EFFECT OF MOOTRAL AND FORAGE AMOUNT ON METHANE EMISSIONS, GROWTH AND CARCASS CHARACTERISTICS OF FEEDLOT STEERS

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Animal Sciences

West Lafayette, Indiana

December 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL

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ACKNOWLEDGMENTS

Earning my master's degree would not have been possible without the help and guidance of my friends, family, and professors. I would like to first thank my advisor, Dr. Jon Schoonmaker, for giving me the opportunity to pursue a master's degree at Purdue University which has become my home away from home, especially during the pandemic. I have always appreciated your open-door policy, being present at the farms to work cattle, and willing to help whenever I had any questions or concerns about any issues that I had. I would also like to thank my committee members, Dr. Boerman and Dr. Henry, for helping me when I had questions regarding ruminant nutrition and helping with the methane collection.

I would like to thank the Beef Unit crew, Brain DeFreese and Charlie Huelsenbeck, for feeding and monitoring all the steers involved in the project. More thanks to the Henry Lab for driving all the way from Lubbock, Texas to Purdue to help in the methane collection process. Andrea, Sergio, and Kenneth were a huge help and made the process of working cattle and collecting methane smooth and easy. I would like to thank the Purdue Meats Lab, especially Blaine Brown and Gary Watters, for processing our steers for the methane project. I would also like to thank the friends, especially Danielle Willson, Tabitha Steckler-Hurst, and Conor McCabe, I made here while at Purdue who without their help and guidance I would not have been able to complete the journey I have been through.

I would like to thank the SLOAN Foundation and the Gates Millennium Scholars Program for supporting me financially while being at Purdue. I would also like to thank my family for their support in me being at Purdue and their advice when it came to make academic decisions.

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ABSTRACT

Methane (CH_4) production from enteric fermentation in ruminant animals is a contributor to global greenhouse gas emissions. Because CH_4 has an impact on increasing global temperatures, there is a push for government regulations to reduce CH_4 from livestock animals. At 1.9% of U.S. CH_4 emissions beef cattle are a large contributor to agricultural CH_4 emissions or (EPA, 2020). Enteric CH_4 emissions are also a loss of energy for the animal, accounting for 2-12% of energy loss from the ruminant animal (Johnson & Johnson, 1995). This energy loss from the diet is contingent upon forage content, where increasing forages in the diet increases CH_4 yield (g/kg of gross energy intake; van Gastelen et al., 2019). Mootral is a feed supplement that contains garlic (*Allium sativum*) and bitter orange (*Citrus aurantium*) extracts. The organosulfur compounds in garlic the flavonoids found in bitter orange extracts are known to decrease CH_4 production, (Busquet et al., 2005a; Balcells et al., 2012; Seradj et al., 2014). However, it is unclear how the forage content and Mootral inclusion will interact to effect CH_4 production and animal performance. Because feedlot cattle are fed a range of forage:concentrate ratios while in the feedlot, it is important to know how effective mitigation strategies are in different forage:concentrate diets. Therefore, the objective of the current study was to quantify CH_4 production and determine growth, intake, and carcass characteristics of feedlot steers fed Mootral in diets with a low, medium, and high forage content. Knowing the effect of garlic and flavonoids on methanogenesis, we hypothesized that Mootral would decrease CH_4 emissions without impacting growth, intake, and carcass characteristics of feedlot steers. We expect that the CH_4 mitigating ability of Mootral will be greatest in the diet with the most forage. For the experiment, 144 Angus x Simmental steers were allotted by body weight (BW; 363 kg), breed composition, and farm origin to a 3 x 2 factorial arrangement of 6 treatments (4 pens per treatment) to determine the effect of Mootral (garlic + citrus extract; 0.25% of the diet

DM vs. 0.0%) on methane emissions, growth and carcass characteristics of feedlot cattle. During the first 84 days, cattle were fed three different forage concentrations in the diet (15, 41.5, or 68% corn silage) with or without Mootral. From day 85 to slaughter, corn silage was included at 15% of the diet DM with or without Mootral. Methane emissions were measured on day 42-46 and day 203-207. Data were analyzed using the GLIMMIX procedure of SAS. There was an interaction ($P = 0.03$) between forage content and Mootral for DMI from d 0 to 84, where Mootral decreased DMI of steers fed 15% corn silage but did not affect DMI of steers fed 41.5 or 68% corn silage. There were no effects ($P \geq 0.22$) of forage content or Mootral on BW or average daily gain at any time, or on DMI from d 84 to slaughter and overall. Intake from d 0-84 was lower and gain:feed from d 0-84 and overall was greater ($P = 0.04$) for steers fed 68% compared to 15 or 41.5% corn silage. On d 42-46, steers fed 41.5 and 68% corn silage had increased ($P \leq 0.02$) methane emissions compared to steers fed 15% corn silage. Mootral did not affect methane emissions on day 42-46 ($P \geq 0.47$), but there was a forage effect, where steers fed the 41.5 and 68% corn silage diets emitted more methane on a g/d ($P = 0.05$) and a g/kg of DMI ($P = 0.007$) basis and tended ($P = 0.07$) to produce more methane on g/kg BW basis compared to steers fed the 15% corn silage diet. Steers fed Mootral emitted less ($P \leq 0.03$) methane on a g/d, g/kg DMI, and g/kg BW basis on d 203-207 compared to steers not fed Mootral. Mootral tended to decrease ($P \leq 0.09$) fat thickness and yield grade. In conclusion, increasing forage content increased methane emissions and Mootral decreased methane production in 15% corn silage diets and improved carcass leanness.

CHAPTER 1. REVIEW OF LITERATURE

1.1 What Are Different GHG?

Greenhouse gases (GHG) are known for trapping heat in the Earth's atmosphere because they have the ability to absorb and emit radiation within a thermal infrared range (FAO, 2014). This means that the ability of GHG to trap heat is related to the wavelength of infrared radiation that a particular gas absorbs. The ability of a GHG to trap heat is also dependent on the amount of time the gas is confined in the atmosphere. Major greenhouse gases include methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O), fluorinated gases such as hydrofluorocarbons (HFC), perfluorocarbons (PFC), sulfur hexafluoride (SF_6), and nitrous trifluoride (NF_3) (EPA, 2020; IPCC, 2014). Greenhouse gases are main sources for global warming (Johnson & Johnson, 1995) and are a major global concern for today's environment and economy (Johnson & Johnson, 1995; Beauchemin et al., 2019; Thompson & Rowntree, 2020). It is well known that fossil fuels and production agriculture contribute to greenhouse gas emissions (IPCC, 2014). Animal agriculture is a concern because it contributes to atmospheric CH_4 and N_2O. Methane has a much shorter lifetime than CO_2 in the atmosphere, which makes it an attractive amelioration target for short-term gains in global warming abatement (Beauchemin et al., 2020).

The ability of a GHG to trap heat is expressed as its global warming potential (GWP), which is determined by the amount of heat a GHG can trap within the Earth's atmosphere relative to CO_2 and allows comparisons with other gases (IPCC, 2014; EPA, 2021). Specifically, GWP is a measure of how much energy the emissions of 1 ton of a gas will absorb over a given amount of time, relative to the emissions of 1 ton of CO_2. The unit of measurement for GWP is expressed in million metric tons (MMT) of CO_2 (IPCC, 2014). For example, CO_2 has a 100-year GWP of 1, CH_4 has a 100-year GWP of 25, and N_2O has a 100-year GWP of 298 (EPA, 2021). This means

that over 100 years, CH_4 could trap 25 times as much heat as CO_2, whereas N_2O could trap 298 times as much heat as CO_2. Even though CO_2 is more abundant in the atmosphere and can remain in the atmosphere longer compared to CH_4 or N_2O, CH_4 and N_2O retain more heat compared to CO_2 (FAO, 2006). The net effect of the shorter lifetime and higher energy absorption is reflected in the GWP. Methane remains in the atmosphere 12.4 years and N_2O remains for 121 years; however, CO_2's lifetime cannot be represented with a single value because the gas is not destroyed over time, but instead moves among different parts of the ocean–atmosphere–land system (EPA, 2021). Some of the excess CO_2 is absorbed quickly (for example, by the ocean surface), but some will remain in the atmosphere for thousands of years, due in part to the very slow process by which carbon is transferred to ocean sediments. Greenhouse gases have been increasing since pre-industrial times (EPA, 2021) and since 1975 there has been an increase in greenhouse gases emitted into the atmosphere of approximately 75% (IPCC, 2014). Although total GHG emissions have been decreasing since 2007.

1.2 US Greenhouse Gases

The overall total US greenhouse production in 2019 from all sectors of the economy was 6558.3 MMT CO_2 Eq. (EPA, 2020). CO2 comprised 80% of that GHG production, CH_4 was 10%, N_2O was 7%, and fluorinated gases made up 3% of all GHG emissions in 2019 (US GHG Inventory, EPA., 2020). The U.S. EPA categorizes all greenhouse gas emitters by their industry sector: energy, industrial process & product use, agriculture, and waste (US GHG Inventory, EPA., 2020). Within those categories, further GHG are broken up by economic sector where transportation accounted for 28% of all GHG emissions, electricity contributed 27%, industry was responsible for 22%, commercial and residential contributed 12%, and agriculture was responsible for 10% of all greenhouse gas emissions (618.5 MMT CO2 eq.) in the United States in 2019 (US

GHG Inventory, EPA., 2021). The greenhouse gases that agriculture emits are CO_2 (1.24%), CH_4 (41%), and N_2O (57.8%). Most CO_2 emissions from agriculture are not considered to contribute to global warming because they are part of a closed cycle, meaning that the atmospheric CO_2 is absorbed into plants, stored as carbohydrates, released as CO_2 during digestion, then re-used by plants. Exceptions to this closed loop cycle are CO_2 emissions from urea use (fertilization and feeding) and liming because urea and lime production use energy and produce CO_2 (US GHG Inventory, EPA., 2020). Agricultural CH_4 is produced from livestock enteric fermentation, livestock manure, rice cultivation, and field burning. Nitrous oxide originates from agricultural soil management, livestock manure management, and field burning. Livestock enteric fermentation generates 178.6 MMT CO_2 Eq. as CH_4 and livestock manure produces 62.4 MMT CO_2 Eq. as CH_4 and 19.6 MMT CO_2 Eq as N_2O. Thus, in terms of US agriculture, livestock enteric fermentation is responsible for 28.7% of all agricultural GHG produced and livestock manure is responsible for 13.1% of all agricultural GHG produced. When accounting for all other sectors of the U.S. economy, livestock are estimated to be responsible for 3.9% of all GHG produced (US GHG Inventory, EPA., 2020).

Ruminant animals are the primary livestock emitters of enteric CH_4 because CH_4 is a byproduct of ruminal fermentation (EPA, 2021). Beef cattle, which includes cows, bulls, calves, heifer replacements, heifer and steer stockers and feedlot cattle, emitted 129.1 MMT CO_2 Eq. of enteric CH_4 in 2019, which is equivalent to approximately 72.7% of all enteric CH_4 produced from livestock in US agriculture. Dairy cattle were the second leading source of enteric CH_4 produced from US livestock and emitted 43.2 MMT CO_2 Eq. which was approximately 24.3% of all enteric CH_4 from livestock animals. However, GHG emissions from sectors including livestock are estimates based on multiple samples taken from multiple sources and mathematical modeling.

Sampling techniques and mathematical models vary across research groups, resulting in different estimates. For example, Gerber et al. (2013) estimates that total global GHG emissions from livestock (animals, manure, feed production and expansion of lands into forested areas) account for 14.5% of total anthropogenic emissions with enteric CH_4 from ruminants contributing approximately 6% of global anthropogenic GHG emissions. The majority livestock CH_4 is also part of a closed loop system: atmospheric CO_2 into plants, plant CO_2 converted to CH_4in the rumen, and then ruminant CH_4 converted back into CO_2 which goes back into plants (Liu et al., 2021). Greenhouse gases emitted from other sectors come from carbon sources that had been sequestered (e.g., oil, coal; Liu et al., 2021)

1.3 Measuring Methane

There are multiple measuring techniques for CH_4 and other gases produced by ruminants. Sampling of gaseous emissions can be accomplished by enclosure methods or tracer techniques. Some of the most common techniques for measuring CH_4 include, but are not limited to, respiration chambers (Hammond et al., 2016; Storm et al., 2012), sulfur hexafluoride tracer technique (SF_6), *in vitro* gas production method (Storm et al., 2012), and laser measurement.

1.3.1 Respiration Chamber

The open-circuit indirect respiration technique allows outside air to flow throughout the chamber (Figure 1.1) with the animal inside and the expired air is collected (Johnson & Johnson, 1995). Quantifying CH_4 using the respiration chamber technique is achieved by comparing the entering air flow with exiting air flow and calculating the concentration differences between the two (Johnson & Johnson, 1995). Air is constantly entering and exiting through intake and exhaust ducts and is measured in g/d by infrared CH_4 and CO_2 sensors (Ku Vera et al., 2018; Hill et al.,

2016; McGinn et al., 2006). Respiration chambers are known as the gold standard because all outputs of gases can be measured for the experiment and the environment is controlled. When measuring livestock gas emissions, chambers do require significant labor inputs and training of animals to collect accurate data for the studies, however, placing animals in respiration chambers decreases dry matter intake (DMI) and alters the behavior of the animal since the animal is not in a natural environment (Hill et al., 2016). Therefore, estimates may not be reflective of actual gas emissions.

1.3.2 Portable Static Chambers

Portable accumulation chambers (Figure 1.2) can identify differences in CH_4 production within 2 hours of sampling (J.P. Goopy et al., 2012). The chamber is made of polymethyl methacrylate (plexiglass) sealed to the floor (Goopy et al., 2011). Methane is sampled through silicon sampling ports and analyzed using a portable flame ionization detector fitted with a silicon tube. The portable accumulation chamber is effective for short term CH_4 measurements and is cheaper than a standard respiration chamber (Goopy et al., 2011).

