THE ROLE OF THE FECAL MICROBIOME

IN PREDICTING METHANE EMISSION IN CATTLE

BY

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ABSTRACT

Six mature non-lactating Holstein dairy cows were offered one of three diets with forage to grain ratios of 100:0 (G0), 75:25 (G25), and 50:50 (G50). The forage portion of the diet consisted of 80% grass hay and 20% alfalfa hay (on a Dry Matter (DM) basis). The experiment was a replicated 3x3 Latin Square Design, each animal received each of the three diets over the course of the three 5-week periods.

A statistical model was created combining 23 bacterial members in the feces, chosen due to their significant Variable Influence on Projection (VIP) values, along with ADF, NDF and starch formulated a basic predictive model for overall CH_4 production (L d^{-1}). The model had an R^2 value of 0.51 and a Q^2 value of 0.49. These 23 bacterial members, along with ADF, NDF, and starch can detect an increase or decrease from mean CH_4 production levels.

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Also, thank you to those in the department who helped with everything along the way. In particular, Ehsan and Kees for giving me this opportunity. I would also like to thank those who helped along the way. In particular, to Shucong and the barn staff for all of their help during the experiment. It was much appreciated. To Hooman for all of his help both in the lab and understanding the microbial analysis. To Hein for all of his help with the microbial analysis, and my officemates as well.

Finally, I want to acknowledge my friends. If it wasn't for you guys I would have a lot more confidence in fantasy football skills going into next year.

DEDICATION

Grandpa, your sudden loss was tremendously difficult for all of us to deal with. You were such an inspiration to all of us and are missed every day. Our early morning card games and fishing trips taught me many important things about life and are memories I will forever cherish. In particular, you have taught me that regardless of what happens keep taking the next step forward and everything will become easier over time. Thank you for everything, save a couple seats in the boat up there for dad and me. I love you.

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ABBREVIATIONS

AGGP	Agricultural Greenhouse Gas Program
BCM	Bromochloromethane
BES	2-Bromoethanesulfonate
BMP	Best Management Practices
DM	Dry Matter
DMI	Dry Matter Intake
ECM	Energy Corrected Milk
EPA	Environmental Protection Agency
GEI	Gross Energy Intake
GHG	Greenhouse Gas
GRA	Global Research Alliance on Agricultural Greenhouse Gases
GTP	Global Temperature Change Potential
GWP	Global Warming Potential
IPCC	Intergovernmental Panel on Climate Change
LCA	Life Cycle Assessment
LPS	Livestock Production System
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PPM	Parts per Million
UNEP	United Nations Environment Program
VFA	Volatile Fatty Acid
VIP	Variable Importance in Projection
WMGHG	Well-Mixed Greenhouse Gas
WMO	World Meteorological Organization

FOREWARD

The primary authors of the contents of this thesis are:

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KP Conceived, designed, and acquired funding for the experiment

1 General Introduction

1.1 Methane and the Environment

Until the Kyoto protocol, which extended the United Nations Framework Convention on Climate Change in 1992, climate change was known primarily to those who studied the environment, and was seldom mentioned by any major media source. That can no longer be said, as climate change concerns have become mainstream and more entrenched in individuals minds throughout the world. Following the White House releasing their Climate Action Plan Strategy to Decrease methane (CH_4) Emissions in March of 2014 (2), this topic has been gained momentum.

Greenhouse gas (GHG) is the term given to gases that are capable of causing radiative forcing. There are six major groups of GHG's, they are CO_2, CH_4, N_2O, Hydrofluorocarbons (HFC), Perfluorocarbons (PFC), and SF_6. Radiative forcing is the term given to a gas in the atmosphere which absorbs and emits radiation within the thermal infrared range, rather than allowing the energy to continue on to space (3). Not all GHGs have the same radiative forcing ability, as some are better able to prevent loss of energy to space, and some are more stable and thus are able to stay in the atmosphere for longer periods of time than others. It is because of these differences, that a compound's overall ability to function as a greenhouse gas is often converted into its global warming potential (GWP) and judged based over a set time frame comparing equal masses of the gas in question to that of carbon dioxide (CO_2), the predominate reference GHG (3). The most commonly used GWP timeframes are 20 and 100 years, respectively.

The GHG most commonly linked with livestock production is CH_4 (4, 5). It is the simplest member of the alkane family. The compound CH_4 is a colorless gas, produced naturally

upon breakdown of feed in the rumen by microbes referred to as methanogens. According to the most recent Intergovernmental Panel on Climate Change (IPCC) publication, CH_4 has a GWP of 84 over a 20 year period, and 28 over a 100 year period, and has been estimated to have a lifespan of 12.4 years in the atmosphere (3). These GWP values indicate that on an equal mass basis comparison between CO_2 and CH_4, CH_4 is 84 times as harmful to the environment over a 20 year period and 28 times as harmful to the environment over a 100 year span than CO_2 (3). Adding to the complexity of the issue of GHG production by livestock is the fact that the world's population is expected to significantly increase by 2050. A population somewhere between a conservative estimate of 8.3 billion and an upper-end estimate of 10.9 billion by 2050 can be expected (6). Thus, increasing ruminant production without harming the environment will be paramount in providing adequate nutrition to the world's growing population.

There are multiple international efforts which have begun in earnest to better understand the role that livestock production has on climate change and to mitigate GHG production while ensuring food security for the future. One such effort is the Global Research Alliance on Agricultural Greenhouse Gases (GRA) (5), which began as a 33 country alliance working to increase research cooperation and stimulate investment in the development of new GHG mitigation technology (7). The ultimate goal of the GRA is to develop more efficient and productive agricultural systems and make them available to farmers. Livestock is one of the GRA's three major research focus groups. Since its inception in December 2009, the GRA has grown to a total of 45 countries, and has representatives from all populated continents as of May 2015. Although the level of commitment varies among countries, some nations such as Canada have adopted further measures to supplement their efforts with those of GRA. The Agricultural Greenhouse Gases Program (AGGP) is an initiative set up by the Canadian government in

accordance with its commitment to the GRA. The goal of this program is to promote the development of on-farm GHG mitigation techniques by encouraging the development and promotion of best management practices (BMP) aimed at decreasing the environmental impact of farming activities on the environment while maintaining the necessary level of production.

In an attempt to better understand climate change, the United Nations Environment Program (UNEP) and the World Meteorological Organization (WMO) created the Intergovernmental Panel on Climate Change in 1988 (8). The IPCC's manifest states their dedication to prepare assessments on all aspects of climate change based on available scientific information and discuss possible response strategies. Many countries have made efforts on their own accord to better understand, monitor, and discuss strategies pertaining to the mitigation of GHG production. For example, in North America, the United States Environmental Protection Agency (EPA) and Environment Canada both publish data on environmental issues, including GHG emissions and their ramifications.

The major issue with current CH_4 mitigation strategies is their lasting effects, or lack thereof. While CH_4 production can be temporarily lowered through dietary changes or feed additives, a sustained decrease in CH_4 production has proved to be the main hurdle (9). There is optimism that overall CH_4 production can be decreased long term after some groups noted sustained decreases in CH_4 production (10, 11). With that said, potential CH_4 mitigation strategies for livestock do exist, such as new generations of probiotics, bateriocins, phages that target rumen archaea, immunizations, and reductive acetogenesis (9, 12). Any future strategies to deal with CH_4 mitigation must be both economically feasible for the producer, while simultaneously not being a burden for producers to implement or maintain.

Currently, the best CH_4 mitigation strategy available to livestock producers is to increase the milk production or growth of each animal. While higher producing animals produce more CH_4 overall, when CH_4 values are adjusted for the parameters of intake and production, less CH_4 is produced on a per unit basis of these parameters than lower producing animals (9, 12, 13). From a producer standpoint, an increased production per animal is an attractive proposition, as housing and labour costs would decrease, and health and longevity are maintained.

1.2 Methane Production in Ruminants

The production of CH_4 is multi-faceted with genetics, gross energy intake, and animal body weight, being among the factors affecting it (14). The production of acetate and butyrate as starch, cellulose, and sugars are lowered in the rumen leads to the formation of hydrogen as an end product, which is utilized by methanogens to create CH_4 (15). Methanogens are obligate anaerobes which only grow in environments with redox potentials below -300 mV (16). Currently two major groups of methanogens are known, hydrogenotrophs and methloytrophs, with hydrogenotrophs being responsible for the CH4 production in the rumen environment (17). Most hydrogenotrophs rely primarily on the reduction of CO_2 to CH_4 with H_2 being the dominant electron donor for this process (17).

The pathway involved in the formation of propionate competes with methanogens for excess hydrogen molecules. Thus, if there is an increase in propionate production relative to acetate and butyrate production, a decrease in CH_4 production should be observed (18). The following stoichiometric equations can be considered for the reduction of glucose ($C_6H_{12}O_6$) into propionate ($C_3H_6O_2$), acetate ($C_2H_4O_2$), and butyrate ($C_4H_8O_2$).

$$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2 + 2H_2O$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 8H$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 4H$$

The production of propionate competes for H with CH_4 production by utilizing H in the rumen environment. The same cannot be said for the production of acetate and butyrate which both have endpoints of CO_2 and H when produced. Thus, increases in production of acetate and butyrate lead to an increase in CH_4 production. Methanogens can utilize CO_2 and H to produce CH_4 by the following equation:

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O$$

The vast majority of the CH_4 produced by livestock is produced in the rumen (87%) and is expelled via eructation (16). The large intestines represent the remainder of CH_4 production (13%) (16). The production of CH_4 can represent up to a 12% cost of energy from feed depending on the diet, which will be lost to the animal (19). This is a significant extra cost to the producer through increased feed costs, while congruently being harmful to the environment. Being able to reduce the production of CH_4 would, therefore, be beneficial for both ruminant production systems and the environment.

The goal of our experiment was to examine the rumen and fecal microbiomes in animals fed diets varying in grain inclusion rates of 0%, 25%, and 50% (G0, G25, and G50) that are designed to alter CH_4 production and ultimately determine if the composition of the fecal microbiome can be used to predict CH_4 emissions. Enteric CH_4 production was monitored as the rumen is the primary source of CH_4 production in ruminants. CH_4 production was cross-referenced with microbiome results to identify prevalent trends pertaining to the bacterial

communities at various taxonomic levels and to use these bacterial members to predict enteric CH_4 emissions.

2 Literature Review

2.1 Introduction

It is widely believed that between 11,000-12,000 years ago, animal domestication began in conjunction with basic agriculture in areas of southwest Asia, and Mesoamerica (20). The ancients observed the ability of some ruminant animals to utilize forages as a feed source. We now know that the process occurs via microbial fibre breakdown. Since humans lack the necessary enzymes to digest cellulose, assuming grain could not be grown instead of forage, there would be no competition between humans and animals over feed sources. Essentially, so long as a source of forage was made available to the animal; the growth of the animal, the protein source associated with the animal's growth, and its secondary products (milk, eggs, etc.) could be utilized as a food source for humans. These animals provided a steady food source, something which was inherently difficult to find at the time, making it of paramount importance for human survival. This practice of animal husbandry would go on to shape the history of the world, as communities now had the ability to become more permanent and less nomadic given a consistent food source (20). The consistent food source also had a variety of additional benefits including what many anthropologists believe to be the creation of more permanent establishments, ultimately leading to the cities and way of life we are so familiar with now. Rather than spending the whole day hunting and gathering, a fraction of the individuals could cultivate and prepare food for the masses, while other individuals worked on different tasks benefitting the community in other ways (21).

As the human population grew, so too did the number of domesticated animals required to feed the population. Animal production, in particular ruminant livestock production, creates CH_4 as a by-product upon breakdown of feed components; this process is harmful to the environment.

Ruminant livestock production was not a major environmental issue while production was prevalent on a small scale, in fact the manure functioned as a fertilizer source adding nutrients to the soil thereby increasing soil quality.

Currently, there are an estimated 1.4 billion cattle in the world (22) which are responsible for producing 17% of total global CH_4 emissions (13). The global human population continues to increase, with estimations for a 2050 population between a conservative estimate of 8.3 billion and an aggressive estimate of 10.9 billion people, up from approximately 7 billion today (6). An increase in ruminant production efficiency will be required to handle the increased number of mouths to feed with the same, if not less land area allocated to production to accommodate the increase in population. As a result, improving production in a manner which does not cause harm to the environment will be paramount in providing adequate nutrition to the world's growing population without harming the environment, and thereby further exacerbating the problem.

CH_4 is the simplest member of the alkane family. It is a colourless, odorless, tasteless gas comprised of one molecule of Carbon, and four molecules of Hydrogen (23). All four C-H bonds are equivalent, and the compound is present in a tetrahedron molecular shape. CH_4 is flammable and with a heat of combustion value of -890.8 kJ mol^{-1}, and it can be used as a fuel source commonly referred to as natural gas (23). Methanogens naturally produce methane in areas deprived of oxygen, such as the rumen of livestock, as well as wetlands and rice paddies (24-26). The ability of CH_4 to function as a GHG has put it under increasing scrutiny throughout much of the world, especially of late.

Efforts aimed at mitigating CH_4 emission have been ongoing, with variable amounts of success (2, 13, 27). Efforts in the past have focussed primarily on manipulating the rumen environment and nutritional strategies (12, 13). The advent of new molecular techniques has

allowed for investigation into this subject at a level that was previously unseen (28). Rather than focussing on the tangible aspects of methane production, such as production numbers based on feed parameters, with high throughput sequencing, researchers can now investigate the microbes, methane producing pathways both acetogenic and hydrogenotrophic, and key cofactors associated with methane production under a variety of factors, such as change in feed level, diets, and the age of animals (28).

The production of CH_4 is closely related to feed intake and the availability of carbohydrates , as well as volatile fatty acid (VFA) production as shown in Figure 1 below (19, 29).

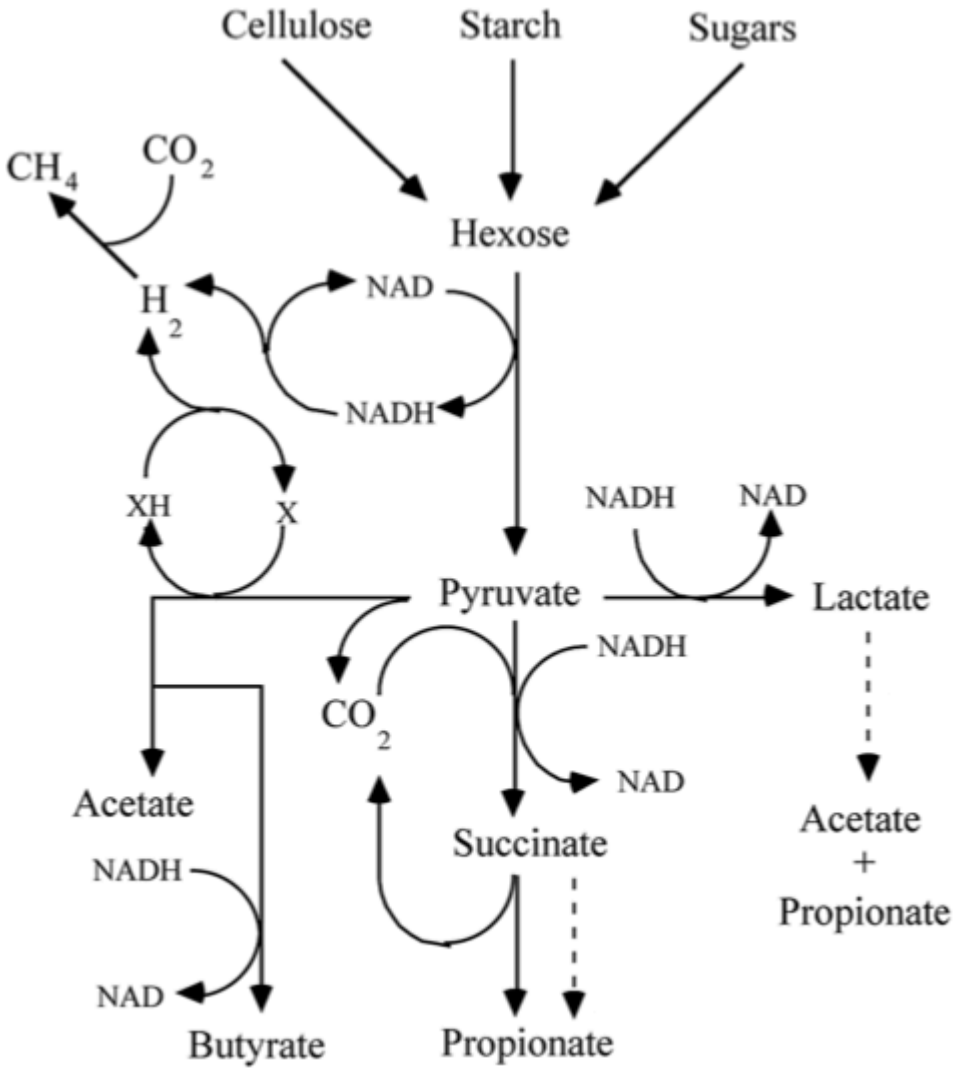


Figure 1: Schematic showing the reduction of carbohydrates as it pertains to VFA and CH_4 production (From Russel and Rychelik (2001) used with permission)

Figure 1 shows the starch, cellulose, and sugars present in the feed are first reduced by rumen microbes to pyruvate (1). Following this, the production of both acetate ($C_2H_4O_2$), and butyrate ($C_4H_8O_2$), among others, occurs. The production of acetate and butyrate from glucose ($C_6H_{12}O_6$) is linked to CH_4 production as both productions have hydrogen as an end-product:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 8H$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 4H$$

Conversely, the production of propionate ($C_3H_6O_2$) competes with methanogens for excess Hydrogen in the environment; thus, if there is an increase in propionate on a molar basis, a decrease in CH_4 production should be observed (18).

$$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2 + 2H_2O$$

Methanogens require excess Hydrogen in the environment to help fuel the reaction below to make methane (CH_4).

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O$$

Keeping the above equations in mind, some CH_4 mitigation techniques in the past have focused on increasing the production of propionate, thereby decreasing the Hydrogen concentration in the rumen environment (13, 18). Any potential CH_4 mitigation strategy must also be conducted in accordance with environmental stewardship and with a holistic focus. If enteric CH_4 is lowered at the expense of increased downstream emissions, such as manure excretion, then to the environment on a holistic level, the benefit is lessened (30). In addition, the financial aspect of the mitigation strategy must be attractive initially, and the cost must remain sustainable. Any deviations from the current costs associated with production must be absorbed by either the producer, consumer, or both and could jeopardize the practicality of the mitigation technique. Finally, strategies should be viewed upon positively by both consumers and businesses to reach long-term viability and thus should strive to avoid invasive procedures or contamination of the final product for the consumer.

2.2 Greenhouse Gases and the environment

Common GHG's include CO_2, N_2O, CH_4, and various forms of fluorocarbons (3). Although CH_4 is not as potent as some fluorocarbons that have GWP values exceeding 10,000, the ability of CH_4 to cause harm to the environment is still evident by its GWP value of 84 over a 20 year period (3). Additionally, CH_4 is exponentially more prevalent in the atmosphere than most other anthropogenic, human created, GHG's such as fluorocarbons (3).

The term well-mixed greenhouse gases (WMGHG) refers to a GHG which adequately disperses within the troposphere such that measurements of the gas's concentration at a few remote surface sites has the ability to characterize the burden of the gas in the atmosphere to the climate (3). These gases are so prevalent that they are considered mixed within the atmosphere. Examples of WMGHG's include CO_2, CH_4, N_2O and SF_6. Recently the IPCC has stated that there is unequivocal evidence that the anthropogenic increase in WMGHG's have substantially increased the greenhouse effect (3). Current values of WMGHG are measured via observations at a few different sites. Pre-industrial values of WMGHG are determined at sites where the air has not been disturbed, a good example of this would be the air trapped inside polar ice which can be analyzed and cross referenced based on time of ice formation (3). Among GHG's which are not considered as WMGHGs are predominantly GHG's of anthropogenic origin such as fluorocarbons which were widely used in aerosols (3).

The GWP unit functions to predict the amount of heat a GHG can retain in the atmosphere on an equivalent mass basis with CO_2, which is considered the gold standard of GHG's (3, 30). It takes into account the compounds stability and its ability to prevent energy loss to space. The unit GWP is commonly portrayed in values over 20 and 100 year spans. Other commonly used units also include Global Temperature Change Potential (GTP), which, as the

name suggests, functions to calculate the ratio of the difference between global surface temperature at one point in time between the desired compound relative to CO_2 (3). It is important to note that both GWP as well as GTP are model based, and as such do have limitations, particularly with regards to their dependence on time horizon and metric type (31). Both limitations are dependent on the application of the metric and the relevant aspects of climate change considered, given the context in question (3).

Coinciding with the Industrial Revolution in the 1700s, humans began adding a large amount of GHG's into the atmosphere through avenues such as the burning of fossil fuels for electricity generation, clearing forests, and for the powering of transportation (32). Consequences of GHG buildup include increased air temperatures near the surface of the earth, alterations in weather patterns, rises in sea levels, and amplifications of the water cycle. Essentially, CH_4 enters the atmosphere and causes an overall change in energy balance within the earth system, as a result of the change it creates in the atmospheric makeup (3). Over time, GHG's are removed from the atmosphere at different rates via chemical reactions, or emission sinks, such as oceans or vegetation (32). Throughout the past 250 years, approximately 28% of CO_2 produced by fossil fuel burning has been absorbed by the oceans (3).

Another major conundrum with regards to CH_4 production in ruminant livestock is which value should be used to portray it most accurately. One way to portray CH_4 production is by using overall production values. These values, expressed in L d^{-1} or kg d^{-1} are commonly used on the basis that they most accurately display the burden of CH_4 production on the environment. Other interpretations of CH_4 production are based on production parameters, and factor in milk production or animal growth.

The portrayal of climate change in overall surface temperature is one of many options currently available to display the ramifications of climate change. Recently, due to increased understanding and research on climate change, auxiliary indicators have been made available (33). Such indicators include surface GHG concentration, degree of glacier loss, water cycle amplification, and major weather events. When used in combination these indicators function to provide a more holistic view of the effects of climate change (33).

On a global scale, net anthropogenic GHG emissions have increased 35% from 1990 to 2010, and when these values are corrected for natural fluctuations, current GHG levels are higher than at any time in recorded history (32). Currently, some key tangible examples of climate change include increased sea levels, increases in glacier melt, and changing plant and animal life cycles (32). The resulting effects of climate change cause fundamental disruptions in the ecosystem that affect the ecosystem's biodiversity, and the plant and animal populations which comprise it (32).

2.2.1 Production estimates and the challenges they present

The increased prominence of non-CO_2 emissions increases the likelihood that warming thresholds will continue to increase in the 21st century (34). It is estimated that non-CO_2 GHG's will comprise approximately one third of the total CO_2 equivalent emissions in the 21st century (34).

In 2006, an FAO article entitled livestock's long shadow caused immense controversy when they stated that livestock accounted for 18% of total anthropogenic GHG production making it the largest single source of anthropogenic GHG emissions (5). In an effort to view livestock emissions in a holistic outlook, the life cycle assessment (35) which functions as a beginning to end of life assessment of environmental impacts associated with livestock, both

directly and indirectly has become the emission reporting method of choice (31, 35). The values reported by LCA's are often reported in CO_2-equivalent emissions. By examining the environmental impact of a product throughout all stages of its production, use, and disposal, a more holistic picture is created. This is the basis for the increasing attention life cycle analyses are currently receiving in both research and policy creation (35). Garnett, (2009) argues that the conclusions garnered from LCAs must include three additional perspectives. Firstly, there should be more documentation regarding the emissions associated with land use change away from livestock production, and the indirect effect livestock production has on an area (36). Secondly increased knowledge pertaining to opportunity costs of alternate land use that does not include animal production should be in included (36). Finally, the degree to which livestock products are needed, both from a food and a secondary source perspective would make for a more accurate LCA (36).

