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THE EFFECT OF DIET ON THE BOVINE RUMEN MICROBIAL COMMUNITY STRUCTURE AND COMPOSITION AND ITS EFFECTS ON METHANE PRODUCTION IN GROWING AND FINISHING CATTLE

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**THE EFFECT OF DIET ON THE BOVINE RUMEN MICROBIAL
COMMUNITY STRUCTURE AND COMPOSITION AND ITS EFFECTS ON
METHANE PRODUCTION IN GROWING AND FINISHING CATTLE**

by

Allison L. Knoell

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**THE EFFECT OF DIET ON THE BOVINE RUMEN MICROBIAL
COMMUNITY STRUCTURE AND COMPOSITION AND ITS EFFECTS ON
METHANE PRODUCTION IN GROWING AND FINISHING CATTLE**

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University of Nebraska, 2016

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Methane is a potent greenhouse gas (GHG) that contributes to global warming. A natural by-product of ruminant fermentation is the production and eructation of methane. Methane is produced by a small unique group of microorganism's called methanogens that belong to the domain *Archaea*. Enteric methane represents 2-12% energy loss in ruminants. It is well established that diet affects the microbial community structure and composition. Fermentative products of the mixed microbial population (bacteria, fungi, and protozoa) become the substrates for methanogens. These substrates influence which microorganisms will thrive. However, the effect of diet on the microbial community while simultaneously calculating methane production by expired breath sample from the cattle has never been explored.

Two studies were conducted under commercial feedlot production systems. A growing study utilizing 120 steers placed initially on a common diet and then transferred to various growing diets observing the effects of forage quality, MDGS supplementation, with or without Rumensin® (Elanco Animal Health, Greenfield, IN). Community structuring was observed between amounts of MDGS supplementation and forage

quality. A finishing study was also conducted utilizing 60 steers placed on a common diet followed by various finishing diets. Finishing diets evaluated the effect of DRC or MDGS supplementation, lipid additions, with and without Rumensin®. Community structuring was observed between DRC and MDGS supplementation however, structuring due to lipid addition was not observed. Utilizing dietary intervention strategies to mitigate methane production may be more suited to the growing phase rather than the finishing phase.

Dedication

I dedicate this work to my family, friends and everyone who assisted on this project.

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CHAPTER 1
LITERATURE REVIEW

Issue with methane production and why research needs to be conducted

Methane (CH₄) is a colorless, natural gas that is produced, often as a byproduct, from a range of sources including wetlands, landfills, oceanic thermal vents, termites, and livestock (EPA, 2015; Hedderich and Whitman, 2006). Methane is classified as a greenhouse gas (GHG) due to its ability to trap heat in the form of solar radiation resulting in increasing surface temperatures of the Earth's atmosphere (Hook et al., 2010; Moss et al., 2000). Until recently, methane was known to have a Global Warming Potential (GWP) of 21, however, in September of 2015, the Environmental Protection Agency (EPA) updated the GWP of methane to 28-36. This suggests that methane is able to trap and emit radiation back to the earth's surface 28 times more than carbon dioxide (CO₂), a gas with a GWP of 1. The atmospheric lifetime of methane is 12 years compared to the lifetime of CO₂ of 100 years (EPA, 2015; Hook et al., 2010). These GHG are necessary for life on Earth by trapping solar radiation and providing heat. However, with the rapid increase in GHG emissions more heat is being retained in the lower atmosphere resulting in a global warming crisis (Moss et al., 2000).

Methane is the second most predominant anthropogenic greenhouse gas emitted in the United States after CO₂ (EPA, 2015). In addition to natural sources of GHG emissions, anthropogenic activities greatly contribute to rising levels of GHG emissions in the atmosphere. Such activities account for nearly 60% of total emissions worldwide (EPA, 2015). Anthropogenic methane is a result of human related activities of natural sources of methane production. These activities have greatly contributed to the increase of GHG emissions. One such source of anthropogenic methane production is enteric fermentation by ruminants, more specifically, domesticated cattle (Monteny et al., 2006).

During fermentation, carbon dioxide (CO₂) and hydrogen (H₂) are produced as by-products in the rumen. These byproducts are utilized by a group of microbes known as methanogens, for methane production. Efforts have been explored at mitigating methane emissions from cattle (Attwood et al., 2011; Buddle et al., 2010; McAllister and Newbold, 2008; Hook et al., 2010). Diet composition is a significant aspect of methane production from ruminants. Manipulation of diet to decrease methane emissions have been explored (Johnson and Johnson, 1995; McAllister and Newbold, 2008; Beauchemin et al., 2007). In addition, byproduct inclusion and processing methods (Hook et al., 2010; Johnson and Johnson, 1995), ionophore supplementation (Hook et al., 2010) and chemical additions (i.e. sulfate and nitrate) (Zijderveld et al., 2010) have also been evaluated as methane mitigation methods.

However, these strategies have displayed varying results with respect to methane mitigation. The reason for such results may be due to the fact that an important variable in the rumen has not been measured. The microbial community produces methane, however, the community change and the methane produced from a community change has not been identified. Identifying interactions between microbial species composition, methane, and diet would help develop dietary intervention and management strategies towards methane mitigation.

Rumen microbes and their host

Interest in the methanogenic population within the rumen has increased greatly in recent years especially due to increased methane emissions from anthropogenic sources, such as ruminants. Ruminants fill a niche by consuming cellulose (Buddle et al., 2010),

and producing consumable products. Cellulose is the most abundant plant polymer on Earth and is only degraded by the enzymes secreted by the mixed microbial communities (Fontes and Gilbert, 2010). In ruminants, release of energy from cellulose is accomplished by a diverse microbial community that thrives in a symbiotic relationship with its host, the ruminant animal. The rumen, where a significant portion of the microbes are found, offers a warm, moist, dark, and anaerobic environment (Hungate, 1960) with nutrients, in turn the microbes help extract energy from low quality diets, to be used by the animal (Hungate, 1960). The complex microbial ecosystem in the rumen is composed of bacteria, protozoa, fungi, archaea, and viruses. This microbial community within ruminants provide the animal with nutrients by converting poor quality cellulose rich diets to usable substrates for the animal. The complex microbial community within the rumen encompass a wide variety of niches and are involved in utilization of carbohydrates, fiber, protein, and lipids. In the anaerobic rumen, a microbial food chain exists where the microbial population degrades the raw feed particles, producing intermediate substrates that can then be utilized by other populations in the rumen to fuel the host animal as well as the residing microbial populations. The feed particles reaching the rumen are broken down by cellulolytic, proteolytic, and amylolytic bacteria and produce simple sugars, alcohols, and volatile fatty acids (VFAs), (acetate, propionate, and butyrate) (Hedderich and Whitman, 2006; Boadi et al., 2004) as well as CO₂, and H₂. The molecular hydrogen and CO₂ are the necessary substrates for methanogenesis. The VFAs produced are absorbed and utilized as an energy source by the animal.

In addition to utilizing fermentation products and by-products, methanogens are also involved in energetic biochemical processes that benefit the ruminant animal. Glucose released from starch or plant polymers, proceeds through the Embden-Meyerhof-Parnas (EMP) pathway under anaerobic conditions within the rumen, producing reduced cofactors (i.e. NADH). In order to continue glycolysis, NADH produced must be re-oxidized to NAD in the rumen (Moss et al., 2000). The regeneration of NAD^+ , under anaerobic conditions is achieved by using the electron transport chain using carbon dioxide, sulfate, nitrate, and fumarate as the terminal electron acceptor (Moss et al., 2000). While hydrogen production occurs, traces of hydrogen in the rumen inhibit continued hydrogenase activity (McAllister and Newbold, 2008). To mediate the hydrogenase activity by prohibiting hydrogen ions to buildup, hydrogen that is produced is often utilized to produce propionate or methane via “inter-species hydrogen transfer” (Moss et al., 2000).

Members of rumen microbial community

Bacteria

Members of the ruminal microbial community constitute cellulolytic, amylolytic, and proteolytic organisms in a mixed microbial community colonizing the fluid, the feed particles, and the rumen epithelium. Bacteria ferment the feed reaching the rumen into volatile fatty acids (VFAs), which are utilized by the animal for energy. Bacteria comprise the most abundant group of microbes in the rumen. Direct microscopic counts estimate that 10^9 - 10^{11} bacteria per mL reside within the rumen (Jouany and Ushida, 1999). Unfortunately, mammals cannot digest cellulose due to cellulose being composed of β 1,4 glycosidic bonds and lack of enzyme production. Bacterial organisms are equipped to secrete enzymes to digest cellulose. A diverse population of bacterial

organisms inhabit the rumen converting plant material into usable forms of energy. Important cellulolytic bacteria isolated from the rumen are *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes* (Flint et al., 2008) producing endoglucanases, exoglucanases, and β -glucosidases, and hemicellulases (Cai et al., 2010). Predominant hemicellulose-degrading bacteria in the rumen are *Butyrivibrio fibrosolvans* and *Prevotella ruminicola*, yet these microbes are unable to degrade cellulose but have the capability to digest xylan and pectin and utilize those products as substrates for energy (Cai et al., 2010).

Bacteria contain multi-enzyme complexes called cellulosomes that aid in bacterial attachment to cellulose (Bayer et al., 2004) and digestion of cellulose, xylan, and hemicelluloses. The cellulosomes are composed of cellulases and hemicellulases that act synergistically to degrade various plant material that is consumed (Fontes and Gilbert, 2010). Fontes and Gilbert (2010) hypothesized that these structures were created due to the anaerobic selection imposed upon by the rumen environment. The most complex cellulosome characterized to date is from *R. flavefaciens* (Fontes and Gilbert, 2010). This complex includes diverse enzymes including glycosidic hydrolases, carbohydrate esterases, and polysaccharide lyases (Fontes and Gilbert, 2010). However, not all bacteria in the rumen have cellulosomes. For example, *F. succinogenes* does not house its cellulolytic enzymes in a cellulosome (Cai et al., 2010).

Protozoa

Protozoa populations in the rumen are seen in concentrations of 10^6 ml⁻¹, yet are larger in size and account for a significant portion of the rumen biomass (Jouany and Ushida, 1999) compared to bacterial populations. Ciliated protozoa are known to engulf starch granules (Jouany and Ushida, 1999) and help in digestion. Additionally, archaeal

populations have been seen attached intra- or extracellularly (Hook et al., 2010) to protozoa and are thought to be involved in interspecies hydrogen transfer. Protozoa are also associated with methane production from cattle. Protozoa are important H₂ producing organisms (Morgavi et al., 2012). Protozoa are H₂ producers, and are involved in interspecies hydrogen transfer which will provide the necessary substrates for methane production in the rumen (Hook et al., 2010).

Protozoa can be removed from the rumen ecosystem through a process known as defaunation (Hook et al., 2010). It is a process by which chemical or dietary agents are used to eradicate the protozoal population in the rumen (Boadi et al., 2004). It is an encouraging method, however, it does have its drawbacks. Chemical agents that are used can be toxic to the animal (Boadi et al., 2004). Also, defaunation is difficult to achieve and maintain in a production setting and can also be diet dependent (Johnson et al., 1995).

Fungi

While the existence of bacteria and protozoa have been known for over a century, fungi were only recently isolated from the rumen (Orpin, 1975, 1977). Partly the reason for the late identification was the thought that fungi are aerobic organisms and could not survive under anaerobic conditions in the rumen (Krause et al., 2013). Fungi are found in small amounts, 10⁴ ml⁻¹ (Jouany and Ushida, 1999) accounting for roughly 10% of the microbial biomass (Krause et al., 2013) and function to attach to cellulose particles and physically break apart the polymer, increasing surface area for bacterial attachment (Dashtban et al., 2010; Gordon and Phillips, 1998). In addition to cellulose, lignin is another plant structural component that is virtually impenetrable to bacterial enzymatic degradation and is the second-most abundant plant polymer on Earth (Dashtban et al.,

2010). Plant cell walls are composed of cellulose, hemicellulose, and lignin. In anaerobic environments, such as the rumen, it is thought that anaerobic fungi are the preliminary colonizers of lignocellulose aiding in increased fiber digestion by enhancing cohesion with bacteria and the rest of the mixed microbial population (Fontes and Gilbert, 2010). Fungi also possess lignin modifying enzymes which function to secrete various oxidases and peroxidases to degrade and breakdown lignin (Martinez et al., 2005). The breakdown of various plant polymers by ruminants (i.e. cellulose and lignin) is accomplished by the complex and mixed group of cellulases, hemicellulases, and ligninases (Bayer et al., 1998; Ljungdahl, 2008; Sanchez, 2009; Weng et al., 2008)

Methanogens

A small select group of organisms that exist in the rumen belong to the domain Archaea (Janssen and Kirs, 2008). Through sequencing of the 16S gene, it has been determined that methanogens had differentiated themselves evolutionarily and subsequently diverging from other forms of life early in evolution (Boadi et al., 2004). With this information, a new domain was called Archaea (that was amended from archaeobacterial in the Euryarchaeota kingdom) (Boadi et al., 2004; Baker, 1997, 1999). Archaea genus and species have various physical characteristics and morphologies (Moss et al., 2000). Archaeal cell walls do not contain a peptidoglycan layer and their intracellular triacylglycerol is replaced by ether linkages between glycerol and polyisoprenoid chains (Moss et al., 2000). Therefore, archaea are able to thrive in the harshest of environments, ranging from thermal vents in the largest depths in the ocean to the ice glaciers in the northernmost of regions (Hedderich and Whitman, 2006; Franzmann et al., 1992; Franzmann et al., 1997; Jones et al., 1983; Kurr et al., 1991). One harsh environment inhabited by archaea is the bovine rumen. Methanogen

populations within the rumen from animals fed a primarily concentrate diet is 10^7 to 10^9 /g of rumen contents and 10^9 to 10^{10} in grazing ruminants (Joblin, 2005). A defining characteristic of methanogens is the ability to produce methane through methanogenesis, however, this trait is not exhibited among all archaeal members (Liu and Whitman, 2008; Whitford et al., 2001).

A majority of the methanogens possess the ability to reduce CO_2 to methane (CH_4), as a majority of the methane that is produced in the rumen is accomplished from the reduction of CO_2 by H_2 to CH_4 (Hedderich and Whitman, 2006). This pathway is energetically more favorable with approximately 82% of the methane being produced from carbon dioxide and hydrogen (Garcia et al., 2000). Several electron donors exist for methanogenesis, with the chief donors being H_2 and formate (HCO_2^-) (Hedderich and Whitman, 2006; Boadi et al., 2004) for the reduction of CO_2 to CH_4 . This conversion occurs as a terminal step in the methanogenesis pathway (Poulsen et al., 2013). The production of methane is biologically advantageous to the ruminant animal (Krause et al., 2014) as it helps recycling NAD^+ without using pyruvate.

Rumen methanogens are abundant in the rumen, and are found in the floating fluid portion, attached to particulates, attached to protozoa, and attached to the rumen epithelium, or rumen wall (Janssen and Kirs, 2008). Growth rate of the methanogens in the various portions of the rumen is variable, as removal is dependent on location within the rumen (Janssen and Kirs, 2008).

Viruses

Ruminal viruses are under studied with little known of their role in rumen environment. Viruses, or phages, are antagonistic to the bacterial cells within the rumen and help shape the rumen microbial community, more specifically bacteria (Gilbert and

Klieve, 2015). Phages are typically dense in population, being found in populations ranging from 10^7 to 10^9 particles per mL (Berg Miller et al., 2012).

Status of the rumen methanogen population

A portion of microbial populations within the rumen have been discovered via culturing, yet those identified are only a fraction of what is believed to be present within the rumen. The rumen methanogen population has been difficult to study due to the community being low in number and difficult to isolate, culture, and identify (Buddle et al., 2011). Poulsen et al. (2013) determined that the rumen methanogens belong to the limited genera of the orders *Methanobacteriales* and *Methanomicrobiales*. These orders have been depicted as H₂ utilizers (hydrogenotrophic) reducing CO₂ or methanol in anaerobic plant degradation (Poulsen et al., 2013). Janssen and Kirs (2008) analyzed a global data set and observed a significant portion (>90%) of the rumen archaeal population to belong to *Methanobrevibacter* (61%), *Methanomicrobium* (15%), and RCC (uncultured archaea; 16%) genera.

Buddle et al. (2011) and Janssen and Kirs (2008) determined through 16S rRNA amplification and sequencing that a majority of the methanogenic archaea belong to the genus *Methanobrevibacter*, mostly being associated with *M. ruminantium* (the most well-known and commonly found species in the rumen) and *M. gottschalki*. In addition, possibly eight more species have been reported (Buddle et al., 2011). This includes four methanogenic species belonging to the genus *Methanosphaera* and one belonging to the genus *Methanomicrobium*. Other genera present within the rumen include

Methanosarcina, *Methanomicrococcus*, *Methanobacterium*, and *Methanohanoculles* (Buddle et al., 2011).

Only four strains of methanogens are found to be common in ruminants:

Methanobrevibacter, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina* (Whitford et al., 2001), additionally, only five species of methanogens have been isolated from the rumen. These species include *Methanobrevibacter ruminantium*, *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanobacterium formicium*, and *Methanomicrobium mobile* (Boadi et al., 2004). *Methanobrevibacter ruminantium* and *M. barkeri* have been found in large numbers in the rumen and are presumed to play a role in methanogenesis (Boadi et al., 2004). While many methanogens have been identified using molecular methods, phylogenetic results are different than previously sequenced isolates, implying that more methanogens are present in the rumen that have not been identified due to limitations in methodology (Boadi et al., 2004). All methanogens in the rumen are categorized into six Orders: Methanococcales, Methanopyrales, Methanobacteriales, Methanosarcinales, Methanomicrobiales, and Methanocellales based on their inherent characteristic of production of methane during energy metabolism (Borrel et al., 2013).

In addition to the above mentioned methanogens, a novel group of archaea have been identified to inhabit the rumen with unknown function and is entitled Rumen Cluster C (RCC) and is remotely related to Thermoplasmatales (Janssen and Kirs, 2008; Buddle et al., 2011). This group was recently found by Poulsen et al. (2013) to be present in sizeable concentrations in ruminants by using 16S rRNA sequence techniques. Buddle et al. (2011) has indicated that microbial counts of Rumen Cluster C can range

from 15-20% of the total archaeal composition within the rumen, yet it has been seen to reach greater than 80% abundance. The methanogenic role in the symbiotic relationship between Thermoplasmata and the rumen microbes is yet to be established (Buddle et al., 2011). However, members of RCC have yet to be isolated, adding to the scope of additional members of this branch that have gone without isolation (Poulsen et al., 2013). Kemnitz et al. (2005) had previously described this group as Rice Cluster C Thermoplasmata. Poulsen et al. (2013) discovered RCC being associated with methane production; however, their biochemical pathways for methane production are absent. In this analysis, transcripts of mRNAs signature of Methanogenesis, including those found matching to mono-, di-, and trimethylamine and methanol were also identified as being essential for some rumen methanogens. This is interesting to note as it was previously thought that only *Methanosarcinaceae* were able to utilize methylamines as an energy source. In addition to this conclusion, the enzymes in the methylamine pathways are only remotely related to *Methanosarcinaceae*. This study suggests that RCC is a new order of methanogens who derive their energy from being methylotrophic from choline and betaine degradation, along with methanol.

Methane Biochemistry

The partial pressure of the rumen can dictate the processes that function as well as the microbial community's structure and function. When methanogens are present in the rumen, the hydrogen ions are quickly utilized and the partial pressure is maintained at 10^{-3} to 10^{-4} atmospheres, making the production of VFAs thermodynamically favorable (Hedderich and Whitman, 2006). At these conditions, the pool of VFAs are metabolized keeping rumen function progressing and impeding potential fatal conditions.

Methanogens are able to utilize excess hydrogen ions in their energy metabolism aiding in keeping the concentration of hydrogens stable so as to not disrupt rumen pH (Boadi et al., 2004). The redox potential of the rumen must be near -300mV for the methanogens to perform energy metabolism (Boadi et al., 2004; Jones et al., 1987; Moss et al., 2000).

Methanogens are the only organisms that show the presence of three proteins specific to methane production: coenzyme 420, coenzyme M, and factor B (Boadi et al., 2004; Jones et al., 1987; Baker, 1999). Coenzyme 420 replaces ferredoxin in electron transfer, while coenzyme M transfers methyl groups, and factor B is a heat-stable coenzyme that aids in the formation of methane from coenzyme M (Boadi et al., 2004; Jones et al., 1987; Baker, 1999).

Hedderich and Whitman (2006) state that conversion of CO₂ to CH₄ tends to be the method often used by the rumen methanogens where electron donors consisting of H₂ and formate, with -130ΔG and -120 ΔG free energies for CO₂ and formate, respectively. Eight electrons are required for the reduction of CO₂ into methane, consuming four molecules of H₂ and formate. Formate is first oxidized to CO₂ prior to methane conversion, even though it is already reduced.

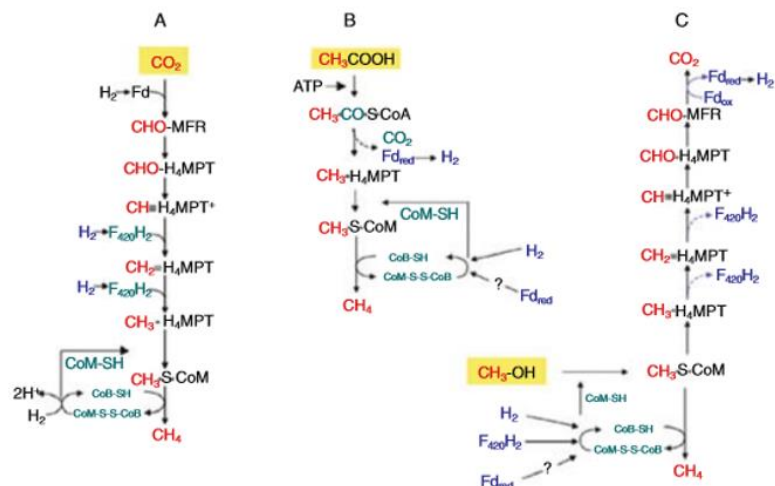
Three one-carbon carrier-bound intermediates are required for the reduction of CO₂ to CH₄ (Hedderich and Whitman, 2006), which includes methanofuran (MFR), tetrahydromethanopterin (H₄MPT), and derivatives, and 2-mercaptoethanesulfonate (i.e. coenzyme M and CoM-SH) (DiMarco et al., 1990). Hedderich and Whitman (2006) describe the reduction to methane from CO₂ begins with an electron reduction of CO₂ and MFR to generate formyl-MFR, with the formyl-group being bound to the amino-group of

the coenzyme. H₄MPT then receives the transferred formyl-group, creating the formyl-H₄MPT. This compound cyclizes to form methenyl-H₄MPT, and is reduced to methyl-H₄MPT. The thiol group of coenzyme M receives the methyl-group in a transfer forming methylthioether, which is reduced to CH₄ in the final step of the sequence.

Hedderich and Whitman (2006) state the pathway of methane production with substrates consisting of one-carbon compounds attached to an O, N, or S (i.e. methanol). These compounds enter the pathway at the coenzyme M step, which is reduced to methane by oxidizing an additional methyl group to CO₂ by reversing the steps of the C₁-pathway of reduction.

When acetate is utilized as a substrate, the second methyl carbon is reduced to methane via electrons optimized from the oxidation of the first methyl carbon in the molecule, thus being termed the acetoclastic reaction. This reaction yields methane and CO₂ from acetate, with the methyl group entering the metabolism pathway at methyl-H₄MPT as stated by Hedderich and Whitman (2006).

Although the three pathways vary from each other, a common step is present in the pathways, methyl-coenzyme M reaction with a thiol coenzyme, coenzyme B forming methane (Hedderich and Whitman, 2006).



(Reproduced from Hedderich and Whitman, 2006)

There are three pathways that are utilized in the generation of methane, consisting of H_2/CO_2 (hydrogenotrophs), acetoclastic methanogenesis (acetate), and methylotrophic methanogenesis (C_1 compounds) all including a group of specific enzymes that are represented throughout all orders of methanogens (Borrel et al., 2013; Zinder, 1993). A common pathway for methane production is the reduction of CO_2 to CH_4 using H_2 (Boadi et al., 2004). The ability for methanogens to utilize the excess hydrogen is a benefit for the animal, as the regulation of pH is affected by the production of hydrogen ions, and an increase or decrease of concentration of H^+ ions reflects upon the pH (Boadi et al., 2004). One method of obtaining hydrogens for energy metabolism and subsequent methane production is through inter-species hydrogen transfer. This interaction allows methanogens to utilize excess hydrogen that is produced by H_2 -producing bacteria, protozoa, or fungi for their metabolism (Boadi et al., 2004; Hegarty and Gerdes, 1998). With this relationship the concentrations of hydrogens stays in a range that allows for the H_2 -producing bacteria to continue fermenting the feed particles (Hegarty and Gerdes, 1998). The benefit of this interaction is that the hydrogen partial pressure stays low

enough for the bacteria and archaea to function. In the breakdown of cell wall polymers, the transfer of hydrogen to methanogens is beneficial (Wolin and Miller, 1988). Methanogens help to re-oxidize NADH without leading to the less efficient and potentially detrimental ethanol or lactate (Moss et al., 2000).

Acetoclastic methanogenesis is another method of methane production. It is derived from acetate being converted to acetyl-CoA, then a methyl group is transferred into the methanogenic pathway (Ferry, 1992). To date it is believed that this process is executed entirely in the rumen by the order *Methanosarcinales* (Ferry, 1992).

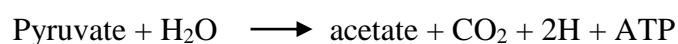
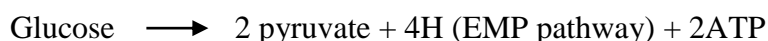
Methylotrophic methanogenesis members apparently have an ecological advantage over other methanogens as their requirement for single carbon compounds such as methanol and methylamines eludes competition with bacteria that are sulfate-reducers (Oremland and Polcin, 1982; Oremland et al., 1982b).

Formate usage is also commonly used in the reduction of CO_2 to CH_4 however, it is not as common as the H_2/CO_2 conversion (Boadi et al., 2004; Boadi et al., 1991). Other substrates that are used, yet not as commonly, are acetate, methanol, methylamines, dimethyl sulfide, and some alcohols (Boadi et al., 2004; Jones et al., 1991; McAllister et al., 1996). Methanols, methylamines, and acetate are utilized by *Methanosarcina* for energy metabolism, contributing to methane production (Boadi et al., 2004). One carbon compounds are also utilized as substrates of methane production (i.e. methanol) (Hedderich and Whitman, 2006).

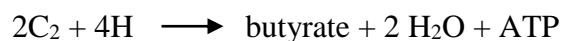
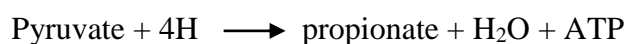
Glucose fermentation under anaerobic conditions (from starch or plant sources) is accomplished through the oxidative process of the Embden-Meyerhof-Parnas pathway yielding reduced co-factors such as NADH, ATP, and pyruvate (Moss et al., 2000). In

order for the ruminant host to obtain energy from VFAs and to keep glycolysis occurring in the microbial cell, the reduced co-factors need to be re-oxidized back to NAD (Moss et al., 2000). In the rumen, methanogens help recycle NADH back to NAD for glycolysis (Moss et al., 2000). The production and utilization of hydrogens in anaerobic pathways is shown below (Moss et al., 2000) as:

Producing reactions:



Utilization reactions:



Cattle contributions to methane levels

It is expected that the world's population will double by the year 2050, thus meat and milk will become increasingly needed (FAO, 2008). Cattle typically lose 6% of the ingested energy in the form of eructated methane (Johnson and Johnson, 1995). Methane that is expelled by cattle is a contributor to global atmospheric methane levels.

Approximately 90% of the methane produced originates from enteric fermentation by ruminants (Boadi et al., 2004).

Methane production in cattle varies due to functionality of the animal. Beef steers typically undergo a growing phase where more forage based diets are fed. However, during the finishing phase, typically beef cattle receive more energetic diets (Johnson and

Johnson, 1995). Methane production varies based on these stages (Johnson and Johnson, 1995) in addition to other factors described in previous sections.

In dairy cattle, there is a range of methane emissions as the methane levels are dependent on the dietary content and stage of production for the cow (Monteny et al., 2006). The nutrient profiles of the consumed feeds also dictate methane production (Monteny et al., 2006). The portion of gross energy that is converted and lost as methane is decreased as higher intakes have an almost linear relationship with methane production (Monteny et al., 2006). Dairy cows at peak lactation are able to surpass beef cows in methane production (Cottle et al., 2011).

Diet and Methane

Growing diets and methane

Cellulose is the most abundant organic compound in the world. Mammalian enzymes are unable to hydrolyze the β -1,4 glycosidic bonds in cellulose, making cellulose indigestible to mammals. However, microbes possess the ability of hydrolyzing these bonds and releasing the energy from the feed particles. This characteristic allows ruminants to graze and consume various plant feeds, thus fulfilling a niche (Buddle et al., 2011). Methane that is released due to respiration or eructation can equal nearly 10% of the caloric content of the feed ingredients (Hedderich and Whitman, 2006). Ruminant feed sources that contain pectins with esterified methoxyl groups are metabolized producing methanol that can be used as a substrate for methane production (Neumann et al., 1999). Neumann et al. (1999) examined the effect of methanol on methanogenesis and fermentation using rumen simulation technique. Methane production increased with the addition of methanol from 16.0 to 23.6 mmol/day ($P < 0.001$). They attributed the

increase in methane to increased total gas production. The VFA profile for acetate, propionate, and butyrate were unaffected by the addition of methanol. Methanogens aid in the breakdown of feed particles and hydrogen utilization, without these organisms organic matter degradation would be inefficient (McAllister et al., 1996).

The cell wall structure of plant material requires extensive fermentation when compared to that of soluble sugars, thereby increasing methane production for cell wall fermentation (Johnson et al., 1996). During acetate production, H₂ are released. Structural carbohydrates ingested affect the rate of fermentation in addition to passage out of the rumen due to increased time needed for digestion, which can favor a higher acetic:propionic acid ratio (Boadi et al., 2004; Hegarty and Gerdes, 1998). Forages that are at a lower maturity stage are more easily digestible, requiring less for digestion so methane production increases with increasing maturity of plants (McAllister et al., 1996; Moss et al., 2000). Feeding high grain diets that accompany high intakes are inclined to a faster rate of fermentation and passage that contributes to increased propionic acid production (Hegarty and Gerdes, 1998). Propionate is thought to act as a hydrogen sink and subsequently decrease methane production potential (Monteny et al., 2006).

Forage based diets leads to more acetate production and increases in methane production from fermented organic matter in the rumen compared to grain diets that produce propionate (Carberry et al., 2014). Carberry et al. (2014) quantified the relative abundance of total methanogens and species including *M. smithii*, *M. ruminantium*, and *M. stadtmanae* in the rumen fluid of cattle divergent for RFI fed a high energy, low forage (LF) and low energy, high forage (HF) diets. Methanogen abundance, animal performance, diet digestibility, and rumen fermentation variables were also examined.

Diets were fed *ad libitum* for 86 yearling Limousin x Friesian heifers for 112 d with the LF diet comprising 30% corn silage and 70% pelleted concentrate. This study concluded that feed efficiency did not significantly affect the abundance of total or specific methanogens at the species level, and further displayed the influence of type of dietary substrate on abundance of methanogen species as well as the complete methanogenic population being controlled by dietary changes.

There are various confounding factors when looking at methods of mitigation of methane in ruminants. The rumen microflora are constantly changing. Environment, diet composition, and antibiotic usage all affect the rumen microbial community (Stewart et al., 1997). The microflora are also affected based on animal species (Boadi et al., 2004; Mathison et al., 1998; Moss et al., 2000). Thus there is great opportunity to change the rumen microbiota composition to reduce methane emissions by using the diet.

Finishing diets and methane

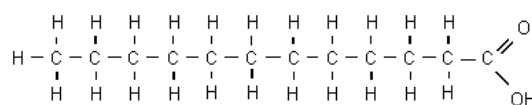
Fermentation products consist of acetate, propionate, butyrate, CO₂, and H₂. To meet host energetic needs, the VFAs are absorbed across the rumen wall leaving products that then become potential substrates for other populations within the rumen to utilize for their specific metabolisms. Feed type and the animals' status will control the rate of digesta passing through the rumen (Mathison et al., 1995). In a review by Johnson and Johnson (1995), starch inclusion of a diet typically will favor propionate production and subsequently have a decreased amount of methane produced by fermentation of organic matter in the rumen. The production of propionate will cause a shift in the microbial population (Monteny et al., 2006), primarily those species associated with a lower pH. A review by Johnson and Johnson, (1995), reports that on a limited intake of highly digestible carbohydrates, a high methane loss occurs, however, with highly digestible

carbohydrates with high intakes, low methane loss occurs. Propionate is considered a carbon sink so the increased production of this VFA will not contribute to the pool of hydrogen in the rumen. While highly digestible carbohydrates may contribute to the pool of available hydrogen, it can be short lived because the carbohydrates will have a faster passage rate and a lower retention time.

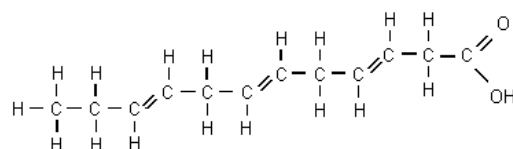
Mitigation strategies

Fat supplementation and methane

A method of reducing methane emissions is the addition of fat to a diet (Boadi et al., 2004). This is a nutritional management strategy that can be accomplished with fat or oil additions. Any unsaturated oil or fat that enters the rumen undergoes biohydrogenation. This is a process in which the microbial population hydrolyzes the double bonds then secretes isomerases in order to place the hydrogens in a trans position in order for each chain to be cleaved from the glycerol backbone (Boadi et al., 2004). Lipid additions and methane are considered to be hydrogen sinks due to the number of hydrogens that can be added to the molecule to keep the rumen at a neutral pH.



