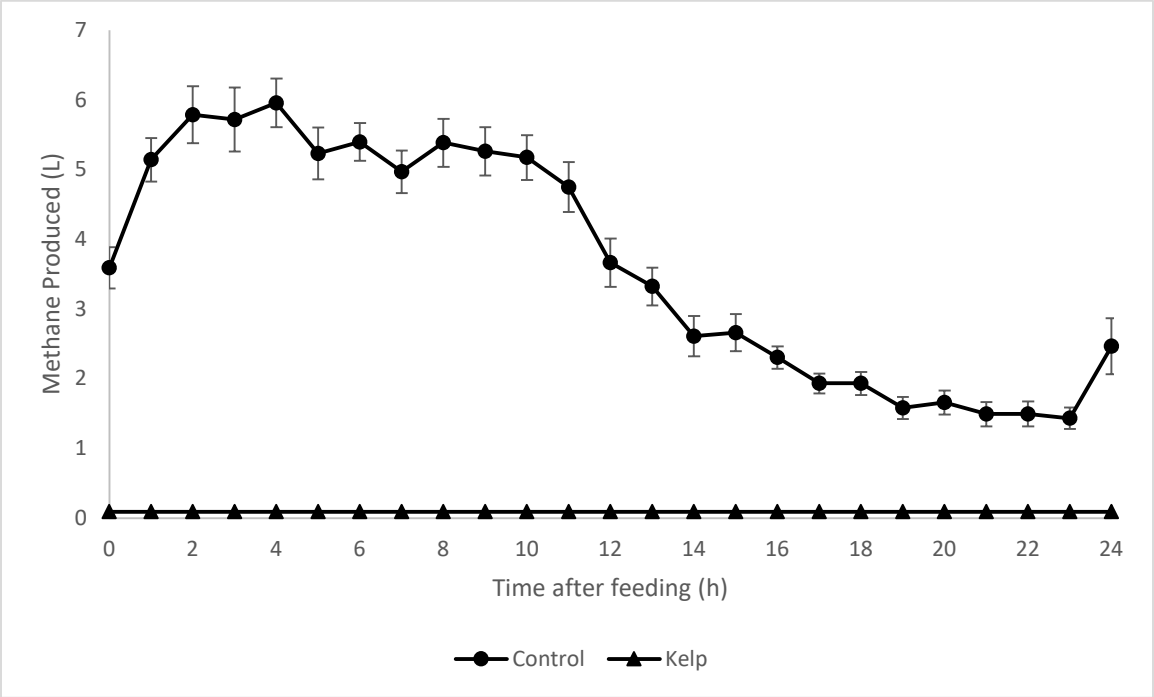
[a] Data are presented as least square means ± the standard error of the mean; n=6 and n=5 for the control and kelp supplemented treatment, respectively.

Table 3-3 Effects of dietary treatment on respiratory gas production (L/d) and oxygen consumption (L/d) in steers

	Treatment			p-value	
Item[a]	Control	Kelp	SE[a]	Block	Treatment
CH_4, L/d	88.7	<2.2[b]	7.5	0.33	<0.001
O_2, L/d	1473	1606	65.9	0.41	0.20
CO_2, L/d	1500	1595	60.8	0.28	0.31

[a] Data are presented as least square means ± the standard error of the mean; n= 6 and n= 5 for the control and kelp supplanted treatment, respectively.
[b]Estimated maximal production based on a sensitivity of 2 ppm.

Figure 3-1 Methane production during 24-hour measurements of steers



[a] Methane production during 24-hour measurements of steers consuming corn-silage based diet supplemented with either control (n=6) or kelp (n=5). Methane concentration in exhaust air and thus production, was below detectable limits for all time points in steers supplemented with kelp.

CHAPTER 4. DURATION OF EFFECTS OF KELP CONTAINING BIOSYNTHESIZED BROMOFORM ON ENTERIC METHANE PRODUCTION IN CATTLE

4.1 Abstract

Many mitigation strategies have been tested for their potential for reducing ruminant livestock CH_4 production. Bromoform, the active compound of kelp has been shown to generate the greatest mitigation potential when included at 1% in ruminant diets. In Chapter 3, the inclusion of kelp at 0.5% of DM reduced CH_4 production to levels below detection. Thus, this study was conducted to investigate the duration of the reduction in CH_4 emissions in response to the inclusion of kelp containing biosynthesized bromoform (2 mg bromoform/ g product) in growing cattle. Twelve Holstein steers with an average initial BW of 342 ± 10.5 kg were randomly assigned to treatments (control, 0.5% and 1.0% DM inclusion of kelp) for a 17-d methane recovery experiment. A significant treatment x day interaction was observed for CH_4 production (L/d; P=0.0003) and CH_4 yield (g/g DMI; P=0.0002). A cubic response was observed for the 0.5%, and a quadratic relationship was observed for the recovery of 1% supplemented steers (Figure 4-2 and 4-3). On each day of the gas collection period, 1% treated steers had lower CH_4 production ($P \leq 0.06$) compared to 0 and 0.5% treatment groups which did not differ ($P > 0.10$). Steers supplemented with 1% kelp reached maximum recovery by day 4 of the gas collection period, after which CH_4 plateaued at approximately 75% of control values for the remaining 6 days. Oxygen consumption (L/d; P=0.0091) and CO_2 (L/d; P=0.0043 and L/kg BW; P=0.0217) were significantly reduced by the 1% treatment (Figure 4-4), however RQ was unaffected by treatment (P=0.7678). The results of this study suggest that the use of bromoform for the reduction of CH_4 is effective for greater than 10 days

after cessation of treatment when kelp was supplemented at 1% of DM (Figure 4-2 and 4-3).

4.2 Introduction

Due to the importance of livestock in climate change, cattle CH_4 production has become a particular focus of research. It is also important to note that the generation of CH_4 is also tied to a 2-12% loss of gross energy intake (GEI) which results in a loss of productivity in cattle. Although CO_2 is the primary atmospheric greenhouse gas, CH_4 is of particular concern because it is estimated to have 25 x the global warming power of CO_2, making it much more effective at trapping heat within the atmosphere (EPA, 2022). Previous research has shown that bromoform supplementation can reduce enteric CH_4 production by greater than 97%. Bromoform is a competitive inhibitor of methyl coenzyme M reductase (MCR), an essential step in methanogenesis (De Bhowmick and Hayes, 2023). When MCR is inhibited, the formation of CH_4 is blocked, and methanogen metabolism is halted, causing death of methanogenic bacteria (De Bhowmick and Hayes, 2023). Due to the slow regeneration time of methanogens (i.e., the target of bromoform containing products), it is likely that the reduction in CH_4 is sustained for a period of time after cessation of supplementation. If it is determined that the kelp maintains reduced CH_4 levels, it would reduce costs associated with administering bromoform as well as exposure to bromoform. Chronic exposure to bromoform has been associated with increased risk of health complications (Risher et al., 2005). Thus, this experiment was

designed to determine the duration of the inhibitory effects of bromoform on CH_4 production and yield in growing steers.