It is often very difficult to compare one LCA method to another, as their scopes, underlying assumptions, extrapolations used, and interpretation of data vary based on their indented outcomes (31). Furthermore, regional differences in animal species, diets, as well as production systems make it very difficult for CH_4 emission values to be accurate on a global scale as a result of the extreme variety of parameters required to be accounted for by extrapolations (31). Indirect emissions are defined as emissions which are not directly derived from livestock, but rather from the secondary processes associated with livestock production, such as feed crops used for animal feed, feed processing and transportation, transportation and processing of livestock, and refrigeration of meat prior to its consumption by the consumer (36). The degree to which indirect emissions are incorporated into various assessments varies based on

the studies' intended goals, such as whether a study is global, national, or regional in nature. The livestock production systems and consumer habits will also affect indirect emission estimation.

Livestock production systems vary greatly throughout the world. According to Steinfeld et al, (2006), there are two major livestock production systems (LPS), landless LPS's and grassland-based LPS's (5). Landless LPS's are primarily found in developed nations and are characterized by their dense livestock populations. Landless LPS's are defined as systems in which under 10% of the feed on a dry matter (DM) basis is produced at the farm with stocking rates above 10 livestock units per hectare of agricultural land (5). Grassland-based LPS's are characterized by their low density of livestock population, with more than 10% of feed on a DM basis being produced at the farm and stocking rates being lower than 10 livestock units per hectare of agricultural land (5). Grassland-based LPS's are estimated to occupy approximately 26% of the earth's ice-free land surface; they are commonly prevalent on land which is otherwise unfit for cropping such as arid or semi-arid lands (5). The lower feed quality prevalent at grassland based LPS's rather than landless LPS's leads to greater enteric CH_4 production on a per animal basis, but has lower indirect emissions than landless LPS's which require a greater amount of transport yet varies on an operation-to-operation basis.

Additionally, ruminants do provide some benefits to society, which are often understated or ignored in prediction equations. For example, ruminants are able to utilize poorer quality grazing land, such as grazing land effected by drought, thereby making an efficient resource from land which might otherwise have to be ploughed (36). The ploughing process of undisturbed land releases CO_2 upon its disturbance, the machinery used for the ploughing process and the fertilizing of the soil also function to release GHG's (36). These secondary products of animals often do not factor into many production models. In addition to providing

food, secondary products such as leather, wool, and manure for fertilization are all benefits of livestock production. With regards to manure, if applied in proper amounts, it is a natural nitrogen source and also contains minerals and organic matter. It can also aid in lowering dependence on energy-intensive synthetic fertilizers, and the transport associated with it (36). Furthermore, research has shown that manure can improve soil fertility and quality while being more biologically active at the microbial level and fertile making it more conducive to the growth of the desired product than soil supplemented only with mineral fertilizers (37). These value-added goods and services should be incorporated into models to more accurately predict the carbon footprint of livestock relative to non-livestock equivalents, for example manure versus fertilizer, leather versus vinyl, and wool versus microfiber (31).

Currently, there is no accounting for default emissions, a value which would represent the difference between the decrease or removal of livestock production systems relative to whatever is to be produced in their place (31). According to Pitesky, (2009) if animal production is decreased, anthropogenic GHG emissions will not be decreased by the full 18% value quoted in livestock's long shadow in 2006, since many resources currently dedicated to livestock production, as mentioned above, would cause greater climate change impacts if they are implemented in other fashions (31).

2.3 Methane production in livestock

With regards to total global CH_4 production, current estimates suggest that agriculture accounts for 29% with enteric fermentation from ruminants representing over half of that total at 17% of the overall anthropogenic CH_4 production (13). These values are estimated, and the accuracy of these values has been a source of debate, however, it is agreed that the values are

still high enough to make lowering enteric CH_4 emissions a priority for the livestock industry (31, 38).

From a producer standpoint, there are tangible benefits to lowering CH_4 production by ruminant livestock. First off, CH_4 is an excellent GHG to target a decrease in emissions, due to the relative cost savings associated with doing so. The energy in emitted CH_4 does not benefit the animal, yet depending on the diet it can represent between 2-12% of the total energy of the ration (19). A decrease of 10% in feed costs would be very beneficial from a financial standpoint for producers, making CH_4 mitigation an enticing proposition.

Certain conundrums appear to be prevalent in CH_4 production. With all other variables being equal, there is still high animal-to-animal variation in CH_4 production (39). It is believed that approximately 28% of variation in CH_4 production can be attributed to variation in the retention time of feed in the rumen (9). Cows with a lower feed retention time in the rumen produce less CH_4 enterically (39). Generally, the retention time in the rumen is in the range of 20 to 30 hours (40). Part of this is due to rumination; a process which returns feed from the rumen back to the mouth where it is re-masticated and reintroduced to the rumen environment. This process increases the surface area available for cellulose degradation by 10^6 allowing for more rapid degradation by microbes (41).

2.3.1 Physiology of livestock with emphasis on the rumen and hindgut

The stomach of ruminants is comprised of four components, the rumen, reticulum, omasum, and abomasum. Forages are chewed vigorously to decrease the particle size of the ingested feed, and increased chewing and ruminating increases the amount of added saliva on a DM basis for digesta (42). The beneficial properties of saliva, including lubrication to assist mastication and deglutition in ruminants have been known since the 1950s (43). Saliva

production has been shown to increase as the physically effective fibre content in the diet increases (43). Saliva also contains buffers that function to prevent large changes in rumen pH while allowing rumen microorganisms to flourish; it is also vital in nitrogen recycling, especially of urea allowing for a constant nitrogen source for the microbes to utilize in the rumen environment (43).

Ruminants are able to derive between 60 and 70% of their necessary amino acids from ruminal microorganisms (44). As the rumen breaks down its feed components, methanogens utilize the excess protons at the end of the cycle to create CH_4.

2.3.2 Past, present, and future mitigation strategies

It is important to note that although producers and consumers can benefit from a decrease in CH_4 production, there are a variety of parameters which must be met prior to the implementation of methane mitigation strategies on a large scale. First off, the procedure must not be invasive or otherwise harmful to the animal. It is entirely plausible that the key to decreasing CH_4 emissions will occur in large part due to the implementation of many strategies which decrease CH_4 emissions by a smaller amount rather than finding one idea to substantially lower CH_4 prediction. At this point in time, efforts toward CH_4 mitigation can be sequestered into three groups: feed management and nutrition, rumen modifiers, and genetics and other management approaches.

With regards to feed management and nutrition, the general basis of these strategies is to create a feed formula in which high quality feeds are fed, thus creating an increase in feed efficiency, reducing the need for concentrate supplementation and thereby decreasing CH_4 production as a proportion of feed intake. It is believed that changes in nutritional management to more closely meet the energy and other demands of milk production could decrease the

CH_4/Energy Corrected Milk ratio from 2.5 – 15% while maintaining milk production levels, thereby leaving the bottom line of the business minimally affected (13). While formulating feed, it is important to remember that an increase in propionate production leads to lower CH_4 production, as the production of both CH_4 and propionate compete for excess hydrogen in the rumen environment. Conversely, a decrease in acetate and butyrate production would create less hydrogen for methanogens to use in the rumen environment; thereby lowering CH_4 production. This basic principle forms the basis of the majority of feed management and nutrition management practices with regards to CH_4 production.

There are a few major issues with feed management and nutrition strategies as they pertain to lowering CH_4 output. One of the main benefits of ruminant production is the ability to turn a fibrous food source that is not suitable for human consumption, into a high-quality source of nutrition. If animal production and the high-quality protein food that it creates uses a high-quality grain-based diet or other feeds that can be consumed by humans, a delicate balance must be struck between environmental stewardship and utilization of potential human food sources. The practise of feeding grains to livestock has been investigated in depth. At only approximately 10% efficiency in the transfer of plant material into energy which can be utilized by the animal for growth, a substantial amount of grain would need to be fed to meet livestock nutritional requirements (5). Further compounding the issue is the rate of concentrate inclusion in the diet which is required for a significant decrease in CH_4 emissions to occur on an intake basis. Past analysis shows that 40% concentrate supplementation in a ration is required to significantly decrease CH_4 emissions on a per intake basis. With 40% concentrate inclusion rate there are significant financial hurdles associated with the implementation, particularly for producers in free range situations (45). Keeping that in mind, there are still some strategies that can mitigate

CH_4 emissions. Research has shown that starch-based diets will favour propionate production when measured as a unit of fermentable matter in the rumen, thereby lowering relative CH_4 production (9, 46). Diets with high starch relative to high fiber contents have a decreased retention time in the rumen, leading to a decrease in CH_4 production (47). With an increased understanding of the microbial reactions and their role in the rumen environment comes the potential to increase the predictive power of CH_4 emission prediction models in the future. An increase in CH_4 production was found to coincide with increases in the maturity of the forages as well as with feeding of grasses rather than legumes (48). Meanwhile, diets which are roughage based favour the production of acetate, thereby increasing the production of CH_4 on an organic matter intake value when compared to diets high in grain content (19). Altering practices to favour a decrease in CH_4 production utilizing the above principles is certainly a practical option. Other perhaps less practical management changes have also shown to decrease CH_4 production. One such example is lowering meal frequencies, as this has been linked to elevated propionate production and decreased acetate production (49). Based on the principles described above, this would cause a decrease in CH_4 emission, however, the likely cause of the changes in CH_4 production would be due to diurnal variation in the pH in the rumen as feed breakdown coincides with the introduction of feed into the rumen environment. It has been shown that any practices which involve the fluctuation of pH should be avoided as they may affect digestion and milk production (9).

Finally, another strategy shown to have the potential to reduce CH_4 emissions is the grinding or pelleting of feed when feed is offered at a high intake level (19). Although CH_4 emissions were shown to be decreased, this strategy is not economically attractive to a producer, and the associated decrease in effective fibre content as a result of the fine grinding process has

also been linked to increased incidences of acidosis, as well as to a decrease in milk fat (9). Research into almost all aspects of feed, feed management and nutrition have occurred to attempt to mitigate CH_4 production. With that said, the metabolic demands on livestock, especially dairy cows in milking are very high, so it is not advisable to deviate away from these demands. The overall outlook with regards to decreasing CH_4 production through the grinding or pelleting of feed is at this point a longshot, in large part due to the impracticality from a producer standpoint and also due to the underwhelming results produced thus far. While modest gains can be expected, at this point it is unlikely that dietary manipulations can be anything more than a part of a larger effort to decrease CH_4 emissions.

Rumen modifiers to alter the rumen environment into a state which is less conducive to CH_4 production has been attempted with varying levels of success. The reason for modifying the microbial population in the rumen environment is that if one can lower the methanogen population, or the substrates that they rely on, then CH_4 emissions should decrease as a result. Boadi et al. (2004) discussed several rumen modification techniques in detail, and they show that addition of fats, ionophores, and defaunation can mitigate CH_4 production (9). With regards to fat supplementation in diets, it has been shown that this has the ability to increase energy density in the diet, while increasing milk yield and modifying the fatty acid composition of the milk fat (50, 51).

One rumen modification strategy involves direct inhibition of methanogens via chemicals, commonly referred to as inhibitors. With regards to inhibitors, the most successful inhibitors as determined by in vivo testing were bromochloromethane (BCM), 2-bromoethane sulfonate (BES), chloroform, and cyclodextrin (38). These compounds are commonly designated as chlorinated CH_4 analogues and their mode of action is competitive inhibition. These compounds

are structural analogues of co-factor mercaptoethanesulfonic acid, more commonly referred to as Coenzyme M (52). This cofactor is exclusive to methanogens, and thus prevents the production of CH_4. It has been shown that BES decreases CH_4 production by 71% (53). Its administration did not affect organic matter digestibility, or changed VFA concentrations in an artificial rumen (53). Chlorinated CH_4 analogues have been shown to decrease CH_4 production in studies by up to 50% in in ruminant species such as goats, sheep, and cattle (54-56). Upon investigation, BCM lowered CH_4 production for multiple months; however, the same could not be said for the other compounds such as BES, chloroform, and cyclodextrin (35, 57, 58). While BCM showed a sustained decrease in CH_4 emission, it is currently a banned compound, as it is an ozone depleting agent, and thus cannot be recommended as a CH_4 reduction agent at this point. In the future, compounds with similar modes of action that are not harmful to the environment may offer opportunities to help lower CH_4 emissions.

Another CH_4 reduction strategy involves dietary supplementation with ionophores. Boadi et al. (2004) defined ionophores as "highly lipophilic substances which are able to shield and delocalize the charge of ions, and facilitate their movement across membranes" (9). Their use has been shown to decrease CH_4 production on a per unit product and per unit intake basis (52, 59). Monensin is a common ionophore; it is also referenced under the trade name Rumensin in the livestock industry. Monensin is the most commonly utilized and studied ionophore in both the beef and dairy industries. When Monensin was given at a concentration of 24 parts per million (ppm), feed intake of dairy cows on a dry matter basis was lowered from 16.1 kg cow^{-1} to 14.5 kg cow^{-1} while milk production increased from 27.6 kg day^{-1} cow^{-1} to 31.5 kg day^{-1} cow^{-1} (60). It is accepted that CH_4 mitigation strategies which decrease DMI are not considered to be practical over an extended period of time. Another study showed that daily administration of 24 mg of

Monensin lowered CH_4 production significantly while simultaneously decreasing dry matter intake (1), however parameters associated with milk quality and quantity such as milk yield, milk fat, and milk protein decreased as well (7). The ability of Monensin to increase propionate production and thus decrease CH_4 is believed to be affiliated with a selective reduction in gram-positive bacteria, such as Ruminococci, and the concurrent proliferation of Gram negative bacteria; the result of which is a shift in fermentation products from acetate to propionate (61). Past research has shown that methanogen strains vary with regards to their susceptibility to Monensin (62). Therefore, extended Monensin treatment may lead to selection towards non-susceptible strains thereby decreasing its long-term potency. Another major issue affiliated with ionophores, is the inconsistency in the results they produce (38). The use of ionophores is currently banned in the EU, citing fears of antibiotic resistance. To date, there has been no evidence of genes coding for resistance. Until both of these issues are addressed, large scale ionophore use to decrease CH_4 emissions will be delayed.

A third rumen modification technique to decrease CH_4 production is defaunation. Defaunation refers to the elimination of ruminal protozoa via dietary or chemical means. The link between protozoa and methanogens is well established. Ruminal protozoa are said to provide a habitat for up to 20% of rumen methanogens, accounting for 37% of the total CH_4 production (63, 64). The logic behind defaunation is based on the strong evidence of correlation between cross-feeding of protozoa and archaea (65). While the exact mode of action causing the decrease in CH_4 production appears to allude researchers, it is believed that there are two major factors governing the decrease. First, a shift in digestion to the hindgut from the rumen is witnessed (66); and secondly, the close relationship between methanogens and protozoa is shifted during defaunation with lower CH_4 production being the result (67). Results associated

with defaunation are extremely variable. The practice is associated with a decrease in ruminal fibre digestibility, which is linked to increased acetate and CH_4 production (68). Currently, the results of defaunation are too variable for this to be promoted as a legitimate CH_4 mitigation practice (38). That is not to say that in the future, as microbial analysis of the relationship between protozoa and bacteria, along with other microorganisms in the rumen continues to expand this will still be the case. In the future, our understanding of the interactions between the protozoa, methanogens, viruses, and bacteria which constitute the rumen environment will grow potentially leading to more specific targets of defaunation (69).

While temporary decreases in CH_4 production have been noted for all of the rumen modifiers discussed, the inability of these modifiers to sustain decreased CH_4 production has been too variable and inconsistent to justify their implementation on a large scale. This is a major issue which should be addressed for rumen modifiers to succeed in the dynamic rumen environment consisting of bacteria, archaea, fungi, and protozoa where microbial succession appears to occur. Increased knowledge with regards to interactions in the rumen between bacteria, fungi, protozoa, and archaea will help the endeavor, and will at the very least provide more detailed insight into the intimate relationships in the rumen at the microbial level as it relates to CH_4 production.

The third and final category of CH_4 mitigation strategies is an increase in animal production through genetic or farm management alterations. Of all approaches, genetics and farm management alterations is currently the most widely adopted, and the greatest gains with regards to CH_4 mitigation have been seen from the implementation of the two. At its core, the concept is that if production can remain the same, but be reached with fewer animals, overall emissions will be lowered as a result. This decrease in total CH_4 production occurs as a result of the amount of

feed energy associated with animal maintenance being between 70% and 75% for beef cattle, and approximately 50% in dairy animals with the remainder of the energy going towards production (52). When combined with nutritional and feeding approaches, the combination of genetic and management approaches have the ability to decrease CH_4/ECM values by 15 to 30% (13). Examples of such managerial approaches include disease and fertility management through checks from veterinarians, change in facility designs, heat abatement, and performance enhancing technologies (13). Managerial approaches to decrease CH_4 emissions are also enticing to the producer as the cost and time with regards to implementation is less than other CH_4 mitigation strategies. This strategy stands to see the greatest gains in developing countries where increases in diet quality via elimination of nutrient deficiencies, utilization of growth promotants, and other strategies to ensure the animals' metabolic needs are properly met would lead to a decrease in CH_4 emissions from feed fermentation associated with animal maintenance (19). In fact, the IPCC has indicated that approximately 70% of the global CH_4 mitigation potential in agriculture lies in developing countries, making an increase in genetic selection based on production traits as well as the implementation of managerial approaches listed above in a timely fashion paramount in these countries (70). As of 2005, North America, non-European former USSR countries, and Europe, accounted for 46.3% of total global meat and milk energy production, while accounting for only 25.5% of enteric CH_4 emissions (71). In contrast, Asia, Africa, and Latin America accounted for 47.1% of meat and milk energy with near 69% of enteric CH_4 emissions (71). By utilizing a combination of improved animal genetics which cannot directly be determined on the farm, optimization of feed for the livestock, increased reproduction rates, increased herd health, and other managerial approaches pertaining to the animal operation, a decrease in CH_4 production on a large scale is likely to be possible (72). One

of the major issues associated with implementation is that geographical areas of livestock production have substantially different climates and feed quality available, meaning that a variety of implementation strategies must be considered. The overall health of the animal must be strictly monitored when implementing changes to ensure adequate animal health rather than focussing solely on production parameters (72). Thus, any changes implemented in one climate cannot be assumed to be functional and promote good health to animals in another climate.

Future techniques are likely to expand on genetic selection for lower CH_4 producing animals, perhaps by traits such as residual feed intake, as well as expanding on efforts to alter the microbial population, with particular focus on protozoa, in the rumen to mitigate CH_4 production (12). It is also likely that vaccines with high specificity designed to promote an environment in which the prevalence of microbes involved in CH_4 production would be mitigated will be utilized on a large scale. The theory is that upon administration, the vaccines would produce salivary antibodies to neutralize the activities of microbes known to be associated with CH_4 in the rumen (73, 74). Trials have shown that live vaccines have induced significant *Streptococcus bovis* antibody responses in both the serum as well as the rumen fluid. *Streptococcus bovis* is a bacteria with known CH_4 inhibition properties, with the mode of action being anti-microbial antibody production in the saliva of the vaccine administered animals to target bacteria known to be associated with CH_4 production (75). These results have increased optimism associated with vaccinations to decrease CH_4 production and their viability in the future (74).

As the use of next generation sequencing (NGS) continues to grow, it is likely that the microbes, pathways, intermediates, and all aspects governing CH_4 production will continue to be investigated in depth for the development of potential CH_4 mitigation options. The major hurdle

for all the above strategies remains the same, producers are running a business, and thus any procedure which is not practical financially will not be considered.

2.4 The Rumen Environment

The rumen is a fermentation chamber present in ruminants such as cattle, giraffes, deer, sheep, goats, camels, yaks, antelopes, and llamas. It is a dynamic environment allowing for the breakdown of cellulose and other components such as starches and sugars, into products such as VFA's and energy sources by the microbes present in the rumen environment, such as bacteria, fungi, archaea, and protozoa. Although the rumen environment is dynamic in its ability to change to optimally breakdown feed, the abundance of the microbes according to Kumar et al. (2013) for bacteria is $10^{10\text{-}11}$ /mL rumen fluid, methanogens are prevalent at a population of $10^{8\text{-}9}$ /mL rumen fluid, while both ciliate protozoa and anaerobic fungi have populations of 10^{6} /mL rumen fluid (76). Methanogens in large part rely on ciliates and fungal mycelia for the protons they produce (77). This relationship is said to account for 9-25% of CH_4 production in the rumen of cattle (61). Protozoa release CO_2 and H_2 during the breakdown of fibre, starch, and sugars, which ultimately end up producing compounds such as butyrate or acetate and others (78).

To this point, much of the study with regards to the rumen microbial environment has been conducted with the bacteria and archaea being the emphasis. These microbes, in conjunction, help breakdown plant material and the cellulose and fiber it contains into a variety of VFAs, namely acetate, propionate and butyrate which can provide energy for the animal. The microbes also function as the building blocks for the production of gases such as CO_2 and CH_4 which are produced in the rumen and expelled enterically via eructation. The process of fermentation of feed in the rumen is oxidative, as NADH, NADPH, and FADH are oxidised into NAD^+, $NADP^+$, and FAD^+ (39). The excess protons sequestered in the rumen are primarily utilized by

methanogens predominantly by the equation $CO_2 + 8H \rightarrow CH_4 + 2H_2O$ thereby creating CH_4 from excess hydrogen present in the rumen environment. This process prevents the over accumulation of hydrogen in the rumen which has been linked to inhibition of dehydrogenase activity (39). Dehydrogenase is involved in the oxidation of reduced cofactors (39). A constant optimal hydrogen concentration, rather than fluctuations in pH, in the rumen is also beneficial for fiber breakdown (79). Efficient H_2 removal has also been linked to a VFA production pattern which is beneficial to the animal, and an increased fermentation rate through eliminating the inhibitory effect H_2 has on the microbial fermentation in the rumen (62, 80).

2.4.1 Methanogens

Archaea are single-celled organisms considered to be extremophiles, owing to their ability to survive in extreme conditions pertaining to one or more of a combination of temperature, salinity and pH (81, 82). Past research has shown that archaea contribute between 0.3 – 3.3% of microbial small subunit (16S and 18S rRNA) genomic matter when rumen fluid samples are sequenced (83-85). Although separate from Prokaryotes, archaea do have some similarities with them, namely their information processing system in which DNA is replicated, transcribed, and translated (81). One distinguishing trait of archaea is their incorporation of ether-linked isoprenoid lipids with a glycerol-1-phosphate backbone. They also lack a nucleus or any membrane-bound organelles (81). Together, there is an estimated 1 billion tons of CH_4 produced by methanogenic archaea in anoxic environments around the world annually (17).