1.3.3 Automated Head Chamber System (GreenFeed)

Automated head chamber systems (AHCS) (Figure 1.3), the most common of which is the GreenFeed system (C-Lock Inc, Rapid City, SD, USA), can measure CH_4, CO_2 and O_2 consumption by sampling eructated gasses and breath samples (Gunter et al., 2016; Huhtanen et al., 2019). The GreenFeed system uses a bait feed to attract cattle into the automated head chamber system and automatically records the animal's ID using a RFID reader that records the start and end time (Histrov et al., 2015). To obtain an accurate sample, animals must be trained and comfortable around the GreenFeed system and need to visit the GreenFeed at least 2 to 4 times a

day (Gunter and Beck, 2018) for 3 to 8 minutes each time. The GreenFeed captures the animals' emitted gases by pulling air (26 L/min) over the animal's head moving CH_4 and CO_2 through the feeder to an infrared sensor where the sample is analyzed for CH_4 and CO_2 (Histrov et al., 2015). Sample data is uploaded to a server, evaluated, and converted to a daily emission and the consumption of gas using a proprietary algorithm (Gunter and Beck., 2018). Background gas is also measured through the exhaust air stream (Cottle et al., 2015). The GreenFeed method of measuring CH_4 is less labor intensive and less expensive ($50-60K) than chamber techniques and allows for more animal sampling (Thompson and Rowntree., 2020)

1.3.4 Tracer Techniques

Tracer techniques are more feasible when working with range cattle or other extensive livestock systems. Tracer techniques are a way to have a marked type of material that can be used to determine properties of an element in biological systems (Ponnuvel, 2016). How the tracer technique works is an inert gas is utilized, such as sulfur hexafluoride or isotope labelled CH_4, and a known amount is mixed in the rumen. When the inert gas is eructated or respired out it is measured, compared to collected CH_4, and a CH_4 release amount is calculated. Tracer techniques are more economical compared to open-circuit chambers and allow researchers to utilize more animals in a single trial (Berndt et al., 2014). Isotopic or non-isotopic tracer techniques can be used to estimate CH_4 emissions from ruminants (Johnson & Johnson, 1995). Isotopic tracer technique uses cannulated animals dosed with ^{3}H or ^{14}C methane directly into the rumen through an infusion line and gas samples are also collected directly from the rumen and calculated through mathematical models (Johnson & Johnson, 1995). The non-isotopic tracer technique does not require cannulated animals since it uses an inert tracer gas that is orally dosed into the rumen and has a known release rate (Johnson & Johnson, 1995).

1.3.5 Sulfur Hexafluoride (SF_6)

Sulfur hexafluoride is an inert tracer gas placed in the rumen inside a permeation tube (Figure 1.4) that has a known release rate. The SF_6 technique (Figure 1.5) does not account for hindgut CH_4 production which can account for 1 to 15% of all CH_4 produced (Hill et al., 2016; Gunter and Beck., 2018). However, it is estimated that up to 90% of CH_4 that is created in the large intestine is absorbed into the bloodstream and released through respiration (McGinn et al., 2006), which the SF_6 technique can capture. The site of digestion either in the rumen or hindgut determines where CH_4 will be produced. Size of feed particles along with level of intake can increase post-ruminal digestion (McGinn et al., 2006). The permeation tube has a brass tube body with nylon washers and a teflon membrane that is fitted with a porous stainless-steel frit and brass nut (Henry et al., 2020; Zimmerman., 1993). The permeation tube is filled with a designated amount of SF_6 gas and the teflon membrane that controls the release of SF_6 (McGinn et al., 2006). After permeation tubes are filled, they are kept at 39^{o}C to replicate the rumen environment and weighed multiple times before and after experiments to determine the daily release rate of SF_6 (Henry et al., 2020; Zimmerman., 1993). The release rate is important because the ratio of CH_4 to SF_6 gas is used to calculate CH_4 emissions from the animal. Permeations tubes need to have a constant flow for at least 3 months prior to inserting them into the rumen (McGinn et al., 2006). It has been noted that permeation tubes with high or low release rates can show high or low CH_4 (Storm et al., 2012). Therefore, the release rate must be measured before and after the experiment to have accurate representation between treatments (Storm et al., 2012). When the ruminant animal eructates, CH_4 and SF_6 are released and collected into a vacuum sealed cannister constructed of PVC pipe located on the animal's neck (Berndt et al., 2014). As the vacuum in the cannister slowly dissipates, a sample of air around the nose and mouth of the animal is drawn in. A capillary tube fitted to the animal's halter controls the flow rate of gases into the cannister. Flow rates described

in the literature range from 1.13 to 4.97 mg/d (Dorich et al., 2015; Henry et al., 2020; McGinn et al., 2006). The vacuum on cannisters last 36 hours and remain attached to the animals for a period of at least 24 hours, to accurately represent a feeding cycle, before replaced with another cannister (Berndt et al., 2014). The cannister should have half the vacuum remaining after 24 hours in order for consistent sampling to occur. Damage to animal collection units (cannisters and halters) is common, thus gases should be collected for 5 days in order to collect at least 3 days of usable data for accurate CH_4 emission values. Methane that exists in the animal's environment is accounted for by sampling the ambient air that is surrounding the animals involved in the study (Johnson et al, 1994).

Gases within the cannisters are later analyzed by gas chromatography. The emission rate of CH_4 from the animal is calculated using the equation of (Johnson et al, 1994; Berndt et al., 2014):

$$Q_{CH4} = Q_{SF6} \times ([CH_4]_\gamma - [CH_4]_\beta) \div ([SF_6]_\gamma - [SF_6]_\beta)$$

Where Q_{CH4} represents the emission rate of CH_4, Q_{SF6} is the emission rate of SF_6, and $[CH_4]$ and $[SF_6]$ are the measured concentrations in the individual animal's and environmental canisters (Berndt et al., 2014).

1.3.6 Laser Methane Detector

Laser CH_4 detectors (Figure 1.6) are an inexpensive and convenient way to measure CH_4 emissions from livestock (Grobler et al., 2014). The laser CH_4 detector was originally designed for detecting CH_4 leaks for industrial purposes such as landfill sites where there could be possible CH_4 leakage (Chagunda et al., 2013). Laser CH_4 detectors are handheld gas detector that uses a semi-conductor laser using infrared absorption spectroscopy (Grobler et al., 2014). Values

obtained from the laser detector are measured as ppm-m (parts per million-meter) and can operate in 0-40^{o}C temperatures with 20-90% humidity (Grobler et al., 2014). Measurements can be taken at least 3 to 150 meters away from the animal while the laser is directed at the animal's nostrils (Grobler et al., 2014; Chagunda et al., 2013). These CH_4 measurements can be taken without disturbing the animals and can record when eating, drinking, feeding, ruminating, standing and lying down for the cows (Sorg et al., 2018; Chagunda et al., 2013). The measurements can detect 10-10,000 ppm-m and it is best to use the device while the animals are eating and drinking because CH_4 emissions seem to be greatest at these times compared to when animals are walking or sleeping (Chagunda et al., 2013). The device can only take spot measurements since no gas is collected (Chagunda et al., 2013). Drawbacks to spot measurement is that CH_4 production amount and emission rates cannot be determined and the laser CH_4 detector is sensitive to ambient conditions, such as wind speed, pressure and wind direction, and humidity when animals are in outside environments (Chagunda et al., 2013). Therefore, the laser CH_4 detector should only be used to measure CH_4 where animals are in an enclosed area and only ambient concentration is desired.

1.4 Ruminal Enteric Methane Production

Enteric CH_4 is a natural by-product of the anaerobic microbial fermentation in the digestive tract of ruminants. Ruminal microbes consist of protozoa, bacteria, archaea, and fungi (Parish et al, 2014). When a ruminant animal ingests feed, the feed is fermented by ruminal microbes (Morgavi et al., 2010) that break down feed and derive energy. The end-products (Figure 1.7) of microbial digestion are primarily microbial protein and volatile fatty acids (VFA; primarily acetate, butyrate, and propionate) that the ruminant uses to meet its own metabolic needs (Morgavi et al., 2010). By-products of ruminal microbial fermentation include CH_4, CO_2, H_2, and H_2S

(Parish et al, 2014). Most enteric CH_4 production occurs in the rumen and is emitted through eructation; however, hindgut methanogenesis also occurs with approximately 1-15% of CH_4 released as flatulence (Hill et al., 2016; McGinn et al., 2006). Up to 90% of CH_4 that is created in the large intestine is absorbed into the bloodstream and released through respiration (McGinn et al., 2006). Methanogenesis is important because it limits hydrogen accumulation in the rumen, which could result in accumulation of lactic acid and an overall inhibition of rumen fermentation (Wolin et al., 1997).

Methanogenic archaea (methanogens) are responsible for producing CH_4 through a process called methanogenesis (Figure 1.8) (Morgavi et al., 2010). Three major pathways of methanogenesis are known: hydrogenotrophic, methylotrophic, and acetoclastic. Hydrogenotrophic methanogens use H as an electron donor and CO_2 as an electron acceptor. Hydrogenotrophic methanogenesis is the most widespread pathway for archaea to produce CH_4, and is the pathway used by ruminal archaea (Morgavi et al., 2010; Hungate., 1967). Other types of methanogenic archaea found elsewhere in nature also use methylotrophic or aceticlastic pathways to create CH_4 which uses methyl groups or acetate, respectively, as substrates (Morgavi et al., 2010; Liu and Whitman., 2008). Hydrogenotrophic methanogens reduce CO_2 to CH_4 in six steps via the Wood–Ljungdahl pathway (WLP), which is also known as the reductive acetyl-CoA pathway (Nitschke and Russell, 2013). Acetogens also use this pathway to produce acetate, although the carbon molecule carrier differs and the H_2 threshold is much greater (Conrad et al., 1983; Nitschke and Russell, 2013). In the WLP, H_2 or sometimes formate, is used as an electron donor. To conserve energy, methanogenic hydrogenotrophs couple the WLP to methanogenesis using the enzyme S-methyltransferase (Mtr), which transfers the methyl group from the WLP to coenzyme M.

The rumen contains a complex microbial community that includes methanogenic species that differ from methanogens in other populations because they lack cytochrome proteins responsible for electron transfer (Knapp et al., 2014; Munoz-Tamayo et al., 2019). In most ecosystems this would be an energetic disadvantage, but in the rumen this lack of cytochrome protein allows the methanogens to survive in the comparatively low H_2 environment and cooperate with H_2 generating microbes (Munoz-Tamaya et al., 2019). There are three major and three minor methanogenic genera present in ruminants: *Methanobrevibacter, Methanomicrobium, Methanosphaera, Methanosarcina, Methanobacterium* and genera in the order *Thermoplamatales* (rumen cluster C) (Whitford et al., 2001, Hill et al., 2016, Beauchemin et al., 2020). Microbial fermentation of cellulose, hemicellulose, and pectin by fiber digesting bacterial results in greater amounts of acetate and butyrate production, whereas fermentation of starch in concentrates results in more propionate and butyrate production. Fermentation of fiber into acetate results in greater amounts of CO_2 and H_2 in the rumen compared to fermentation of starch into propionate, which in turn eventually results in greater CH_4 production. Propionate production consumes H_2, therefore competing with methanogenesis (Beauchemin et al., 2020).

1.5 Modifying Ruminal Microbial Fermentation

1.5.1 Alternative Hydrogen Sink

1.5.1.1 Forage

Forage content of diets influence the amount of structural versus non-structural carbohydrates which affects digestibility and therefore influences ruminal CH_4 production (Histrov et al., 2013). Non-structural carbohydrates, such as starch, found in grains are more digestible and yield more propionate during microbial degradation compared to structural carbohydrates (i.e., cellulose, hemicellulose) found in forages. Fermentation of carbohydrates to

propionate consumes H_2 from the ruminal environment, limiting electrons needed for methanogenesis. A meta-analysis of studies investigating forage amount on CH_4 emissions suggests that increasing forage at the expense of concentrate decreased DMI for dairy and beef cattle (van Gastelen et al., 2019). The same meta-analysis reported that increasing forage:concentrate decreased CH_4 production (g/d) in dairy cattle but increased CH_4 production (g/d) in beef cattle. However, CH_4 yield (g/kg of DMI) generally increased as did CH_4 intensity (g/kg of product) in both dairy and beef cattle as forage:concentrate increased in the studies used in the meta-analysis. Methane yield (as % of gross energy intake [GEI]) increased for dairy and beef cattle as forage:concentrate increased (van Gastelen et al., 2019). Lovett et al. (2003) reported that increasing the forage:concentrate ratio in finishing beef cattle diets from 10:90 to 40:60 and 65:35 linearly increased CH_4 production measured in L of CH_4/day, CH_4/kg DMI, and CH_4 as % GEI and reduced feed conversion efficiency over an 11-week period. Aguerre et al. (2011) observed that increasing forage:concentrate ratios (47:53, 54:46, 61:39, 68:32) in Holstein cows caused an increase in CH_4 emissions from 538 to 648 g/d.

Forage type, maturity, and quality also influence rates of digestion and H_2 production. It is estimated that 75% of CH_4 from ruminants is from animals grazing low quality feeds (Knapp et al., 2014). Higher quality forages are more digestible and will increase the production efficiency of animals (Histrov et al., 2013). Higher quality of forage causes greater DMI, which is correlated to a higher passage rate from the rumen, which in turn results in a decrease in CH_4 produced per unit of dry matter intake (Beauchemin et al., 2019). Pinares-Patino et al. (2003) used Charolais cows grazing timothy grass at 4 different maturity stages (early vegetative, heading, flowering, senescence) for 14-day periods and measured CH_4 emissions during the last 7 days. They reported that timothy grass at the heading stage produced more CH_4 (g/d) than the other stages, most likely

because cows were able to consume it at the greatest amount. Holtshausen et al., (2012) compared grass silages harvested at early maturity, mid maturity, and late maturity, and concluded that during 24 and 48 h in vitro incubations, total CH_4 production and CH_4 production per gram of NDF digested decreased as maturity rose. Methane production per g of DM digested did not differ. However, DM and NDF digestibility decreased with increasing maturity (Holtshausen et al., 2012), suggesting that animal performance would decrease, and a greater number of days would be required to achieve a similar weight, thus potentially increasing CH_4 production in a live animal setting.