Keywords: bromoform, cattle, methane recovery

4.3 Materials and Methods

This experiment was conducted in an environmentally controlled, thermoneutral environment at the University of Kentucky C. Oran Little Beef Research Unit. This experiment was conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

4.3.1 Animal Management

Twelve Holstein steers with an average initial body weight of 342.35 ± 10.45 kg were housed individually under thermoneutral conditions. Each stall was equipped with a feed bunk and automatic waterer which allowed for ad libitum access to water for a 17-d study including a respiratory gas collection period (Figure 4-1). During d-14 through d-1, steers were adapted to their basal diet plus the control supplement. On days 0- 10 steers were fed the basal diet with their experimental treatments (either 0, 0.5, or 1% kelp). On d 8, after consuming their daily allotment of feed, steers were transferred into headbox style respiratory calorimeters for d 8-17. Steers were fed assigned experimental treatments through daily feedings on d 10. Starting on the morning of d 11 steers received control supplement, marking the start of the recovery period (Figure 4-1).

4.3.2 Experimental Design

During d 0 to 10 each period, steers received the basal diet plus their appropriate treatment supplement, formulated to supply 0, 0.5, or 1.0% of kelp in the total ration (DM basis; bromoform content= 2 mg/g product). Beginning on d 11, and through the remaining portion of the experiment, all steers were switched back to the control supplement to measure residual effects of the treatment. Regardless of formulation, each supplement was top dressed at a rate of 5% of the ration DM. Supplement was prepared prior to the beginning of each experimental period by mixing kelp with a ground corn and distillers dried grain carrier in a commercial stainless-steel chopper (Mandeville Company, Inc., Minneapolis, MN, USA) for 10 minutes until uniform distribution of kelp was achieved (Table 4.2). Following mixing, top dress was stored at -20 ºC in plastic containers (Rubbermaid Commercial Products, Sarasota Spring, NY, USA) with a plastic cover and snap on lid. A corn-silage based diet was top dressed and fed at 1.5-times the net energy requirement for maintenance to each animal once per day at 0800. The diet ingredients were subsampled daily and composited weekly for DM analysis (55 ºC forced air oven). For each collection, a 200 g sample (in duplicate) was collected, weighed and dried for DM analysis. Feed refusals were collected daily, weighed, subsampled (duplicate 100-200 g samples) and dried for DM analysis. Dry matter was determined by weighing samples daily until sample weight remained constant for two consecutive days. Values for refusals were used in calculations for DMI. Animals were weighed on d -7 and d -1, weights for d -1 were used to set intake for each steer for the remaining experiment.

4.3.3 Respiratory gas measurement

Methane and CO_2 production and O_2 consumption were measured by moving steers within the same facility to metabolism stalls that had been fitted with stainless steel, headbox style indirect respiratory chambers. As previously described by Koontz et al. (2009) each headbox style respiration chamber (0.90 m x 1.5 m x 0.60 m) was composed of stainless-steel with three plexiglass windows (.030 m x 0.60 m on sides and 0.90 m x 0.60 m on rear) and a shroud which was secured at the base of the animal's neck (Koontz et al., 2009). Each respiration chamber was fitted with a continuous water basin which allowed for ad libitum intake of water. A door at the rear of the chamber (0.90 m x 0.60 m) allowed for access to the animal during feeding. Each chamber was designed around a stall designed for intensive metabolic studies including an adjustable track to allow for extension of the bedding area (1.84-2.44 m x 1.68 m) which was covered in a rubber mat. Each respiration chamber was also equipped with an air conditioning unit to maintain temperature (21 ºC) and humidity (35%).

Prior to use, the zero point of O_2, CO_2 and CH_4 gas analyzers was calibrated with pure N_2 gas (American Welding & Gas, Lexington, KY, USA) followed by setting of the span point of each gas analyzer with a custom calibration gas (American Welding & Gas, Lexington, KY, USA) containing 19.90% O_2, 0.70% CO_2 and 0.065% CH_4 that is accurate within 1% tolerance. Prior to beginning the experiment, the recovery for each respiration chamber is determined by combusting lab grade propane gas (99% purity) over a 120 min period followed by determining the mass of propane combusted relative to the amount of CO_2 and O_2 measured within the respiration chamber. All recoveries were > 94% prior to beginning experiment.

Air flow was maintained at 600 L/min during measurements via flow controller (Flow Max XL; Columbus Instruments, Columbus, OH, USA). Air flow from each respiration chamber is measured by HFM-200 mass flow meters (Teledyne Hastings Instruments, Hampton, VA, USA) with vertically imposed LS-4F laminar flow elements (Teledyne Hastings Instruments, Hampton, VA, USA). Air from the chambers passed through a 10-channel expansion interface, system sample pump (0.5 L/min) and sample drier prior to entering each of the gas analyzers. Prior to entering analyzers, sample gases pass through sample drying tubes filled with desiccant indicator (W.A. Hammond Drierite Co., LTD, Xenia, OH, USA) to ensure that minimal water enters the gas analyzers. Samples are then analyzed for O_2 via paramagnetic sensor (Columbus Instruments, Columbus, OH, USA), CO_2 (Columbus Instruments, Columbus, OH) and CH_4 (VIA-510; Horiba Ltd., Kyoto, Japan) via infrared sensors. Data is communicated to a computer interface through a CI-Bus Serial Interface (Columbus Instruments, Columbus, OH, USA) at 4 or 8-min intervals each measuring background and chamber gas concentrations. Data are presented using Oxymax software which provides a series of gaseous measurements (version 4.8.5; Columbus Instruments, Columbus, OH, USA). The Oxymax program allows for respiration chambers to be excluded from sampling, and due to the design of this experiment only three of the four available headboxes were used throughout the experiment. To ensure animal safety during gas measurement periods, the computer interface and animals were monitored continuously to ensure CO_2 concentrations remained between 0.4-0.7% and power supply remained available. This range has been established based on the sensitivity of the analyzers, and respiratory values which minimize the effects of the headbox environment on animal health.

Following collection, O_2 consumption, CO_2 production and CH_4 production were calculated as total volume of gas consumed/produced during 24-hour increment between feedings and were averaged over the 72-h period. All openings of the headboxes were recorded and data measured during this time is excluded from calculations. Openings are restricted to feeding and occur following a sample timepoint from the headbox being opened.

4.3.4 Statistical Analysis

Data was analyzed using the MIXED procedure of SAS v 9.4 with a repeated measures model to account for autocorrelation within animal measurements A first order autoregressive correlation structure was assumed. Linear, quadratic, and cubic contrasts were included for both time and linear and quadratic contrasts included for treatment. Treatment means on specific days were also separated using Fishers LSDs through use of the LSMEANS diff option. Response variables included 1) DMI, 2) CH_4, 3) O_2 and 4) CO_2. Significance was set at $P \leq 0.05$.