There are three traditional categories of archaea. The first category are thermoacidophiles, which require a combination of high temperature (usually exceeding 60°C) and a very low pH (usually below 3) for survival. The second category are extreme halophiles, also referred to as haloarchaea, which require environments with high salt concentrations (usually exceeding 1M

NaCl) to survive. The third category of archaea, and most relevant to the rumen environment, are the methanogens. Methanogens are anaerobic organisms that generate CH_4 through the reduction of acetate, CO_2, or other one-Carbon compounds such as methanol, or methylamines (81, 82). Methanogenesis in the rumen is a syntrophic process involving anaerobic bacteria, protozoa, and fungi, who hydrolyze biopolymers to monomers and lipids to glycerol and long chain fatty acids and ferment these with syntrophic bacteria forming acetate, CO_2, and H_2 (86). Methanogenic archaea are the only known life form possessing the ability to carry out methanogenesis, and this process is the only known mechanism of ATP synthesis for methanogens (87). Ruminants can utilize VFA's including acetate as an energy source via resorption from the intestinal tract, thus there is competition for acetate in the rumen environment. According to Janssen and Kirs, (2008), methanogens are found in numerous locations throughout the rumen, including attached to or as endosymbionts within rumen protozoa, attached to particulate matter, in rumen fluid, and also attached to the rumen epithelium (88).

Currently, seven orders of methanogenic archaea have been identified; these orders include *Methanopyrales*, *Methanococcales*, *Methanobacteriales* (class I), *Methanomicrobales* (class II), *Methanosarcinales* (class III), *Methanocellales*, and *Methanoplasmatales*. With regards to early colonization of the rumen environment by archaea populations, *Methanobrevibacter* species appear to be dominant (88). With regards to the prevalence of archaea at the genus level in a mature rumen environment, 92.3% of rumen archaea belong to three major genus level groups (88). At 61.6%, *Methanobrevibacter* is the most prevalent genera, with a total of 15.8%; *Rumen Cluster C* is the second most prevalent genera in the rumen environment (88). Finally, comprising 14.9% is the genera *Methanomicrobium* (88). Research has shown a general negative correlation between the archaea *Methanobrevibacter ruminantum* and *Methanobrevibacter*

gottschalkii (R = -0.51, Spearman's rank coefficient P=0.023) suggesting that there is competition between the two in the rumen environment (89).

There are a variety of different methanogens, each with differences conferring advantages in specific ecological niches; As such, the pathways and substrates they use to produce CH_4 vary among these species. Methanogen populations will also vary based on a variety of factors. One such factor is the composition of the diets. Since the rates of passage in the rumen for different feed vary, the methanogen population based on the ruminal fraction of the feed at any particular time also varies (90). The changes in rumen fraction resulting from feed intake, based on passage time of the feed may help to explain part of the phylogenetic diversity in the rumen (88). Changes in the diet can also lead to rumen pH changes and the formation of other compounds which are toxic to the methanogen population such as 2-bromoethanesulphonate and nitroethane thereby affecting the ability of methanogens to function (88).

Hydrogenotrophic methanogens primarily utilize H_2 as the dominant electron donor in the reduction of CO_2 to CH_4 (17). Hydrogenotrophic methanogenesis is believed to have occurred early in the evolution process, possibly coinciding with the beginning of life on earth (17). As their predominant source of energy coupling, Hydrogenotrophic methanogens utilize electron bifurcation (17). The process of electron bifurcation entails the coupling of the reduction of a high chemiosmotic potential substrate, and utilizing the energy harvested in the reduction to help drive a low potential substrate (17). Electron bifurcation is used in the coupling of the final heterodisulfide-reducing step to the initial reduction of CO_2 thereby forming a cycle (17).

Other methanogens create CH_4 via methyl groups. These methanogens are called methylotrophic methanogens. The process of electron bifurcation is not a requirement in methylotrophic methanogenesis (17). $\Delta G^{\circ\prime}$ is a measurement of spontaneity of a reaction, the

lower the value the higher the likelihood of the reaction occurring relative to a reaction with a higher $\Delta G^{\circ\prime}$value. Some examples of reactions of methylated 1-carbon containing reactions them as discussed by Liu, 2008 include:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O(\Delta G^{\circ\prime} = -105\ kJ\ mol\ CH_4^{-1})$$

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O\ (\Delta G^{\circ\prime} = -113\ kJ\ mol\ CH_4^{-1})$$

$$2(CH_3)_2 - S + 2H_2O \rightarrow 3CH_4 + CO_2 + H_2S\ (\Delta G^{\circ\prime} = -49\ kJ\ mol\ CH_4^{-1})$$

$$4CH_3 - NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3(\Delta G^{\circ\prime} = -75\ kJ\ mol\ CH_4^{-1})$$

$$2CH_3 - NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3N(\Delta G^{\circ\prime} = -73\ kJ\ mol\ CH_4^{-1})$$

$$4(CH_3)_3 - N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3\ (\Delta G^{\circ\prime} = -74\ kJ\ mol\ CH_4^{-1})$$

$$4CH_3NH_3Cl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4Cl\ (\Delta G^{\circ\prime} = -74\ kJ\ mol\ CH_4^{-1})$$

A heterodisulfide reaction occurs via a membrane-bound electron transport chain which exports protons, thereby creating a chemiosmotic membrane potential which powers the reaction (17). When CO_2 is the primary carbon substrate, the required electrons for the reduction are provided by an energy-converting hydrogenase, which is membrane associated, at the expense of its chemiosmotic potential (17).

Members of the order *Methanosarcinales* are considered methylotrophic methanogens and contain cytochromes and methanophenazine (86). *Methanosarcinales* possess the ability to use any or all three of the following substrates for CH_4 production, depending on the species, giving them the widest substrate range of all known methanogens. These substrates include, CO_2 reduction with H_2, dismutatation of methyl compounds, and the splitting of acetate in the equation below (91).

$$CH_3COOH \rightarrow CH_4 + CO_2\ (\Delta G^{\circ\prime} = -33\ kJ\ mol\ CH_4^{-1})$$

The genus *Methanosaeta*, in which acetate splitting is the only catabolic pathway, and the genus *Methanosarcina* which uses acetate, along with other substrates; are able to use electron acceptors other than CO_2 in the production of CH_4 (91). The K_m, the substrate concentration where the rate of reaction is at ½ of the maximum rate for the system, for *Methanosaeta* is lower for acetate than for *Methanosarcina*, as such, it is often able to outcompete *Methanosarcina* in environments with slow turnover and low acetate concentrations (91). Acetate is not believed to be a significant contributor to CH_4 production in the rumen (92). This is assumed to be a result of the rumen passage rate of feed being shorter than the growth rate of acetate-utilizing methanogens leaving no viable substrates for the acetate-utilizing methanogens (62). One defining trait of *Methanosarcinales* is that all its members contain cytochromes (86, 91). Methanogens with cytochromes have shown a large increase in both growth yields and threshold concentrations for H_2 as compared to methanogens which do not contain cytochromes (86). Methanogens with cytochromes dominate in environments with lower temperatures (<4°C); however in environments with higher temperatures (>60°C) methanogens without cytochromes are more prevalent (93, 94). Methanogens with cytochromes have not been found in environments with temperatures exceeding 60°C (93, 94). Evidence to this point in time suggests that cytochrome containing methanogens do not grow on H_2 and CO_2, on the basis that they are outcompeted by non-cytochrome containing methanogens and they prefer environments with higher ΔGo´ where reactions are less spontaneous and they can compete better, and thus they are believed to be largely outcompeted in the rumen environment (86).

2.4.2 Ruminal Bacteria

Ruminal bacteria are paramount for ruminants. They assist in the breakdown of feed, in particular cellulose, but also hemicellulose, non-structural carbohydrates and rumen degradable protein, and provide the animal with viable products including VFA, and amino acids upon the completion of digestion. Ruminants rely on microbial protein as a major source of amino acids, as such, the amount of bacterial mass produced in the rumen is paramount for the overall functioning of the animal (95). Many of the bacteria closely attach to the feed particles, making the adhered populations difficult to exactly enumerate or extract DNA from.

There are relatively few phyla present in the rumen environment with most sequences belonging to two major phyla. The first phylum of interest is the Gram negative *Bacteroidetes*, primarily represented by the genus *Prevotella*. The second phyla of particular interest in the rumen in the Gram positive *Firmicutes*, noted for their low guanine-cytosine content (96). Both the genus *Prevotella* associated with the phylum *Bacteroidetes* and the phylum *Firmicutes* are cellulolytic bacteria, involved in the breakdown of cellulose, as well as bacteria which primarily utilize soluble components already in the diet, and take advantage of solubilized products of cell wall breakdown (96).

With regards to cellulolytic bacteria in the rumen, there are three species of paramount interest. The first, belonging to the phylum *Fibrobacteres* is *Fibrobacter succinogenes*. The second and third belong to the phylum *Firmicutes*, they are *Ruminococcus flavefaciens*, and *Ruminococcus albus* respectively. Culture studies have shown in both batch (97, 98) and continuous (99) cultures that ruminal cellulolytic species are under intense competition for both cellulose and cellobiose. A number of factors govern the interactions among the cellulolytic species, including the affinity for cellodextrin products of cellulose hydrolysis (100),

bacteriocins (101), the adherence rate to cellulose (102), and the presence of non-cellulolytic bacteria in the environment (99).

The name *Fibrobacter succinogenes* is derived from succinogenes, which is the name given to *Fibrobacter* strains that are isolated in the rumen. *Fibrobacter succinogenes* binds very tightly to the feed and is said to be the most active cellulolytic bacterium isolated from the rumen (41). Due to its tight binding, it is not easy to isolate. There are two major strains of *Fibrobacter succinogenes*, *Fibrobacter succinogenes* S85, as well as *Fibrobacter intestinalis* NR9; however S85 is more predominant in rumen studies (41).

Ruminococcus flavefaciens was first discovered in the rumen environment over 50 years ago (103). One of its unique features is that its strains have a narrow range of sugars which can be utilized for growth (104). It is largely believed that this might be due to the energetic advantage imparted by possessing a cellobiose phosphorylase (105). The phosphoric cleavage upon transportation into the cell, yields both glucose and glucose-1-phosphate. Upon the conversion of glucose-1-phosphate into glucose-6-phosphate, both substrates can enter directly into glycolysis without an ATP consuming kinase step, making it tremendously efficient from an energy production standpoint (96). *Ruminococcus flavefaciens*, along with other cellulolytic bacteria employ the use of cellulosomes, appendages that connect to the surface of cellulose via attachment mechanisms and through a concoction of enzymes function the breakdown the cellulose (96). Cellulosomes function to assist in the breakdown of cellulose and contain numerous cellulases, and other polysaccharide hydrolases which are arranged on the cell surface to optimize the hydrolysis of the plant cell wall (106).

Another important bacterial group in the rumen are *Ruminococcus albus* and *Ruminococcus flavefaciens*, both belonging to the phylum *Firmicutes*. Both species are able to

utilize hemicellulose as a source of energy, leading to suggestion regarding their ability to grow on oligomeric products of enzyme hydrolysis (107). *Ruminococcus albus* has the ability to produce ethanol, acetate, formate, and hydrogen, while *Ruminococcus flavefaciens* exclusively produces succinate rather than ethanol (41).

There are bacteria present in the rumen which are unable to degrade intact plant cell walls, yet still produce multiple polysaccharides capable of degrading a variety of polysaccharides such as pectins, xylose, and amorphous (non-crystalline) varieties of cellulose (96). One such example is *Prevotella bryantii*, this non-cellulolytic bacteria functions to utilize the soluble compounds present within the diet, and take advantage of the products solubilized upon plant cell wall breakdown. There are also a large number of non-cellulolytic bacteria present in the rumen. How different species of these bacteria obtain their requirements vary; some examples include, metabolic cross-feeding, gaining energy from the breakdown of carbohydrate substrates released by primary cellulose degrading species, or fermentation of products (108-110).

Amylolytic bacteria function to create an energy source by employing the use of amylase to breakdown starch containing compounds. Starch is particularly prevalent in diets of both feedlot steers, as well as lactating dairy cows as their energy demands and starch supplementation is often used to meet these demands. Some examples of bacterial species noted for their high amylase production include *Streptococcus bovis*, *Ruminobacter amylophilus*, *Butyriovibrio fibrisolvens*, and *Bacteroides ruminicola* (111).

Finally, some bacteria possess the ability to produce acetate from both H_2 and CO_2 utilizing the Wood-Ljungdahl pathway; these phylogenetically, and metabolically diverse

bacteria are referred to as homoacetogens, however these bacteria are not believed to be major contributors to CH_4 production in the rumen at this point in time (112).

2.5 The use of high-throughput sequencing to characterize microbial communities and their dynamics

Next-generation sequencing (NGS) methods have improved DNA preparation protocols to a genome-wide scale and enhanced resolution precision to be reliable to a single base level thereby increasing genomic precision (113, 114). NGS functions to amplify single strands of a fragment library and platform sequencing reactions on the amplified strands, essentially taking a strand of DNA and amplifying it exponentially (113). Through the process of annealing blunt-ended fragments of interest to platform-specific linkers, fragment libraries are formed (113, 114).

The ability of NGS platforms to open previously unknown areas of biological discovery, such as ancient genome investigation and ecological diversity characterization has substantially widened metagenomic analysis as it pertains to samples derived from a variety of environments (113, 114). This was made evident by the number of peer reviewed literature manuscripts published that were relevant to NGS, which immediately surpassed 100 (113).

Another benefit of NGS platforms is the streamlined sample preparation process, which allows for a substantial amount of time savings along with minimal additional equipment relative to the highly automated, multistep pipelines required for clone-based high-throughput sequencing (113). As a result of long run times and streamlined sample preparation, to keep NGS instruments at full capacity is attainable with a single operator.

2.5.1 The Illumina Mi-Seq sequencing process

From a production standpoint, Mardis, (2008) explains that Illumina's platform employs a sequencing-by-synthesis approach whereby simultaneously, DNA polymerase and all four nucleotides are presented to the flow cell channels to be incorporated into the oligo-primed cluster fragments (113). The added nucleotides have a base-unique fluorescent label on the 3'OH that ensures that via chemical blocking, each nucleotide base incorporation is a unique event (113, 115). The amplification step of a single molecule for the Illumina Genome Analyzer begins with an Illumina-specific adaptor library and occurs on an oligo-derivatized surface of a cell; an automated device referred to as a cluster station performs the necessary steps (113, 115). Following all base incorporation steps, all flow cell lanes undergo an imaging step whereby imaging on the three 100-tile segments occurs via instrument optics at a cluster intensity of 30,000 (113). Upon completion of the imaging step, the 3' blocking group is removed via chemicals to prepare each of the strands for the next incorporation step using DNA polymerase (113, 115).

The reactions occur in an 8-channel sealed glass micro-fabricated device called a flow cell, which enables bridge amplification of fragments on its surface and enables multiple DNA copies or clusters by using DNA polymerase (113). Each amplification represents the single molecules which initiated the cluster amplification process (113). Each of the clusters contain approximately one million copies of the original fragment, this represents a satisfactory amount for reporting the incorporated bases at the required signal intensity for detection during the sequencing (113).

The sequencing quality provided by NGS can ascertain important information pertaining to the accuracy of every step in the process, such as library preparation, base calling, read

alignment, and variant calling. With regards to base calling accuracy, measurement according to Phred quality score, more commonly known as Q-Score, is the most common accuracy determinant of a sequencing platform (116). The Q-Score assesses the probability that a given base will be incorrectly called by the sequencer. The Q-Score parameter is defined as a property which is logarithmically related to the probability of a base-calling error, $(P)^2$, making Q = -10 log P. A major reason Q-Score is so commonly used is its accuracy across a range of sequencing chemistries and instruments, allowing it to be the quality scoring standard for commercial NGS technologies (117). Q-Scores assist in revealing the amount of data from a given run, which is usable in a resequencing, or assembly, experiment. Lower Q-Scores prevalent in sequencing data can lead to a significant portion of reads which are unable to use due to poor quality. Additionally, low Q-Scores can increase false positive variant calls, leading to higher costs for validation experiments, and inaccurate conclusions. Generally, a Q-Score of Q30 represents perfect reads, which are considered to have no errors or ambiguities, making it a benchmark for quality in NGS.

There are three major NGS platforms, each with their own set of advantages and disadvantages relative to the others. Along with Illumina, who has adopted a sequencing-by-synthesis approach, there is also the Ion Torrent Personal Genome Machine which uses a semi-conductor chip and measures pH of the synthesis reaction to determine whether a base was correctly incorporated into the reaction. Finally, the Pacific Biosciences RS system uses a magnetic bead system and ZMW flow cells to determine the sequence of a sample.

There are two major types of sequencing bias, coverage bias and error bias. Coverage bias is defined as a deviation from the uniform distribution of reads from throughout the genome, it is considered the most damaging sequence filter (118). It is also referred to as relative

coverage, and is determined by enumerating the read bases mapped to it in an alignment, calculated as: $\frac{coverage\ of\ a\ given\ reference\ base\ in\ a\ genome}{mean\ coverage\ of\ all\ reference\ bases}$ (118). When the relative coverage is equal to 1, the coverage of a given base is at the expected average rate; when the value is above 1, the coverage rate is higher than expected, and below 1 the coverage rate is lower than expected (118).

Sequencing bias hinders genomic data applications, such as genome assembly and variation discovery, as such, applications rely on genome wide coverage to properly function (118). Nakamura et al, 2011 (119) stated that strand specific errors in Illumina can lead to coverage biases by impairing alignment performances (48, 119). Error bias functions to account for the deviation from the expectation of deletion and insertion rates, as well as uniform mismatch in reads throughout the genome (48). In addition to coverage bias and error bias, bias can also be introduced by erroneous computational steps in the sequencing pipeline.

With regards to measuring bias, there are two primary methodologies, per-base bias measurements, and motif bias measurements. Per-base bias measurements rely on deep-coverage sequencing, and are hypothesis free, making them ideal for the discovery of new bias types (118). On the contrary, motif bias measurements require only shallow-coverage sequencing, and as such, they are optimal for comparisons across a variety of experimental conditions, as well as overseeing ongoing sequencing pipeline performance in areas possessing known bias-prone sequencing contents and locations (118). Motif bias measurement functions as a crucial metric for determining and improving the sources of sequencing bias (118). When used in combination, per-base bias measurement and motif bias measurement can be used to analyze various NGS platforms, determine the utility of combining data from multiple platforms, and to measure the degree of coverage bias as described by the statistics of known motifs (118).

Bias, however is not the only important metric when evaluating a NGS platform, as accuracy, throughput, speed, cost, and other factors will all be essential in determining an appropriate platform for an experiment (118).

Currently, all existing sequencing technologies fall short of uniform read distribution throughout the genome without sequence dependant variations in quality. Some common areas where loss of coverage occurs are regions of high or low GC content, repetitive regions, and regions of low complexity; this has also been linked to an increased likelihood of ambiguous mapping (118). Some reads cannot be mapped to a single locus, as the probability of ambiguous mapping will increase as reads become shorter or less accurate (118). As such, deep sequencing is necessary to identify bases more accurately with lower relative coverage.

The ongoing goal for both sequencing investigators and individual investigators is to increase DNA sequencing product quality by means of increasing read yield and length, overall base quality, as well as other measurements which denote how the NGS platform technology functions on sections of the genome with no known difficulties associated with their sequencing. Although such measures do not make the difficult to sequence part of the genomes prevalent, all NGS platforms are constantly working to update their product. For example, when Illumina updated their Hi-Seq platform to version 3.0, this increased coverage of high-GC motifs and increased coverage of promoters (118).

Of the bacterial data sets tested by Ross et al, (2013), interpretation of the bias curves suggest that data provided by Illumina's HiSeq from a human genome perspective provides the most even coverage when compared to Ion Torrent PGM and Pacific Biosciences RS. Evenness is calculated by dividing standard deviation of coverage breadth with the average coverage

breadth. Furthermore, because of its lower cost per sequencing rates than other NGS platforms, interest in amplicon sequencing using Illumina is increasing (120-122).

As NGS continues to expand in both popularity and prevalence, the hope in the future is that its quality will also continue to increase. Ross et al. (2013) believe it is paramount that a list of hard to sequence motifs, which are short recurring patterns present in DNA that are known to be difficult to sequence, be made available publicly (118). Known trouble spots such as low and high guanine and cytosine content areas, and ATAT runs where the bases adenine and thymine are repeated numerous resulting in sequencing difficulty should also be included in the published list. The underlying belief behind making a list of known difficulties is the belief that if portions of DNA which are known to cause issues with sequencing are made known, the ability to eradicate them would be made significantly easier. Ross et al. (2013) believe that in the future, monitoring assays will be important in improving NGS quality. Assays functioning to minimize bias as well as monitor process changes, both unintentional and intentional, which may affect bias would help achieve more optimal sequencing results. In the future, refined and more comprehensive motifs would allow for increased knowledge of bias-prone contexts. The above improvements would function to increase data quality, thereby improving the accuracy of the genome assemblies while simultaneously decreasing the probability that biologically important members would be poorly represented in the sequencing data (118).

2.5.2 Microbial analysis

The acronym PICRUSt stands for phylogenetic investigation of communities by reconstruction of unobserved states. It is a program which functions on the premise that correlation between gene content and phylogeny, expect in rare cases such as laterally transferred elements, may allow for the possibility to predict the approximate functional potential of

microbial communities based on phylogeny (123-125). The correlation coefficient between inferred and metagenomically measured gene content approached 0.9 on occasion, with an average of approximately 0.8 (126).

PICRUSt employs a computational approach that allows for prediction of the functional composition of a metagenome via marker gene data, while cross-referencing a database of reference genomes (126, 127). PICRUSt takes the information obtained from 16S rRNA sequencing and cross-references the information with some of the noteworthy findings pertaining to genomic composition from the human microbiome project and goes on to predict the abundance of gene families in environmental and host-associated communities with quantifiable uncertainty (126). Ultimately, the accuracy of PICRUSt relies on several factors, such as reference database coverage, gene functional category and its potential to laterally transfer genes, phylogenetic error, ancestral state reconstruction method, microbial taxonomy, and the depth of 16S sequencing to name a few (126).

To provide functional insight into sequencing data, PICRUSt uses a two-step algorithm process. By using an extended algorithm to reconstruct the ancestral-state allowing for the prediction of the prevalence of gene families, then combining the gene families present to estimate the composite metagenome present (126, 127). The first step of the process is the initial gene content interference, where the gene content for each organism is precomputed in a phylogenetic tree for reference (126, 127). Subsequently, a table of each organism's predicted gene family abundances in the 16S-based phylogeny is reconstructed (126). The second step of the PICRUSt algorithm is referred to as metagenome interference, which combines the predicted gene content for all microbial taxa and the relative abundance of 16S rRNA genes in at least one microbial community sample, corrected using a correction algorithm to account for the expected

16S rRNA gene copy number with the goal being to create estimated abundances of the gene families throughout the entire community (126, 127). According to Langille et al. (2013), the metagenome interference step is essentially the use of ancestral state reconstruction to infer genes in each relevant bacteria's ancestors, thereby inferring traits of ancestral organisms via fitting evolutionary models to the distribution of observed traits in living organisms using criteria such as Bayesian posterior probability or maximum likelihood (126). Additionally, by extending existing ancestral state reconstruction methods, PICRUSt predicts the traits of extant organisms, which according to Langille et al. (2013) accounts for the propensities of lateral transfer by gene families, as well as the degree to which a gene family is conserved within particular microbial clades (126). Following that step, the gene content of each of the reference genomes and the inferred ancestral genomes are used to predict a reference phylogenetic tree for all microorganisms (126).

The metagenome inference step relies on the user to provide an operational taxonomic unit (OTU) table for each sample with associated Greengene identifiers (126, 127). Langille et al. (2013) describe that OTU abundances normalized then multiplied by the calculated gene family abundances for all relevant taxa during the gene content inference step (126).

In the first step of PICRUSt, gene content inference, genes present in organisms that have yet to be sequenced based on genes by using existing annotations of gene content are predicted (126). Additionally, the 16S copy number from the reference genomes for bacteria and archaea present are in the integrated material genome database (126). PICRUSt uses a combination of ancestral state reconstruction and a weighting method created by PICRUSt developers to make predictions of gene content accounting for estimates of uncertainty for all organisms in the Greengene phylogenetic tree of 16S sequences (126).