Saturated Fatty Acid



Unsaturated Fatty Acid

During the isomerization, the microbes will place free floating hydrogen ions onto the carbons in order to create a fully saturated fatty acid. This process aids in decreasing the

toxicity that fatty acids have upon the rumen environment (Poulsen et al., 2013). It is in this method that the oil and fat additions act as a hydrogen sink, decreasing the amount of H⁺ left in the rumen to be fixed as methane. Unsaturated fatty acids in the rumen are able to be reduced and act as electron acceptors. Poulsen et al. (2013) looked at metatranscriptomic approach to examine the method of methane mitigation by supplementing rapeseed oil (RSO) on the rumen microbiota of lactating Holstein cows, focusing primarily on the methanogenic archaeal population. They observed that *Methanobrevibacter* and *Methanosphaera* genera from the *Methanobacteriales* order and the RCC Thermoplasmata were the predominant archaea that was detected, with the RCC showing the only significant decrease in population with the RSO dietary supplementation. The RCC clade was determined to be accountable for the decrease in methane production as their numbers were significantly decreased with the addition of RSO to the diet.

Ionophores and methane

Another method of methane mitigation was the inclusion of ruminal ionophores to cattle diets. Often the benefit of adding ionophores to the diet is the decrease of the acetate:propionate ratio and the decrease in methane production (Mathison et al., 1998; Moss et al., 2000). The role of ionophores are to make ions unrestricted and enable their passage across membranes (Mathison et al., 1998). Monensin (Elanco Animal Health, Greenfield, IN) is a frequently used ionophore and subsequently the most studied ionophore (Boadi et al., 2004). Monensin inclusion is often correlated with a selective reduction in Gram-positive ruminococci, and an increase in abundance of Gram-negative bacteria with a parallel shift in propionate production (Newbold et al., 1988; Van Nevel and Demeyer, 1995). The studies detected an increase in the production of propionate

and a reduction in methane production, a trend that is frequently seen in the addition of ionophores (Wallace et al., 1980).

Methane production appears to be unresponsive to prolonged supplementation of ionophores in bovine studies (Van Nevel and Demeyer, 1995; McCaughey et al., 1997; Sauer et al., 1998). Johnson and Johnson (1995) stated that per unit of grain or forage diet fed to cattle, methane levels returned to previous levels within 14 days, indicating the adaptation of the microbial community. Boadi et al. (2004) described that the effect of monensin on decreasing methane production may be due to an increase in potential strains that have adapted to the antibiotic and thus become resistant. Conversely, methanogen resistance to monensin has yet to be tested (Hegarty, 2001). Sustained use of monensin may select for the strains of methanogens not susceptible (Boadi et al., 2004). The use of ionophores in diets and their success in improving feed efficiency may outweigh their long-term effects on methane production. Reduction of methane is hypothesized to be from the lower amount of intake compared to an effect on the methanogenic population (Johnson et al., 1995).

Nitrate and sulfate addition

The addition of nitrates and sulfates to the diet have gained some attention as potential methane mitigation agents. Nitrate is reduced to nitrite and finally to ammonia producing more energy than the reduction of carbon dioxide to methane (Ungerfeld and Kohn, 2006). If nitrate was provided in sufficient amounts in the rumen, this would provide an ideal route of hydrogen disposal (Zijderveld et al., 2010). In this process, eight electrons are utilized, so with 1 mole of nitrate reduced to ammonia methane production would decrease by 1 mole (Zijderveld et al., 2010). The generated ammonia from this process would be available for anabolic purposes and would provide a nitrogen

source for rumen microbes if diets are deficient in rumen degradable protein (Dijkstra et al., 1998, Leng and Nolan, 1984).

The negative role of nitrate supplementation is a limiting factor. The microbial community needs to be adapted to nitrate in their diet in order to reduce nitrate to nitrite effectively (Lewis, 1951). The accumulation of nitrite increases in the rumen (Zijderveld et al., 2010). Nitrite is easily absorbed across the rumen wall and alters blood hemoglobin from ferrous to ferric (Morris et al., 1958; Zijderveld et al., 2010). Methemoglobin, termed from the ferric form of hemoglobin, leaves the compound incapable of bringing oxygen to the tissues (Morris et al., 1958). This can reduce animal performance and be fatal (Ozmen et al., 2005).

Sulfate can be added to the diet to decrease methane production (Zijderveld, et al., 2010). Hydrogen sulfide can also function as an electron donor in the reduction of nitrite to ammonia by nitrate-reducing, sulfide-oxidizing bacteria (Hubert and Voordouw, 2007). Supplementing the diet with sulfur molecules (Leng, 2008) may reduce nitrite abundance in the rumen (Zijderveld et al., 2010). Sulfate acts as a reductant (Ungerfeld and Kohn, 2006) and will vie for electrons and potentially lower methane production (Zijderveld et al., 2010). However, hydrogen sulfide gas buildup in the rumen is a limiting factor additionally (Zijderveld et al., 2010). Feeding above maximum inclusion rates increases the risk of polioencephalomalacia, a condition in which hydrogen sulfide gas (H_2S) builds up in the rumen and can be inhaled (Gould, 1998).

According to Hedderich and Whitman (2006), environments abundant in sulfate are catalyzed by sulfate-reducing bacteria due to the oxidation of H_2 and sulfate as the electron acceptor being thermodynamically more favorable compared to CO_2 as the

electron acceptor. Methanogens then fail at competing for H₂ substrate, in addition to being outcompeted for formate. The same occurs in environments with nitrate. However, these organisms will consume all of the substrate (i.e. sulfate and nitrate) indirectly increasing the concentration of CO₂ for methanogenesis then to proceed.

Conclusion

Cattle produce methane as a method of maintaining pH and recycling NADH. Many factors play a role in methane production. These factors often depend on stage of production for the animals. Growing diets fed are typically composed of forages whereas finishing diets fed are comprised of concentrates. Methane production differs among these two stages as do the microbial populations. Various dietary components fed as a whole diet and the corresponding microbial populations and their reflection upon methane production has not been explored in detail. Studies looking at diet composition, methane production, and community structure simultaneously are needed to develop effective mitigation strategies, thus the studies described in this thesis attempt to better understand these interactions.

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CHAPTER 2

Effect of diet on the rumen microbial community composition and methane emissions in growing cattle

Abstract

Methane (CH₄) is a potent greenhouse gas that is able to trap heat from solar radiation 28 times more than carbon dioxide (CO₂). In the rumen, methane is produced as a by-product of fermentation, by methanogens, and is greatly dependent on the rumen microbial community composition and the diet fed to the animal. However, the interactions between diet composition, microbial community composition, and methane production are poorly understood. To better understand these interactions, methane emission and microbial community composition were evaluated on a common diet and under 10 different dietary conditions (high and low quality forage, with and without monensin supplementation, and different amounts of modified distillers grain plus solubles (MDGS) supplementation) in growing cattle. Samples were collected for microbial community analysis via esophageal tubing, and the microbial community structure was analyzed by sequencing the V3 region of the 16S rRNA gene using the Ion Torrent personal genome machine (PGM) at a depth of 5153 sequences for bacteria and 3055 sequences for archaea. Bacteroidetes and Firmicutes comprise the bacterial community at > 87% and Thermoplasmata and Methanobrevibacter were highly abundant for the archaea (> 97% for both time points). Microbial community shifts are greatly influenced by diet and forage rich growing diets provide an opportunity for methane mitigation utilizing dietary intervention strategies.

Introduction

The rise in greenhouse gas emissions (GHGs) in the atmosphere has raised concerns regarding global warming. Anthropogenic methane produced by ruminants contributes toward global methane emissions (EPA, 2016). Thus, various mitigation

strategies are being explored to reduce methane emissions from ruminants. Next to carbon dioxide, methane is the second greatest GHG that is emitted in the United States, including anthropogenic sources (EPA, 2016). The Global Warming Potential (GWP) of methane is 28, compared to carbon dioxide which has a GWP of 1, indicating that methane is able to trap solar radiation more efficiently than carbon dioxide contributing to the gradual warming of the Earth (Moss et al., 2000; EPA, 2016).

Methane is produced in the gastrointestinal tracts of various animals as well as peat bogs, rice paddies, thermal vents deep within the ocean, in addition to gastrointestinal tracts of various animals (Hedderich and Whitman, 2006; Thauer et al., 2008). The ruminant gastrointestinal tract is a major producer of methane (Hook et al., 2010; McAllister et al., 1996), producing 25% of the US methane emissions (Poulsen et al., 2013; EPA, 2016). Ruminants and their microbes fill an important niche which is the ability to consume and digest large amounts cellulose-rich plant fiber via fermentation, however as a by-product of fermentation produce significant amounts of methane (Poulsen et al., 2013).

The production of methane in ruminants is the fermentation product of a small group of rumen inhabitants known as the methanogens that belong to the domain Archaea (Hook et al., 2010). The methanogens play an important role in maintaining glycolysis in the rumen by recycling NADH produced during glycolysis (Moss et al., 2000), which is essential for rumen function. The methanogens utilize the hydrogen (H₂) and carbon dioxide produced during bacterial fermentation to produce methane, a natural end product of ruminal enteric fermentation (McAllister and Newbold, 2008).

Many studies have demonstrated that methanogens are influenced by diet due to the concentrations of H₂ produced during rumen fermentation (Johnson and Johnson, 1995). Using this concept, many studies have evaluated nutritional intervention methods to reduce methane (Buddle et al., 2011; Johnson and Johnson, 1995; Kumar et al., 2014). Such investigations have suggested that forage quality, type, and intake have the greatest influence on methane production. Additionally, ionophore supplementations has also been utilized as a tool for decreasing methanogenesis as well as boosting performance (Schelling, 1984; Wallace et al., 1980). However, the utilization of monensin to reduce methane may be brief (Johnson and Johnson, 1995). Corn by-products have also been utilized in diets as a method to boost nutrient availability (Johnson and Johnson, 1995). Nutritional mitigation strategies for decreasing methane production in cattle are focused on alterations of fermentation processes by targeting the microbial populations. Carbohydrate type fed is a major determinant of potential methane production (Johnson and Johnson, 1995). This is likely due to its effects on rumen pH, availability of intermediates for methane production and subsequent influence on the rumen microbial community composition (Johnson and Johnson, 1995). Forage quality will affect fermentation time and rate of passage in the rumen (Boadi et al., 2004). Studies have shown that feeding higher quality forage such as alfalfa may reduce methane production compared to lower quality forages (i.e. cornstalks) (McCaughey et al., 1999). Forage diets will favor acetate and butyrate production, leading to increased methane production (Boadi et al., 2004; Johnson and Johnson, 1995; Moss et al., 2000). Whereas, feeding concentrate leads to increased propionate production, which is a hydrogen sink that reduces methane, as this pathway competes for hydrogen that is available for methane

production (Moss et al., 2000). Therefore, the forage:concentrate ratio impacts the acetate:propionate ratio which in turn impacts methane production (McAllister and Newbold, 2008). These dietary changes also impact passage rate out of the rumen, which will impact the type of VFA produced as well as methane produced (Boadi et al., 2004; Mathison et al., 1998).

Various studies have concluded that diet impacts (or determines) the microbial community structure and composition of the rumen; however, studies investigating the microbial community composition have failed to measure relevant metadata such as methane production. In this study, 16S rRNA gene amplicon sequencing was utilized to explore the rumen microbial community under various growing diets representing the various factors known to affect methane production (forage quality, varying amounts of by-product supplementation, and ionophore supplementation), while measuring methane production (Pesta et al., 2014) to identify potential interactions between diet, microbial community composition, and methane emissions from growing cattle.

Experimental Procedures

An 84-d growing study was conducted utilizing 120 steers in an individually-fed, semi-confinement barn utilizing a Calan® gate system (American Calan Inc., Northwood, NJ) at the UNL Agriculture Research and Development Center (ARDC) near Mead, NE. Steers were placed on a common (basal) diet for 21 d, consisting of 50% Sweet Bran® (wet corn gluten feed, Cargill Corn Milling, Blair, NE) and 50% alfalfa hay to create a baseline for microbial community composition and methane emissions and to reduce animal-to-animal variation when weighing (Watson et al., 2013). The steers were then

assigned randomly to one of 10 treatment diets (Table 1) with 12 steers per treatment. The growing treatment diets were formulated to evaluate forage quality, level of by-product inclusion, and ionophore supplementation on methane production in growing cattle in a randomized block design as described by Pesta et al. (2014) (APPENDIX II). All animal procedures were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Rumen sampling and DNA Isolation

Sampling was performed on the common basal diet after 21 d adaptation and treatment sampling was performed on days 21 and 63 prior to feeding on the treatment diets. A representative sample of rumen contents (solid particles and rumen fluid) of 40 mL was collected by esophageal tubing. To ensure a representative sample collection, the particles retained on the filter were added to the collection tube. The samples collected were snap frozen in liquid nitrogen and placed in a -80°C until used for DNA extraction. DNA was extracted from 1 - 2 g of rumen contents using the MoBio PowerMag™ Soil DNA Isolation Kit (Optimized for KingFisher® Flex protocol) (MoBio Laboratories, Carlsbad, CA) according to the manufacture's protocol with the following modifications: approximately 1 - 2 g of raw sample were added to a sterile 2.0 mL Safe-Lock tube (Eppendorf, North America, Inc. USA) with 0.5 g of acid washed beads (Scientific Asset Management, Basking Ridge, NJ); between the two rounds of bead beating, the samples were placed in a > 85°C water bath for 5-8 min. The samples were centrifuged (4500 X G) and then the supernatant was transferred into sterile 1.5 mL tubes (Fisherbrand, Fisher Scientific, USA). Lastly, 130 µL of elution buffer was used to

elute the DNA. Quality of the DNA was evaluated using gel electrophoresis and was stored at -20°C until used for community analysis.

16S rRNA library preparation and sequencing of the V3 Bacteria and V6 Archaea regions

Eubacterial 16S rRNA library prep

The V3 region of the 16S rRNA gene was amplified using extracted total rumen DNA using universal eubacterial 16S primers 341F and 518R as described by Whiteley et al. (2012). The V3 region of the 16S rRNA gene was amplified in a 15 µL reaction volume. A PCR reaction consisted of 1X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life Technologies™, Massachusetts, USA), 1.7 µM of 341F and 0.2 µM of 518R primer, approx. 50 ng of extracted total DNA. Quantitative PCR (qPCR) conditions for amplification of the 16S rRNA gene included: 95°C for 10 min for initial denaturation; followed by 25 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s, with a dissociation curve following the amplification. Following amplification, 5 µL of amplicon product was run on a 1.8 % agarose gel using gel electrophoresis (QD LE Agarose, Green Bio Research, Baton Rouge, LA) at 120 V for 55 minutes for size verification and to ensure amplification. PCR products were normalized using the Invitrogen Sequal Prep™ Normalization Plate kit (Frederick, Maryland) to 1 – 2 ng/µL according to manufacturer's protocol and was pooled. Library qPCR preparation, normalization, and pooling was conducted using the Eppendorf epMotion (M5073, Germany). The pooled library, 300-500 µL, was column purified using PCR cleanup procedure (DNA, RNA, and protein purification Clontech Laboratories, Inc, California) as described by the manufacturer with the modification of eluting into 40 µL. The

purified concentrated libraries were size selected using the Pippin Prep (Sage Science, Inc., USA) to remove any spurious PCR fragments. Finally, the PCR product size and quantity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using High-Sensitivity DNA chips. Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) according to the manufacturer's protocol with emPCR, bead deposition and sequencing was performed as described by the manufacturer.

Archaea 16S rRNA library prep

The V6 region of the 16S rRNA gene was amplified using extracted total rumen DNA using universal archaeal specific primers 751F and 934R (Whiteley et al., 2012). The primers were synthesized to have adapters and barcodes as described by Whiteley et al. (2012). The V6 region of the 16S rRNA gene was amplified in a 20 μ L volume. The common diet samples utilized Terra Polymerase mix and buffer (Clontech Laboratories, Inc, California) at 1X with primer concentrations of 1.25 μ M 751F and 0.15 μ M 934R, 1.0 – 2.0 g (approx.. 50 ng/ μ L) while the rest of the samples utilized 1X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life Technologies™, Massachusetts, USA. Each reaction contained, 1.25 μ M 751F and 0.15 μ M 934R primer, approx. 50 ng of extracted total DNA. Quantitative PCR (qPCR) conditions for amplification of the 16S rRNA gene for the common diet included: 95°C for 10 min for initial denaturation; followed by 10 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s, followed by 20 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s with a dissociation curve following the amplification. The treatment diet samples followed a slightly different method of 95°C for 10 min for initial denaturation; then 30 cycles of 95°C

for 30 s, 52°C for 30 s, and 72°C for 45 s with a dissociation curve following the amplification. Following amplification, the product was run on a 1.8 % agarose gel using gel electrophoresis (QD LE Agarose, Green Bio Research, Baton Rouge, LA) at 120 V for 55 minutes for initial size verification and to ensure amplification. Following amplification, a 0.6X SPRI was conducted according to manufactures protocol (Agencourt® AMPure®) to remove primer dimers. SPRI products were normalized using Invitrogen Sequel Prep™ Normalization Plate kit (Frederick, Maryland) to 1 – 2 ng/ according to the manufacturer’s protocol and pooled. Library qPCR preparation, normalization, and pooling was conducted using the Eppendorf epMotion (M5073, Germany). The pooled library, 300-500 µL, was column purified using PCR cleanup procedure (DNA, RNA, and protein purification Clontech Laboratories, Inc, California) as described by the manufacturer. Size select elution of libraries was conducted by using the Pippin Prep (Sage Science, Inc., USA). Product size and quantity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using its High-Sensitivity DNA chips. Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) according to manufacturer’s protocol with emPCR, bead deposition and sequencing was performed as described by the manufacturer.

Microbial community analysis

The .fastq file that is generated from the PGM was converted into a .fasta file and were de-multiplexed utilizing the barcode on the reverse primer and the mapping file utilizing the platform Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Raw reads from Ion Torrent PGM sequencing were first analyzed for quality (Anderson et al., 2015). Briefly, reads were removed if 1) an incomplete forward primer

sequence was present, 2) barcode was not identified, 3) sequence length was less than 100 or greater than 250 nucleotides, and 4) if ambiguous bases (“N”) were present. Additional quality control checks included removing sequences with one or more errors within the forward primer, two or more errors in the reverse primer, and two or more errors in the barcode which were performed using Qiime (ver.1.9.1) (Caporaso, et al., 2010). After this primary quality control, reads that met these requirements were concatenated into a single file (one for bacteria and one for archaea). Reverse primers were removed. Resulting sequences were further processed using Mothur (Schloss et al., 2009) and the FASTX-TOOLKIT to remove and trim to a fixed length of 130 bp for bacteria and 140bp for archaea to improve OTU classification (Edgar, 2013). The sequences were reverse complemented in Mothur (Schloss et al., 2009). Utilizing a custom pipeline within the Fernando Lab, chimera identification and removal, and OTU picking based on 97% sequence similarity. Sequences less than 96% are considered phylogenetically a different species. This was conducted using UPARSE composed by Edgar (2013) using a batch script. Taxonomic classification was determined using Qiime using the GreenGenes database (ver. 13_8). The OTU sequences generated were aligned using Ribosomal Database Project (<https://pyro.cme.msu.edu>). OTUs aligning outside the 16S gene were eliminated. The phylum Cyanobacteria were removed from the OTU table as it is a photosynthetic phylum and the rumen environment is anaerobic and is dark. The cyanobacterial reads are most likely a result of the 16S copies present in the chloroplast of the forage portion of the diet. Subsequent analyses were conducted separately on the bacteria and the archaea samples, however the same steps occurred in both bacteria and archaeal analyses, as different primers were utilized to sequence

different regions but each set contained all diets. Singletons OTUs were eliminated as a single sequence may have been generated due to sequencing error, even if the single read is real, the abundance will have little biological meaning. Scripts can be found in Appendix 1.

Statistical analysis

Total reads from each sample were subsampled to the sample with the lowest number of reads to achieve an equal sampling depth rarefaction (bacteria, 5153) and (archaea, 3055). Global bacterial and archaea community composition changes were evaluated using the unweighted unifracs distance matrices (Lozupone et al., 2011). To evaluate the effect of diet on bacterial and Archaeal community structuring, 2 way Non-Parametric MANOVA test was utilized, where diet was used as a main effect and animal was used as a random effect (MatLab, 2015). *P*-values of < 0.05 were considered significant. Pairwise tests were conducted on a one way comparison using R (ver. 3.2.1) to identify diets that resulted in significant changes in community composition. Principle coordinate analyses were performed to visualize structuring of eubacterial and archaeal community shifts (Qiime, ver. 1.9.1). Each dot within the plots represents a community from an animal. It is generated based on the factors of phylogenetic relationships and abundance. Fluctuations in OTU (Operational Taxonomic Unit) abundances were identified using the `differential_abundance.py` command within Qiime (1.9.1), choosing the *P* adjusted values. The sequences were rarefied (bacteria, 5153 and archaea, 3055) and used for calculation of diversity using the Chao1 index and to generate rarefaction curves (Kuczynski et al., 2011). To visually observe shifts in the community, principle coordinate analyses was performed utilizing the unweighted UniFrac distances from

subsampled OTU tables (Lozupone et al., 2011). Scripts and procedures used for analysis are shown in Appendix 1.

Heatmaps were created to visualize significantly differential OTUs using R heatmap.2 function (Ploner et al., 2014) with the OTU relative abundance as input. Bray-Curtis dissimilarity matrix was used to estimate the distance between samples and dendrograms were created by hierarchical clustering of OTUs and samples.

Results

The bovine rumen microbial community structuring and composition still remains somewhat of a mystery when trying to utilize dietary intervention strategies to mitigate methane. Diet has been shown in previous research to affect the community; however, measuring the changes the community undergo with various dietary substrates available, and comparing those changes to simultaneous methane emissions has not been previously explored in a production setting. Observing the change in community structure and composition from a common diet to various growing diets can provide an insight into the community and the interactions involved can be utilized to develop dietary intervention strategies to decrease methane emissions without sacrificing animal performance can be achieved.

Bacteria

All animals were placed on a common basal diet to establish a baseline for comparisons and reducing animal to animal variation in microbial community structure. For the bacterial community, globally, compared to the common diet, there was an effect due to Diet, Time, and Animal ($P = 0.001$), establishing that the diet, time, and animal

were affected. A Diet \times Time interaction was observed ($P = 0.001$) (Table 1a).

Establishing that time and diet did have an effect on the community, analyzing the treatments separate from the common diet is necessary to see if there is a difference between the two growing time points (Table 1b). By comparing the two growing time points (d 21 and d 63), Diet, Time, and Animal were observed as being significantly different ($P = 0.001$). A Diet \times Time interaction was not significantly different ($P = 1.00$).

Global effects indicated that time was significant so each day was analyzed separately to identify if barn location had an effect on the dietary treatments. No effect was observed on d 21 ($P = 0.288$) and a Diet \times Barn interaction was not observed ($P = 0.413$) (Table 2a). On d 63, no effect of Barn was observed ($P = 0.514$) and a slight Diet \times Barn interaction was observed ($P = 0.047$) (Table 2b).

Dietary difference are apparent between diets, within each time point (21 d or 63 d). Table 3a contains the pairwise comparisons between the bacterial communities on day 21 on the treatments. All diets were significantly different from the common ($P < 0.001$). The diets 20Deoil and 40Deoil and 20Norm and 40Norm were significantly different from each other ($P < 0.0001$). The diets looking at the effect of Rumensin® with high quality forage at 40% MDGS inclusion without Rumensin® did not change the community structure ($P = 1.00$) but the same diets with Rumensin® did change the community ($P < 0.0001$). Diets to test if Rumensin® supplementation had an effect on the community (HQNoRum and HQRum) were not significantly different ($P = 0.914$), however, the dietary comparison of High Quality forage with 40% MDGS inclusions with and without Rumensin® were significantly different ($P = 0.002$).

The dietary treatments were different on d 63 of the study. Pairwise comparisons of all diets for the bacteria community are represented in Table 3b. Similarly, all diets are significantly different from the common ($P < 0.0001$). However, not all of the treatments reflected the community changes observed on 21 d. Diets observing the effect of high quality and low quality at 40% MDGS inclusion without Rumensin® displayed a community change ($P < 0.0001$). Another comparison observing high quality and low quality at 40% MDGS inclusion with Rumensin® were also significantly different ($P = 0.001$). The dietary comparisons observing the level of MDGS supplemented with low quality forage (20 and 40 Deoil and Norm) with Rumensin® were significantly different ($P < 0.0001$) and ($P < 0.0001$) as observed on day 21. However, two dietary comparisons observing the effect of Rumensin® on community changes with high quality forage and 40% MDGS inclusion and 0% MDGS inclusion with and without Rumensin® were not observed ($P = 0.393$) and ($P = 0.054$) respectively.

The taxonomic distribution of the bacterial community at the phylum level for the entire study is represented over several figures (Figures 1, 2a, and 2b). The prominent phyla were Firmicutes, primarily in the common at 37.5%, Bacteroidetes over both time points at 51%, and Proteobacteria increasing throughout the growing time points at 3.5% (Figure 1). These phyla represented approximately 92% of the community taxonomic distribution, with 17 other phyla as well as those unassigned, comprising the remaining 8% of the community. Figure 2a shows the taxonomic distribution at the phylum level on day 21 of the study. There are 17 phyla represented, along with 2 other unassigned groups however, the top three abundant were Bacteroidetes at 60.3%, Firmicutes at 29.5%, and Proteobacteria at 3.4% totaling 93.2% of the community with the other phyla

and unassigned groups comprising the other 6.8%. Figure 2b shows the same contents as Figure 2a. This figure shows the taxonomic distribution on day 63 on the treatments. Slight differences in abundance are observed, however the pattern at 21 d is evident in the 63 d community.

Figures 3-7 provide Principle Coordinate Analyses results and is consistent with the statistical results for the bacterial community structure clustering against the common diet and the diets respective to each time point as discussed previously. Briefly, clustering separate from the common diet was apparent (Figure 3). After 21 d on the treatments, clustering differences were due to level of MDGS inclusions for low quality diets, 0% or 40% for high quality diets, Rumensin® has no effect on community structuring (Figures 4-7). After 63 d on the treatments, clustering is apparent for MDGS inclusion amounts of 0% and 40% for high quality and low quality diets with either 20% or 40% MDGS supplementation, and low quality diets clustering separate from high quality diets, and Rumensin® appeared to have no effect on the community (Figures 8-11).

Archaea

For the Archaeal community, globally, compared to the common diet, Diet, Time, and Animal were significantly different ($P = 0.001$), additionally, a Diet \times Time interaction was observed ($P = 0.001$) (Table 4a). After establishing a difference between diet, time, and animal on the archaeal community, analyzing the two time points, omitting the common diet, is essential to establishing what differences were present, that will change the community on treatment diets. The archaea mirrored the bacterial

community, with significant factors being Diet, Time, Animal ($P = 0.001$). The only not significantly different interaction was Diet \times Time ($P = 0.959$) (Table 4b).

Global effects indicated that time was significant so each day was analyzed separately to identify if barn location had an effect on the dietary treatments. No effect was observed on d 21 ($P = 0.797$) and a Diet \times Barn interaction was not observed ($P = 0.084$) (Table 5a). On d 63, no effect of Barn was observed ($P = 0.066$) and a Diet \times Barn interaction was not observed ($P = 0.886$) (Table 5b).

Pairwise comparisons were made to identify which dietary treatments are different. On day 21 of treatment diets (Table 6a), no significantly different dietary changes on the community were observed with the exception of two diets: 20Norm ($P < 0.001$) and HQ40Rum ($P = 0.006$). The level of normal MDGS supplementation at 20% or 40% was observed to be significantly different ($P = 0.022$) for the archaeal community. A difference was also observed for 20% MDGS supplementation between normal and deoiled MDGS added. Dietary comparisons observing the effect on community on day 63 of treatment (Table 6b), only displayed a significant effect with Rumensin® on High Quality forage with 40% MDGS ($P = 0.001$).

Figure 12 shows the archaeal community distribution between the common diet and the treatment diets for the entire study at the genus level. Twenty-nine groups were present however, the genera *Methanobrevibacter* at 35.2% (found primarily in higher abundance on the common diet) and Thermoplasmata at 62.0%, and the family Methanobacteriaceae at 1.4% (found in higher abundances in the treatment diets) were the dominant groups present throughout the entire study representing 98.6% of the community with the other 26 groups comprising 1.4%. Figure 13a shows the taxonomic

distribution of the archaeal community 21 d on the study. The same dominant genera and family presented in Figure 12, are present, combining to make 99.3% of the community leaving the other 26 groups to make up 0.7%. Figure 13b shows on day 63 of the treatments, the taxonomic distribution of the archaeal community. The same three groups are present as in Figure 14 and 15a, however, *Methanobrevibacter* and Thermoplasmata changed significantly in abundance, 23.3% and 74.6% respectively, while Methanobacteriaceae remained at 1.2%. The other 26 groups combine to make up the other 0.9%.

Figures 14-17 provide Principle Coordinate Analyses results, confirming the statistical results obtained for the archaeal community structure against the common diet and the diets respective to each time point as discussed previously on 21 of treatment. Briefly, between the low quality diets, the level of MDGS supplementation of 0% or 40% is apparent (Figure 15), the high quality diets cluster separate from the low quality diet with 0% and 40% MDGS with and without Rumensin® (Figure 16). No apparent clustering between forage quality when 40% MDGS is supplemented with Rumensin® (Figure 17).

Figures 18-21 provide principle coordinate analyses of the community data and responses to diet. Slight clustering is evident between 20% and 40% MDGS supplementation (Figure 19). Slight clustering is also apparent (Figure 20) between the low quality diets and high quality diets as well as 40% MDGS supplementation. No effect of Rumensin is present in Figure 21.

Rarefaction curves in Figures 22 and 23 confirm that sequencing depth was sufficient in order to characterize this community data. Goods coverage test concluded that 73% of the bacterial community and 93% of the archaeal community were characterized.

Tables detailing dietary composition, VFA profiles, and methane emissions are described briefly in APPENDIX II and in detail in Pesta et al. (2014).

Discussion

Diet is known to affect the microbial community composition within the bovine rumen. The dietary ingredients offered are fermented into various substrates by the mixed microbial population including bacteria, fungi, protozoa, and archaea. Initial breakdown is due to the bacteria, fungi, and protozoa communities. These groups provide products for the archaeal community to utilize and a byproduct of their metabolism is methane (Attwood et al., 2011). However, methane measurements taken simultaneously with ruminal dietary samples to measure the effect of diet on the community structure and composition and its effects on methane production are limited.

Sampling depth of bacterial and archaeal communities was verified using the rarefaction curves (Figures 23 and 24 respectively). This plot shows whether the sequencing depth allows for a proper characterization of the microbial community. Additionally the Chao1 index was used to evaluate microbial diversity by observing the number of rare or significantly different OTUs based on 97% similarity. When comparing the treatment diets against the common diet, diet and time are significant, this is mainly due to feeding different diets during the two sampling periods. However, when observing the treatment diets, diet is significant but only between the diets within a time

point. The two time points are statistically different but diet is not driving that difference. This difference in microbial community structure in day 21 and day 63 may be due to other factors such as environmental factors (temperature, humidity etc.) For instance, the sampling date for day 21 took place in March of 2013 whereas, the sampling date for 63 d took place in May of 2013, a substantial environmental change in Nebraska. Additionally, dietary characteristics that affect the microbial community structure and composition that are most commonly utilized are feed intake levels, carbohydrate type, forage quality, and ionophore additions (Johnson and Johnson, 1995; Guan et al., 2008). These dietary factors has various effects on the microbial population within the rumen. Each of these factors can affect one or more microbial communities.

The additions of MDGS to the diets has interesting effects on the microbial community. Adding MDGS to forage diets can often overshadow the effects of another factor. For instance, on day 21, bacteria and days 21 and 63 for archaea, had no statistical community difference when comparing high and low quality with 40% MDGS supplementation without Rumensin®. This could be due to effects of the higher level of MDGS fed and the role it has in shaping the community's structure by impacting rumen pH and substrate availability (Johnson and Johnson, 1995). However, other factors such as environment should be considered due to this study not reflecting this outlook for the bacterial community after day 63. Pesta et al. (2014) stated that 40% MDGS increased molar proportions of propionate and butyrate. This may indicate the two way role of 40% MDGS, not only a hydrogen sink but also contributing to increased methane emissions by the increasing butyrate.

The addition of ionophores in cattle diets are multipurpose. These additives increase performance and control methane production by targeting the Gram-positive microorganisms present (Johnson and Johnson, 1995). Ionophores are speculative in their precise role in methane mitigation. Johnson and Johnson (1995) speculate that a decrease in methane production is related to a decrease in intake and subsequent concentrations of substrate availability rather than directly effecting methanogenesis. However, it has been previously reported that the effect of ionophores on the microbial community can be short-lived as it is hypothesized that the community becomes trained and adapts to the additive (Johnson and Johnson, 1995).

Other factors that need to be taken into consideration when identifying mitigation strategies for methane production are factors such as protozoa, fungi, and viruses. These factors all impact methane production directly or indirectly. Protozoa and viruses have recently been thought to contain organelles called hydrogenosomes (Williams, 1986). The function of this structure is to produce hydrogen ions, contributing to the hydrogen ion pool. Protozoa community members also engulf starch granules, leading to a potentially decreased concentration of substrate. Archaea members have also been identified as being symbiotic and attached to the protozoa potentially contributing to the inter-species hydrogen transfer idea (Moss et al., 2000). Fungi physically attach and break apart fiber particles within the rumen allowing for an increase in surface area for bacterial attachment to increase fermentation of the ingested feeds and increase the hydrogen pool within the rumen.

The results of the taxonomic distribution for bacteria for this study is similar to a study conducted by Thoetkiattikul et al. (2013) where Holsteins-Friesian dairy cows

were utilized to observe bacterial population structures in the dairy cow rumen when fed three different fiber and starch diets. The top three dominant phyla consisted of Bacteroidetes being the most abundant, Firmicutes, and Proteobacteria being extremely low in abundance.

The taxonomic distribution results for the archaeal community for this study are slightly different as previously reported in a review by Hook et al. (2010). The genus *Methanobrevibacter* is present in a significant portion, however it is not the dominant archaeal taxa found in growing diets.

The genus Thermoplasmata belongs to the Order Thermoplasmata. Thermoplasmata were previously named Rumen Cluster C and prior to this name, Rice Cluster C (Poulsen, et al., 2013). This group as a member of this Order is present in high abundance in the growing diets utilized within this study. This novel group has a unique niche within the archaeal community and that is its ability to utilize methylamines for their source of carbon for energy (Poulsen, et al., 2013). Poulsen et al. (2013) performed an experiment with lactating Holstein cows feeding rapeseed oil focusing on decreasing the methanogenic archaea. In this study, the major contributors are the genera *Methanobrevibacter*, *Methanosphaera*, and Thermoplasmata. The results of the aim of this paper coincide with these findings as well. The exact pathway of methanogenesis from this group is unknown as no isolates have been obtained for further examination (Poulsen et al., 2013). With the identification of Thermoplasmata, this increases the microorganisms that utilize methanol as a substrate, in particular, broadening the number of groups for this substrate from *Methanosarcinaceae*. Methylamines are derived from

betaine and choline in plants (Poulsen et al., 2013; Neill et al., 1978; Mitchell et al., 1979).