For forage type, Meale et al. (2012) collected rumen fluid from Holstein cows and found that non-leguminous shrubs produced less CH_4 compared to leguminous shrubs and grasses during an *in vitro* fermentation study. Because tannins inhibit fiber digestibility (described later), legumes with high tannin content have decreased digestibility and CH_4 production. Thus, CH_4 production was lower for animal fed high tannin legumes versus the animals fed low tannin legumes (Archimede et al., 2011). Chaves et al. (2006) showed that Angus heifers emitted more CH_4 when fed alfalfa (162.8 g CH_4/head) *ad libitum* compared to the grass (113.5 g CH_4/head) *ad libitum*. In addition, CH_4 per unit of DMI was 39% lower for the grass pastures versus the alfalfa pastures (Chaves et al., 2006). Hassanat et al., (2013) noticed that replacing alfalfa silage with a complete 100% corn silage diet in lactating Holstein cows decreased CH_4 over a 32-d period. There was also no impact on pH, acetate:propionate ratio, and protozoal populations at the lower concentration of 50% corn silage diet but an increase in pH, decrease in acetate:propionate ratio, and a decrease in protozoal populations when Holstein cows were on a 100% corn silage diet (Hassanat et al., 2013).

Gislon et al., (2020) analyzed diets consisting of corn silage, alfalfa silage, wheat silage, and an alfalfa/Italian ryegrass mix diet fed to lactating Italian Friesian cows and reported no difference in CH_4 production when expressed as g/kg of DMI or g/kg of milk. However, there was a difference in digestible energy intake (CH_4 energy loss) where the alfalfa/Italian ryegrass mix diet was the highest at 8.67% of digestible energy intake and the corn silage with the lowest value of 7.70% on digestible energy intake (Gislon et al., 2020). Chung et al. (2011) reported lower ruminal pH, greater propionate, 31% less CH_4 produced in g/hd/d and 30% less CH_4 per kg of DM in Holstein cows when whole crop barley silage in a 50:50 forage:concentrate diet was replaced with chopped grass hay. Benchaar et al., (2014) saw a decrease in CH_4 in g/kg of DMI, % GE intake, and %DE intake along with a decrease in the acetate:propionate ratio, improved milk production, and a decrease in urinary N loss when corn silage was at 54% of the diet and fully replaced barley silage in dairy cow diets (Benchaar et al., 2014).

Cattle fed C4 grasses emit more CH_4 compared to cattle fed C3 grasses because C3 grasses have decreased fiber and lignin content and are of a higher quality versus the C4 grasses (Thompson and Rowntree., 2020). Archimede et al. (2011) conducted a meta-analysis where they found that cattle fed C4 grasses emitted 17% more CH_4 than C3 grasses. Methane production was greatest in cattle fed C4 grass diets vs. C3 diets for L/kg DMI, L/kg OMI, and L/kg of DOM (Archimede et al., 2011). Also, CH_4 produced from warm season legumes was 7-22% lower than the cold season legumes (Archimede et al., 2011). It was also noted that cattle fed warm season legumes produced 20% less CH_4 compared to warm season C4 grasses (Archimede et al., 2011).

1.5.1.2 Lipids

Microbial biohydrogenation of unsaturated fatty acids to saturated fatty acids competes with methanogens as a hydrogen sink and therefore methanogenesis is slightly inhibited when diets contain a greater amount lipids, specifically unsaturated fatty acids (Beauchemin et al., 2019). Lipids also inhibit methanogenesis by replacing the organic matter in the diets, which in turn decreases the amount of methanogens and protozoa (Beauchemin et al., 2019). However, supplementing fats have an inconsistent effect on CH_4 production and can have a negative impact on DMI, fiber digestibility, and subsequent milk production by causing milk fat depression in lactating dairy cows (Histrov et al., 2013; Beauchemin et al., 2019). The main fatty acids responsible for low CH_4 output are short and medium chain fatty acids and polyunsaturated 18 carbon fatty acids (Chilliard et al., 2009). These fatty acids are responsible for decreasing protozoa, cellulolytic bacteria, and archaea methanogens and with polyunsaturated carbon fatty acids having a toxic effect directly on methanogens, protozoa and cellulolytic bacteria that involves fiber digestion and hydrogen production (Chilliard et al., 2009).

Beauchemin et al., (2007) observed that compared to a control diet, feeding tallow (predominantly saturated fatty acids) and sunflower oil (predominantly unsaturated fatty acids) to 16 Angus heifers fed barley silage, barley grain, limestone, salt and a vitamin and mineral premix diet decreased CH_4 15 and 25%, respectively, when corrected for GEI. There was also a trend for higher average daily gain for heifers fed sunflower oil and sunflower seeds versus heifers fed the control diet and sunflower seeds and there was no difference in ruminal pH, total VFA or individual VFA concentration (Beauchemin et al., 2007). Beauchemin et al. (2006) found that canola oil, which is a blend of fatty acids that are predominantly unsaturated, decreased CH_4 (g/heifer) by 32%, increased propionate, and decreased ammonia-N in Angus steers fed a high

forage diet, but the decrease could be the result of 10% decrease in DMI and a 15% decrease in total tract digestibility (Beauchemin et al., 2006).

Carvalho et al (2016) reported that Nellore steers fed 80 g/kg on a DM basis of linseed oil grazing *Brachiaria brizantha* pastures supplemented with ground corn and soybean meal diet had a 38% decrease in CH_4 on a mg/kg BW basis compared to Nellore steers fed a control diet. Inclusion of palm oil (80 g/kg on DM basis) had no effect on CH_4 production, but protected fat (calcium salts of fatty acids; 90 g/kg on DM basis) showed a 12% reduction in CH_4 and whole soybeans (400 g/kg on DM basis) showed a 24% reduction in CH_4 on a mg/d/kg BW basis. None of the lipids affected dry matter, organic matter, average daily gain or NDF intake; however, NDF digestibility decreased with linseed oil and palm oil compared to the control (Carvalho et al., 2016).

Caprylic acid (C8:0) is a medium chain fatty acid, and its mode of action to inhibit methanogenesis is by inhibiting substrate transport in microbes and decreasing microbial growth (Rajaraman et al., 2017). Adding increasing doses (0, 0.17, 0.33, 0.67 g/L) of caprylic acid + a stabilizer (beta cyclodextrin) to rumen fluid and incubating for 9 hours increased propionate and total VFA, decreased acetate, increased total gas production, and decreased CH_4 production (ml/incubation) from 21 to 28% compared to the control (Rajaraman et al., 2017). In the *in vivo* study, Rajaraman et al., (2017) observed that feeding 104 g/d of caprylic acid + beta cylclodextrin to Hanwoo steers fed timothy hay (4.85 kg), ground corn grain (1.2 kg), and corn gluten meal (0.13 kg) diet only numerically decreased CH_4 by 10% compared to the control suggesting that it may need to be supplemented at a higher dose to have an effect on CH_4 production.

1.5.1.3 Dicarboxylic Organic Acids (Propionate Precursors)

Dicarboxylic organic acids are propionate precursors in the succinate-propionate pathway that require H_2 for propionate production. By acting as alternative H_2 sinks in the rumen, dicarboxylic organic acids have the potential to decrease ruminal methanogenesis (Newbold et al., 2005). The potential of dicarboxylic organic acids such as fumaric acid and malic acid as inhibitors of methanogenesis is well documented *in vitro* (Carro and Ranilla, 2003; Newbold et al., 2005) and *in vivo* (Lila et al., 2004; Wallace et al., 2006). Diallyl maleate is a liquid ester form of maleic acid, which is a precursor to fumaric acid. Diallyl maleate contains double bonds that act as a hydrogen sink (Lila et al., 2004). Due to its hydrophobic internal cavity, cyclodextrin can stabilize compounds that are readily oxidized, like organic acids. It was reported that feeding diallyl maleate in a cyclodextrin matrix (CD-M) increased total volatile fatty acid production, *in vitro*, as the level of CD-M was increased (Lila et al., 2004). Propionate and butyrate concentrations increased but it was noticed that there was a decrease in acetate as CD-M inclusion increase (Lila et al., 2004). The use CD-M had no effect on ruminal protozoa but did decrease CH_4 (ml/incubation) up to 75% *in vitro* with increasing levels of CD-M (Lila et al., 2004). Supplementing CD-M to Holstein steers fed chopped sudangrass hay:concentrate mixture (1.5:1) decreased acetate 10.2%, increased propionate 28.6%, decreased ammonia-N 19.4%, and decreased CH_4 (L/kg DMI) by 18% compared to the control (Lila et al., 2004).

Tatsuoka et al. (2008) reported that inclusion of fumaric acid at 20 and 30 mmol with cyclodextrin in an *in vitro* study using rumen fluid caused a 42.9 and 73.1% decrease in CH_4 production (mmol/60 mL), respectively. Inclusion of malic acid at 20 and 30 mmol with cyclodextrin in an *in vitro* study using rumen fluid caused a 13.4 and 39.1% decrease in CH_4 production, respectively.

1.5.2 Ionophores

Ionophores are antimicrobial feed additives that can act as CH_4 inhibitors that change the rumen microbial populations to produce more propionate. Ionophores inhibit gram positive bacteria and protozoa, which produce greater proportions of acetate and H_2. By denying methanogens of substrates, such as H_2, CH_4 production is decreased (Honan et al., 2017). Ionophores are widely used in the cattle industry and can decrease CH_4 in the rumen (Histrov et al., 2013). Common ionophores include monensin, lasalocid and laidlomycin propionate. In a review of multiple study with the use of monensin, propionate levels generally increase, and acetate and butyrate concentrations decrease across a wide range of diets with different concentrate:forage ratios (Ellis et al., 2012). Monensin is dose dependent and has the ability to increase feed efficiency by decreasing DMI in feedlot steers while maintaining ADG (Beauchemin et al., 2009; Histrov et al., 2013; Duffield et al., 2012).

Monensin and lasalocid, when fed alone, decreased CH_4 by 30% after 2 weeks in steers fed a high concentrate diet and decreased CH_4 27% by week 4 in steers fed a low concentrate, both in units of L/kg of dry matter intake and % of GEI (Guan et al., 2006). Monensin and monensin + lasalocid did not affect methanogen populations, but total ciliate protozoa were decreased by 82.5% in the 2^{nd} week in steers fed a high concentrate diet and 78.6% in the 4^{th} week while on a low concentrate diet (Guan et al., 2006). Monensin, lasalocid, or monensin + lasalocid did not change total VFA production in low and high concentrate diets, but they did decrease the acetate:propionate ratio and ammonia-N concentration (Guan et al., 2006). Brahman steers fed Rhodes grass hay *ad libitum* and supplemented with 60 or 250 mg of monensin for 40 days had decreased methanogens by 42%, as an average, and showed a decrease in CH_4 production on a g/d and g/kg of DMI basis compared to control (Tomkins et al., 2015). For rumen fermentation, there were no differences in pH or total volatile fatty acid concentration. However, steers fed 250 mg

monensin had a decreased intake by 18% and did have lower acetate:propionate compared to control and had a significant increase in propionate compared to all groups (Tomkins et al., 2015). Monensin supplemented to dairy cows at 15-20 mg/kg of DMI had no effect on CH_4 production but monensin added in a diet at 25-35 mg/kg of DMI showed a 3-8% reduction in CH_4 on a g/kg of DMI basis (Ellis et al., 2012).

1.5.3 Microbial Products – Use Methanogenic Substrates (H and CO_2) In Other Ways

Microbial products (probiotics, cultures, direct fed microbials) are microorganisms that are added in animal diets for the purpose of improved digestion (Honan et al., 2017). Lactate utilizing or lactate producing bacteria aid in rumen health by creating a consistent rumen pH which will cause a possible decrease in the amount of methanogens present due to high pH or a consistent pH in the rumen (Histrov et al., 2013). Feeding cattle propionate producing bacteria is another option because these bacteria can consume H_2 as a reducing equivalent, competing with methanogenesis (Honan et al., 2013; Ungerfeld 2013). Another option to reduce and/or outcompete methanogenesis is by feeding acetogenic bacteria, which use CO_2 and H_2 as substrates to make acetate (reductive acetogenesis) as a way of H_2 disposal (Honan et al., 2013; Joblin 1999).

The mode of action of yeast is to scavenge for oxygen, making the rumen a more anaerobic environment, which stimulates ruminal microorganism growth and in turn balances out ruminal pH, and increases fiber digestibility (Chung et al., 2010). Feeding two different strains of the yeast *Saccharomyces cerevisiae* for 35 days did not affect DMI, body weight (BW), or apparent tract digestibility in dairy cows consuming a diet that contained 50% barley, 19.5% steam-rolled barley grain, and a 30.5% pellet (Chung et al., 2010). Strain 2 (novel strain to aid in *in vitro* fiber degradation and ammonia utilization) decreased g of CH_4/kg of DMI by 10%, decreased ruminal pH and acetate concentration, and increased propionate compared to strain 1 (Levucell SC;

enchances fat corrected milk production and feed efficiency) and the control. However, strain 2 did have an increase in acidosis (Chung et al., 2010). In contrast, McGinn et al. (2004) reported that 2 strains of yeast (Levucell SC and Procreatin-7) had no effect on CH_4 production in Holstein steers fed 75% barley silage with 19% steam rolled barley grain diet compared to the control group that were not fed yeast. More research needs to be conducted to evaluate the extent of yeast supplementation on CH_4 production because a meta-analysis of studies suggested that there was minimal to no effect of yeast supplementation on CH_4 emissions in dairy or beef cattle (Darabighane et al., 2019).

