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UNDERSTANDING RUMEN MICROBIAL COMMUNITY STRUCTURE AND FUNCTION TOWARDS DECREASING METHANE EMISSIONS

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UNDERSTANDING RUMEN MICROBIAL COMMUNITY STRUCTURE AND FUNCTION TOWARDS DECREASING METHANE EMISSIONS

by

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UNDERSTANDING RUMEN MICROBIAL COMMUNITY STRUCTURE AND FUNCTION TOWARDS DECREASING METHANE EMISSIONS

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Ruminants are vital to feeding an emerging population. Resource use is continually being limited due to this growth, therefore, production of high-quality animal protein sources, such as meat and milk, are challenged. Meeting the demands for these consumables, while utilizing fewer resources, is a task the livestock industry is currently facing. The rumen microbiome is extensive and serves to provide several metabolic requirements for the animal for growth. Recently, a significant amount of research is being driven towards understanding the rumen microbiome due to its large effect on metabolic requirements.

A study was conducted to replace alfalfa with nonforage fiber sources in dairy cows. It was determined milk yield and intake are maintained when nonforage fibers replace forage sources, while decreasing methane levels. Water consumption decreased when cows were fed a straw and dried distillers grains and solubles mixture in replacement of alfalfa. The microbial community observed no differences in alpha diversity measures, despite the abundance of some taxa being correlated with methane production. These dietary treatments do not alter microbial community composition to determine performance differences. Examination into substrate production by these

microbes may provide insight into how energy is diverted in dairy cows fed nonforage fiber sources.

A metagenomic analysis was conducted characterizing the genomic capacity within the microbial community in beef cattle fed diets based on forage quality to evaluate methane mechanisms within the microbial community. In high-quality forage diets, the propionate pathway becomes enhanced, acting as a hydrogen sink for methanogenesis. Betaproteobacteria genes were identified to be present in the propionate pathway, which becomes enhanced in high-quality forage-based diets, indicating a syntrophic relationship may be occurring to reduce methane emissions in beef cattle.

Keywords: beef cattle, dairy cow, nonforage fiber, metagenomic, methane, rumen microbiome

Dedication

I dedicate this work to my family and to the graduate students who helped in these experiments.

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“when will you be done?”, well Dad, I am finally done. I wish you were here to see it and celebrate. I know you are with me in spirit.

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DISSERTATION INTRODUCTION

Resource availability for cattle production is becoming increasingly limited due to the increase in human global population growth. One challenge of the livestock industry is to produce high-quality meat and milk products to sustain this growing population. Cattle are viewed as a significant source of greenhouse gas production (Johnson and Johnson, 1995; Moss et al., 2000). Rumen microbes play a pivotal role in the degradation of plant matter as well as providing energy substrates for the animal (Flint, 2004). Characterizing how the rumen microbial community adapts to changes in dietary composition, as well as their ability to impact feed efficiency in cattle represents a knowledge gap that may provide answers to maximizing returns on meat and milk production.

Conversion of consumed feed into tissue (meat) and milk components determines feed efficiency in cattle. Historically, investigation into feed efficiency in beef cattle focused primarily on host genetics, but primarily made cattle bigger and not more efficient. However, these traits may have moderate heritability, and may impede efforts to improve feed efficiency due to genomic selection (Koch et al., 1963; Archer et al., 1999; Snelling et al., 2011; Berry and Crowley, 2013; Lu et al., 2013; Seabury et al., 2017). With the rumen microbe's role in enteric fermentation and energy production, evaluating the extent of the microbial community on feed efficiency measures may provide opportunities to improve feed efficiency.

Enteric fermentation produces methane (CH_4) from a select group of rumen microbes (Janssen and Kirs, 2008). Methane represents an energetic loss up to 12% of energy intake (Johnson and Johnson, 1995), impacting feed efficiency. This molecule is

also implicated in greenhouse gas (GHG) emissions and influencing climate change, making ruminants a unique species to examine. However, with land diminishing in availability and together with rainwater droughts, feeding cattle nontraditional feed ingredients, such as coproducts (Klopfenstein et al., 2008; Foth et al., 2015) is a management strategy to help the industry to circumvent these issues. These coproducts are produced as a waste product from traditional crops grown for human needs. It is unclear how the rumen microbiome is altered in structure and composition, as well as the impact on energy utilization, by these feed ingredients. Opportunities are available to examine how utilizing these coproducts in dairy rations impacts methanogenesis and energy partitioning.

To elucidate solutions for improvement in dairy cattle energy partitioning and microbial metabolic routes of methane production in beef cattle, this dissertation contains three objectives:

1) determine how the replacement of high-quality forage (alfalfa) with a mixture of straw and coproducts influences energy utilization
2) characterize the effect of replacement of forages with coproducts on the microbial community to better understand methane production
3) identify the functional profile of the bacterial community based on forage quality to elucidate impact on CH_4 production.

CHAPTER 1

REVIEW OF LITERATURE

Introduction

Providing high-quality protein sources to feed the increasing global population is a current challenge for the livestock production sector. The world population is anticipated to exceed 9 billion people in the next 30 years (FAO, 2006). The ability to meet this anticipated demand for meat and milk products while resources such as land and water are the same or less, requires the beef and dairy sectors to improve efficiency of feed use for animal production. One method of answering this concern is by using dietary interventions to improve animal efficiency as well as using rumen microbiome manipulations to improve production measures. Increasing production efficiency through dietary strategies allows for increased animal products for consumers while minimizing the carbon footprint of the livestock sector.

Methane (CH_4) is a greenhouse gas (GHG) that contributes to global warming (Moss et al., 2000) with a designated global warming potential of 25, meaning that CH_4 traps solar radiation heat 25 times more efficiently than carbon dioxide over a century (IPCC, 2007). Methane is produced in a variety of anaerobic environments including deep ocean thermal vents, soil, and termites (Liu and Whitman, 2008). Agriculture sector in general is often criticized for its contribution to GHG emissions. The Environmental Protection Agency (2018) stated that agriculture contributes nearly 10% of all U.S. GHG emissions coming from human sources and is the most significant contributor of CH_4 emissions in the U.S. Most of the anthropogenic CH_4 emissions attributed to agricultural

production is traced to enteric fermentation in cattle by rumen microbes. Methane production in cattle constitutes an energetic loss to the animal, limiting energy availability to the animal. As the rumen microbes are the engine of enteric fermentation, targeting this group of microbes to develop novel intervention strategies to decrease CH_4 production and improve feed efficiency will provide new opportunities to improve performance using microbiome manipulation. This knowledge can be applied to operations to allow for increased efficiency while minimizing energetic losses.

Members of the Rumen Microbial Community

The rumen microbial community constitutes bacteria, archaea, protozoa, fungi, and bacteriophages (Morgavi et al., 2010) thus representing every domain of life. These organisms work collectively to breakdown complex carbohydrate plant polymers, proteins, and other organic material. This microbial community and its interactions play an important role in complex carbohydrate degradation and rumen ecosystem efficiency. As cattle are categorized as pre-gastric fermenters, the rumen microbial community is responsible for generating approximately 70% of the energetic needs for the animal based on the diet consumed (Flint, 2004).

Bacteria

The predominant microbes in the rumen are bacterial in origin, with counts estimating from $10^9 – 10^{11}$ cells per mL of rumen fluid (Jouany and Ushida, 1999). This faction of microbes is highly diverse (Cai et al., 2010), encompassing several taxonomic lineages. Bacteria are considered primary fermenters as they outnumber other populations

of microbes. Broadly, rumen microbes are classified based on location found in the rumen, which includes free-floating microbes and microbes adhered to either other microbial cells or feed particles (Nagaraja, 2016). The free-floating microbial component accounts for approximately 30% of the bacterial population, while the adherent bacterial population constitutes the majority of the rumen bacteria at nearly 70% (Nagaraja, 2016). The complex organic material that enters the rumen is digested by bacteria, protozoa, and fungi into volatile fatty acids (VFAs), CO_2, and H_2 (Morgavi et al., 2010). These microbes actively digest the plant carbohydrates into oligomers and monomers for utilization. Cross-feeding of metabolic end-products helps microbes that lack the enzymes to break down complex carbohydrates to still obtain energy from partially digested organic matter. Therefore, for efficient digestion of complex carbohydrates these microbes have developed specialized structures called cellulosomes that contain enzymes such as amylases, cellulases, proteases, and lipases (Huws et al., 2018). Principal cellulytic bacteria in the rumen include: *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes*, producing endoglucanases, exoglucanases, β-glucosidases, and hemicellulases (Flint et al., 2008). *Butyrivibrio fibrosolvens* and *Prevotella ruminicola* are two principal hemicellulose-digesting bacteria; however, they cannot ferment cellulose, but instead convert xylan and pectin into soluble sugars for their substrate requirements (Bryant et al., 1958; Dehority, 2003).

Select bacteria members have developed cellulosomes, a multi-enzyme complex that increases the digestion of plant structural carbohydrates (Artzi et al., 2017). This specialized structure is present in a select set of bacteria to improve enzymatic digestion of complex carbohydrates under anaerobic conditions. These cellular structures provide

the ability to separate cellulytic components enabling increased surface area access for other enzymes and microbes (Artzi et al., 2017). There are two types of components enabling attachment to cellulose material: dockerin enzymes and cohesion-containing structural proteins. These two protein categories bind tightly to one another and facilitate the inclusion of this unit onto cellulose particles to enhance digestion (Artzi et al., 2017). The most complex cellulosome characterized to date originates from *R. flavefaciens*, with a complex containing glycosidic hydrolases, carbohydrate esterases, and polysaccharide lyases (Fontes and Gilbert, 2010).

Archaea

Methanogens, a specialized group of microbes inhabiting the rumen, belong to the Domain *Archaea* (Janssen and Kirs, 2008; Hook et al., 2010). Archaeal community members are found in diverse environments from ocean thermal vents, ice glaciers, and marshes (Kurr et al., 1991; Franzmann et al., 1992; Franzmann et al., 1997; Hedderich and Whitman, 2006). One commonality between the archaeal members in these environments is their ability to produce CH_4. Methanogens, produce CH_4 as a byproduct of their own metabolic processes, yet, this trait is not exhibited among all archaeal members (Whitford et al., 2001; Liu and Whitman, 2008). As humans domesticated ruminant species for food consumption, the CH_4 produced by these animals is considered anthropogenic CH_4 (EPA, 2018). Rumen methanogens are strict anaerobes that comprise less than 4% of the total microbial 16S rDNA in the rumen (Janssen an Kirs, 2008). The production of CH_4 by this select group enables healthy rumen function by recycling energy equivalents (Moss et al., 2000). Rumen methanogens are found in virtually all

areas of the rumen, including free floating in the fluid, attached to particulates and protozoa, and attached with the rumen epithelium (Janssen and Kirs, 2008).

Currently, seven orders of methanogens have been identified by scientists (Borrel et al., 2014): Methanococcales, Methanopyrales, Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanocellales, and more recently Methanomassiliicoccales. Members of Methanopyrales, Methanococcales, and Methanobacteriales are classified as hydrogenotrophs (Lang et al., 2015), reducing CO_2 to CH_4 via H_2 or utilizing one-carbon compounds, such as formate, as electron donors (Liu and Whitman, 2008). This pathway is also found in the orders Methanomicrobiales and Methanocellales (Lang et al., 2015). Members that utilize these substrates have been known to lack cytochromes (Morgavi et al., 2010).

Protozoa

The ruminal protozoal community accounts for 50% of the rumen biomass (Williams and Coleman, 1997). These organisms are eukaryotic in origin, possessing a 18s rRNA subunit (Newbold et al., 2015). Protozoa are known to impact fermentation. In spite of this their influence on nutrition remains relatively unclear (Newbold et al., 2015). Protozoal members are known to engulf starch granules, aiding in digestion and animal health (Jouany and Ushida, 1999). In cattle fed high amount of concentrates, metabolic issues can arise creating severe health issues in these animals, such as acidosis (Nagaraja and Titgemeyer, 2007). Normally during fermentation, substrate availability to the microbes can influence VFA production and absorption by the animal, and occurs at rates that maintain healthy rumen function. However, when cattle are fed large amounts of

readily fermentable carbohydrates, the production of VFAs occurs quickly, increasing the concentration, and removal from the rumen by absorption is greater due to increased protonation (undissociated form) due to a lowered rumen pH (Bergman, 1990; Nagaraja and Titgemeyer, 2007). Protozoa offer a means to control acidosis by engulfing starch particles to limit substrate for community degradation (Nagaraja and Titgemeyer, 2007).

Protozoa produce H_2 through complexes known as hydrogenosomes (Morgavi et al., 2010), contributing to inter-species hydrogen transfer (Hook et al., 2010). This process aids other organisms in the rumen requiring H_2 substrates by providing a source of H_2 to carry out other biological processes. One such process is methanogenesis. Archaeal members have been reported to be attached to protozoa (Hook et al., 2010; Huws et al., 2018). The production of H_2 from protozoa provides a source for archaea to utilize for the production of CH_4 (Huws et al., 2018). Additionally, the removal of protozoal populations from the rumen has been studied and discussed in a review from Newbold et al. (2015). While the presence of protozoa has shown beneficial aspects to nutrition, rumen protozoa are nonessential organisms to the rumen environment (Newbold et al., 2015), meaning the removal of this group does not impact the host animal. The process of protozoal removal by either chemical or physical techniques is called defaunation, and has been used as a tool to study the role of protozoa in ruminant nutrition (Boadi et al., 2004b; Newbold et al., 2015). With the removal of ruminal protozoal members, CH_4 production has been shown to be affected, often reducing methanogenesis (Hegarty et al., 1999a,b; Morgavi et al., 2010).

Fungi

This group of rumen inhabitants is eukaryotic in origin similar to protozoa and, prior to distinct discovery, fungi were thought to be a type of protozoa (Nagaraja, 2016). Broadly, ruminal fungi are delegated into two classifications: molds and yeasts (Nagaraja, 2016). Yeasts are single-celled organisms in the rumen while molds form complex filament linkages called hyphae (Nagaraja, 2016). Previous thought was that fungi cannot exist in the rumen as the rumen is an anaerobic environment (Krause et al., 2013). Recent advances in methodology have allowed for five genera and several species to be identified in several host species including cattle (Nagaraja, 2016). Fungi, while being minimal in mass (Nagaraja, 2016), are known for being extremely effective at fiber degradation due to their plethora of structural polymer-degrading enzymes (Soloman et al., 2016). Some enzymes possess the power to breach structural polymers (Orpin, 1977a,b), increasing the surface area for attachment of other microbial members for increased digestion (Huws et al., 2018). Fungal members have the capability of producing enzymes belonging to the categories of cellulases, hemicellulases, pectin lyases, amylases, and proteases (Nagaraja, 2016). Interesting to note, fungal degradation activity is enhanced in the presence of methanogens (Cheng et al., 2009). Despite this knowledge, the impact of the fungal community remains relatively elusive.

Bacteriophages

Viruses in the rumen comprise a unique and intriguing group of organisms that is attracting significant attention recently. In the rumen, viruses are mainly present in the form of phages, with the task of infecting bacteria. Phages are dense in population with

enumeration ranging from 10^7 to 10^9 particles per mL (Berg Miller et al., 2012). With the ability to attack and integrate their DNA into host bacterial cells, phages possess the ability to alter the bacterial community (Gilbert and Klieve, 2015). Phages have also been identified as a source for genetic repository. Phages that remain lysogenic, will infect a cell and pass along their genetic content to the host cell. This gene pool can become incorporated into the bacterial genome (Nagaraja, 2016). This contributes to increased genetic diversity and genomic capability within the microbial community. Phages can also enter into a lytic phase. During the lytic phase, replicated phages are released into the rumen environment. With phages being a repository for a gene pool, viral community research has become a lucrative yet challenging component to improving the knowledge of the relationship of phages to the microbial community structure and composition.

Investigating the Rumen Microbial Community

Since the turn of the century, microbial identification methods have greatly improved. These methods have allowed for a significant increase in the identification of new taxa and functional capability. High-throughput sequencing methods such as 16S rRNA amplification and metagenomic analyses provide a clearer and more accurate representation of the dynamics within the rumen, allowing for increased understanding how diet impacts the microbial community, and in turn, how the community impacts CH_4 production and efficiency. By knowing this information, diets can be formulated for optimal animal production measures, including feed efficiency and CH_4 production.