The presence of certain taxa and their relative abundances can be seen in the heatmaps generated for various dietary combination observing the effects of diet on the microbial community of bacteria and archaea. The family *Prevotellaceae* is present in some level of abundance in virtually all diets as described by Purushe et al. (2010). It is composed of four genera consisting of *Prevotella*, *Paraprevotella*, *Alloprevotella*, and *Hallella* (Rosenberg, 2014). The *Prevotella* strains are Gram-negative singular cells that thrive under anaerobic conditions (Rosenberg, 2014). The *Prevotella* sp. are responsible for the breakdown of cellulose lacking polysaccharides and protein as well as the breakdown and utilization of starch, xylan, and pectin (Thoetkiattikul et al., 2013). The *Prevotella* genus includes two widely known species, *P. bryantii* and *P. ruminicola* and are capable of utilizing starch and other cellulose lacking polysaccharides producing succinate, which is able to be decarboxylated into propionate (Purushe et al., 2010). The family *Lachnospiraceae* was present in the diet consisting of high quality forage with 40% MDGS supplementation without Rumensin®. This family contains 24 genera that have been identified along with several uncharacterized strains (Meehan and Bieko, 2014). Several members share a trait of producing short-chain fatty acids, however, further research into this family's influence and role in the rumen regarding methane production needs to be explored (Meehan and Bieko, 2014). The family Veillonellaceae was observed in the diet containing low quality forage 40% MDGS with Rumensin®. This group has some characterized members, however, further research regarding this family is necessary. A member of this family includes *Megasphaera elsdenii*, which

ferments lactate producing CO₂, H₂ and VFAs, however, this trait may not be observed in every strain (Marchandin and Jumas-Bilak, 2014). The inclusion of MDGS and ionophore supplementations possibly allow for a more diverse microbial population to be present.

The archaea are far less in abundance comprising only 3% of the total mixed microbial population (Janssen and Kirs, 2008). The archaeal population within the rumen is extremely low in abundance. While there can significantly different OTUs between diets, the abundance may be far below 1.0%. However, two families are present in both archaeal heatmaps (Figure 31 and 32). One family is Methanobacteriaceae, whose members are strictly anaerobic belonging to the class Methanobacteria (Boone et al., 2001). According the Bergey's Manual of Systematic Bacteriology Volume I (Boone et al., 2001), this group's metabolism consists of oxidizing H₂, formate, and CO₂. They are also able to reduce methanol and sulfur, however, sulfur production inhibits growth. Again, various OTUs comprise each family leading to abundances in different diets. The other family present is *Methanomassiliicoccaceae* belonging to the class Thermoplasmata. This microbial family is novel with very little characterization within the rumen. Thermoplasmata are microorganisms that inhabit extreme environments and thusly are still widely uncharacterized (Iino, et al., 2013). A study conducted by Iino et al. (2013) wanted to phylogenetically classify Thermoplasmata methanogens and proposed family classification within Thermoplasmata called *Methanomassiliicoccaceae*. In this study, members of *Methanomassiliicoccaceae* were observed growing in environments with methanol as the metabolite. However, further

examination of this family is required to obtain an accurate characterization for this methanogenic family.

Various OTUs were observed being significantly different between the diets however, these were represented at the family level and the various OTUs are still uncharacterized due to the rumen microbial community being extensive and the isolation of the uncharacterized groups have proved challenging. Also, the read length utilized in this study for both bacteria and archaea were relatively short. If longer read lengths were utilized in the future, the potential for classification of some of these uniquely present OTUs may provide enhanced insight into the rumen microbial community's symbiotic relationship and lead to novel mitigation strategies.

Conclusion

Methane production by ruminants is a controversial topic without an absolute answer on methane mitigation. Diet does change the community structure, and therefore can be potentially used to control methane production. Both bacterial and archaeal communities change due to diet and substrate availability. However, simultaneous measurements of diet, methane and microbial community are critical to understand how dietary intervention can be used for methane mitigation and to develop science-based intervention strategies.

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Table 1a. Global bacterial statistics
against the common.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	0.001

Table 1b. Global bacterial statistics for the treatment diets.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	1

Table 2a. Effect of barn at d 21
on the bacterial community.

Diet	0.623
Barn	0.288
Animal	0.001
Diet × Barn	0.413

Table 2b. Effect of barn at d
63 on the bacterial community.

Diet	0.346
Barn	0.514
Animal	0.001
Diet × Barn	0.047

Table 3a. Pairwise comparison statistics against the common diet on 21 d for the bacterial community.

	Treatments ³									
	Comm ¹	20Deoil ²	20Norm ²	40Deoil ²	40DRC	40Norm ²	HQ40NoRum ²	HQ40Rum ²	HQNoRum	HQRum
20Deoil ²	< 0.0001	-	-	-	-	-	-	-	-	-
20Norm ²	< 0.0001	0.0029	-	-	-	-	-	-	-	-
40Deoil ²	< 0.0001	< 0.0001	< 0.0001	-	-	-	-	-	-	-
40DRC	< 0.0001	0.69512	0.05151	< 0.0001	-	-	-	-	-	-
40Norm ²	0.00041	< 0.0001	< 0.0001	1.00	< 0.0001	-	-	-	-	-
HQ40NoRum ²	< 0.0001	0.00148	1.00	< 0.0001	0.38302	< 0.0001	-	-	-	-
HQ40Rum ²	< 0.0001	< 0.0001	0.15344	< 0.0001	< 0.0001	< 0.0001	0.00215	-	-	-
HQNoRum	< 0.0001	0.68176	0.15344	< 0.0001	1.00	< 0.0001	0.6567	< 0.0001	-	-
HQRum	< 0.0001	1.00	0.0011	< 0.0001	1.00	< 0.0001	0.00613	< 0.0001	0.91434	-
LQ40NoRum ²	< 0.0001	< 0.0001	1.00	< 0.0001	0.00746	< 0.0001	1.00	0.38302	0.0372	0.00015

¹Common diet fed before start of trial consisting of 50:50 blend of alfalfa and Sweet Bran®

²Diets containing Modified Distillers Grains plus Solubles

³Diets fed consisting of Low quality forages (ground corn stalks) with 20% and 40% Normal and Deoiled MDGS with and without Rumensin®, DRC at 40% with Rumensin®, High Quality forages (alfalfa) with 0% and 40% Deoiled MDGS with and without Rumensin®

Table 3b. Pairwise comparison statistics against the common diet on 63 d for the bacterial community.

	Treatments ³									
	Comm ¹	20Deoil ²	20Norm ²	40Deoil ²	40DRC	40Norm ²	HQ40NoRum ²	HQ40Rum ²	HQNoRum	HQRum
20Deoil ²	< 0.0001	-	-	-	-	-	-	-	-	-
20Norm ²	< 0.0001	1	-	-	-	-	-	-	-	-
40Deoil ²	< 0.0001	< 0.0001	< 0.0001	-	-	-	-	-	-	-
40DRC	< 0.0001	0.7491	1	0.001	-	-	-	-	-	-
40Norm ²	< 0.0001	< 0.0001	< 0.0001	0.003	< 0.0001	-	-	-	-	-
HQ40NoRum ²	< 0.0001	1	1	< 0.0001	0.7163	< 0.0001	-	-	-	-
HQ40Rum ²	< 0.0001	0.5599	1	0.0017	1	< 0.0001	0.3937	-	-	-
HQNoRum	< 0.0001	0.0586	0.0212	< 0.0001	< 0.0001	< 0.0001	0.1345	< 0.0001	-	-
HQRum	< 0.0001	1	1	< 0.0001	0.7491	< 0.0001	1	0.7163	0.0548	-
LQ40NoRum ²	< 0.0001	0.0149	0.1972	0.1695	1	< 0.0001	0.0144	1	< 0.0001	0.0169

¹Common diet fed before start of trial consisting of 50:50 blend of alfalfa and Sweet Bran®

²Diets containing Modified Distillers Grains plus Solubles

³Diets fed consisting of Low quality forages (ground corn stalks) with 20% and 40% Normal and Deoiled MDGS with and without Rumensin®, DRC at 40% with Rumensin®, High Quality forages (alfalfa) with 0% and 40% Deoiled MDGS with and without Rumensin®

Table 4a. Global archaeal statistics
against the common diet.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	0.001

Table 4b. Global archaeal statistics
for the treatments diets only.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	0.959

Table 5a. Effect of barn at d 21
on the archaeal community.

Diet	0.671
Barn	0.797
Animal	0.001
Diet × Barn	0.084

Table 5b. Effect of barn at d
63 on the archaeal community.

Diet	0.216
Barn	0.066
Animal	0.001
Diet × Barn	0.886

Table 6a. Pairwise comparison statistics against the common diet on 21 d for the archaeal community.

	Treatments ³									
	Comm ¹	20Deoil ²	20Norm ²	40Deoil ²	40DRC	40Norm ²	HQ40NoRum ²	HQ40Rum ²	HQNoRum	HQRum
20Deoil ²	1	-	-	-	-	-	-	-	-	-
20Norm ²	0.00012	0.01004	-	-	-	-	-	-	-	-
40Deoil ²	1	1	0.01229	-	-	-	-	-	-	-
40DRC	1	1	1	1	-	-	-	-	-	-
40Norm ²	1	1	0.02291	1	1	-	-	-	-	-
HQ40NoRum ²	1	1	0.03981	1	1	1	-	-	-	-
HQ40Rum ²	0.00669	0.09732	1	0.25366	1	0.31591	0.604498	-	-	-
HQNoRum	1	1	0.19038	1	1	1	1	1	-	-
HQRum	1	1	1	1	1	1	1	1	1	-
LQ40NoRum ²	1	1	0.00192	1	0.90276	1	1	0.04731	1	1

¹Common diet fed before start of trial consisting of 50:50 blend of alfalfa and Sweet Bran®

²Diets containing Modified Distillers Grains plus Solubles

³Diets fed consisting of Low quality forages (ground corn stalks) with 20% and 40% Normal and Deoiled MDGS with and without Rumensin®, DRC at 40% with Rumensin®, High Quality forages (alfalfa) with 0% and 40% Deoiled MDGS with and without Rumensin®

Table 6b. Pairwise comparison statistics against the common diet on 63 d for the archaeal community.

	Treatments ³									
	Comm ¹	20Deoil ²	20Norm ²	40Deoil ²	40DRC	40Norm ²	HQ40NoRum ²	HQ40Rum ²	HQNoRum	HQRum
20Deoil ²	0.75577	-	-	-	-	-	-	-	-	-
20Norm ²	1	1	-	-	-	-	-	-	-	-
40Deoil ²	1	1	1	-	-	-	-	-	-	-
40DRC	1	0.56273	1	1	-	-	-	-	-	-
40Norm ²	1	1	1	1	1	-	-	-	-	-
HQ40NoRum ²	< 0.0001	< 0.0001	0.00026	0.0102	1568	0.00026	-	-	-	-
HQ40Rum ²	1	1	1	1	1	1	0.00124	-	-	-
HQNoRum	< 0.0001	< 0.0001	< 0.0001	0.00089	0.00062	< 0.0001	1	< 0.0001	-	-
HQRum	0.05946	0.00411	0.08163	0.80272	1	0.0814	1	0.40218	0.61728	-
LQ40NoRum ²	0.76763	0.08565	0.52558	1	1	0.84077	0.82123	1	0.20576	1

¹Common diet fed before start of trial consisting of 50:50 blend of alfalfa and Sweet Bran®

²Diets containing Modified Distillers Grains plus Solubles

³Diets fed consisting of Low quality forages (ground corn stalks) with 20% and 40% Normal and Deoiled MDGS with and without Rumensin®, DRC at 40% with Rumensin®, High Quality forages (alfalfa) with 0% and 40% Deoiled MDGS with and without Rumensin®

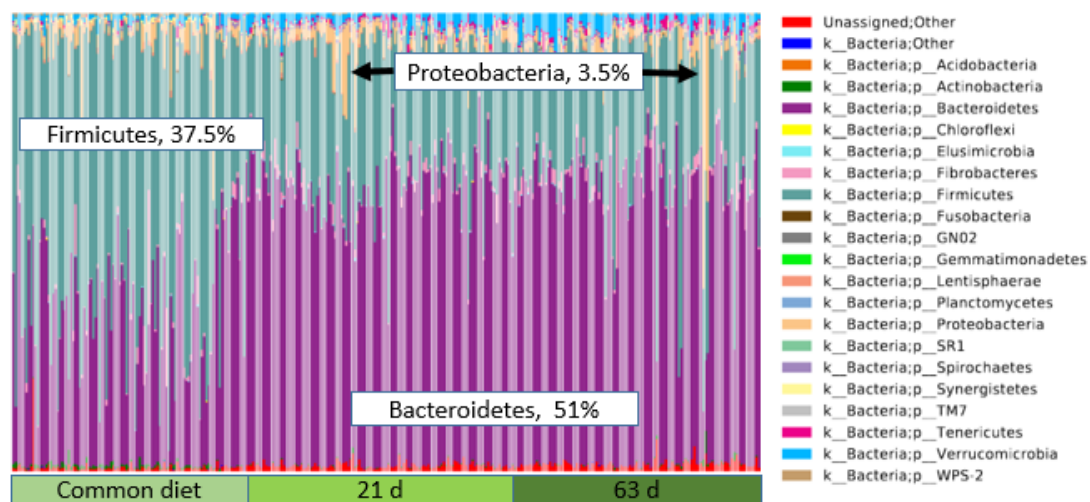


Figure 1. Bovine bacterial community taxonomic distribution at the phylum level with the abundance of the top three genera present.

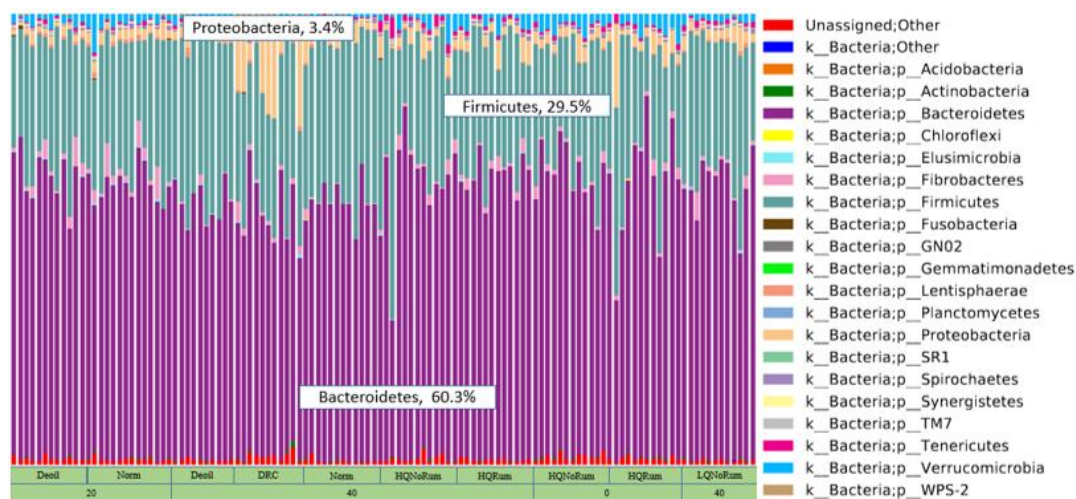


Figure 2a. Bovine ruminal bacterial community taxonomy at the phylum level with the corresponding abundance 21 d on the study.

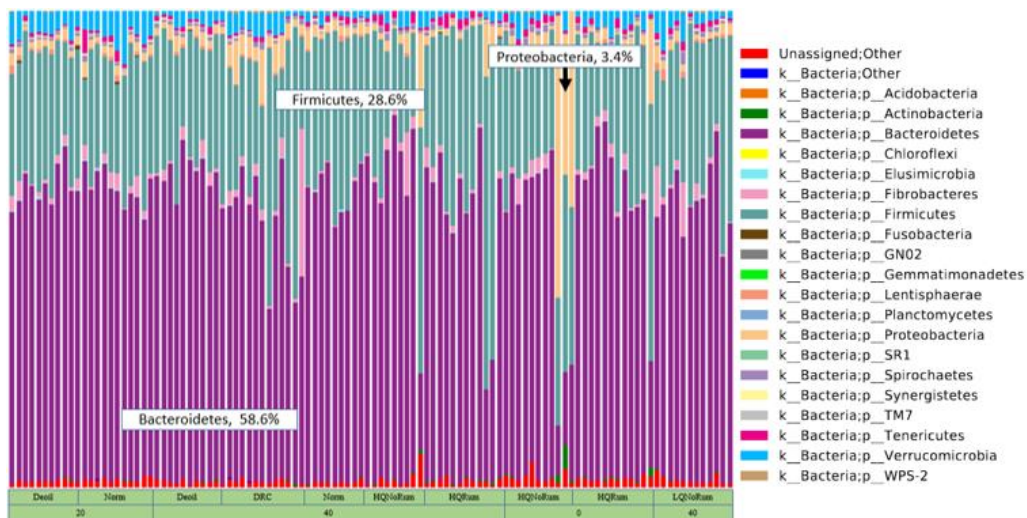


Figure 2b. Bovine ruminal bacterial community taxonomy at the phylum level with the corresponding abundance 63 d on the study.

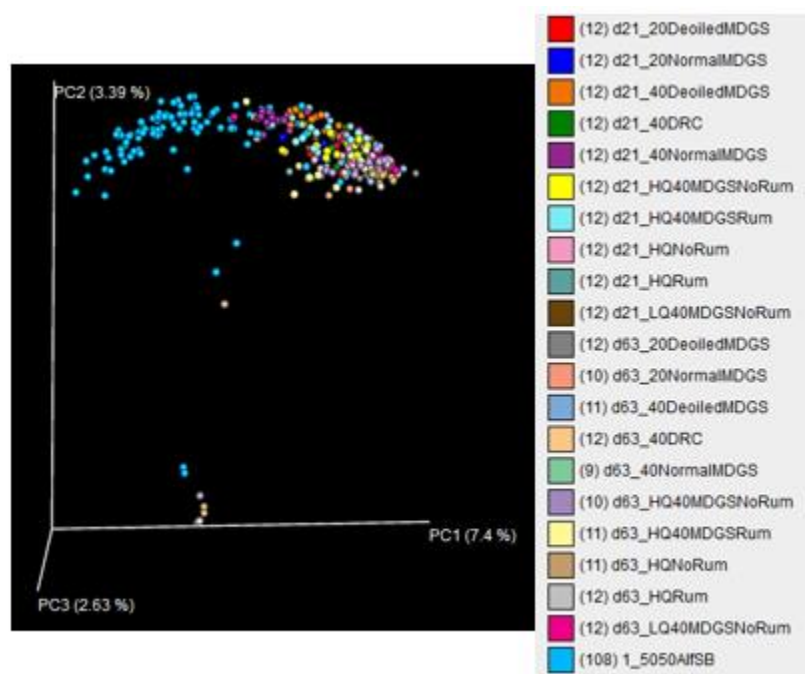


Figure 3. Principle Coordinate Analysis of the bovine ruminal bacterial community structure between the common diet and both growing time points combined. The common diet is identified as 1_5050AlfSB. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 or 63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clear clustering of the common diet separate from both growing time points, with both time points clustering near each other.

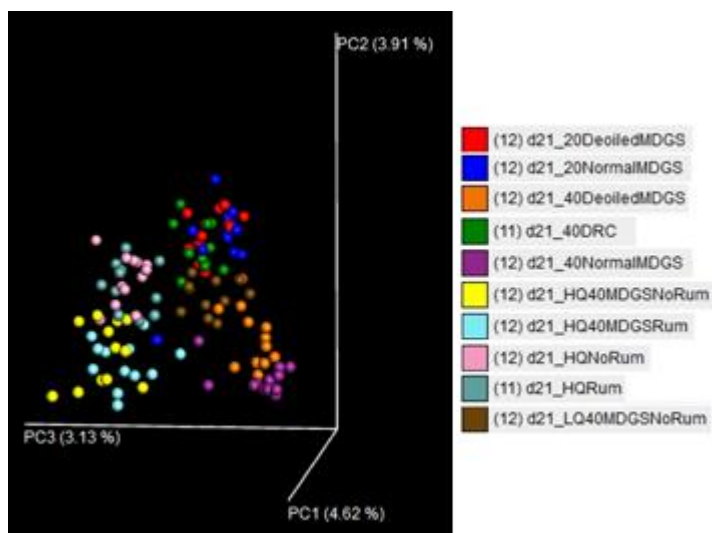


Figure 4. Principle Coordinate Analysis of the bovine bacterial community clustering after 21 d on study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows all ten diets on 21 d clustering. Patterns are evident with low quality diets clustering separate from the high quality diets.

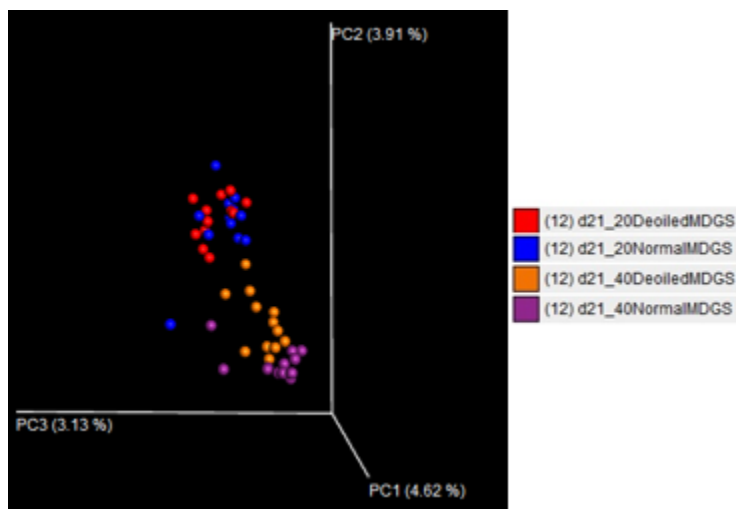


Figure 5. Principle Coordinate Analysis of the bovine bacterial structure 21 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. MDGS inclusion level of 20% clusters separate from 40% of both deoiled and normal.

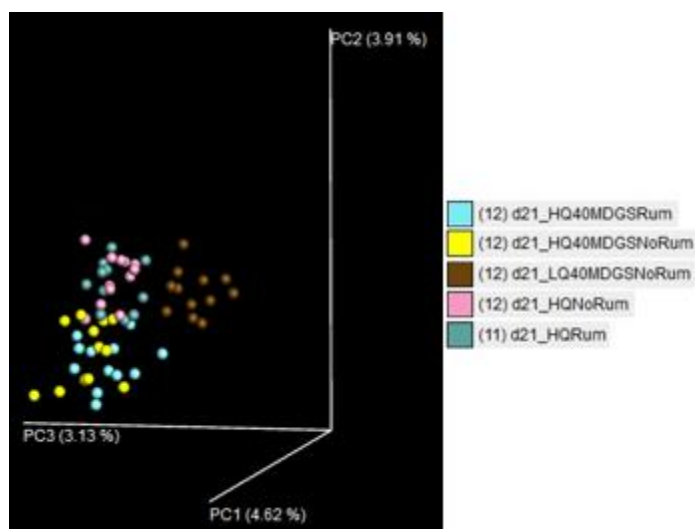


Figure 6. Principle Coordinate Analysis of the bovine bacterial structure 21 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to high and low quality forage as well as 0 or 40% MDGS inclusion with and without Rumensin®.

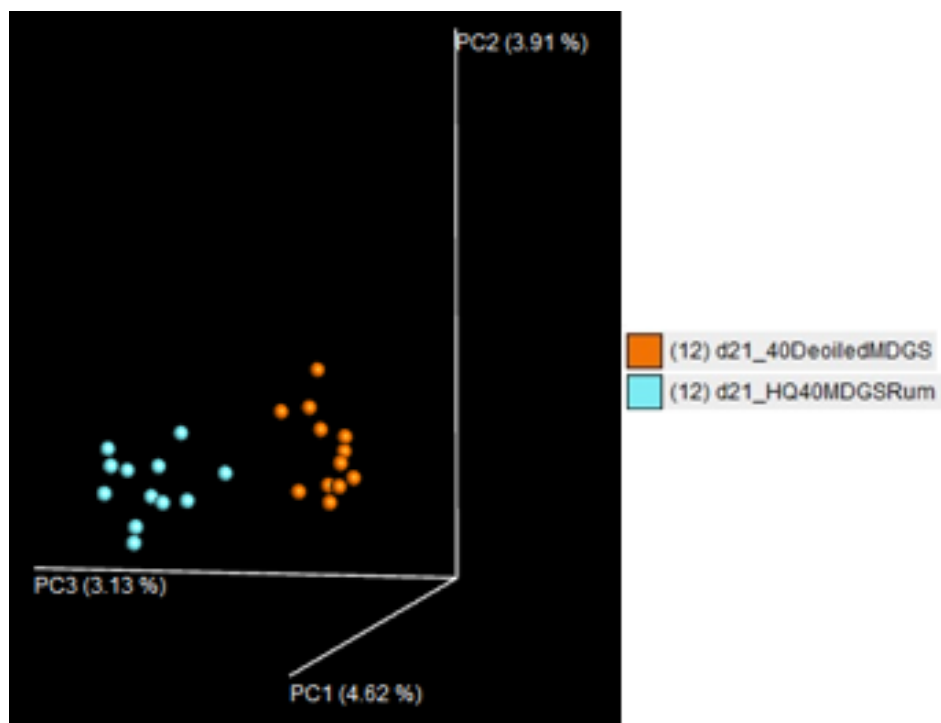


Figure 7. Principle Coordinate Analysis of the bovine bacterial structure 21 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to high and low quality forage.

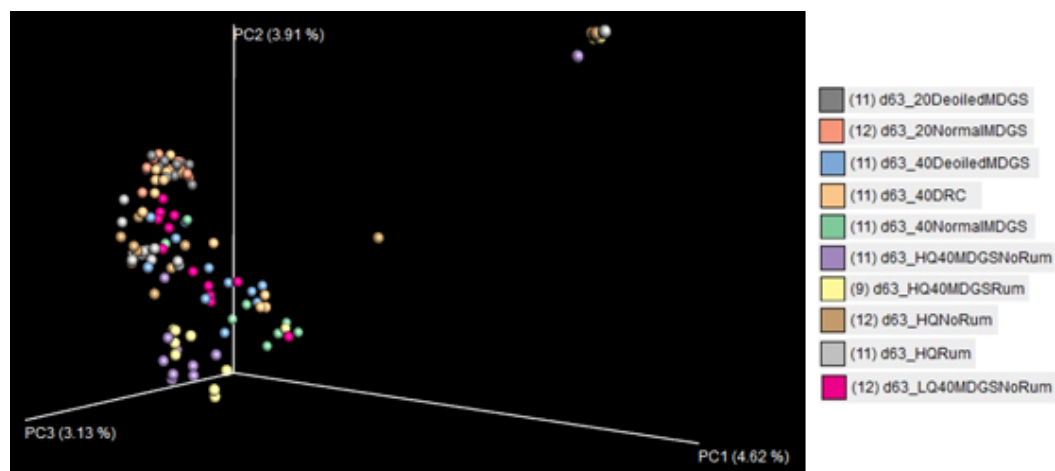


Figure 8. Principle Coordinate Analysis of the bovine bacterial community structuring 63 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows all ten diets after 63 d on the treatments with clustering apparent due to high and low quality diets.

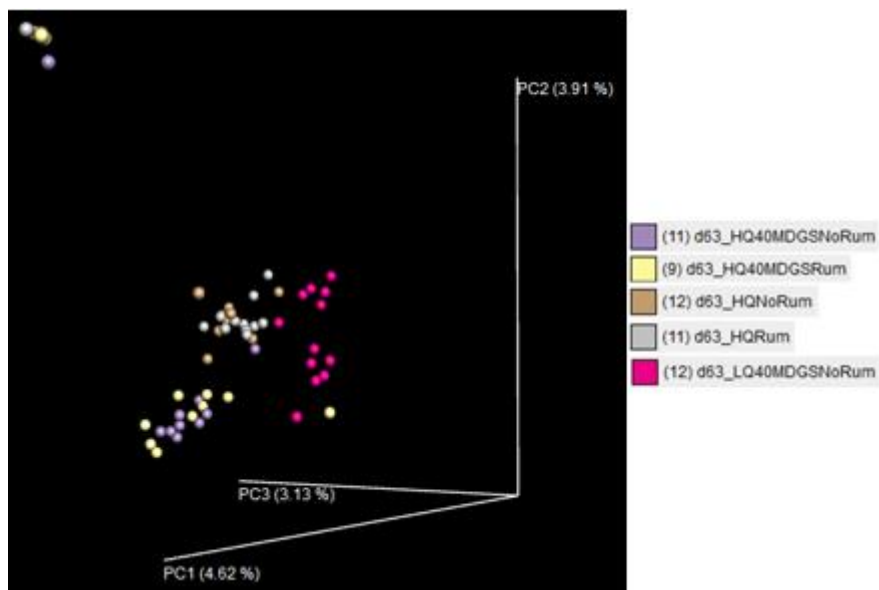


Figure 9. Principle Coordinate Analysis of the bovine bacterial structure 63 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to high and low quality forage and either 0 or 40% MDGS supplementation with and without Rumensin®.

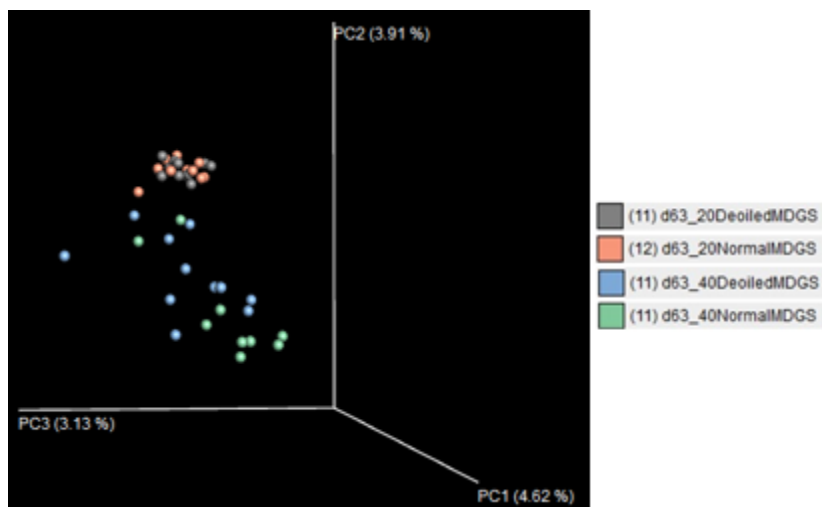


Figure 10. Principle Coordinate Analysis of the bovine bacterial structure 63 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to level of MDGS supplementation of either 20 or 40% inclusion with low quality forage.

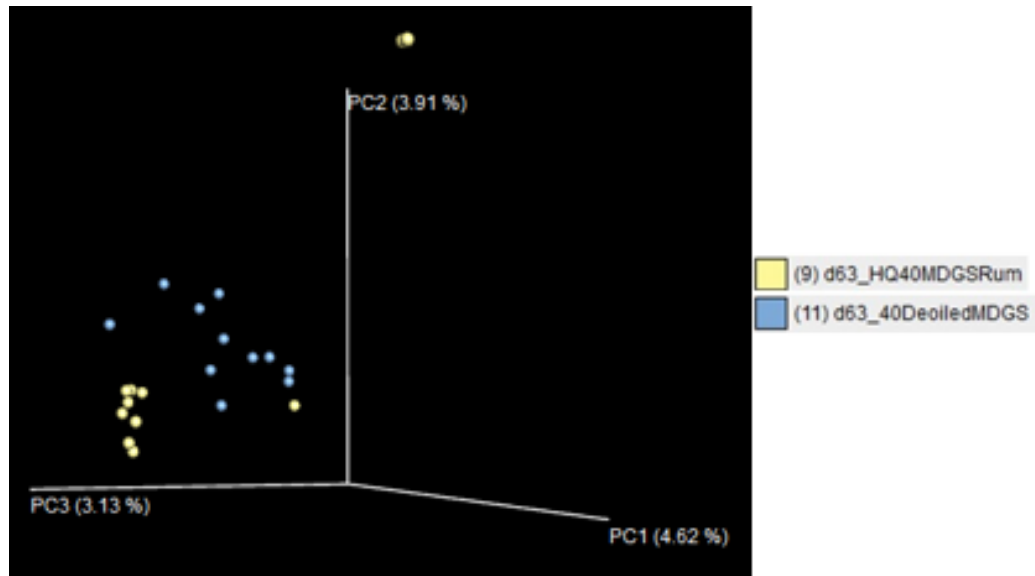


Figure 11. Principle Coordinate Analysis of the bovine bacterial structure 63 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. This figure shows clustering between high and low quality forage at 40% MDGS with Rumensin® inclusions.

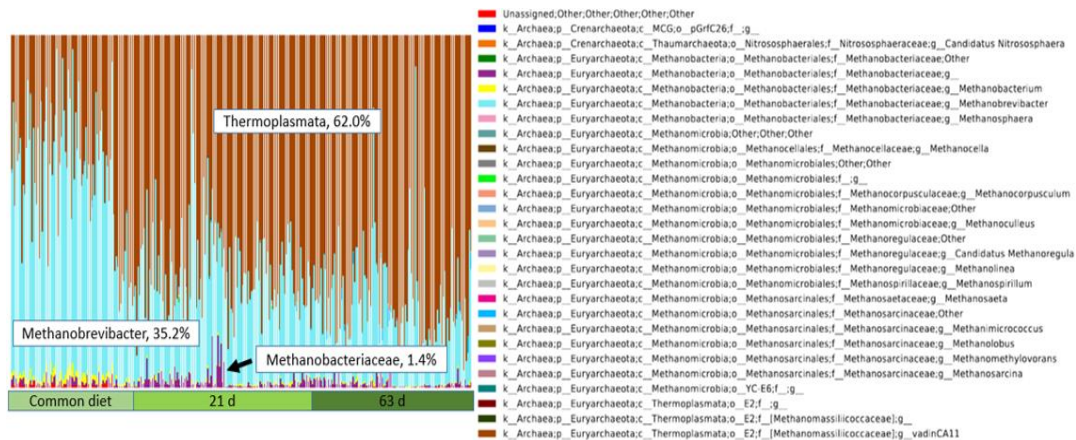


Figure 12. Bovine archaeal community taxonomic distribution at the genus level with the abundance of the top three genera present.