4.4 Results and Discussion

4.4.1 Intake

The amount of feed offered was limited to 1.5 times the net energy required for maintenance of growing steers. Due to this, all feed was consumed throughout the entire adaptation and respiratory gas collection period. Similar to our earlier study as well as

Kinley et al. (2020), intake was not altered due to supplementation of kelp to the diet of growing steers when bromoform containing kelp was (6.55 mg/g product) included at 0.05, 0.10, or 0.20% of OM.

4.4.2 Methane and Respiratory Gases

A treatment x day interaction ($P \leq 0.0003$) was observed for both enteric CH_4 production (L/d; Table 4-3 and Figure 4-3) and yield (g/g DMI; Table 4-3 and Figure 4-2). Steers consuming kelp at the 0.5% inclusion rate had similar ($P \geq 0.3$) rates of CH_4 production as those consuming the control diet throughout the measurement period. In contrast, CH_4 production was lower ($P \leq 0.01$) for steers consuming the 1% kelp treatment compared with those fed control and 0.5% kelp throughout the measurement period; except for d 8 and 9 where a tendency ($P \leq 0.1$) for lower production was observed for the 1% kelp treatment. Similarly, CH_4 yield throughout the measurement period was lower ($P \leq 0.03$) for steers consuming 1% kelp treatment compared with those receiving the control or 0.5% treatment. However, CH_4 yield was similar ($P \geq 0.2$) at all time points for steers receiving the 0.5% and control diets.

On d 1 and 2 of the gas measurement period steers overlaps with the final days of kelp supplementation, whereas on d 3 through 10 all steers received the control supplement. Therefore, the initial two days of the measurement period represent the impact of kelp supplementation on CH4, whereas the remaining time points reflect post-kelp supplementation, or a recovery period. Methane production during the supplementation period suggests that although the bromoform concentration of this study

was lower (2 mg/g vs 10 mg/g in the previous study), 1% inclusion completely eliminated CH_4 production after 8 d of supplementation. The reduction in bromoform concentration in the supplement from the previous study likely contributed to the lack of response observed in the 0.5% inclusion treatment. Other studies have shown that high bromoform inclusion can cause decreased DMI, however, changes in DMI were not observed when kelp containing 2 mg bromoform/g product was supplemented at 1% of DM. Methane production and yield was unaffected ($P \geq 0.08$) by d of measurement for the control treatment. Steers consuming the 0.5% kelp treatment exhibited a cubic ($P=0.02$) response over time for both CH4 production and yield. This response is largely reflective of a slight increase from d 1 and small inflection points at d 5 and 6 and 10. A quadratic response ($P<0.0001$) over time was observed for steers receiving 1.0% kelp, where CH_4 production and yield increased from d 2-4, followed by a plateau through d 10 of the recovery period. The plateau for both CH_4 production and yield was approximately 75% of the value for control and 0.5% kelp treatment. To our knowledge, this is the first data available on recovery of CH_4 following bromoform supplementation. The active compound of kelp, bromoform, targets methyl coenzyme M reductase (MCR), which interrupts the metabolism of methanogens causing cell death (Krizsan et al., 2023). Methanogens are also known to be slow growing, thus, substantial reductions in methanogen presence in the rumen should take an extended period of time to recover (Buan, 2018). 3-nitrooxypropanol (3-NOP), an antimethanogenic compound is believed to have a similar mechanism as bromoform, via targeting methyl coenzyme M reductase (MCR) (Duin et al., 2016). Romero-Perez (2015) investigated the potential of 3-NOP to sustain mitigation following 112 days of 3-NOP supplementation (2g/d) to a total mixed

ration (60% barley silage, 35% barley grain, 5% vitamin-mineral supplement. Following 112 days of supplementation CH_4 production was reduced 59.2%, although there were no residual effects of 3-NOP during the recovery period (Romero-Perez et al., 2015). These results differ from the findings of this experiment which show sustained reductions in CH_4 following bromoform supplementation, however, the reason for the differences in findings is unclear. These results suggest that kelp supplementation may not be needed daily. Thus, research should focus on developing a protocol of repeated delivery of bromoform, that does not require daily supplementation to maintain maximum effectiveness against CH_4 production. Future studies are also needed to focus on bromoform effectiveness at reducing CH_4 production may be altered by inclusion in scenarios which allow for commingling of cattle which can contribute to bacterial exposure which would allow for reinoculation of the rumen.

A quadratic response was observed for O_2 consumption (L/d) in steers supplemented 1.0% kelp (P=0.05). While CO_2 production (L/d) was quadratically reduced in steers supplemented with 1.0% kelp (P=0.004). The same trend was observed for CO_2 production when expressed on a body weight basis (P=0.08), while O_2 consumption (L/kg BW) was linearly reduced (P=0.04). A reduction in CO_2 and O_2 to the extent observed in this study suggests that a depression in digestibility was observed. However, in preliminary unpublished in-vitro studies, VFA production was not affected by the inclusion of kelp, suggesting that a depression in digestibility did not occur. These findings are similar to Terry et al. (2022) who observed no treatment effects on total VFA in response to the supplementation of kelp at 0, 1, and 2% to heifers consuming barley silage and barley straw (52 and 44%). Concurrently, a linear decrease (P=0.002) in total-

tract DM digestibility was observed, but no changes in organic matter digestibility occurred (Terry et al., 2023). Roskam et al. (2022) observed a decrease in total VFA production (mM/d) from 91.16 to 73.14 mM/d in vitro when *A. taxiformis* (P=0.01) was included at a rate of 10g/kg DM (bromoform content=4.35mg/g). Although there was a reduction in total VFA production, there were no changes observed in DM disappearance, or OM components (Roskam et al., 2022). However, a curvilinear effect on *in vitro* OM digestibility (P=0.043) was observed when *A. taxiformis* was supplied across 25 studies, while *in vitro* digestibility of crude protein was linearly decreased (P=0.029) with a decrease in gas production when red macroalgae species were supplied compared to brown (Sofyan et al., 2022). Differences in gas production between red and brown macroalgae species is due to the availability of active compounds such as bromoform (Sofyan et al., 2022). Respiratory quotient (RQ) was not affected by treatment (P≥0.1). O_2 consumption (L/kg BW) was different from control and 0.5% treatment on days 7-10, whereas CO_2 production (L/kg BW) in 1.0% supplemented steers was statistically different from control and 0.5% supplemented steers on d 2, 3, 5, 6, and 7 (P≤0.05). The observed differences in CO_2 production in this study were not consistent with Roque et al. who observed no changes in CO_2 production (g/d) or intensity (g/kg ADG) when supplemented *A. taxiformis* at 0.5% DM (bromoform content= 7.82 mg/g) while consuming high, medium, or low forage diets (P≥0.7) (Roque, 2021). Differences between the findings of this study and Roque et al. (2021) are likely due to decreases in DMI which were not observed in this study. Without changes in DMI it would be expected CO_2 production to increase as CH_4 production decreased, however, this was not

observed in this study, rather we observed a concurrent decrease in CO_2. At this time the reason for decreasing CO_2 production observed in this study is not clear.