Pathways of Enteric CH_4 Production in the Rumen

A current global concern is the contribution of GHG to climate change. Livestock production, more specifically cattle production, receives significant negative attention on this issue due to cattle producing CH_4. In 2018, according the US EPA, agriculture accounted for 10% of total US greenhouse gas emissions. Of that 10%, approximately one third is attributed to enteric fermentation by cattle. While rumen methanogens produce CH_4 naturally, to the animal it represents an energetic loss ranging from 2-12% (Johnson and Johnson, 1995). Several reviews and novel investigative studies in beef and dairy cattle have investigated diet fermentation characteristics that influence CH_4 production (Johnson and Johnson, 1995; Boadi et al., 2004b; Guan et al., 2006; Beauchemin et al., 2008; Hook et al., 2010; Morgavi et al., 2010; Van Zijderveld, 2010; Carberry et al., 2012; Hristov et al., 2013; Poulsen et al., 2013; Knapp et al., 2014; Henderson et al., 2015; Haque, 2018). This research covers aspects of ruminant nutrition that can be altered or that induce alterations within fermentation characteristics and substrates availability. The factors studied at great length include feed intake and composition, forage quality, lipid supplementation, concentrate inclusion, ionophore addition, and alternative hydrogen sinks. Additionally, many of these elements have been studied to determine what concentration of addition is most beneficial. Changes in these aspects can alter rumen function and influence fermentation products. Some strategies mentioned above have produced varied results, such as ionophore addition (Guan et al., 2006) and lipid supplementation (Machmüller and Kreuzer, 1999). However, some methods listed above have resulted in decreases in CH_4 production, such as feeding higher quality forages or concentrates (Boadi et al., 2002; Johnson and Johnson, 1995).

As feed ingredients are altered or improved, characterization of how the microbial community is impacted by these alterations requires further investigation.

Biochemical pathways of methanogenesis in ruminants

The production of CH_4 by rumen microbes is a natural end-product, as it is the most effective way to rid the rumen of excess H^+ to maintain healthy function (Thauer, 1998; Janssen and Kirs, 2008; Liu and Whitman, 2008). Enteric CH_4 production occurs via three pathways: hydrogenotrophic, methylotrophic, and acetoclastic (Hedderich and Whitman, 2006; Liu and Whitman, 2008). These pathways benefit the microbial community by allowing for decreased competition for substrates and help to increase community diversity, while still eliminating excess H^+. These pathways also aid with the consumption of a variety of feed sources as a diverse community can break down a wide range of feeds and maintain healthy rumen function. The figure below is a schematic of the three methanogenesis pathways published in Hedderich and Whitman (2006), showing step by step how metabolically different substrates are converted into CH_4. Pathway labeled A represents CH_4 production via the hydrogenotrophic pathway. Pathway B is utilized when the substrate is acetate, and finally pathway C is used when the substrate available is a one-carbon methyl group such as methylamines and methanol. With an abundance of H_2 in the rumen, the predominant pathway utilized is the hydrogenotrophic pathway (A). Several steps depicted require the input of H_2 as an electron donor for the reduction of CO_2 into CH_4 (Hungate, 1967; Morgavi et al., 2010). In order for the reduction of CO_2 to CH_4 to occur, eight electrons are required and are acquired from the consumption of H_2, formate, or 2-propanol at four molecules each

(Hedderich and Whitman, 2006). Formate, while a one-carbon compound, is already in a reduced form and can be used by hydrogenotrophic methanogens as an electron donor (Hedderich and Whitman, 2006). McAllister and Newbold (2008) stated that the major methanogens in the rumen of cattle utilize H_2 and CO_2 for CH_4 production. In this pathway, four molecules of H_2 and formate are utilized (Hedderich and Whitman, 2006). The aceticlastic pathway (B) utilizes acetate as the substrate for metabolic energy needs. This pathway is used by methanogens belonging to the Methanosarcinales order (Liu and Whitman, 2008). However, this pathway is not used extensively by methanogens due to acetate being used by the host animal for its energy needs, as well as, the growth rate of

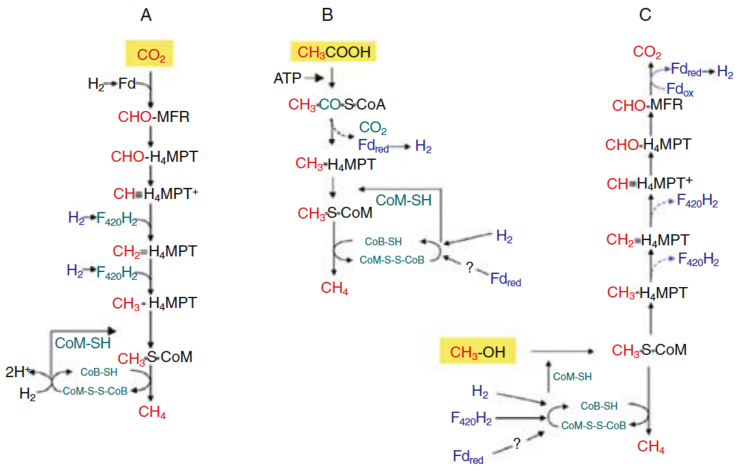

Figure 1. A reproduced schematic of the three pathways for methanogenesis from Hedderich and Whitman (2006). A – hydrogenotrophic pathway detailing how CO_2 is converted into CH_4. B – aceticlastic pathway showing the reaction of acetate conversion into CH_4. C – methanol and methylamine type substrates pathway indicating the steps for conversion of C_1 compounds and methylamine compounds into CH_4.

methanogens involved in aceticlastic methanogenesis is quite slow compared to the passage rate of contents in the rumen (Nagaraja, 2016). According to Hedderich and Whitman (2006), this metabolic pathway reduces acetate to CH_4 and CO_2 by splitting acetate into CH_4 and CO_2. The resulting methyl compound enters the pathway for one-

carbon compounds at the second-to-last step of this pathway. Finally, remaining pathway (C) uses methyl compounds as the substrate. Methyl compounds are sourced from methylamines, methanol (Morgavi et al., 2010) and other compounds of a methyl group bound to O, N, or S (Hedderich and Whitman, 2006).

The last three steps of all three pathways are similar. Hedderich and Whitman (2006) describe in great detail the molecular and chemical steps that occur in methanogenesis. Briefly, methyl-tetrahydromethanopterin (CH_3-H_4MPT), a derivative of H_4MPT, transfers the methyl-group to a thiol coenzyme called mercaptoethanesulfonate (CoM-SH, coenzyme M) forming a methylthioether (methyl-coenzyme M; CH_3-S-CoM), that is reduced to CH_4. This is the common intermediate in all pathways. The final step in all pathways involves methyl-coenzyme M reacting with coenzyme B (CoB-SH), a thiol, creating CH_4 and a heterodisulfide (CoM-S-S-CoB) acting as the terminal electron acceptor. In pathways A and B, CoM-SH converts CH_3-H_4MPT to CH_3S-CoM; however, in pathway C, CoM-SH converts methanol to CH_3S-CoM. Additionally, the reaction of CH_3S-CoM with a thiol coenzyme (coenzyme B, CoB-SH) forms a heterodisulfide (CoM-S-S-CoB) by CH_3S-CoM reductase (Mcr), the essential enzyme in methanogenesis. Coenzyme F420, acts as an electron donor like H_2, and is known for fluorescing at a wavelength of 420, giving off a blue-green appearance to the methanogens.

With the intermediates involved in methanogenesis, these steps may provide potential targets for methane mitigation. Providing dietary sources as terminal electron acceptors for methanogenesis redirection may serve as a viable method, that may target the last step of methanogenesis and the incorporation of sulfur (S). More detail can be

found in the following section for nutritional strategies of CH_4 mitigation under nitrate and sulfate.

The fermentative capacity within the rumen microbial community produces various fermentative products that are utilized by other microbial community members for growth in addition to the host animal. Methanogens are at the bottom of the complex food chain occurring in the rumen (Morgavi et al., 2010). The final process of methanogenesis serves to maintain efficient rumen function by regenerating energy equivalents utilized during glycolysis, the TCA cycle, and other energy-utilizing pathways (Moss et al., 2000; Morgavi et al., 2010). During glycolysis, hexose and pentose compounds from fiber or starch origins (Moss et al., 2000) are fermented imparting the rumen with reduced glycolytic cofactors such as NADH (Moss et al., 2000). In order for glycolysis to continue and complete sugar fermentation, NADH must be reoxidized back to NAD^+ (Moss et al., 2000). If this reaction does not occur, NADH levels accrue in the rumen and fermentation is reduced (Morgavi et al., 2010). In the rumen, this process occurs anaerobically, and therefore electron transfer to an electron acceptor other than oxygen is needed to regenerate NAD+ (McAllister and Newbold, 2008). End-products of rumen fermentation, such as volatile fatty acids (VFAs) can also be used as an electron acceptor (Ungerfeld, 2020). However, VFAs are not generally used as the terminal electron acceptor, as the process for oxidation into CO_2 and H_2 is extensive and inhibited by rumen turnover (Janssen and Kirs, 2008). The production of H_2 from fermentation impacts rumen pH and H_2 partial pressure (Hegarty and Gerdes, 1999; Morgavi et al., 2010). This too can impede rumen function and inhibit the regeneration of NAD+ (Moss et al., 2000; Morgavi et al., 2010). The production of CH_4

serves as the most efficient method of removing excess H+ without compromising rumen microbial function (Sharp et al., 1998).

Moss et al. (2000) and Janssen and Kirs (2008) suggest that a large portion of rumen methanogens belongs to a novel uncultured group of methylotrophic archaea. These archaeal members are suggested as a target for reduction of CH_4 in ruminants, and have yet to be cultured (Poulsen, et al., 2013). A majority of methylotrophic methanogenic archaea belong to the orders Methanosarcinales, Methanobacteriales, and Methanomassiliicoccales (Enzmann et al., 2018). Currently, the role and the impact of these substrates to CH_4 production is poorly understood (Morgavi et al., 2010). With increased sequencing capabilities and technical advances, investigation of these novel archaeal groups may provide new opportunities for CH_4 mitigation.

Nutritional Strategies for CH_4 Mitigation

It is well known that diet impacts the rumen microbial community (Carberry et al., 2012; Jami et al., 2014). Due to the multiple factors that intersect with diet (intake, additive inclusion, oil type and inclusion, forage type and inclusion), understanding the effect of dietary ingredients on the microbial community may provide insight into ways to mitigate CH_4 without compromising production. The most significant component of plant matter is cellulose (Weimer, 1996). Cellulose is a structural carbohydrate that is not digestible by mammals as they do not possess the enzymatic capability to break the β-1,4 glyosidic bonds of the glucose monomers that make up the crystalline structure of cellulose. Digestion of cellulose requires prokaryotic and fungal enzyme. The members of the rumen microbial community possess the enzymes need to digest plant

carbohydrates, allowing ruminants to utilize organic plant matter. Various factors regarding plant and dietary components alter the microbial community and affect CH_4 production by different routes. The chemical composition of feed ingredients available to producers for rations dictates the type and concentration of substrates produced, influencing VFA patterns, energy extracted from those feeds, as well as CH_4 produced (Knapp et al., 2014).

Effect of forage-based diets and quality on CH_4 production

Cellulytic rumen microflora are predominantly responsible for the digestion of cellulosic forage in the rumen; therefore, understanding how forage-based diets and forage quality alter the community will help the industry improve feed utilization by the rumen microflora and improve animal performance. Moe and Tyrell (1979) reported that fermentation of various carbohydrate types influences CH_4 production. Forage-based diets that are composed of higher amounts of cellulose, hemicellulose, and lignin produce higher amounts of acetate and butyrate when fermented by rumen microbes compared to concentrates (Moe and Tyrrell, 1979). One dietary component of forage-based diets that impacts CH_4 production levels is forage quality (Boadi et al., 2002). Higher-quality forage, such as immature plants and highly digestible fiber material, can reduce CH_4 levels by improving digestibility and passage rate due to lesser amounts of structural carbohydrates (Beever et al., 1986). Typically, more soluble forages have increased digestibility, whereas mature forages have decreased digestibility, which favors CH_4 production (Milich, 1999). Increasing feed quality can have positive results of increased feed efficiency and animal performance and decreased CH_4 production (Knapp et al.,

2014). Structural carbohydrate fermentation results in losses in the form of CH_4 due to diversion of gross energy consumed into an unusable product to be eructated (Boadi et al., 2004a).

The most abundant natural plant polymer in the world is lignocellulose (Matthews et al., 2019). It is composed of cellulose, hemicellulose, and lignin. Mammalian enzymes do not possess the ability to break the β-1,4 glyosidic bonds that composes cellulose, and the β-1,4 linked xylose from hemicellulose. However, rumen microflora are uniquely adapted to digest this complex carbohydrate (Koike and Kobayashi, 2009; Matthews et al., 2019). Using a culture-based approach, Hungate (1966) examined the rumen microbial community and concluded that in cattle on forage-based diets, the bacterial community was composed primarily of Gram-negative microbes. There are three major cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* (Dehority, 1991; Weimer, 1992) in the rumen. These species are highly specialized to utilize cellulose, xylan, and pectin (Hungate, 1966). The results of fermentation from these species include acetate, butyrate, propionate, H_2, and CO_2 that get utilized quickly in the rumen (Nagaraja et al., 1997; Matthews et al., 2019). Fibrolytic bacteria in the rumen produce CO_2 and H_2 in addition to VFAs, with H_2 being the main fermentation end-product (Morgavi et al., 2010). Fermentation of plant structural components is known to yield higher acetate:propionate ratios and increased CH_4 production (Moe and Tyrell, 1979; Johnson and Johnson, 1995). Therefore, utilizing forage quality as a strategy for CH_4 mitigation can be beneficial for animal productivity and the environment.

Effect of concentrate-based diets and quality on CH_4 production

Blaxter and Clapperton (1965) determined that the implementation of higher amounts of concentrate in cattle diets decreases percentage of energy consumed in the diet that is converted to CH_4. This is due to a shift in fermentation substrates from a more fibrous content to a higher starch content (Beauchemin et al., 2008). Concentrates are composed of starch and soluble sugars, which are more readily fermented by rumen microbes. Lovett et al. (2003) suggest that concentrate inclusion into diets should be greater than 80% for observing a decrease in CH_4 production levels, as inclusion amounts of less than 60% did not result in a reduction in methane. Johnson and Johnson (1995) stated that in feedlot cattle fed 90% concentrate, loss of CH_4 was observed at 2-3% of energy intake. Diets that contain concentrates can be associated with increased intake, increased passage rate, and a shift in microbial composition (Martin et al., 2010). Generally, an increase in dry matter intake (DMI) increases passage rate while decreasing digestibility (Boadi et al., 2004a), with CH_4 losses decreasing to almost 1.6% of energy intake (Johnson and Johnson, 1995).

Fermentation of starch shifts the fermentation pattern from more acetate and butyrate to higher amounts of propionate (Johnson and Johnson, 1995). More propionate is produced due to increased proliferation and metabolic activity of propionate-producing bacteria that is reflected with diets high in starch and cereal grain-based diets (Ominski and Wittenberg, 2005; Martin et al., 2010). To produce propionate, pyruvate and four hydrogens are consumed (Hungate, 1966; Czerkawski, 1986; Moss et al., 2000), which decreases the availability of H_2 for the reduction of CO_2 to CH_4 (Knapp et al., 2014). However, hemicellulose is composed of 5- and 6-C sugars, providing a different

fermentation pattern of VFA and CH_4 production than cellulose and starch, which are composed of 6-C sugar units (Knapp et al., 2014). The hemicellulose:cellulose ratios of distillers byproducts and brewers grains are higher compared to grasses and legumes (1.5-1.6:1 vs 0.35 to 0.76 :1, respectively). Moe and Tyrrell (1979) concluded that hemicellulose digestion yields only 37% CH_4 relative to digested cellulose. This indicates that diets that are higher in hemicellulose may change the microbial community structure, thereby changing VFA production patterns and decreasing methanogenesis.

The predominant amylolytic bacteria in the rumen on starch-based diets are *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis*, and members of *Lactobacillus* and *Bifidobacterium* species (Nagaraja et al., 1997). Protozoa are also present and ingest starch granules (Nagaraja et al., 1997), which decreases the amount of starch for bacterial fermentation and may serve as a method of reducing CH_4 production and preventing a low-pH environment. When feeding concentrate-based diets, it is imperative to adapt cattle to inhibit metabolic diseases that arise with feeding large amounts of starch. When cattle are switched from a forage-based diet to a concentrate-based diet, a lowered ruminal pH frequently occurs (Fernando et al., 2010). Low ruminal pH can impede the growth and functionality of methanogens and ciliate protozoa as they are intolerant to low pH (van Kessel and Russell, 1996; Hegarty, 1999a). While this is a method of methane mitigation, producers need to adapt the rumen microflora as metabolic disorders can escalate, becoming severe and potentially fatal. The sudden increase in glucose levels in the rumen due to highly fermentable carbohydrates can result in a decline in pH. This event provides a near-perfect environment for *S. bovis* and other *Lactobacillus* species to proliferate when a highly fermentable carbohydrate is fed

(Nagaraja et al., 1997). During times of acidosis, *S. bovis* abundance increases, further contributing to the acidotic state (Nagaraja and Titgemeyer, 2007). However, when cattle were adapted to high-concentrate diets, very little concentrations of *S. bovis* have been observed (Nagaraja and Titgemeyer, 2007).