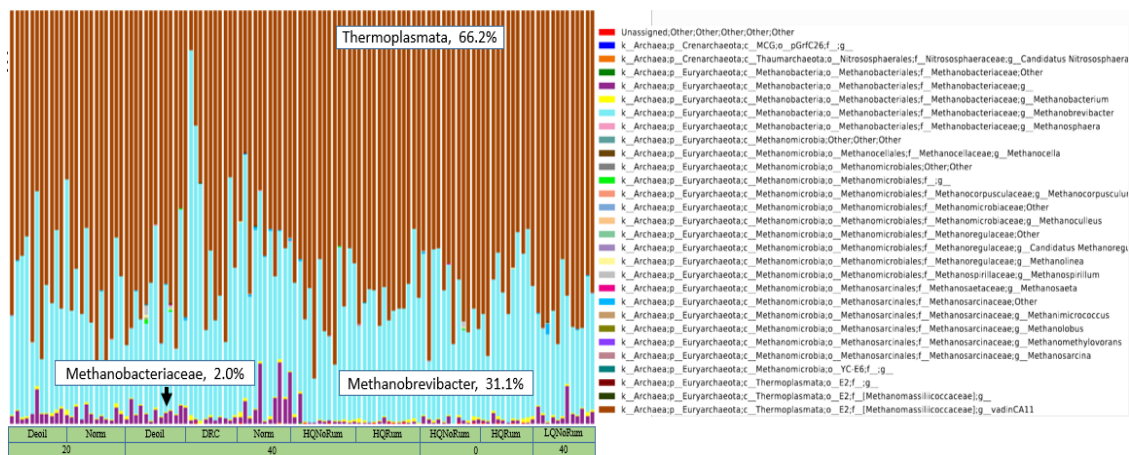


Figure 13a. Bovine ruminal archaeal community taxonomy at the genus level with the corresponding abundance 21 d on the study.

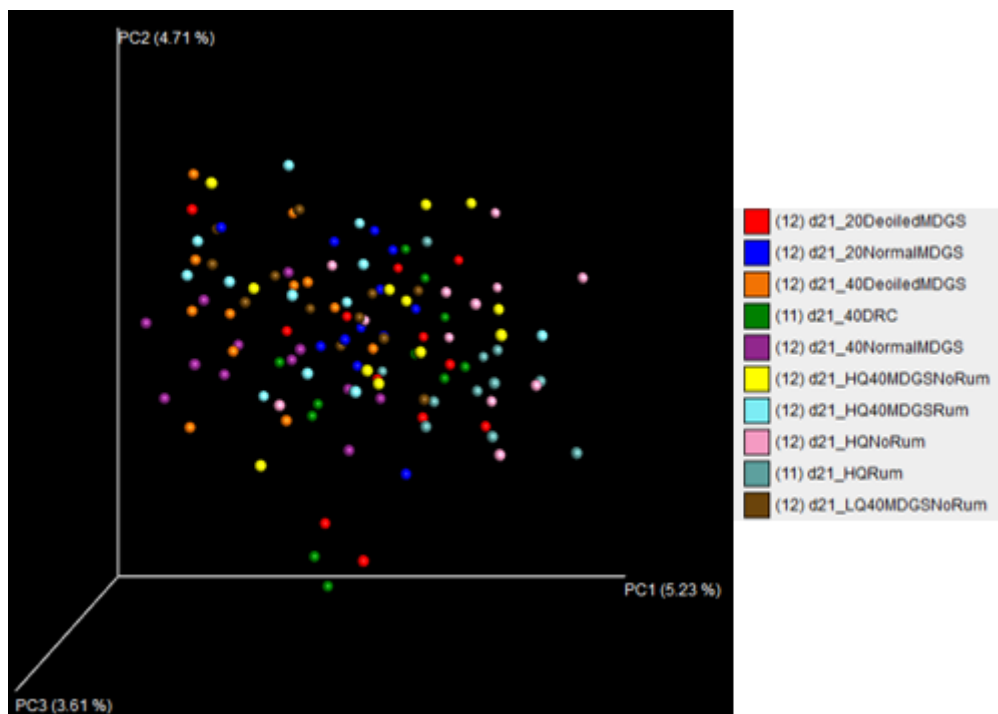


Figure 14. Principle Coordinate Analysis of the bovine archaeal community clustering 21 d on study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows all ten diets clustering together after 21 d.

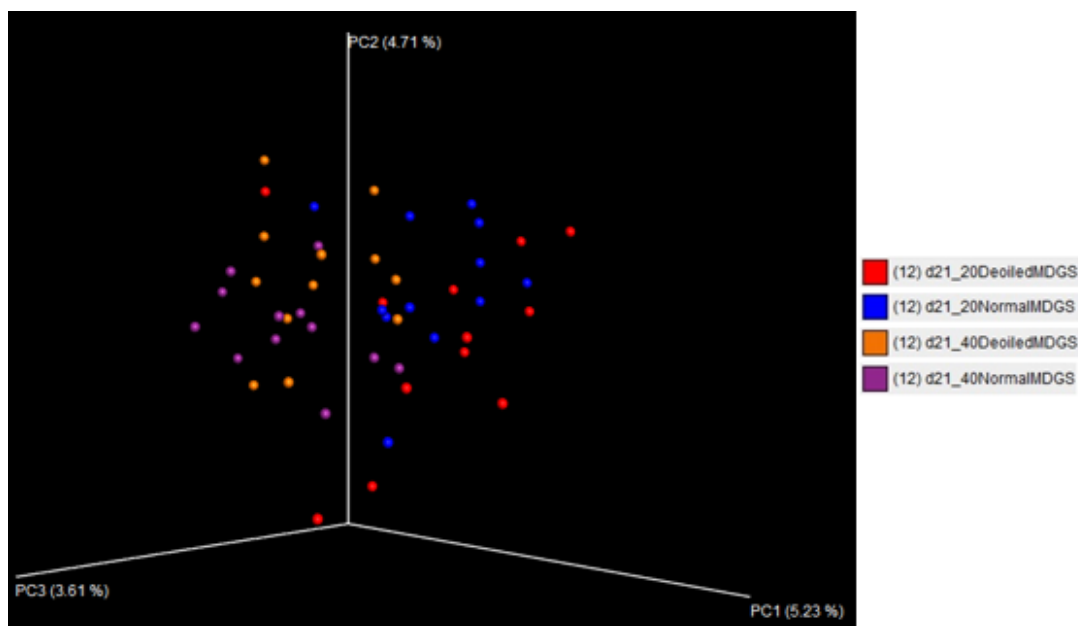


Figure 15. Principle Coordinate Analysis of the bovine archaeal structure 21 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to 20% MDGS supplementation separate from 40% MDGS supplementation.

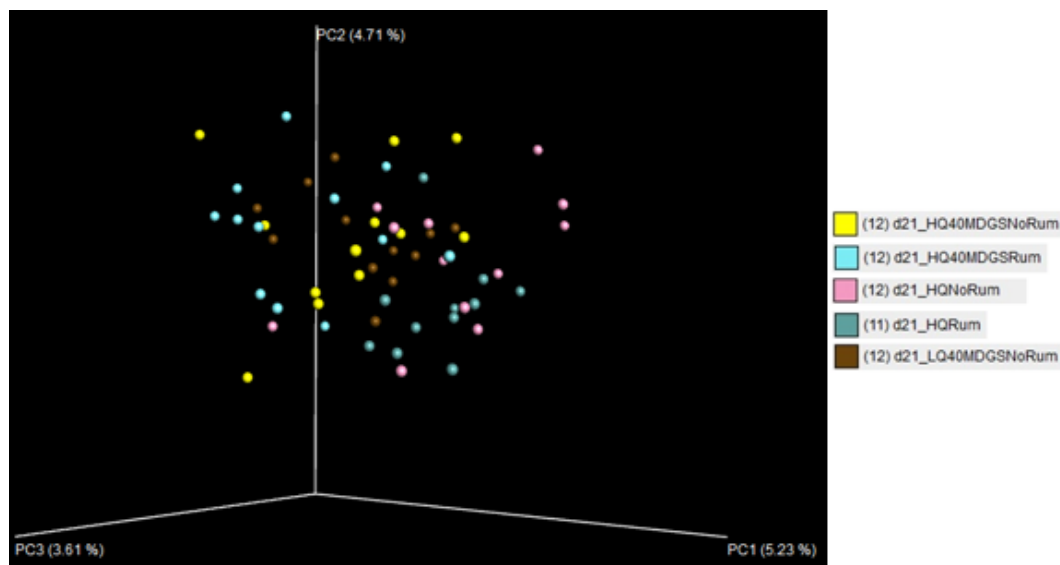


Figure 16. Bovine archaeal structure 21 d on the study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering due to high quality forage at 0 and 40% MDGS supplementation with and without Rumensin® and high and low quality forage with 40% MDGS without Rumensin®.

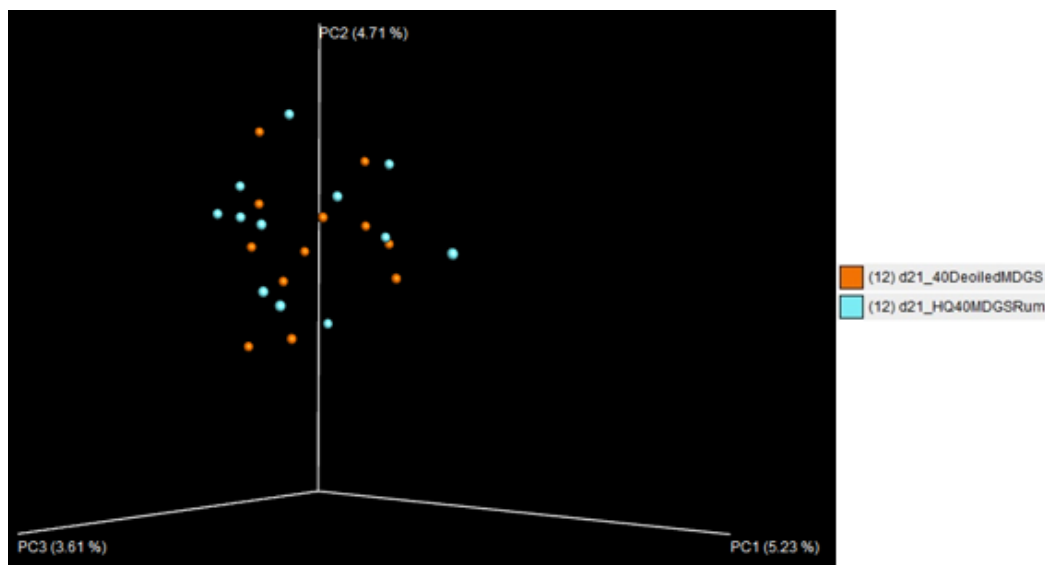


Figure 17. Principle Coordinate Analysis of the bovine archaeal structure 21 d on the study consisting of Low Quality 40% Deoiled and High Quality 40% Deoiled Modified Distillers Grains plus Solubles (MDGS) with Rumensin®. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (21 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows no difference in clustering due to high and low quality forage with 40% MDGS with Rumensin supplementation®.

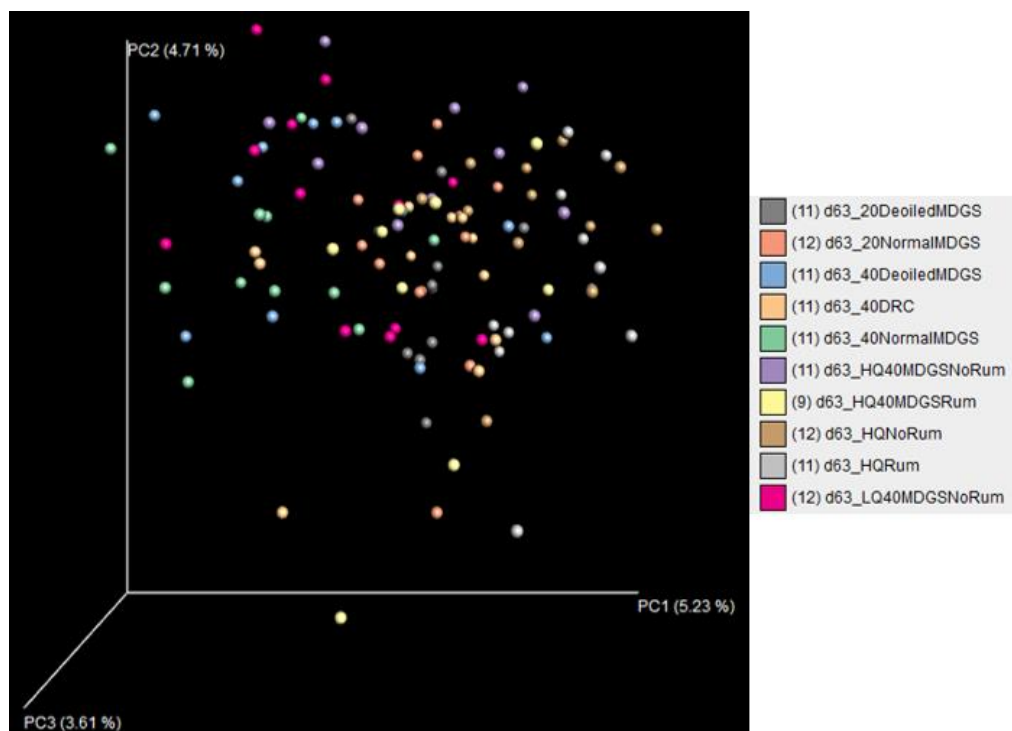


Figure 18. Principle Coordinate Analysis of the bovine archaeal community clustering 63 d on study. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows all ten diets clustering together after 63 d.

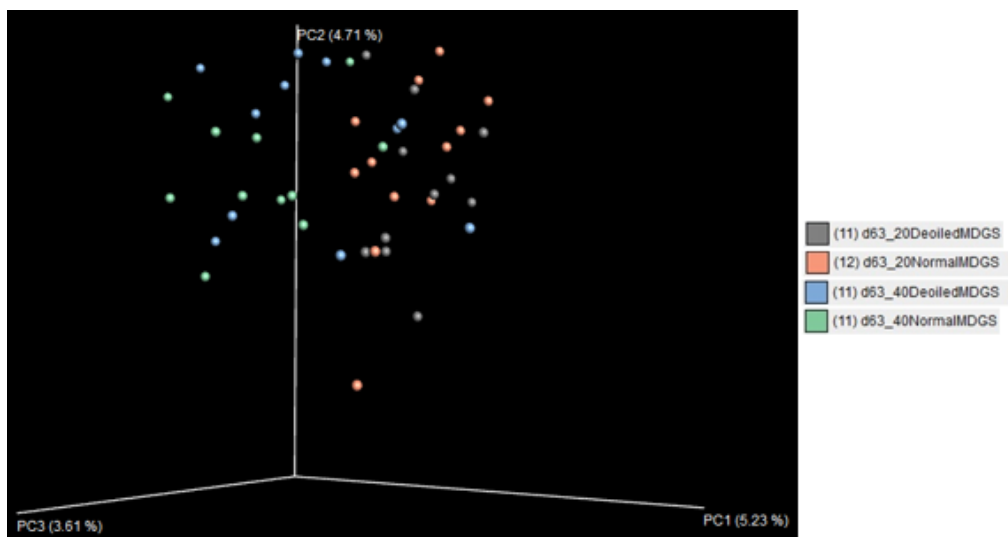


Figure 19. Principle Coordinate Analysis of the bovine archaeal structure 63 d on the study consisting of Low Quality 20% and 40% Deoiled and Normal Modified Distillers Grains plus Solubles (MDGS) with Rumensin®. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows no clustering between 20 and 40% MDGS.

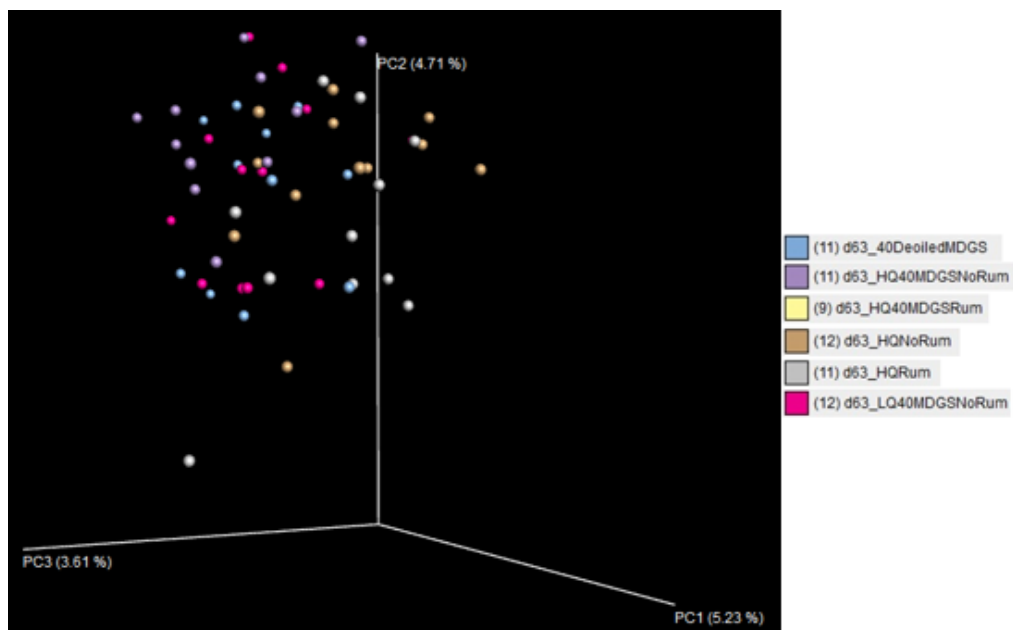


Figure 20. Principle Coordinate Analysis of the bovine archaeal structure 63 d on the study consisting of High and Low Quality 40% Modified Distillers Grains plus Solubles (MDGS) with and without Rumensin® and High Quality with and without Rumensin®. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows no clustering between high and low quality forage with 0 or 40% MDGS with and without Rumensin supplementation®.

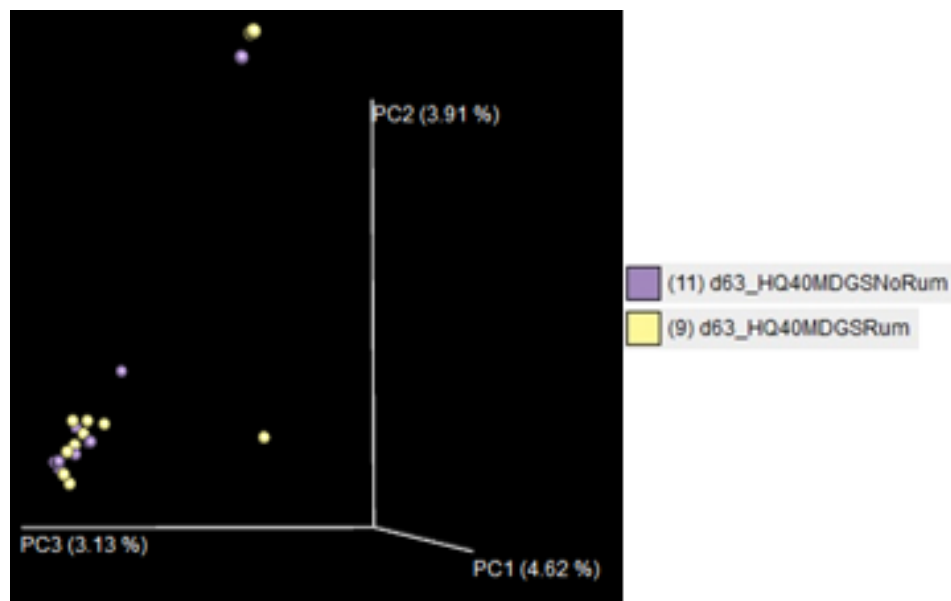


Figure 21. Principle Coordinate Analysis of the bovine archaeal structure 63 d on the study consisting of High Quality 40% Modified Distillers Grains plus Solubles (MDGS) with and without Rumensin®. The number in parentheses refers to how many animals are within each diet. The “d_” refers to day (63 d), referencing time point. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering between high quality forage with 40% MDGS supplementation with and without Rumensin®.

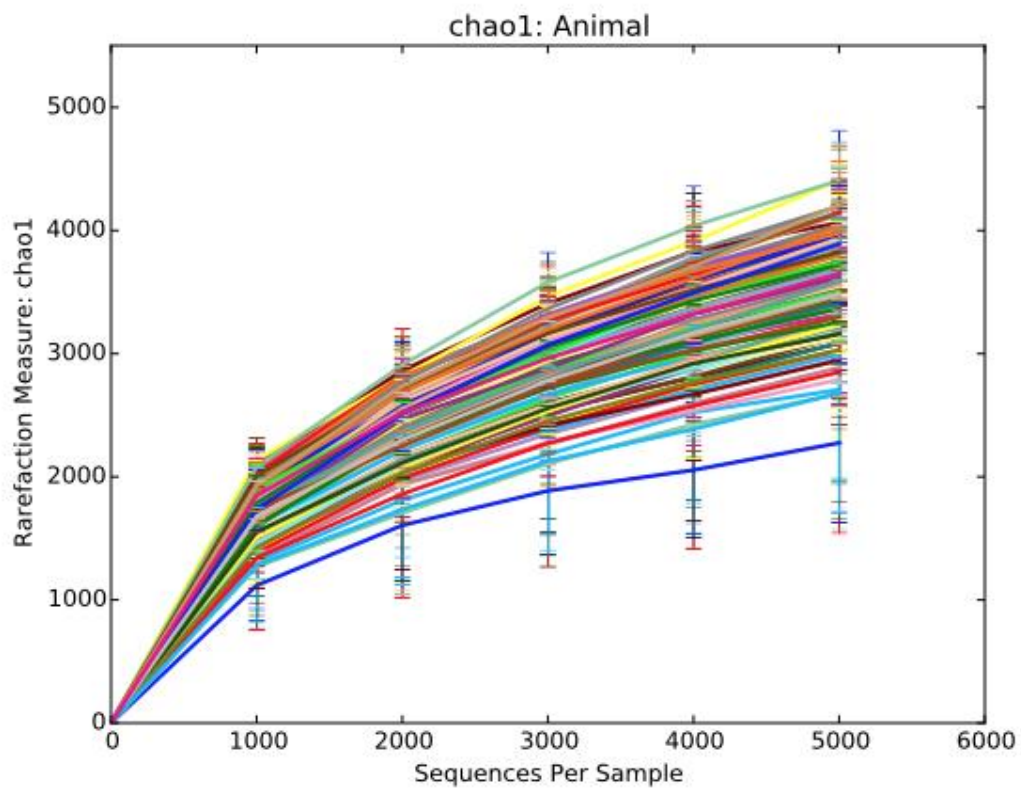


Figure 22. Bacterial alpha rarefaction curve for sequencing depth verification (5153 sequences).

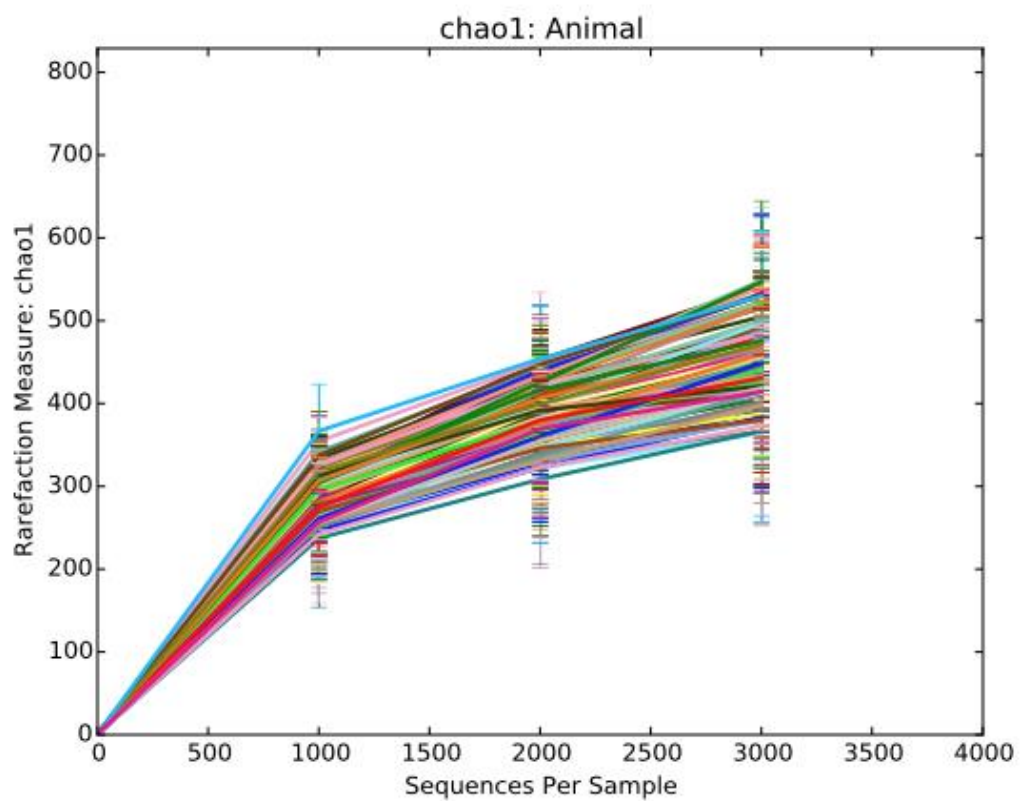


Figure 23. Archaeal alpha rarefaction curve for sequencing depth verification (3055 sequences).

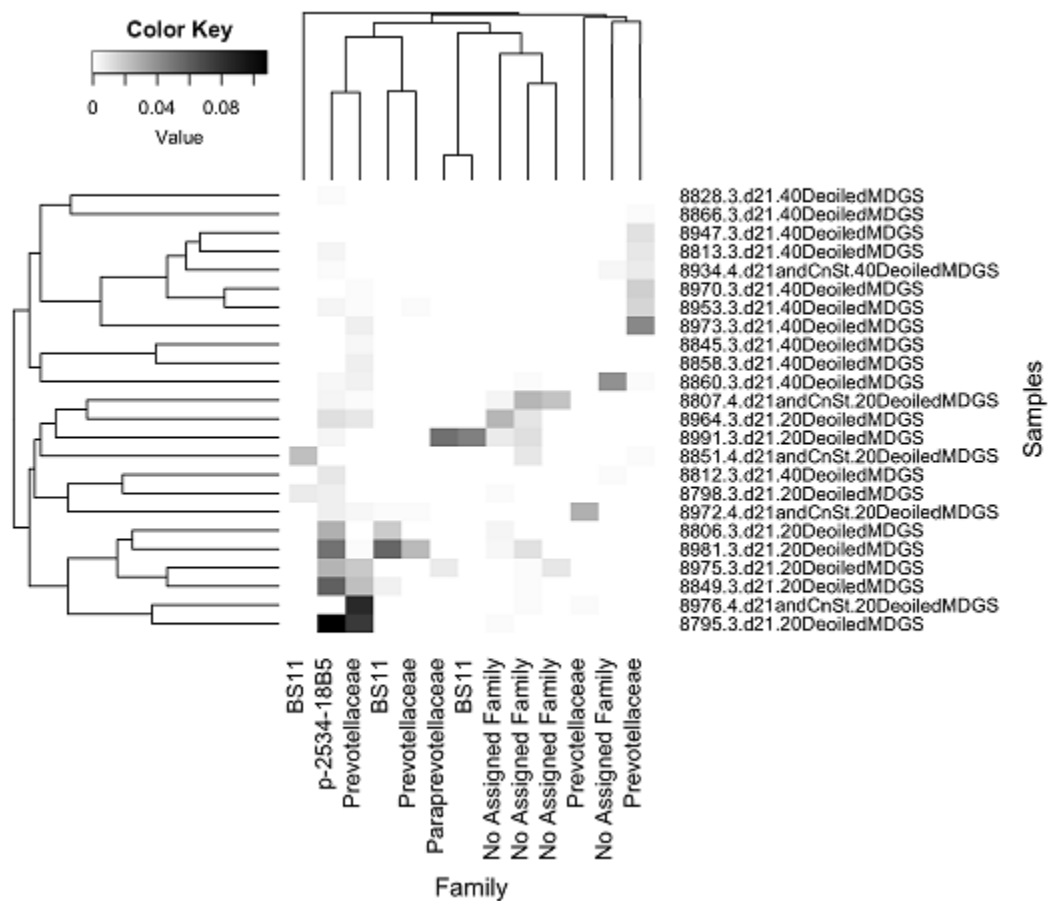


Figure 24. Bacterial heatmap of 20 v 40 Deoiled MDGS at 2.0% on d 21. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

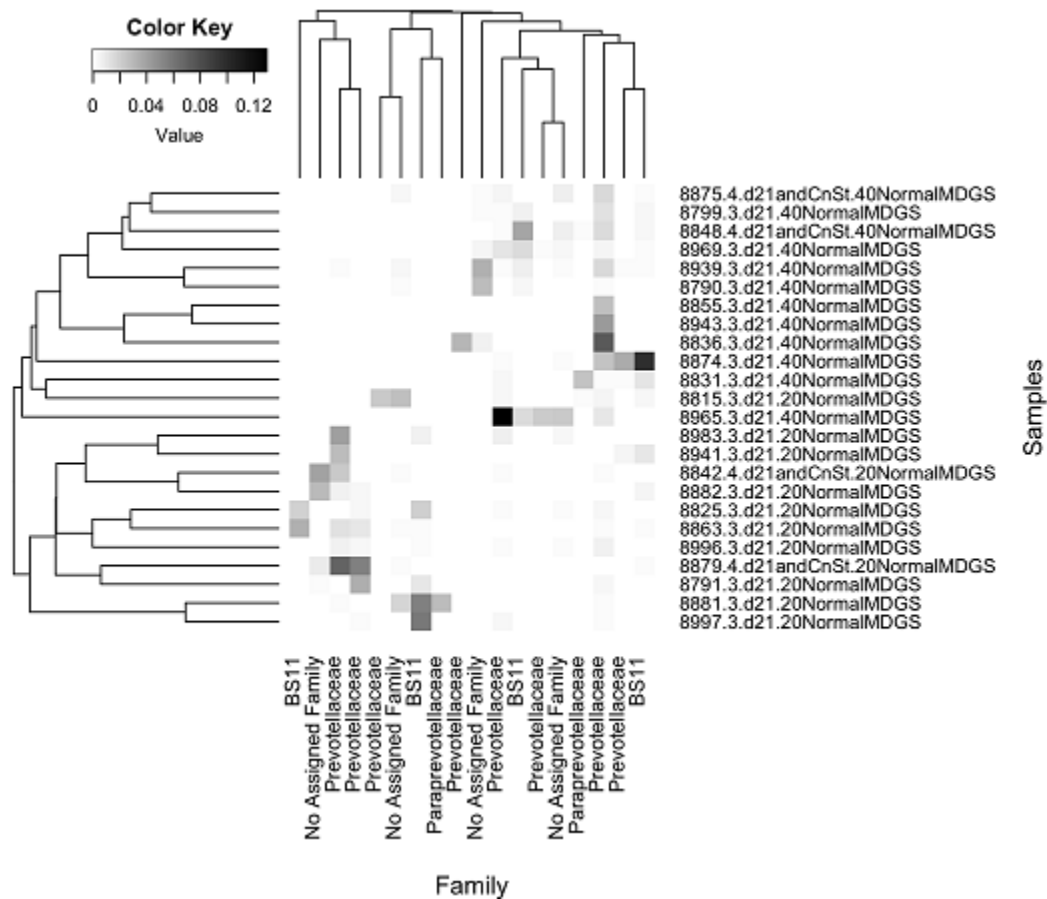


Figure 25. Bacterial heatmap of 20 v 40 Normal MDGS at 2.0% on d 21. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

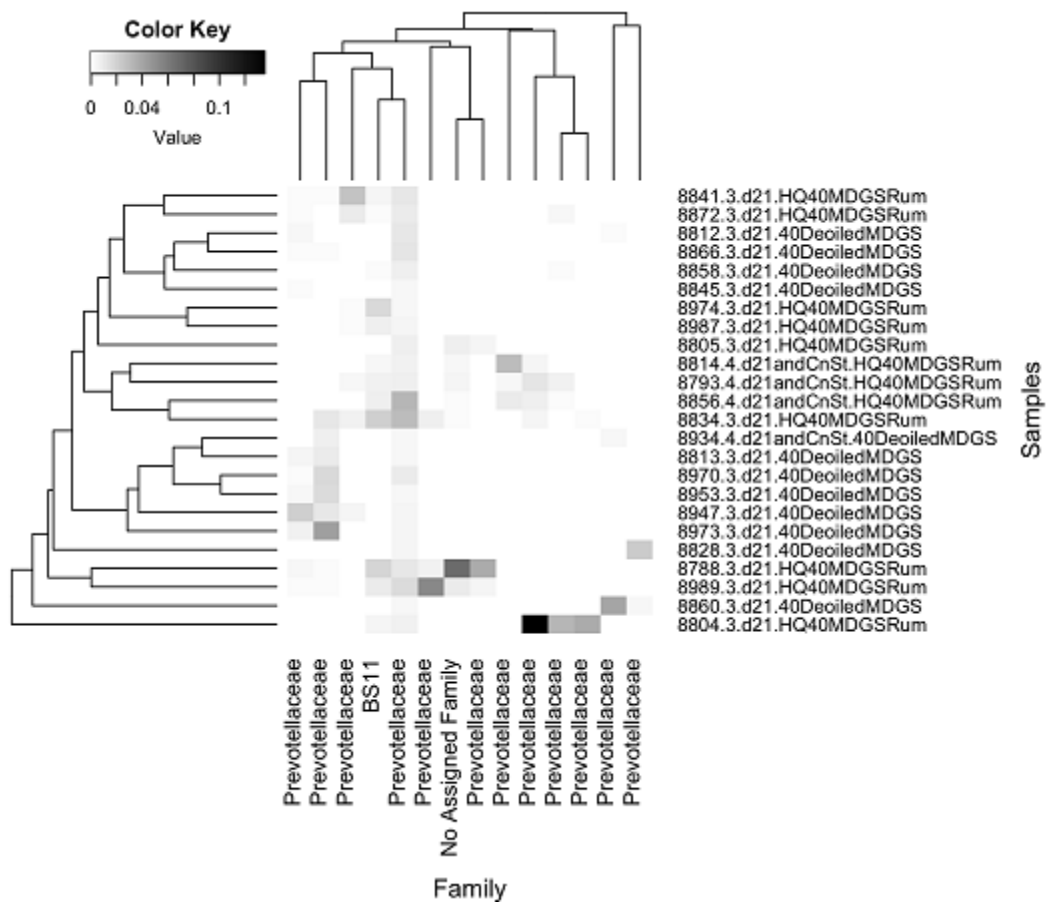


Figure 26. Bacteria heatmap of HQ40MDGSRum v 40 Deoiled MDGS at 2.0% on d 21. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