2.6 The Relationship between Bacteria and CH_4 Production

The conversion of digesta into CH_4 in the rumen environment involves a variety of microbial species targeting various functions of feed breakdown. The final step in the conversion process is the production of CH_4, which is carried out by methanogenic archaea (128, 129). Methanogens predominantly utilize H_2, but also a small number of simple compounds, such as, CO_2, formate, acetate, various methyl compounds, and alcohols to produce CH_4 (130). Therefore, methanogens in the rumen are dependent on the products created by the conversion of other substrates for their metabolism and ultimately for their survival.

Within the rumen, bacteria involved in fermentation hydrolyse and ferment carbohydrates, proteins, and lipids to produce propionate, butyrate, acetate, and long chain fatty acids (LCFA) as well as H_2 and CO_2 (78). The production of acetate and butyrate, largely via the fermentation process of structural carbohydrates and starch, results in the production of H_2, the major substrate for methanogens in the reduction of CO_2 into CH_4 (59, 131).

Bacteria are also one of the major sources of nitrogen for protozoa in the rumen environment (132). Protozoa also release H_2 and CO_2 into the rumen environment during the breakdown of starches and fibres, and are also involved in acetate and butyrate production. The relationship between protozoa and methanogens is believed to be responsible for between 27 to 37% of the CH_4 produced in the rumen environment and would not be possible without ruminal bacteria (64). Bacteria are vital to the rumen environment's overall function, such as feed digestion, and the bacterial population is dynamic and therefore able to best digest the diet provided. Rumen bacteria are intimately involved in the breakdown of feed components such as fiber, sugar, and starch. The production of hydrogen is a common product of many chemical reactions in the rumen, particularly from cellulolytic bacteria. This hydrogen is then utilized by methanogens to

create CH_4, thereby ensuring that hydrogen concentrations in the rumen do not significantly fluctuate. Many of the bacteria that are intimately involved in CH_4 production are carried into the hindgut as it is believed that bacterial communities present in the rumen and those found in the feces are believed to be similar and ultimately expelled in the feces (133). By determining the composition of the microbiome in the hindgut as represented by the feces, biomarkers of CH_4 production may be identified, which may have the ability to ascertain whether the animal is a high or low CH_4 producer.

2.7 General Summary

The production of the greenhouse gas CH_4 by livestock is one of the foremost hurdles facing sustainable agriculture. It is estimated that the 1.4 billion head of cattle in the world account for approximately 14.5% of total anthropogenic GHG emissions (134). The production of CH_4 can represent an energy loss for the animal of between 2-12% leading to a significant increase in feed cost for the producer while being harmful to the environment (19).

Current strategies to predict livestock CH_4 emissions are costly and time intensive for both initiation and perpetuation. Strategies such as the SF_6 tracer technique or a chamber hood calorimetric device takes weeks to prepare, days to initiate, and requires constant supervision while the equipment is in use. This makes them impractical for a producer to implement as a predictor of herd health, as it affects the bottom line of their business. As a result, current estimates for CH_4 are shifting towards a life cycle assessment in conjunction with microbiome analysis, which allows for a more holistic evaluation on the basis of the assessment's intended goal, analyzes multiple GHG's involved in the process and reports in CO2-equivalents. In the case of CH_4 production, it can provide a total environmental cost of animal production from birth to consumption. The combination of microbiome analysis and life cycle assessments allows for a more accurate estimate regarding the ultimate effect animal production and any intervention aimed at lowering CH_4 emission have on the environment.

Recent advances in microbial analysis through next generation sequencing have allowed for the comprehensive investigation of the microbial communities in the rumen as well as in other environments pertaining to livestock at previously unseen levels. We know that the rumen environment is dominated by the two phylum *Bacteroidetes* and *Firmicutes*. We also know that there are certain keystone functional groups of bacteria in the rumen, such as fibrolytic bacteria

(135). We also know that as a by-product of fermentation, hydrogen is produced. Thus, with equal intake of fibre relative to starch, the cellulose will produce more CH_4 due to the increased propionate production from starch production. As ruminal hydrogen levels continue to increase, hydrogen sinks function to utilize the excess ruminal hydrogen in the environment. An over accumulation of H^+ in the rumen environment as a result of inefficient H^+ removal has been linked to inhibition of dehydrogenase activity involved in the oxidation of reduced cofactors (39). An optimal H^+ concentration (pH) in the rumen is also beneficial for fibre breakdown and increased fermentation rates (79, 80). Methanogens are thermodynamically the most efficient hydrogen sinks in the rumen environment, they utilize CO_2 and H and produce CH_4.

The goal of this experiment was to investigate microbial trends pertaining to species richness and diversity in both the rumen and hindgut as they pertain to CH_4 production to better understand the relationship between the fecal microbiome and CH_4 emission, and ultimately predict CH_4 emission based on the prevalence of certain bacteria. This was accomplished by comparing the abundance, richness and diversity of species in the rumen and fecal microbiome in non-lactating dairy cows with varying concentrate inclusion rates in the diet to differ CH_4 production.

3 Hypotheses

A. We hypothesized that an increase in forage-to-concentrate ratio of the diet of dairy cattle increases the CH_4 production when corrected on dry matter intake parameters.

B. The composition and functionality of microbial communities in the hindgut of dairy cattle (as represented by the feces) is a predictor of rumen microbial community composition and function.

C. Composition and function of microbial communities in the hindgut of dairy cattle (as represented by the feces) is a predictor of enteric CH_4 production in the rumen.

4 Objectives

A. To assess the associations between the fecal and rumen microbiomes in dairy cattle fed diets with different forage-to-concentrate ratios.

B. To assess the associations between rumen microbiome composition and function and enteric CH_4 emission levels in dairy cattle fed diets with different forage-to-concentrate ratios.

C. To determine if the composition of the fecal microbiome can predict enteric CH_4 emission levels by cattle.

To achieve this, we employed NGS techniques to assess the rumen and fecal microbiota composition and function in dairy cattle fed diets of varying forage-to-concentrate ratios while maintaining consistency throughout other experimental factors, and measuring the rumen CH_4 production levels. Various physiological parameters were recorded simultaneously. These physiological parameters, such as DMI, and pH of the rumen and feces provide an assessment of overall rumen health (39, 79, 80) and used to ensure that microbial data were not the result of dysbiosis of microbiomes due to other confiding factors.

Diurnal fluctuations in enteric CH_4 production were also determined using an open-chamber hood calorimetric device on a real-time basis.

5 Material and Methods

This experiment was conducted in accordance with Canadian Council on Animal Care guidelines (136) and was approved by the University of Manitoba Animal Care Committee prior to commencing.

5.1.1 Setup and experimental design

Six mature, non-lactating, non-cannulated Holstein dairy cows housed in a tie-stall facility at the University of Manitoba's Glenlea Research Station were fed diets of varying grain inclusion rates of 0, 25, and 50% (G0, G25, G50) ad libitum for a period of five weeks. The most prevalent ingredients in the grain ration were 34.6% wheat middlings, 30% barley 10% corn, and 10% wheat. Various minerals were included in the ration, including calcium (1.63%), Phosphate (0.770%), sodium (0.407%), chloride (0.665%), potassium (0.648%), magnesium (0.562%), and sulfur (0.208%) at a percent inclusion rate and iron (248.5 mg/kg), manganese (65.5 mg/kg), zinc (45.7 mg/kg), copper (7.9 mg/kg), selenium (0.34 mg/kg), cobalt (0.17 mg/kg), and iodine (0.035 mg/kg), at a mg/kg inclusion rate.

Three of the cows were primiparous and three were multiparous. The animals were familiarized with the chamber hoods prior to the beginning of the experiment. The experiment was set up as a replicated 3 × 3 Latin Square design and consisted of three periods, each of which were five weeks long. A detailed description of the diets can be found in Table 1.

Table 1: Dietary Components and Chemical Composition

Items	Treatment[1]		
	G0	G25	G50
Ingredient Composition, % DM			
Grass Hay	80.0	60.0	40.0
Alfalfa Hay	20.0	15.0	10.0

Barley-Corn Based Ration	0.0	25.0	50.0
Chemical Composition			
Dry Matter	95.2	95.4	95.6
Crude Protein (% DM)	13.6	13.5	13.4
ADF (% DM)	36.6	30.0	23.3
NDF (% DM)	56.0	47.3	38.7
Starch (% DM)	0.5	10.0	19.5

[1]Treatment: G0, 100% Hay; G25, 25% Grain, 75% Hay ; G50, 50% Grain, 50% Hay

In the first week of every period the G25 diet was fed to all animals. This functioned to decrease the possibility of rumen acidosis during the transition to diet G0 or G50. Especially for the G50 diet this approach reduced the chance of rumen acidosis associated with a large increase in dietary grain from G0 to G50 over a short period of time. In the following 3 weeks of each experimental period (weeks 2-4) the animals were offered the diet corresponding to their treatment in that period. During the fifth week of each period, fecal and rumen fluid samples were taken at 0830 h prior to AM feeding, and at 1500 h, 6 hours after initial feeding for the day. Rumen fluid samples were collected using an oral stomach tube (137). Fecal grab samples were collected using a sterile glove (137). The pH of samples was determined with a pH probe (Fisher Scientific, Toronto, ON, Canada). Prior to samples being collected, the pH meter was calibrated using standard solutions of pH 4 and 7 (Fisher Scientific, Toronto, ON, Canada). Following pH determination, two subsets of the rumen and fecal samples were taken and prepared as described in 5.1.2 and 5.1.3.

The day following sampling, the animals were placed for a period of 24 h in an open-hood calorimetric system to measure enteric methane as described by Odongo et al. (2007) (7) with some modifications as described in section 5.1.4.

5.1.2 Fecal sample preparation

A representative subset of the fecal sample (5 g) was put in a 60 g whirltop bag (Fisher Scientific, Toronto, ON, Canada) and placed in liquid nitrogen for two minutes. The sample was then removed and stored at -80°C until microbial analysis. Another portion of the representative fecal sample (~20 grams) was placed in a 50 mL falcon tube (Corning Life Sciences, Fisher Scientific, Toronto, ON, Canada). A saline solution (Bimeda-MTC NAC, Cambridge, ON, Canada) was added to the fecal sample in the falcon tube at a 1:1 g:mL ratio and the sample was

centrifuged at 1200 × *g* for 15 min. Upon completion, 4 mL of supernatant was removed into a sterile culture tube with cap (Simport, Beloeil, QC, Canada) in duplicate and 1 mL of 0.6 M Metaphosphoric acid was added to the sample for VFA analysis. Duplicates were taken for all sample types collected.

5.1.3 Rumen fluid sample preparation

A subset of the rumen fluid sample (10 mL) was collected, and thoroughly mixed before being put in a 15 mL conical centrifuge tube (Corning Life Sciences, Fisher Scientific, Toronto, ON, Canada) and placed in liquid nitrogen for 2 min. The sample was then removed and stored at -80°C until microbial analysis. To further analyze the rumen fluid sample, 40 mL of the representative rumen fluid sample was placed in a 50 mL falcon tube (Corning Life Sciences, Fisher Scientific, Toronto, ON, Canada) and was centrifuged at 1200 × *g* for 15 min. Upon completion, 4 mL of supernatant was removed into a sterile culture tube with cap (Simport, Beloeil, QC, Canada). To the supernatant, 1 mL of 0.6 M Metaphosphoric acid was added to the sample for VFA analysis. Duplicates were taken for all sample types collected.

5.1.4 Methane production determination technique

Enteric CH_4 production values were determined using a modified open-hood calorimetric system similar to that from Odongo et al. (2007) which allowed for real-time data analysis to be displayed and stored on a windows-based laptop computer using LabVIEW software (version 11.0 Professional, National Instruments, Vaudreuil-Dorion, QC, Canada) (138). Major modifications were conducted from what is documented by Odongo et al. (2007) (7). Modifications to the chamber hood included amendments to the calibration frequency which now occurred daily and to the procedure which involved adding known concentrations of CH_4, CO_2, and O_2 to calibrate the analyzers, the addition of valves to ensure consistent flow rate for both the

chambers and the background air, and the installation of gauges to ensure that there was an optimum amount of each gas for the sensor to function. In brief, analyzer calibration of CH_4, CO_2, and O_2 occurred daily preceding the animal's entry into the chamber. The CH_4 recovery rate of both chambers used in this study was measured at the end of each sample period by adding a known amount and concentration of CH_4 into the chamber hood and comparing that to the amount of CH_4 determined by the analyzer.

Measurements were gathered for a span of 100 seconds sequentially from both chambers and background air then cycling back to the chambers again. For the background measurements, the tubing was changed to 8.5 cm to ensure a flow rate consistent with both chambers. Since the measurements occur in a negative pressure system, the values in the total production numbers were subtracted from the value of CH_4 present in the background air, which the animal was not directly accountable for producing enterically, but rather was a function of the CH_4 values present in the experimental environment.

To determine the value for CH_4 production in L d^{-1} at standard pressure, the calculations below were required to account for changes in temperature and pressure which effect the number of molecules flowing through the system. The analyzers determined values for temperature (°C), relative humidity (%), absolute pressure (PSI), flow rate (L m^{-1}), as well as the values of O_2 (%), CO_2 (%) and CH_4 (ppm).

The saturation pressure of H_2O vapor (p_{ws}) in kPa, which was the maximum amount of moisture the air could carry had to be calculated with the temperature in Kelvin (K):

$$p_{ws} = \frac{exp\left(77.3450 + 0.0057T - \frac{7235}{T}\right)}{T^{8.2}}$$

Subsequently, the absolute pressure (p_{abs}) was converted from pounds per square inch (PSI) to Pascal (Pa) with the conversion factor of 1 PSI = 6894.745 Pa.

Then, using pws from the first equation, vapor pressure (p_w) was calculated using the relative humidity (RH) value determined by the analyzer and the equation:

$$Vapor\ Pressure(p_w) = \frac{(RH \times pws)}{100}.$$

Using Dalton's Law and combining vapor pressure calculated in the last step (p_w) with the dry absolute pressure value (p_{abs}) calculated earlier, the total pressure of the air was determined using:

$$p\ (total\ pressure\ of\ air) = p_{abs} + p_w.$$

The next step functioned to determine the volume of dry gas pumped through the system during a 60 sec interval. Prior to exposure to the analyzers, moisture present in the air was removed using a commercial desiccant (W. A. Hammond Drierite Company Ltd, Xenia, OH, USA). With observations from the analyzers occurring at 10 sec intervals, the flow rate of the air is measured with humidity via display gauges. The calculation then consisted of:

$$Dry\ vol\ of\ gas = (\frac{measured\ flow\ rate \times partial\ pressure\ of\ dry\ air}{p_{abs}} \times 60\ \text{sec}\ span)$$

The next step determined the standard temperature and pressure (STPD) for the volume of gas. This step combined Charles' Law which states that at a constant pressure, the volume of a given gas is directly proportional to the absolute temperature $\left(\frac{V1}{T1} = \frac{V2}{T2}\right)$ with Boyle's Law which states that at a constant temperature, the volume of a given quantity of gas is directly proportional to its pressure($P1 \times V1 = P2 \times V2$). Both Charles' Law and Boyle's Law were

utilized and the volume of the gas was standardized over the analyzer at a standard temperature (237.15 K), and pressure (100 kPa), thus:

$$\text{STPD } V2 = \frac{(P1\ (Pabs))}{1000} \times V1\ (dry\ volume\ of\ gas \times \frac{T2\ (standard\ T\ of\ 273.15\ K)}{T1(from\ display) \times P2(from\ display}$$

The final calculation was to determine CH_4 production in $L\ min^{-1}$. This consisted of taking the difference between CH_4 production in the chamber hood and CH_4 prevalent in the background air over a 60 sec span, and multiplying it by STPD which was calculated in the last step. Taking into account CH_4 values need to be converted from µL to L:

$$STP(CH_4\ L\ min^{-1}) = [CH_4 production\ (\ \mu L)] - [CH_4 in\ background\ air\ (\ \mu L)] \times STPD(l)/10^6$$

The values were then individually reviewed using a MIXED procedure in SAS (ver 9.3, 2011) to ensure no outliers were present in the dataset.

The open-hood calorimetric system was calibrated daily at 0830, prior to the introduction of the animal into the chamber hood calorimeter, which began at 0900 during sample weeks. The calibration process occurred for each of the CO_2, O_2, and CH_4 gases. Known volumetric concentrations of each gas, 0.15% M for CH_4, 20% M for O_2, and 2.5% M for CO_2 were dried using a commercial grade desiccant and directly sent through the sample pump to the analyzers which display the readings. The concentration of gases used for calibration was based on production values from literature and reflect approximate expected concentrations of CH_4, CO_2, and O_2 present in the chamber hood based on published production values (52). These values were cross referenced with the values obtained from the adjustment period which occurred immediately prior to the animal trial during which the animals were introduced to the open-hood calorimetric system. Based on the readings, the known concentrations of the reference gases which were previously determined and documented on the certificate of analysis would then

allow for the calculation of offset and gain enabling the software to correctly analyze the concentration of CH_4, CO_2, and O_2 gases for the chambers and background air. Following each sample period, recovery rate tests for CH_4 were conducted. This involved a three-decimal point scale to hold the CH_4 canister. The initial weight of the canister was documented. Following this, CH_4 was released into the airtight chamber for a duration of 1 min. Following this duration, the CH_4 canister was again weighed. The difference between the initial and final weights was calculated, then knowing the CH_4 concentration, the amount of CH_4 inserted into the chamber was calculated. The calculated amount of CH_4 presented to the chamber was then cross referenced with the values determined by the analyzer over the same time span. Any deviation between the calculated values based on the amount of CH_4 released into the chamber and the values displayed by the analyzer was considered an error on the part of the analyzer.

5.1.5 DNA extraction and quality check

Approximately 200 mg of rumen fluid and feces sample was used for DNA extraction. The day preceding DNA extraction, samples were removed from the -80°C freezer and placed in a 4°C refrigerator overnight to thaw. The samples were then cryogenically homogenized with a Geno/Grinder® 2010 (SPEX SamplePrep, Metuchen, NJ, USA), subsequently, the DNA was extracted using a ZR-96 Fecal DNA Kit (Zymo Research, Irvine, CA, USA) that included a bead-beating step for mechanical lysis of bacterial cells. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA was then normalized to a concentration of 20 ng/µl, and the quality of the amplified DNA was verified by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as

described by Khafipour et al. (139). The amplicons were quality checked via agarose gel electrophoresis.

5.1.6 Library construction and Illumina sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. (140). In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers (141). The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. Duplicates were run for all PCR reactions, with the contents of each reaction being 1.0 µL of pre-normalized DNA, 1.0 µL of both forward and reverse primers (10 µM), 12 µL HPLC grade water (Fisher Scientific, Ottawa, ON, Canada) and 10 µL 5 Prime Hot MasterMix (5 Prime, Inc., Gaithersburg, MD, USA). The PCR reactions occurred in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany) and was comprised of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 secs, 50°C for 60 sec, and 72°C for 90 sec. upon completion of the amplification cycles the final step was an extension step which occurred at 72°C for a duration of 10 min. Upon completion, the PCR products were purified using the ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA) to remove primers, dNTPs and reaction components. A 200 ng of product from each sample was pooled into the allocated V4 library. The pooled samples were then quantified fluorometrically using Picogreen dsDNA (Invitrogen, Burlington, ON, Canada). This was followed by multiple dilution steps using a pre-chilled hybridization buffer (HT1; Illumina, San Diego, CA, USA) in an effort to bring the pooled amplicons to a final concentration of 5 pM. Following this, the final concentration of the pooled amplicons was measured using a Qubit 2.0 Fluorimeter (Life technologies, Burlington, ON,

Canada). Finally, 15% of PhiX control library was administered into the amplicon pool to enhance the composition of unbalanced and biased bases, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3′) were synthesized, and subsequently purified via polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq reagent kit V2 (300-cycle; Illumina, San Diego, CA, USA). The 150 paired-end sequencing reaction was performed using the MiSeq platform (Illumina) at the Gut Microbiome and Large Animal Biosecurity Laboratories (Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada). The sequencing data were deposited into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed via accession number SRR3202872.

5.1.7 Bioinformatic analyses

The FLASH assembler (142) was employed to merge all paired-end Illumina fastq files which overlapped. Any sequences possessing mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then further analyzed by downstream computational pipelines of the open source software package QIIME (143). Assembled reads were de-multiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with ambiguous calls and those with phred quality scores (Q-scores) below 20 were discarded. Chimeric reads were filtered using UCHIME (144) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (145) at

97% pairwise identity threshold using an open-reference OTU picking process (146). Taxonomies were assigned to the representative sequence of each OTU using RDP classifier (147), and were aligned with the Greengenes Core reference database (ver 13.5) (148) using PyNAST algorithms (149).

To allow for further microbial community comparisons, a phylogenetic tree was built using FastTree 2.1.3. (150). Within community diversity (α-diversity) was calculated using QIIME. An Alpha rarefaction curve was generated using Chao 1 estimator of species richness (151) with ten sampling repetitions at each sampling depth. An even depth of approximately 15,700 sequences per sample was used for calculation of richness and diversity indices.

To compare the microbial composition between communities in the rumen and fecal samples, β-diversity was measured by calculating the weighted and unweighted UniFrac distances (152) using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots using PRIMER v6 software (153). Permutational multivariate analysis of variance (PERMANOVA) (154) was employed to determine *P*-values and test for significant differences of β-diversity among treatment groups. Finally, the open source software PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states; v. 1.0.0-dev) (126) was used to predict the functional capacity of microbiome using 16S rRNA gene sequencing data and Greengenes (v. 13.5) reference database (155). To make our open-reference picked OTUs compatible with PICRUSt, all de-novo OTUs were removed and only those that had matching Greengenes identifications were retained. The new OTU table was then used to generate metagenomic data after normalizing the data by copy numbers, and to derive relative Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway abundance (156). Predicted genes were clustered hierarchically and categorised on the basis of KEGG (157) orthologs (KO's) and pathways (level 1-3). The KEGG data was analyzed using STAMP (STatistical Analysis of Metagenomic Profiles) (158).

5.1.8 Statistical analysis

Statistical analyses were performed as described by Li et al. (159). In brief, for DMI, pH, VFAs, CH_4, microbiota richness and diversity as well as microbiota composition at the phylum level, statistical calculations were conducted using a completely randomized design using MIXED procedure of SAS (v. 9.3, 2011). The model included period, cow, diet, and period×diet interaction. The fixed factors of the model included diet, period, period×diet. Cow (square), and square were considered random with square being whether the animal was assigned to the first or second of the replicated Latin square at random during experimental setup. For the production data, the effect of day within period also was considered as fixed. Differences between means were determined using Tukey's test, and the UNIVARIATE procedure of SAS was used for testing the normality of residuals. For non-normally distributed data, Poisson and negative binomial distributions were fitted in the SAS GLIMMIX procedure and the goodness of fit for different distributions was determined using Pearson chi-square /DF (closer to 1 is better) when necessary. The differences between treatments were considered significant at $P < 0.05$. Trends were discussed at $P < 0.1$.