Alternative hydrogen sinks

The addition of different electron acceptors can be used to mitigate CH_4 emission by shifting the majority of CH_4 production from CO_2/H_2 to other acceptors (Morgavi et al., 2010). Briefly, this method utilizes electron acceptors other than CO_2 that are more thermodynamically favorable, such as nitrate- and sulfate-reduction. Typically, the microbes that perform these unique reactions are low in abundance in the rumen. With the inclusion of the terminal electron acceptor as a dietary component, the abundance of these populations can be increased (Morgavi et al, 2010). However, the final product formed must be considered as some end-products and intermediates formed may pose a health risk to the host animal. Thermodynamically, the reduction of CO_2 and H_2 to CH_4 is -131 kJ/mol, while the reduction process of nitrate (NO_3^-) to nitrite (NO_2^-) is -163.2 kJ/mol, and sulfate (SO_4^{2-}) to sulfide (HS^-) is -152.2 kJ/mol (Thauer et al., 1977; Latham et al., 2016). The singular reduction process of NO_3^- to ammonia (NH_3) is -599.6 kJ/mol (Thauer et al., 1977; Latham et al., 2016). Incidentally, the reduction of CO_2 to acetate is -95 kJ/mol (Thauer et al., 1977). Thermodynamically, SO_4^{2-} and NO_3^- can be used as terminal electron acceptors to reduce CH_4 emissions in ruminants. However, understanding the products of these reduction processes is imperative to animal health. Methods to illustrate alternative hydrogen sinks with potentially lethal reduced products

of NO_3^- and sulfate reduction are discussed below in addition to other methods such as ionophores and lipid supplementation.

NO_3^- and SO_4^{2-} supplementation

Shifting the production of CH_4 to NH_3 is more thermodynamically favorable (Morgavi et al., 2010) and may serve as a fundamental and practical means of reducing methanogenesis. In addition, the increase of nitrogen concentrations may promote increased microbial growth (Latham et al., 2016). In the rumen, conversion of nitrate to nitrite ($NO_3^- + H_2 \rightarrow NO_2^- + H_20$) is rapid; however, the rate of NO_2^- reduction to ammonium (NH^{4+}) ($NO_2^- + 3H_2 + 2H^+ \rightarrow NH_4^+ + 2H_20$) is comparably slow (Iwamoto et al., 1999; Olijhoek et al., 2016). As a result, the accumulation of NO_2^- in the rumen leads to increased absorption of NO_2^- leading to methemoglobinemia (Lewis, 1951), where NO_2^- binds to hemoglobin and decreases oxygen flow. This is often seen in animals that are not adapted to higher inclusion of NO_3^-. However, adaptation by feeding incremental amounts of NO_3^- has shown to increase the abundance of nitrite-reducing bacteria, resulting in decreased CH_4 production and NO_2^- toxicity events (Morgavi et al., 2010). Simon (2002) stated that *Wolinella succinogenes* has the capability to convert NO_3^- to NH_3 without contributing to an increase in NO_2^- concentration as part of its respiration (Morgavi et al., 2010). The reduction processes of NO_3^- to NO_2^-, and NO_2^- to NH_3 are more thermodynamically favorable compared to the reduction of CO_2/H_2 (Ungerfeld and Kohn, 2006). In addition to decreased CH_4, the subsequent increased supply of NH_3 would be available for utilization by microbes in diets lacking RDP protein (Dijkstra et al., 1998). Alaboudi and Jones (1985) gradually adapted sheep over a 10-week period by

stepwise introduction of NO_3^- into diets at 1.5g of NO_3^-/kg of BW daily. The sheep displayed no signs of methemoglobinemia with the stepwise adaptation. Olijhoek et al. (2016) also conducted a study increasing dietary NO_3^- in the form of calcium ammonium nitrate at 5.3, 13.6, and 21.1 g NO_3^-/kg DM to lactating Holstein dairy cows. The study concluded that CH_4 production decreased linearly with the linear increase of NO_3^- in the diet. Hydrogen levels emitted were also higher for cows fed the higher NO_3^- diets. This was concluded to be due to assimilation of hydrogen to CH_4 or NO_2^-/NH_3 was not occurring. One suggestion for this reason was the toxicity to the methanogen population in the rumen. Overall, if animals are adapted, NO_3^- could be used as a dietary supplement to reduce CH_4 emissions and increase nitrogen availability in the rumen. However, concerns of toxicity to certain members of the rumen microflora due to increased levels of NH_3 should be considered, as NO_3^- and NO_2^- levels have been linked to toxicity and inhibition of fibrolytic community members (Olijhoek et al., 2016).

Sulfur can also be used as a supplement in diets to decrease methanogenesis. The supplementation of S can be performed in parallel to feeding nitrates in diets as it can be used as a method to reduce NO_2^- accumulation in the rumen (Leng, 2008). The reduction of SO_4^{2-} to sulfide (HS^-) occurs as follows $SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_20$ (Thauer et al., 1977). A majority of the reduced HS^- produced is converted to hydrogen sulfide (H_2S) in a pH-dependent process that is usually observed in cattle on high-concentrate diets (Beauchamp et al., 1984). According to Hubert and Voordouw (2007), H_2S has been observed in anaerobic environments and acts as an electron donor for reducing NO_2^- to NH_3 by nitrite-reducing, sulfide-oxidizing bacteria. The SO_4^{2-} fed in the diet acts as a reductant that may outcompete hydrogens and decrease CH_4 levels as it is more

thermodynamically favored (Ungerfeld and Kohn, 2006; Van Zijderveld et al., 2010). Sulfate-reducing organisms have been observed to function at lower partial pressures compared to methanogens, therefore, the competition of the hydrogen pool may favor SO_4^{2-} reducers with H^+ in a more acidic environment. Additionally, S in the diet is potentially toxic to methanogens, unless adaptation occurs (Mathison et al., 1998). However, the reduction of S yields H_2S, a highly toxic compound to ruminants when respired into the lungs. Polioencephalomalacia, PEM, is a neurologic disorder that arises due to alterations in thiamine status within the animal as a result of high S intake from either water or dietary sources (Gould, 1998). For the purposes of this review, dietary S sources will be discussed. Often, cattle consume higher levels of S on concentrate-based diets due to feeding of corn coproducts (Merck, 2016). Sulfate is non-toxic to cattle; however, the fermentation end product of H_2S and other ionic forms are toxic and can interrupt and inhibit energy metabolism, especially impacting the central nervous system (Merck, 2010). Sulfur requirements for beef cattle are relatively unclear, especially when feeding wet or dry distillers that contain different energetic values (Sarturi et al., 2011). Sarturi et al. (2011), suggested that dry distillers, when compared to the wet, contains decreased energy content and reduced S availability. Currently, the recommended concentration of S is 0.15% (NASEM, 2016). Rumsey (1978) stated that cattle fed high-concentrate diets with increasing amounts of S improved feed efficiency, while Zinn et al. (1997) observed S in the form of ammonium sulfate in finishing diets containing more than 20% steam-flaked corn decreases performance in feedlot heifers. Rumsey (1978) observed supplementing substantially higher than 0.15% of S to have no impact on performance and decrease DMI in finishing cattle.

Van Zijderveld et al. (2010) performed a 2 x 2 factorial study to investigate the effect of dietary NO_3^- and SO_4^{2-} supplementation on decreasing CH_4 production in sheep. Treatments consisted of a control treatment without NO_3^- or SO_4^{2-}, a NO_3^- treatment with inclusion at 2.6% of dietary DM, a SO_4^{2-} treatment with inclusion at 2.6% of dietary DM, and a combination treatment including both NO_3^- and magnesium sulfate at 2.6%, with a four-week adaptation to the dietary treatments. The results indicated that the NO_3^- dietary treatment decreased CH_4 production by 32% and the SO_4^{2-} dietary treatment decreased CH_4 production by 16% compared to the control. No interaction of the combination treatment was observed for DMI or CH_4 (L/d) suggesting that adding both as a combination may have a lesser impact on intake and CH_4 compared to feeding either the nitrate or sulfate. The rumen protozoal population was not impacted in this study, yet sulfate-reducing bacteria counts were increased when SO_4^{2-} was supplemented. The increase was more apparent on the combination dietary treatment, as noted previously (Morgavi et al., 2010). Methemoglobinemia was observed to be only slightly elevated in this study in sheep. This study concluded that when NO_3^- and SO_4^{2-} are fed together, a reduction of CH_4 occurs; however, ration formulations must ensure for adequate adaptation and minimize toxicity levels for both ingredients. Hibberd et al. (1994) indicated that clinical signs of nitrate toxicity can appear when 40% of the total hemoglobin is in the methemoglobin form. Hibberd et al. (1994) also indicated that increasing microbial fermentation can lead to higher amounts of nitrate reduction to nitrite in the rumen.

Ionophores

Ionophores are widely utilized in beef and dairy cattle diets to influence rumen fermentation with the goal of improving feed efficiency. Two commonly used ionophores are monensin and lasalocid (Russell and Strobel, 1989). Russell (2002) explains that monensin is a carboxylic polyether ionophore antibiotic. Addition of monensin in beef cattle diets has been shown to decrease feed intake 5-6%, reduce the acetate:propionate ratio, and subsequently decrease CH_4 production (Goodrich et al., 1984). This can be observed in cattle consuming high-concentrate diets (Johnson and Johnson, 1995). Johnson and Johnson (1995) consider the potential of ionophores indirectly impacting CH_4 production through intake reductions versus directly inhibiting methanogenesis.

Guan et al. (2006) investigated the characteristics of ionophore supplementation on CH_4 emissions, duration of reduced CH_4 emissions, rotation of ionophore supplementation on CH_4 emissions, and determined the effects of supplementation on ciliate protozoal abundance. The dietary treatments were a low-concentrate without ionophore, low-concentrate with monensin, and low-concentrate with monensin and lasalocid rotation. A high-concentrate diet was also fed, with the same ionophore supplementation design. It was observed that reductions in CH_4 production were observed for two to four weeks, depending on dietary energy content. It was reported that the decline in CH_4 was due to a decrease in protozoal abundance. Additionally, the rotation of monensin and lasalocid biweekly did not extend the duration of CH_4 reduction. In low-concentrate diets, the addition of monensin and the rotation of monensin and lasalocid did not impact DMI, ADG, and F:G. Cattle fed the high-concentrate diet with monensin and the rotation of monensin and lasalocid significantly

decreased DMI and improved F:G, with no impact on ADG. When ionophores were supplemented into the diets, CH_4 production decreased by 27% in the first two weeks when expressed as L/kg DMI for high-concentrate diets. In low-concentrate diets supplemented with ionophores, a 30% reduction of CH_4 was observed over a 4-week period. In spite of the initial reductions of CH_4, emission amounts did return to baseline levels, indicating results can be short-term. The rotation of the two ionophores did not affect the amount of CH_4 reduced or the duration of the decrease. This study suggests that ionophores can reduce enteric CH_4 emissions; however, this reduction is short-lived and is not sustained over long periods of time. However, ionophore supplementation is still recommended as it improves feed efficiency.

Odongo et al. (2007) investigated the long-term impact of supplementing monensin on enteric CH_4 emissions in dairy cows. Dietary treatments investigated included a normal TMR with a forage:concentrate ratio of 60:40 and served as the control treatment. The ionophore treatment consisted of the normal TMR control diet plus 24 mg of Rumensin Premix. The premix was incorporated into soyhulls at 2.56 g of premix with 997.44 g soyhulls at 17.3% DM basis. This study reported no impact on DMI or milk yield due to monensin addition, yet observed a sustained six month decrease in CH_4 emissions by 7%. It has been proposed in the past (Chen and Wolin, 1979) that ionophores can alter the microflora to shift toward more Gram-negative bacteria while shifting the fermentation pattern to more propionate production. Appuhamy et al. (2013) performed a meta-analysis on the effect of monensin supplementation on CH_4 emissions in dairy and beef cattle and reported that the impact of ionophores on CH_4 production in

dairy cattle was contradictory among studies. However, this study reported the responses are more consistent among beef cattle although the magnitude still varied.

Lipid supplementation

Lipid is added to cattle diets to increase dietary energy density, improve milk yield, and to alter the fatty acid profile in milk fat in dairy cattle or increase fat deposition in beef cattle (Murphy et al., 1995; Ashes et al., 1997; Enjalbert et al., 2017). Additionally, it has also been observed to decrease methanogenesis in the rumen (Dohme et al., 2004). The variations observed in CH_4 production could be due to the type of lipid supplemented in the diet and how available it is to the rumen microbiome (Boadi et al., 2004b). One significant concern with fat supplementation is the impact on fiber digestibility in the rumen. Typically, when supplementation of lipid in the diet exceeds 6% of dietary DM, a decrease in fiber digestibility is observed (Boadi et al., 2004b). Lipid supplementation is shown to suppress fiber degradation (Van Soest, 1994) and decrease DMI, along with an increased risk of milk fat depression (Knapp et al., 2014). With high levels of lipid supplementation, reduction in CH_4 production could reach as high as 40% (Machmüller and Kreuzer, 1999). In practice, reductions are closer to 10-25% (Beauchemin et al., 2008). Lipids can decrease CH_4 production through redirecting fermentation to produce more propionate than acetate (Johnson and Johnson, 1995). Several factors are known to influence CH_4 production when fat supplementation is used. This includes concentration of fat, fat source, fatty acid profile, and diet type (Beauchemin et al., 2008; Knapp et al., 2014). Beauchemin et al. (2008) conducted a meta-analysis to examine the concentration of fat added and its effect on decreasing CH_4

emissions in beef and dairy cattle, and lambs. This analysis demonstrated that CH_4, when calculated as (g/kg DMI), decreased by 5.6% with each 1% addition of supplemental fat. Conversely, Winders et al. (2020) and Hales et al. (2017) reported reductions in CH_4 (g/kg DMI) by 4.3% and 4.5%, respectively. While substantial decreases in CH_4 have been observed, the inclusion level of fat should not exceed more than 6-7% (Beauchemin et al., 2008).

Anaerovibrio lipolytica and *Butyrivibrio fibrosolvens* are common lipolytic bacteria in the rumen (Nagaraja, 2016). One product of lipolysis is glycerol, which is quickly fermented to VFAs by these bacteria (Nagaraja, 2016). Another lipolytic product are fatty acids. Lipid supplementation can have a negative effect on the rumen microflora, and it can inhibit fibrolytic digestion by bacterial species (Brooks et al., 1954; Enjalbert et al., 2017). As an adaptation mechanism to prevent toxicity issues, the microbes adapted for survival by hydrogenation of unsaturated fatty acids (Reiser, 1951). Briefly, biohydrogenation is the process of converting an unsaturated fatty acid ($C_{18:2}$) in a double bond cis- formation into hydrogenated $C_{18:0}$ in the trans- formation using isomerases and reductases (Jenkins, 1993). This process reduces the amount of H_2 available for methanogenesis as it gets redirected towards saturation of unsaturated fatty acids (Poulsen et al., 2013). Methanogens are also known to be inhibited with lipid supplementation (Machmüller and Kreuzer, 1999). Poulsen et al. (2013) used metatranscriptomics to examine the effect of rapeseed oil supplementation on the microbial community, especially methanogens, in lactating Holstein cows. This study observed that a novel group of methylotrophic archaea, Thermoplasmata, were inhibited with the supplementation. Additionally, the effect of fat on the protozoal population has

been inconsistent with reports of no effect to decreasing the protozoal population (Enjalbert et al., 2017).

Alfalfa replacement in lactating dairy cattle diets

With increasing climate variability, resources such as land and water, may become limiting for livestock production. In order to meet nutrient chemical composition requirements during drought conditions, crop production usually relies on irrigation practices for growing crops (Djaman et al., 2020). Reliance upon irrigation practices to raise crops, such as alfalfa, is imperative to providing a high-quality feed ingredient. However, there are areas of the world that are extremely arid, characterized by drought-like conditions, where irrigation may be impractical. For dairies to be resilient and maintain production levels in these arid locations, ration formulations will need to be flexible to account for ingredients unavailable or too expensive.