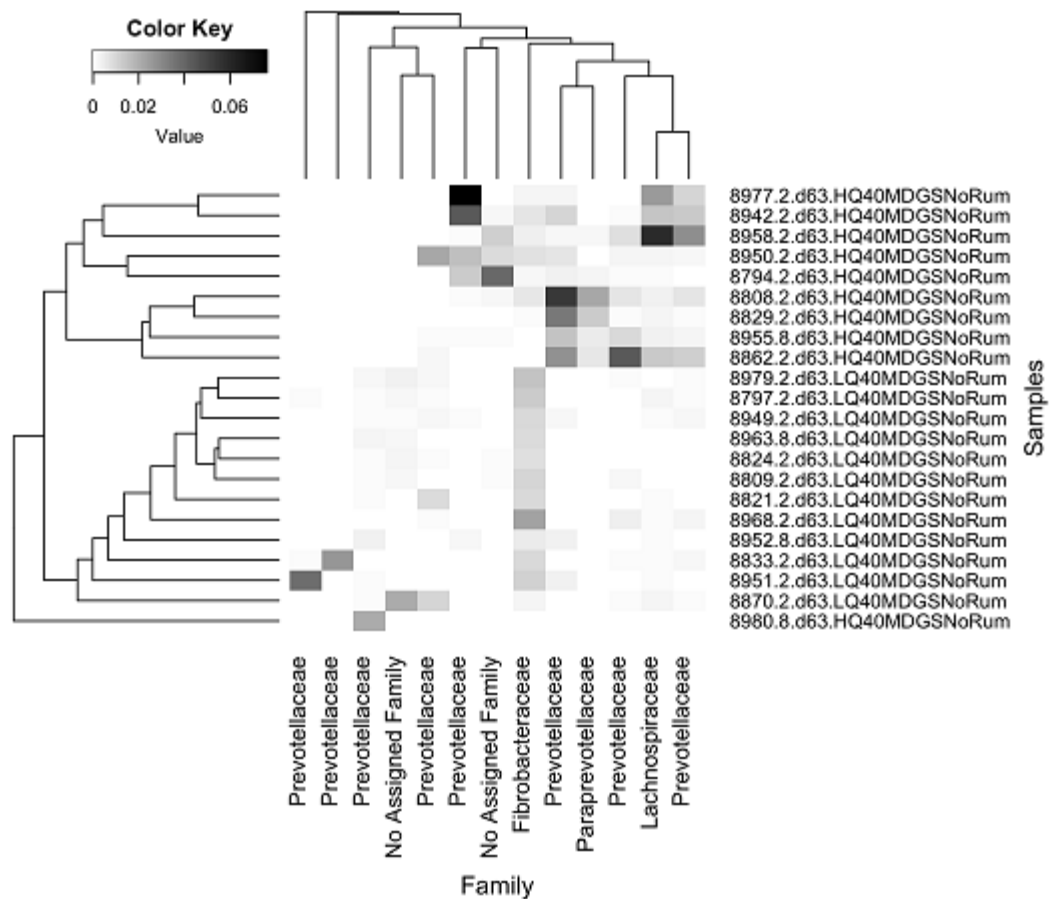


Figure 27. Bacteria heatmap of HQ40MDGSNoRum v LQ40MDGSNoRum at 2.0% on d 63. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

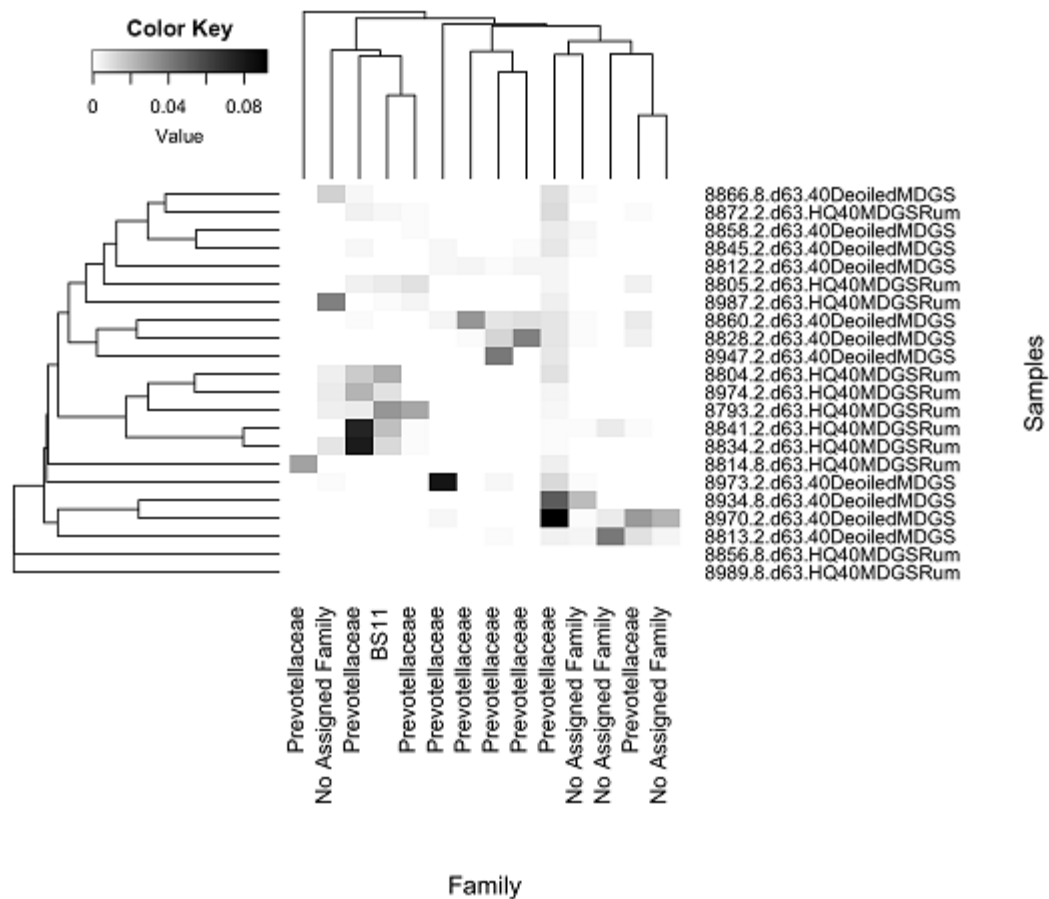


Figure 28. Bacteria heatmap of HQ40MDGSRum v 40 Deoiled MDGS at 2.0% on d 63. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

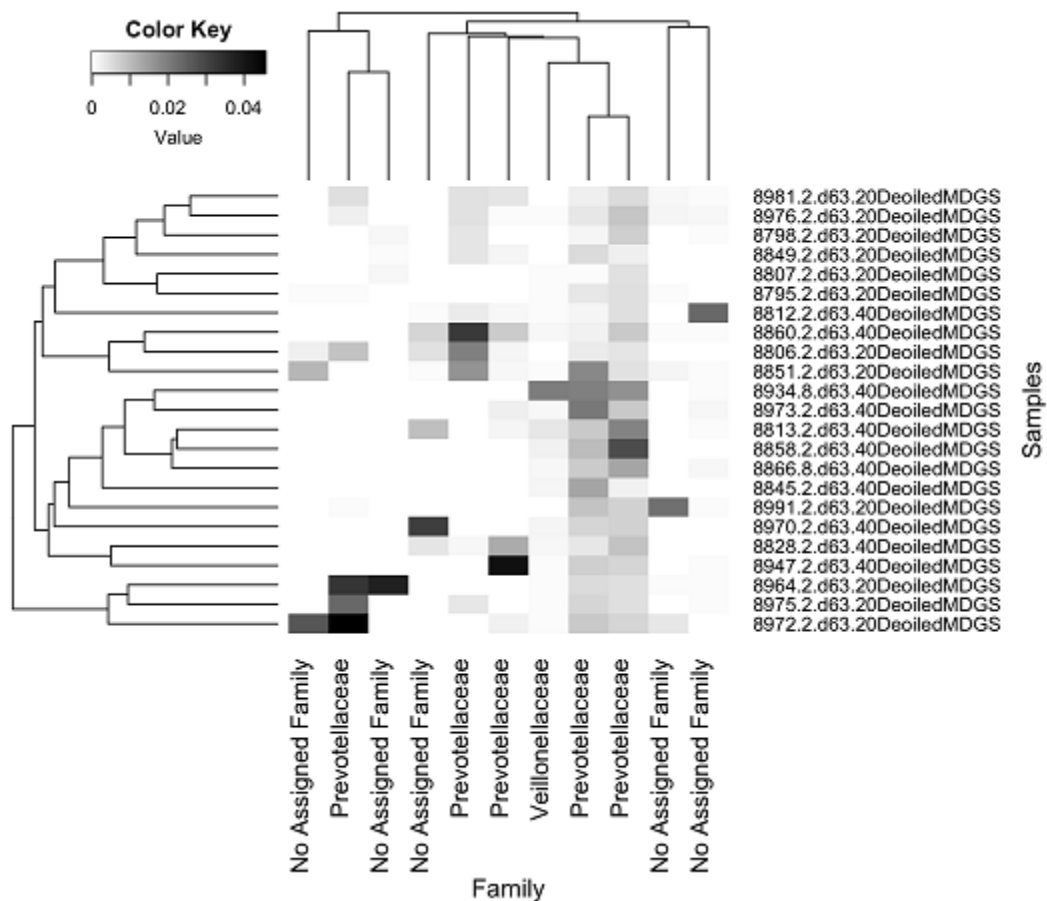


Figure 29. Bacteria heatmap of 20 v 40 Deoiled MDGS at 2.0% on d 63. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

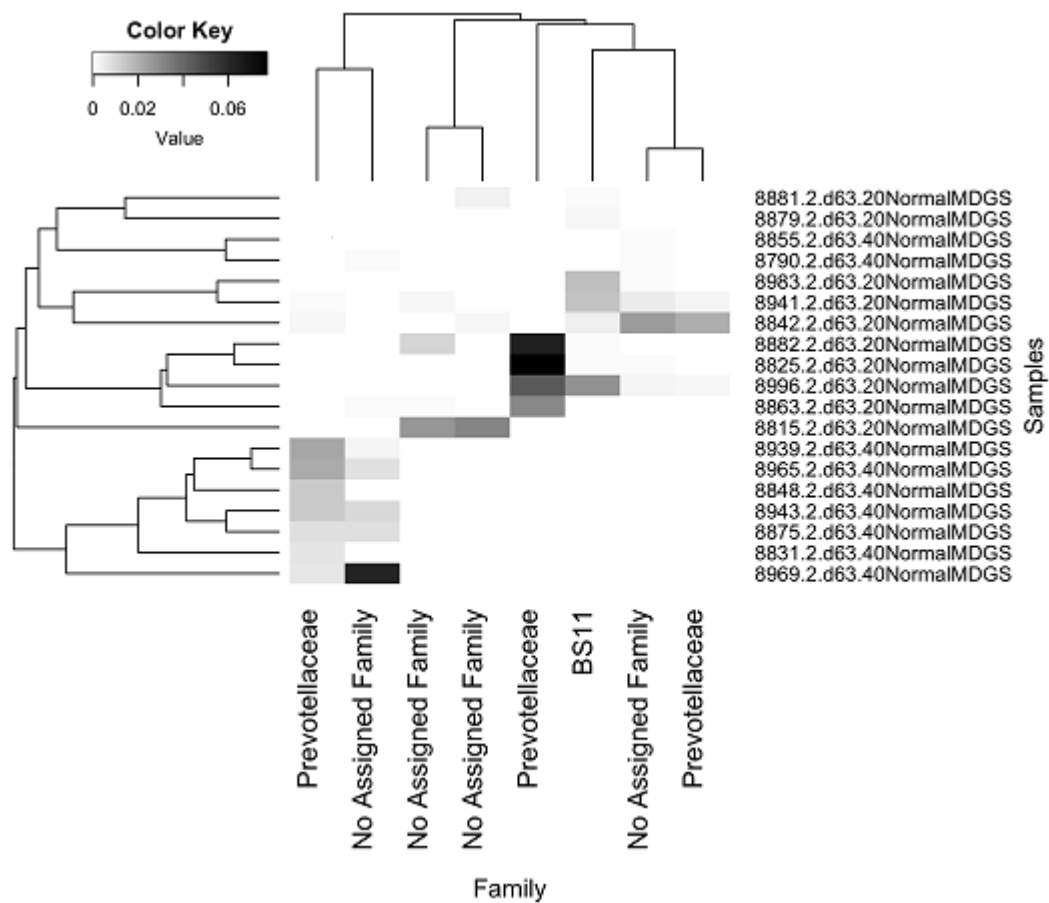


Figure 30. Bacteria heatmap of 20 v 40 Normal MDGS at 2.0% on d 63. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

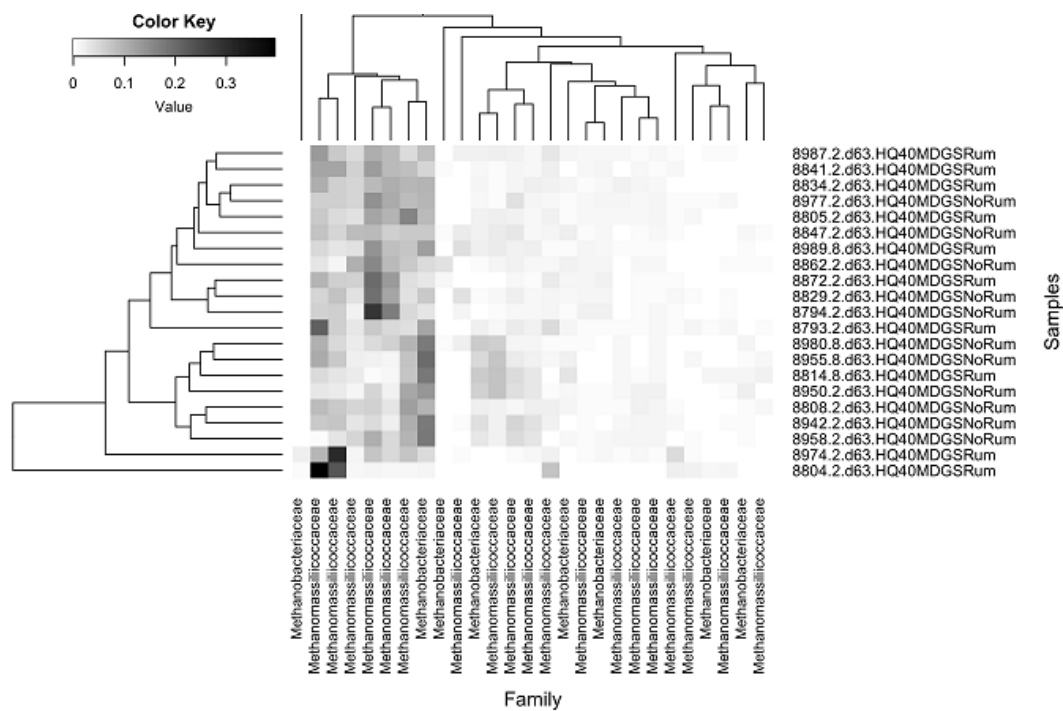


Figure 32. Archaeal heatmap of HQ40MDGSNoRum v HQ40MDGSRum at 1.5% on d 63. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

CHAPTER 3

The effect of fat source supplementation on microbial community composition and reflection upon methane emissions in finishing cattle

Abstract

At the heart of enteric methane production in ruminants is a microbial food chain that is greatly influenced by diet. The interactions between diet, microbial community composition and methane emissions are poorly understood. To evaluate the influence of diet on microbial community composition and methane emission, 120 animals were fed six finishing diets consisting of different fat sources (corn oil, tallow, and distillers) with and without monensin supplementation. Microbial community composition and methane emissions were monitored. Rumen contents were collected via esophageal tubing for microbial community analysis. The V3 and V6 region of the 16S rRNA gene was sequenced to evaluate bacteria and archaea community structure respectively using the Ion Torrent personal genome machine (PGM) at a depth of 5153 sequences per sample. Community structure varied slightly due to diets however, the composition of the communities, bacteria and archaea respectively, showed little change. No effect on methane production was observed due to diet. The diets imposed on this study do not provide evidence for potential dietary intervention strategies to mitigate methane in finishing cattle.

Introduction

The increase in greenhouse gases (GHGs) is a current research focus. Potent GHGs include nitrous oxide (N₂O), carbon dioxide (CO₂), ozone (O₃), and the focus of this research, methane (CH₄) that are of particular concern. Methane is the second most predominant GHG released in the United States from anthropogenic sources (USEPA, 2016), and is capable of absorbing and re-emitting infrared radiation contributing to the Global Warming effect. The Global Warming Potential (GWP) of methane is at least 28

times more, with a 12 year lifetime atmospheric presence, compared to carbon dioxide, which has GWP of 1, with no definite lifespan (Attwood et al., 2011; Moss et al., 2000; USEPA, 2016).

Methane is produced in various environments, ranging from oceanic thermal vents, swamps, rice paddies, and gastrointestinal tracts of termites and other animals (Hedderich and Whitman, 2006; Thauer et al., 2008). In addition to these environments, the ruminant animal is an anthropogenic source of methane production (Attwood et al., 2011; Buddle et al., 2011; Hook et al., 2010). The unique and advantageous niche of ruminants is the ability to consume cellulose-rich polysaccharides (Buddle et al., 2011) for energy. However, as a by-product of fermentation, ruminants produce large amounts of methane.

This methane production is attributed to a select group of rumen microorganisms called methanogens that belong to the domain *Archaea* (Hook et al., 2010).

Methanogenesis by this unique group of microbes is a product of normal enteric fermentation in the process of recycling energy substrates and maintaining rumen function (Attwood et al., 2011; Hook et al., 2010; McAllister and Newbold, 2008; Moss et al., 2000).

Methane mitigation strategies utilizing dietary intervention have been widely explored (Beauchemin et al., 2007; Buddle et al., 2011; Johnson and Johnson, 1995; Hook et al., 2010; McAllister and Newbold, 2008; and Monteny et al., 2006). Boadi et al. (2004) showed extensively that lipid supplementation of cattle diets can reduce methane emissions. Lipids have been shown to be toxic to methanogens and if unsaturated would be a H₂ sink competing with methanogens for H₂ (Poulsen et al.,

2013). Ionophore supplementations have also been utilized as a tool for decreasing methanogenesis as well as boosting performance (Schelling, 1984; Wallace et al., 1980). However, the utilization of monensin to reduce methane may be short lived (Johnson and Johnson, 1995). Corn by-products have also been solicited for incorporation into diets for its nutrient composition and its potential for lowering methane production in finishing cattle. All of these methane abatement strategies by nutritional intervention influence the rumen microbial community structure and composition, which are the direct drivers of methanogenesis in ruminants. However, most studies have failed to evaluate the microbial community composition within the rumen during dietary intervention to reduce methane emissions.

Many studies have demonstrated that diet effects microbial community composition of the rumen (Fernando et al., 2010; Hook et al., 2010; Johnson and Johnson, 1995; McAllister et al., 1996); however, studies evaluating the microbial community composition have failed to simultaneously measure methane production to evaluate impact of diet on microbial community structure and vice versa. In this study, 16S rRNA gene amplicon sequencing was utilized to observe the changes in rumen microbial community under various widely used finishing diets with fat supplementation with simultaneous methane sampling (Pesta et al., 2015) to better understand the interactions between diet, microbial community composition, and methane emissions from finishing cattle.

Experimental Methods

A 125 d finishing study was conducted utilizing sixty crossbred steers with an initial body weight of 300 kg \pm 25 kg. The animals were individually fed in a Calan® gate semi-confinement barn at the UNL Agriculture Research and Development Center (ARDC) near Mead, NE. At the initiation of the study, the steers were placed on a basal diet (common diet) consisting of 50:50 blend of Alfalfa and Sweet Bran® for 21 days to establish a similar community between the steers and limit animal to animal variation (Watson et al., 2013). The steers were assigned randomly to one of six treatment diets (Table 1) with 10 steers per treatment. The diets were formulated to evaluate lipid source and type of carbohydrate on methane production in finishing cattle in a completely randomized design with an additional 2 \times 2 factorial as described by Pesta et al. (2015) (APPENDIX II). All animal procedures were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Rumen sampling and DNA isolation

Sampling was performed on days 0 (basal diet) and day 55 (both samplings occurred prior to feeding). A representative sample of rumen contents (solid particles and rumen fluid) was collected by esophageal tubing. To ensure collection of a representative sample, the particles retained on the filter were added to the collection tube. The samples collected were snap frozen in liquid nitrogen and placed in a -80°C until used for DNA extraction.

DNA was extracted from 1 - 2 g of rumen contents using the MoBio PowerMag™ Soil DNA Isolation Kit (Optimized for KingFisher® Flex protocol) (MoBio Laboratories, Carlsbad, CA) according to the manufacture's protocol with the following modifications: approximately 1 - 2 g of raw sample was added to a sterile 2.0 mL Safe-Lock tube

(Eppendorf, North America, Inc. USA) with 0.5 g of acid washed beads (Scientific Asset Management, Basking Ridge, NJ); between the two rounds of bead beating, the samples were placed in a $> 85^{\circ}\text{C}$ water bath for 5-8 min. The samples were centrifuged (4,500 x G) and then the supernatant was transferred into sterile 1.5 mL tubes (Fisherbrand, Fisher Scientific, USA). Lastly, 130 μL of elution buffer was used to elute the DNA. Quality of the DNA was evaluated using gel electrophoresis and was stored at -20°C until used for community analysis.

16S rRNA library preparation and sequencing of the V3 Bacteria and V6 Archaea regions

Eubacterial 16S rRNA library prep

The V3 region of the 16S rRNA gene was amplified using extracted total rumen DNA using universal eubacterial 16S primers 341F and 518R as described by Whiteley et al. (2012). The V3 region of the 16S rRNA gene was amplified in a 15 μL reaction volume. A PCR reaction consisted of 1X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life Technologies™, Massachusetts, USA), 1.7 μM of 341F and 0.2 μM of 518R primer, approx. 50 ng of extracted total DNA. Quantitative PCR (qPCR) conditions for amplification of the 16S rRNA gene included: 95°C for 10 min for initial denaturation; followed by 25 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s, with a dissociation curve following the amplification. Following amplification, 5 μL of amplicon product was run on a 1.8 % agarose gel using gel electrophoresis (QD LE Agarose, Green Bio Research, Baton Rouge, LA) at 120 V for 55 minutes for size verification and to ensure amplification. PCR products were normalized using the Invitrogen Sequal Prep™ Normalization Plate kit (Frederick, Maryland) to 1 – 2 ng/ μL

according to manufacturer's protocol and was pooled. Library qPCR preparation, normalization, and pooling was conducted using the Eppendorf epMotion (M5073, Germany). The pooled library, 300-500 μL , was column purified using PCR cleanup procedure (DNA, RNA, and protein purification Clontech Laboratories, Inc, California) as described by the manufacturer with the modification of eluting into 40 μL . The purified concentrated libraries were size selected using the Pippin Prep (Sage Science, Inc., USA) to remove any spurious PCR fragments. Finally, the PCR product size and quantity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using High-Sensitivity DNA chips. Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) according to the manufacturer's protocol with emPCR, bead deposition and sequencing was performed as described by the manufacturer.

Archaea 16S rRNA library prep

The V6 region of the 16S rRNA gene was amplified using extracted total rumen DNA using universal archaeal specific primers 751F and 934R (Whiteley et al., 2012). The primers were synthesized to have adapters and barcodes as described by Whiteley et al. (2012). The V6 region of the 16S rRNA gene was amplified in a 20 μL volume. The PCR reaction contained, X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life Technologies™, Massachusetts, USA). Each reaction contained, 1.25 μM 751F and 0.15 μM 934R primer, approx. 50 ng of extracted total DNA. Quantitative PCR (qPCR) conditions for amplification of the 16S rRNA gene included: 95°C for 10 min for initial denaturation; followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s, with a dissociation curve following the amplification. Following

amplification, the product was run on a 1.8 % agarose gel using gel electrophoresis (QD LE Agarose, Green Bio Research, Baton Rouge, LA) at 120 V for 55 minutes for initial size verification and to ensure amplification. Following amplification, a 0.6X SPRI was conducted according to manufactures protocol (Agencourt® AMPure®) to remove primer dimers. SPRI products were normalized using Invitrogen Sequal Prep™ Normalization Plate kit (Frederick, Maryland) to 1 – 2 ng/ according to the manufacturer’s protocol and pooled. Library qPCR preparation, normalization, and pooling was conducted using the Eppendorf epMotion (M5073, Germany). The pooled library, 300-500 µL, was column purified using PCR cleanup procedure (DNA, RNA, and protein purification Clontech Laboratories, Inc, California) as described by the manufacturer. Size select elution of libraries was conducted by using the Pippin Prep (Sage Science, Inc., USA). Product size and quantity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using its High-Sensitivity DNA chips. Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM) according to manufacturer’s protocol with emPCR, bead deposition and sequencing was performed as described by the manufacturer.

Microbial community analysis

The .fastq file that is generated from the PGM was converted into a .fasta file and were de-multiplexed utilizing the barcode on the reverse primer and the mapping file utilizing the platform Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Raw reads from Ion Torrent PGM sequencing were first analyzed for quality (Anderson et al., 2015). Briefly, reads were removed if 1) an incomplete forward primer sequence was present, 2) barcode was not identified, 3) sequence length was less than

100 or greater than 250 nucleotides, and 4) if ambiguous bases (“N”) were present. Additional quality control checks included removing sequences with one or more errors within the forward primer, two or more errors in the reverse primer, and two or more errors in the barcode which were performed using Qiime (ver.1.9.1) (Caporaso, et al., 2010). After this primary quality control, reads that met these requirements were concatenated into a single file (one for bacteria and one for archaea). Reverse primers were removed. Resulting sequences were further processed using Mothur (Schloss et al., 2009) and the FASTX-TOOLKIT to remove and trim to a fixed length of 130 bp for bacteria and 140bp for archaea to improve OTU classification (Edgar, 2013). The sequences were reverse complemented in Mothur (Schloss et al., 2009). Utilizing a custom pipeline within the Fernando Lab, chimera identification and removal, and OTU picking based on 97% sequence similarity. Sequences less than 96% are considered phylogenetically a different species. This was conducted using UPARSE composed by Edgar (2013) using a batch script. Taxonomic classification was determined using Qiime using the GreenGenes database (ver. 13_8). The OTU sequences generated were aligned using Ribosomal Database Project (<https://pyro.cme.msu.edu>). OTUs aligning outside the 16S gene were eliminated. The phylum Cyanobacteria were removed from the OTU table as it is a photosynthetic phylum and the rumen environment is anaerobic and is dark. The cyanobacterial reads are most likely a result of the 16S copies present in the chloroplast of the forage portion of the diet. Subsequent analyses were conducted separately on the bacteria and the archaea samples, however the same steps occurred in both bacteria and archaeal analyses, as different primers were utilized to sequence different regions but each set contained all diets. Singletons OTUs were eliminated as a

single sequence may have been generated due to sequencing error, even if the single read is real, the abundance will have little biological meaning. Scripts can be found in Appendix 1.

Statistical analysis

Total reads from each sample were subsampled to the sample with the lowest number of reads to achieve an equal sampling depth rarefaction (bacteria, 5153) and (archaea, 3055). Global bacterial and archaea community composition changes was evaluated using the unweighted unifracs distance matrices (Lozupone et al., 2011). To evaluate the effect of diet on bacterial and Archaeal community structuring, 2 way Non-Parametric MANOVA test was utilized, where diet was used as a main effect and animal was used as a random effect (MatLab, 2015). *P*-values of < 0.05 were considered significant. Pairwise tests were conducted on a one way comparison using R (ver. 3.2.1) to identify diets that resulted in significant changes in community composition. Principle coordinate analyses were performed to visualize structuring of eubacterial and archaeal community shifts (Qiime, ver. 1.9.1). Each dot within the plots represents a community from an animal. It is generated based on the factors of phylogenetic relationships and abundance. Fluctuations in OTU (Operational Taxonomic Unit) abundances were identified using the `differential_abundance.py` command within Qiime (1.9.1), choosing the *P* adjusted values. The sequences were rarefied (bacteria, 5153 and archaea, 3055) and used for calculation of diversity using the Chao1 index and to generate rarefaction curves (Kuczynski et al., 2011). To visually observe shifts in the community, principle coordinate analyses was performed utilizing the unweighted UniFrac distances from

subsampled OTU tables (Lozupone et al., 2011). Scripts and procedures used for analysis are shown in Appendix 1.

Heatmaps were created to visualize significantly differential OTUs using R heatmap.2 function (Ploner et al., 2014) with the OTU relative abundance as input. Bray-Curtis dissimilarity matrix was used to estimate the distance between samples and dendrograms were created by hierarchical clustering of OTUs and samples.

Results

The rumen microbial community composition is poorly characterized when identifying methane mitigation strategies. The ability to identify microbial community structure while simultaneously measuring methane will provide a better understanding of the microbial composition on various commonly fed finishing diets and provide a better understanding of potential dietary intervention strategies in finishing feedlot cattle.

Bacteria

The global microbial community was significantly affected by diet, time, and animal between the common basal diet and the treatment diets ($P = 0.001$) with a Diet \times Barn interaction ($P = 0.001$) (Table 1a). Microbial community structure between the six treatment diets (removing the common diet), however, were not significantly different ($P = 0.553$) but animal was significantly different ($P = 0.001$) (Table 1b) indicating that dietary treatment had no significant effect on the community structure and potential animal to animal variation is apparent.

Global effects indicated that Diet was not significantly different, however, the effect of barn location had not been identified. Table 2 indicates the effect of Barn was not observed ($P = 0.121$) and a Diet \times Barn interaction was not observed ($P = 0.676$).

However, pairwise comparisons indicated that all diets are statistically different from one another with the exception of 87DRCRumControl, $P = 0.1468$) (Table 3).

To better ascertain a visual model of the community structure and its influence by diet, Principle Coordinate Analyses was performed using rarefied OTU tables to account for unequal read depth. The PCoA plots were generated by utilizing unweighted unifrac as a measure of β -diversity (Lozupone et al., 2011). Phylogenetic relationship and abundance are considered when the plots are generated indicating a close evolutionary relationship by the distance between each community. Figure 1 provides clear clustering of the bacterial community based on diet type. Sampling the animals on the common diet allows for a baseline to measure a quantitative difference between communities, thus the treatment diets cluster separate from the common diet, suggesting that the treatment diets did change the microbial community from the basal common diet. Figure 1 shows the common and all six treatment diets clustering together. Figure 2a shows all treatment diets cluster together, however within this plot, slight patterns do emerge. Figures 2b and c display slight clustering by the presence of Dry Rolled Corn (DRC), Modified Distillers Grains plus Solubles (MDGS) presence, respectively. Figures 2d and e show how minor Rumensin® effects on the bacterial community structure. The diets that were not supplemented with Rumensin® tended to have a tighter grouping than those diets that were supplemented with Rumensin®.

The predominant phyla detected in the finishing diets are Bacteroidetes, Firmicutes, and Proteobacteria. These phyla represent approximately 93% of the bacterial community. The additional 7% of the community is composed of 17 other phyla with relatively small abundances. The primary phyla present on the common diet are Bacteroidetes and Firmicutes. When the animals were placed on the treatment diets, the presence of Proteobacteria becomes increasingly more abundant (Figures 3 and 4).

Heatmaps were generated to show changes in bacterial community composition at OTU level. Due to the read length the OTUs are classified at family level and OTUs with greater than 2.0% relative abundance is shown. Figure 5 shows that families S24-7, *Prevotellaceae*, *Veillonellaceae*, and a few unassigned taxa, are abundant in the diet containing 50MDGSNoRum. Additionally, families RFP12, *Prevotellaceae*, *Lachnospiraceae*, *Spirochaetaceae*, as well as other unassigned taxa, are significantly more abundance, yet less than 2.0% in abundance in the diets containing 50MDGSRum. Figure 6 displays the relative abundance between 87DRCNoRumControl and 87DRCRumControl. *Prevotellaceae* and *Lachnospiraceae* are shown to be more abundant in the two control diets that have no Rumensin supplemented. The control diet that contains supplemented Rumensin appears to have *Veillonellaceae*, S24-7, and unassigned taxa as the higher abundant taxa, in addition to the presence of *Prevotellaceae*. In Figure 7 families *Veillonellaceae* is more abundant in the diet containing oil, however it is also abundant, to a lesser extent, in the control diet containing Rumensin. *Prevotellaceae* is abundant in both diets however, it is seemingly more abundant in the control diet. However, animal-to-animal variation is evident. Figure 8 shows that *Succinivibrionaceae*, RF16, and *Veillonellaceae* are higher in

abundance in the control diet. The diet containing tallow has another strain of *Veillonellaceae* and S24-7 as the most highly abundant taxa, yet animal-to-animal variation in community composition is apparent. Figure 9 shows taxa highly abundant in the tallow diet being *Succinivibrionaceae*, *Veillonellaceae*, and *Lachnospiraceae*, while the diet containing oil shows RF16 being the most abundant family present.

Archaea

The method utilized for eubacterial analysis was mimicked for archaeal analysis. Global effects of total microbial community composition demonstrated diet, time, and animal as significantly different when compared to the common diet ($P = 0.001$) with a Diet \times Barn interaction observed ($P = 0.001$) (Table 4a). Global effects between the six treatments show that diet ($P = 0.306$) and animal ($P = 0.468$) are not significantly different (Table 4b) indicating that the dietary treatment had no significant effect on total microbial community composition and little animal-to-animal variation occurred as to the archaea populations.