1.5.4 Methane Inhibitors

Methane inhibitors are chemical compounds known to decrease the production of CH_4 by disrupting the process of methanogenesis. Examples of CH_4 inhibitors include halogentated aliphatic compounds, which inhibit the corinoid enzymes and cobamide-dependent methyl group transfer in methanogenesis, hydroxymethylglutaryl-CoA (HMG-S-CoA) reductase inhibitors, which inhibit HMG-S-CoA reductase and the growth of methanogens by blocking the creation of mevalonate, and nitrooxy compounds, which inhibit the last step of methanogenesis that transfers the methyl group to methyl coenzyme M reductase (MCR) to produce CH_4 . Plant secondary metabolites are also CH_4 inhibitors and directly inhibit methanogens, act as hydrogen sink, lower H_2 production, and inhibit growth of protozoa and H_2 producing bacteria (Patra et al., 2017). Methane inhibitors are known to decrease CH_4 by up to 50% when applied to ruminant animals (Histrov et al.,2013).

1.6 Halogenated Aliphatic Compounds

Compounds such as bromocholormethane (BCM), 2-bromoethane sulfonate, bromoform, and chloroform are halogenated aliphatic compounds known to inhibit CH_4 production by blocking the function of cobamide-dependent methyl group transfer enzymes in methanogenesis (Patra et al., 2017). Halogenated compounds can also serve as electron acceptors and competitively inhibit CH_4 production (Patra et al., 2017). However, many halogenated compounds are known to be greenhouse gases, ozone depleting agents (bromochloromethane), or classified as a carcinogen (Histrov et al., 2013), and high doses can have toxic effects on the microbiome and rumen fermentation (Patra et al., 2017). Seaweeds accumulate halogenated aliphatic compounds and are an emerging area of CH_4 mitigation research.

1.7 Seaweeds

Seaweeds are classified as macroalgae and can be red, brown, or green. *Asparagopsis armata* is a red seaweed that accumulates bromoform which as a halogenated compound inhibits the cobamide-independent methyl transferase in the terminal step of methanogenesis (Roque et al., 2019). *Asparagopsis armata* is known to be anti-methanogenic without having negative effects on ruminal fermentation. *Asparagopsis taxifroms* has been recorded to decrease CH_4 production by 80% in sheep (Beauchemin et al., 2019; Roque et al., 2019). However, the bromoform found in *Asparagopsis* is an ozone depleting compound and there is concern that it could be harmful for human health and safety when consuming meat from animals that have been fed seaweed that contain high amounts bromoform, above the standard set by the EPA (Beauchemin et al., 2019). However, studies that have actually measured residues have reported no significant bromoform concentrations found in milk (Roque et al., 2019) or meat (Kinley et al., 2020; Roque et al., 2021).

Asparagopsis taxiformis decreased *in vitro* CH_4 by 65% after 24 hours and 74% after 48 hours and *Zonaria farlowii*, a brown seaweed with lower bromoform concentrations, decreased CH_4 by 11.5% after 24 hours and 10.5% after 48 hours (Brooke et al., 2020). Roque et al. (2021) observed that inclusion of 0.25 and 0.50% of the red seaweed *Aspargopsis taxiforms* in low, medium, and high forage diets fed to Anugus x Hereford steers decreased CH_4 production (g/d) by 81.8, 86.7, and 58.7%, respectively, and decreased CH_4 yield (g/kg of DMI) 80.0, 79.7, and 51.9%, respectively, compared to no seaweed inclusion. There was also a forage amount x seaweed interaction for CH_4 yield (g/kg of DMI), where seaweed supplementation decreased CH_4 yield to a greater extent in high forage compared to low forage diets (Roque et al., 2021). There was also an increase by 7% in the low seaweed and 14% in the high seaweed treatments for feed conversion efficiency (Roque et al., 2021). There was no difference in BW, total gain, carcass weight, rib eye area, shear force resistance, or sensory attributes (Roque et al., 2021). Kinley et al. (2020) observed that CH_4 was decreased by 9, 38, and 98% and hydrogen production increased when *Asparagopsis taxiformis* was included in the diet of grain-fed Brahman x Angus steers at 0.05, 0.10, and 0.20% of the diet DM, respectively. Average daily gain increased by 26 and 22% in the 0.10 and 0.20% seaweed levels but was not significant for the 0.05 level (Kinley et al., 2020). Seaweed increased DMI by 7.5% in the 0.10% seaweed treatment with no effect at low or high levels of seaweed inclusion (Kinley et al., 2020). Feed conversion ratio, total VFAs, and carcass characteristics did not differ because of seaweed inclusion (Kinley et al., 2020).

Using the GreenFeed system, Roque et al. (2019) observed that compared to no inclusion, CH_4 production was decreased by 26.4 and 67.2% on a g/d basis when *Asparagopsis armata* seaweed was supplemented at 0.5 and 1.0%, respectively to lactating Holstein cow diets. On a g/kg of intake basis, CH_4 was decreased by 20.3 and 42.7% at the low and high seaweed inclusion rates

and H_2 production increased by 55.5% at low seaweed and 78.9% at high seaweed inclusion (Roque et al., 2019). In addition, there was no effect on BW, milk fat %, lactose %, solids non-fat %, milk urea nitrogen (mg/dl), and somatic cell count; however, DMI did decrease 10.8 and 38% in the 0.5 and 1.0% seaweed treatments, respectively and cows fed 1% of seaweed produced 11.6% less milk compared to the control cows (Roque et al., 2019).

1.8 3-nitrooxypropanol (3NOP)

A CH_4 inhibitor currently being researched is 3-nitrooxyproponal (3-NOP) (Figure 1.9), which mimics methyl-coenzyme M, a cofactor involved in the last step of methanogenesis that transfers a methyl group to methyl-coenzyme M reductase. Instead of methyl-coenzyme M binding to methyl-coenzyme M reductase, 3-NOP does and as a result blocks the last step of methanogenesis (Martinez-Fernandez et al 2014; Duin et al., 2016 Kim et al., 2019). The extra H_2 released when methanogenesis is inhibited could theoretically be diverted to other VFA, such as propionate, which can be used as an energy source by the animal.

Methane emissions (g/d) measured 3 consecutive days were linearly decreased as 3-NOP supplementation increased in Angus heifers fed a high forage diet supplemented with 4 different levels of 3-NOP (0, 0.75, 2.25, 4.50 mg/kg BW) for 28 days (Romero-Perez et al., 2014). The highest dose (4.50 mg/kg BW) of 3-NOP decreased CH_4 (g/kg DMI or % GE intake) by 33% compared to the control. The number of methanogens was not affected by supplementation with 3-NOP. Dry matter intake was reduced by 5.8% when heifers were supplemented with 2.25mg/kg 3-NOP. Dry matter digestibility decreased for heifers fed 0.75 and 2.25 mg/kg BW 3-NOP, but increased for heifers fed 4.5 mg/kg BW 3-NOP (Romero-Perez et al., 2014). Acetate was decreased 9 and 15% when heifers were supplemented with 2.25 and 4.5 mg/kg BW, respectively and propionate was increased by 22% at the greatest dose of 4.5 mg/kg of BW 3-NOP (Romero-Perez

et al., 2014). There was a decreased acetate:propionate ratio by 3% (0.75 mg/kg), 17% (2.25 mg/kg), and 38% (4.5 mg/kg). In another 112-day study with periods split up into four 28-day intervals, 2 g/d of 3-NOP supplemented to Angus heifers fed a 60% forage diet decreased CH_4 (g/kg DMI) by 59.2% compared to heifers fed the control diet, and CH_4 was decreased as soon as 2 to 4 hours after feeding (Romero-Perez et al., 2015).

The effectiveness of 3-NOP seems to depend on dosage and forage content of the basal diet. Methane (g/kg DMI) emissions were decreased and H_2 emission increased in steers fed an 87% dry-rolled barley finishing diet supplemented with 200 mg/kg BW of 3-NOP for 238 days (Vyas et al., 2016). In the finishing diet, the low inclusion level (100 mg/kg BW of 3-NOP) had no effect on CH_4 emissions (g/d) or CH_4 intensities (g/kg DMI & % of GE intake). Methane (g/d) decreased and H_2 (g/d) emission increased when steers were fed a backgrounding diet of 70% barley silage diet supplemented with 100 and 200 mg/kg BW of 3-NOP for the first 105 days. When corrected for DMI and GEI in the backgrounding diet, only the high (200 mg/kg BW of 3-NOP) dose decreased CH_4 (Vyas et al., 2016). While cattle were fed the 70% barley silage backgrounding diet, 200 mg/kg BW of 3-NOP tended to reduce intake as well in the finishing diet (Vyas et al., 2016). The 200 mg/kg BW dose of 3-NOP improved feed efficiency in the backgrounding diet, but ADG and feed efficiency tended to be reduced in the finishing diet (Vyas et al., 2016). Both doses of 3-NOP (100 and 200 mg/kg of BW) reduced dressing percentage but there was no effect on HCW, fat, LM area, marbling score or saleable meat (Vyas et al., 2016). Kim et al. (2019) also reported that 3-NOP has varying degrees of effectiveness depending on forage content of the diet. Steers fed a 64% corn silage produced 18% less CH_4 (g/d) and greater amounts of ruminal acetate and propionate when supplemented with 100mg/kg of 3-NOP in their diet compared to the control, whereas there was no difference in CH_4 yield (g/kg DMI) or VFA

when steers were fed high grain diets (Kim et al., 2019). Cattle fed 3-NOP on the high forage diet had no differences in DMI, BW, feed consumption rates compared to the control, but 3-NOP did cause a decrease in ruminal pH (Kim et al., 2019). For steers fed the high grain diet there were no differences in DMI, BW, feed consumption, or rumen pH due to 3-NOP (Kim et al., 2019).

1.9 Plant Secondary Metabolites

Plant bioactive compounds such as tannins, saponins, and essential oils have antimicrobial properties against several types of microorganisms, including methanogens (Patra et al., 2017). Tannins are commonly found in browse and warm climate forages, saponins are found in legume plants, and essential oils are extracted through steam distillation of plants such as cinnamon, lemongrass, garlic and eucalyptus (Honan et al., 2021).

1.9.1 Tannins & Saponins

Tannins are also soluble, phenolic compounds in plant tissues that help with the defense of the plant (Honan et al., 2021). Condensed and hydrolysable tannins are 2 types of tannins fed to livestock. Condensed tannins have monomers that are connected by C-C or C-O-C bonds and hydrolysable tannins have hydrolysable ester bonds that are between the main chemical core structure (Bule et al., 2020). Condensed tannins could have an indirect effect of decreasing CH_4 by decreasing fiber digestion to decrease H_2 production, but the mechanism of action is still not fully understood (Odongo et al., 2010). Hydrolysable tannins can inhibit the growth of methanogens (Odongo et al., 2010). Condensed tannins are known to decrease soluble protein and ammonia-N in rumen fluid (McMahon et al., 2000). Tannins can also increase nitrogen retention and reduce urea excretion (McMahon et al., 2000). A combination of chestnut (hydrolysable) and quebracho (condensed) tannins fed to steers on a high forage diet (47.5% alfalfa silage and 47.5%

barley silage) at 1.5% inclusion in their diet decreased ruminal ammonia, and decreased CH_4 yield (CH_4 g/kg DMI) by 6.4% compared to the control but had no effect on rumen pH, total VFA, rumen protozoa, molar proportions of acetate and propionate, average daily gain, or efficiency (Aboagye et al., 2018). Chestnut alone at 1.5% inclusion did decrease CH_4 by 1.3% (Aboagye et al., 2018). Beauchemin et al. (2007) observed that red quebracho tannin, a condensed tannin, when fed to steers and heifers in a 70% forage diet at 1 or 2% of the diet DM had no effect on CH_4 production, BW, gain, or intake. However, there was a decrease in ruminal NH_3 as the quebracho tannin concentration increased in the diet and apparent digestibility of crude protein decreased by 5 and 15% when quebracho was added as at 1 or 2% of the diet, respectively (Beauchemin et al., 2007). Although tannins can decrease CH_4 production, they can have a negative effect of decreased digestion. For example, Hristov et al. (2013) observed that while CH_4 was decreased significantly by up to 30% when tannins were fed, milk production was also reduced by 10% (Histrov et al., 2013).

Bos taurus x Bos indicus steers fed *Pennistetum purpureum* forage with 20%, 40%, 60% and 80% of dry matter in the diet replaced with a high condensed tannin legume forage, *Leucaena leuococephala* had decreased CH_4 (L/kg of DMI) 26.2, 36.3, 45.4, and 61.6%, respectively, compared to 0% *Leucaena leuococephala* inclusion (Pineiro-Vasquez et al., 2018). *Leucaena leuococephala* condensed tannins had no effect on DMI or organic matter intake, but crude protein intake did increase as the level of *Leucaena leuococephala* increased in the diet (Pineiro-Vasquez et al., 2018). There was no difference in rumen pH, molar proportions of fatty acids, or numbers of ruminal protozoa with addition of *Leucaena leuococephala,* but rumen ammonia-N increased as condensed tannins increased up to 138% when the diet contained 80% of *Leucaena leuococephala* (Pineiro-Vasquez et al., 2018).

Saponins affect rumen fermentation by reducing the number of protozoa which in turn reduces the H_2 availability and CH_4 production (Cieslak et al., 2013; Honan et al., 2017). Saponins come from legume plants such as chick peas, green peas, kidney and soya beans (Honan et al., 2017). Although saponins can be used to decrease CH_4 production, they have been reported to decrease organic matter digestibility (Histrov et al., 2013).