Alfalfa use in lactating dairy cow rations

Alfalfa forage is a significant dietary component incorporated into lactating dairy cow diets, which typically contain approximately 20% CP (NRC, 2001). The longer particle size observed with alfalfa hay (Beauchemin et al., 2003) is a beneficial source of physically effective fiber (pe) (Mertens, 1997). This concept refers to the physical attributes of feed ingredients that may describe their effect on rumen pH based on chewing activity or particle length (Beauchemin et al., 2003). Beauchemin (1991) reported average times for dairy cows to spend consuming a ration and ruminating as 4 -7 h/d and 5-9 h/d, respectively. This action serves to maintain a healthy and efficient rumen

in high-producing dairy cattle (Beauchemin, 2018). Reduction of particle size through remastication increases saliva production, which is critical in maintaining an optimal rumen environment (Beauchemin, 2018). Rumination allows for increased microbial attachment to feed particles as well as optimizing passage rate out of the rumen. Increased rumination time is beneficial to inhibit metabolic disorders such as acidosis, with the increased salivary production enhancing the buffering capacity during conditions that can favor acidotic conditions (Beauchemin, 2018). Furthermore, this action also serves to maintain high intake levels to help maintain production capabilities of the high-producing dairy cow. Consequently, feeding a bulky forage such as alfalfa, can be associated with a decrease in DMI, possibly due to gut fill (Hall and Chase, 2014), as bulkier forages require increased chewing and rumination.

Renewable resource usage, especially in the production livestock sector, has garnered significant attention for using copious amounts of water for growing crops and feeding livestock. According to Mekonnen and Hoekstra (2010), growing animal feed accounts for most of the water usage in the animal product supply chain. The water requirement when irrigating alfalfa is high (Saeed and El-Nadi, 1997), which, when coupled with an extended growing term, makes alfalfa a high water-consuming crop (Djaman et al., 2020). In Nebraska, alfalfa can require nearly ten inches of water for the third and fourth cutting, with an overall range of 30-38 inches for the season (Irmak et al., 2007) compared to soybeans that require overall less water at 26 inches for the growing season (Kranz and Specht, 2012). The potassium (K) content of alfalfa is approximately 2.37% (NRC, 2001). Silanikove et al. (1997) noted that increased consumption of dietary ions, such as K, corresponds to increased water demand in the animal. Fisher et al. (1994)

fed 1.6, 3.1, and 4.6% K to cows in early lactation. The 4.6% K treatment yielded increased water intake, indicating an increase in ion consumption can have impacts on the animal's water balance to maintain ion homeostasis. This also serves to increase water usage in the dairy industry; therefore, identifying other feed sources or combinations of feed sources that require less water input would greatly benefit producers in certain areas of the world where water access is limited.

Coproduct use in dairy cattle rations

Dried distillers grains plus solubles (DDGS), a coproduct from corn ethanol production, is a common feed ingredient used in dairy cattle rations in the United States (Foth et al., 2015). It is produced with the material remaining after fuel is extracted from corn ethanol production (Kim et al., 2008), and if not utilized for feed may be considered a waste product. The lignin content is approximately 4.3% and 38% NDF (NRC, 2001), and nearly 68% of the NDF content is digestible (Getachew et al., 2004). The benefit of incorporating coproducts like DDGS into rations is the increased energy and protein content without compromising rumen function that is often associated with high-starch based diets (Schingoethe et al., 2009). The starch that is in the corn gets fermented to ethanol, leaving stillage that when water is removed produces DDGS (Klopfenstein et al., 2008). A 3-fold increase in fat, protein, fiber, and P concentrations are observed (Klopfenstein et al., 2008). Protein is increased to nearly 30%, fat to 12%, NDF heightened to 36%, and P to 0.9% of DM. The fat content of this product fluctuates between 10-12%, therefore inclusion in ration formulations has been around 10% of dietary DM in dairy cattle (Janicek et al., 2008) due to the potential of high

concentrations of dietary fat impairing fiber digestion (Van Soest, 1994). However, Ranathunga et al. (2010) included DDGS at 21% and observed no decreases or compromises in yield or milk components. Anderson et al. (2006) reported rations that contain DDGS at 20% to replace corn and soybean meal can maintain or increase milk yields. Janicek et al. (2008) incorporated DDGS from 0 to 30% in replacement of soybean meal, cottonseed, and some forage, and observed a linear increase in milk yield. These studies indicate that DDGS can be included in rations at higher than 10% without impeding milk yield.

Inclusions of greater than 30% can run the risk of developing metabolic disorders. Milk fat depression (MFD) is a metabolic disorder (NRC, 2001) that occurs usually when the fat content of rations is high, resulting in nearly a 50% reduction of milk fat yields (Bauman and Griinari, 2001). Interestingly, the reduction is only observed for the milk fat component and not milk yield, protein, or lactose (Bauman and Griinari, 2001). This disorder is not directly caused by a single factor, rather the interactions occurring during fermentation when readily fermentable carbohydrates increased concurrently with decreased inclusions of fibrous components (Lock, 2010). The lack of fibrous components reduces physically effective fiber, which aids in rumination and buffering to mitigate metabolic disorders.

Berger and Singh (2010) reported a product called reduced-fat DDGS (RFDDGS) is produced when a fraction of the oil is removed from corn grain leftover from corn ethanol production. The fat content is decreased nearly 60% that of DDGS (Ramirez-Ramirez et al., 2016) at 5.5% according to Castillo-Lopez et al. (2014). The increase in corn ethanol production has coincidently increased the amount of DDGS available.

Therefore, more DDGS are available for oil removal, producing larger amounts of RFDDGS for incorporation into cattle diets (Janicek et al., 2008). With the decrease in fat in this ingredient, concern for using it as an energy source is apparent. This product has been evaluated to determine the optimum inclusion into rations for maximum milk yield and milk components subsequently evaluating the risk potential for MFD (Castillo-Lopez et al., 2014; Foth et al., 2015; Ramirez-Ramirez et al., 2016). These research studies reported either no difference or an increase in intakes, milk yield, and milk components of fat and protein.

Using coproducts as a source of energy may serve as a means of CH_4 mitigation. Moe and Tyrell (1979) state that digested hemicellulose generates only one-third of the CH_4 compared to cellulose. Diets containing coproducts such as DDGS can favor increased propionate production compared to forage-based diets containing alfalfa (Johnson and Johnson, 1995) due to higher levels of fat. As mentioned previously, DDGS have a higher ratio of hemicellulose:cellulose (Knapp et al., 2014). Hemicellulose is fermented slightly differently due to the mixture of odd-numbered and even-numbered sugars, theoretically providing a different VFA pattern, along with a high NDF content making these feeds more digestible (Janicek et al., 2008). This fermentation pattern may serve as an avenue towards redirecting energy availability and decreasing CH_4 levels.

Replacement of dietary ingredients in dairy rations

With the increase in availability of DDGS, most research in replacing feed ingredients with DDGS has been linked to corn-based products and soybean ingredients (Anderson et al., 2006; Kleinschmit et al., 2006; Mjoun et al., 2010; Ranathunga et al.,

2010; Foth et al., 2015; Ramirez-Ramirez et al., 2016). These studies showed either no effect or an increase in milk production. This effect has been observed with up to 22% (DM basis) replacement of corn and soybean products (Ranathunga et al., 2018). In diets containing 50% forage, Schingoethe et al. (2009) recommends including DDGS at 20% of the diet DM for maximum performance in lactating dairy cows. With the decreased fat content in DDGS, this becomes somewhat of an ideal feed ingredient with a low-risk potential for milk fat depression (Bauman and Griinari, 2003; Ramirez-Ramirez et al., 2016).

Hall and Chase (2014) evaluated performance of lactating dairy cows fed combinations of forage substitutes in diets that were low in forage and given only DDGS for supplementation. Wheat straw was used in this study as the source of effective fiber. Decreases in milk production were observed most likely due to dietary energy supply. Inclusion of wheat straw showed a tendency to decrease milk production and percent of lactose in the milk. Ranathunga et al. (2018) investigated the effect of DDGS at 18% of diet DM with high- and low-forage contents and hypothesized that NDF would not impact production parameters. Milk production in this study increased on the low-forage diets that contain higher starch or contain DDGS, which are known to increase propionate proportions in the rumen (Ramirez-Ramirez et al., 2016; Ranathunga et al., 2018). However, little investigation into substituting DDGS as a complete replacement for alfalfa has been conducted, providing an opportunity to elucidate energy partitioning, fermentation dynamics within the microflora, and CH_4 production for this ingredient substitution.

As technology continues to advance, changes to common feed ingredients for dairy cows and beef steers most likely will occur. Investigation into understanding how these new ingredients impact production for the dairy and beef industry is vital for the livestock sector in general to continue to provide high-quality animal products for consumption by the global population. By examining these feed sources, we can begin to further characterize fermentation characteristics of these various diet scenarios.

Feed Efficiency in Beef Cattle

Beef production today is under great scrutiny due to the potential impact on climate, land and resource use, and rising costs of feed sources. In order to meet future animal product demands, producing more meat with less resources, is a goal the beef industry is pushing towards. With the cost of feed being the single largest expense for producers (Lowe and Gereffi, 2009), phenotypic efficiency characteristics may provide insight into making animals more efficient (Bergman, 1990; Seabury et al., 2017). Increasing efficiency of animal and plant production will enhance the ability to meet future demands that will occur as a result of population increase (Berry and Crowley, 2013).

Measures of feed efficiency in beef cattle

Feed efficiency can be described as how well an animal can convert the energy consumed as feed into tissue mass or milk production. This trait complex impacts producer variable costs. Koch et al. (1963) recognized disparities in cattle with feed conversion to tissue gains and determined the necessity of estimating heritability of feed

efficiency phenotypes and other quantifiable traits. Genetics can influence other production traits, such as feed intake, in dairy cattle (Korver, 1988), and intake and gain for beef cattle (Koch et al., 1963). Feed efficiency is a quantitative trait described as a calculation rooted in body weight gain for a given amount of feed (Koch et al., 1963), otherwise known as the feed conversion ratio (FCR). Several factors that limit the exactitude of this calculation includes type of gain (bone, fat, or lean tissue), as well as the animal-to-animal variation in maintenance requirement (Koch et al., 1963). Traditionally, feed efficiency has been reported as the ratio of G:F (Koch et al., 1963). Koch et al. (1963) also indicated that two components can be used to describe feed intake, which includes the expected feed intake and the residual feed intake. Recently, an alternate measure of feed efficiency is gaining more widespread usage (Seabury et al., 2017), and is described as residual feed intake (RFI) (Koch et al., 1963). This measure is defined as the difference between the observed and expected feed intake of an animal compared to the animal's body weight gain within a designated time period (Archer et al., 1999). The residual variable can be used to identify animals that may diverge from their expected intakes (Archer et al., 1999), shedding light on potential variation on metabolic capabilities that may influence feed efficiency (Brelin and Brannang, 1982; Korver, 1988). Simply, RFI can be discussed as the difference between the actual consumption of feed and what was predicted. The unique aspect of RFI is its independence of any covariate found in the model (Seabury et al., 2017).

Historically, increasing feed efficiency was achieved by evaluating host animal genetics to identify heritable traits, such as growth rates, weaning weights, gain, and feed consumption (Koch et al., 1963; Archer et al., 1999; Snelling et al., 2011; Berry and

Crowley, 2013; Lu et al., 2013). Koch et al. (1963) conducted a path analysis of feed efficiency and concluded that 38% of the variation in gain could be a result of genetic differences in feed efficiency. Feed efficiency measures show a significant variation in heritability ranging from 0.06 to 0.62 (Berry and Crowley, 2013), indicating that feed efficiency traits can be heritable. Conversely, when using these selection criteria, it is critical to understand that when selecting for a trait, there may be other physiological and phenotypic changes that may or may not benefit the host. For example, if using higher growth rate selection parameters, an associated increase in cost can occur for producing and maintaining larger animals (Seabury et al., 2017).

Microbiome influencing feed efficiency measures

The rumen microbiome is responsible for producing a significant portion of the animal's energetic requirements, indicating that targeting this group and their metabolic capabilities may provide insight into microbial strategies to improve feed efficiency. This area is a new avenue in exploration into improving feed efficiency.

A study by Myer et al. (2015) evaluated the rumen bacterial community to identify the association of the community composition relating to feed efficiency in steers with a variation in intake and growth by evaluating the microbial community using 16S rRNA gene sequencing. Results of ADG regressed over ADFI were plotted on a cartesian plot, and extreme samples in the plot distribution were used for further analysis. Bacteriodetes and Firmicutes were the most predominant phyla, ranging from 53-63% and 23-33%, respectively. Seven taxa and 25 OTUs identified were associated with the phenotype of ADG. The phylum Lentisphaerae, families Veillonellaceae, Victivallaceae,

and Lachnospiraceae, and genera *Dialister* and *Acidaminococcus* were associated with gain. Of the 25 OTUs associated with performance, only three were associated with intake. The family Veillonellaceae was observed for both gain and intake. While significant information is obtained from 16S studies, performing metagenomic studies on rumen microbial community samples will further enhance the understanding of why certain taxa are present and at a particular abundance by elucidating the genomic potential within the community.

In 2018, Paz et al. investigated how the bacterial community structure impacts feed efficiency measures of average daily feed intake (ADFI), average daily gain (ADG), and G:F in steers and heifers, similar to Myer et al. (2015). Moving a step beyond Myer et al. (2015), Paz et al. (2018) used forward stepwise regression models at different taxonomic levels. Feed efficiency measures were plotted on Cartesian plots to identify the extreme communities for the feed efficiency measures. Bacteriodetes, Firmicutes, and Proteobacteria were found to be the most abundant phyla for both groups, with a combined percentage of nearly 86% for heifers and nearly 95% for steers. When corrected for breed, the ADFI model for heifers and steers explained nearly 19% and 30% of the variation, respectively. The heifer cohort observed OTUs associated with an increase in intake belonging to the families of Ruminococcaceae and Victivallaceae, which are known to digest cellulose (White et al., 1993) and cellobiose (Zoetendal et al., 2003), respectively. Prevotellaceae OTUs were associated with decreased intake; however, this family is highly abundant in the rumen with roles in polysaccharide (Matsui et al., 2000) and protein digestion (Wallace, 1996). The steer cohort OTUs associated with increased intake belong to the families Bifidobacteriaceae,

Prevotellaceae, and Paraprevotellaceae. The families Lachnospiraceae and Veillonellaceae had OTUs that were associated with both positive and negative influence on intake. The model of ADG for steers and heifers was able to explain 33% and 25%, respectively, of the variation in gain for the cohorts, when corrected for breed. The families Lachnospiraceae, Prevotellaceae, Ruminococcaceae, and Veillonellaceae had OTUs present and were associated with gain increases. Lachnospiraceae, Prevotellaceae, and Erysipelotrichaceae were associated with decreased gain. In steers, members of Lachnospiraceae were classified to genus Butyrivibrio, which is known to carry a variety of digestion enzymes encompassing hemicellulytic, proteolytic, and uricolytic (Cotta and Hespell, 1986; Kelly et al., 2010). The model for G:F was able to explain nearly 20% and 27% of the variation observed for heifers and steers, respectively. Lachnospiraceae, Prevotellaceae, and Spirochaetaceae families had OTUs that were present and associated with a decrease in the ratio. Lachnospiraceae and Veillonellaceae have been observed with inefficient dairy cattle based on transcriptome data (Li et al., 2017), while Prevotellaceae has been observed in both inefficient and efficient cattle, potentially indicating the multitude of enzymatic capabilities and functional redundancy. The heifers on the forage diet experience a higher number of significant OTUs most likely due to the forage characteristics of the diet increasing microbial diversity compared to diets with increased amounts of readily fermentable carbohydrates. With the rumen microbiome being responsible for producing a significant portion of the animal's energy in the form of VFAs, coupled with the range of heritability for feed efficiency measures, suggests that the host animal may have control in shaping its own gut microbiota composition.