Global effects indicated diet was not significant, however the effect of barn location has been identified. Table 5 shows no effect of barn ($P = 0.117$) and a Diet \times Barn interaction was not observed ($P = 0.758$).

Pairwise comparisons indicated that 50MDGSRum is statistically not different from 87DRCNoRumControl ($P = 0.1097$) and 87DRCRumControl ($P = 0.2165$) (Table 6), however, all other diet combinations were statistically different.

Archaeal β -diversity mirrored that of the Bacteria. This analysis provides evidence that visually the community does not change and is not affected by fat source supplementation. Figure 10 shows clear clustering when sampled on the common diet

and on the treatment diets. Figure 11a shows the clustering of the communities for all six treatment diets. Figures 11b and c show how the community is affected by the lack of supplementation of Rumensin®, showing a somewhat tighter configuration, and those diets supplemented with Rumensin® did not show a tight grouping, rather a wider grouping, respectively.

The taxonomic distribution of the archaea on the finishing treatments has 29 total classifications. Within those 29 classifications, not all OTUs are represented at a genus level. However, at the genus level, the two genera that comprise the majority of the entire community throughout the study are Thermoplasmata (Class, Thermoplasmata) and *Methanobrevibacter* with 44% and 53%, respectively, characterizing over 97% of the entire archaeal community. While the animals were on the common diet, the primarily abundant genus was Thermoplasmata (Figure 12). While the animals were on the treatment diets, *Methanobrevibacter* became increasingly abundant (Figure 13). The distribution of the OTUs abundance and taxonomy can be seen in Figure 14. One or more OTUs in this figure have 1.5% abundance or greater. The family *Methanomassiliicoccaceae* appears to be more abundant with the diet containing oil.

Tables detailing dietary composition, VFA profiles, and methane emissions are described briefly in APPENDIX II and in detail in Pesta et al. (2015).

Discussion

Beef cattle contribute to methane levels through enteric fermentation, producing over 55 million metric tonnes (Tg) a year (McMichael et al., 2007). Anthropogenic methane sources contribute virtually 40% of the agricultural sector (Steinfeld et al.,

2006). Due to evolution and the function of the rumen, cattle are able to consume various low-quality plant fiber and convert that energy into products for the human consumption. In the process of converting unusable substrates into products that can be used for human consumption, ruminants produce various products via enteric fermentation. One important product is Hydrogen (H_2) (Hungate, 1967). The concentration of H_2 increases due to type of feed component used as well as how much is ingested in a period of time (Buddle et al., 2011). The loss of H_2 as methane leads to an energy loss to the animal, 2-12% (Johnson and Johnson, 1995). Thus decreasing methane production would have beneficial effects to the animal. Lipid supplementation, by-product supplementation, and ionophore addition are methods previously researched to lower methane production. However, the archaeal community that is directly involved in methanogenesis in the rumen is poorly characterized and the interactions are poorly understood. This study shows a glimpse into such interactions that occur in the rumen.

In the bacterial community, *Bacteroidetes* and *Firmicutes* are the two major phyla in the rumen when the cattle are a common diet that closely resembles a growing diet. The common diet contained alfalfa which is composed of xylan, an abundant plant cell polymer second to cellulose. Dodd et al (2011) determined that the degradation of xylan (a five carbon sugar) is advantageous and favors healthy rumen function due to its ability to be highly degraded (Van Soest, 1994). *Bacteroidetes* are typically correlated with large amounts of carbohydrate fermentation in the rumen, along with *Firmicutes*, which prefer polysaccharides (Hanning and Diaz-Sanchez, 2015). The phyla Proteobacteria became increasingly abundant on the six finishing diets, making it the third dominant

phyla in this study, indicating its potential role in increased utilization of more simple polysaccharides such as starch (Fernando et al., 2010).

The dietary combinations explored within this study do provide evidence of animal to animal variation, regarding family level distribution and abundance of the bacterial community in the heatmaps shown. However, the relatively higher number of animals used per treatment, and the common diet fed to all steers at the start of the study helps identify significant microbial community shifts in this study.

To confirm sampling depth was sufficient, rarefaction curves were generated (Kuczynski et al., 2011) utilizing a rarefied OTU table for bacteria (5153) and archaea (3055) sequences. Both curves show that species richness has been concluded and the rare OTUs have been sequenced (Figures 15 and 16, respectively) thus providing adequate sampling depth for characterization of the rumen bacterial and archaeal communities, characterizing 73% of the bacterial and 93% of the archaeal community.

Due to the length of reads, taxonomic classification of the rumen microorganisms in this study are at the family level classification. The family *Succinivibrionaceae* are commonly found in the rumen, yet fairly uncharacterized in the rumen (Stackebrandt and Hespell, 2006). Strains of this family have been identified in cattle that are fed grain diets (Stackebrandt and Hespell, 2006). Stackebrandt and Hespell (2006) determined that this family are Gram-negative various shaped rods. This family primarily ferments carbohydrates (i.e. glucose) producing succinate and acetate. This group can potentially contribute to methane mitigation by producing succinate, which feeds into the propionate producing pathway, and it can also contribute to increasing methane production by

producing acetate. This family is seemingly most abundant in the corn control diets containing Rumensin also the diet containing Tallow.

The family *Prevotellaceae* is profoundly present in communities that are fed diets that contain unprocessed grain rather than forage rations (Shanks et al., 2011). This family is commonly found in the rumen as this family have a high affinity for hemicellulytic and proteolytic substrates (Thoetkiatikul et al., 2013). For this characteristic, this group was found to be abundant on diets that contained MDGS and DRC at 87% inclusion, in addition to corn oil. *Lachnospiraceae* are fairly uncharacterized within the bovine rumen. This family has a trait (however it is not represented throughout all strains), of producing short-chain fatty acids including butyrate (Meehan and Beiko, 2014). *Veillonellaceae* is another family that is present more abundantly in the lipid containing diets of oil and tallow, as well as the DRC control diet without monensin. This family is Gram-negative and due to variation within the various strains, carbohydrates are fermented yet in some strains may not be fermented (Marchandin and Jumas-Bilak, 2014). However, a well-known member of this family that present in the rumen is *Megasphaera elsdenii*.

Archaeal community composition is the primary focus of this experiment. Archaea are responsible for the production of methane as the terminal step in the ruminant fermentation process. It is this community primarily that is being targeted by utilizing dietary intervention methods, either directly (ionophore utilization) or indirectly (changing concentrations of substrate availability). Dietary comparisons explored diets with and without Rumensin in both MDGS and DRC diets, as well as the addition of two lipid sources (oil and tallow), which contain different levels of saturation.

The archaeal community composition shifts from the common to the treatment diets showing a decrease in the genus *Thermoplasmata* (Class *Thermoplasmata*) and increase in the genus *Methanobrevibacter* (Figures 11 and 20, respectively). However, this trend is not apparent for all samples. Microorganisms belonging to the Order *Thermoplasmata* are characterized as utilizing methylamines as their primary substrate for methane production (Poulsen et al., 2013). Methylamines levels are in response to nitrogen levels in the soil and can alter plant physiology by decreasing starch concentrations in the shoot and increasing starch concentrations in the root of the plant (Shiraishi et al., 2002). This group in general decreases in abundance once the animals are switched to the treatment diets, indicating that high energy diets that contain different levels of unsaturated fatty acids can be employed into decreasing methylamine utilization for methane production in the rumen (Poulsen et al., 2013). *Methanobrevibacter* increased in abundance generally for all the treatment diets and is commonly found in the bovine rumen on various diets as this genus is responsible for methane production by using CO₂ and H₂ and formate (Leahy et al., 2010).

Significant OTUs were identified between the five dietary combinations mentioned above, however, the abundances associated with these OTUs are less than 1.5% in total therefore it can be concluded that these OTUs play an insignificant role in contributing very little to methane production and community structuring due to low abundance. The two families that are assigned to these significantly different OTUs are *Methanobacteriaceae* and *Methanomassiliicoccaceae*. *Methanobacteriaceae* members are known to reduce CO₂ with H₂ producing methane as part of their energy growth

(Oren, 2014). Various members are also able to utilize formate in the production of methane (Oren, 2014).

Ionophores are added to diets to decrease the methane production and improve efficiency (Johnson and Johnson, 1995), however their effects are inconclusive (Johnson and Johnson, 1995). In this experiment, the addition of Rumensin to the diets had no effect on methane emissions, and had minimal changes in the community structure amounting to significance.

Nutrient composition, VFA profiles, and CH₄:CO₂ ratios for this study can be found in Pesta et al. (2015). In short, there was no impact on performance due to dietary fat. No interaction of diet x monensin were observed, as well as no change in the VFA profile due to fat source, MDGS, or monensin supplementation. In the aim of this study, it can be deduced that the microbes are not able to identify different lipid sources due to the factors not being significantly different as their additions were the same amount and the diets still provided 6.5% dietary fat. The results of this study fall in line with performance data presented by Pesta et al. (2015) in that the microbial community was not changed due to fat source type, MDGS supplementation, or monensin supplementation.

Conclusion

Various strategies are being utilized to abate methane production in ruminants. Previous research has indicated that nutritional mitigation exist to decrease methane production including by-product supplementation, lipid supplementation, as well as ionophore additions. From this research study, the effect of fat sources have no effect

overall on the community structure and composition, however, individual dietary effects on community structuring are observed. Finishing diets produce less methane on a gain basis and may not be an option for using dietary intervention strategies to mitigation methane emissions.

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Table 1a. Global eubacterial results
between the common and treatment diets.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	0.001

Table 1b. Global eubacterial
results between treatments only.

Diet	0.553
Animal	0.001

Table 2. Effect of barn on the finishing treatments on the bacterial community.

Diet	0.214
Barn	0.121
Animal	0.001
Diet × Barn	0.676

Table 3. Bacteria pairwise comparison against basal and treatment diets.

	Treatments ³					
	Com ¹	50NoRum ²	50Rum ²	84DRCRumOil	84DRCRumTallow	87DRCNoRumCon
50NoRum ²	< 0.0001	-	-	-	-	-
50Rum ²	< 0.0001	< 0.0001	-	-	-	-
84DRCRumOil	< 0.0001	< 0.0001	< 0.0001	-	-	-
84DRCRumTallow	< 0.0001	< 0.0001	< 0.0001	< 0.0001	-	-
87DRCNoRumCon	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	-
87DRCRumCon	< 0.0001	< 0.0001	< 0.0001	0.0022	0.001	0.1468

¹Common diet fed before treatments, consisting of a 50:50 blend of alfalfa and Sweet Bran®.

²Diets containing Modified Distillers Grains plus Solubles

³Common diet of 50:50 alfalfa and Sweet Bran®, MDGS at 50% without Rumensin®, MDGS at 50% with Rumensin®, DRC at 84% with Rumensin® and Corn Oil at 3%, DRC at 84% with Rumensin® at Tallow at 3%, Control diet of DRC at 87% without Rumensin®, Control diet of DRC at 87% with Rumensin®.

Table 4a. Archaea global results between the common and the treatment diets.

Diet	0.001
Time	0.001
Animal	0.001
Diet \times Time	0.001

Figure 4b. Archaea global results
between the treatment diets.

Diet	0.306
Animal	0.468

Table 5. Effect of barn on the finishing treatments on the archaeal community.

Diet	0.304
Barn	0.117
Animal	0.003
Diet \times Barn	0.758

Table 6. Archaea pairwise comparison between the common and the treatment diets.

	Treatment ³					
	Com ¹	50NoRum ²	50Rum ²	84DRCRumOil	84DRCRumTallow	87DRCNoRumCon
50NoRum ²	< 0.0001	-	-	-	-	-
50Rum ²	< 0.0001	0.01075	-	-	-	-
84DRCRumOil	< 0.0001	0.00032	< 0.0001	-	-	-
84DRCRumTallow	< 0.0001	< 0.0001	< 0.0001	< 0.0001	-	-
87DRCNoRumCon	< 0.0001	< 0.0001	0.10973	< 0.0001	< 0.0001	-
87DRCRumCon	< 0.0001	0.11	0.2165	< 0.0001	< 0.0001	0.00237

¹Common diet fed before treatments, consisting of a 50:50 blend of alfalfa and Sweet Bran®.

²Diets containing Modified Distillers Grains plus Solubles

³Common diet of 50:50 alfalfa and Sweet Bran®, MDGS at 50% without Rumensin®, MDGS at 50% with Rumensin®, DRC at 84% with Rumensin® and Corn Oil at 3%, DRC at 84% with Rumensin® at Tallow at 3%, Control diet of DRC at 87% without Rumensin®, Control diet of DRC at 87% with Rumensin®.

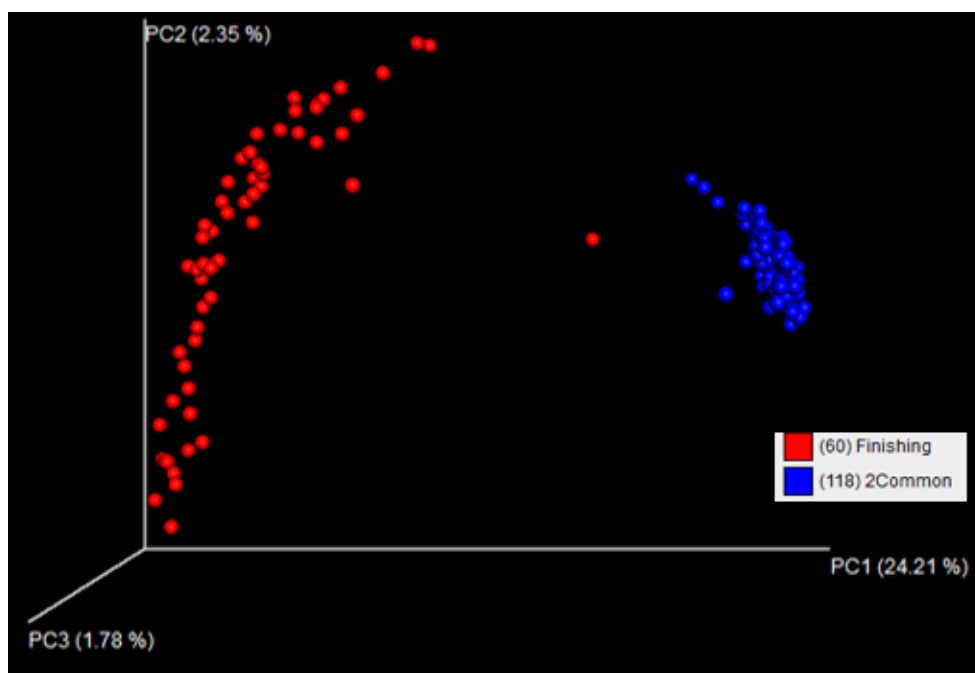


Figure 1. Principle Coordinate Analysis of the bovine ruminal bacterial community structure between the common diet and the finishing treatments. The common diet is identified as 2Common and all six finishing treatments are noted as Finishing. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering of the common diet separate from the finishing diets.

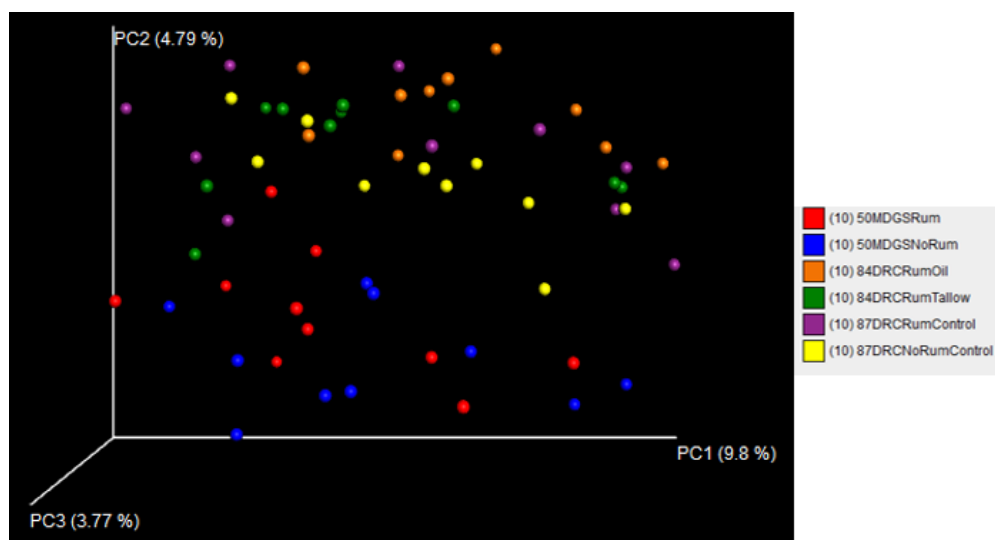


Figure 2a. Principle Coordinate Analysis of the bovine ruminal bacterial community structure between all six of the treatments. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows ultimately no clear and distinct clustering due to diet, however, slight clustering is evident between diets that contain MDGS and diets that contain DRC.

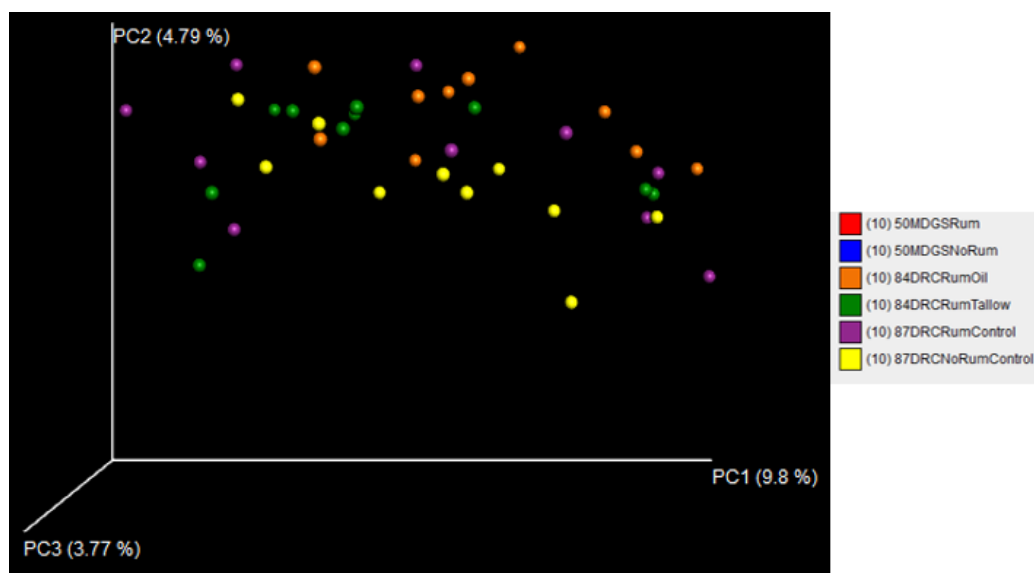


Figure 2b. Principle Coordinate Analysis of the bovine ruminal bacterial community structure between the treatment diets containing DRC. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows the diets containing DRC clustering together higher in the figure. However, no clustering is evident between the diets containing DRC.

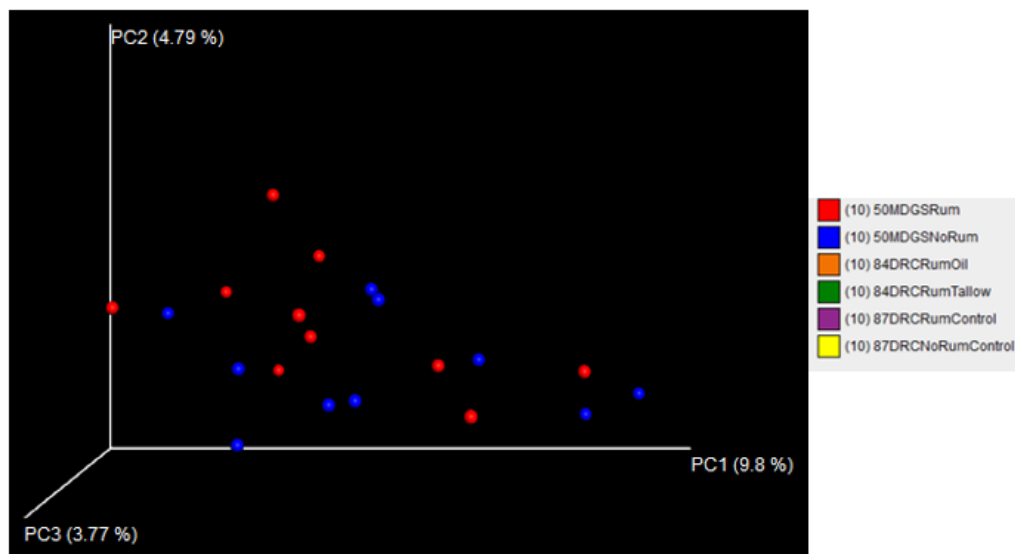


Figure 2c. Principle Coordinate Analysis of the bovine ruminal bacterial community structuring between diets containing MDGS. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows slight clustering of diets containing MDGS on the lower portion of the figure.

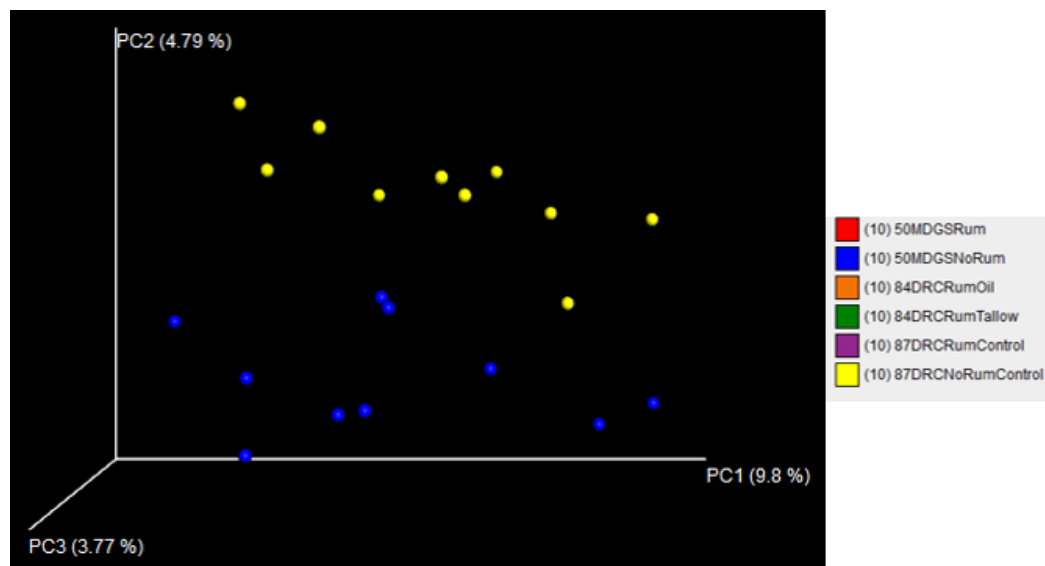


Figure 2d. Principle Coordinate Analysis of the bovine ruminal bacterial community structuring between diets containing no Rumensin clustering together. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows slight clustering of two diets containing no Rumensin®.

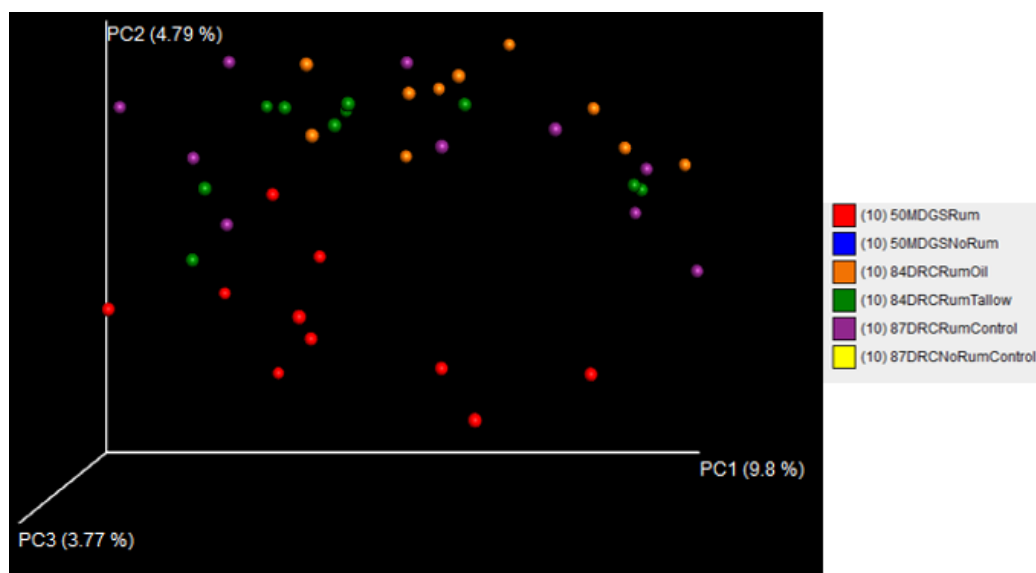


Figure 2e. Principle Coordinate Analysis of the bovine ruminal bacterial community structuring between diets containing Rumensin® having a wider clustering appearance. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows the diets containing Rumensin® having a wider clustering appearance.

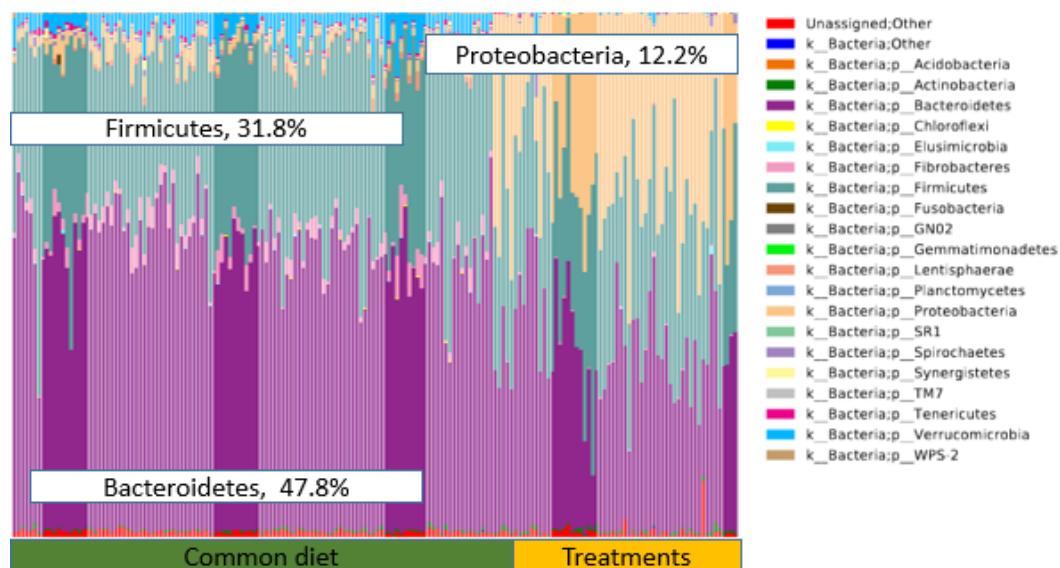


Figure 3. Bacterial taxonomy distribution of the common and treatment diets at the phylum level, representing approximately 93% of the community. Many taxa are present, however, the combined abundance of those taxa only represent 8% of the community, therefore, most likely contribute very little to community change with methane by providing substrates for the methanogens.

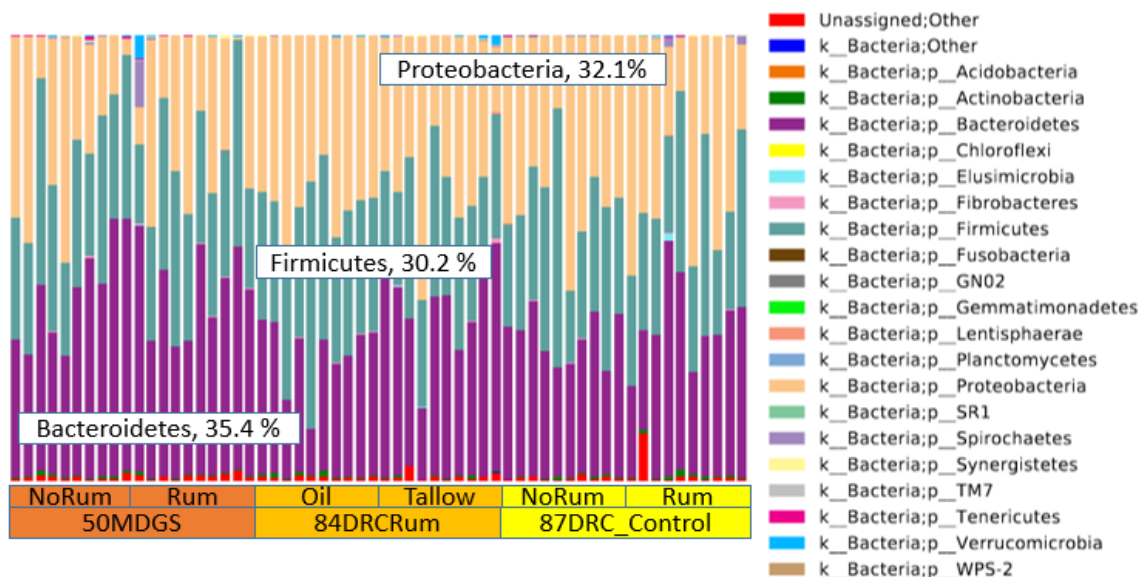


Figure 4. Bacterial taxonomy distribution of the treatment diets at the phylum level, representing approximately 97% of the community. Many taxa are present, however, the combined abundance of those taxa only represent 2.3% of the community, therefore, most likely contribute very little to community change with methane by providing substrates for the methanogens.