1.9.2 Flavonoids

Flavonoids are known to be anti-inflammatory, antioxidative and antimicrobial and can interfere with bacterial enzymes, toxins, and signal receptors (Ku-Vera et al., 2020). As such, flavonoids may have an impact on improving gut health and having a beneficial effect in certain metabolic diseases such as obesity and diabetes (Jimenez-Ocampo et al., 2021). Flavonoids are labeled as polyphenols with a C6-C3-C6 skeleton, derivatives of benzo-L-pyrone from fruits, vegetable and seeds and are also known to prevent bloat and acidosis in cattle that are placed on high concentrate diets (Seradj et al., 2014). Citrus plants are a rich source of flavonoids that include naringen, naringenin, nobelitin, narirutin, and hesperidin; naringin being responsible for the distinctive sour flavor and bitter taste of grapefruit, bitter orange, and other citrus fruits (Jimenez-Ocampo et al., 2021). Addition of 4.5% (DM basis) of either of the flavonoids naringin or querectin to ruminal fluid from cows fed a 60:40 grass:concentrate decreased methane and suppressed methanogen and protozoa populations (Oskoueian et al., 2013). Bioflavex, a commercial product that contains flavonoid components from citrus, added to rumen fluid incubations from steers fed a 90% concentrate diets with 10% barley straw reduced methanogens by 13% and decreased methane by 26% (Seradj et al., 2014). Bioflavex also decreased pH, increased molar proportion of propionate and lowered concentrations of acetate (Seradj et al., 2014). Adding naringen (1.5 and 3.0 g/kg) to *in vitro* fermentations from cows fed a 70:30 forage:concentrate diet was reported to

increase propionate and decrease acetate but had no effect on CH_4 production (Jimenez-Ocampo et al., 2021). However, an in vivo trial with *Bos taurus* x *Bos indicus* crossbred heifers fed a 70:30 forage:concentrate diet with rumen cannulas demonstrated no effect of naringin on rumen pH, acetate:propionate ratio, or CH_4 production (Jimenez-Ocampo et al., 2021).

1.9.3 Essential Oils

Essential oils are chemical compounds, terpenoids and phenylpropanoids, that are extracted from plants where they have been responsible for odor, color, and spices of the plant (Calsamiglia et al., 2007). Essential oils have also been reported to decrease CH_4 due to their antimicrobial properties and ability to positively affect ruminal fermentation by increasing total VFA production and decreasing the rate of deamination (Castillejos et al., 2005; McIntosh et al., 2003). Essential oils can also have a potential effect by directly inhibiting methanogens (Cieslak et al., 2013). Some examples of essential oils and the plants that they come from are cinnamaldehyde (cinnamon), zingiberene (ginger), allicin (garlic), cadinene (juniper), eucalyptol (eucalyptus), carvacrol (oregano), 1,8-Cineole (rosemary), thymol (thyme), capsaicin (paprika), terpinene-4-ol (tea tree), anethol (anise), eugenol (clove) (Honan et al., 2017).

Essential oils that showed promise to influence ruminal fermentation and possibly decrease CH_4 production were first identified in *in vitro* studies. On days 2 through 5 of an *in vitro* study where fermenters were dosed a diet of 52:48 concentrate ratio, total VFA and ammonia N were not affected, acetate was higher for garlic, cinnamon, anise and oregano essential oils, and propionate was lower for cinnamon, garlic, anise, and oregano and oregano oils (Cardozo et al., 2004). These results suggest possible modifications of acetate and propionate levels from garlic, cinnamon, oregano, and anise extracts which in turn could affect rumen pH, methanogen populations and CH_4 production.

Eucalyptus essential oil + cyclodextrin (10 mg) added to rumen fluid increased *in vitro* propionate production from 23.7 to 30% and decreased *in vitro* CH_4 production 40% (Tatsuoka et al., 2008). Wasabi essential oil + cyclodextrin (10 mg) added to rumen fluid increased *in vitro* propionate production from 23.7 to 30%, decreased acetate from 53.2 to 48%, and decreased *in vitro* CH_4 production by 85% (Tatsuoka et al., 2008). Cineol + cyclodextrin (5 mg) added to rumen fluid decreased *in vitro* propionate from 21% to 17-18% and doubled CH_4 production (Tatsuoka et al., 2008). The other essential oils investigated (thyme, peppermint, and menthol) did not cause significant changes in CH_4 production (Tatsuoka et al., 2008).

A 30 ppm blend of coriander + geranyl acetate + eugenol essential oil blend showed a 17% decrease in CH_4 production during a 72 h *in vitro* experiment (Castro-Montoya et al., 2015). When the same essential blend was fed to dairy cows consuming an 83% corn and grass silage diet at 0.2 g/d for 8 weeks, CH_4 production (g/d) was decreased by 15% and CH_4 produced on a g/kg of DMI basis was decreased by 14%. Supplementing beef cattle, fed *ad libitum* maize silage, with 200mg/d of coriander +geranyl acetate + eugenol blend decreased CH_4 (BW basis) between 13 and 20% for weeks 2 thru 6 (Castro-Montoya et al., 2015). Cinnamon oil (250 mg/L), juniper berry oil (20 mg/L) and p-cymene (20 mg/L) decreased *in vitro* CH_4 production by 72, 49 and 30%, respectively when the diet was 46.6% whole crop barley silage (Chaves et al., 2007). Cinnamaldehyde added to a continuous culture fermenter at high (312 mg/L) and low (31.2 mg/L) concentrations decreased acetate and branched chain VFAs, and increased propionate and butyrate concentrations (Busquet et al., 2005a), suggesting that CH_4 has the potential to be decreased.

A mixture of thymol, eugenol, vanillin and limonene (Vertan; IDENA, Sautron, France) fed at 2 g/d increased feed efficiency in Angus x Hereford steers fed a 75% grass/legume silage with 24% rolled barley compared to steers not fed essential oils or monensin (Benchaar et al.,

2006). However, increasing supplementation of the essential oil blend to 4 g/d decreased feed efficiency compared to the control treatment. Steers fed monensin did not differ in feed efficiency compared to steers fed the essential oil blend or the control supplement (Benchaar et al., 2006). Dry matter intake did not differ between steers fed monensin and control in terms of % BW (Benchaar et al., 2006). Dry matter intake was greater for steers fed essential oil compared to steers fed the control diet, and dry matter and organic matter digestibility did not differ among treatments (Benchaar et al., 2006). The essential oil supplementation caused a higher N digestibility compared to monensin and control, however the retention of N, % of N intake, and % of N digested were not affected (Benchaar et al., 2006). An essential oil blend that contains a mixture of thymol, eugenol, vanillin, guaiacol, and limonene (CRINA Ruminants, DSM) fed to Angus steers on a 75% barley silage diet had no effects on CH_4 or ruminal fermentation after 21 days, but did decrease digestibility compared to the control (Beauchemin et al., 2006). In agreement, the same commercial mixture of essential oils (CRINA, DSM) fed at 1 or 2 g/d for 26 days to Brahman steers consuming Rhodes grass hay *ad libitum* had no effect on gain, intake, ruminal pH, ruminal VFA concentrations, CH_4 production, or methanogen populations compared to the control (Tomkins et al., 2015).

It is hypothesized that garlic may act as a CH_4 inhibitor through its organosulfur compounds inhibiting 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is a thiol disulfide exchange reaction involved in methanogen membrane synthesis (Busquet et al., 2005a). Garlic oil added to a continuous culture fermenter at a concentration of 312 mg/L decreased acetate and increased propionate and butyrate (Busquet et al., 2005a) indicating that there was a shift in H_2 consumption away from CH_4 to propionate. There were no significant changes that were observed at the lower concentration of garlic oil of 31.2 mg/L (Busquet et al.,

2005a). Busquet et al. (2005b) reported that 300 mg/L of garlic oil or compounds found in garlic oil including diallyl disulfide, and allyl mercaptan decreased CH_4 by 73.6, 68.5, and 19.5%, respectively in a 24 h incubation with diluted ruminal fluid with a 50:50 forage:concentrate diet. Garlic oil and allyl mercaptan each at 300 mg/L increased proportions of propionate, all compounds at 300 mg/L increased butyrate and decreased acetate proportions. However, garlic oil, diallyl disulfide, and allyl mercaptan, all showed a decrease in total VFA concentration, lower DM disappearance, and lower ADF and NDF digestibility compared to the control (Busquet et al., 2005b).

Garlic oil supplemented at 100 and 250 mg/L decreased *in vitro* CH_4 by 70% for both doses compared to the control (Chaves et al., 2007). Garlic oil also reduced the number of methanogens, from 20.3 in the control vs. 6.4 and 6.3 in the 100 and 250 mg/L garlic oil treatments and decreased propionate (Chaves et al., 2007). Patra et al. (2009) reported that a combination of garlic (1% of DMI) and harad (Terminalia Chebula), which is an herb used in traditional medicine in Iran and India, at 1% of DMI, increased NDF, ADF, DM, and OM digestibility and decreased CH_4 production (L/kg of digested DMI) by 23.6% compared to control but did not affect DMI in sheep fed a 1:1 forage to concentrate diet for 27 days. When harad was fed alone at 1% of DMI there was a 23.9% decrease in CH_4 (L/kg digested DMI) compared to control and when garlic was fed alone at 1% of DMI there was a 11.9% decrease in CH_4 (L/kg digested DMI). Allicin, an oxygenated sulfur compound found in garlic, increased apparent digestibility of organic matter, nitrogen, NDF and ADF, and decreased CH_4 production by 5.95% when supplemented at 2 g/head/day to crossbred ewes fed 68.7% forage diets (Ma et al., 2016). Ma et al. (2016) also observed that allicin decreased methanogen populations by 104%, tended to decrease protozoa,

and increased cellulolytic bacteria. Supplementing allicin did not affect ruminal pH, decreased ammonia and total VFA, and showed greater nitrogen intake and retention (Ma et al., 2016).

1.9.4 Mootral

Mootral is a commercial feed additive that contains garlic extract high in allicin content and citrus extracts from orange processing high in flavonoid content. When Mootral was supplemented to batch fermenters at 10 or 20% of a 50:50 grass:concentrate substrate, CH_4 emissions were decreased 22% and 54.4%, respectively (Ahmed et al., 2021). Mootral increased *in vitro* production of propionate, decreased acetate and the acetate:propionate ratio, increased VFA production, and had no effect on pH, digestibility, or ammonia-N of batch fermenters (Ahmed et al., 2021). Mootral decreased the population of *Methanobacteriaceae* for both doses compared to the control but increased *Methanomassiliicoccaceae,* which are major methanogenic groups (Ahmed et al., 2021). An increase in *Prevotellaceae,* a family that produces propionate, and an increase in *Veillonellaceae* was observed when Mootral was fed at the 20% of substrate dose and was suggested to be responsible for the increase in propionate (Ahmed et al., 2021). Although 15 g/d of Mootral fed to Angus x Hereford steers fed 90% concentrate, 10% hay diet had decreased the CH_4 yield (g/kg DMI) throughout a 12-week study, it was not statistically decreased until the 12^{th} week of the study, when Mootral decreased CH_4 yield by 13.3% (Roque et al., 2019). There was no difference in BW, average daily gain, or feed conversion efficiency and there was no difference in CO_2 and O_2 emissions between treatments (Roque et al., 2019).

Vrancken et al., (2019) observed a 38.3% decrease in CH_4 (ppm) and 5% increase in milk yield for jersey cows and a 20.7% decrease in CH_4 and a 7.8% increase in milk yield for Holstein-Friesian cows when Mootral was added to a 55% grass silage diet for 12 weeks with Mootral being

supplemented as 3% of diet. Differences in CH_4 production are based on a measurements taken prior to Mootral feeding, at the end of Mootral feeding (12 weeks), and a post-Mootral feeding measurement taken 4 weeks after Mootral supplementation concluded (16 weeks). Compared to the pre-Mootral feeding period, Mootral decreased bulk tank somatic cell counts by 32.6% for Holstein-Friesian cows at 12 weeks. Feed efficiency increased 13% during the 12 weeks of Mootral feeding compared to the pre-Mootral feeding period and increased feed efficiency 24% after Mootral withdrawal compared to the pre-feeding period in Jersey cows. In the Holstein-Friesian cows there was a 1.6% increase in feed efficiency during Mootral feeding compared to the pre-feeding period and an 8% increase in feed efficiency after Mootral withdrawal compared to the pre-Mootral levels (Vranken et al., 2019).

Feeding 4 g/d of Mootral for 70 days to Holstein bull calves fed milk replacer and a 52.3% concentrate starter was found to decrease CH_4 by 22.8% on a g/d basis and by 32.3% on a g/kg of BW basis compared to no Mootral inclusion (Brand et al., 2021). The amount of CO_2 produced on a g/d basis was not affected by Mootral. There was also no difference in DMI, BW, carcass weight, daily gain, carcass gain, carcass conformation, or carcass fat compared to the control calves (Brand et al., 2021).

1.10 Conclusion

Livestock are estimated to be responsible for 3.9% of all GHG produced (US GHG Inventory, EPA., 2020). Because CH_4 is a byproduct of ruminal fermentation, ruminant animals (dairy and beef cattle) are the primary livestock emitters of CH_4, responsible for 172.3 MMT MMT CO_2 Eq. or 97% of all livestock enteric CH_4 in 2019 (EPA, 2021). Methane yield (g of CH_4 per kg DMI) increases as forage content increases and many mitigation strategies have different effects depending on forage:concentrate of the diet. Thus, there is a need to develop mitigation strategies

that are effective in a range of forage:concentrate diets. Mootral, a combination of organosulfur and flavonoid compounds, which effectively mitigate CH_4 production to varying degrees in ruminants, is a promising dietary additive. However, it is unclear how forage content and Mootral inclusion interact to effect CH_4 production and animal performance. Therefore, the objective of the current study was to quantify CH_4 production and determine growth, intake, and carcass characteristics of feedlot steers fed Mootral in diets with varying forage content.