Factors that influence the microbiome composition

Several factors have been examined as playing a role in shaping the rumen microbiome. These factors include diet, host genetics, breed, sex, and environment (Li et al., 2019; Abbas et al., 2020). Diet has been studied extensively to evaluate dynamic changes in microbial community composition. (Johnson and Johnson, 1995; Hook et al., 2010; Fernando et al., 2010; Morgavi et al., 2010; Henderson et al., 2015; Anderson et al., 2016; Huws et al., 2018). Animal performance is based off of the products produced by the extensive rumen microbial community, and understanding environmental and host genetic factors that shape the rumen microbial community composition can indicate areas to target for manipulation to impact animal performance and efficiency. To understand how host genetics influences the microbiome, it is important to examine environmental factors that are shown to impact gut microbial community regardless of genetics. A "top-down" model for the assembly of microbial structure and composition by the host is suggested (Benson et al., 2010). Benson et al. (2010) investigated how environmental factors and host genetic factors shape microbial community composition using a murine model in which genetic background can be evaluated and environmental conditions controlled. It was demonstrated that host genetics affect lower orders of classification indicating that the microbiome composition is a polygenetic trait that is heritable. Extrapolating these concepts to ruminants, it has been shown that host genetics influence the rumen microbiome composition (Li et al., 2019; Abbas et al., 2020). Li et al. (2019) conducted an extensive study investigating the additive genetic effects of the host on microbial features in beef cattle. They hypothesized that there are host SNPs that are influencing the differences in microbial composition. The bacterial and archaeal

communities were different based on breed, sex, and diet in this study. Hernandez-Sanabria et al. (2013), Yurkovetskiy et al. (2013), and Asnicar et al. (2017) have implicated that sex can alter the rumen microbiome, and this was observed by Li et al. (2019). Results from Li et al. (2019) indicated that additive genetic variation in relative abundance occurred primarily in bacterial taxa. Moderate heritability of the bacterial abundance was observed in bacteria but not in archaeal abundance. A majority of the heritable microbial features had a significant contribution to variations observed in FCR, ADG, and intake. However, it did not relate to RFI. Heritable microbial features were also correlated to VFA parameters, more specifically acetate and propionate amounts. Unclassified Clostridiales, Christsenellaceae, and Mogibacteriaceae were positively correlated with acetate production and negatively correlated to propionate concentrations. Succinivibrionaceae was positively correlated with propionate production as well. Overall, the study verified that the rumen microbial community is susceptible to host genetics, breed, sex, and diet for shaping the composition, indicating a level of genetic control over the microbiome not previously discovered.

Abbas et al. (2020), evaluated the effect of host genetic factors in shaping species composition of bacteria in the rumen on a large cohort of beef cattle from different locations. A genome-wide association study was performed to identify host chromosomal regions and SNPs that can influence the composition and subsequently function within the rumen. When heritability was assessed for phylogenetic classification, several taxa exhibited higher heritability values, such as Methanobacteriaceae and Succinivibrionaceae. Phenotypic variation was observed at phylum, family, and OTU levels on more than one chromosome indicating that different taxonomic levels are

governed by the host genetics making it polygenic. This study indicates that effect of host genetics on shaping the composition can be highly specific to the species level. The Succinivibrionaceae family was associated to chromosome 9. This family has been connected to methane emissions, that was previously described to be heritable in dairy cows (Wallace et al., 2019). Additionally, the genus Succiniclasticum was associated with two chromosomes, with the only known species being highly specialized in converting succinate to propionate as its only method of energy metabolism. Having this organism in the rumen is valuable for energy metabolism dynamics in the rumen, also providing evidence of preferential selection of an organism for its role in supply energy metabolites to the host. *Prevotella* genus was also found to be associated with multiple loci on different chromosomes. This could be due to the genus' known capability for protein and polysaccharides digestion of various carbohydrate material. These loci could be impacting certain species composition within this genus in the rumen.

Feed efficiency in dairy cattle

Shabat et al. (2016) used the species taxa and the genetic content from the microbiome to predict the feed efficiency phenotype at 91% accuracy. Additionally, in efficient cow samples, VFAs propionate, butyrate, valerate, and isovalerate were observed to be abundant in the rumen. This study observed a difference of 10% between the efficient and inefficient animals, with the total concentration of VFAs being higher in the efficient animals. It was suggested that this could play a significant role in utilization of nutrients and metabolites as microbes are responsible for providing nearly 70% of the animal's energetic needs (Seymour et al., 2005). The results from this study determined

that a more diverse rumen microbiome is observed in inefficient cows, thereby resulting in a wider range of metabolite types and concentrations that may have a negative impact on energetic efficiency or may not be utilized directly by the animal. In the efficient cows' microbiomes, *Megasphaera elsdenii* was the dominant species of the enriched annotated genes. It was suggested that this species was observed as being dominant due to the metabolic capacity it contains with the ability to convert lactate to propionate. This species and other closely related taxa were not observed in the inefficient cows' microbiomes. This study also demonstrated that the inefficient cows' microbiomes were not dominated by a single taxa or related taxa and more governed by a variety of species, suggesting that functional taxa for a given function are more abundant in cattle identified as efficient. Members of Lachnospiraceae were observed in both efficient and inefficient cohort. *Methanobrevibacter ruminantium*, a prominent methanogen, was found to be highly abundant in inefficient cows. The reasoning for the enriched genome of this methanogen is that methane production is a loss in energy to the animal, so exhibiting higher methanogen numbers may lead to an inefficient microbiome, in effect making the animal less efficient. In the efficient cohort, the acrylate pathway was the only propionate production pathway that was enhanced. Incidentally, Prabhu et al. (2012) noted that *M. elsdenii* contains this pathway in its genome. Gene prediction of functional features are able to associate taxa with metabolic pathways. Genomic sequence data can then be applied to the above-mentioned pathways, identifying pathways relating metabolic functions. As such, differences in feed efficiency can be ascertained, allowing nutritionists and producers to provide the best dietary ingredients for greatest performance returns.

While several studies have examined different taxa and their relationship with RFI (Jami et al., 2014; Jewell et al., 2015) these studies utilized 16S rRNA sequencing to describe associations. This provides only taxonomic evidence for associations, where functional redundancy may overshadow deeper knowledge at the gene level. By incorporating metagenomic testing, a more comprehensive knowledge on the microbial composition can be achieved.

Conclusion

Providing high-quality protein food sources to feed an expanding population is an on-going challenge for agriculture. The rumen microbiome is responsible for utilizing consumed feed and providing resources for the animal. This indicates that examining how various dietary ingredients impact the microbial structure may lead to identifying methods to increase the amount of meat and milk products by reducing methanogenesis, a source of inefficiency. In doing so, the impact of the cattle industry on GHG production and climate change will be decreased.

Previous genomic selection measures have been used to identify animals with a high genetic merit to improve feed efficiency measures. One component gaining significant attention is the genetic capacity retained with the rumen microbiome. By examining what fermentation genes are present regarding energy harvest and utilization, as well as, identifying their heritability within the host genome, may provide novel insights into why these potential genes are selected by the host.

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CHAPTER 2

ENERGY UTILIZATION IN LACTATING JERSEY COWS CONSUMING A MIXTURE OF DDGS AND STRAW IN REPLACEMENT OF ALFALFA HAY

INTERPRETIVE SUMMARY. Knoell et al. (20XX). "Energy utilization in lactating Jersey cows consuming a mixture of DDGS and straw in replacement of alfalfa hay." Four diets increasing amount of dried distillers grains and solubles (DDGS) and wheat straw while decreasing alfalfa amounts were fed to lactating Jersey cows. Increasing the DDGS and straw mixture did not impact dry matter intake (DMI) or milk yield (MY). Methane (CH_4) production decreased with the lowest amounts of CH_4 coming from the highest DDGS and straw diet. The results from this study indicate that production parameters can be maintained while decreasing methane production when feeding diets including DDGS and straw.

RUNNING HEAD: DISTILLERS GRAINS AND SOLUBLES, STRAW, AND ALFALFA EFFECT ON ENERGY UTILIZATION

Energy utilization in lactating Jersey cows consuming a mixture of DDGS and straw in replacement of alfalfa hay

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ABSTRACT

Some forages require significant amounts of water to grow, causing the dairy industry to be dependent on a limited resource. Feeding crop residues and feed coproducts in dairy rations may represent opportunities when alfalfa is not readily available, and to reduce the industry's use of water. A study using indirect calorimetry and 12 multiparous lactating Jersey cows (BW = 447.5 ± 43.7 kg; DIM = 71 ± 11 d, mean ± SD) was conducted to determine the effect of feeding dried distillers grains and solubles (DDGS) and straw in replacement of alfalfa hay on milk production and energy utilization. A triplicated 4 × 4 Latin square design was used to evaluate the replacement of alfalfa hay with a coproduct mixture (COP) containing approximately 1/3 wheat straw and 2/3 DDGS. The experimental treatments were as follows (proportions on a DM basis): a control diet (CON) containing 18.2% of alfalfa hay, a low-coproduct diet (LCOP) that contained 8.1% of COP, a medium-coproduct diet (MCOP) that contained 16.3% of COP, and a high-coproduct diet (HCOP) that contained 24.3% of COP. No differences were observed for dry matter intake (DMI) and milk yield (mean ± SEM) 19.5 kg ± 0.60, 29.6 kg ± 1.03, respectively. Milk protein concentration increased linearly from 3.54% on the CON to 3.65% on the HCOP diet. A linear decrease in total water consumption was observed on the HCOP diet, consuming only 91.4 L/d, as well as a 19.7% linear reduction in methane emissions on the HCOP diet. The digestibility of CP increased linearly on the HCOP diet. A tendency for increased N intake from the CON to the HCOP diet was detected while fecal N excretion was significantly decreased on the HCOP diet. A linear decrease in time spent ruminating at 5.7 h was detected for the HCOP diet. No differences were observed in gross, digestible, and metabolizable energy.

Results of this study indicate that alfalfa hay with a mixture of straw and DDGS can maintain milk production and DMI, but the replacement with the COP mixture may result in differences in energy utilization mostly driven by effects on methane reduction.

Key Words: dairy cow, coproducts, energy utilization, indirect calorimetry

INTRODUCTION

With the increasing global population, the demand for water for food production has increased (Fereres and Soriano, 2007); consequently, there is a need to seek methods to produce food in ways that requires less water. Use of irrigation varies around the world, but in some regions, policies are being enacted that may potentially limit the production of forage for dairy cows. For example, Saudi Arabian policy-makers (Cision PR Newswire, 2018) recently embarked on a plan to conserve water resources by limiting domestic forage production, and practical solutions to replace this common feed are needed. One such method is to develop diet formulation strategies that includes feed-quality biomass that is either unwanted or unsuitable for human consumption (Takiya et al., 2019).

Feed coproducts are produced by the fuel-ethanol industry and these feeds are incorporated into many U.S. dairy rations (Foth et al., 2015). Cattle are unique in their ability to consume food sources that are inedible to humans, such as fibrous coproducts of ethanol production, and turn these into nutritionally rich food products for human consumption. Milk production from the dairy cows enables productive use of otherwise inedible product for humans (Garnett, 2009; White and Hall, 2017). Utilizing coproducts

such as DDGS that require no added water to produce, may contribute to decreasing agricultural water usage. Previous research has been conducted on replacing corn or grain sources with coproducts as well as simply decreasing alfalfa hay in the diet, yet a knowledge gap exists with full replacement of alfalfa hay with coproducts on energy partitioning and production measures.

Limited water supply in some regions is resulting in producers looking for alternatives to feeding irrigation-dependent alfalfa. If the alfalfa supply is limited or too expensive, dairy producers need effective options for balancing rations, while maintaining performance. However, this can be troublesome as DDGS has a smaller particle size compared to alfalfa (Beauchemin, 2018). Beauchemin et al. (2008) also reported that cows consume concentrates faster than rough forages and, as a result, chewing activity is impacted and less saliva is produced, which can disrupt effective rumination (White et al., 2017). Physically effective fiber is a method to characterize the effects of rumen function with consumption of roughages (Mertens, 1997; White et al., 2017). Dietary formulations must be unique to provide the necessary physically effective fiber to maintain chewing activity and rumination, as rough forages are imperative to rumen health (Cole and Mead, 1943). The addition of straw to diets will provide the necessary effective fiber to promote healthy rumen activity, as Beauchemin et al. (2008) indicated that straw stimulated double the amount of saliva production due to it having a higher NDF content and slower rate of consumption by the cows. A mixture of DDGS and straw will supply the effective fiber requirement and nutrients required for milk production. In this current study, we plan to look at such formulations with a focus on whole animal energy and N utilization, as well as effective fiber.

MATERIALS AND METHODS

Twelve multiparous lactating Jersey cows averaging 71 ± 11.0 DIM and 447.5 ± 43.7 kg of BW were utilized for this study. All cows were housed in a temperature-controlled facility at the Dairy Metabolism Facility at the Animal Science Complex at the University of Nebraska-Lincoln and milked twice daily at 0700 and 1800 h in individual tiestalls equipped with rubber mats. All animal care and experimental procedures were approved by the University of Nebraska-Lincoln Animal Care and Use Committee. At the conclusion of the last experimental period, all cows were less than 90 d pregnant; thus, no energetic adjustments were made for conceptus growth. One cow was removed from the study due to inability to acclimate to the headbox.

The experimental design was a 4 × 4 triplicated Latin square. Cows were blocked by milk production and assigned randomly to 1 of the 4 dietary treatments, control (CON), low-coproduct (LCOP), medium-coproduct (MCOP), or high-coproduct (HCOP) according to Kononoff and Hanford (2006). Animals were blocked into each square by milk production (kg/d). Treatments were alternated over 4 experimental periods and measurements were collected on each animal consuming each treatment within the same period. The study was conducted with a total of 4 experimental periods, each being 35 d in duration and cows were fed ab libitum with approximately 5% refusal.

The 4 treatment diets were formulated with treatments containing different concentrations of DDGS (POET Nutrition LLC, Sioux Falls, SD) and wheat straw (Table 1). The DDGS for this study was delivered all at once in one load. It was sampled periodically throughout the study for a total of three samplings (n = 3). All dietary

treatments contained corn silage and a concentrate mixture that was combined as a TMR. Replacement of alfalfa hay was achieved by incrementally increasing a mixture of DDGS and wheat straw. In an attempt to balance the treatment diets for similar concentrations of bypass protein, fat, and energy, small proportions of the non-enzymatically browned soybean meal, lard, ground corn were also reduced. The control (CON) diets did not contain either DDGS or straw, but the inclusion of these ingredients increased in LCOP (6.0 and 2.1%), MCOP (12.1 and 4.2%), and HCOP (18.1 and 6.2%) treatments (Table 2.1). Straw was a 3-inch grind size for ration mixing and alfalfa was a 7-inch grind size. Diet compositions for all treatments are presented in Table 2.1. The TMR was mixed in a Calan Data Ranger (American Calan Inc., Northwood, NH) and fed once daily at 1000 h to the cows.

Individual feed ingredients were sampled on the first day of each collection period and frozen at -20°C. A subsample was sent to Cumberland Valley Analytical Services Inc. (Hagerstown, MD) for complete nutrient analysis. The DM content of forages was determined by drying at 135°C (Method 930.15, AOAC International, 2000). Additionally, nitrogen (Leco FP-528 N Combustion Analyzer, Leco Corp., St. Joseph, MI), NDF with sodium sulfate (Van Soest et al., 1991) and ∝-amylase, ADF (method 973.18; AOAC International, 2000), acid detergent lignin (Goering and Van Soest, 1970), NFC [100 – (% NDF + % CP + % Fat + % Ash)], sugar (DuBois et al., 1956), starch (Hall, 2009), crude fat (2003.05; AOAC International, 2006) and minerals (985.01; AOAC International, 2000) were determined. Total mixed rations were sampled on each day of each collection period and were frozen at -20°C. The samples were then composited by period and treatment. A subsample was sent to Cumberland Valley

Analytical Services Inc. (Waynesboro, PA) for nutrient analysis using the same laboratory processes as the individual feed ingredients. The TMR was used to determine particle size according to Heinrichs and Kononoff (2002) using the Penn State Particle Separator. Each day of the collection period, refusals were sampled and frozen at -20°C. The samples were composited by period and individual cow. A subsample was sent to Cumberland Valley Analytical Services Inc. for nutrient analysis of DM (AOAC International, 2000), N (Leco FP-528 N Combustion Analyzer, Leco Corp.), NDF with sodium sulfite (Van Soest et al., 1991), starch (Hall, 2009), and ash (943.05; AOAC International, 2000).

Body weight was taken at on the first day of the collection period and on the last day of the period. Body condition scoring was done at the same time as body weight.