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was conducted on the genus data to ascertain the effects of treatments. The PLS-DA is a specific case of partial least square regression analysis in which Y is a group of variables characterizing the categories of a categorical variable on X (160). For this situation, the X

variables were bacterial genera and the Y variables were diets varying in grain concentration (G0, G25, and G50). Data was scaled using Unit Variance in SIMCA (SIMCA P+ 13.0, Umetrics, Umea, Sweden). To ascertain the number of significant PLS components, cross-validation was formed, permutation testing was also conducted to validate the model. The variable influence on projection value (VIP) was utilized in an effort to prevent the over parameterization pertaining to the model (160). All genus and genera with VIP values <0.50 were discarded from the final model to ensure genera prevalence throughout samples (161, 162). The R^2 estimate was used to estimate the goodness of fit. To evaluate the predictive value of the model, Q^2 estimates were used. The PLS-regression coefficients were used to identify genera that were most characteristics of each treatment group. The significant shifts of taxa were determined when the error bars of each component were above or below x axis of coefficient plot (Wang et al., 2016). Results were visualized by PLS-DA loading scatter plots.

5.1.9 Predictive Analysis

The cumulative prediction model was determined using the PLS method in SIMCA (SIMCA P+ 13.0, Umetrics, Umea, Sweden). The taxa were filtered so selection occurred if the taxa were present in over 10% of the samples, had significant coefficients values for CH_4 production, and had a variable importance in projection (VIP) value greater than 0.6. The CH_4 production values, and identification parameters were cross referenced with the 16S rRNA gene sequencing results analyzed by QIIME, as well as dietary ADF, NDF, and starch concentrations to determine the bacterial correlation as it relates to overall CH_4 production (L d^{-1}). For the model, the Y variable is CH_4 production (L d^{-1}) and the X variable included ADF, NDF, starch and bacteria taxa that met the above criteria.

Results

6.1.1 Dry Matter Intake

As shown in Table 2, the average DMI, calculated in kgDMI d^{-1} for the animals over all 3 periods for diets G0, G25, and G50, respectively, were 11.4, 13.7, and 15.6 ±0.7 kgDMI d^{-1} ($P \leq 0.01$). DMI is reflective of the total sampling week and there was no significant difference in intake coinciding with sampling days.

6.1.2 pH

As seen in Table 2, the pH values of both the rumen and fecal samples decreased with increasing concentrate supplementation from G0, to G25, to G50. The pH of the rumen fluid samples collected in the AM was not significantly different ($P = 0.1$) among treatments with values of 6.84, 6.77, 6.74 for G0, G25, and G50, respectively. There was a trend downwards ($P \leq 0.01$) for the pH of the rumen fluid samples for G0, G25, and G50 collected in the PM, with values of 6.80, 6.68, 6.46.

With regards to the fecal samples, there was a significant difference in pH between the three diets in both the AM and PM samples. Again, absolute values were higher ($P \leq 0.01$) in the AM than PM, with pH values of 7.38, 7.02, and 6.87 for diets G0, G25, and G50 in the AM. The pH values in the PM were 7.19, 6.88, and 6.78 for G0, G25, and G50, respectively ($P \leq 0.01$). For fecal samples, increased concentrate inclusion in the diet decreased the pH ($P \leq 0.01$) in both the AM and PM sampling periods.

Table 2: Experimental Results: DMI and pH

	Treatment[1]				
	G0	G25	G50	SEM	*P* Value
DMI (kg)	11.4	13.7	15.6	0.7	<0.01
Rumen pH					
0830	6.84	6.77	6.74	0.05	0.1

1500	6.80	6.68	6.46	0.05	<0.01
Fecal pH					
0830	7.38	7.02	6.87	0.06	<0.01
1500	7.19	6.88	6.78	0.07	<0.01

SEM = Standard error of means of samples

Statistical Analysis was conducted using MIXED procedure of SAS.

0830 and 1500 = Time of sample collection in 24-hour time

(1) Barley-Grain ration inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%)

6.1.3 CH_4 Production

As seen in Table 3, with regards to total CH_4 production (L d^{-1}), the production values increased ($P \leq 0.01$) from G0, to G25 and G50 with values of 354.5, 423.3, 445.0 L d^{-1}, respectively. There was no significant effect pertaining to the sequence of bringing animals from different treatments in the hood.

When CH_4 production was expressed per kg DMI^{-1}, the opposite effect was observed, as values numerically decreased coinciding with increased grain supplementation in the diet. The CH_4 production values, when adjusted to account for adjusted DMI showed the value associated with G50 was significantly different ($P = 0.01$) than those of G0 and G25. The values were of 30.3, 30.1, and 27.6 L kg adjusted DMI^{-1} for G0, G25 and G50, respectively. When CH_4 production was expressed on a %GEI basis, there was no difference ($P = 0.5$) between diets G0, to G25, to G50 with values of 8.0, 7.6, 7.1 L $\%GEI^{-1}$ respectively.

Table 3: Experimental Results: CH_4 Production

Methane Production	Treatment[1]			SEM	*P* Value
	G0	G25	G50		
CH_4 (L d^{-1})	354.5	423.3	445.0	10.2	<0.01
CH_4 (L kg Adjusted DMI^{-1}) [2]	30.3	30.1	27.6	1.1	0.01

CH_4 (L %GEI^{-1}) [3]	8.0	7.6	7.1	0.7	0.5

SEM = Standard error of means of samples

Statistical Analysis was conducted using MIXED procedure of SAS.

(1) Barley-Grain ration inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%)
(2) Adjusted DMI was an average of DMI on sample collection and CH_4 collection days.
(3) CH_4 production as a percentage of the animal's gross energy intake

As seen in Figure 2, a diurnal change in CH_4 production ($P \leq 0.01$) was observed throughout the 24 h period as determined by a time contrast calculation. Figure 2 took the average CH_4 production values from all animals on of each of the treatments and averaged them to coincide with the time the CH_4 was produced with standard error being labelled. Additionally, there was an increase ($P \leq 0.01$) in CH_4 production coinciding with feeding events (1300, 1700), while the lowest CH_4 production in the period occurred at duration between feeding which was the longest.

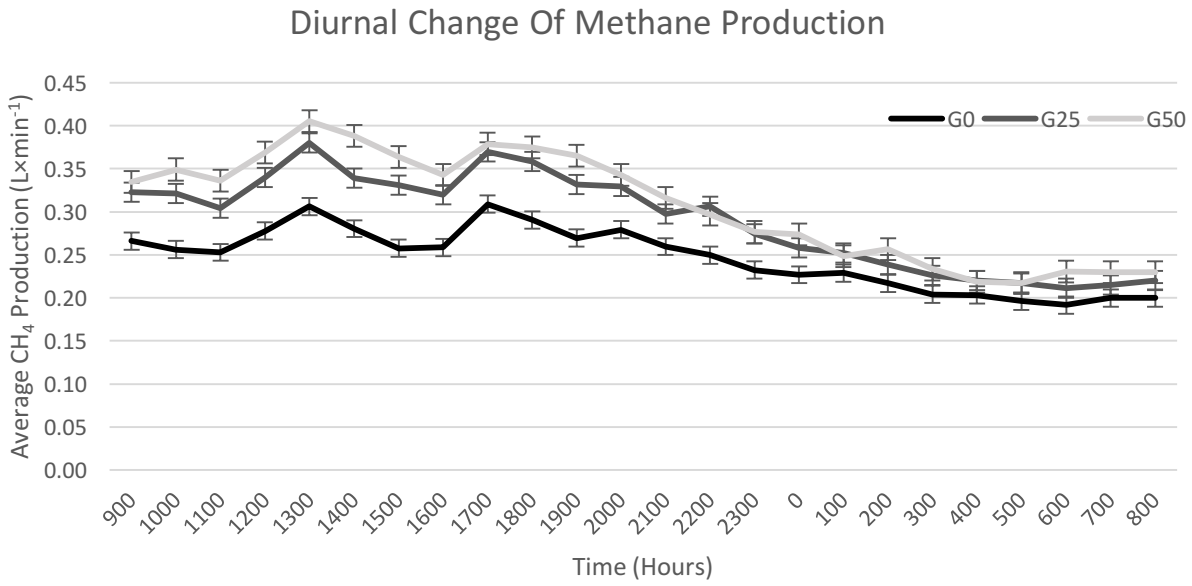


Figure 2: Average CH_4 production for G0, G25, and G50 and the time corresponding to the production throughout all 3 periods. Error bars are standard error of the means.

6.1.4 Volatile Fatty Acid Production

The concentration of VFA in most cases increased coinciding with the increase of grain inclusion in the diet (Table 4). In the rumen fluid, the concentration of acetate increased (P = 0.01) from 47.5 to 53.0 and 55.3 mM in diets G0, G25, and G50, respectively. The propionate concentrations also increased ($P \leq 0.01$) with increasing grain inclusion in the diet, from 11.0 and 12.7 to 15.3 mM for G0, G25, and G50, respectively. Butyrate concentrations also increased ($P \leq 0.01$) coinciding with increased grain inclusion rates in the diet, from 6.2, 8.5, and 9.8 mM for each of diets G0, G25, and G50, respectively. Finally, there was also an increase ($P \leq 0.01$) in total VFA concentrations in the rumen from 67.1, to 76.8, to 83.0 mM.

Table 4: Experimental Results: Rumen VFA Results

	Treatment[1]				
Volatile Fatty Acids	G0	G25	G50	SEM	*P* Value
Acetate (mM)	47.5	53.0	55.3	1.7	0.01

Propionate (mM)	11.0	12.7	15.3	0.5	<0.01
Iso-Butyrate (mM)	0.9	0.9	0.7	0.1	0.3
Butyrate (mM)	6.2	8.5	9.8	0.5	<0.01
Iso-Valerate (mM)	0.8	0.8	1.0	0.07	0.2
Valerate (mM)	0.7	0.7	0.9	0.06	0.01
Total VFA (mM)	67.1	76.8	83.0	2.5	<0.01
Acetate:Propionate	4.3	4.2	3.6	0.08	<0.01

SEM = Standard error of means of samples

Statistical Analysis was conducted using MIXED procedure of SAS.

(1) Barley-Grain ration inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%)

The fecal VFA concentrations are shown on Table 5. There was a similar trend of increased VFA concentrations coinciding with an increase in grain inclusion in the diet for the fecal samples with a pronounced difference between diet G50 and the other two diets. The concentration of acetate was 17.8, 18.6, and 26.8 mM ($P = 0.01$) for each of diets G0, G25, and G50, respectively. The concentration of propionate was 3.6, 3.6, 5.5 mM ($P = 0.02$) for diets G0, G25, and G50, respectively. For diets G0, G25, and G50, butyrate concentration values were 2.1, 2.1, 3.5 mM ($P = 0.04$). Finally, the total VFA concentration increased from 26.1 and 26.5 to 38.5 mM ($P = 0.02$) for diets G0, G25, and G50, respectively.

Table 5: Experimental Results: Fecal VFA Results

	Treatment[1]				
Volatile Fatty Acids	G0	G25	G50	SEM	*P* Value
Acetate (mM)	17.8	18.6	26.8	2.9	0.01
Propionate (mM)	3.6	3.6	5.5	0.6	0.02
Iso-Butyrate (mM)	1.1	1.0	1.3	0.2	0.4
Butyrate (mM)	2.1	2.1	3.5	0.4	0.04
Iso-Valerate (mM)	0.9	0.7	0.8	0.2	0.6
Valerate (mM)	0.6	0.5	0.6	0.1	0.6
Total VFA (mM)	26.1	26.5	38.5	4.2	0.02
Acetate:Propionate	5.1	5.1	4.9	0.2	0.5

SEM = Standard error of means of samples

Statistical Analysis was conducted using MIXED procedure of SAS.

(1) Barley-Grain ration inclusion rate in the diet: G0 (0%), G25 (25%), or G50 (50%)

6.2 Rumen Microbial Analysis

The reads were quality filtered on the basis of the quality and length of the reads. The OTU's were aligned at a 97% similarity threshold against the Greengenes database. While the majority of sequences are classified at the genus level, some could only be assigned to the phylum, class, order, and family levels. The average sequencing lengths for the V4 region was 253 nucleotides, and there was an average of 35,600 sequences per sample.

6.2.1 Within Community Diversity

Table 6 shows the numerical values pertaining to the within community diversity found in the rumen fluid samples. At a cut-off of 10,000 sequences per sample, the G25 treatment had the highest number of observed species ($P \leq 0.001$) compared to G0 and G50. The Chao1 estimator of richness values were 1307, 1418, and 1325 ($P = 0.001$) for the diets G0, G25, and G50 respectively. The values for the Shannon diversity index for G0, G25, and G50 were 8.14, 8.43, and 8.14 ($P = 0.003$). Finally, the Simpson diversity index showed values of 0.96, 0.99, and 0.99, ($P = 0.03$) for G0, G25, and G50 respectively.

Table 6: Statistical summary for diversity indices in rumen communities based on treatment

	Treatment						
	G0	G25	G50	SED[3]	*P* (diet)	*P* (period)	*P* (diet×period)
Number of sequences per sample	10,000	10,000	10,000	---	---	---	---
Observed number of species	1043[b]	1122[a]	1051[b]	19.22	<0.001	<0.001	<0.001
Goods-Coverage (%)	96.9[b]	96.6[a]	96.8[ab]	<0.001	0.007	0.004	0.02
Chao1[4]	1307[a]	1418[b]	1325[a]	28.50	0.001	<0.001	0.001
Shannon[5]	8.14[a]	8.43[b]	8.15[a]	0.09	0.003	0.01	<0.001
Simpson[5,6]	0.986[ab]	0.991[b]	0.986[a]	0.001	0.03	0.09	<0.001

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

[1] Based on Chao1 estimator of species richness

[2] Based on Shannon and Simpson diversity estimators

[3]SED = Standard error of difference between least square means of treatments

[4] Species richness estimate provided by the Chao1 index

[5] Species diversity index provided by the Shannon-Wiener index and the Simpson index

[6] Simpson Diversity Index was calculated using GLIMMIX model

6.2.2 Taxonomic Classification

At the phylum level, there were 21 phyla present in the rumen fluid of animals offered diet G0. Among those, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Fibrobacteres*, and *Tenericutes* were the most abundant in the rumen fluid. Investigation into the genus level showed there was 185 genera present. Among those, there were 106 classified genera, and 79 unclassified genera. There were 22 phyla present in the rumen fluid of animals offered diet G25. Among those, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fibrobacteres*, and *Tenericutes* were the most abundant. With regards to the genera present in the rumen fluid at the G25 level, there

were a total of 182 genera present. Of those 182 genera, 109 were classified, while 73 were unclassified. Finally, in the rumen fluid collected from animals offered diet G50, there was 22 phyla present. Among those, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, and *Tenericutes* were the five most abundant phyla. For the rumen fluid collected from animals offered diet G50, there were also a total of 171 genera present. Of those 171, there were 100 classified genera as well as 71 unclassified genera present.

Additionally, as seen in Figure 3, the microbial composition of communities of the three treatments differed ($P \leq 0.01$).

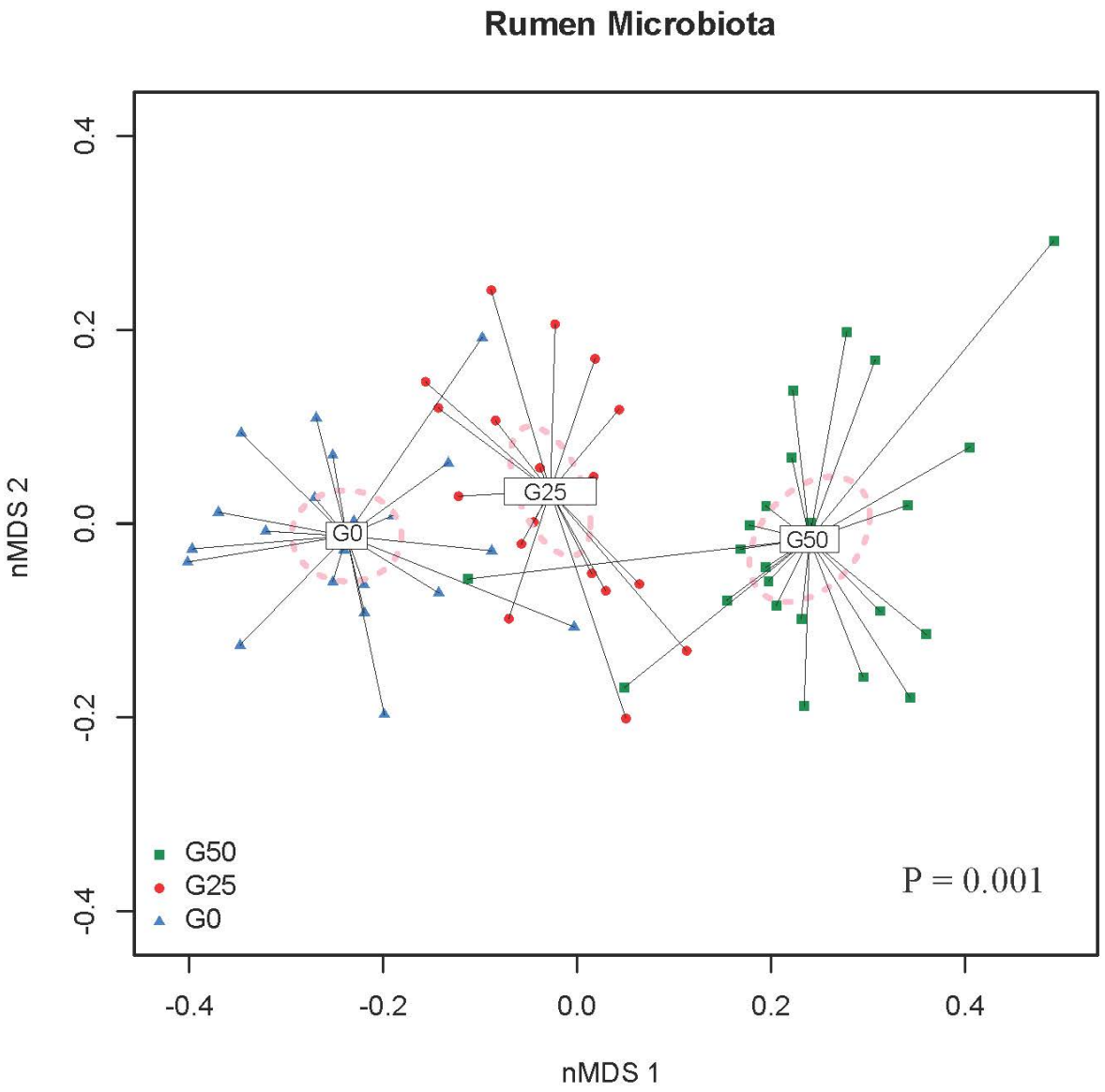


Figure 3: Between Community Diversity of the Rumen Environment

6.2.3 PLS-DA Analysis

Multivariate regression analysis was conducted to determine the differences in microbial communities in the rumen fluid for G0 vs. G25, G0 vs. G50, and G25 vs. G50. The greatest differences in microbial community composition in the rumen fluid were found in samples for animals fed G0 and G50, the results are shown on Figure 4. There were no significant differences

between the regression analysis of G25 vs. G50. With regards to the analysis of G0 vs. G25, the R^2 and Q^2 estimates were 0.880, and 0.779, respectively. There were 6 bacterial members, which were positively correlated to G0 and negatively correlated to G25. Members of the phyla *Bacteroidetes* (order *Bacteroidales* and genera *BF311*), Cyanobacteria (order *YS2*), *Tenericutes* (order *ML615J-28*) and the phylum *Lentispaherae* were all among the bacterial members positively correlated to G0 and negatively related to G25 in the rumen fluid samples of animals offered G0 and G25 respectively. There was one group whose abundance was positively correlated to G0 only, it belonged to the phylum *Tenericutes* (order *Acholeplasmatales*). Another bacterial member whose abundance in rumen fluid was negatively correlated to G25 only, belonged to the phylum *Tenericutes* (genera *Aeroplasma*). There were 5 bacterial members which were positively correlated in rumen fluid samples in animals offered the G25 diet, and also negatively correlated with animals offered G0. The bacterial members included the phyla *Bacteroidetes* (family *S24-7*), *Firmicutes* (genera *Ruminococcus*), and *Proteobacteria* (family *Succinivibrionaceae*, genera *Ruminobacter*, and *Succinoibrio*). There was also one bacterial member found in the rumen fluid, which was positively correlated to animals offered G25 belonging to the phylum *Firmicutes* (genera *Shuttleworthia*). There were two groups that were negatively correlated to G0 also belonging to the phylum *Firmicutes* (family *Clostridiaceae*, genera *Succiniclasticum*).

Figure 4 shows the comparison of bacterial members found in the rumen fluid in animals offered diets G0 vs. G50. The R^2 and Q^2 estimates were 0.876, and 0.810, respectively. There were 7 bacterial members, which were positively correlated to G0 and negatively correlated to G50. The bacterial members belonged to the phylum *Cyanobacteria* (order *YS-2*), *Proteobacteria* (class *Alphaproteobacteria*), *Bacteroidetes* (genera *BF311*), *Tenericutes* (order

ML615J-28, and *RF-39*), *Firmicutes* (genera *RFN20*), and the phyla *Lentisphaerae* itself. There were 6 bacterial members showing positive correlation to G50, while being negatively correlated to G0. They included members belonging to the phylum *Elusimicrobia* (class *Endomicrobia*), *Proteobacteria* (genera *Ruminobacter*), *Bacteroidetes* (family *S24-7*), and *Firmicutes* (genera *Shuttleworthia*, *Oscillospira*, and *Succiniclasticum*).

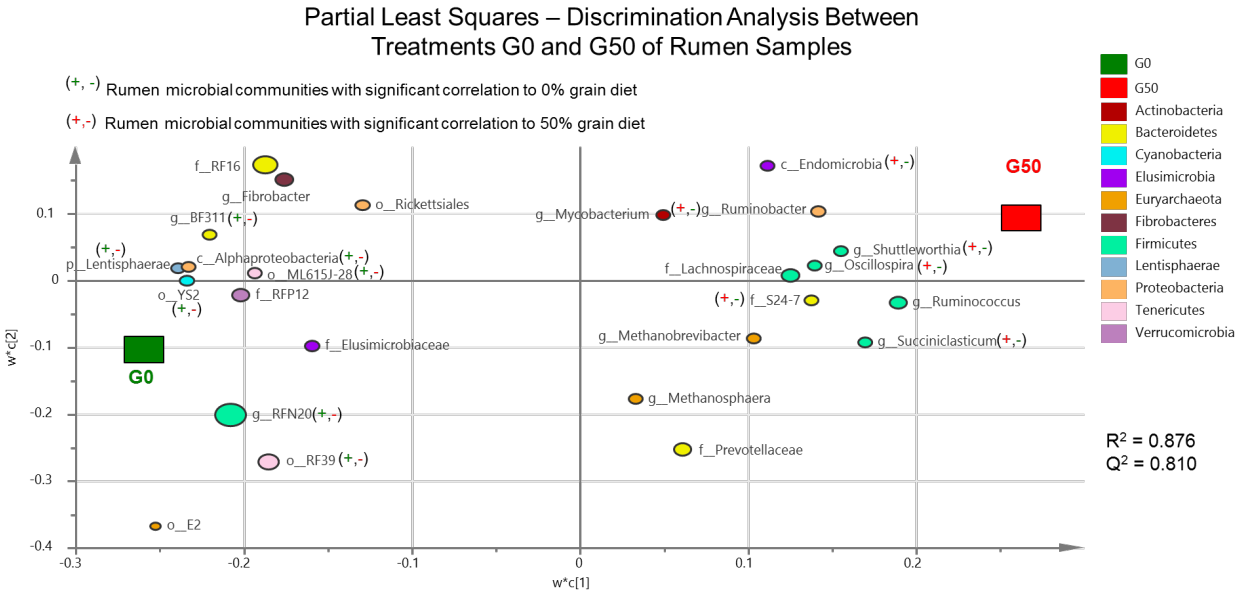


Figure 4: Partial Least Squares - Discrimination Analysis between G0 and G50 of Rumen fluid Samples

6.2.4 Bacteria Showing Correlation Between CH_4 kgDMI^{-1} and Treatment in Rumen Fluid Samples

Upon analysis of the bacteria positively and negatively correlated with both CH_4 kgDMI^{-1} and each of treatments G0, G25, and G50, there were bacteria correlating both positively and negatively based on their Spearman's Rho coefficient.