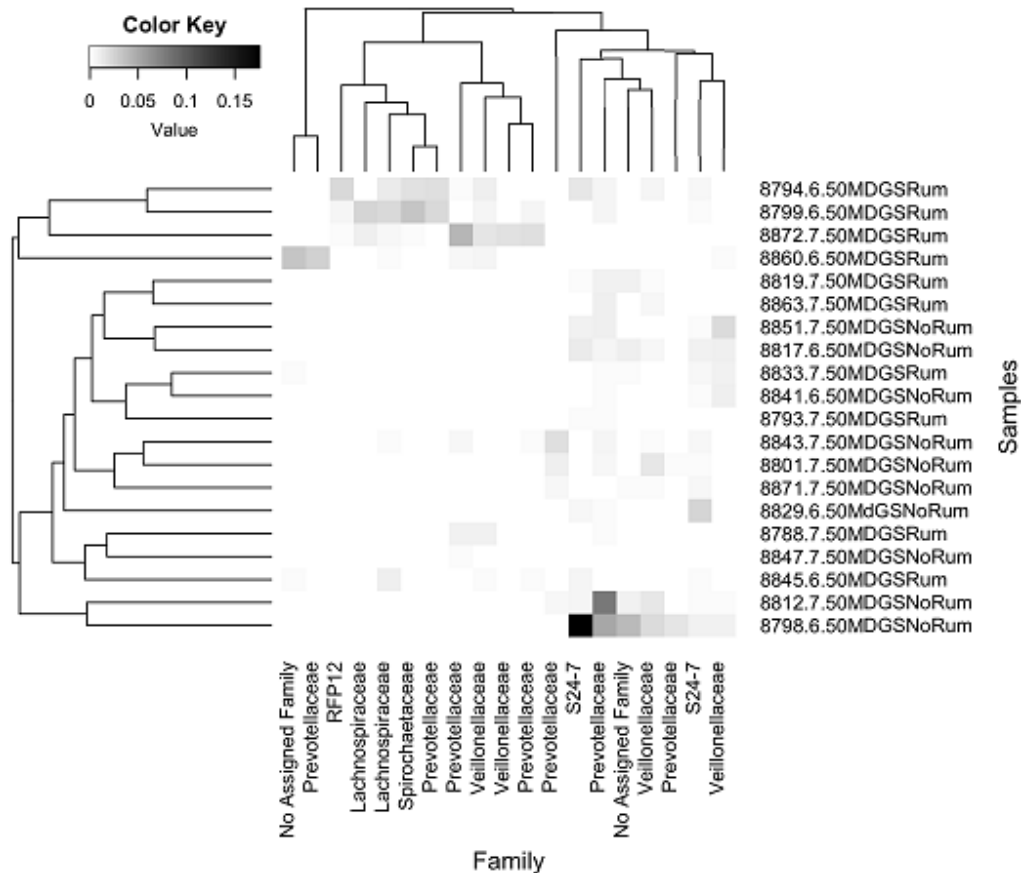


Figure 5. Bacterial heatmap showing diets containing MDGS at 50% inclusion with and without Rumensin® at 2.0%. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

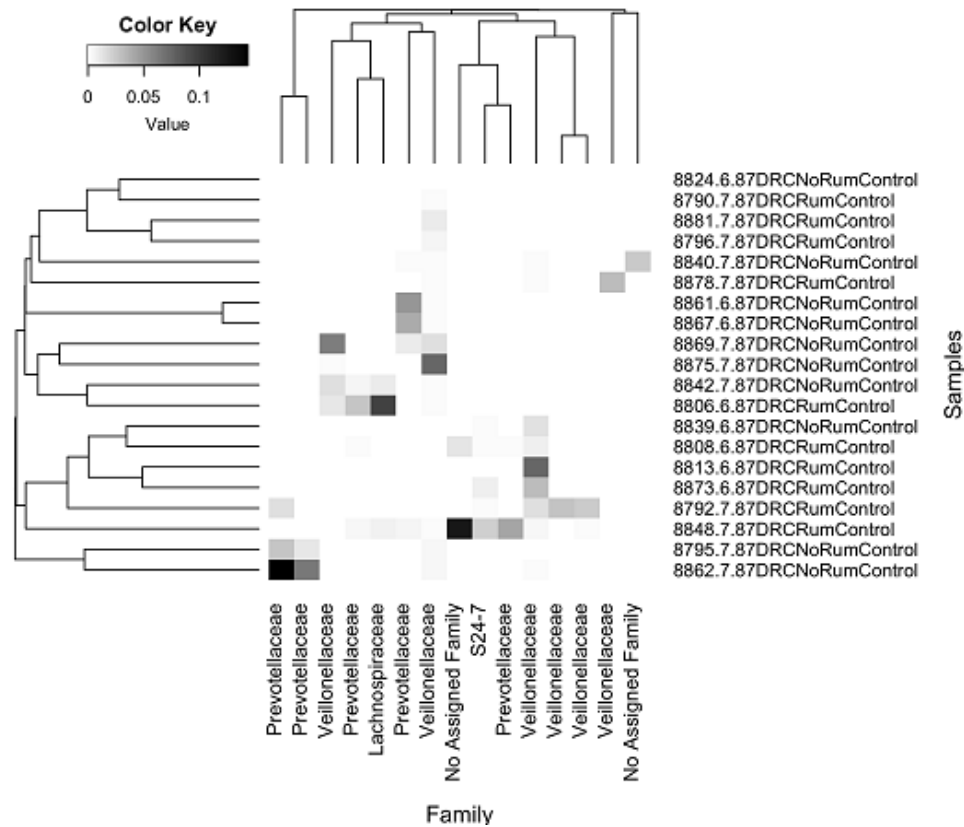


Figure 6. Bacterial heatmap of diet containing DRC at 87% with and without Rumensin® at 2.0% abundance. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

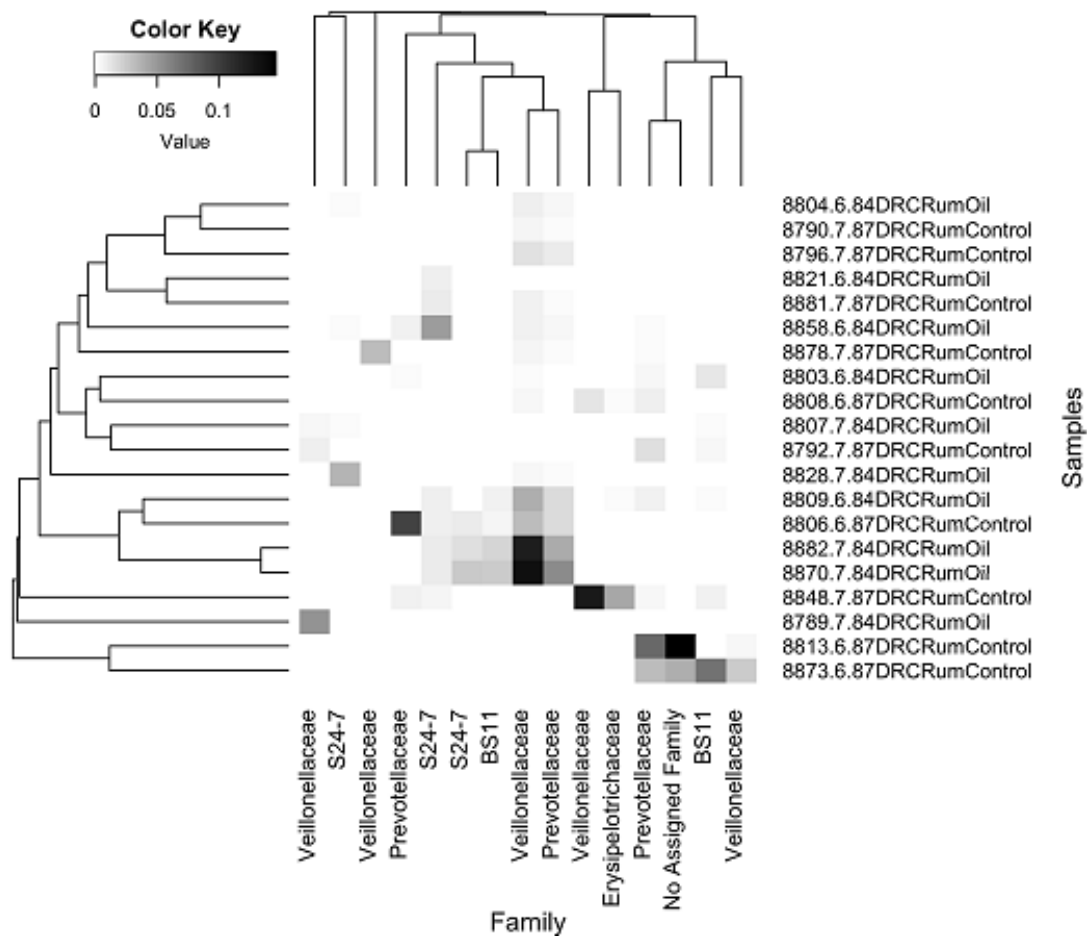


Figure 7. Bacterial heatmap of diets containing DRC at 84% with Rumensin® with Oil and 87% with Rumensin® at 2.0%. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

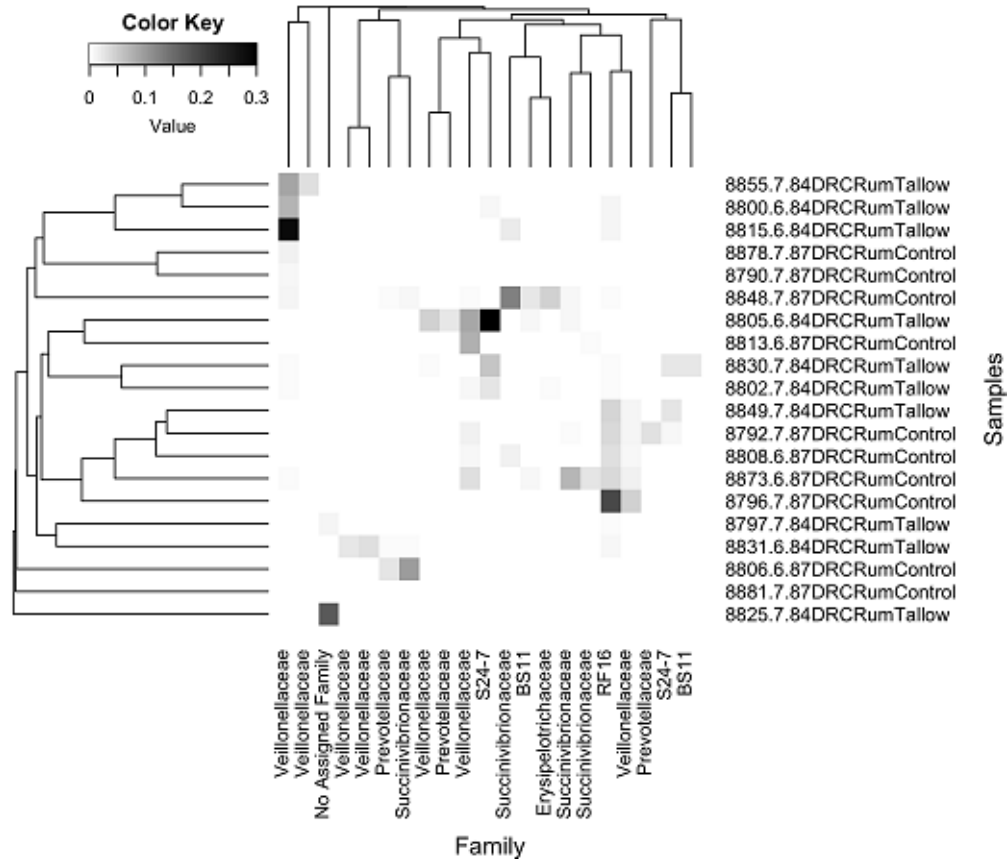


Figure 8. Bacterial heatmap of diets containing DRC at 84% with Rumensin® and Tallow and 87% without with Rumensin® at 2.0%. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

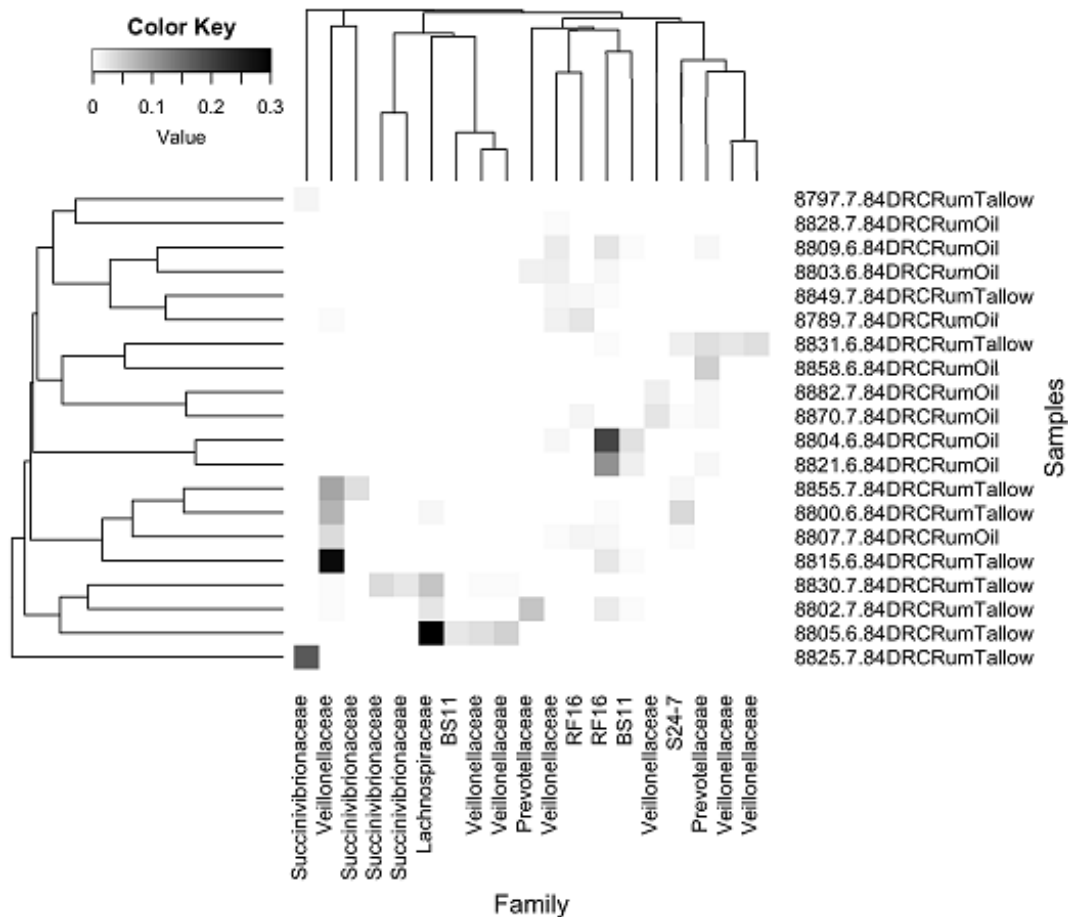


Figure 9. Bacterial heatmap of diets containing DRC at 84% with Rumensin® and either oil or tallow at 2.0%. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

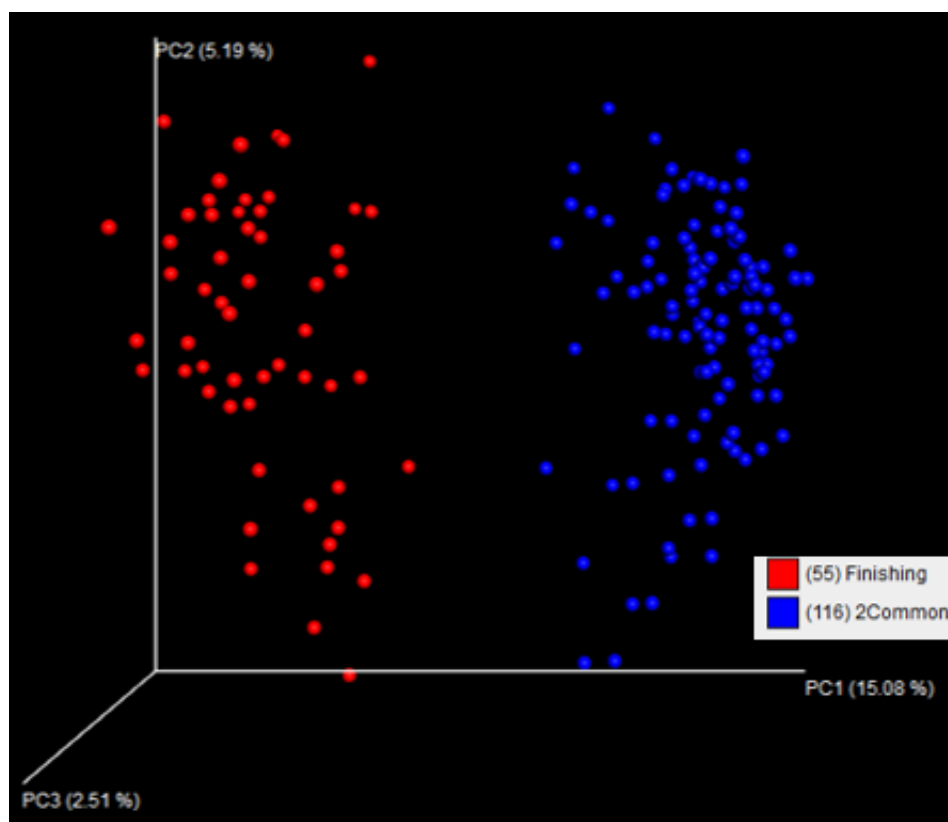


Figure 10. Principle Coordinate Analysis of the bovine archaeal community structuring between the common diet and the treatment diets. The common diet is identified as 2Common and all six finishing treatments are noted as Finishing. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows clustering of the common diet separate from all six treatment diets.

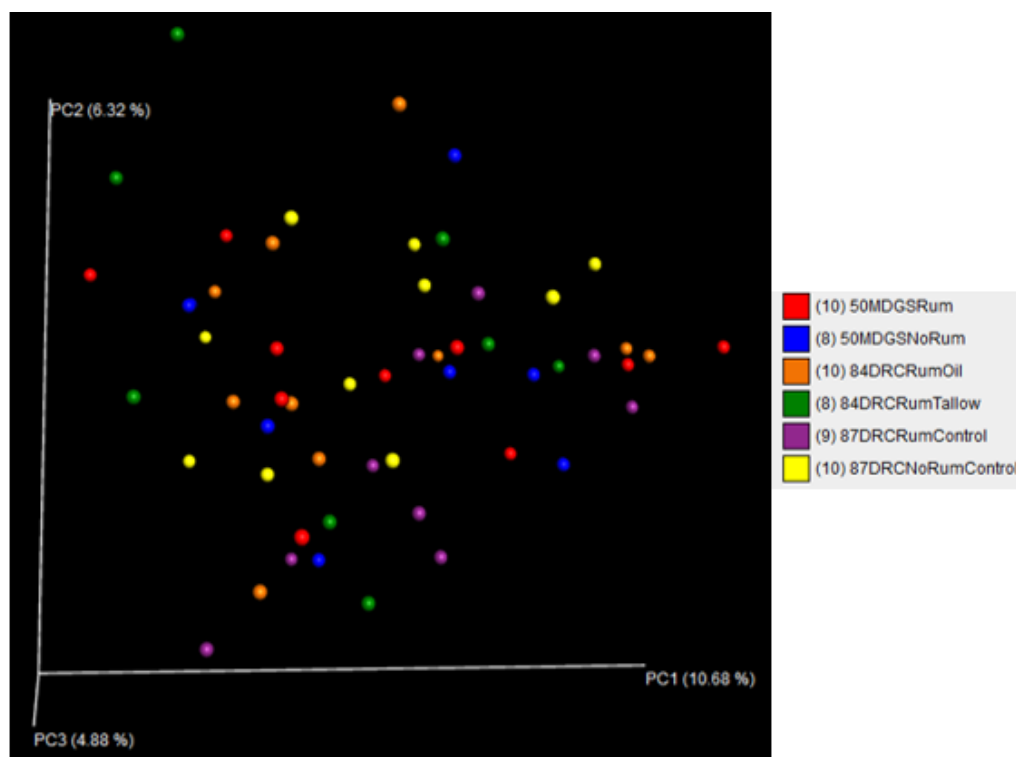


Figure 11a. Principle Coordinate Analysis of the bovine archaeal community structuring between all six treatment diets. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows very little clustering of the community due to diet, however, slight clustering can be seen between the diets that do not contain Rumensin®.

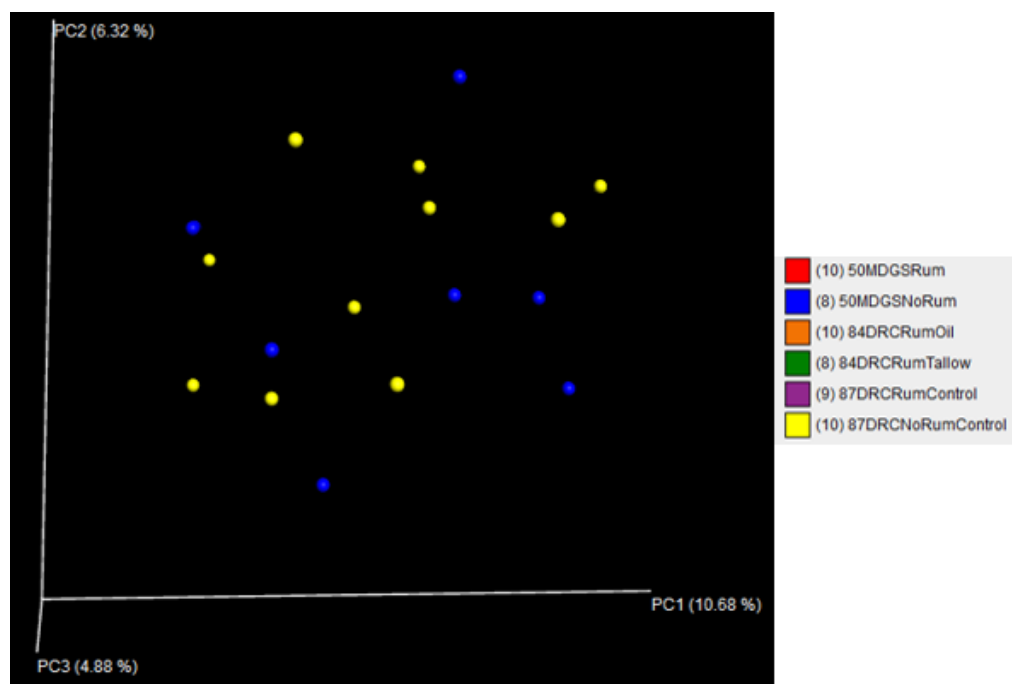


Figure 11b. Principle Coordinate Analysis of the bovine archaeal community structuring between diets without Rumensin. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows slight clustering of the two diets that do not contain Rumensin®.

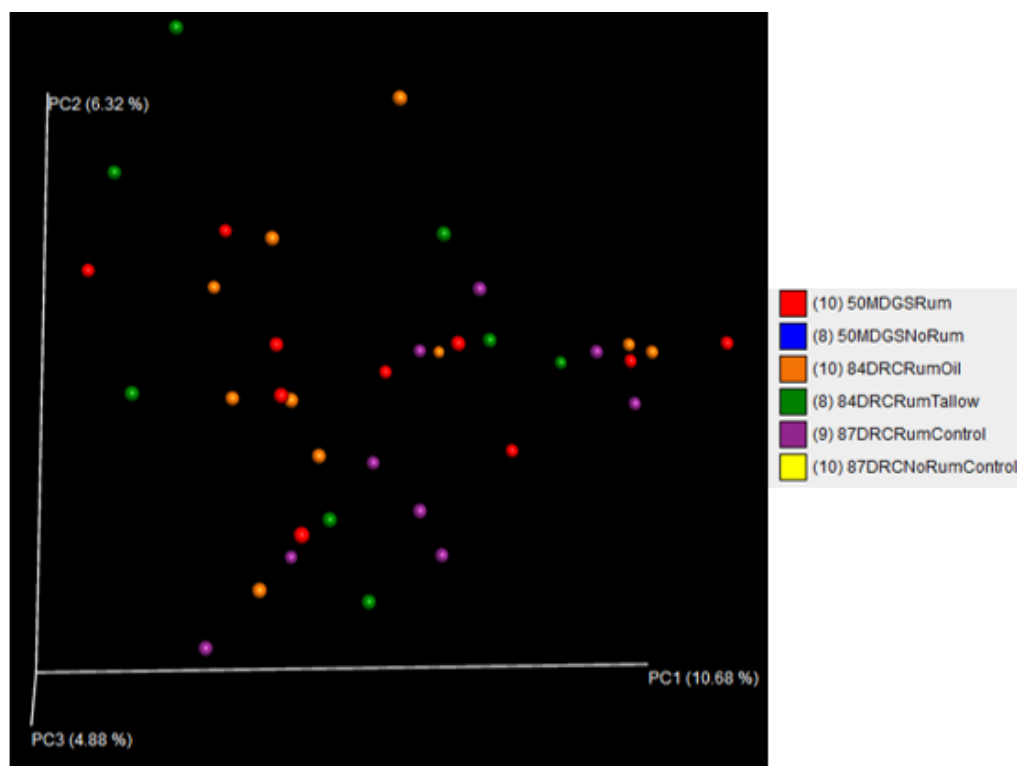


Figure 11c. Principle Coordinate Analysis of the bovine archaeal community structuring between diets with Rumensin added is not as tight as those with Rumensin added. The number in parentheses refers to how many animals are within each diet. Each dot represents a community from animal. The three axes are to provide a 3D visualization with variance between the axes measured. This figure shows that the diets containing Rumensin® show no clustering.

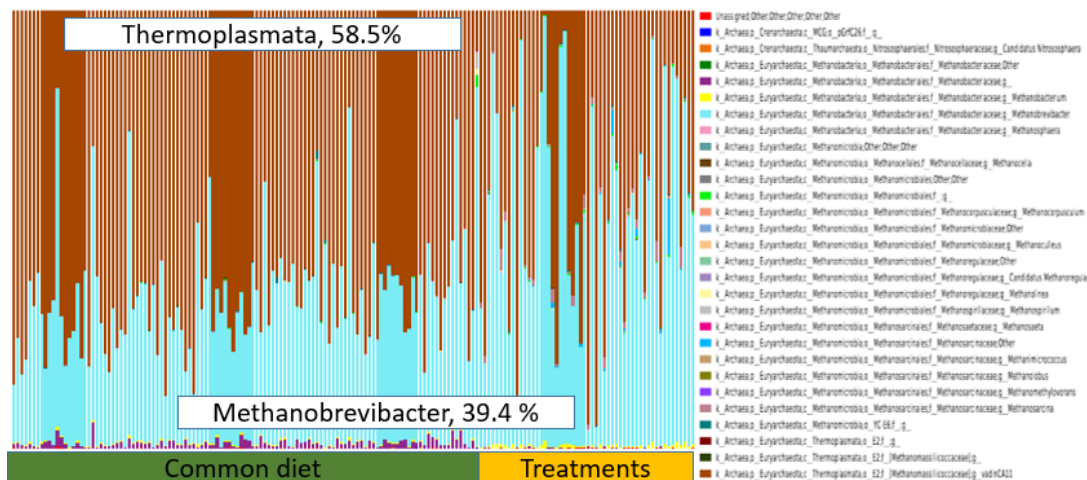


Figure 12. Archaeal taxonomy distribution of the common and treatment diets at the genus level, representing approximately 98% of the community. Many taxa are present, however, the combined abundance of those taxa only represent 2% of the community, therefore, most likely contribute very little to community change with methane.

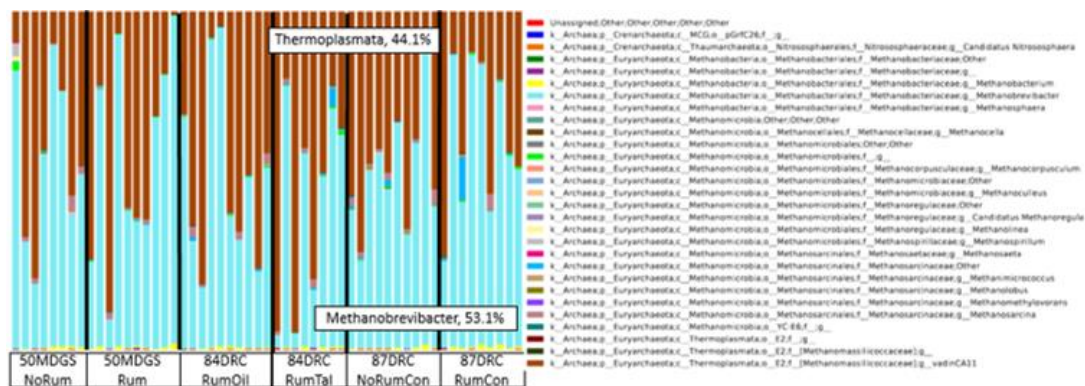


Figure 13. Archaeal taxonomy abundance on the treatment diets at the genus level, representing over 97% of the community. Other taxa are present, however, the combined abundance of those taxa only represent 2% of the community, therefore, most likely contribute very little to community change and methane.

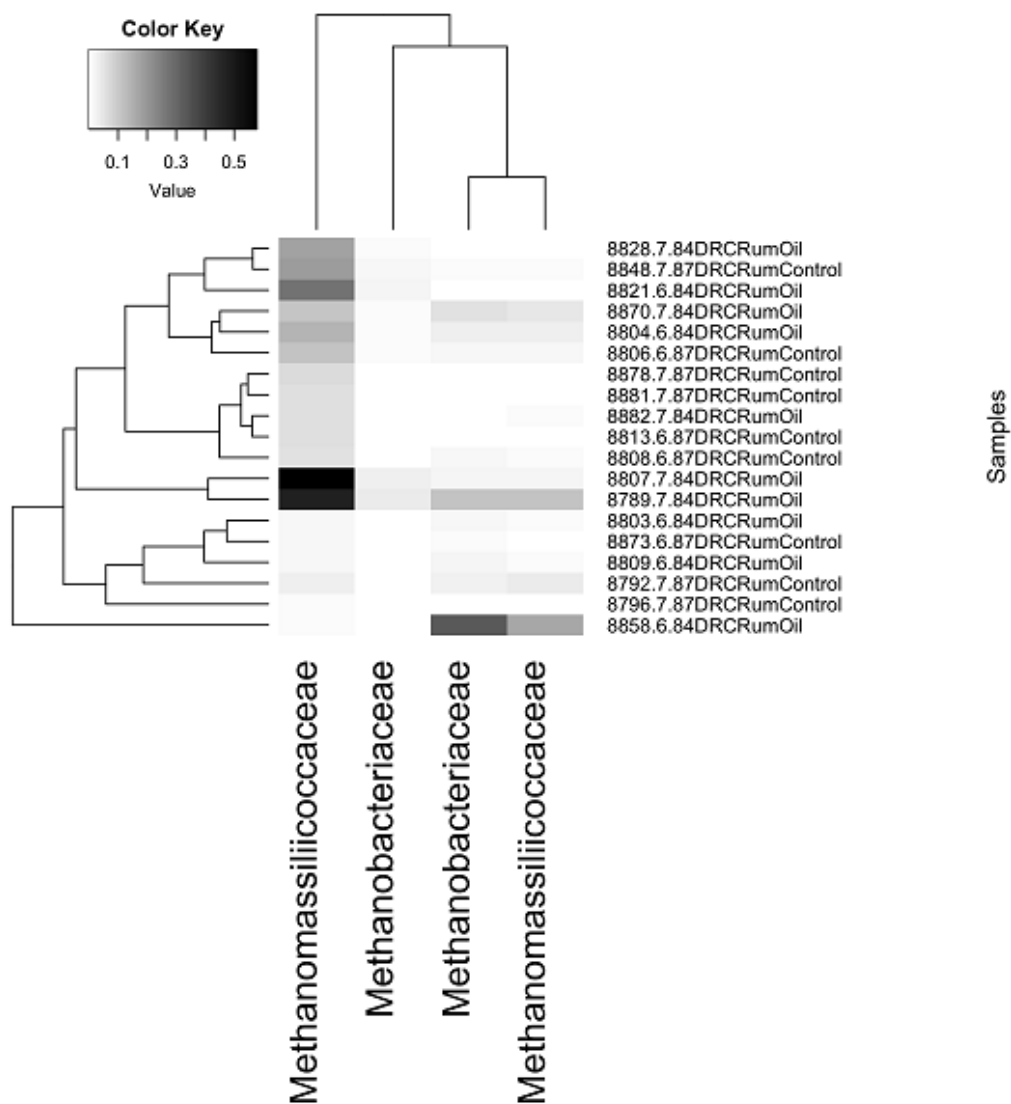


Figure 14. Archaeal heatmap of diet containing DRC at 84% with Rumensin® with and without the supplementation of oil at 1.5% abundance. The Families consists of various OTUs that are phylogenetically linked by hierarchical linkage. The darker the shading indicates the range of abundance of that Family of OTUs within a particular diet.

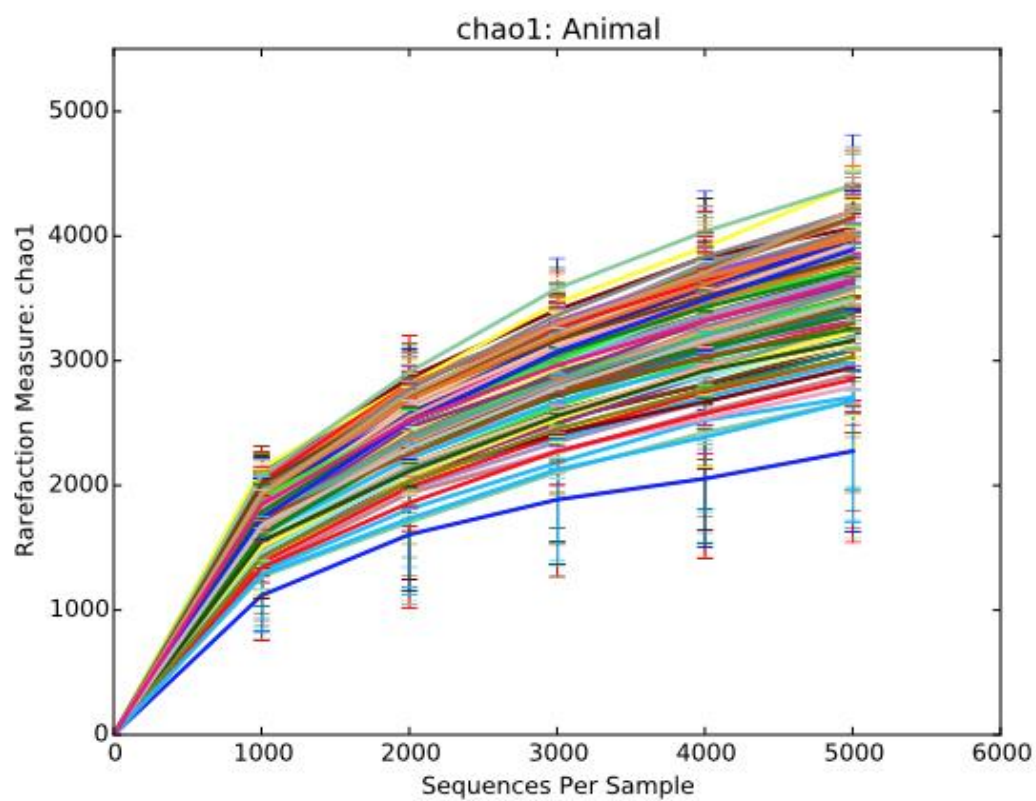


Figure 15. Bacterial alpha diversity rarefaction curve showing all samples sequenced provided enough depth to characterize the rumen constituents (5153 sequences).

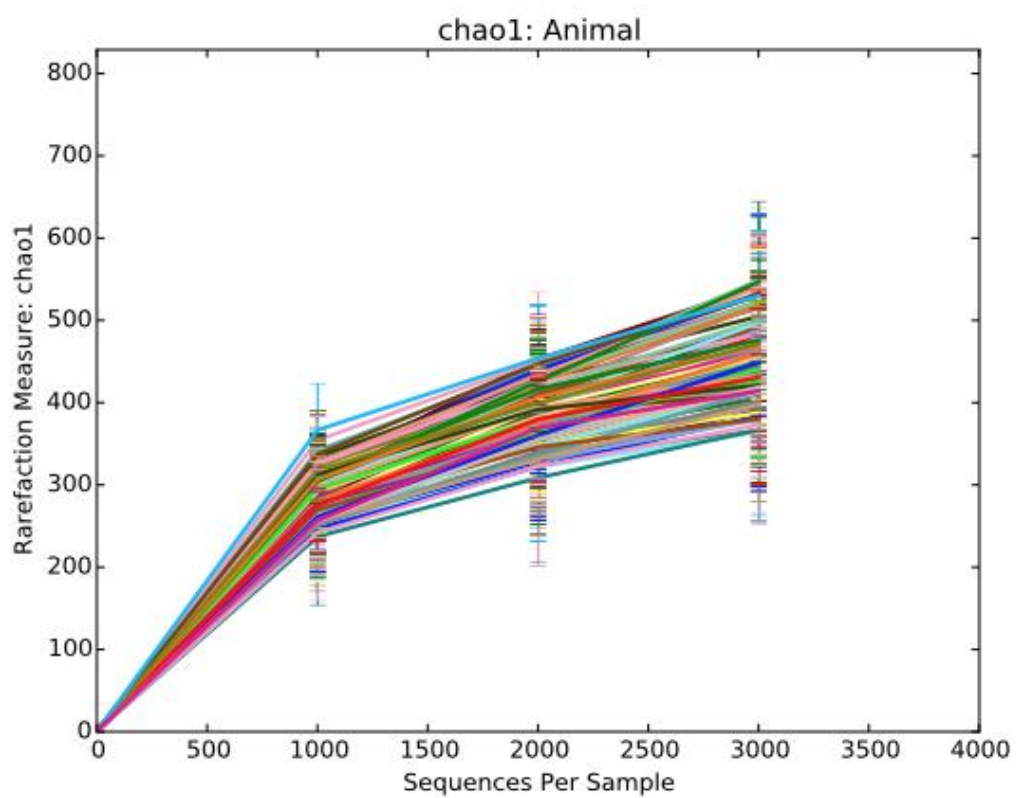


Figure 16. Archaeal alpha diversity rarefaction curve showing all samples sequenced provided enough depth to characterize the rumen constituents (3055 sequences).

Chapter 4

CONCLUSIONS

Concluding remarks and implications

Diet affects the microbial community and ultimately methane production. However, it may involve extreme differences in the community such as forage quality and level of MDGS supplementation. If the diets contain similar nutrient composition profiles, it can be hypothesized that the community that the community may not recognize exact differences and respond to these feeds in the same fashion.

Methane production from ruminants cannot be narrowed to one factor, due to multiple components affecting methane production ranging from environment, diet, and breed. Therefore, more extensive research needs to be explored including multiple time points, increased accuracy of methane production in production settings, and utilizing a wider variation in feeds, particularly in the finishing phase can potentially begin to illustrate novel methods in utilizing dietary intervention strategies to mitigate methane production from ruminants.