1.11 References

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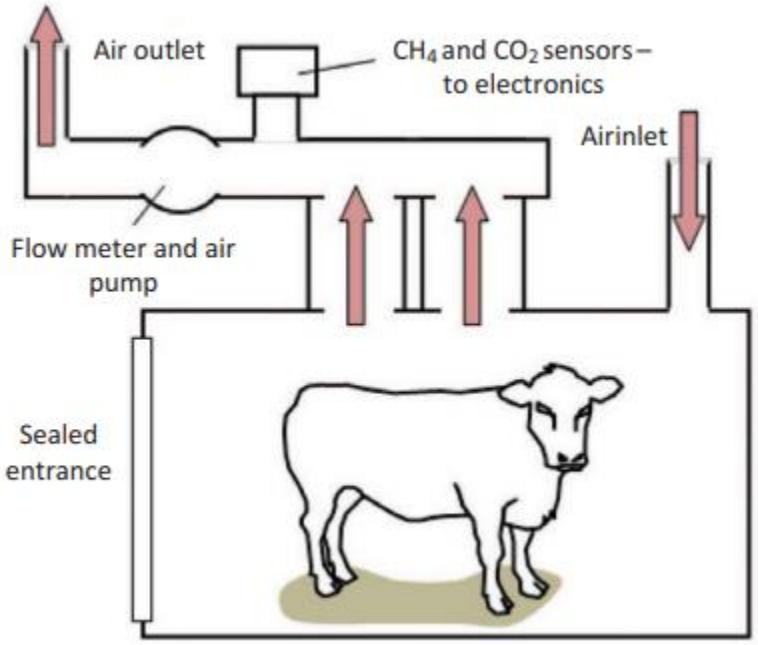


Figure 1.1. Respiration Chamber (Hill et al., 2016)

Figure 1.2. Portable Accumulation Chamber (J.P. Goopy et al., 2011)

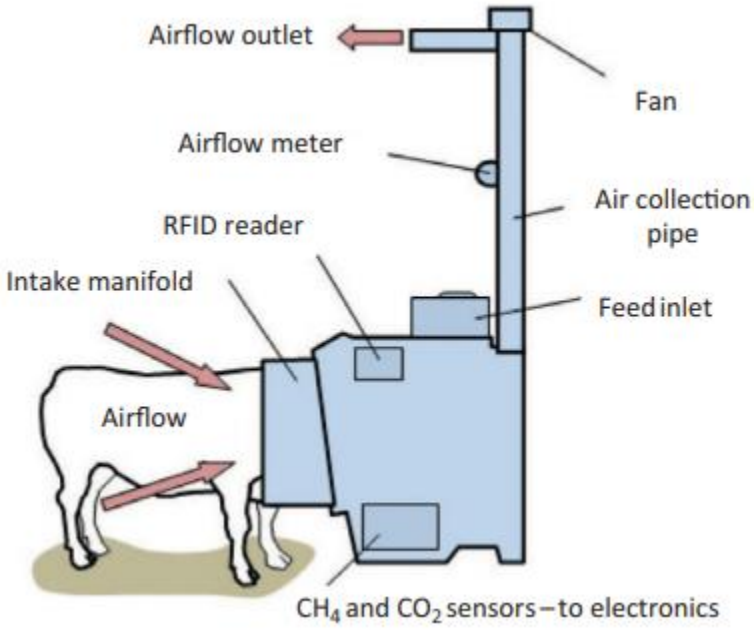


Figure 1.3. Automated Head Chamber System (Hill et al., 2016)

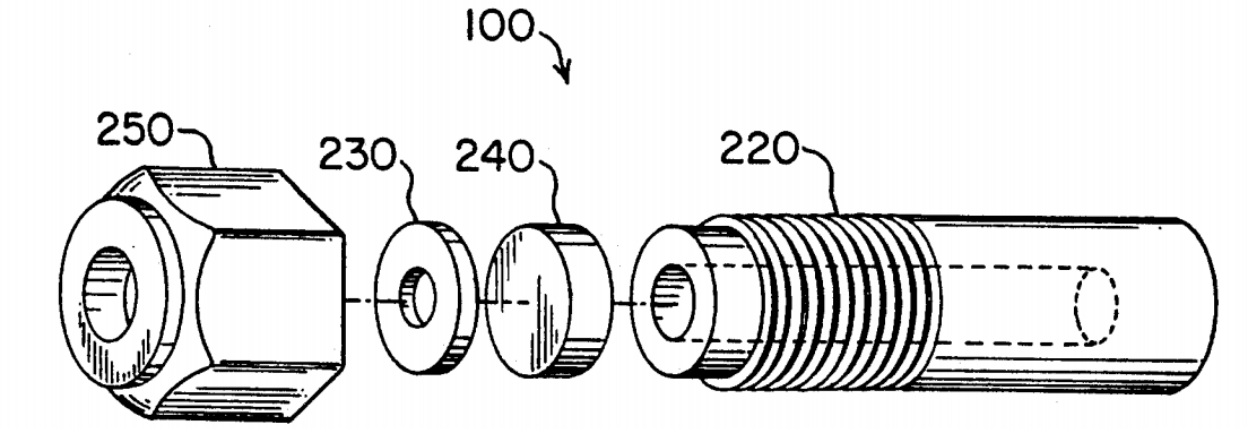


Figure 1.4. SF6 Permeation Tube (Zimmerman 1993)

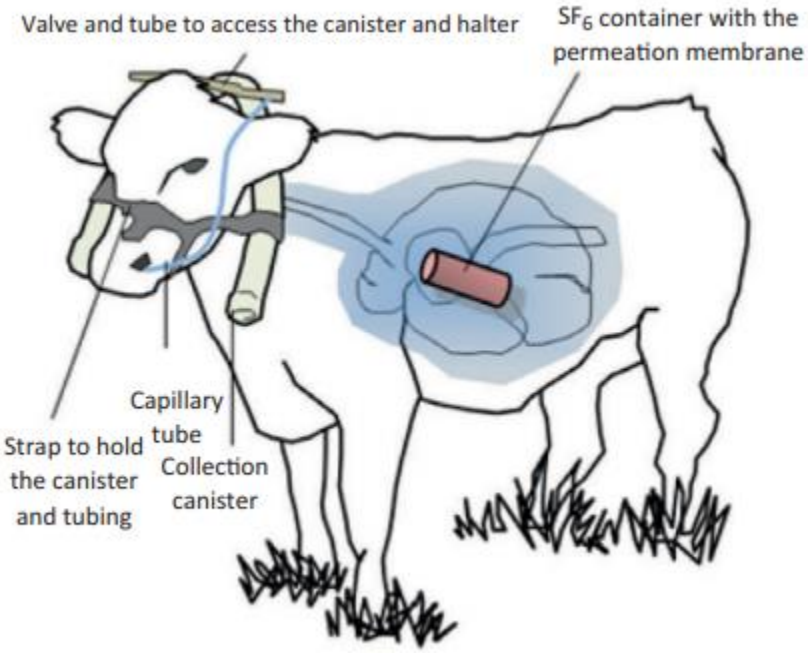


Figure 1.5. SF6 Tracer Technique (Hill et al., 2016)

Figure 1.6. Laser Methane Detector (Chagunda 2013)

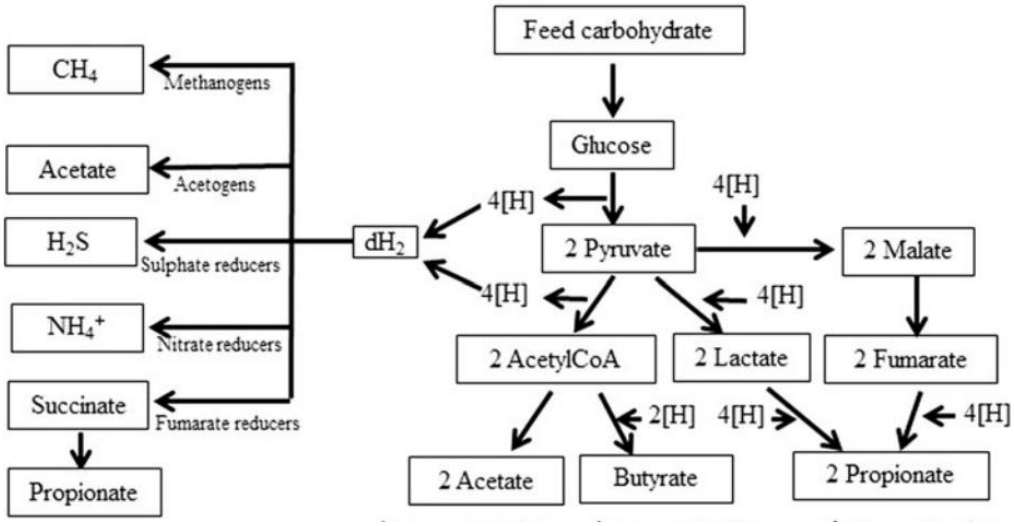


Figure 1.7. Pathways of Rumen Fermentation (Beauchemin et al., 2020)

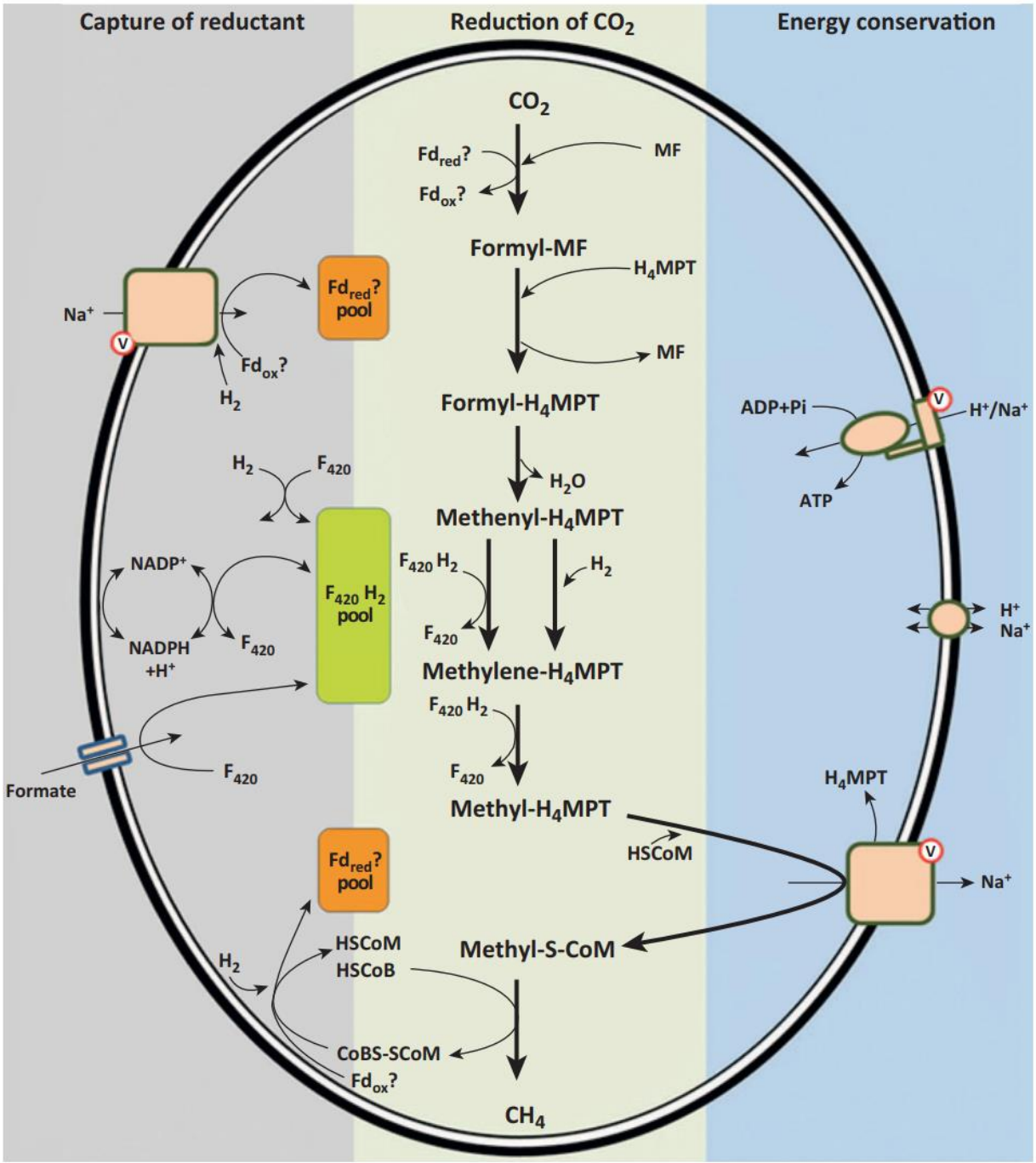
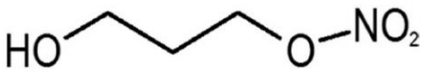


Figure 1.8. Methane Pathway (Hill et al., 2016)

Methyl-coenzyme M



3-Nitrooxypropanol

Figure 1.9. Methyl-coenzyme M & 3-NOP (Romero-Perez et al.,2014)

CHAPTER 2. EFFECT OF MOOTRAL AND FORAGE AMOUNT ON METHANE EMISSIONS, GROWTH AND CARCASS CHARACTERISTICS OF FEEDLOT STEERS

2.1 Introduction

Methane production from enteric fermentation in ruminant animals is a contributor to global CH_4, which is a greenhouse gas. Livestock are currently estimated to produce 3.9 to 14.5% of global anthropogenic CH_4 emissions (Gerber et al., 2013; US EPA, 2020). Beef cattle in the US are responsible for approximately 72.7% of livestock methane emissions or 1.9% of U.S. CH_4 production (EPA, 2020). Because CH_4 has an impact on increasing global temperatures, there is a push for government regulations to reduce CH_4 from livestock animals. Enteric CH_4 accounts for 2-12% of energy loss from the ruminant animal (Johnson & Johnson, 1995). Energy loss from the diet is contingent upon forage content, where increasing forages in the diet increases CH_4 yield (g/kg of GEI) up to approximately 12% for dairy cattle and 32% for beef cattle (van Gastelen et al., 2019).