Table 7 depicts rumen bacteria under diet G0 that were significantly correlated with CH_4 kgDMI^{-1}. Orders *Clostridiales* (Spearman's Rho -0.548, $P \leq 0.01$) and *Bacteroidales* (Spearman's Rho -0.531, $P \leq 0.01$) as well as family *Coriobacteriaceae* (Spearman's Rho -

0.650, $P \leq 0.01$) and genus *Anaeroplasma* (Spearman's Rho -0.555, $P \leq 0.01$) were negatively correlated with CH_4 kgDMI^{-1}. There was a positive correlation between the genus *Blastococcus* (Spearman's Rho 0.438, $P = 0.03$) and CH_4 kgDMI^{-1}.

Table 7: Bacterial Correlation between CH_4kgDMI^{-1} and treatment G0 in the rumen environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Order Actinomycetales	-0.435	0.03
Family Coriobacteriaceae	-0.650	< 0.01
Family Patulibacter	-0.449	0.03
Genus Corynebacterium	-0.492	0.02
Genus Bifidobacterium	-0.466	0.02
Genus Brevibacterium	-0.462	0.02
Genus Brachybacterium	-0.456	0.03
Genus Glycomyces	-0.435	0.03
Genus Atopobium	-0.417	0.04
Genus Blastococcus	0.438	0.03
Bacteroidetes		
Order Bacteroidales	-0.531	< 0.01
Family Prevotellaceae	-0.487	0.02
Family Chitinophagaceae	-0.506	0.01
Genus Dyadobacter	-0.435	0.03
Firmicutes		
Order Clostridiales	-0.548	< 0.01
Genus RFN20	-0.700	< 0.01
Genus Succiniclasticum	-0.522	< 0.01
Genus Streptococcus	-0.455	0.03
Genus Staphylococcus	-0.417	0.04
Genus Butyrivibrio	-0.417	0.04
Proteobacteria		
Class Betaproteobacteria	0.458	0.03
Family Aurantimonadaceae	-0.446	0.03
Genus Erwinia	-0.511	0.01
Genus Methylobacterium	-0.454	0.03
Genus Bibersteinia	-0.433	0.04
Genus Methylobacillus	0.435	0.03
Spirochaetes		
Genus Treponema	-0.442	0.03
Tenericutes		
Genus Anaeroplasma	-0.555	< 0.01
WPS-2		
Phyla WPS-2	-0.456	0.03

Table 8 shows the bacterial members with significant correlation between CH_4kgDMI^{-1} fed diet G25. Family *Erysipelotrichaceae* (Spearman's Rho -0.756, $P \leq 0.01$) and genus *Anaerovibrio* (Spearman's Rho -0.653, $P \leq 0.01$) and *Succinoclasticum* (Spearman's Rho -0.640, $P \leq 0.01$) were negatively correlated with CH_4 kgDMI^{-1}. Positive correlation with CH_4 kgDMI^{-1} was found in order *Burkholderiales* (Spearman's Rho 0.646, $P \leq 0.01$) and family *Oxalobacteraceae* (Spearman's Rho 0.599, $P \leq 0.01$).

Table 8: Bacterial Correlation between CH_4 kgDMI^{-1} and treatment G25 in the rumen environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Family Coriobacteriaceae	-0.520	0.02
Family Nocardiopsaceae	-0.448	0.04
Genus Saccharopolyspora	-0.448	0.04
Elusimicrobia		
Class Endomicrobia	-0.453	0.04
Fibrobacteres		
Genus Fibrobacter	-0.514	0.02
Firmicutes		
Family Erysipelotrichaceae	-0.756	< 0.01
Genus Anaerovibrio	-0.653	< 0.01
Genus Succiniclasticum	-0.639	< 0.01
Genus Pseudobutyrivibrio	-0.628	< 0.01
Genus Butyrivibrio	-0.568	< 0.01
Genus L7A_E11	-0.515	0.02
Genus Schwartzia	-0.445	0.04
Lentisphaerae		
Family Victivallaceae	-0.446	0.04
Planctomycetes		
Family Pirellulaceae	-0.498	0.02
Proteobacteria		
Class Betaproteobacteria	0.664	< 0.01
Order GMD14H09	-0.467	0.03
Order Burkholderiales	0.646	< 0.01
Family Succinivibrionaceae	-0.561	< 0.01
Family 0319-6G20	-0.443	0.04
Family Oxalobacteraceae	0.599	< 0.01
Genus Balneimonas	-0.562	< 0.01

Genus Bradyrhizobium	0.459	0.04
Genus Novosphingobium	0.510	0.02
Synergistetes		
Genus TG5	-0.535	0.01
Tenericutes		
Order Acholeplasmatales	-0.557	< 0.01
Family Anaeroplasmataceae	0.466	0.03
Genus Anaeroplasma	-0.502	0.02
Verrucomicrobia		
Order LD1-PB3	-0.502	0.02
WPS-2		
Phylum WPS-2	0.054	0.01

Table 9 shows the bacterial members in the rumen fluid sample with significant correlation between CH_4kgDMI^{-1} and diet G50. Genera *Bifidobacterium* (Spearman's Rho -0.500, $P \leq 0.01$) and *Sanguibacter* (Spearman's Rho -0.463, $P = 0.02$) were negatively correlated with CH_4 kgDMI^{-1}. Positive correlation with CH_4 kgDMI^{-1} was found in the family *Anaeroplasmataceae* (Spearman's Rho 0.468, $P = 0.02$).

Table 9: Bacterial Correlation between CH_4 kgDMI^{-1} and treatment G50 in the rumen environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Family Coriobacteriaceae	-0.534	< 0.01
Genus Bifidobacterium	-0.500	< 0.01
Genus Sanguibacter	-0.463	0.02
Firmicutes		
Genus Butyrivibrio	-0.412	0.04
Genus Oscillospira	0.411	0.04
Proteobacteria		
Order GMD14H09	-0.509	< 0.01
Order RF32	0.424	0.03
Family Phyllobacteriaceae	-0.420	0.03
Tenericutes		
Family Anaeroplasmataceae	0.468	0.02

6.2.5 Functional Analysis of Microbial Populations

To provide functional insight into the microbial analysis, PICRUSt software was used. Upon comparing the diets G25 and G50, there was only one significant finding as shown in Figure 5, as the biosynthesis of secondary bile acid ($P = 0.032$) was greater in diet G50 than G25.

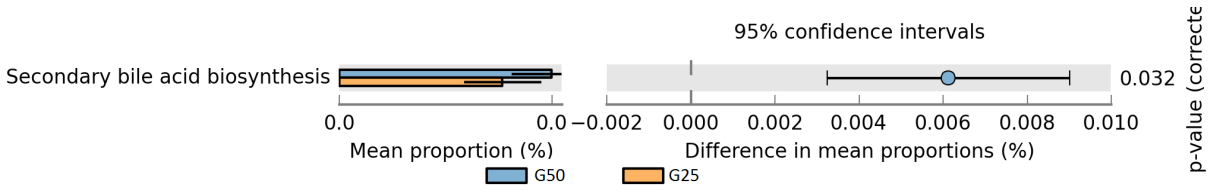


Figure 5: Experimental Results: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Rumen Environment for differing treatments for treatments G25 and G50

Pertaining to the differences between diets G0 and G25, there were 11 significant functional differences, as shown in Figure 6. There was increased metabolism of glycerolipids (P ≤ 0.01), phosphonate and phosphinate ($P \leq 0.01$), and linoleic acid ($P = 0.03$) in the diet G25 vs G0. Also, included among the 11 functional differences was the metabolism of riboflavin ($P \leq 0.01$), which was elevated in diet G0 compared to G25. The G0 treatment also showed an increase in the biosynthesis of folate ($P \leq 0.01$), as well as ubiquinone and other terpenoid-quinones ($P = 0.02$), and lipopolysaccharide ($P = 0.03$) relative to G25.

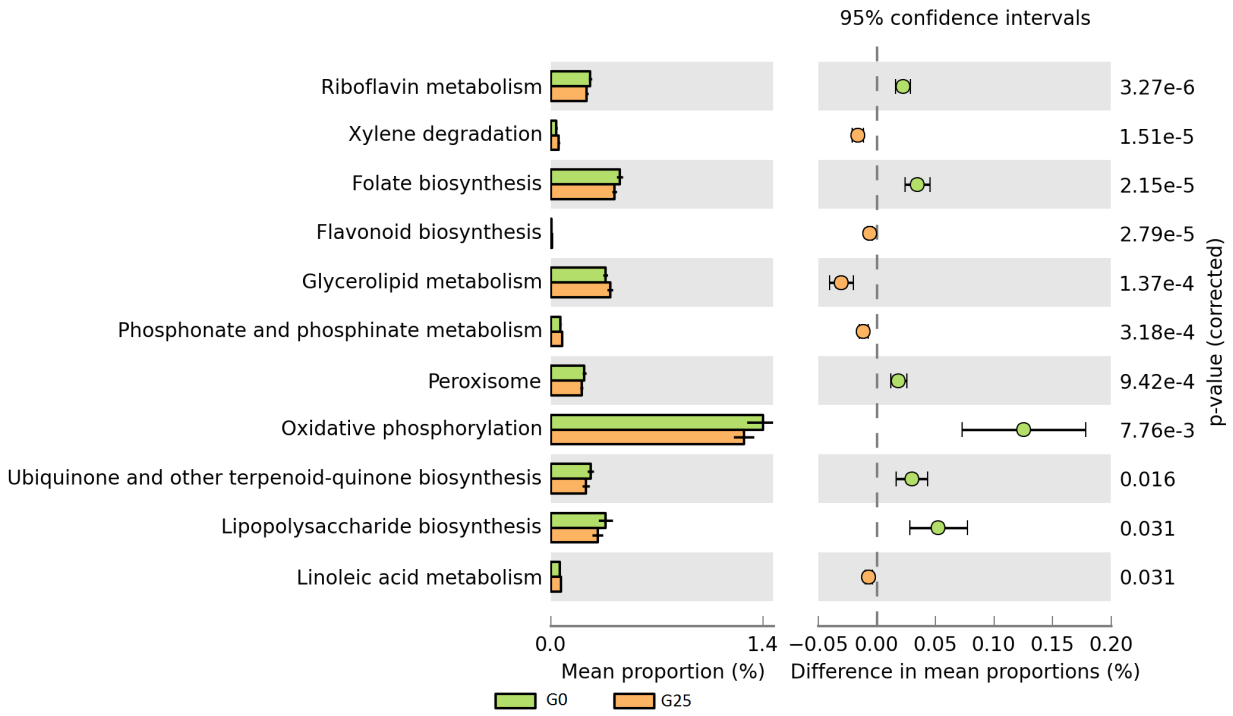


Figure 6: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Rumen Environment for differing treatments for treatments G0 and G25

Finally, there were 38 significant functional differences between diets G0 and G50 as seen in Figure 7. The diet G0 showed an increase in the metabolism of riboflavin ($P \leq 0.01$), taurine and hypotaurine ($P \leq 0.01$), sulfur ($P \leq 0.01$), glycerophospholipids ($P = 0.02$), biotin ($P = 0.04$), and lipoic acid ($P = 0.05$). The diet G0 also showed increases in the biosynthesis of folate ($P \leq 0.01$), steroid hormones ($P \leq 0.01$), ubiquinone and other terpenoid-quinones ($P \leq 0.01$), lipopolysaccharides ($P \leq 0.01$), steroids ($P \leq 0.01$), isoquinoline alkaloids ($P \leq 0.01$), and isoflavanoids ($P = 0.01$) relative to the G50 diet. The G50 diet showed increased metabolism of linoleic acid ($P \leq 0.01$), glycerolipids ($P \leq 0.01$), phosphonate and phosphinate ($P \leq 0.01$), starch and sucrose ($P \leq 0.01$), pyruvate ($P \leq 0.01$), fructose and mannose ($P = 0.02$), and galactose ($P = 0.02$) relative to G0 diet. There was also an increase in the biosynthesis of secondary bile acid ($P \leq 0.01$), primary bile acid ($P \leq 0.01$), and flavonoids ($P \leq 0.01$) in the diet G50 relative to diet

G0. Additionally, the pentose phosphate pathway ($P \leq 0.01$), and glycolysis/gluconeogenesis ($P \leq 0.01$) values were elevated in the G50 diet relative to the G0 diet.

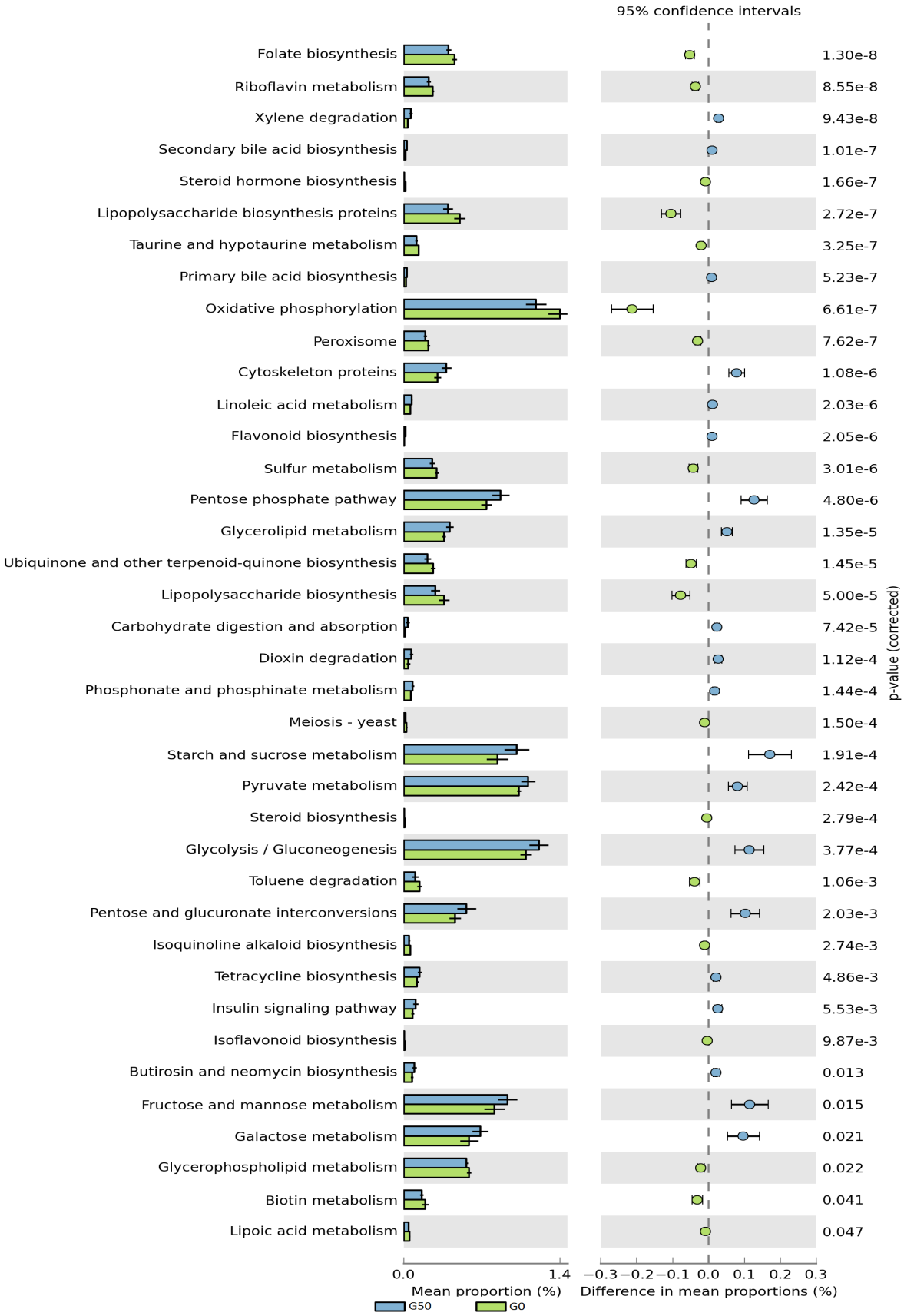

95% confidence intervals
Folate biosynthesis
Riboflavin metabolism
Xylene degradation
Secondary bile acid biosynthesis
Steroid hormone biosynthesis
Lipopolysaccharide biosynthesis proteins
Taurine and hypotaurine metabolism
Primary bile acid biosynthesis
Oxidative phosphorylation
Peroxisome
Cytoskeleton proteins
Linoleic acid metabolism
Flavonoid biosynthesis
Sulfur metabolism
Pentose phosphate pathway
Glycerolipid metabolism
Ubiquinone and other terpenoid-quinone biosynthesis
Lipopolysaccharide biosynthesis
Carbohydrate digestion and absorption
Dioxin degradation
Phosphonate and phosphinate metabolism
Meiosis - yeast
Starch and sucrose metabolism
Pyruvate metabolism
Steroid biosynthesis
Glycolysis / Gluconeogenesis
Toluene degradation
Pentose and glucuronate interconversions
Isoquinoline alkaloid biosynthesis
Tetracycline biosynthesis
Insulin signaling pathway
Isoflavonoid biosynthesis
Butirosin and neomycin biosynthesis
Fructose and mannose metabolism
Galactose metabolism
Glycerophospholipid metabolism
Biotin metabolism
Lipoic acid metabolism
1.30e-8
8.55e-8
9.43e-8
1.01e-7
1.66e-7
2.72e-7
3.25e-7
5.23e-7
6.61e-7
7.62e-7
1.08e-6
2.03e-6
2.05e-6
3.01e-6
4.80e-6
1.35e-5
1.45e-5
5.00e-5
7.42e-5
1.12e-4
1.44e-4
1.50e-4
1.91e-4
2.42e-4
2.79e-4
3.77e-4
1.06e-3
2.03e-3
2.74e-3
4.86e-3
5.53e-3
9.87e-3
0.013
0.015
0.021
0.022
0.041
0.047
p-value (corrected)
0.0
1.4
Mean proportion (%)
−0.3 −0.2 −0.1 0.0 0.1 0.2 0.3
Difference in mean proportions (%)
G50
G0

Figure 7: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Rumen Environment for differing treatments for treatments G0 and G50

6.3 Fecal Microbial Analysis

The reads were quality filtered based on the quality and length of the reads. The OTU's were aligned at a 97% similarity threshold against the Greengenes database. Some sequences could only be determined to the phyla, class, order, and family levels rather than the genus level. The average sequencing lengths for the V4 region was 253 nucleotides, and there was an average of 28,500 sequences per sample.

6.3.1 Within Community Diversity

Table 10 shows the numerical values pertaining to the within community diversity found in the rumen fluid samples. At a cut-off of 10,000 sequences per sample, the number of observed species for diets G0, G25, and G50 were 1004, 1035, and 961 ($P \leq 0.01$) respectively. With regards to the Chao1 estimator of richness, the values were 1244, 1289, and 1182 ($P \leq 0.01$) for the diets G0, G25, and G50 respectively. The Good's coverage estimate values were 97.2%, 97.1%, and 97.4% ($P \leq 0.01$) for the diets G0, G25, and G50, respectively The Shannon diversity index values for G0, G25, and G50 were 8.39, 8.49, and 8.31 ($P \leq 0.01$), respectively, showing the highest diversity in diet G25.

Table 10: Statistical summary for diversity indices in fecal communities based on treatment

	Treatment						
	G0	G25	G50	SED[3]	*P* (diet)	*P* (period)	*P* (diet×period)
Number of sequences per sample	10,000	10,000	10,000	---	---	---	---
Observed number of species	1004[b]	1035[c]	961[a]	14.55	<0.01	<0.01	<0.01
Goods-Coverage (%)	97.2[a]	97.1[a]	97.4[b]	0.001	<0.01	0.35	0.68
Chao1[4]	1244[b]	1289[c]	1182[a]	15.0	<0.01	0.09	0.11
Shannon[5]	8.39[b]	8.49[c]	8.31[a]	0.03	<0.01	0.05	0.02
Simpson[5,6]	0.689 [a]	0.690 [b]	0.689 [a]	0.0002	<0.01	0.05	0.2

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

[1] Based on Chao1 estimator of species richness

[2] Based on Shannon and Simpson diversity estimators

[3]SED = Standard error of difference between least square means of treatments

[4] Species richness estimate provided by the Chao1 index

[5] Species diversity index provided by the Shannon-Wiener index and the Simpson index

[6] Simpson Diversity Index was calculated using GLIMMIX model

6.3.2 Taxonomic Classification

At the phyla level, there were 16 phyla present in the diet G0. Among those, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes*, and *Verrucomicrobia* were the most prevalent in the fecal samples. Investigation at the genus level showed there were 197 genera present. Among those, there were 114 classified genera, and 83 unclassified genera. There were 16 phyla present in the diet G25. Among those, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Tenericutes* and *Cyanobacteria* were the most abundant. With regards to the genera present at the G25 level, there were a total of 182 genera present. Of those 182 genera, 109 were classified, while 73 were

unclassified. Finally, in the diet G50, there were 17 phyla present. Among those, *Firmicutes*, *Bacteroidetes*, *Tenericutes*, *Spirochaetes*, and *Cyanobacteria* were the five most abundant phyla. For the diet G50, there were also a total of 171 genera present. Of those 171, there were 100 classified genera as well as 71 unclassified genera present. Various sequences were only identified at the Phylum (P), Class (C), Order (O), and Family (F) levels.

Additionally, as seen in Figure 8, the microbial composition of communities of the three treatments differed ($P \leq 0.01$).