4.5 Conclusion

1% supplementation of biosynthesized bromoform containing kelp reduced CH_4 production and maintained lower CH_4 production throughout the 10-d period following cessation of supplementation. Although changes were observed in CO_2 production and O_2 consumption (L/d), and in O_2 consumption (L/kg BW) at 1% supplementation, no changes were observed in respiratory quotient, thus, at this time we do not believe that the observed changes are detrimental to the animals. No changes in DMI were observed, all animals consumed 1.5 times the determined net energy of maintenance throughout the experiment, so kelp supplementation does not appear to impact intake.

Table 4-1 Diet and Topdress Composition

	Treatment		
	0	0.5	1
Item[a]	% DM Basis		
Corn Silage	50.0	50.0	50.0
Cracked Corn	20.0	20.0	20.0
Soybean Meal-49	10.0	10.0	10.0
UKARC Generic Supplement	10.0	10.0	10.0
Top dress			
Distillers Dried Grains	5.0	5.0	5.0
Ground Corn	5.0	4.5	4.0
Kelp	0.0	0.5	1.0

Table 4-2 Topdress Composition

	Treatment		
Item[a]	0	0.5	1
Distillers Dried Grains	50	50	50
Ground Corn	50	40	30
Bromform Product	0	10	20

[a] Data represents percentages of each ingredient within the topdress. Kelp was mixed at 0, 10 and 20% within the topdress and added to the ration at 5% of ration DM to provide 0, 0.5, or 1% bromoform product.

Table 4-3 Effects of bromoform supplementation on CH_4 and CO_2 production, O_2 consumption and RQ

	Treatment				p-value			
Item[a]	0	0.5	1.0	SE	Linear	Quadratic	Day	Trt x Day
Initial BW, kg	359	354	314	10.5	-	-	-	-
DMI, kg/d	6.6	6.6	6.7	-	-	-	-	-
CH_4, L/d	187	193	106	10.7	0.007	0.03	<0.0001	0.0003
CH_4, g/g DMI	21.4	21.6	12.1	0.8	0.0009	0.007	<0.0001	0.0002
O_2, L/d	3055	3033	2473	81.2	0.006	0.05	0.30	0.53
CO_2, L/d	3099	3077	2495	75.2	0.003	0.004	0.38	0.56
O_2, L/kg BW	8.5	8.6	7.9	0.2	0.04	0.15	0.29	0.49
CO_2, L /kg BW	8.6	8.7	7.9	0.1	0.02	0.08	0.25	0.50
RQ	1.01	1.02	1.01	0.01	0.57	0.69	0.11	0.21

[a] Data are presented as least squares means ± the standard error of the mean; n=2 for control, n=2 for 0.5% kelp, n=2 for 1% biosynthesized bromoform product. %

Figure 4-1 Experimental Timeline

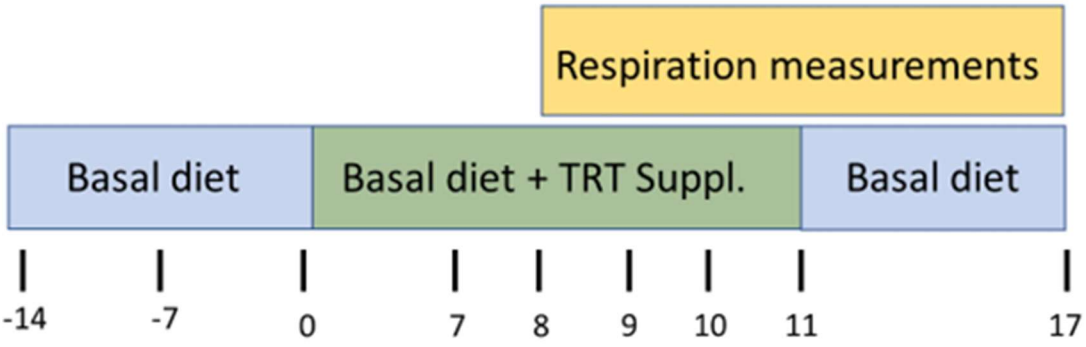
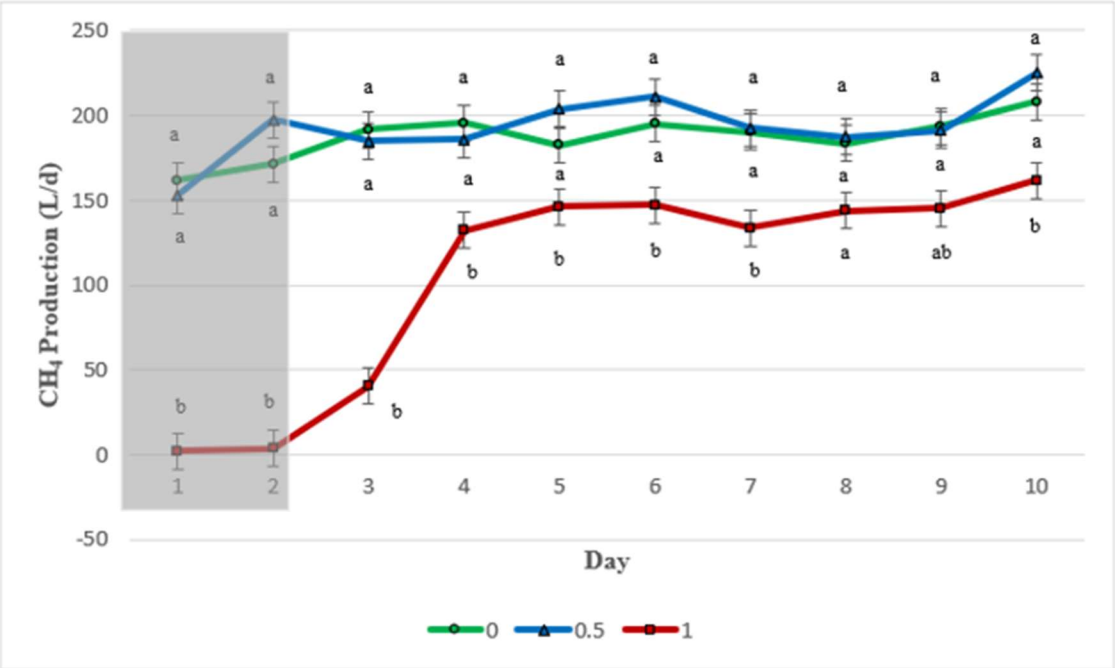
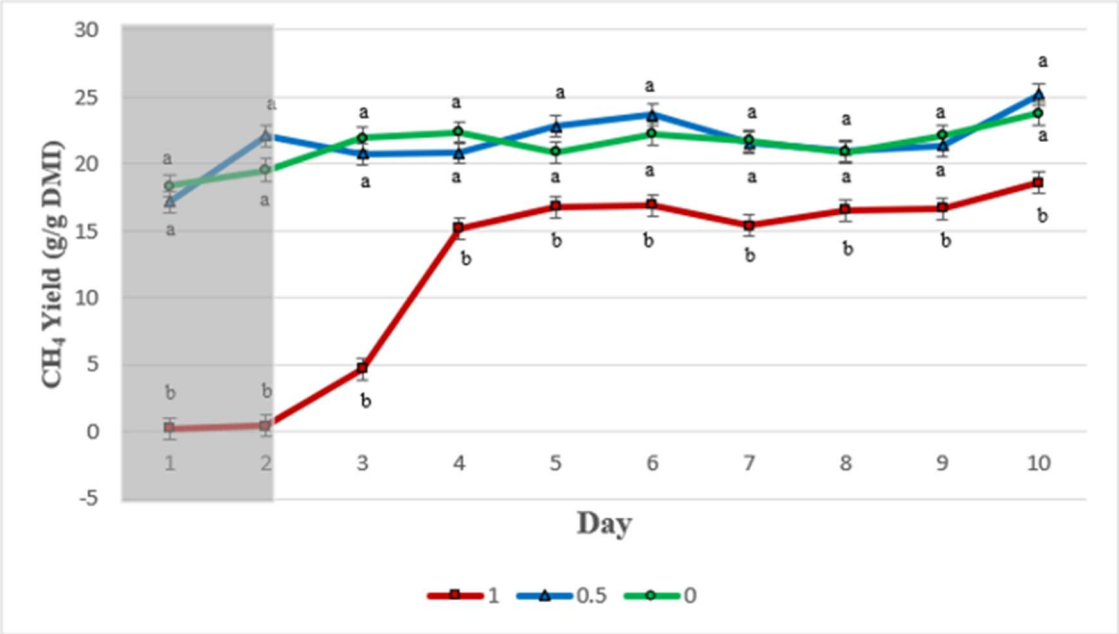