There are currently strategies being researched to decrease enteric CH_4 such as changes in diet and addition of supplements to alter rumen fermentation. Mootral is a feed supplement that contains garlic (*Allium sativum*) and bitter orange (*Citrus aurantium*) extracts. The organosulfur compounds in garlic are known to decrease methane production by inhibiting 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is a thiol disulfide exchange reaction involved in the membrane synthesis of methanogens (Busquet et al., 2005a). Garlic addition to ruminant diets reduces methanogens, increases propionate, and decreases acetate concentrations (Chaves et al., 2007; Busquet et al., 2005). Flavonoids are known to be anti-inflammatory, antioxidative and antimicrobial and can interfere with bacterial enzymes, toxins, and signal receptors (Ku-Vera et

al., 2020). Flavanoids are labeled as polyphenol benzo-L-pyrone derivatives from fruits, vegetable and seeds. Citrus plants are a rich source of flavonoids that include naringin, naringenin, nobelitin, narirutin, and hesperidin; naringin being responsible for the distinctive sour flavor and bitter taste of grapefruit, bitter orange, and other citrus fruits (Jimenez-Ocampo et al., 2021). The flavonoids found in bitter orange extracts are known to decrease ruminal methanogen populations, inhibit CH_4 production, and reduce acidosis in cattle through their anti-microbial properties (Balcells et al., 2012; Seradj et al., 2014). Mootral has been reported to decrease CH_4 production in ruminant animals (Roque et al., 2019; Brand et al., 2021), reduce the number of methanogens, and allow rumen fermentation to continue (Eger et al., 2018; Ahmed et al., 2021). However, it is unclear how the forage content and Mootral inclusion will interact to effect CH_4 production and animal performance. Because feedlot cattle are fed a range of forage:concentrate ratios while in the feedlot, it is important to know how effective mitigation strategies are in different forage:concentrate diets. Therefore, the objective of the current study was to quantify CH_4 production and determine growth, intake, and carcass characteristics of feedlot steers fed Mootral in diets with a low, medium, and high forage content. Knowing the effect of garlic and flavonoids on methanogenesis, we hypothesize that Mootral will decrease CH_4 emissions without impacting growth, intake, and carcass characteristics of feedlot steers. We expect that the CH_4 mitigating ability of Mootral will be greatest in the diet with the most forage.

2.2 Materials and Methods

All procedures performed in this study were approved by the Purdue University Animal Care and Use committee (protocol number 19080019361) and were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS,

2010). The experiment was conducted at the Purdue University Animal Sciences Research and Education Center (ASREC) in West Lafayette, IN.

2.2.1 Animals and Diets

One Hundred and forty-four Angus x Simmental steers (363 ± 0.09 kg) from ASREC or Feldun Purdue Agricultural Research Center were used to determine the effect of Mootral and forage content on methane emissions, growth and carcass characteristics of feedlot steers. Steers were stratified by body weight, breed composition (% Simmental), and farm origin and allotted to a 3 x 2 factorial arrangement of treatments with 3 concentrations of corn silage (15, 41.5, or 68%) and 2 concentrations of Mootral (0 or 0.25% of the diet DM). All diets (Table 1) contained 5% vitamin/mineral supplement and Mootral replaced a portion of dried distillers grains with solubles (DDGS). The balance of the 15% corn silage diets consisted of 58% dry rolled corn and 22 or 21.75% DDGS, the balance of the 41.5% corn silage diets consisted of 29% dry rolled corn and 24.5 or 24.25% DDGS, and the balance of the 68% corn silage diets consisted of no corn and 27 or 26.75% DDGS. Steers were housed in 24 pens (4 pens/treatment; 6 animals/pen; 24 animals/treatment) in 2 barns. Pens (6.1 x 3.7 m) were located in a slatted floor, curtain-sided finishing barn and provided 0.55 m of bunk space and access to water. On day 85 of the experiment all animals were switched to the 15% corn silage diet with the 2 concentrations of Mootral consisting of 0.25% or 0.0% of the diet DM. Diets were formulated to meet or exceed NASEM (2016) requirements for CP, vitamins, and minerals. Feed was offered once daily at 0900 h, and steers were allowed *ad libitum* access to feed and water. Daily feed deliveries were adjusted using a 4-point bunk scoring system to allow for *ad libitum* feed intake with little or no accumulation of feed (Pritchard, 1993). Feed delivery was recorded daily, and feed samples were collected every two weeks for DM analysis. Diet ingredient subsamples were taken, composited, and analyzed by

wet chemistry for CP, NDF, P, and Ca (Cumberland Valley Analytical Services, Waynesboro, PA).

Steers were previously vaccinated and given a booster against Infectious Bovine Rhinotracheitis, Bovine Viral Diarrhea Types I and II, Parainfluenza-3, Bovine Respiratory Syncytial Virus, *Mannheimia haemolytica*, and *Pasturella multocida* (Vista Once, Merck Animal Health, Summit, NJ), against *Clostridia* and *Haemophilus somnus* (Vision-7 Somnus; Merck Animal Health), treated with a pour-on (Cydectin, Bayer, Shawnee Mission, KS) for external parasites, and drenched with a de-wormer (Safeguard, Merck Animal Health, Madison, NJ) for internal parasites. All steers were implanted with Revalor-XS (trenbolone acetate and estradiol, Merck Animal Health, Madison, NJ) at feedlot entry. Steers were weighed twice on consecutive days at the initiation of the study and at slaughter and weighed once approximately every 21 days to monitor growth and health. Scales (480 Legend, Rice Lake Weighing Systems, Rice Lake, WI) weighed to the nearest 0.5 kg and were checked for accuracy at each weigh date. Performance data was analyzed for the first half (d 0 to 84), second half (d 85 to slaughter), and overall (d 0 to slaughter).

Individual steers within pens were selected for slaughter based on body weight with 5 steers per pen transported 400 km to a commercial abattoir (Tyson Foods Inc., Joslin, IL) and 1 steer per pen transported 7 km to the Purdue University meats laboratory. Steers sent the commercial abattoir were slaughtered at 3 different time points (155, 182, and 209 d) and steers sent to Purdue were slaughtered at 3 different time points (162, 169, and 176 d). The weighted average of days on feed (pen days) was 182 days for all animals. All carcasses were chilled for 24 h, and qualified University personnel measured subcutaneous fat thickness between the 12th and 13th rib, *Longissimus dorsi* area via direct grid reading between the 12th and 13th rib, kidney-pelvic-heart

fat as a percent of hot carcass weight, marbling score, and USDA quality and yield grades (USDA, 1997).

2.2.2 Methane Emissions Measurement

Enteric CH_4 emissions were measured using the sulfur hexafluoride (SF_6) technique (Johnson et al., 1994) two times for five days from days 42 to 46 and days 203 to 207. Forty-eight steers, 2 from each pen that represented the average body weight and breed composition of each pen, were orally dosed with brass permeation tubes containing SF_6 using a balling gun 3 days before the start of the first methane collection period. Four to 6 weeks prior to the second methane collection, 1 steer per pen with a permeation tube was slaughtered at Purdue (see slaughter protocol), the permeation tube was removed and was placed in another lighter steer from the same pen. Permeation tubes were built from brass cylinders (length = 4.4 cm, outside diameter = 1.43 cm, inside diameter = 0.79 cm, inside depth = 3.8 cm, volume = 1.86 mL), fitted with nylon washers and a Teflon membrane secured with a porous (2-um porosity) stainless steel frit and a brass nut. Permeation tubes were filled with ~3.1 g of SF_6 and had an average release rate of 8.7 mg/d of SF_6. Permeation tubes were kept at 39^{o}C and weighed twice a week for 6 weeks prior to the study to determine the release rate. Steers were fitted with gas collection cannisters constructed of polyvinyl chloride pipe to have a volume of 2 L. The gas cannisters were evacuated to 68.6 cmHg, creating a vacuum that draws eructed gases and respired breath through a crimped capillary tube connected to a silicone loop positioned near the animals' nostrils (McGinn et al., 2006). The volume of the collection cannisters and extent of crimping in the capillary tubes were designed to allow half of the vacuum to remain after 24 h. The gas cannister, capillary tube, and silicone loop were fastened to a halter secured around the ears and nose of the animal. Gas collection cannisters were replaced every 24 h in order to take a sub-sample of collected gases and record methane

emission/production. Sub samples of gas from collection cannisters were collected by pressurizing the collection cannisters with nitrogen to one atmosphere then extracting samples from cannisters using a syringe and transferring them to an evacuated glass collection tube (~60 ml). Haisan et al. (2014) proposed to only include animals in the data set with at least 2 d of valid CH_4 measurements. For the current experiment, only steers with at least 3 successful days of collection and measurement were considered in the final analysis.

2.2.3 Nutrient Analyses

Diet samples were collected from all bunks starting at 0700 h on days 1 thru 4 of gas collection. Fecal samples were collected from the surface of each pen starting at 0600 on days 2 thru 5 of gas collection and were frozen at -20^{o}C. Fresh feed samples and frozen fecal samples were dried at 60^{o}C for 48 h to calculate dry matter content. Feed and fecal samples were then ground in a Wiley mill (Retsch GmbH & Co. KG, 42781 Hann, Rheinische Str. 36, Germany) through a 2mm screen for subsequent analysis of CP, NDF, and ADF. Samples of feed and feces were weighed (0.5 g) into F57 bags (Ankom Technology Corp., Macdedon, NY) and analyzed for NDF, using a heat stable α-amylase and sodium sulfite. Subsequent ADF analysis was performed sequentially as described by Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technology Corp.). Feed and fecal samples were analyzed for total N using the Dumas dry combustion method with a N analyzer (LECO Corporation; St. Joseph, Michigan). Crude protein was then calculated by multiplying the N concentration of the dry sample by 6.25.

2.2.4 Methane and SF_6 Analysis

All methane and SF_6 gases in the collection cannisters were analyzed by gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA). A flame and

ionization was used for CH_4 and an electron capture detector was used for the SF_6 with a capillary column (Plot Fused Silica 25 m x 0.32 mm, Coating Molsieve 5A, Varian CP7536; Varian Inc., Lake Forest, CA). The injector, column, and detector temperature for CH_4 and SF_6 analysis were 80, 160, and 200^{o}C respectively. For SF_6, 50, 30, 300^{o}C were the temperatures for the injector, column, and detector, respectively. The carrier gas for the CH_4 and SF_6 was N_2. Methane emitted by the steers was determined in relation to the SF_6 tracer gas that was captured in the collection cannisters. The equation used was:

$$Q_{CH4} = Q_{SF6} \text{ x } (([CH_4]_\gamma - [CH_4]_\beta) \div ([SF_6]_\gamma - [SF_6]_\beta))$$

where Q_{CH4} is the methane emissions from the individual steer (g/d), the Q_{SF6} is the SF_6 release rate (mg/d), the $[CH_4]_\gamma$ is the concentration of the CH_4 in the in the steer's collection cannister, $[CH_4]_\beta$ is the concentration of methane in the ambient cannisters, $[SF_6]_\gamma$ is the concentration of SF_6 in the steer's cannister, and $[SF_6]_\beta$ is the concentration of SF_6 in the ambient cannisters.

2.2.5 Statistical Analysis

Data were analyzed as a complete randomized design with 3 x 2 factorial arrangement of treatments using GLIMMIX procedure of SAS, with pen as the experimental unit. Periodical performance data were analyzed as repeated measures over time by comparing five covariance structures for each variable (variance components, compound symmetric, heterogenous compound symmetric, spatial power, and unstructured). Spatial power consistently yielded the lowest Bayesian Information Criteria was used for all results. The repeated measures model included random effects of pen and fixed effects of forage amount, Mootral inclusion, time, as well as the forage amount × time, Mootral inclusion × time, forage amount × Mootral inclusion, and forage amount × Mootral inclusion × time interactions. The Satterthwaite approach was used to estimate denominator degrees of freedom. The SLICEDIFF function of SAS was used to analyze only the

within time pairwise comparisons that were meaningful and the SLICE function of SAS was used to determine the simple effects of treatments within time, which is what is presented in the results section. Treatment comparisons were corrected using the Tukey adjustment and the least square means difference was used to calculate adjusted means. Methane emissions, overall performance, and carcass characteristics were analyzed using the GLIMMIX procedure of SAS as a randomized design without repeated measures. The non-repeated measures model included the random effects of pen and the fixed effect of forage amount and Mootral content. Treatment comparisons were made using Fisher's protected least significant difference, and the least square means statement was used to calculate adjusted means. Simple effect means (forage x Mootral) are presented for days 0 to 84 and main effect means (forage, Mootral) are presented for days 85 to slaughter. Significance was declared at $P \leq 0.05$ and a tendency was declared $0.05 < P \leq 0.10$.

2.3 Results

Neither forage content or Mootral affected ($P \geq 0.24$) body weight on day 0 or 84 (Table 2). Average daily gain from day 0 to 84 did not differ among forage contents or between Mootral treatments ($P \geq 0.22$). There was an interaction effect for dry matter intake from day 0 to 84 ($P = 0.03$) where Mootral decreased intake in the 15% corn silage diet, but not in the 41.5 or 68% corn silage diets. Steers fed the 68% corn silage diet had a greater gain:feed from day 0 to 84 ($P = 0.04$) compared to steers fed the 15 and 41.5% corn silage diets. Mootral did not affect methane emissions on days 42 to 46 ($P \geq 0.47$), but there was a forage effect. Steers fed the 41.5 and 68% corn silage diets emitted more methane on a g/d ($P = 0.05$) and a g/kg of DMI ($P = 0.007$) basis compared to steers fed the 15% corn silage diet. On days 42 to 46, steers fed the 41.5 and 68% diets tended ($P = 0.07$) to produce more methane on g/kg BW basis compared to steers fed the 15% corn silage treatment.

After steers were all switched to the 15% corn silage diets (Table 3), Mootral did not affect body weight ($P = 0.99$), average daily gain ($P = 0.36$), dry matter intake ($P = 0.59$) or gain:feed ($P = 0.11$). Mootral decreased the amount of methane produced on a g/d, g/kg DMI, and g/kg BW basis by 25.6, 24.6, and 26.4% on days 203 to 207 compared to the control treatment ($P \leq 0.03$).

Overall (Table 4), Mootral did not affect daily gain, daily intake, or gain:feed ($P \geq 0.12$). Forage content did not affect gain or intake ($P \geq 0.24$), but steers fed the 68% corn silage treatment showed a higher gain:feed compared to steers fed the 15 or 41.5% corn silage diets ($P = 0.04$). For days on feed there was no Mootral or forage effect ($P = 0.58$). There was a tendency for Mootral to reduce fat depth and yield grade ($P \leq 0.09$) and another tendency for forage to reduce kidney, pelvic, heart fat % ($P = 0.07$), but other carcass characteristics did not differ ($P \geq 0.12$).