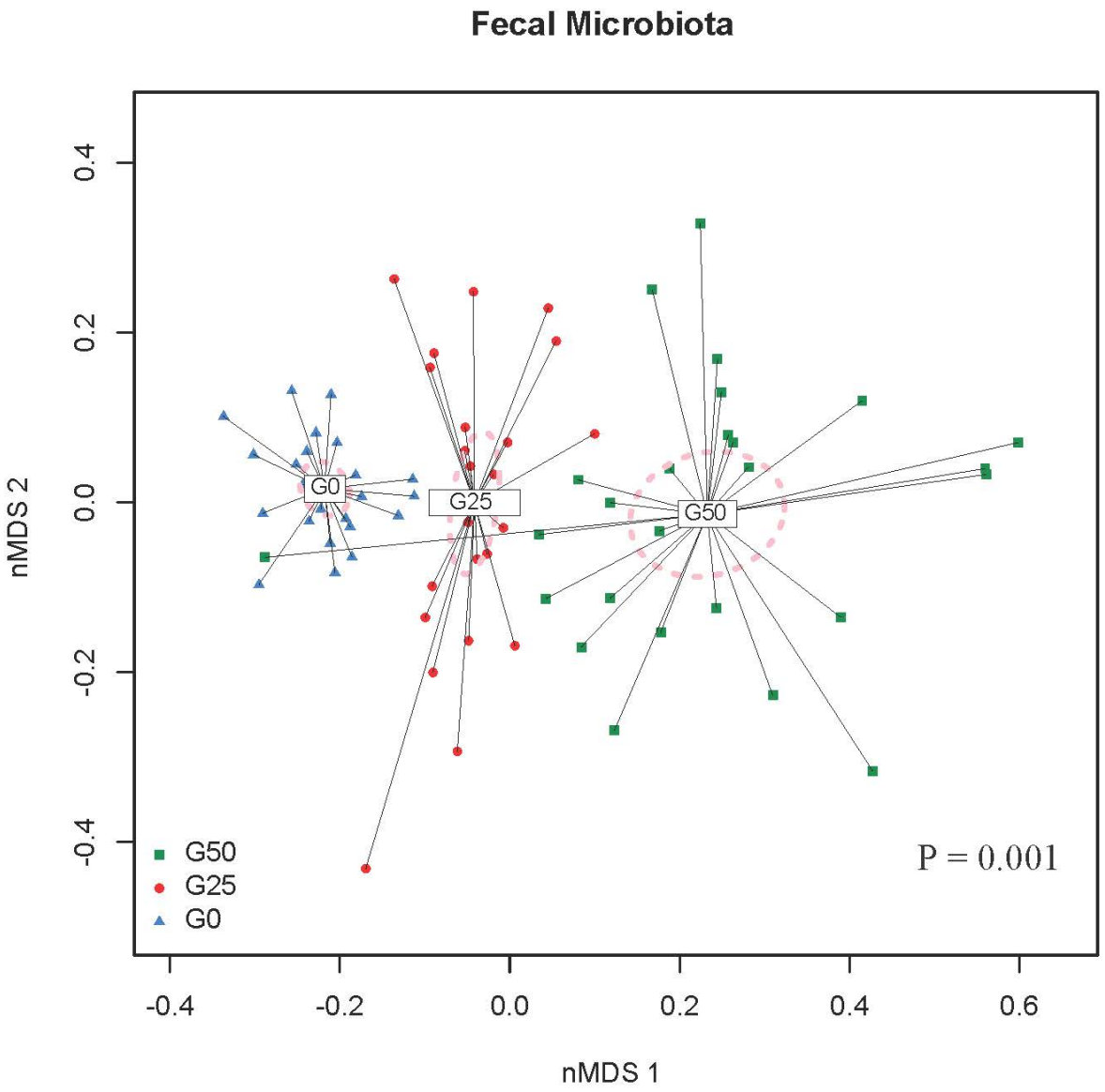


Figure 8: Experimental Results: Between Community Diversity of the Fecal Environment

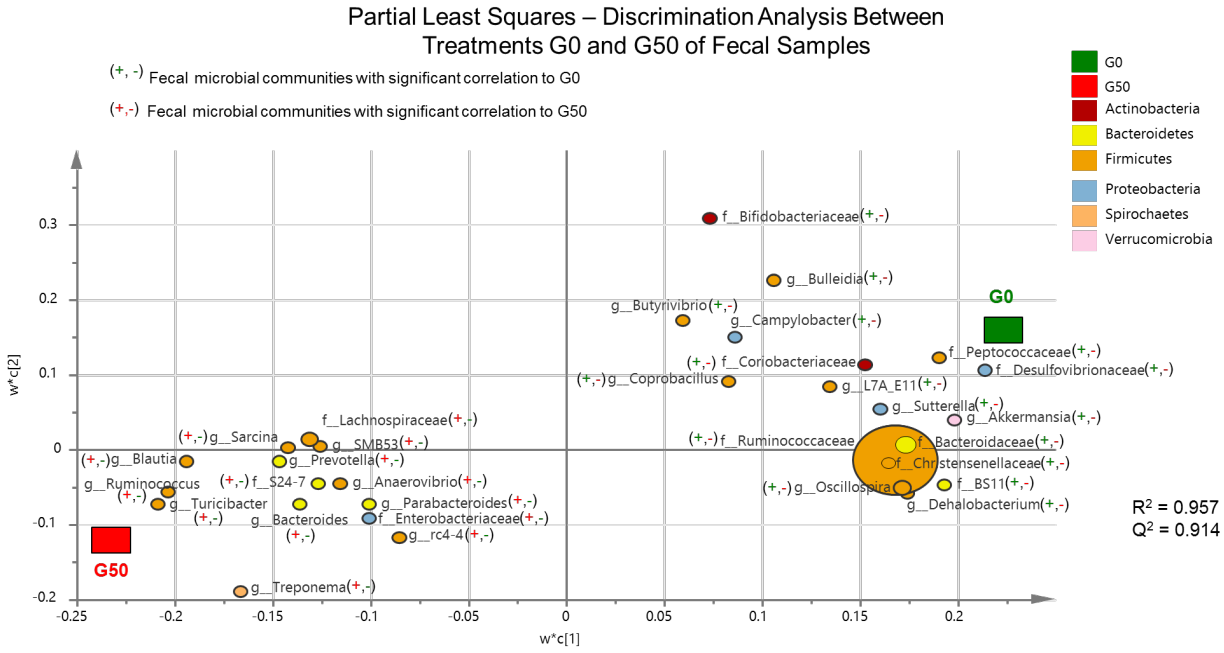
6.3.3 PLS-DA Analysis

Multivariate regression analysis was conducted to compare G0 v. G25, G0 v. G50, and G25 v. G50. With regards to the analysis of G0 v. G25, the R^2 and Q^2 estimates were 0.921, and 0.773 respectively. There were 5 taxa which were positively correlated to G0 and negatively correlated to G25. They included members of the phyla *Tenericutes* (order ML615J-28),

Actinobacteria (family *Coriobacteriaceae*), and *Firmicutes* (family *Peptococcaceae*, genera *Coprobacillus*, and *Dehalobacterium*). There were 10 findings which were positively correlated with G25, and also negatively correlated with G0. Among them were members belonging to the phyla *Bacteroidetes* (genera *Prevotella*), *Spirochaetes* (genera *Treponema*), *Proteobacteria* (order *RF-32*), and *Firmicutes* (family *Lachnospiraceae*, genus *Coprococcus*, *Sarcina*, *Blautia*, *Turicibacter*, *Anaerostipes*, and *Ruminococcus*).

Figure 9 shows the analysis of the comparison between G0 v. G50. The R^2 and Q^2 estimates were 0.957, and 0.914 respectively as determined by PERMANOVA. There were 16 groups that were positively correlated to G0 and negatively correlated to G50. They included members belonging to the phyla *Actinobacteria* (family *Bifidobacteriaceae*, and *Coriobacteriaceae*), *Proteobacteria* (family *Desulfovibrionaceae*, genera Campylobacter, and *Sutterella*), *Verrucomicrobia* (genera *Akkermansia*), *Bacteroidetes* (family BS11, and *Bacteroidaceae*), and *Firmicutes* (family *Ruminococcaceae*, *Christensenellaceae* and *Peptococcaeace*, and genera *Bulledia*, *Butyrivibrio*, *L7AE11*, *Oscillospira*, and *Dehalobacterium*). There were 13 groups that were positively related to G50, while being negatively correlated to G0. They included members of phyla *Proteobacteria* (family *Enterobacteriaceae*), *Bacteroidetes* (family *S24-7*, genus *Bacteroides*, *Prevotella*, and *Parabacteroides*) and *Firmicutes* (family *Lachnospiraceae*, genera *Blautia*, *Ruminococcus*, *Turicibacter*, *Sarcina*, *SMB53*, *RC4-4* and *Anaerovibrio*).

Figure 9: Partial Least Squares - Discrimination Analysis between G0 and G50 of Fecal Samples



6.3.4 Bacteria Showing Correlation between CH_4kgDMI^{-1} and Treatment in Fecal Samples

The analysis of the correlation between CH_4 kgDMI^{-1} and treatment showed that, there were bacterial members with both positive and negative correlations as reflected by their Spearman's Rho coefficient values.

Table 11 depicts fecal bacteria under diet G0 that were significantly correlated with CH_4 kgDMI^{-1}. Genus *Anaerostipes* (Spearman's Rho -0.497, $P = 0.01$) and *Butyrivibrio* (Spearman's Rho -0.455, P = 0.03) were negatively correlated with CH_4 kgDMI^{-1}. There was a positive correlation between the genus *Streptococcus* (Spearman's Rho 0.545, $P \leq 0.01$) and *Anaerofustis* (Spearman's Rho 0.537, $P \leq 0.01$) and CH_4 kgDMI^{-1}.

Table 11: Bacterial Correlation between CH_4 kgDMI^{-1} and treatment G0 in the fecal environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Family Coriobacteriaceae	-0.412	0.05
Bacteroidetes		
Family Barnesiellaceae	-0.490	0.02
Genus YRC22	-0.508	0.01
Firmicutes		
Family Christensenellaceae	-0.445	0.03
Genus YRC22	-0.508	0.01
Genus Bacillus	0.509	0.01
Genus Solibacillus	0.488	0.02
Genus Streptococcus	0.545	≤ 0.01
Genus Anaerofustis	0.537	≤ 0.01
Genus Anaerostipes	-0.497	0.01
Genus Butyrivibrio	-0.455	0.03
Genus rc4-4	-0.636	≤ 0.01
Proteobacteria		
Family Desulfovibrionaceae	0.422	0.04
Genus Shewanella	-0.435	0.03
Spirochaetes		
Genus Treponema	0.436	0.03

Table 12 depicts fecal bacteria under diet G25 that were significantly correlated with CH_4 kgDMI^{-1}. Family *Succinivibrionaceae* (Spearman's Rho -0.588, $P \leq 0.01$) and *Bifidobacteriaceae* (Spearman's Rho -0.505, $P = 0.02$) and genus *Akkermansia* (Spearman's Rho -0.487, P = 0.02) and *Odoribacter* (Spearman's Rho -0.529, $P = 0.01$) were negatively correlated with CH_4 kgDMI^{-1}. There was a positive correlation between the genus *Treponema* (Spearman's Rho 0.576, $P \leq 0.01$) and *Campylobacter* (Spearman's Rho 0.526, $P = 0.01$) and CH_4 kgDMI^{-1}.

Table 12: Bacterial Correlation between CH_4 kgDMI^{-1} and treatment G25 in the fecal environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Family Bifidobacteriaceae	-0.505	0.017
Bacteroidetes		
Family RF16	0.437	0.042

Family p-2534-18B5	-0.660	≤ 0.01
Genus Paludibacter	0.596	≤ 0.01
Genus Odoribacter	-0.529	0.01
Genus CF231	-0.505	0.02
Firmicutes		
Family Ruminococcaceae	0.498	0.02
Family Mogibacteriaceae	-0.467	0.03
Genus Lactobacillus	-0.444	0.04
Genus Streptococcus	0.453	0.03
Genus Garciella	-0.452	0.04
Genus Blautia	-0.433	0.04
Genus Butyrivibrio	-0.433	0.04
Genus Anaerovibrio	-0.455	0.03
Genus Coprobacillus	0.452	0.04
Genus RFN20	0.441	0.04
Proteobacteria		
Family Succinivibrionaceae	-0.588	≤ 0.01
Genus Limnohabitans	0.483	0.02
Genus Campylobacter	0.526	0.01
Spirochaetes		
Genus Treponema	0.576	≤ 0.01
Tenericutes		
Order ML615J-28	0.505	0.02
Verrucomicrobia		
Genus Akkermansia	-0.487	0.02

Table 13 depicts fecal bacteria under diet G50 that were significantly correlated with CH_4 kgDMI^{-1}. Family *Succinivibrionaceae* (Spearman's Rho -0.485, $P = 0.01$) and *Shuttleworthia* (Spearman's Rho -0.438, $P = 0.03$) were negatively correlated with CH_4 kgDMI^{-1}. There was a positive correlation between the family *Enterobacteriaceae* (Spearman's Rho 0.433, $P = 0.03$) and *Ruminococcaceae* (Spearman's Rho 0.392, $P = 0.05$) and CH_4 kgDMI^{-1}.

Table 13: Bacterial Correlation between CH_4 kgDMI^{-1} and treatment G50 in the fecal environment based on phyla

	Spearman's Rho	P-Value
Actinobacteria		
Family Coriobacteriaceae	-0.3934	0.047
Genus Bifidobacterium	-0.5826	0.002
Bacteroidetes		

Family p-2534-18B5	-0.6254	0.001
Firmicutes		
Family Ruminococcaceae	0.392	0.048
Family Mogibacteriaceae	-0.4139	0.036
Genus Pseudoramibacter Genus Eubacterium	-0.4768	0.014
Genus Butyrivibrio	-0.4948	0.010
Genus Shuttleworthia	-0.4375	0.025
Genus Sharpea	-0.5376	0.005
Proteobacteria		
Family Succinivibrionaceae	-0.4854	0.012
Family Enterobacteriaceae	0.4325	0.027

6.3.5 Functional Analysis of Microbial Population

To provide functional insight into the microbial analysis, PICRUSt software was used. There were 7 significant findings pertaining to the differences between diets G50 and G25 as summarized in Figure 10. In terms of metabolism, the metabolism of starch and sucrose ($P \leq 0.01$), and galactose ($P = 0.02$) showed higher levels in G50 than in G25. The diet G25 showed greater levels than G50 in the biosynthesis of betalain ($P \leq 0.01$), indole alkaloid ($P \leq 0.01$), and isoflavinoid ($P \leq 0.01$). The metabolism of inositol phosphate ($P = 0.02$) was also greater in diet G25 than G50.

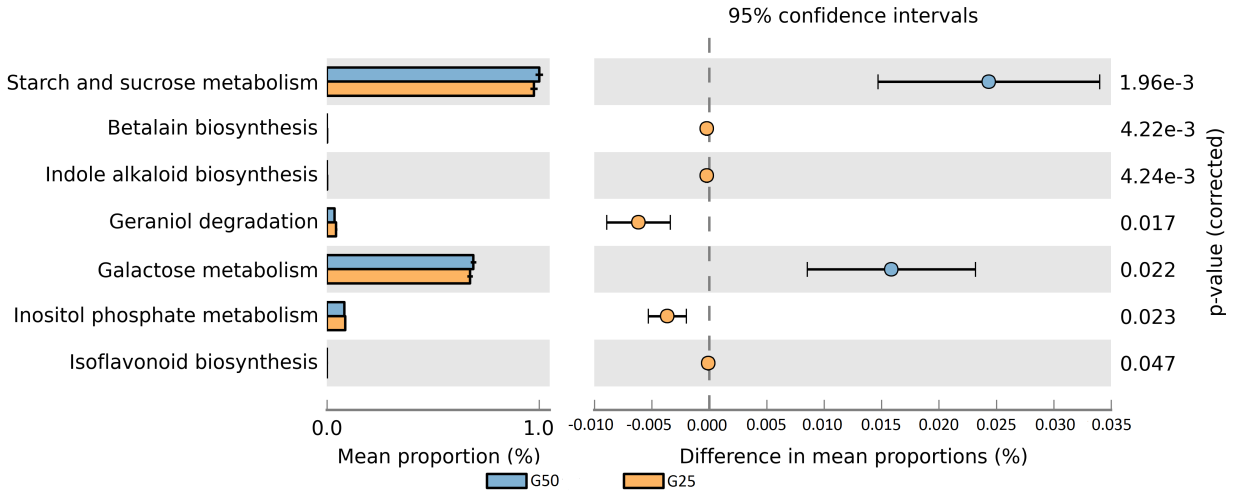


Figure 10: Experimental Results: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Fecal Environment for differing treatments for treatments G25 and G50

There were 4 significant differences between the diet G0 and G25 as summarized in Figure 11. The level of carbohydrate digestion and absorption ($P \leq 0.01$), primary bile acid biosynthesis ($P = 0.03$), metabolism of glycerolipids ($P \leq 0.01$), linoleic acid metabolism ($P \leq 0.01$) all displayed elevated values in diet G25 relative to G0.

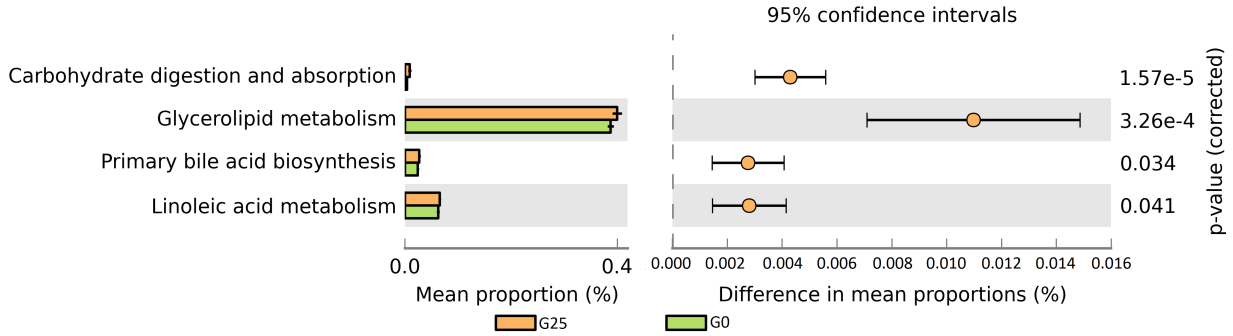


Figure 11: Experimental Results: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Fecal Environment for differing treatments for treatments G0 and G25

There were 27 significant differences between diets G0 and G50 as summarized in Figure 12. In diet G0, there were elevated levels in the metabolism of riboflavin ($P \leq 0.01$), porphyrin

and chlorophyll ($P \leq 0.01$), as well as the metabolism of taurine and hypotaurine ($P \leq 0.01$), and nitrogen ($P \leq 0.01$). The biosynthesis of steroid hormones ($P \leq 0.01$), and indole alkaloid ($P \leq 0.01$) were also higher in the feces of the diet G0 than in that of G50 as was mineral absorption ($P \leq 0.001$). The diet G50 showed elevated levels in the metabolism of glycerolipid ($P \leq 0.01$), linoleic acid ($P = 0.01$), and the metabolism of nicotinate and nicotinamide ($P = 0.01$) relative to diet G0. Tetracycline biosynthesis levels ($P \leq 0.01$), were also elevated for diet G50. Elevated levels of carbohydrate digestion and absorption ($P \leq 0.01$), and pathways pertaining to the pentose phosphate pathway ($P \leq 0.01$) were found in G50.

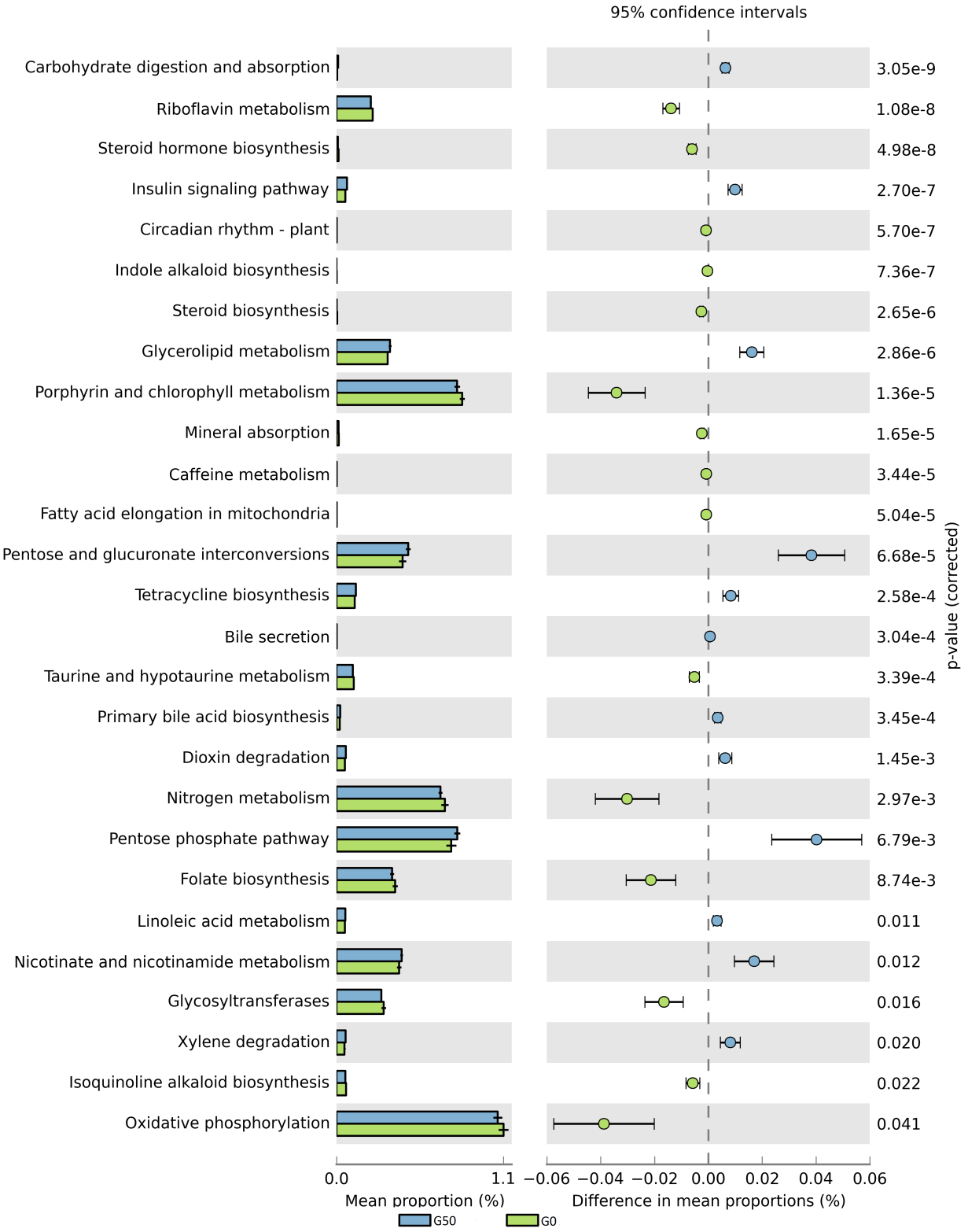


Figure 12: Experimental Results: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of the Fecal Environment for differing treatments for treatments G0 and G50

6.3.6 Predictive Model for CH_4 Emissions

To determine which bacterial and dietary models should be included in the model the criteria, significance was determined using SIMCA. Members are selected based on the effect they had on CH_4 production, with required Variable Influence on Projection (VIP) values exceeding 0.6 being necessary for inclusion in the model in addition to prevalence in over 10% of the samples taken. Figure 13 shows the VIP plot, the VIP plot functions to describe which of the X variables characterize the X variable well and are related to the Y variable. In this case, the X variable represents bacterial members sampled from the hindgut environment as represented by fecal samples at the genus level, and the Y variable being compared to the X variable is total CH_4 production (L d^{-1}). It is these bacterial members combined with ADF, NDF, and starch which were used in the prediction model.