Figure 4-2 Effects of Bromoform Supplementation on CH_4 yield (L/d)



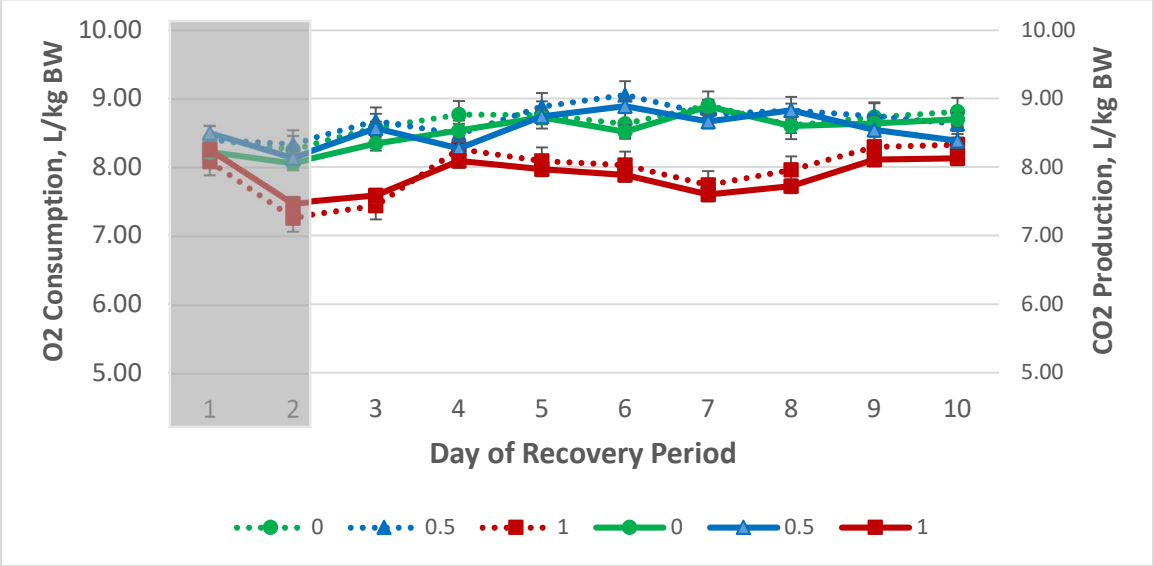
[a] Daily CH_4 production of steers consuming corn-silage based diet supplemented with either control (n=2), 0.5% kelp (n=2), or 1% kelp (n=2). Treatment x Day interactions were observed (P=0.0003). No effect of time was observed for the control (P>0.10), however, cubic (P=0.02) and quadratic (P<0.0001) were observed for 0.5 and 1% inclusion, respectively. Differences between LSMEANS within day are represented with different super scripts (P≤0.05). Steers given the 1% treatment were different from control throughout the recovery period with the exception of d 8, on d 9, the 1% treatment is different from the control but not from the 0.5% treatment. Shaded area represents overlap of treatment period and respiratory gas collection period and are used to establish baseline measures for the effectiveness of kelp supplementation.

Figure 4-3 Effects of Bromoform Supplementation on CH_4 production (g/g DMI)



[a] Daily CH_4 yield of steers consuming corn-silage based diet supplemented with either control (n=2), 0.5% kelp (n=2), or 1% kelp (n=2). Treatment x Day interactions were observed (P=0.0002). No effect of time was observed for the control (P>0.10), however, cubic (P=0.02) and quadratic (P<0.0001) were observed for 0.5 and 1% inclusion, respectively. Differences between LSMEANS within day are represented with different super scripts (P≤0.05). 1% treatment is different from control on d 1-10. 0.5% treatment is not different from control throughout recovery period. Shaded area represents overlap of treatment period and respiratory gas collection period and are used to establish baseline measures for the effectiveness of kelp supplementation.

Figure 4-4 Effects of Bromoform Supplementation on CO_2 production and O_2 consumption (L/kg BW)



[a]Daily CO_2 production and O_2 consumption of steers consuming corn-silage based duet supplemented with either control (n=2), 0.5% (n=2), or 1% kelp (n=2). Oxygen consumption of steers treated with 0.5% kelp was not different than the control (P>0.4), however, 1% steers had lower O_2 consumption compared to the control on d7 (P=0.01) Oxygen consumption of steers receiving 1% treatment were lower than 0.5% steers on d 3, 7, and 8 (P<0.03). Carbon dioxide production of steers treated with 0.5% kelp was not different than the control (P>0.3), however, 1% treated steers had lower CO_2 production compared to the control on d 2, 3, and 7 (P<0.04). Carbon dioxide production of steers receiving 1% treatment was lower than 0.5% treatment on d 2, 3, 6, 7, and 8 (P<0.04). A linear decrease (P=0.04and P=0.02) were observed for O_2 consumption and CO_2 production, respectively. Shaded area represents overlap of treatment period and respiratory gas collection period and are used to establish baseline measures for the effectiveness of kelp supplementation.