Total fecal and urine output were collected from each individual cow during the collection period for 4 consecutive days. A 137 × 76 cm rubber mat was placed behind the cow to collect feces. The feces were deposited multiple times a day from the rubber mats into a large garbage container (Rubbermaid, Wooster, OH) with a black garbage bag covering the top to reduce nitrogen losses before subsampling. The feces were subsampled (~250g as is) every day for 4 consecutive days on a wet-weight basis, dried at 60°C in a forced-air oven for 48 h, and then composited by cow and period before being ground to pass through a 1-mm screen (Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA). The ground feces samples were sent to Cumberland Valley Analytical Services Inc. for nutrient analysis of DM (AOAC International, 2000), N (Leco FP-528 N Combustion Analyzer, Leco Corp.), NDF with sodium sulfite (Van Soest et al., 1991), starch (Hall, 2009), and ash (943.05; AOAC International, 2000). Total urine was

collected by inserting a 30 French foley catheter into each cow's bladder with a stylus. The balloon was inflated 55mL with physiological saline, and Tygon (Saint Gobain, La Defense, Courbevoie, France) tubing drained into a plastic carboy (15 quart) behind the cow. Using the funnel spout of the plastic carboy, urine was deposited into a 55-L plastic container multiple times daily and was acidified with HCl before subsampling (500mL) on a wet-weight basis and freezing at -20°C every day of the collection period. Prior to analysis, urine was thawed and boiled to remove the water content. To boil the urine, 2 thawed 250-mL bottles of urine were poured into a 600-mL beaker. Twelve urine-filled beakers were placed into a boiling water bath (Ankom Technology, Macedon, NY) underneath a hood. The water bath was turned on in the morning and off in the afternoon, for approx. 8 h/d, to reduce the chance of sample being overheated and burned. The brown paste was then lyophilized (Freezemobile 25ES, VirTis, Gardiner, NY). The lyophilized urine samples were analyzed at the University of Nebraska-Lincoln for laboratory-corrected DM (100°C oven for 24 h), N (FlashSmart N/ Protein Analyzer CE Elantech, Inc., Lakewood, NJ) and GE (Parr 6400 Calorimeter, Moline, IL).

Milk production was measured daily and milk samples were collected during both the morning and evening milking's for 5 consecutive days or d 29 to 33 of the entire period. Four tubes were collected each milking (150 mL); two 50-mL conical tubes were frozen at -20°C and two tubes were sent to Heart of America DHIA (Kansas City, MO) preserved with 2-bromo-2-nitropropane-1,3 diol. Milk samples were analyzed for fat, protein, lactose, SNF, MUN, and SCC using a Bentley FTS/FCM Infrared Analyzer (Bentley Instruments, Chaska, MN). The conical tubes were lyophilized and composited by cow and period for nutrient analysis. Milk samples were analyzed at the University of

Nebraska-Lincoln for laboratory-corrected DM, N, and GE. To determine the DM content of individual feed ingredients, TMR, refusals, feces, and urine samples were dried at 60°C in a forced-air oven for 48 h and then composited by treatment, then by cow and period. Milk samples were lyophilized to determine DM. Feed ingredients, refusals, and feces were ground as previously described with the feces and for laboratory-corrected DM and GE. The GE of the diet was measured using an isoperibol bomb calorimeter (Parr 6400 Calorimeter, Moline, IL).

Indirect calorimetry is used to indirectly estimate heat production, which represents a loss of energy from the animal (Blaxter, 1962). Measuring the ratio of CO_2 produced to O_2 consumed is known as the respiratory quotient (RQ), and is known to be influenced by diet composition, metabolic rate, physiological status, and intake (van Ouverkerk and Pedersen, 1994). Heat production was determined through the headbox-type indirect calorimeters, described by Foth et al. (2015) and Freetly et al. (2006), that were built at the University of Nebraska-Lincoln. Prior to collections, headboxes were used to test the rate of recovery of gas by burning 100% concentration of ethyl alcohol in the sealed headbox and comparing this measure with calculated gas concentrations. For each cow, a collection of one 23-h interval measured oxygen consumption and carbon dioxide and methane production. The design of the headboxes allowed for feed to be placed in the bottom of the box, and ad libitum access to water was available for the cows from a water bowl placed inside the headbox. Free water intake was measured using a DLJGHT garden hose water meter (DLJ Meter, Hackensack, NJ) while each cow was inside the headbox, whereas water from feed was measured from moisture contents of feed consumed. Within the headbox, dry-bulb and dew-point temperatures were recorded

every minute for a 23-h interval using a probe (model TRH-100, Pace Scientific Inc., Mooresville, NC) that was connected to a data logger (model XR440, Pace Scientific Inc.). Fifteen minutes before the start of the collection, the doors were closed and the motor was started to initialize headbox function. Line pressure was measured using a manometer (item # 1221-8, United Instruments, Westbury, NY). Barometric pressure of the room was also recorded using a barometer (Chaney Instruments Co., Lake Geneva, WI) and uncorrected for sea level. Total volume of gas in the headbox was measured using a gas meter (model AL425, American Meter, Horsham, PA). From the headbox, continuous amounts of outgoing and incoming air were diverted to 2 different collection bags (61 × 61 cm LAM-JAPCON-NSE, 44 L; PMC, Oak Park, IL) using glass tube rotameters (model 1350E Sho-Rate "50," Brooks Instruments, Hatfield, PA). Collection bags with gas samples inside were analyzed (Emerson X-stream 3-channel analyzer, Solon, OH) at the University of Nebraska-Lincoln. Heat production was estimated through calculation of oxygen consumption, and carbon dioxide and methane production with correction for urinary N loss according to Brouwer (1965; Equation 1). The gaseous products were reported in liters and the mass of urinary N in grams. Respiratory quotient was calculated using the ratio of carbon dioxide produced to the oxygen consumed and was not corrected for nitrogen. Volume of methane produced was multiplied by a constant of 9.45 kcal/L to estimate the amount of energy formed from the gaseous products. Energy balance was calculated for each cow according to Freetly et al. (2006) using the Brouwer equation [1]:

Heat Production (Mcal/d) = $3.866 \times O_2$ L + $1.200 \times CO_2$ L – $0.518 \times CH_4$ L – $1.431 \times$ N g [1]

Metabolizable energy (ME) (Mcal/d) = intake energy Mcal/d – fecal energy Mcal/d – urinary energy Mcal/d – methane energy Mcal/d [2]

Surplus energy was presumed to characterize tissue energy, expressed on an NE_L basis:

Recovered energy (RE) (Mcal/d) = ME – HP [3]

Tissue Energy (TE) (Mcal of NE_L/d) = positive TE × k_L/k_G or negative TE × k_T [4]

Where k_T is the efficiency of utilizing body reserve energy for milk production, k_G is the efficiency of utilizing ME intake for tissue gain, and k_L is the efficiency of utilizing ME intake for milk production. Values of 0.89, 0.75, and 0.66 were used for k_T, k_G, and k_L, respectively (Moraes et al., 2015).

Tissue Energy (TE) (Mcal/d) = RE – milk energy Mcal/d [5]

TE as body protein (g/d) = (N balance g/d) × (5.88 kg of protein/ kg of N) × (5.7 Mcal/kg of protein)/1000 [6]

Rumination, eating, standing, and laying behaviors were recorded simultaneously by visual assessment of each animal every 10 min for 24 h for the first three days of the collection period (Kononoff et al., 2002). The third day for periods 2 and 3 was calculated out of 23 h. Behaviors from the first day of period 4 were not included. If the animal was marked as performing the behavior (eating, ruminating, standing or laying), and subsequently not performing, the assumption was made that the animal maintained that behavior for the duration of the 10 minutes. For rumination behavior, the cow was marked for eating if actively consuming feed. Rumination was marked if the cow was chewing and not actively eating. The cows were also marked if not eating or ruminating. For standing behavior, totals for the day were summed and subtracted from total minutes

a day to obtain each behavior. Total time spent doing each of the rumination behaviors were summed and averaged across the three days.

Statistical Analysis

Data were analyzed using the GLIMMIX procedure of Version 9.3 of SAS (SAS Institute Inc., Cary, NC). Factors of treatment, square, and period within square were considered as fixed effects. Cow within square was considered as a random effect. Using the LSMEANS option, the least square means of the treatments were determined. The linear, quadratic, and cubic effects of the coproduct mixture were tested using CONTRAST statements of SAS. Significance was declared at $P < 0.05$ and tendencies at $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Diet Composition

Chemical composition of the treatment diets as well as individual ingredients are presented in Tables 2.1 and 2.2. All treatment diets were formulated for similar chemical composition. The CP increased across treatments from 16.9 on the CON, 17.3 on the LCOP, 17.8 on the MCOP, and finally to 18.3% DM on the HCOP with the inclusion of DDGS. A similar trend was observed in crude fat, which increased from 4.1 to 4.7% of DM, mostly due to the inclusion of DDGS, which contained 7.5% crude fat. While percent hemicellulose was not measured directly, calculated values of ADF subtracted from NDF indicated an increase in percent hemicellulose from 9.8% in the CON to 11.3% in the HCOP diet. Similarly, percent cellulose was not measured directly,

calculated values of lignin subtracted from ADF indicated a decrease in percent cellulose from 15.6% in the CON and 14.8% in the HCOP.

Dry Matter and Water Intake

Schuler et al. (2013) suggest that when feeding lactating dairy cows increasing amounts of forages, intake may become limiting. This is because feeding bulky forages can be associated with a reduction in DMI, possibly due to gut fill (Hall and Chase, 2014). Additionally, increasing the inclusion of DDGS in the diet has also been observed to increase DMI (Castillo-Lopez et al., 2014). Despite the fact that alfalfa was removed from the diet and the inclusion of DDGS increased, DMI was not different between diets averaging 19.5 ± 0.60 kg/d across treatments (Table 2.3). We suggest that this is because the addition of straw resulted in similar particle size (Table 2.4) across treatments and that NDF intake was also similar, averaging 5.7 kg/d, neither particle size nor fiber content affected feed intake. The feed refusals indicated a tendency to decrease in NDF, indicating a potential for sorting of feed particles (Table 2.4).

Potassium has many functions within the body such as a role in maintaining osmotic pressure and water balance (NRC, 2001), nonetheless, it is not stored in the body, therefore daily intake of potassium is required (NRC, 2001). Once the potassium requirement has been met in the animal, excess potassium in the body gets excreted in the urine. According to the NRC (2001), alfalfa typically contains approximately 2.37% K. In the current study, the alfalfa hay contained 3.58% potassium (Table 2.2). The potassium content of both straw (1.42%) and DDGS (1.38%) was lower and as a consequence, the potassium content of the total diet decreased from 1.81 to 1.39% as the

concentration of alfalfa was reduced and the straw and DDGS mixture increased (Table 2.1). Similarly, water consumption was observed to be greatest for cows consuming the CON diet containing the greatest proportion of alfalfa hay and linearly decreased to almost 17 L less per d (Table 2.3) for the HCOP. We speculate that this response was due to a reduction in potassium consumption. Increasing dietary cations, such as potassium, coincides with the increase in water demand for the animal (Silanikove et al., 1997). The response observed in the current study is similar to studies by others who have evaluated the effect of potassium on water intake (Fisher et al., 1994; Nennich et al., 2006; Fraley et al., 2015). It should be noted that potassium was greatest in the control diet, with sodium being similar across treatments.

Nutrient Digestibility

Apparent digestibility of the diets is listed in Table 2.5. No differences were observed in dry matter and organic matter digestibility ($P \geq 0.35$) across treatments. A linear increase in CP digestibility was observed from the CON to HCOP diet ($P < 0.01$), increasing from 64.0 to 70.4 ± 0.95%. This could be attributed to the increase in protein in the diets from the DDGS being broken down by the microbes in the rumen for growth, and bypassing into the small intestine for digestion. Kononoff et al. (2007) states that feeding a dry distillers product in dairy rations results in higher RUP content. Additionally, hindgut fermentation may have been reduced. Castillo-Lopez et al. (2014) also fed increasing DDGS to lactating dairy cattle and observed a tendency of increased protein digestibility. A tendency for a quadratic response in starch digestibility was observed potentially due to the HCOP diet having a higher amount of starch in the diet by

ingredient. Dietary ADF decreased from 19.4% on the CON diet to 18.1% on the HCOP diet, allowing for increased hemicellulose digestion. The NDF digestibility remained similar ($P = 0.63$) across diets averaging 46% DM. While dietary NDF was not different, the amount of dietary lignin decreased from 3.8 in the CON diet to 3.3 in the HCOP diet. With a decrease in lignin content from the CON to the HCOP diet, digestibility of cellulose and hemicellulose should increase, yet no change was observed in ADF digestibility. Hemicellulose digestibility increased from roughly 9.8% to 11.3%. The addition of straw often decreases digestibility as it is a low-quality forage and can limit intake due to the lower digestibility and gut fill. By removing alfalfa and increasing DDGS in the diets, the concern for increased passage rate and metabolic occurrences were apparent. Including straw in the diet can help to increase gut fill (Hall and Chase, 2014) without adding greatly to nutrient density (Janovick et al., 2011) contributing to maintaining healthy normal rumen function. The mixture of straw and DDGS maintained digestibility with the decrease in inclusion of alfalfa.

O_2 Consumption, Production of CO_2, CH_4, and Heat

Gas production data are represented in Table 2.6. The RQ observed in this study did not differ between treatments indicating diet did not change the volumes of O_2 consumed and CO_2 produced. Recovery rates of oxygen and carbon dioxide averaged 99.0 $\pm$ 5.64% and 93.6 $\pm$ 8.02%, respectively. Consumption of O_2 did not differ ($P = 0.82$) averaging 4617.2 ± 166.2 L/d (Table 2.5) while production of CO_2 also did not differ ($P = 0.60$) averaging 4587.4 ± 188.3 L/d. Interestingly, methane production linearly decreased from 429.4 to 345.0 L/d as the proportion of alfalfa was reduced and

replaced with straw and DDGS. The observed decrease in CH_4 may have been due to a linear increase in fat content from 4.1 to 4.7% across these treatments. Several factors have been noted to depress methanogenesis in the rumen. Higher fat inclusion has been implicated in decreased fiber digestion (Huhtanen et al., 2009; Knapp et al., 2014) preventing microbial attachment. A decrease in fiber digestion implies rumen fermentation has been reduced. Fat may also have an impact on the methanogen population in the rumen with reduced substrates from bacterial fermentation. With increased fat in the diet, biohydrogenation may become more extensive, serving to redirect fermentation through a hydrogen sink (Blaxter and Czerkawski, 1966; Jenkins, 1993). The NRC (2001) reports that the average sulfur (S) content of DDGS is 0.44%. In the current study, alfalfa had 0.18% S content, straw had 0.08% S, while DDGS had 1.08% S (Table 2.2). The S content of the diets increased from 0.23 on the CON to 0.38 on the HCOP treatment, allowing for the potential for an overall increase in S in the rumen which has been noted in feedlot cattle (Nichols et al., 2012; Sarturi et al., 2012). This could permit sulfate-utilizing bacteria in the rumen to divert excess H^+ to making H_2S gas, a more energetically favorable pathway for recycling reducing equivalents (van Zijderveld et al., 2010; Ungerfeld and Kohn, 2006).

Efficiency measures can be indicative of strategies to reduce methane production from lactating dairy cattle. Determining the rate of CH_4 release per unit of milk yield (CH_4/MY), per unit DMI (CH_4/DMI), and per unit energy corrected milk (ECM) (CH_4/ECM), each in L/kg/d, can provide insight into the expected effectiveness of a methane mitigation strategy. Johnson and Johnson (1995) state that in vitro, distillers coproducts result in nearly 33% less CH_4 per kg DM digested. Furthermore, distillers

coproducts have ratios of hemicellulose to cellulose of greater than 1:1, while grasses and legumes have ratios less than 1:1 (Knapp et al., 2014), providing continued evidence of digested hemicellulose producing nearly 37% less methane compared to the more indigestible cellulose component (Moe and Tyrell, 1979). In this current study, CH_4/MY, CH_4/DMI, and CH_4/ECM linearly decreased ($P \leq 0.01$) from the CON diet to the HCOP diet. Methane per unit of digested NDF (dNDF) also linearly decreased ($P = 0.03$), most likely due to increasing hemicellulose (Knapp et al., 2014; Drehmel et al., 2018). Despite not being measured directly, the hemicellulose content of feeds can be grossly estimated by subtracting ADF from NDF (Goering and Van Soest, 1970). Hemicellulose increased from 9.8% on the CON diet to 11.3% on HCOP diet.

Chewing Behaviors and Effective Fiber

Fiber contained in feed is believed to have several important functions. The first is to supply energy to rumen microbes. The second is to physically stimulate chewing activities, which increases salivary buffer flow to the rumen reducing the risk of acidosis. The third is to contribute to the floating mat of large particles in the rumen and ultimately aid in maintaining normal rumen function. Replacing forages with nonforage fiber may reduce effective fiber in the diet (Bradford and Mullins, 2012). Although corn milling coproducts contain a high concentration of fiber similar to forages, DDGS are finer in particle size than forages. In addition to replacing alfalfa hay with DDGS, the proportion of straw in the diet was also increased. Heinrichs and Kononoff (2002) described particle size recommendations for the different sieve sizes while using the Penn State Particle Separator. The addition of straw to these diets could increase feed bunk sorting activity.