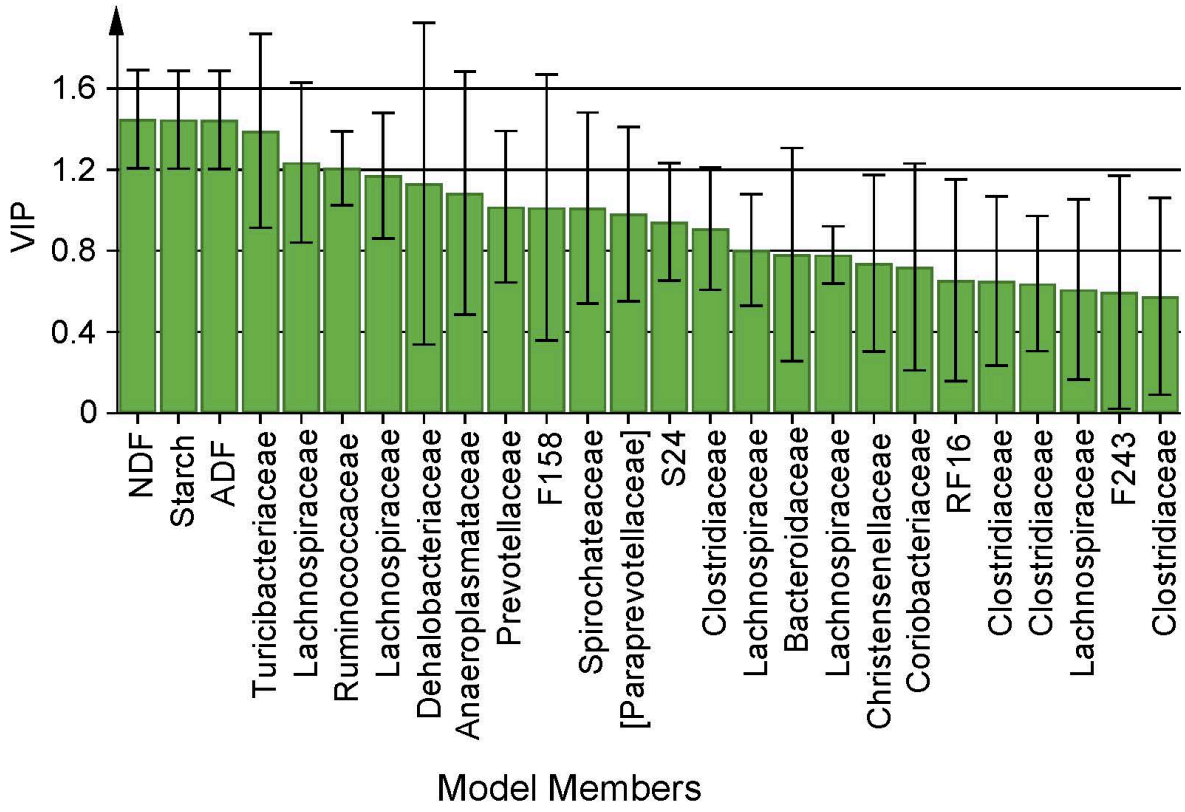


Figure 13: Variable Influence On Projection (VIP) Plot To Determine Members For The Prediction Model

Figure 14 incorporates all members of Figure 13 to depict the predictive value of the members of the model to predict total CH_4 production (L d^{-1}). The Root Mean Squared Error of Estimation (RMSEE) value represents one standard deviation point for the model, in this case that value is 41.03 L d^{-1} of CH_4. The Q^2 value of the predictive model was 0.49 and the R^2 value of the model was 0.51.

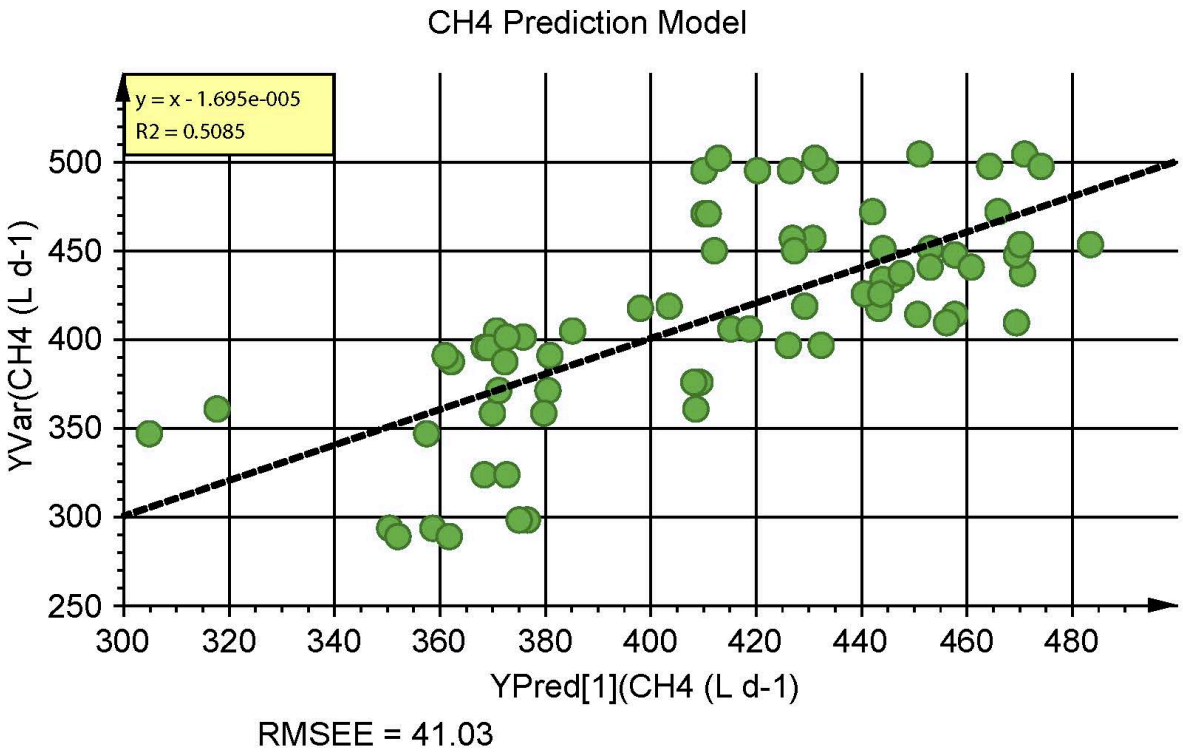


Figure 14: Enteric CH_4 Prediction Model Based on Representative Hindgut Sampling

7 General Conclusion

To conclude, there was a significant diurnal variation in CH4 production throughout the 24 hour period of measurement. Additionally, the 23 bacterial members chosen based on their VIP value exceeding 0.6 and a prevalence of the microbial community in over 10% of the fecal samples. The purpose of this was to ensure that the bacteria selected had both a significant outcome on CH_4 production and that the bacterial members were not outliers or only prevalent in some of the samples, ensuring the model can properly quantify CH_4 production in as many samples as possible. The R^2 of the model was 0.51, approximately 0.12 higher than the model with ADF, NDF and starch alone. In many cases, the bacterial members of the model that have been researched coincide with digestion in other mammals, and in many cases with fiber or starch digestion in cattle, further adding validity to the model. The model allows for the prediction of an increase or decrease from mean CH_4 production from a fecal sample. We believe a combination of these bacterial members have the potential to function as biomarkers in the future.

8 Discussion

As concentrate inclusion rate in the diet increased, so too did overall CH_4 production (L d^{-1}), however CH_4 production on an intake basis decreased (L $kgDMI^{-1}$). Coinciding with these changing CH_4 values were changes in both within community diversity and between community diversity in both the rumen fluid and fecal samples. Extensive analysis into bacterial correlations for rumen fluid and fecal samples was conducted to ensure a broad knowledge of the role the various treatments had on bacterial prevalence within each treatment in isolation pertaining to CH_4 production. For the CH_4 prediction model, the bacterial members selected in addition to ADF, NDF, and starch values were selected based on significance in CH_4 production as determined by the VIP graph using SIMCA. In addition to having VIP values exceeding 0.6, only taxa prevalent in over 10% of the samples were selected. Essentially, we believe that the members comprising the model in the hindgut environment as determined by fecal samples and under similar dietary conditions can function as a tool to predict CH_4 production from the rumen environment. Based on these results and an R^2 value of 0.51, the ability of these bacteria, used in conjunction with ADF, NDF, and starch values in the diet to function to predict an increase or decrease in CH_4 production relative to mean CH_4 production values.

Therefore, we can accept the hypothesis that these bacterial members, in addition with the ADF, NDF, and starch values of the diet can be a predictor of an increase or decrease from mean CH_4 emissions as determined in L d^{-1} under similar dietary conditions; however further investigation into these bacteria on a larger scale would be needed to consider these bacterial members as biomarkers for CH_4 production under a variety of different dietary parameters.

Among the bacteria in the feces correlated with overall CH_4 production at the family level, some of the members showing correlation to CH_4 production and therefore included in the

prediction model include, *Prevotellaceae*, *Christensenellaceae*, *Coriobacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. In these cases, past research has shown these bacteria to be involved in the digestion and fermentation of various components of feedstuff. One such bacteria at the family level is *Prevotellaceae*, which according to past research has been shown to be among the most abundant microbes found in past research of the rumen and hindgut of ruminants (164). Its main function in ruminants is to assist in the breakdown of both protein and carbohydrates in the rumen environment (164). The bacterial family *Christensenellaceae* is strongly affected by genetics in mammals, as it is the most heritable bacteria in all mammals, it was found to be negatively correlated to CH_4 production (165). It has also been found to be more abundant in lean people as it has been shown to decrease weight gain in mice (165). Another family prevalent in the model was *Coriobacteriaceae*. It is commonly found in the gastrointestinal tract of mammals and functions to convert bile salts and steroids and has been shown to activate dietary polyphenols (166). *Clostridiaceae*, which was found to be significantly positively correlated to decreased CH_4 production in the model is commonly found in the rumen environment (167). *Clostridiaceae* is also known for its ability to fixate Nitrogen and has also demonstrated the ability to degrade monocarbohydrates and polymers, including starch, chitin, xylans, and cellulose, which were present in varying levels in G0, G25, and G50 (167). The family *Lachnospiraceae* is among the most commonly isolated bacteria from the gastrointestinal tracts of mammals (168, 169). The production of butyrate and the ability to degrade xylans are common properties observed by members of *Lachnospiraceae* (168). Along with being directly related to CH_4 production, the production of butyrate has been linked to both colon cancer protection and obesity in mammals such as humans (169). The family *Ruminococcaceae* was also found to be significantly associated with CH_4 production; it is

among the most abundant of all *Firmicutes* members found in the gut environment (170). *Ruminococcaceae* is very common in fibrolytic communities, communities involved in the breakdown of complex plant polysaccharides including cellulose and hemicellulose, and research has proved the ability of *Ruminococcaceae* to degrade a wide variety of substrates that are difficult to digest (170). Additionally, *Ruminococcaceae* is highly specialized for the degradation of complex plant materials including hemicellulose and cellulose, which are in turn fermented and converted into short chain fatty acids which can be absorbed by the host (170). *Ruminococcaceae* is also well known, with one species of *Ruminococcus*, *Ruminococcus bromii* is believed to be a keystone species in the hindgut for the degradation of starch in the colon of humans (171). Essentially, the majority of the bacterial findings function to explain why certain bacterial members continually appear when bacterial correlation as it relates to CH_4 production and treatment is analyzed.

To this point, the majority of work pertaining to the understanding of CH_4 production and prediction has favoured the rumen environment due to the fact that the vast majority of the CH_4 produced by livestock is produced in the rumen (87%) and is expelled via eructation (16). The large intestines represent the remainder of CH_4 production (13%) (16). With that said, sample collection from the fecal environment is significantly less invasive and time consuming than sample collection from the rumen environment. After the rumen, the next digestive step occurs in the omasum, where contents are filtered on the basis of particle size (172, 173). In the event the feed particles are too large, they are rejected from entering the reticulum environment and are regurgitated. The feed is then transferred into the omasum, which acts to filter the feed and any H_2O in the feed (172, 173). The next stage in the digestion process occurs in the abomasum before the feed is released from the body in feces. The abomasum environment has a low pH and

enzymes such as pepsin which assist in digestion of proteins prevalent in feed; the environment is not unlike that of a monogastric intestine(172, 173). Digesta that is not absorbed enters the reticulum, is passed to the rumen, the omasum, the abomasum, and ultimately the hindgut, thus leading to our use of the hindgut, the endpoint for feed in the digestion process, as a CH_4 indicator.

Biomarker is a term which is often ambiguously defined, however one definition according to Strimbu and Tavel, 2010 is; a broad subcategory of medical signs, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly (174). According to the WHO, a biomarker could range from basic chemistries such as pulse or blood pressure, to complex laboratory tests of tissues or blood (174). Essentially, a biomarker is a quantifiable characteristic of a biological process. Currently, biomarkers are very beneficial in medicinal biology as they can provide a simple and accurate diagnosis, via a blood or urine sample for example, for a variety of diseases (175). This sentiment has been echoed by the American Food and Drug Administration, who state that biomarkers are important to pharmaceutical innovation and personalized medicine (175). As such, the potential of bacterial biomarkers involved in livestock CH_4 prediction is possible.

Two common types of biomarkers include the introduction of a biomarker into an organism as a means of examining internal functions, such as the organ, known as an imaging biomarker (176). Secondly, biomarkers can also be characteristic biological properties or molecules which can be detected and measured in certain parts of the body such as blood or tissues, as a means to indicate a state or normality or disease, known as a molecular biomarker (174). Biomarkers can consist of genes or gene products, molecules, enzymes, specific cells, and hormones (176). Some more commonly known biomarkers include temperature to detect fever, blood pressure as a

biomarker for the risk of stroke, C-reactive protein to detect inflammation, and cholesterol as a biomarker and risk factor for coronary and vascular disease (177). The biomarker Her2 is one of the more well known genetic biomarkers and is an established indicator of breast cancer, it has also been validated for cardiovascular toxicity in patients administered certain drugs (175).

Biomarkers are known to play major roles in medicinal biology. For a biomarker to be a candidate for clinical use, good sensitivity and specificity, is necessary. There are numerous uses for biomarkers. Three common ones include measuring disease progress, to establish long-term susceptibility to cancer, or its recurrence, and the evaluation of the most effective therapeutic regime for a particular type of cancer. It is believed that currently biomarkers in general are the most objective and quantifiable medical signs which modern laboratory science allows us to measure reproducibly and accurately (174).

In the future, biomarkers have the potential to detect predisposition for disease in a population and screen for its presence, as targets to discover new drugs, to determine if future research on an entity is necessary, to better detect potential side effects of prescription drugs, to reduce treatment overhead by optimizing dosage and measure responses to get results more rapidly and with more accuracy, and also to identify subjects suitable for initial human testing potentially decreasing side effects of initial drug testing including mortality (175) Further investigation is needed to determine biomarker involvement in the CH_4 production process.

Pioneering investigation into CH_4 production in livestock began in the 1950s (178, 179). These rudimentary production estimates functioned as the pillar for modern rumen microbiology studies. Since then, knowledge pertaining to the rumen environment, as well as the number of livestock itself has increased, both trends are expected to continue to increase to meet future demands. Current attention with regards to research and policy is focused on microbiome

analysis as it pertains to lifecycle assessments (35) which functions to examine a products environmental impact from all stages of production, use, distribution, and disposal (36).

Many current CH_4 estimation techniques are labour intensive and time consuming, making them largely impractical from the standpoint of a producer. The two major CH_4 estimation techniques on a per animal basis are the SF_6 tracer technique, and the chamber-hood calorimeter method. With regards to the SF_6 technique, the setup time is substantial as the permeation tubes must be precisely weighed weekly for approximately 6 weeks to determine the permeation rate (13, 180). The SF_6 permeation tube is then inserted into the rumen, and remains in the rumen for the duration of the animal's life. Additionally, the results given from the SF_6 technique are not given in real time. The chamber hood calorimeter method is another CH_4 measurement technique, and like the SF_6 tracer technique, it also requires a substantial initial setup time. Unlike the SF_6 technique, chamber hood calorimeters require a large monetary investment for setup, and continual monitoring. Furthermore, the chamber hood should be calibrated daily while in use. The animal must also be adjusted to the surroundings of the chamber hood which also takes time and constantly monitoring must occur while the chamber hood is in use. Overall, these techniques, although beneficial from a research standpoint are not practical from a producer standpoint.

It should be noted that current studies into the role animal genetics plays in CH_4 production continue, with the exact genetic markers affecting livestock CH_4 production proving difficult to determine and genetically select for (181). Currently, the primary targeted traits for genetic selection are production based (181). The logic behind selecting based on production parameters is that if production can remain the same, with fewer animals being used to reach these values, then on a production basis CH_4 emissions will decrease.

The treatments, as seen in Table 1, were formulated in accordance with the ultimate goal of inducing significant differences in CH_4 production and the composition and functionality of microbial communities present in the rumen and fecal environments among treatments. The grain inclusion rate in G50 was high for a dry cow, but was designed to avoid rumen acidosis via excess grain inclusion, which would have had a negative impact on the rumen and fecal microbiomes for the duration of the trial (182). The CP levels were also designed to be consistent across treatments and be above 10% DM so as to avoid being considered excessively low (183). Although the ADF values were greatest in G0 and decreased in G25 and G50, the at 23.3% in G50, the value is still above the minimum requirement of approximately 20% DM (183); the same can be said for NDF values in which G50 had a value of 38.7% DM which is still above the minimum requirement of approximately 35% DM (183).

As seen on Table 3, the DMI increased coinciding with an increase in grain inclusion rate in the diet. There are a variety of factors influencing intake, however physical fill and a decreased passage time in the rumen for grain relative to grasses are considered the chief factors affecting this (184, 185). A decrease in DMI is one of the major symptoms of acidosis, with another symptom being a drop in pH below approximately 5.6 (186). These symptoms were not witnessed at any time during the trial. The rumen pH values were collected via an oral tube rather than a cannula, the reason for this will be discussed later.(187).

The CH_4 production data, as shown on Table 4 shows an increase in overall CH_4 production coinciding with an increase in grain inclusion rate in the diet. When CH_4 production was adjusted to account for intake parameters, the opposite trend was observed, as CH_4 expressed in L $kgDMI^{-1}$ showed a decreasing trend. This is common among studies which measure total CH_4 production as well as CH_4 values corrected for intake parameters (13).

Pertaining to VFA concentration in the rumen, VFA concentrations increased coinciding with an increase in grain inclusion rate in the diet as seen in Table 5. According to past literature, a total VFA concentration value below 120 mM is to be expected when feeding diets similar to those used in our study (188). On a per dry matter intake basis, grain is more digestible and fermentable in the rumen than forages; therefore an increase in VFA associated with an increase in grain intake is to be expected if the VFA cannot be absorbed quickly enough (189). Also consistent with other studies was a decrease in the ratio of the concentrations of acetate:propionate coinciding with an increase in grain inclusion in the diet. The production of acetate and butyrate produce excess hydrogen in the rumen environment, as such, methanogens are able to utilize this excess to create more CH_4. The production of propionate is less linked to the production of CH_4; as such an increase in grain inclusion rates increases propionate production thus providing validity to the decrease in overall CH_4 production (41).

The VFA production trends prevalent in the fecal samples were similar to those in the rumen fluid samples, as seen in Table 6. As expected, the values in the fecal samples were lower than in the rumen, likely due to the fact that less fermentation occurs in the hindgut relative to the rumen per volume of digesta (189). The VFA values were similar to literature values (189). The differences in VFA concentrations between G0 and G25 were minimal and in some cases there was no difference (189).

The within community diversity in the rumen fluid samples was shown on Table 7. The sequencing coverage in the rumen fluid samples for all 3 treatments was approximately 97%. The largest number of observed species was found in G25, this is likely due to the fact that G25 was comprised of a substantial amount of both cellulose, hemi-cellulose and starch which the bacteria would have to digest rather than diets dominated by either fibre (G25) or starch (G50).

Pertaining to species richness in the rumen, that of the G25 diet was significantly higher than both G0 and G50 as determined by the Chao1 index. Diet G25 was also significantly higher in terms of species diversity present in the community than G50 or G0 as determined by the Shannon index. Therefore, the greatest number of species, as well as the largest number of different species were found in the rumen fluid of the G25 treatment. The results were very similar in the fecal samples, where the G25 treatment significantly had the largest values in observed species, species richness as determined by the Chao1 index, and species diversity as determined by the Shannon index.

One methodological limitation of this experiment is that separate investigation into the both the solid and the liquid fractions of the rumen could not occur using an oral stomach tube, likely resulting in slightly conservative reporting for cellulolytic bacteria in the rumen environment. Rumen digesta was collected via oral stomach tube, rather than a cannula for three major reasons. Firstly, to collect samples, the rumen cannula is removed thereby allowing oxygen to enter the rumen environment, subsequently altering the rumen environment. Secondly, the rumen cannula, although tightly fastened is not completely air tight. A key objective of the experiment was to measure enteric CH_4 output as precisely as possible, thus any CH_4 leakage from the cannula would compromise the integrity of the CH_4 concentrations that were measures in the chamber. Previous research by Beauchemin et al, 2012 supports this, as it was determined that when emissions were corrected for cannula leakage, 51% of cow-day measurements varied within 10% of actual emissions, whereas 69% of uncorrected measurements were within 20% of actual emissions (190). Finally, since sample collections were set to occur twice in a 24-hour period the day before CH_4 production was measured, an invasive procedure such as ruminocentesis to collect rumen fluid samples had the potential to affect both DMI and CH_4

values and only results in the collection of rumen fluid. In the event the invasive procedure caused harm to an animal or caused a change in DMI, the CH_4 values would not have been representative of a healthy rumen or fecal environment. Based on previous studies, a more detailed investigation into the solid fraction of the rumen would have increased the proportion of cellulolytic bacteria (191). Many cellulolytic microorganisms firmly attach themselves to fiber sources, if they are unable to detach they are unable to be included in the analysis making cellulolytic microorganisms difficult to completely quantify via 16S rRNA sequencing (191).

9 Future Directions

With regards to dealing with a changing environment and the role livestock production plays in it, some basic assumptions must be made. One assumption is that the global population will continue to rise. The exact amount can, should be, and likely will be discussed however the importance of the statement is that barring an unforeseen circumstance food security will present itself as an increasingly difficult task in the future. Another important assumption is that climate change is a process, which will continue, and as such must be addressed. Essentially, under these operating assumptions, animal production, including ruminant production will have to increase numerically while becoming more environmentally friendly.

A better understanding of livestock microbiomes, in particular the rumen microbiome where CH_4 production is most prevalent, would increase knowledge pertaining to lowering CH_4 emissions as well as improve overall animal health. The biochemical pathways responsible for CH_4 production in the rumen, as well as the relationship between feed type and CH_4 production are well known (17, 18, 41) however the functionality and even the exact composition of the communities involved in CH_4 production in the rumen is less well known.

Future investigation into the solid fraction of the rumen environment, perhaps via rumen cannulation in a full body methane capture chamber would allow for increased insight pertaining to CH_4 production in ruminants as cellulolytic bacteria are closely involved with CH_4 production in the rumen. Additional insight into the microorganisms which function to break down the feed particles and how they function will increase our understanding of the CH_4 production puzzle. Additionally, further understanding pertaining to CH_4 production and bacterial correlation both positively, at the phylum level, *Proteobacteria* and at the genera level *Akkermansia*, *rc4*, and *Oscillospirilla* and negatively, at the phylum level *Spirochaetes* and at the genera level

Prevotella, *Turicibacter*, *Blautia*, and *Ruminococcus* as these members have been associated with CH_4 production. This would be of great benefit as we have identified these bacteria to be correlated with CH_4 production in the rumen. As a result of the correlation with CH_4 production it is also entirely likely that these bacteria are also extensively involved in shaping the archaeal population in the rumen. Further investigation into the roles which the bacterial members presented in our model play in the breakdown of various components of feed and the degree to which they affect the archaea population should be the next step.

One important aspect which will be paramount in the future, is that microbiome research, similar to the research conducted here continues to determine the role that microbes and genetics has in CH_4 production. Further research with regards to climate change, and ways to combat it continue to occur. Increased knowledge on the situation will only better enable us to deal with climate change and its ramifications in the future. Just as important as the knowledge garnered via research will be the ability to act on it on a large scale and in a timely fashion, while not harming the economy or further exacerbating the issue via any implementation strategies. The implementation of mitigation strategies must be a co-ordinated effort by all parties involved, including producer, consumer, and government with CH_4 mitigation benefits not coming at the expense of events further down the production line.

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