2.4 Discussion

Throughout the current study there was no difference in body weight (BW), or average daily gain (ADG) as a result of Mootral inclusion which is consistent with results from Brand et al. (2021) who fed 18-week-old Holstein-Friesian bull calves on a milk replacer + grain diet with 4 g of Mootral per head per day and observed no differences in BW or ADG. In yearling Angus x Hereford steers fed a 90% concentrate diet, 12 weeks of Mootral supplementation did not affect body weight, average daily gain, or feed conversion efficiency (Roque et al., 2019). The fact that cattle fed the diet with the greatest concentration of forage had the lowest intake and best gain:feed in the present study is not typical. A meta-analysis of studies investigating forage amount on methane emissions and performance suggests that increasing concentrates increases DMI and decreases gain:feed for beef cattle (van Gastelen et al., 2019). It may be possible that silage was more digestible than anticipated, or that the inclusion of a digestible fiber like DDGS with corn silage created a positive associative effect that enhanced overall digestibility of the diet.

Increased forage content in the present study causing an increase in CH_4 emissions is consistent with other studies. Lovett et al. (2003) reported that increasing the forage:concentrate ratio in finishing beef cattle diets from 10:90 to 40:60 and 65:35 linearly increased CH_4 production measured in L of CH_4/day, CH_4/kg DMI, and CH_4 as % GEI and reduced feed conversion efficiency over an 11-week period. Aguerre et al. (2011) observed that increasing forage:concentrate ratios (47:53, 54:46, 61:39, 68:32) in Holstein cows caused an increase in CH_4 emissions from 538 to 648 g/d. A meta-analysis of studies (van Gastelen et al., 2019) reported that increasing forage:concentrate decreases CH_4 production (g/d) in dairy cattle but increases CH_4 production in beef cattle and could be the result of the quality of forage being fed since the forages being fed varied from corn silage, grass silage, barley silage and others. However, CH_4 yield (g/kg of DMI or as a % of GEI) and CH_4 intensity (g/kg of product) generally increases for beef and dairy cattle as forage:concentrate increases in the diet (van Gastelen et al., 2019).

Mootral causing decreased CH_4 production in the from day 203 to 207 of the current study agrees with past studies, *in vivo* and *in vitro*, where Mootral decreased CH_4 in dairy cows fed high forage diets (Vrancken et al., 2019) and beef cattle fed high concentrate diets (Roque et al., 2019). When Mootral was supplemented at 10 or 20% of an *in vitro* substrate, methane emissions were decreased 22% and 54.4%, respectively (Ahmed et al., 2021). These authors found that Mootral increased propionate, decreased acetate and the acetate:propionate ratio, increased volatile fatty acid production, and had no effect on pH, digestibility, or ammonia-N. In addition, Mootral decreased the population of *Methanobacteriaceae* for both doses compared to the control but increased *Methanomassiliicoccaceae*. An increase in *Prevotellaceae*, a family that produces propionate, and an increase in *Veillonellaceae* was also observed when Mootral was fed at the 20% of substrate dose and was suggested to be responsible for the increase in propionate (Ahmed et al.,

2021). Angus x Hereford steers fed 90% concentrate, 10% hay diet supplemented with 15 g/d of Mootral decreased methane yield (g/kg DMI) 13.3% by the 12th week of the study (Roque et al., 2019). There was no difference in CO_2 and O_2 emissions between treatments (Roque et al., 2019). Brand et al. (2021) observed a 22.8% decrease in methane on a g/d basis and a 32.3% decrease in methane on a g/kg of BW basis in pre-weaned Holstein-Friesian bull calves fed milk replacer and a 52.3% concentrate starter for 70 days.

Mootral decreasing CH_4 emissions in the second half of the current study also agrees with previous work conducted with the ingredients contained in Mootral In ruminal incubations, 100 and 200 mg/L garlic oil both reduced the number of methanogens by 68%, decreased propionate and decreased CH_4 production by 70% compared to the control (Chaves et al., 2007). Busquet et al. (2005b) reported that 300 mg/L of garlic oil or compounds found in garlic oil including diallyl disulfide and allyl mercaptan decreased methane by 73.6, 68.5, and 19.5%, respectively in a 24 h incubation with ruminal fluid from cattle fed a 50:50 forage:concentrate diet. Garlic oil and allyl mercaptan each at 300 mg/L increased proportions of propionate, but all compounds decreased total VFA concentration, lowered DM disappearance, and depressed ADF and NDF digestibility compared to the control (Busquet et al., 2005b). In contrast, allicin, an oxygenated sulfur compound found in garlic, increased apparent digestibility of organic matter, nitrogen, NDF and ADF, and decreased methane production by 5.95% when supplemented at 2 g/head/day to crossbred ewes fed 68.7% forage diets (Ma et al., 2016). These authors also observed that allicin decreased methanogen populations by 104%, tended to decrease protozoa, and increased cellulolytic bacteria. Supplementing allicin did not affect ruminal pH, decreased ammonia and total VFA, and showed greater nitrogen intake and retention (Ma et al., 2016).

Addition of 4.5% (DM basis) of either of the flavonoids naringin or querectin to ruminal fluid from cows fed a 60:40 grass:concentrate decreased CH_4 and suppressed methanogen and protozoa populations (Oskoueian et al., 2013). Bioflavex, a commercial product that contains flavonoid components from citrus, added to rumen fluid incubations from steers fed a 90% concentrate diet reduced methanogens by 13% and decreased methane by 26% (Seradj et al., 2014). Bioflavex also decreased pH, increased molar proportion of propionate and lowered concentrations of acetate (Seradj et al., 2014). Adding naringin (1.5 and 3.0 g/kg) to in vitro fermentations from cows fed a 70:30 forage:concentrate diet was reported to increase propionate and decrease acetate but had no effect on methane production (Jimenez-Ocampo et al., 2021). However, an *in vivo* trial with *Bos taurus* x *Bos indicus* crossbred heifers fed a 70:30 forage:concentrate diet demonstrated no effect of naringin on rumen pH, acetate:propionate ratio, or methane production (Jimenez-Ocampo et al., 2021).

2.5 Conclusion

Increasing forage content of feedlot diets increases methane emissions. Mootral inclusion at 0.25 effectively decreased methane emissions in 15% corn silage (DM basis) diets, tended to reduce fat depth and yield grade. Mootral could be used in commercial feedlots and other grain-feeding scenarios as an effective method to decrease methane emissions.

2.6 References

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Table 2.1. Diet composition (DM basis)[1]

	15% Corn silage		41.5% Corn silage		68% Corn silage	
	Control	Mootral	Control	Mootral	Control	Mootral
Dry rolled corn	58	58	29	29	---	---
Dried distillers grains with solubles	22	21.75	24.5	24.25	27	26.75
Corn silage	15	15	41.5	41.5	68	68
Vitamin/mineral supplement[2]	5	5	5	5	5	5
Mootral supplement	---	0.25	---	0.25	---	0.25
Diet composition[3]						
NEm, Mcal/kg[4]	1.89	1.90	1.86	1.85	1.83	1.83
NEg, Mcal/kg[4]	1.25	1.26	1.23	1.22	1.20	1.20
CP, %	15.4	15.9	15.6	15.7	15.1	15.7
NDF, %	20.7	20.5	26.3	27.2	32.8	31.8
Calcium, %	0.96	0.95	1.01	0.99	1.04	1.03
Phosphorus, %	0.44	0.43	0.41	0.40	0.39	0.37
Sulfur, %	0.23	0.23	0.25	0.25	0.26	0.25

[1]Control = no Mootral inclusion; Mootral = 0.25% Mootral of diet DM as Mootral (Zaluvida Inc.)

[2]Vitamin/mineral supplement contained (DM basis): 18.25% Ca, 1.32% K, 0.44% Mg, 0.18% S, 563.91 ppm Zn, 522.90 ppm Fe, 440.41 ppm Mn, 183.33 ppm Cu, 9.66 ppm I, 4.48 ppm Se, 3.43 ppm Co, 42.19 IU/g vitamin A, 4.98 IU/g vitamin D, 0.155 IU/g vitamin E, 413.6 ppm Rumensin (176.4 g/kg, Elanco Animal Health, Indianapolis, IN).

[3]Analyzed at Cumberland Valley Analytical Services (Waynesboro, PA)

[4]Calculated based on NASEM (2016)

Table 2.2. Effect of Mootral and forage content on performance and methane production (d 0-84)[1]

	15% Corn Silage		41.5% Corn Silage		68% Corn Silage			*P*-values		
	Control	Mootral	Control	Mootral	Control	Mootral	SEM	Forage	Mootral	F x M
Body weight, kg										
Day 0	362.2	363.9	363.3	363.2	362.3	364.5	1.24	0.96	0.24	0.77
Day 84	502.0	493.0	505.0	502.0	500.9	500.6	5.37	0.55	0.37	0.73
Daily gain, kg	1.67	1.54	1.69	1.65	1.65	1.62	0.061	0.54	0.22	0.60
Daily intake, kg	9.5	8.5	9.5	9.3	8.7	8.6	0.27	0.02	0.08	0.03
Gain:feed	0.176	0.181	0.177	0.178	0.191	0.190	0.0051	0.04	0.71	0.20
Methane emissions day 42-46										
per day, g	160.1	128.4	187.1	184.5	197.8	194.3	20.69	0.05	0.47	0.72
per dry matter intake, g/kg	15.8	14.6	20.3	21.8	24.2	27.3	3.32	0.007	0.64	0.76
per metabolic BW, g/kg	1.73	1.40	1.97	1.95	2.10	2.09	0.257	0.07	0.54	0.74

[1]Control = no Mootral inclusion; Mootral = 0.25% Mootral of diet DM as Mootral (Zaluvida Inc.)

Table 2.3. Effect of Mootral and forage content on performance and methane production (d 85-slaughter)[1]

	Forage content			Methane treatment			*P*-values		
	15%	41.5%	68%	Control	Mootral	SEM	Forage	Mootral	F x M
Final body weight, kg	646.0	645.5	646.9	646.2	646.1	4.32	0.97	0.99	0.74
Daily gain, kg/d	1.57	1.45	1.50	1.48	1.53	0.049	0.22	0.36	0.41
Intake, kg/d	11.2	10.9	10.7	11.0	10.8	0.29	0.52	0.59	0.79
Gain:feed	0.141	0.132	0.141	0.134	0.142	0.0039	0.20	0.11	0.19
Methane emissions day 203-207									
per day, g	105.5	129.9	114.3	133.7	99.4	11.78	0.32	0.02	0.08
per dry matter intake, g/kg	10.1	12.3	10.6	12.6	9.5	1.19	0.39	0.03	0.15
per metabolic BW, g/kg	0.84	1.02	0.89	1.06	0.78	0.095	0.35	0.02	0.08

[1]Control = no Mootral inclusion; Mootral = 0.25% Mootral of diet DM as Mootral (Zaluvida Inc.)

Table 2.4. Effect of Mootral and forage content on performance and carcass characteristics[1]

	Forage content			Methane treatment			*P*-values		
	15%	41.5%	68%	Control	Mootral	SEM	Forage	Mootral	F x M
Daily gain, kg/d	1.58	1.55	1.57	1.57	1.57	0.035	0.84	0.99	0.76
Intake, kg/d	10.2	10.2	9.7	10.2	9.9	0.22	0.24	0.27	0.46
Gain:feed	0.156	0.152	0.161	0.154	0.158	0.0024	0.04	0.12	0.28
Days on feed	181.5	183.3	182.2	182.9	181.8	1.61	0.75	0.58	0.80
Hot Carcass Weight, kg	401.9	402.4	400.1	402.1	400.8	2.15	0.73	0.60	0.52
Dressing, %	62.2	62.3	61.9	62.2	62.0	0.21	0.26	0.44	0.38
Fat Thickness, cm	1.41	1.46	1.30	1.45	1.33	0.056	0.12	0.08	0.43
L. Dorsi Area, cm^2	94.5	94.3	94.5	93.9	95.0	0.96	0.99	0.35	0.86
Kidney, Pelvic, Heart Fat, %	2.00	2.08	1.95	2.00	2.01	0.037	0.07	0.85	0.85
Yield Grade	2.96	3.05	2.83	3.03	2.86	0.084	0.20	0.09	0.45
Marbling Score	482.6	475.4	469.1	482.1	469.3	13.84	0.79	0.43	0.55
Quality Grade Distribution									
Choice$^-$, %	14.6	22.9	21.7	18.6	20.9	5.01	0.46	0.70	0.30
Choice ^{0}, %	47.9	33.3	42.5	40.8	41.7	6.00	0.25	0.91	0.41
Choice $^+$, %	25.0	39.6	25.0	29.2	30.6	6.84	0.25	0.86	0.16
Prime, %	12.5	4.2	10.8	11.4	7.0	4.97	0.47	0.45	0.30
Warner-Bratzler shear force, kg	3.00	2.76	2.92	2.90	2.89	0.157	0.56	0.95	0.74

[1]Control = no Mootral inclusion; Mootral = 0.25% Mootral of diet DM as Mootral (Zaluvida Inc.)

VITA

Bryce Bitsie was born in May 1997 in Albuquerque, NM. He completed his Bachelor of Science degree at New Mexico State University in 2019, where he was an active member of the Sigma Chi International Fraternity. Bryce began his graduate studies at Purdue University in August of 2019 under the mentorship of Dr. Jon Schoonmaker where he studied animal nutrition. Bryce's work during his MS was focused on reducing methane emissions through feeding an all natural feed supplement that also aids in growth and carcass characteristics. During his graduate career in the Department of Animal Sciences, Bryce was awarded the SLOAN Indigenous Scholarship (2019) and a current Gates Millennium Scholar. Bryce is also a member of the American Society of Animal Science. After completion of his MS in 2021, Bryce continued studies in human nutrition and dietetics at Purdue University in the Department of Nutrition Science.