CHAPTER 5. EFFECT OF BIOSYNTHESIZED BROMOFORM ON FEEDLOT PERFORMANCE AND BROMOFORM RESIDUES IN ORGAN, ADIPOSE AND MUSCLE TISSUE SAMPLES

5.1 Abstract

Recent studies evaluating the effects of kelp inclusion on methane (CH_4) emissions suggest that *A. taxiformis*, can reduce CH_4 production up to 99% in vitro (Roque et al., 2021). A major concern when supplementing kelp, such as *A. taxiformis,* is the accumulation of potentially harmful residues in tissues. Bromoform, the active compound in kelp, has been identified as a carcinogenic compound, and the EPA has established a safety limit of 0.7 ppm in drinking water (EPA, 2022). Thus, this study was conducted to determine if feeding kelp containing biosynthesized bromoform (bromform content= 2 mg/g) to cattle would result in accumulations of bromoform in organ and tissue samples taken from cattle post-mortem. Twelve Angus steers (avg initial BW 424 ± 12.5 kg BW) were randomly assigned to treatments (control, 0.5% and 1% DM inclusion of kelp; bromoform content 2 mg/g) for a 30-day feeding trial. Steers were fed ad libitum adjusted twice weekly to maintain ad libitum intake with 10% refusals. Dry matter intake (P= 0.25), ADG (P=0.55), and growth efficiency (P=0.82) were not affected by the inclusion of kelp extract. DMI (DMI/BW) (P=0.53) was similar across treatments. Bromoform residues in kidney, liver, adipose and longissimus dorsi muscle samples were below detection for all treatments. The results of this study suggest that the inclusion of kelp containing biosynthesized bromoform does not impact intake or growth and does not contribute to the accumulation of bromoform in tissues.

Keywords: bromoform, residues, cattle

5.2 Introduction

Greenhouse gases (GHG) contribute to the greenhouse gas effect by absorbing infrared radiation. Enteric fermentation from livestock contributes to 6% of annual global GHG production (IPCC, 2023). Since the industrial revolution, atmospheric CO_2 and CH_4 concentrations have increased on average about 1 ppm and 8 ppb/year, respectively (IPCC, 2023). Particular concern is centered around CH_4 emissions because of its enhanced ability to contribute to global warming. It is estimated the CH_4 has 25x the global warming power of CO_2, thus it has become a major focus of climate change research (EPA, 2022). However, the IPCC estimates that the radiative effect of CH_4 is approximately 43x that of CO_2 when considering multiple factors (IPCC, 2023).

Enteric CH_4 production is a natural product of fermentation in the rumen which can account for 2-12% of dietary gross energy (Johnson and Johnson, 1995). However, the production of CH_4 is important for the regeneration of substrates of energy metabolism, such as NAD^+. While many additives have been shown to effectively reduce CH_4 production, few have been as effective as kelp species (i.e., *Asparagopsis*). Roque et al. (2021) reported that A. *taxiformis,* supplementation to cattle reduced CH_4 production by 98% when supplemented at 1% of DM. *Asparagopsis* species, as well as other species of kelp have been shown to contain the bioactive compound bromoform, which interacts with methyl coenzyme M to inhibit methanogenesis. During supplementation, Roque et al. observed that DMI tended to decrease by 8% when *A. taxiformis* was supplemented at 0.5% and by 14% at 1% supplementation (Roque et al., 2021). Stefenoni et al. observed a similar decrease of 7.8% in DMI when *A. taxiformis* was included at 0.5% DM (Stefenoni et al., 2021). Bromoform has been implicated for causing changes to hepatic

and renal function as well as being listed as a possible carcinogen by the CDC, particularly when exposure occurs orally, thus, strict levels have been established for items intended for human consumption (ATSDR, 2005). Given the significant amount of evidence suggesting that bromoform causes health effects in mice and rats, the EPA recommends that drinking water should contain no more than 0.7 ppm of bromoform, and OSHA established limits for the amount that workers can be exposed in air during an 8-hr work day (0.5 ppm) (ATSDR, 2005). Due to the concerns with bromoform, the use of kelp containing halogenated methane analogues, such as bromoform, requires investigation into its effects on the animal ingesting the bromoform, as well as any possible modes of excretion or retention which may threaten the health of consumers. Roque et al. (2021) observed no detectible bromoform residues detected in longissimus dorsi samples from cattle consuming *A. taxiformis* for 21 weeks. A similar study in dairy cows conducted by Stefenoni et al. (2021) did not find bromoform above detection in milk. Thus, the objective of this study was to investigate the potential of kelp containing biosynthesized bromoform on DMI, ADG, growth efficiency (ADG/DMI), and concentration of bromoform in the liver, kidney, subcutaneous fat, and longissimus dorsi muscle tissue.

5.3 Materials and Methods

This experiment was conducted in an environmentally controlled, thermoneutral environment at the University of Kentucky C. Oran Little Beef Research Unit. This

experiment was conducted under protocol approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

5.3.1 Animal Management

Twelve Angus steers with an average body weight of 424 ± 12.5 kg were housed individually in partially covered pens equipped with concrete feed bunks and automatic waterers which allow for ad libitum access to water for a 30-d feeding study. For the duration of the experiment steers were fed a corn silage-based diet (Table 5.1) once daily at 0800. Feed offered was adjusted twice weekly to provide ad libitum intake with minimal residuals. Prior to the beginning of the study animals were adapted to ad libitum intake of an 80% concentrate basal diet for a minimum of 14-d.

5.3.2 Experimental Design

Steers were blocked by weight and assigned randomly to one of three dietary treatments (Table 1). Supplements were composed of a ground corn carrier with distillers dried grains (control) with the addition of 10 or 20% kelp (0.5 and 1% treatment). Each supplement was top dressed at a rate of 0, 0.5 or 1% of the total ration DM. Kelp containing supplements were prepared prior to the initiation of the experiment by mixing company supplied kelp with a ground corn carrier and distillers dried grain for 10-minutes using a commercial stainless-steel chopper (Mandenville Company, Inc., Minneapolis, MN, USA). The control supplement was subjected to the same procedures to minimize differences in particle size. Following mixing, supplements were stored in

plastic containers (Rubbermaid Commercial Products, Sarasota Spring, NY, USA) with a plastic snap on lid in an environmentally controlled room. Individual ingredients of the basal diet were sampled (500 g) weekly and analyzed for DM by weighing duplicate 200-250 g samples which were dried in a 55 ºC in forced air oven until weight of sample remained consistent for two consecutive days. Weekly DM determinations were used to adjust dietary ingredients and to calculate dry matter intake (DMI). The amount of feed offered was adjusted twice weekly to provide ad libitum intake with minimal refusals. Any feed refusals were collected weekly and weighed (duplicate 200-250 g samples at 55℃ in forced air oven) and analyzed for DM. Steers were weighed prior to beginning the feeding period (d 0) and weighed prior to transport to the University of Kentucky Meat Sciences Laboratory (Lexington, KY) (d30). Start dates for the feeding period were staggered across steers to provide for a 30-d feeding period and to accommodate for capacity limitations and scheduling conflicts at the laboratory.