As a result, particle sizes of the experimental diets were similar, yet the addition of DDGS resulted in a lower percentage retained in the 8.0 – 1.18 mm sieve size from 49% in the CON to 43% in the HCOP diet, and the percentage that landed in the catch pan (<1.18 mm) increased from 22.6 to 31.1% (Table 2.4). While dietary NDF composition was similar across treatments, a linear decrease from 35.6 to 27% was observed in the amount of NDF refused (Table 2.3), indicating that while some disparities appear in particle size, a tendency for sorting was apparent, with the greatest amount of potential sorting appearing in the CON diet. The forage NDF decreased also from the CON to HCOP diet, potentially due to the lower number of large particles in the HCOP diet. With straw being a low-quality forage, there were concerns that it would not stimulate enough rumination to maintain healthy rumen function, as well as lead to sorting, which could impact intake.

Results of eating and ruminating behaviors are listed in Table 2.7, and given the similarities in TMR particle size it is interesting to note that total eating time was similar across treatments and that compared to the control, total ruminating time was only reduced in cows consuming the HCOP treatment. Beauchemin (1991) indicated that the average time dairy cows spend eating ranges from 4 to 7 h/d and ruminating ranges from 5 to 9 h/d. In the current study, cows across all treatments spent the same amount of time eating, approximately 3.6 h. Additionally, total time spent eating per kg of intake was not different across treatments. In a study conducted by Suarez-Mena et al. (2013), it was concluded that the forage to concentrate ratios had no influence on rumination time while Zhang et al. (2010) observed a decrease in ruminating time for cows fed a low-forage diet. As the DDGS mixture increased in inclusion of the diet in the present study,

ruminating time decreased linearly from 6.5 h to 5.7 h, indicating that the HCOP diet containing no alfalfa requires less rumination, while still able to maintain rumen function. Adding indigestible or low-quality forages can promote increased ruminating time due to increased need of remastication for particle size reduction (Welch and Smith, 1970) and chewing to enhance buffer secretion (Mertens, 1997; Farmer et al., 2014). In the current study, feeding a lower-quality forage did not lead to increased rumination time. While straw was only included at 6%, this amount was demonstrated to be adequate for rumination.

Energy Partitioning

Energy partitioning values are presented in Table 2.8. Given the nutrient composition of the ingredients and compared to the overall diet chemical composition, it would be most likely that the HCOP diet would have different energy intake compared to the CON. No differences were observed for GE intake, DE, and ME. In the current study, GE per unit of DM was observed to linearly increase from the CON to the HCOP diet (4.35 to 4.41 ± 0.01 Mcal/kg DM). Even though no differences were observed in DMI in this study, the increase in crude fat and crude protein on the HCOP diet may provide additional energy per unit of DM. Tendencies for linear increases for DE and ME were also observed. As a percentage of GE, no differences were observed in DE or ME. While the diets were formulated to be as isoenergetic as possible (Table 2.1), numerically the GE in cal/g increased, likely due to the increased protein and fat contents of diets that contained DDGS.

Energy is lost in feces, urine, CH_4, milk, and heat (Coppock, 1985; Moe, 1981). In the current study, total energy lost as feces, urine, milk, and heat were not different across treatments ($P \geq 0.1$). However, energy lost as CH_4 was significantly affected ($P \leq 0.01$). As the proportion of coproducts increased across treatments, energy lost as CH_4 decreased from 4.06 to 3.26 ± 0.215 Mcal/d in the CON to HCOP diets. This could be due to increased fat in the diet suppressing methanogenesis by increased biohydrogenation. Additionally, excess carbon could have been used to linearly increase milk protein percentage, or to linearly increase tissue protein, as both were observed. As a percentage of GE, CH_4 was reduced linearly ($P \leq 0.01$) from the CON to the HCOP treatment, decreasing from 4.78 to 3.89 ± 0.22%. The energy from the decrease in CH_4 may have been diverted to other metabolic processes and production measures. A 6% increase in urine energy loss was observed from the CON to HCOP diet, indicating that more energy may have been required to eliminate excess protein in the urine.

Moraes et al. (2015) conducted a meta-analysis of energy balance data from lactating dairy cattle and concluded that cows with a superior genetic aptitude have higher fasting heat production, and is also reflected in the increased maintenance requirements observed currently. No difference was observed for NE_L in Mcal/kg of DM indicating that all diets provided the same efficiency for converting ME to milk (k_L). Variable ME has been known to impact milk yield (Moe and Tyrrell, 1974). Nonetheless, this was not observed in the current study as ME was not different among diets. An increase in fat content in diets could increase k_L in those respective diets (Morris et al., 2020); however, this was not observed in this current study. Ranathunga et al. (2018)

increased DDGS from 0 to 18% without replacing any alfalfa hay, but saw no impact on NE_L on both low- and high-forage diets.

Nitrogen Balance

Feeding coproducts that contain greater CP content from forage or grains it replaces leads to an in increase in CP in the rations, leading to increased N excretion (Broderick, 2003; Groff and Wu, 2005). In the current study, N partitioning for treatments is presented in Table 2.9. A tendency for a linear increase in N intake was observed from the CON diet to the HCOP treatment diet. This is a function of the increase in CP when coproducts increased in the diet. Weiss et al. (2009) determined that total N intake affects N excretion. Fecal N excretion exhibited a 12.8% linear decrease from the CON to the HCOP diet, while urinary N excretion was not affected. This could be due to CP digestibility increasing from the CON to the HCOP diet, and that the excess amount of CP may have been routed for milk protein or tissue deposition. Weiss et al. (2009) states that amount of N consumed influences the amount N found in the feces, while the location of elimination of N is determined by forage and carbohydrate type. This can cause variable results for N balance. The CP digestibility increased with coproduct inclusion and is likely why fecal N excretion decreased and N balance increased with coproduct inclusion. Feeding dry distillers products can result in a higher RUP content (Kononoff et al., 2007). This may raise the amount of excess N to be eliminated from the animal. The HCOP treatment diet observed an increase of 47.4 ± 15.0 g/d in N balance from the CON diet. Elimination of excess N may require less water on the HCOP diet compared to the CON.

Milk Production, and Composition

Janicek et al. (2008) observed a linear increase in milk yield with increasing DDGS concentration. In the current study, milk production was not different across treatments averaging 29.6 kg/d ± 1.03 kg/d (Table 2.3). Hall and Chase (2014) conducted a study evaluating different forage substitute combinations impacting performance of lactating dairy cows. In their study, they reported decreases in milk production, although not significant, and speculated this response was due to the dietary energy provided in the diet. The lack of difference observed in DMI may also explain why milk yield was not different between the diets. Milk fat depression is a concern when formulating dairy rations that contain DDGS (Janicek et al., 2008; Ramirez-Ramirez et al., 2016) which contain a high concentration of unsaturated fatty acids that can be biohydrogenated in the rumen resulting in some isomers that have direct and negative effects on fatty acids synthesis. Although the concentration of milk fat was not affected, total yield of milk fat tended to follow a quadratic response with high yield observed on the intermediate treatments. Additionally, milk protein percentage increased linearly from 3.54 to 3.65 ± 0.08% from the CON to the HCOP diet. We speculate that the response observed in milk composition are a result of differences in sorting behavior, and as a result of nutrient intake. In the current study, a linear effect on NDF content of feed refusals was observed. We speculate that as when cows consume the highest proportion of coproduct, that a breakpoint was reached in which the consumption of unsaturated fatty acids had a direct effect on milk fat synthesis (Kadegowda et al., 2008). In contrast the increase in fatty acid

intake supplied additional metabolizable energy that supported milk protein synthesis (Brun-Lefleur et al., 2010).

CONCLUSIONS

The comparison of replacing alfalfa with straw and DDGS directly has not been well characterized. This substitution was able to maintain milk production, and increase milk protein. Energy partitioning with this substitution was also not different but exhibited a decrease in methane production. Water consumption also decreased with this substitution. This information indicates that when water is scarce and forages, such as alfalfa, may be low in availability, coproducts and straw can be used to maintain production measures while decreasing the contribution to methane emissions, and less consumption of water.

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Table 2.1. Chemical composition and analysis of treatments formulated to contain increasing amounts of coproducts

	Treatment[1]			
Item	CON	LCOP	MCOP	HCOP
Ingredient, % DM				
Corn silage	37.5	37.5	37.5	37.5
Alfalfa hay	18.2	12.1	6.1	-
Straw	-	2.1	4.2	6.2
Ground corn	14.7	13.7	12.8	12.0
Soybean meal	11.3	11.3	11.3	11.3
RFDDGS[2]	-	6.0	12.1	18.1
Nonenzymatically-browned soybean meal[3]	5.17	4.12	3.06	2.01
Molasses, dried	2.55	2.55	2.55	2.55
Whey	2.56	2.56	2.56	2.56
Lard	2.01	1.81	1.62	1.43
Soybean hulls	1.44	1.44	1.43	1.43
Calcium carbonate	1.33	1.48	1.63	1.77
Bloodmeal	0.89	0.89	0.89	0.89
Calcium soaps of fatty acids [4]	1.33	1.48	1.63	1.77
Sodium bicarbonate	0.57	0.56	0.56	0.55
Salt	0.33	0.33	0.33	0.33
Magnesium oxide	0.31	0.31	0.31	0.31
Calcium diphosphate	0.22	0.15	0.07	0.00
Rumen protected methionine[5]	0.07	0.07	0.07	0.07
Rumen protected lysine[6]	0.00	0.06	0.11	0.17
Vitamin premix[7]	0.05	0.05	0.05	0.05
Mineral premix[8]	0.04	0.04	0.04	0.04
Chemical Composition, % of diet DM[9]				
DM, %	60.2 (0.02)	59.9 (0.02)	60.1 (0.03)	60.0 (0.02)
CP	16.9 (0.48)	17.3 (0.43)	17.8 (0.38)	18.3 (0.35)
Crude fat	4.1 (0.32)	4.3 (0.29)	4.5 (0.28)	4.7 (0.28)
ADF	19.4 (1.11)	19.0 (0.87)	18.5 (0.67)	18.1 (0.55)
NDF	29.2 (1.41)	29.3 (1.07)	29.3 (0.76)	29.4 (0.50)
fNDF[10]	23.3 (1.49)	22.0 (1.28)	20.7 (1.28)	19.3 (1.02)
Lignin	3.8 (0.31)	3.6 (0.29)	3.5 (0.32)	3.3 (0.39)
Ash	8.4 (0.20)	8.1 (0.23)	7.9 (0.29)	7.6 (0.36)
Starch	24.3 (1.20)	24.3 (1.10)	24.2 (1.01)	24.1 (0.91)
Na	0.39 (0.02)	0.39 (0.01)	0.39 (0.01)	0.39 (0.01)
K	1.81 (0.04)	1.67 (0.04)	1.53 (0.03)	1.39 (0.04)
S	0.23 (0.02)	0.28 (0.02)	0.33 (0.02)	0.38 (0.02)
DCAD, % DM[11]	38.6 (1.71)	32.0 (1.44)	25.4 (1.28)	18.8 (1.25)

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]RFDDGS -Dakota Gold reduced fat distillers grains plus solubles, POET Nutrition LLC, Sioux Falls, SD.
[3]Soypass, LignoTech, Overland Park, KS.
[4]Calcium salts of long-chain fatty acids marketed as Megalac by Church & Dwight Co. Inc. Princeton, NJ.
[5]Smartamine M, Adisseo Inc., Antony, France.
[6]AjiPro-L, Ajimomoto Heartland Inc., Chicago, IL.
[7]Contained 120,000 IU/d vitamin A, 24,000 IU/d of vitamin D, and 800 IU/d Vitamin E in total ration.
[8]Contained 13.9 % Ca, 0.03 % P, 0.42 % Mg, 0.20 % K, 4.20 % S, 0.08 % Na, 0.03 % Cl, 445 mg/kg Fe, 60,021 mg/kg Zn, 17,375 mg/kg Cu, 43,470 mh/kg Mn, 287 mg/kg Se, 527 mg/kg Co, and 870 mg/kg I in total ration.
[9]Values determined by Cumberland Valley Analytical Services, Hagerstown, MD, Mean (SD).
[10]fNDF, % DM – forage NDF fraction, as a percent of DM
[11]Dietary cation-anion difference (mEq/100g of DM = ((Na + K) – (Cl + S))/100 g of DM).

Table 2.2. Feed chemical composition for alfalfa hay, corn silage, straw, distillers grains, and concentrate mixes

	Alfalfa hay (N = 4)[1]		Corn silage (N = 4)		Straw (N = 4)		DDGS (N = 3)		CON Concentrate (N = 4)		BP Concentrate (N = 4)	
Item, % of DM	Mean[2]	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM, % as is	89.0	0.01	38.3	0.03	91.4	0.01	89.7	0.96	91.5	0.01	91.7	0.00
CP	15.8	0.99	8.25	0.41	3.63	0.46	30.8	0.55	24.6	0.61	26.6	0.46
Soluble Protein	5.48	0.22	4.30	0.34	1.40	0.08	5.07	0.91	4.08	0.84	4.95	0.50
ADICP[3]	1.52	0.21	0.77	0.18	1.18	0.11	2.47	0.42	0.66	0.23	1.28	0.28
NDICP	2.35	0.23	0.88	0.21	1.39	0.17	2.74	0.63	2.32	0.65	1.53	0.33
ADF	40.0	2.50	22.9	1.13	57.6	1.43	12.2	1.50	7.95	1.34	10.5	0.65
NDF	49.4	2.06	38.3	1.38	79.9	0.98	33.8	5.25	13.3	3.10	17.9	1.17
Lignin	9.00	0.42	3.06	0.22	9.28	0.51	2.18	0.18	2.26	0.55	2.83	0.52
Starch	1.58	0.50	35.3	2.33	1.18	0.34	5.47	3.18	24.4	0.90	19.2	0.49
Crude fat	1.09	0.29	2.79	0.28	0.80	0.23	7.54	2.91	6.37	0.72	6.35	0.57
Ash	9.67	0.19	4.52	0.61	8.65	0.91	5.21	0.17	11.2	0.37	9.52	0.22
Ca	1.09	0.08	0.21	0.02	0.23	0.02	0.07	0.03	2.23	0.11	1.74	0.07
P	0.37	0.01	0.30	0.02	0.08	0.03	0.92	0.04	0.61	0.09	0.64	0.04
Mg	0.23	0.01	0.13	0.01	0.07	0.01	0.40	0.01	0.69	0.07	0.58	0.01
K	3.58	0.04	1.16	0.09	1.42	0.17	1.38	0.10	1.64	0.08	1.54	0.06
S	0.18	0.03	0.15	0.02	0.08	0.02	1.08	0.24	0.32	0.02	0.56	0.02
Na	0.04	0.00	0.02	0.01	0.02	0.00	0.24	0.12	0.84	0.05	0.68	0.03
Cl	0.11	0.02	0.10	0.01	0.16	0.19	0.21	0.03	0.70	0.06	0.55	0.00

[1]The number of samples that were used to calculate values.

[2]Mean and SD were calculated based on samples of each feedstuff collected during each period and estimated by a commercial feed testing laboratory (Cumberland Valley Analytical Services, Waynesboro, PA).

[3]ADICP = Acid-detergent-insoluble crude protein; NDICP = Neutral-detergent-insoluble crude protein.