APPENDIX I

1. Check mapping for errors:
 - a. `validate_mapping_file.py -m mapping_file.txt -o mapping_file_check`
2. Convert .fastq file to .fasta:
 - a. `convert_fastaqual_fastq.py -c fastq_to_fastaqual -f filename.fastq -o filename.fastaqual`
3. Demultiplex .fasta file using separate mapping file for each plate:
 - a. `split_libraries.py -f fastq_files/file_name_fastaqual/file_name.fna -b variable_length -l 0 -L 1000 -x -M 1 -o split_library_file_name/ -m fastq_files/mapping_file.txt/mapping_file_plate_specific.txt`
*Do this for as many plates are represented
4. Concatenate seqs.fna with sequences:
 - a. `cat seq.fna seqs.fna > concat_file.fna`
5. Count the number of sequences:
 - a. `grep -c ">" file_name_concat.fasta`
*Do for each .fna file to make sure they add up to concat file
6. Remove the reverse primer:
 - a. `truncate_reverse_primer.py -f file_name_concat.fasta -m fastq_files/mapping_file_txt -z truncate_only -M 2 -o file_name_concat_rev_primer_truncated`
7. Trim sequences to desired length (Bacteria, 130 bp, Archaea, 140 bp):
 - a. `mothur >`
`trim.seqs(fasta=/path_to_truncated_file_rev_primer_truncated.fna,minlength=bp_size)`
8. Open fastx_trimmer:
 - a. `/Users/samodha/fastx/./fastx_trimmer -i /path_to_file_truncated.trim.fasta -l bp_size -o /path_to_output_folder_and_file_rev_primer_truncated.trim.trim.fasta`

9. Check for uniform length:

- a. `mothur > summary.seqs(fasta=/path_to_file_name_rev_primer_truncated.trim.trim.fasta)`

10. Reverse complement trimmed file:

- a. `mothur > reverse.seqs(fasta=/path_to_file_name_truncated.trim.trim.fasta)`

11. Open tusker. Go to work folder, \$WORK. Move trim.trim.fasta into tusker.

12. Rename file to test.trim.rc.fasta.

13. `qsub usearch_batch_master.pbs`
 *qstat job_name provides status

14. save otu.table_test.txt and test.otus2.fa

15. Assign taxonomy:

- a. `assign_taxonomy.py -i test.otus2.fa -t /macqiime/greengenes/gg_13_8_otus/taxonomy/97_otu_taxonomy.txt -r /macqiime/greengenes/gg_13_8_otus/rep_set/97_otus.fasta -o file_folder_assign_gg_taxa`

16. Manually copy and paste taxonomy into otu table with header “taxonomy”

17. Convert .txt into .biom format

- a. `biom convert -i test.otu_table.txt -o test.otu_table.biom --table-type "OTU table" --process-obs-metadata taxonomy --to-json`

18. Go to RDP website (listed in Chapter’s 1 and 2 materials and methods section). Upload test.otus2.fa and select corresponding database.

19. Remove otus that align outside of region:

- a. open alignment_summary.txt in excel, sort by start position, then pick otus that are not within the region and copy over to text document
- b. sort by end position and pick otus that are not within region, copy over to text document
- c. save text document as otus_outside_alignment.txt

- d. `filter_otus_from_otu_table.py -i test.otu_table.biom -o filtered_otu.table.biom -e otus_outside_alignment.txt`

20. Remove Cyanobacteria from table:

- a. `filter_taxa_from_otu_table.py -i filtered_otu.table.biom -o cyano_filtered_otu.biom -n p__Cyanobacteria`

21. Remove Bacteria or Archaea from table:

- a. `filter_taxa_from_otu_table.py -i cyano_filtered_otu.biom -o archaea_filtered_otu.biom -n p__Archaea`
*Do this for bacteria, and for archaea, use `p__Bacteria` to filter out any nonmatching phyla

22. Remove singletons:

- a. `Filter_otus_from_otu_table.py -i phylum_filtered_otu.biom -o singletons_removed_otu.biom -n 2`
*At this point, able to decide which samples get resequenced based on sequence threshold.

17. Make phylogenetic tree:

- a. open rdp alignment file in text doc and replace all “.” with “-“
- a. replace “>” with “>AAAAAAAAAAAA”
- b. remove last line of document
- c. `mothur > dist.seqs(fasta=aligned_test.otus2.fa,output=phylip,countends=F)`
- d. `mothur> clearcut(phylip=output_of_prev.phylip.dist)`
- e. remove the A’s from output file, replace with “ “

18. Summarize table to get the number of sequences per sample

- a. `biom summarize-table -i singletons_removed_otu.biom -o singletons_removed_summarized.txt`

19. Remove samples under threshold (Bacteria, 5000 and Archaea, 3000):

- a. `filter_samples_from_otu_table.py -i singletons_removed_otu.biom -o low_samp_filtered_otu.biom -n number`
*Renamed this file to `master_shared_otu_table.biom`

20. Subsampled file to lowest number of sequences present in data set (Bacteria, 5153 and Archaea, 3055):

- a. `single_rarefaction.py -i master_shared_otu_table.biom -d number -o master_shared_rarefied_otu_table.biom`

Component Analysis (Clustering)

21. Split_otu_table based on time and diet:

- a. `split_otu_table.py -i master_shared_rarefied_otu_table.biom -o TimeID_Diet_split -m mapping_file.txt -f TimeID`

22. Merge tables:

- a. `merge_otu_tables.py -i table.biom,table.biom -o merged_otu_table.biom`
*Able to do any combination of groupings

23. Convert .biom to .txt to view sample groupings and taxonomy:

- a. `biom convert -i merged_d21_otu_tables.biom -o merged_d21_otu_tables.txt --header-key taxonomy --table-type "OTU table" --to-tsv`

24. Beta diversity to view data and generate distance matrices:

- a. `beta_diversity_through_plots.py -i TimeID_Diet_split/merged_otu_table.biom -p qiime_parameters_working-1.txt -t file_alignment/aligned_file.otus2.phylip.tre -m mapping-file.txt -o Total_Beta_Diversity/file_diversity`
*Able to do any combination of groupings

25. Summarize taxa:

- a. `summarize_taxa.py -i master_shared_rarefied_otu_table.biom -o Summarize_taxa/`

26. Plot taxa:

- a. `plot_taxa_summary.py -i master_shared_rarefied_otu_table_L2.txt, master_shared_rarefied_otu_table_L3.txt, master_shared_rarefied_otu_table_L4.txt, master_shared_rarefied`

```
_otu_table_L5.txt, master_shared_rarefied_otu_table_L6.txt -l
phylum,class,order,family,genus,species -c pie,bar,area -o taxa_charts
```

Run global statistics in MatLab

27. Open beta diversity folder and open the grouping for a particular diversity.
28. Open the unweighted_unifrac_dm.txt
29. Copy the first column of sample names into new excel spreadsheet
30. Delete the first column of samples in the dm.txt file and the corresponding first row of the samples. Save this file as unweighted_unifrac_dm_edited.txt
31. In the new spreadsheet, numerically categorize samples in columns.
*Note, DO NOT CHANGE the order of the samples as they coincide with the edited dm.txt file
32. Open MatLab. First add:
 - a. `addpath('/Users/samodha/Desktop/Fathom')`
33. Then add:
 - a. `result = f_npManova(unweightedunifracdmedited,[Diet Time Animal],1000,1)`
34. Import the files and select the columns. Make sure those columns are listed in the `result=` line of the script

Identification of significantly different otus based on dietary comparisons and plotting them in heatmaps

35. Identifying significantly different otus between diets:
 - a. `filter_samples_from_otu_table.py -i master_shared_otu_table_no_taxa.json.biom -o master_shared_otu_table_no_taxa.json.diet_1_diet_2.biom -m mapping_file.txt -s 'DiffAbund:diet_1,diet_2'`
36. Filter the otus:

- a. `filter_otus_from_otu_table.py -i master_shared_otu_table_no_taxa.json.diet_1_diet_2.biom -o master_shared_otu_table_no_taxa.json.diet_1_diet_2_filtered.biom -n 1`

37. Determine the significantly different otus between the selected diets:

- a. `differential_abundance.py -i master_shared_otu_table_no_taxa.json.diet_1_diet_2_filtered.biom -o master_shared_Time_diet_split/DiffAbund/diet_1&_diet_2_diff_otus.txt -m mapping_file.txt -a DESeq2_nbinom -c DiffAbund -x diet_1 -y diet_2 -d`

*Do for all dietary comparisons

38. Open each of the comparison .txt files and copy the list of otus into new .txt document. Label this as sig_diff_otus.txt

39. Normalize otu table:

- a. `out_table_normalization.R`
*Follow instructions within program

40. Split out table by time and diet again:

- a. `split_otu_table.py -i master_shared_otu_table_normalized.biom -o TimeID_split -m mapping_file.txt -f TimeID`

41. Merge otu tables:

- a. `merge_otu_tables.py -i master_shared_otu_table_normalized_03092016__TimeID_diet_1.biom, master_shared_otu_table_normalized_03092016__TimeID_diet_2.biom -o merged_diets_1_&_2.biom`

42. Filter the significantly different otus from the merged file:

- a. `filter_otus_from_otu_table.py -i merged_diets_1_&_2.biom -o merged_diets_1_&_2_filtered.biom --negate_ids_to_exclude -e sig_diff_otus.txt`

43. Convert .biom to .txt to check these steps were correct and the correct samples and taxonomy are present:

- a. `biom convert -i o merged_diets_1_&_2_filtered.biom -o merged_diets_1_&_2_filtered.txt --to-tsv --header-key taxonomy`

44. This script utilizes the .txt file to cut the file at the last column of samples. The # is removed before OTU. Also, the family taxa is designated by count five taxa after the column. Ex: 25 columns of samples and the family classification is five classifications from the kingdom:

```
a. awk '{gsub(";", "\t", $0); print;}' merged_diets_1_&_2_filtered.txt | awk
    '{gsub("#OTU", "OTU", $0); print;}' | cut -f-20,25 | tail -n +2 | awk
    '{if(NR==1){print $0, "\ttaxonomy"}else{print }}' > merged_
    diets_1_&_2_filtered_otus_family.txt
```

45. Open heatmap.R and follow the instructions listed in the script.

Generate alpha diversity

46. multiple_rarefactions.py -i master_shared_rarefied_otu_table.biom -o
Rarefactions/ -m 1 -x number -s 1000
*Do for both Bacteria, 5153 and Archaea, 3055

47. alpha_diversity.py -i Rarefactions/ -m chao1 -o adiv_chao1/

48. collate_alpha.py -i adiv_chao1/ -o collated_adiv/

49. make_rarefaction_plots.py -i collated_adiv/ -m mapping_file.txt -o plots/ -d 180 -
g pdf

50. alpha_diversity.py -i master_shared_rarefied_otu_table.biom -o
goods_coverage.txt -m goods_coverage,observed_otus

Miscellaneous information regarding bioinformatics:

- A. To run R scripts, may need to run `chmod 775 path_to_script`
- B. To execute R, `./name_of_script`
- C. Qiime scripts can be found on <http://qiime.org/scripts/>

R scripts

D. Normalization

```
#!/usr/bin/Rscript
#H. Paz
#December 2015
#Normalize OTU table
```



```
#chmod 775 /Volumes/allie_backup/Allie/Yr1_Archaea/11-22-2015/otu_table_normalization.R -will change for file location
#Rscript /Volumes/allie_backup/Allie/Yr1_Archaea/11-22-2015/otu_table_normalization.R -will change for file location
#For R, header line CANNOT have a # at the beginning, does not recognize it and will jump over it to the next line
#For R, output header line must start with a letter so it will input a letter automatically
```

```
#write directory you want to work in within ""
#the directory in which your output will go
setwd("/Volumes/allie_backup/Allie/Yr1_Archaea/11-22-2015/")
```

```
#path you are loading your OTU table from within ""
#the location of your file that you are wanting to use
#a .txt file
otu_table <- read.table("/Volumes/allie_backup/Allie/Yr1_Archaea/11-22-2015/master_shared_otu_table.txt", header = T, sep = "\t")
```

```
#OTU ID is the first column but consensus lineage (or taxonomy) is not, thus change accordingly
#makes rows in file the otu id
#this number is the column number of consensus lineage location
#identifies location of CL and puts everything before that column into a variable
OTU.ID <- otu_table[,1]
taxonomy <- otu_table[,501]
samples_data <- otu_table[,-501]
```

```
row.names(samples_data) <- samples_data$OTU.ID
samples_data <- samples_data[, -1]
samples_data_trans <- as.data.frame(t(samples_data))
samples_propor <- samples_data_trans/rowSums(samples_data_trans)
samples_propor_trans <- as.data.frame(t(samples_propor))
```

```
#write your file name in the option file = within the ""
#name file output
OTU_proportion <- data.frame(OTU.ID, samples_propor_trans, taxonomy)
write.table(OTU_proportion, file =
"master_shared_otu_table_normalized_03092016.txt", sep = "\t", row.names = F,
col.names = T, quote = F)
```

E. Beta_diversity PairWise Comparisons

```
#!/usr/bin/Rscript
```

```
#C. L. Anderson
```

```
args <- commandArgs(trailingOnly = TRUE)
```

```
if(length(args)!=5){
  writeLines("\n\nWrong number of arguments supplied. Provide the following
arguments in this order: \n\npath_to_distance_matrix \npath_to_mapping_file
\nname_of_1st_factor_column_in_mapping_file,
\nname_of_2nd_factor_column_in_mapping_file,
\nname_of_baseline_treatment_in_mapping_file_for_pw_comparisons
\n\nPairwise comparisons will be run on factor1. \n\nExample:
./beta_diversity.R allie/unweighted_unifrac_dm.txt allie/Yr1_Arch_mapping.txt
Diet Animal 1Common\n\n")
  quit()
}
```

```
dm_file <- args[1]
mapping_file <- args[2]
treatment_column <- args[3]
id_column <- args[4]
baseline <- args[5]
```

```
require(vegan)
require(spaa)
```

```
dm <- read.table(dm_file, sep = "\t", header = TRUE)
map <- read.table(mapping_file, sep = "\t", header = TRUE, comment.char = "")
```

```
row.names(dm) <- dm$X
dm <- dm[, -1]
map_sub <- map[map$X.SampleID %in% row.names(dm), ]
```

```
map_sub_dim <- dim(map_sub)
dm_dim <- dim(dm)
if (!(map_sub_dim[1] == dm_dim[1])) {
  writeLines("\n\nNumber of samples in the subset mapping file do not
match the number in the distance matrix. Likely missing samples in provided
mapping file.\n\n")
  quit()
}
```

```
colnames(map_sub)[which(names(map_sub) == treatment_column)] <-
"Treatment"
```

```

colnames(map_sub)[which(names(map_sub) == id_column)] <- "ID"
map_sub$ID <- as.character(map_sub$ID)
map_sub$Treatment <- as.character(map_sub$Treatment)
map_sub <- map_sub[ order(match(map_sub$X.SampleID, row.names(dm))), ]
dm <- as.dist(dm)

```

```

adonis_out <- adonis(dm ~ Treatment + ID, permutations = 999, data = map_sub)
capture.output(adonis_out, file = "beta_div_global_output.txt", append = FALSE)

```

```

sink("beta_div_pw_output.txt", append=FALSE)
cat('Baseline:',baseline,'\ \n\n\n')
sink()
dm_list <- dist2list(dm)
map_base <- map_sub[map_sub$Treatment == baseline, ]
if ((nrow(map_base) < 1)) {
  cat("Error: Make sure you actually used treatments listed in the mapping file
  columns provided.\n\n")
  quit()
}
base <- dm_list[dm_list$row %in% map_base$X.SampleID,]
base <- base[!base$value == 0,]
base$col <- as.character(base$col)
base$row <- as.character(base$row)
for (i in 1:nrow(base)) {
  resort <- sort(c(base$col[i],base$row[i]))
  base$col[i] <- resort[1]
  base$row[i] <- resort[2]
}
base <- unique(base)
names(map_sub)[names(map_sub) == "X.SampleID" ] <- "col"
base <- merge(base, map_sub, by="col")
wilcox_out <- pairwise.wilcox.test(base$value,base$Treatment, p.adj = "holm")
capture.output(wilcox_out, file = "beta_div_pw_output.txt", append = TRUE)

```

```

#####
#pw_names <- unique(map_sub$Treatment)
#dm_list <- dist2list(dm)
#
#sink("adonis_pw_output.txt", append=FALSE)
#cat('Baseline:',baseline,'\ \n\n\n')
#sink()
#
#pw_func <- function(x) {

```

```

#   single_combo <- unlist(x)
#   first_treatment <- single_combo[1]
#   second_treatment <- single_combo[2]
#   map_first <- map_sub[map_sub$Treatment == first_treatment, ]
#   map_second <- map_sub[map_sub$Treatment == second_treatment, ]
#   map_base <- map_sub[map_sub$Treatment == baseline, ]
#
#   map_first$X.SampleID <- as.character(map_first$X.SampleID)
#   map_second$X.SampleID <- as.character(map_second$X.SampleID)
#   map_base$X.SampleID <- as.character(map_base$X.SampleID)
#   dm_list$row <- as.character(dm_list$row)
#   dm_list$col <- as.character(dm_list$col)
#
#   first_base <- dm_list[(dm_list$col %in% map_first$X.SampleID &
dm_list$row %in% map_base$X.SampleID),]
#   first_base <- first_base[!first_base$value == 0,]
#
#   second_base <- dm_list[(dm_list$col %in% map_second$X.SampleID &
dm_list$row %in% map_base$X.SampleID),]
#   second_base <- second_base[!second_base$value == 0,]
#
#   if (first_treatment == baseline) {
#       for (i in 1:nrow(first_base)) {
#           resort <- sort(c(first_base$col[i],first_base$row[i]))
#           first_base$col[i] <- resort[1]
#           first_base$row[i] <- resort[2]
#       }
#       first_base <- unique(first_base)
#   }
#
#   if (second_treatment == baseline) {
#       for (i in 1:nrow(second_base)) {
#           resort <- sort(c(second_base$col[i],second_base$row[i]))
#           second_base$col[i] <- resort[1]
#           second_base$row[i] <- resort[2]
#       }
#       second_base <- unique(second_base)
#   }
#
#   if ((nrow(first_base) < 1)) {
#       cat("\n\n",first_treatment)
#       cat(" Treatment has 0 observations. Make sure you actually used
treatments listed in the mapping file columns provided.\n\n")
#       quit()

```

```

#     }
#
#     if ((nrow(second_base) < 1)) {
#       cat("\n\n",second_treatment)
#       cat(" Treatment has 0 observations. Make sure you actually used
treatments listed in the mapping file columns provided.\n\n")
#         quit()
#     }
#
#     adonis_pw <- wilcox.test(first_base$value, second_base$value, p.adj =
"fdr")
#
#     sink("adonis_pw_output.txt", append=TRUE)
#     cat(first_treatment, second_treatment,':\n')
#     sink()
#
#     capture.output(adonis_pw, file = "adonis_pw_output.txt", append =
TRUE)
#}
#
#combn(pw_names, 2, simplify = FALSE, FUN = pw_func)

```

F. Heatmap

```

#!/usr/bin/Rscript

#chmod 775 /Volumes/allie_backup/Allie/Yr1_Archaea/11-22-
2015/TimeID_split/heatmap.R
#Rscript /Volumes/allie_backup/Allie/Yr1_Archaea/11-22-
2015/TimeID_split/heatmap.R

setwd("/Volumes/allie_backup/Allie/Yr1_Archaea/11-22-2015/TimeID_split")

require(gplots)
require(vegan)
require(Heatplus)
require(RColorBrewer)

#Modify taxonomy column
otus_table <- read.table("87DRCNRConvRCon_filtered_otus_family.txt", header
= T, sep = "\t", fill = TRUE)
otus_table$taxonomy <- sub("f__", "", otus_table$taxonomy)
otus_table$taxonomy <- sub("\\]", "", otus_table$taxonomy)
otus_table$taxonomy <- sub("\\[", "", otus_table$taxonomy)

```

```
otus_table$taxonomy <- sub("^$", "No Assigned Family", otus_table$taxonomy)
```

```
#Change samples names
```

```
colnames(otus_table) <- c("OTUs", "8867.6.87DRCNoRumControl",
  "8824.6.87DRCNoRumControl", "8861.6.87DRCNoRumControl",
  "8839.6.87DRCNoRumControl", "8842.7.87DRCNoRumControl",
  "8840.7.87DRCNoRumControl", "8875.7.87DRCNoRumControl",
  "8869.7.87DRCNoRumControl", "8795.7.87DRCNoRumControl",
  "8862.7.87DRCNoRumControl", "8806.6.87DRCRumControl",
  "8873.6.87DRCRumControl", "8813.6.87DRCRumControl",
  "8808.6.87DRCRumControl", "8796.7.87DRCRumControl",
  "8881.7.87DRCRumControl", "8878.7.87DRCRumControl",
  "8792.7.87DRCRumControl", "8848.7.87DRCRumControl",
  "taxonomy")
```

```
#excel row numbers get replaced with OTU id number (OTUs) without removing
the OTUs column
```

```
row.names(otus_table) <- otus_table$OTUs
```

```
#remove OTUs column
```

```
otus_table <- otus_table[, -1]
```

```
#taxonomy set
```

```
tax_set <- subset(otus_table, select = c(taxonomy))
```

```
#samples set
```

```
samples_set <- otus_table[, -20]
```

```
#transpose
```

```
samples_trans <- as.data.frame(t(samples_set))
```

```
#color of the heatmap
```

```
scalewhiteblack <- colorRampPalette(c("white", "black"), space = "rgb")(100)
```

```
# determine the maximum relative abundance for each column
```

```
maxab <- apply(samples_trans, 2, max)
```

```
#head(maxab)
```

```
# remove the family with less than 2% as their maximum relative abundance
```

```
n1 <- names(which(maxab < 0.0000001))
```

```
data_abun <- samples_trans[, -which(names(samples_trans) %in% n1)]
```

```
#Generates taxonomy classification brackets on the left of the heatmap
(dendogram)
```

```
data.dist <- vegdist(data_abun, method = "bray")
```

```
row.clus <- hclust(data.dist, "aver")
```

```
#Generates taxonomy classification brackets on the top of the heatmap
(dendrogram)
data.dist.g <- vegdist(t(data_abun), method = "bray")
col.clus <- hclust(data.dist.g, "aver")

data_abun_trans <- as.data.frame(t(data_abun))
merge_data <- merge(data_abun_trans, tax_set, by = "row.names")
row.names(merge_data) <- merge_data$Row.names
merge_data <- merge_data[, -1]
data_abun_fam <- subset(merge_data, select = c(taxonomy))

png("heatmap_87DRCNRConvRCon.png", height = 6, width = 9, units = "in", res
= 300)
heatmap.2(as.matrix(data_abun), Rowv = as.dendrogram(row.clus), Colv =
as.dendrogram(col.clus),
          col = scalewhiteblack, margins = c(12, 15), trace = "none", density.info =
"none",
          labCol = data_abun_fam$taxonomy, xlab = "Family", ylab = "Samples",
lhei = c(2, 8))
dev.off()
```

APPENDIX II

Table 1. Composition of growing diets containing high or low-quality forage, 0 or 40% modified distillers grains plus solubles, and presence or absence of monensin (DM basis; Exp. 1).

Item	High-quality Forage ¹				Low-quality Forage ²	
	0 MDGS ³		40 MGDS		40 MGDS	
	+ Mon ⁴	- Mon ⁵	+ Mon	- Mon	+ Mon	- Mon
Alfalfa hay	57	57	33	33	0	0
Sorghum silage	38	38	22	22	0	0
Corn stalks	0	0	0	0	55	55
MDGS	0	0	40	40	40	40
Supplement ⁶						
Fine Ground						
Corn	4.523	4.535	3.968	3.980	3.412	3.424
Limestone	-	-	0.556	0.556	1.111	1.111
Salt	0.300	0.300	0.300	0.300	0.300	0.300
Tallow	0.100	0.100	0.100	0.100	0.100	0.100
Trace Mineral ⁷	0.050	0.050	0.050	0.050	0.050	0.050
Vitamin A-D-E ⁸	0.015	0.015	0.015	0.015	0.015	0.015
Rumensin-90 ⁹	0.012	-	0.012	-	0.012	-
Diet TDN ¹⁰	51.9	51.9	73.2	73.2	66.9	66.9

¹ High-quality forage = 60:40 blend of alfalfa hay and sorghum silage.

² Low-quality forage = ground corn stalks.

³ MDGS = modified distillers grains plus solubles.

⁴ + Mon = Diets containing monensin, formulated to provide 200 mg/steer daily.

⁵ - Mon = Diets containing no monensin.

⁶ Supplement formulated to be fed at 5% diet DM.

⁷ Premix contained 6.0% Zn, 5.0% Fe, 4.0% Mn, 2.0% Cu, 0.29% Mg, 0.2% I, 0.05% Co.

⁸ Premix contained 30,000 IU vitamin A, 6,000 IU vitamin D, 7.5 IU vitamin E per gram.

⁹ Premix contained 198 g/kg monensin.

¹⁰ TDN calculated based on values from NRC (2000) and Ahern et al. (2011).

Dietary table reproduced from Pesta et al., 2014.

Table 6. Effects of forage quality and monensin on methane emissions and VFA profile in diets containing 40% modified distillers grains plus solubles (Exp. 1).

Item	HQ forage ¹		LQ forage ²		SEM	P-value ³		
	+ Mon ⁴	- Mon ⁵	+ Mon	- Mon		Forage	Mon	Forage*Mon
CH ₄ :CO ₂	0.101 ^a	0.101 ^a	0.083 ^b	0.101 ^a	0.003	<0.01	<0.01	<0.01
CH ₄ , L/d ⁶	345 ^a	345 ^a	166 ^c	213 ^b	10	<0.01	0.03	0.03
CH ₄ , L/kg DMI ⁶	33.6 ^a	34.8 ^a	26.6 ^b	32.5 ^a	1.1	<0.01	<0.01	0.04
CH ₄ , L/kg ADG ⁶	215	237	221	267	16	0.26	0.03	0.43
CO ₂ , L/d ⁶	3447	3405	1997	2116	88	<0.01	0.66	0.37
Acetate, mol %	66.9	67.3	70.8	70.8	0.6	<0.01	0.73	0.69
Propionate mol %	17.7	17.1	17.8	17.9	0.3	0.20	0.51	0.24
Butyrate, mol %	8.6	9.7	5.8	6.6	0.2	<0.01	<0.01	0.54
Acetate:Propionate	3.81	3.97	4.01	3.96	0.09	0.30	0.54	0.24

¹ HQ forage = 60:40 blend of alfalfa hay and sorghum silage.

² LQ forage = ground corn stalks.

³ Forage= main effect of forage quality, Mon= main effect of presence of Monensin, Forage*Mon= effect of interaction between forage quality and Monensin

⁴ + Mon = Diets containing monensin, formulated to provide 200 mg/steer daily.

⁵ - Mon = Diets containing no monensin.

⁶ Calculated values based on the equation of Madsen et al. (2010).

^{a,b,c} Means in a row with different superscripts are different ($P < 0.05$).

Dietary table reproduced from Pesta et al., 2014.

Table 7. Effects of MDGS¹ level and monensin on methane emissions and VFA profile in high quality forage diets (Exp 1).

Item	0 MDGS		40 MDGS		SEM	P-value ²		
	+ Mon ³	- Mon ⁴	+ Mon	- Mon		MDGS	Mon	MDGS*Mon
CH ₄ :CO ₂	0.101	0.104	0.101	0.101	0.002	0.52	0.54	0.79
CH ₄ , L/d ⁵	224	223	345	345	12	<0.01	0.95	0.99
CH ₄ , L/kg DMI ⁵	25.3	25.0	33.6	34.8	0.7	<0.01	0.49	0.34
CH ₄ , L/kg ADG ⁵	256	238	215	237	10	0.05	0.86	0.06
CO ₂ , L/d ⁵	2210	2153	3447	3405	113	<0.01	0.66	0.95
Acetate, mol %	71.3	72.8	66.8	67.2	0.5	<0.01	0.04	0.23
Propionate, mol %	15.2	14.5	17.7	17.0	0.4	<0.01	0.11	0.98
Butyrate, mol %	8.4 ^b	7.9 ^b	8.7 ^b	9.7 ^a	0.3	<0.01	0.33	<0.01
Acetate:Propionate	4.78	5.05	3.81	3.99	0.12	<0.01	0.06	0.70

¹ MDGS = modified distillers grains plus solubles.

² MDGS= main effect of MDGS inclusion level, Mon= main effect of presence of Monensin, MDGS*Mon= effect of interaction between level of MDGS and Monensin

³ + Mon = Diets containing monensin, formulated to provide 200 mg/steer daily.

⁴ - Mon = Diets containing no monensin.

⁵ Calculated values based on the equation of Madsen et al. (2010).

^{a,b} Means in a row with different superscripts are different ($P < 0.05$).

Dietary table reproduced from Pesta et al., 2014.

Table 1. Composition of finishing diets varying in fat source and monensin content (DM basis; Exp. 1).

Ingredient	0 MDGS ¹		50 MGDS		Corn Oil	Tallow
	+ Mon ²	- Mon ³	+ Mon	- Mon	+ Mon	+ Mon
Dry-rolled corn	87	87	37	37	84	84
MDGS	-	-	50	50	-	-
Sorghum silage	8	8	8	8	8	8
Corn oil	-	-	-	-	3	-
Tallow	-	-	-	-	-	3
Supplement⁴						
Fine Ground Corn	1.669	1.686	1.734	1.751	1.669	1.669
Urea	1.500	1.500	-	-	1.500	1.500
Limestone	1.315	1.315	2.750	2.750	1.315	1.315
Salt	0.300	0.300	0.300	0.300	0.300	0.300
Tallow	0.125	0.125	0.125	0.125	0.125	0.125
Trace Mineral ⁵	0.050	0.050	0.050	0.050	0.050	0.050
Vitamin A-D-E ⁶	0.015	0.015	0.015	0.015	0.015	0.015
Rumensin-90 ⁷	0.017	-	0.017	-	0.017	0.017
Tylan-40 ⁸	0.009	0.009	0.009	0.009	0.009	0.009
Diet TDN ⁹	84.6	84.6	84.7	84.7	85.4	85.4

¹ MDGS = modified distillers grains plus solubles.

² + Mon = Diets containing monensin, formulated to provide 375 mg/steer daily (Elanco Animal Health, Greenfield, IN).

³ - Mon = Diets containing no monensin.

⁴ Supplement formulated to be fed at 5% diet DM.

⁵ Premix contained 6.0% Zn, 5.0% Fe, 4.0% Mn, 2.0% Cu, 0.29% Mg, 0.2% I, 0.05% Co.

⁶ Premix contained 30,000 IU vitamin A, 6,000 IU vitamin D, 7.5 IU vitamin E per gram.

⁷ Premix contained 198 g/kg monensin (Elanco Animal Health, Greenfield, IN).

⁸ Premix contained 88 g/kg tylosin (Elanco Animal Health).

⁹ TDN calculated based on values from NRC (2000).

Dietary table reproduced from Pesta et al., 2015.

Table 6. Effect of source of dietary fat in the finishing diet on methane production and ruminal VFA profile (Exp. 1).

Item	Treatment ¹				SEM	P-value
	CON	MDGS	OIL	TAL		
CH ₄ :CO ₂	0.047 ^b	0.058 ^a	0.054 ^{ab}	0.049 ^b	0.003	0.07
L CH ₄ /d ²	227	270	249	221	18	0.21
L CO ₂ /d ²	4774	4654	4633	4521	130	0.60
L CH ₄ /kg DMI ²	20.1	24.5	22.3	20.9	1.3	0.13
L CH ₄ /kg ADG ²	141.3	173.7	148.1	149.0	12.1	0.27
Acetate, mol/100 mol	45.2	48.5	45.1	46.4	1.9	0.57
Propionate, mol/100 mol	40.3	36.4	42.7	39.9	2.1	0.22
Butyrate, mol/100 mol	8.1	8.2	6.1	7.3	1.1	0.45
Acetate:Propionate	1.21	1.40	1.08	1.20	0.13	0.42

¹ CON = corn-based diet with no added fat, MDGS = diet containing 50% modified distillers grains plus solubles, OIL = corn-based diet with 3% corn oil, TAL = corn-based diet with 3% tallow.

² Values calculated using equation of Madsen et al. (2010).

^{a,b} Means in a row with different superscripts are different ($P < 0.10$).

Dietary table reproduced from Pesta et al., 2015.

Table 7. Effect of diet type and presence of monensin on methane production and ruminal VFA profile (Exp. 1).

Item	0 MDGS		50 MDGS		SEM	P-value ¹		
	+ Mon ²	- Mon ³	+ Mon	- Mon		Diet	Mon	D *M
CH ₄ :CO ₂	0.047	0.053	0.058	0.056	0.003	0.03	0.56	0.19
L CH ₄ /d ⁴	227	247	270	260	18	0.12	0.77	0.41
L CO ₂ /d ⁴	4774	4610	4654	4780	167	0.87	0.90	0.37
L CH ₄ /kg DMI ⁴	20.1	22.5	24.5	22.5	1.3	0.10	0.81	0.11
L CH ₄ /kg ADG ⁴	141.3 ^b	164.0 ^{ab}	173.7 ^a	149.9 ^{ab}	13.8	0.49	0.97	0.08
Acetate, mol/100 mol	45.2	44.1	48.5	45.3	1.9	0.23	0.24	0.57
Propionate, mol/100 mol	40.3	41.7	36.4	40.2	2.1	0.20	0.20	0.56
Butyrate, mol/100 mol	8.1	7.3	8.2	7.6	1.0	0.85	0.46	0.91
Acetate:Propionate	1.21	1.10	1.40	1.14	0.12	0.34	0.12	0.56

¹ P-value: Diet = main effect of diet (0 or 50% MDGS), Mon = main effect of presence of monensin (Elanco Animal Health, Greenfield, IN), D*M = effect of interaction between diet type and monensin.

² + Mon = Diets containing monensin, formulated to provide 375 mg/steer daily.

³ - Mon = Diets containing no monensin.

⁴ Values were calculated using equation of Madsen et al. (2010).

^{a,b} Means in a row with different superscripts are different ($P < 0.10$).

Dietary table reproduced from Pesta et al., 2015.