Upon completion of each feeding period, steers were transported to the University of Kentucky abattoir where they were humanely harvested under USDA inspection using a captive-bolt followed by exsanguination. Immediately following the removal of the hide and separation of the carcass and organ, approximately 1 g (wet weight) samples from the liver, kidney, subcutaneous fat and longissimus dorsi muscle at the $12^{th}/13^{th}$ interface were harvested using a biopsy needle (Bergström-Stille, Torshälla, Sweden). Tissues were placed into vials labeled with animal ID and tissue type and immediately frozen in liquid nitrogen until all samples were harvested. Once collection was completed, samples were stored at -80 ºC until samples had been collected from all 12 animals. After tissues were harvested from the final group of steers, samples were

shipped to Bigelow Analytical Services (BAS: East Boothbay, ME) for determination of bromoform concentration. Methods developed by Paul et al. (2006), with modifications developed by BAS for the determination of bromoform in tissues harvested in this study. Once samples arrived at BAS, they were immediately placed in a freezer (-80℃) until extractions took place. In summary, samples were weighed and extracted in MeOH with 4 µg/mL naphthalene as an internal standard. The extraction volume was approximately 0.5 mL MeOH extractant/1 g sample, this ratio was established following extensive testing for extraction efficiency (Paul and Steinberg, 2006). The sample and MeOH extractant were then bead-beat for 15 minutes (Retsch Mixer Mill MM40) at 30 Hz, with 2.0 mm Zirconia Oxide beads per sample. Next, samples were centrifuged for 5 minutes at 15,000 rpm (Paul and Steinberg, 2006). Because we did not require esterification, which is only needed for halogenated acetic acids, samples were not frozen (-30℃) for 72 hrs. Samples were then transferred via glass syringe into a separate GC-MS vial. MeOH extracts were then quantified using GC-MS (Shimadzu QP2010 GC-MS, 30-meter RTX 502.2 column). 1 µL injections were performed with a split ratio of 20 with an inlet pressure of 8 psi. The injection port temperature was set to 200℃ and the interface temperature was 220℃. A selected ion monitoring mode (SIM) was used for ions m/z 173 and 128, with the temperature program starting at 65 C and immediately increasing to 220℃ at 30 C/min with a 3 min hold at 220℃ (total run time: 8.17 minutes). All other steps of the Paul et al. (2006) method were used for finalizing sample bromoform quantification. Sample limits of detection were established for each of the tissues prior to quantification of bromoform and are listed in Table 3.

5.3.3 Statistical Analysis

Data were analyzed using the MIXED procedure of SAS v 9.4. Response variables included 1) DMI, 2) average daily gain (ADG), 3) growth efficiency (ADG/DMI), and 4) concentration of bromoform in liver, kidney, subcutaneous fat, and longissimus dorsi muscle tissue. Least square means were separated using orthogonal linear and quadratic contrasts and significance was set at $P \leq 0.05$.

Following initiation of the experiment it was determined that there was insufficient kelp to support ad libitum intake of the kelp treatments throughout the 30-d feeding period. A decision was made to maintain the 30-d feeding period and reduce the number of observations made for the two kelp treatments, resulting in an unequal number of observations between treatments (n=4 for control, n=3 for 0.5% kelp, and n=2 for 1.0% kelp).

5.4 Results and Discussion

5.4.1 Intake and Growth Performance

Dry matter intake was not different (P=0.25) among treatment, averaging 9.34 kg/d for the control treatment, 10.57 kg/d for the 0.5% treatment, and 8.05 kg/d for the 1% treatment (Table 5-2). Expression of DMI on a body weight basis also showed no effect (P=0.53) of treatment. These findings are consistent with our previous study in which growing Holstein steers were supplemented at 0.5 and 1.0% kelp (bromoform= 2 mg/g) where DMI did not differ between treatments. Stefenoni et al. observed no significant

changes in DMI when A. *taxiformis* was supplemented at 0.25 or 0.5% DM, although, the bromoform content of the algae was not defined (Stefenoni et al., 2021). Whereas Roque et al. (2021) observed a decrease in DMI at both 0.5 and 1.0% inclusion of A. armata with a bromoform content of 7.82 mg bromoform/g dry weight. Roque et al. (2021) observed a 10% and 37% decrease in DMI at 0.5 and 1% inclusion, respectively. The findings of this study disagree with Stefenoni et al. who observed a decrease in DMI when A. taxiformis was supplemented at 0.5% of DM when supplemented for 10d (Stefenoni et al., 2021). The bromoform content of that study was not defined, thus, it is difficult to make conclusions on the differences in reported intake. The bromoform content of the product used in the Roque study was much higher (7.82 mg/g) than that used for this study (2 mg/g) which may have contributed to the decrease in intake reported in Roque et al. (2021). However, when bromoform content was reduced to levels similar to those in this study (2 mg/g) the effects on intake were mitigated and DMI was consistent across treatment (P=0.25).

The potential to recapture the energy that would have been lost in the form of CH_4 is a major goal of mitigation studies, however, with detrimental effects on DMI, changes in growth performance could eliminate the potential benefits of inclusion. Average daily gain (kg/d) and growth efficiency (g/kg) were not affected by treatment (Table 5-2; P=0.55 and 0.82). These findings are in agreement with Roque et al. which reported no changes (P=0.72) in average daily gain across treatments when beef cattle were supplemented with 0.25 or 0.5% kelp containing 7.82 mg bromoform/g product (Roque et al., 2021). Growth efficiency (g gain/kg feed) did not change in response to treatment (P=0.82), however, Roque et al. (2021) observed a significant increase in growth

efficiency in response to increasing kelp concentration in the ration. Differences in the findings between Roque et al. (2021) and this study may be due to the short length of this study, the Roque study was 147-d long, allowing for increased response to treatment.

5.4.2 Tissue and Organ Bromoform Residues

Bromoform, the active compound in kelp, as with other trihalomethane (THM) compounds, is readily absorbed through skin contact, breathing and ingestion, although ingestion is the primary route of entry for bromoform (Gad and Pham, 2014). The CDC has identified bromoform as a human carcinogen, thus, it is important that when utilizing bromoform for its CH_4 mitigation potential that residues are not being retained in tissue. In animal studies, repeated oral exposure to bromoform (600 mg/kg/d) resulted in 100% mortality in rats, however, when exposure decreased to 400 mg/kg/d, no deaths were reported (NTP, 1989). Carcinogenic risks of receiving bromoform orally five days per week for two years have also been reported in mice and rats, including, an increase incidence of colon polyps in female, but not male rats (NTP, 1989). Halogenated organic compounds, such as bromoform, tend to be fat soluble and hydrophobic, leading to the accumulation of these substances in fatty substances including the lipid components of blood and milk (Batterman et al., 2001). After ingestion, bromoform is distributed primarily in the stomach, liver and kidneys (Gad and Pham, 2014). Following distribution of bromoform to the liver and kidneys cytochrome p450 aids in the metabolism of bromoform to CO_2 and CO, where both are exhaled through the lungs (Gad and Pham, 2014). Bromoform is predominantly processed by the liver, however, no significant changes in liver function, biochemical or histology were observed in response to

bromoform supplementation at 25-300 µL/kg administered interperitoneally (i.p.) as an oil suspension (corn oil 1mL/kg), (Agarwal and Mehendale, 1983).

Oral bromoform administration during this study did not contribute to detectable accumulations of bromoform within the liver (LOD= 8.5 µg/kg), which suggests that although bromoform is processed in the liver, it may be retained as a metabolite or be metabolized elsewhere. Although bromoform was not detected in liver, kidney, muscle, and adipose tissue collected in this study following 30-d of bromoform supplementation, it is important to consider that bromoform may be broken down into forms such as bromide.

Bromide (Br^-) has been identified in tissues following exposure to bromform, however, few studies have evaluated the retention of Br^- following bromoform supplementation. Bromide is primarily excreted through the renal pathway, however, reabsorption of bromide is commonly seen when chloride intake is low (Palmer and Clarke, 1933; Trepanier, 1955). While bromoform has not been consistently detected in tissues and excretions, it is important to consider possible metabolites. Major metabolites of bromoform include dibromochloromethane, bromochloroacetic acid, and dibromoacetic acid (Vucko et al., 2017).

However, Vucko et al. (2017) observed differences in bromoform concentrations across various processing methods (fresh, frozen or freeze-dried kelp). Kelp biomass that was frozen and then freeze-dried resulted in higher bromoform concentrations than any other processing method tested (Vucko et al., 2017). A negative correlation was observed between vitro CH_4 production and bromform, as expected (r^2= -0.965 ;P <0.001) (Vucko et al., 2017). Concurrently, water used to rinse A. taxiformis had higher bromoform

concentration, suggesting that rinsing kelp can also affect bromoform concentration (Vucko et al., 2017). Given these findings, researchers suggest that bromoform volatilization is affected by processing method.

Initial findings from Pellizzari et al. identified halogenated organic compounds in maternal breastmilk of individuals exposed to bromoform (Pellizzari et al., 1982). Given these findings, bromoform was a subject of concern in lactating cattle, where bromoform was detected in milk collected from lactating dairy cows who were supplemented at low, medium and high levels (67, 133, 333 g DM of *Asparagopsis taxiformis*; bromoform content= 1.26 mg/kg DM) (Muizelaar et al., 2021). Muizelaar et al. (2021) also observed histological changes to the rumen wall of two of the cows harvested during the study, including a reduction in ruminal papillae, increased invasion of inflammatory cells, and formation of ulcers. After d 1 of supplementation, bromoform was detected in the milk of animals in 67 and 133 g DM *A. taxiformis* treatments (bromoform content= 1.26 mg/kg DM), and in urine for all treatments (Limits of detection 6-267 µg/L) (Muizelaar et al., 2021). A lack of residues were detected in feces throughout the study, as well as the presence of bromoform in milk and urine samples, suggests that bromoform does not accumulate in tissues, but can be excreted via urine and milk (Muizelaar et al., 2021). Roque et al. did not report any detectable bromoform residues in milk from lactating dairy cows consuming 0.5% or 1% A. *armata* (bromoform content= 1.32 mg/g dry weight) (Roque et al., 2019). Following 30-d of kelp supplementation in the current study, no significant bromoform residues were detected in any of the tissues (liver, kidney, muscle or adipose) across treatments, limits of detection ranged from 3.7-9.1 µg/kg, lower than those described in previous studies. The findings of present research

suggest that bromoform is not retained in the tissue following kelp supplementation. However, future research should focus on the impact of bromoform supplementation on the accumulation of bromoform metabolites.

5.5 Conclusion

Dry matter intake, ADG and growth efficiency were not affected by treatment, suggesting that the inclusion of bromoform does not contribute to significant changes in growth performance or intake in growing cattle. No detectable changes in bromoform concentrations in kidney, liver, longissimus dorsi muscle tissue and adipose tissue were detected across treatments, suggesting that the supplementation of bromoform for 30-d to growing cattle does not contribute to significant accumulations of bromoform within tissues. Future research should focus on the accumulation of bromoform metabolites, including bromide in tissues.

Table 5-1 Ration Composition

	Treatments		
	Control	Kelp	
Item		0.5%	1.0%
Basal Diet		*% DM basis*	
Corn Silage	50.0	50.0	50.0
Cracked Corn	20.0	20.0	20.0
Distillers Dried Grains	15.0	15.0	15.0
UKARC 105	10.0	10.0	10.0
Top Dress Supplement			
Ground Corn	2.5	2.0	1.5
Distillers Dried Grains	2.5	2.5	2.5
Kelp	0.0	0.5	1.0

Table 5-2 Effects of Biosynthesized Bromoform on Dry Matter Intake and Growth Performance Measures of Angus Steers During the Experimental Period

	Treatment				p-value		
Item	Control	0.50%	1%	SE[a]	Trt	Linear	Quadratic
Initial BW, kg	425	438	408	22.9	0.67	0.62	0.47
DMI, kg	9.34	10.57	8.04	0.89	0.25	0.35	0.13
DMI, kg/kg BW	0.026	0.025	0.028	0.002	0.53	0.55	0.30
ADG, kg/d	1.67	1.41	1.53	0.18	0.55	0.60	0.41
Growth efficiency, g/kg	150.5	148.2	163.5	16.3	0.82	0.60	0.67

[a] Data are presented as least squares means ± the standard error of the mean; n=4 for control, n= 3 for 0.5% kelp, n=2 for 1% kelp.

Table 5-3 Effects of Biosynthesized Bromoform Treatment on Bromoform Residues in Organ, Adipose and Muscle Tissue Samples

	Bromoform, ug/kg[a]			
Item	Control	0.50%	1%	LOD, ug/kg
Liver	<LOD	<LOD	<LOD	8.5
Kidney	<LOD	<LOD	<LOD	9.1
Adipose	<LOD	<LOD	<LOD	3.7
Muscle	<LOD	<LOD	<LOD	4.4

[a] n=4 for control, n=3 for 0.5% kelp, n=2 for 1% kelp. LOD= limit of detection.

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