Table 2.3. The effects of replacing alfalfa hay with a mixture of DDGS and straw on DMI, milk production and composition, body weight and BCS[7], and water intake in lactating Jersey cows

	Treatment[1]					*P*-value			
Item	CON	LCOP	MCOP	HCOP	SEM[2]	Treatment	Linear	Quadratic	Cubic
DMI, kg/d	19.4	19.5	19.6	19.3	0.60	0.979	0.956	0.688	0.884
NDF Intake[3], kg/d	5.71	5.70	5.77	5.65	0.18	0.957	0.892	0.729	0.674
Refusal NDF, %	35.6	29.1	30.6	27.9	2.61	0.132	0.056	0.426	0.249
Milk yield, kg/d	29.4	29.5	30.5	29.1	1.03	0.401	0.909	0.211	0.246
ECM[4], kg/d	37.6	38.6	39.0	36.6	1.06	0.240	0.534	0.059	0.574
Fat, %	5.48	5.64	5.40	5.26	0.26	0.631	0.351	0.463	0.581
Fat yield, kg/d	1.58	1.64	1.62	1.50	0.06	0.282	0.308	0.090	0.929
Protein, %	3.54	3.56	3.60	3.65	0.08	0.187	0.034	0.704	0.938
Protein yield, kg/d	1.03	1.04	1.09	1.05	0.04	0.428	0.307	0.362	0.382
Lactose, %	4.83	4.80	4.82	4.74	0.03	0.014	0.007	0.211	0.068
Lactose yield, kg/d	1.41	1.41	1.46	1.37	0.05	0.213	0.697	0.143	0.122
MUN[5], mg/dL	17.2	17.4	16.7	16.9	0.76	0.807	0.577	0.960	0.437
SCC[6], cells/mL	71.7	78.5	101.3	108.4	41.9	0.345	0.086	0.995	0.660
Body weight, kg	457	462	457	462	14.6	0.129	0.295	0.976	0.031
BCS[7]	3.23	3.26	3.21	3.23	0.05	0.566	0.641	0.969	0.192
Free water intake, L/d	95.2	90.3	86.6	78.8	6.79	0.118	0.019	0.758	0.790
Water from feed, L/d	12.8	12.9	13.0	12.6	0.36	0.845	0.695	0.485	0.661
Total water intake, L/d	108.1	103.2	99.6	91.4	6.85	0.113	0.018	0.723	0.768

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]Lowest standard error of treatment means is shown.

[3]NDF Intake = the amount of NDF consumed.

[4]Energy corrected milk = 0.327 × milk yield [kg] + 7.2 × protein [kg] + 12.95 × fat [kg] adjusted for 3.5% fat and 3.2% total protein (DHI Glossary, 2014).

[5]MUN = Milk urea nitrogen.

[6]SCC = Somatic cell count.

[7]BCS = Body Condition Score 1-5 scale according to Wildman et al. (1982).

Table 2.4. Particle distribution of treatments formulated for increasing coproducts on the total mixed ration (DM basis)[1]

	CON		LCOP		MCOP		HCOP	
Particle Size, %	Mean	SD	Mean	SD	Mean	SD	Mean	SD
> 19.0 mm	4.38	1.77	3.00	1.41	4.50	1.69	3.75	1.75
19.0 - 8.0 mm	24.5	2.51	23.5	2.51	24.0	4.14	22.3	3.37
8.0 - 1.18 mm	49.0	2.39	47.3	1.83	43.4	2.56	42.5	2.62
< 1.18 mm	22.6	2.62	26.1	3.36	27.9	3.52	31.1	2.47

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.
[2]Determined using the Penn State Particle Separator on DM basis (Heinrichs and Kononoff, 2002).

Table 2.5. Apparent DM, OM, CP, NDF, starch, ash digestibility, and urine and fecal outputs of treatments containing no, low, medium, or high amount of coproduct

	Treatment[1]					*P*-value			
Component, %	CON	LCOP	MCOP	HCOP	SEM[2]	Treatment	Linear	Quadratic	Cubic
DM	67.5	68.9	68.7	68.5	0.860	0.601	0.469	0.324	0.612
OM	69.0	70.5	70.4	70.2	0.831	0.532	0.346	0.314	0.678
CP	64.0	67.4	68.5	70.4	0.951	< 0.001	< 0.001	0.378	0.442
NDF	45.6	47.6	46.7	47.0	1.490	0.787	0.625	0.563	0.514
Starch	95.0	95.5	95.6	93.9	0.750	0.228	0.247	0.086	0.587
Fecal output, kg/d									
Wet	42.1	40.0	39.9	37.9	1.56	0.213	0.047	0.993	0.511
Dry	6.36	6.05	6.18	5.98	0.21	0.465	0.223	0.764	0.334
Urine output, kg/d									
Wet	22.8	23.4	21.8	20.4	0.96	0.066	0.020	0.203	0.498
Dry	1.24	1.16	1.21	1.12	0.05	0.159	0.093	0.900	0.214

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]Lowest Standard error of treatment means is shown.

Table 2.6. Oxygen consumption, carbon dioxide and methane productions, methane efficiencies, and heat production for treatments formulated to contain DDGS and straw

	Treatment[1]					*P*-value			
Item	CON	LCOP	MCOP	HCOP	SEM[2]	Treatment	Linear	Quadratic	Cubic
O_2 consumption, L/d	4637.7	4632.3	4582.1	4616.7	166.2	0.982	0.820	0.855	0.789
CO_2 production, L/d	4628.7	4617.5	4571.6	4531.7	188.3	0.945	0.559	0.909	0.942
CH_4 production, L/d	429.4	405.0	391.3	345.0	22.8	0.015	0.002	0.512	0.555
CH_4/MY[3], L/kg/d	14.8	13.8	13.1	11.9	0.96	0.040	0.005	0.910	0.854
CH_4/ECM, L/kg/d	11.4	10.5	10.1	9.4	0.60	0.036	0.005	0.719	0.718
CH_4/DMI, L/kg/d	22.2	20.7	20.0	18.1	1.04	0.019	0.002	0.813	0.599
CH_4/dNDF[4], L/kg	165.9	149.6	147.0	137.8	9.23	0.155	0.033	0.672	0.588
RQ[5]	1.00	1.00	1.00	0.99	0.010	0.330	0.124	0.353	0.547

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]Lowest standard error of treatment means is shown.

[3]MY = Milk yield, L/kg/d.

[4]CH_4/dNDF = Methane per unit digested NDF.

[5]RQ = Respiratory quotient (CO_2 production/ O_2 consumption).

Table 2.7. The effects of replacing alfalfa hay with a mixture of DDGS and straw on standing, lying, eating, and ruminating behaviors in lactating Jersey cows

	Treatment[1]					*P* - value			
Item	CON	LCOP	MCOP	HCOP	SEM[2]	Treatment	Linear	Quadratic	Cubic
Standing, hr	11.6	12.8	12.2	12.5	0.586	0.253	0.329	0.310	0.152
Lying, hr	11.7	10.7	11.3	11.0	0.586	0.253	0.329	0.310	0.152
Eating, hr	3.6	3.5	3.6	3.6	0.172	0.874	0.980	0.869	0.427
Ruminating, hr	6.5	6.7	6.5	5.7	0.220	0.007	0.007	0.011	0.668
Eating/DMI, min/kg	11.2	10.8	11.3	11.1	0.618	0.931	0.896	0.876	0.538
Ruminating/DMI, min/kg	20.0	20.7	20.0	17.8	0.797	0.031	0.021	0.035	0.959

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]Lowest standard error of treatment means is shown.

Table 2.8. Partitioning of energy for treatments formulated to contain DDGS and straw for lactating Jersey cows

	Treatment[2]					*P*-value			
Item[1]	CON	LCOP	MCOP	HCOP	SEM[3]	Treatment	Linear	Quadratic	Cubic
	Mcal/d								
GE intake	84.9	84.7	86.6	84.2	2.67	0.907	0.963	0.650	0.564
DE	57.0	57.9	59.2	57.6	2.13	0.880	0.720	0.548	0.712
ME	50.3	51.6	52.8	51.5	2.00	0.824	0.578	0.505	0.771
Component									
Feces	28.0	26.9	27.4	26.5	0.981	0.584	0.270	0.879	0.413
Methane	4.06	3.83	3.70	3.26	0.215	0.015	0.002	0.512	0.555
Urine	2.60	2.44	2.67	2.73	0.114	0.181	0.162	0.239	0.191
Heat	23.0	23.0	22.8	22.9	0.849	0.982	0.769	0.927	0.800
Milk	20.3	19.6	20.2	18.9	0.631	0.157	0.096	0.452	0.133
Tissue	6.99	8.93	9.86	9.35	1.773	0.623	0.294	0.465	0.954
in body protein	1.45	2.72	2.85	3.04	0.502	0.077	0.025	0.241	0.546
in body fat	5.54	6.21	7.01	6.26	1.362	0.878	0.618	0.589	0.771
	% of GE								
DE	67.1	68.3	68.4	68.3	0.903	0.661	0.341	0.470	0.814
ME	59.3	60.9	61.0	61.1	1.043	0.510	0.227	0.432	0.742
Feces	32.9	31.7	31.6	31.7	0.903	0.661	0.341	0.470	0.814
Methane	4.78	4.50	4.27	3.89	0.216	0.009	0.001	0.769	0.774
Urine	3.07	2.89	3.10	3.26	0.117	0.176	0.132	0.145	0.380
Heat	27.2	27.3	26.4	27.5	0.780	0.616	0.983	0.403	0.300
Milk	23.9	23.3	23.4	22.8	0.832	0.668	0.249	0.998	0.661
Tissue	8.16	10.3	11.3	10.8	1.935	0.579	0.268	0.439	0.966
	Mcal/kg of DM								
GE	4.35	4.35	4.39	4.41	0.011	0.002	< 0.001	0.206	0.264
DE	2.92	2.97	3.00	3.02	0.041	0.354	0.091	0.648	0.996
ME	2.58	2.65	2.68	2.70	0.047	0.287	0.074	0.562	0.879
NE_L[4]	1.63	1.65	1.67	1.62	0.032	0.695	0.980	0.267	0.688

[1]GE = gross energy; DE = digestible energy; ME = Metabolizable energy

[2]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[3]Lowest standard error of treatment means is shown.

[4]$NE_L = 0.080 \times BW^{0.75}$ + milk energy + tissue energy for efficiency of conversion to milk energy (NRC, 2001).

Table 2.9. Partitioning of nitrogen for treatments formulated to contain no, low, medium, or high amount of coproduct

	Treatment[1]					*P*-value			
Item	CON	LCOP	MCOP	HCOP	SEM[2]	Treatment	Linear	Quadratic	Cubic
Mass, g/d									
N intake	527.5	547.2	566.2	565.1	17.2	0.268	0.074	0.508	0.778
Fecal N excretion	190.1	178.1	177.6	165.9	6.49	0.024	0.004	0.977	0.299
Urine N excretion	159.2	145.0	160.1	167.6	8.20	0.161	0.207	0.126	0.231
Milk N concentration	135.0	143.0	143.6	139.3	5.66	0.550	0.537	0.211	0.898
N balance[3]	43.3	81.1	85.0	90.7	15.0	0.077	0.025	0.241	0.546
N, % of intake									
Fecal N	36.0	32.6	31.5	29.6	0.95	< 0.001	< 0.001	0.378	0.442
Urine N	30.3	26.3	28.3	29.9	1.57	0.211	0.911	0.063	0.331
Milk N	25.5	26.2	25.4	24.6	1.02	0.611	0.348	0.375	0.687
N balance	8.11	14.8	14.8	15.9	2.53	0.081	0.032	0.223	0.435

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]Lowest standard error of treatment means is shown.

[3]N balance = intake N – Fecal N – urine N – milk N.

CHAPTER 3

EFFECT OF REPLACING ALFALFA HAY WITH A MIXTURE OF STRAW AND DRIED DISTILLERS GRAINS AND SOLUBLES ON METHANE PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE

INTERPRETIVE SUMMARY: Knoell et al. (20XX). "Effect of replacing alfalfa hay with a mixture of straw and dried distillers grain and solubles on methane (CH_4) production and microbial community structure." Four diets with increasing levels of dried distillers grains and solubles (DDGS) and wheat straw was fed to lactating Jersey cows. These dietary treatments did not impact bacterial and archaeal community structure measures. However, some amplicon sequence variants (ASVs) were identified to be correlated with CH_4 production. The results from this study indicate competition for substrates as well as a greater abundance of hydrogen sinks, which resulted in an overall reduction of substrates for the methanogens.

RUNNING HEAD: DISTILLERS GRAINS AND SOLUBLES, STRAW, AND ALFALFA ON RUMEN MICROBIAL COMMUNITY COMPOSITION AND METHANE PRODUCTION

Effect of replacing alfalfa hay with a mixture of straw and dried distillers grains and solubles on methane production and microbial community structure

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ABSTRACT

The rumen microbial community is responsible for the digestion of complex cellulosic plant fibers, influencing various production measures from intake to enteric methane (CH_4) emissions. However, due to various processing methods available for coproducts, the characterization of the rumen microbial community consuming coproducts in place of more traditional forage remains relatively uncharacterized. A study was conducted using rumen samples collected from 12 multiparous lactating Jersey cows (BW = 447.5 ± 43.7 kg; DIM = 71 ± 11 d, mean ± SD) when replacing alfalfa with a mixture of coproducts and straw, to investigate the microbial community composition of the bacterial and archaeal communities and its effect on methane emission. A triplicated 4 × 4 Latin square design was used to evaluate the replacement of alfalfa hay with a coproduct mixture (COP) containing approximately 1/3 wheat straw and 2/3 DDGS. The treatment diets were as follows (proportions on a DM basis): a control diet (CON) containing 18.2 % of alfalfa hay, a low coproduct diet (LowDDGS) that contained 8.1 % of COP, a medium coproduct diet (HighDDGS) that contained 16.3 % of COP, and a high coproduct diet (HighDDGS) that contained 24.3 % of COP. Bacteroidetes and Firmicutes accounted for the major bacterial phyla identified while the methanogenic archaea were dominated by Methanobacteriaceae. Alpha diversity measures for both bacteria and archaeal communities were not significant, and global community composition was not different among dietary treatments. Results from Bayesian regression analysis (BayesC) identified bacteria belonging to primarilly Prevotellaceae, to have a significant impact on CH_4 production, while the archaea community had fewer identified and belonged to *Methanobrevibacter*. Correlations among bacterial and

archaeal taxa to methanogenesis in the CON diet identified three bacterial ASVs belonging to Ruminococcaceae and Prevotellaceae families to be negatively correlated with CH_4 with higher abundances in HighDDGS diet. Positively correlated ASVs were found primarily in the CON diet and included three members of the Prevotellaceae family. These data indicate that while diet is driver of community composition, rather than methanogen population abundance, the fermentation end products by bacteria may influence methanogenesis in the rumen under these dietary conditions rather than impacting the archaeal community.

Key words: dairy cow, methane, ASV, BGLR, methanogen

INTRODUCTION

As the supply for ethanol in the United States increased, the production of corn ethanol coproducts increased simultaneously. These coproducts are a common feed source for dairy cattle as they are an excellent source of energy and rumen undegradable protein (Kononoff et al., 2007; Schingoethe et al., 2009). However, coproducts are continuously evolving. As a result, different extraction methods are utilized for different plant components resulting in the production of wet or dry distillers grains, with or without the addition of solubles, and removal of oil from distillers grains resulting in reduced-fat distillers grains plus solubles (DDGS) (Foth et al., 2015; Mjoun et al., 2010). Alfalfa hay, while a common feed ingredient in the dairy industry, requires higher water amounts to grow that is met by irrigation practices. As a result, to increase water

conservation, the dairy industry has begun to utilize alternative feed ingredients, such as distillers grains, to develop new feed sources that are readily available as coproducts of other industries. However, as producers develop new feed resources, effects of such ingredients on the rumen microbial population and its effect on rumen metabolism needs to be investigated to identify the beneficial or detrimental effect of coproducts on the rumen microbiome and in-turn animal health and performance.

Methanogens represent less than 5% of total rRNA genes (Lin et al., 1997) present in the rumen. As such, this domain of archaea represents a small proportion of the rumen microbial population. Despite their low abundance, this distinct group of microbes play a major role in methane (CH_4) production by reducing carbon dioxide with the use of H_2, or using formate and other one carbon compounds such as methanol, and methylamines (Hedderich and Whitman, 2006; Janssen and Kirs, 2008; Poulson et al., 2013). The microbial populations within the rumen breakdown ingested feed ingredients, producing a variety of intermediate end products that are then utilized by the host and other microbes within the rumen for energy generation (Bergman, 1990). Methanogens utilize some of these end products as substrates to produce CH_4 and in the process help recycle reducing equivalents to maintain metabolic functions in the rumen ecosystem (Moss et al., 2000). Thus, it is of great importance to understand how new feed sources may influence the rumen microbial population and in-turn animal performance.

A recent study (see Chapter 2) demonstrated that replacing alfalfa hay with linear incremental amounts of straw and DDGS had no effect on milk yield and dry matter intake. However, this study reported a decrease in CH_4 production by nearly 20%. As such, it is interesting to understand how DDGS affected rumen microbial communities to

reduce CH_4 production. Johnson and Johnson (1995) indicate that distillers grains products can be fed to lower CH_4 production. Additionally, it has been noted that fibrolytic bacterial family abundances, such as *Ruminococcus* and *Fibrobacter*, changed due to concentrate feeding and that this change resulted in fermentation intermediates and could impact CH_4 production (Morgavi et al., Knapp et al., 2014; Enjalbert et al., 2017).

Although studies have evaluated the effect of feeding DDGS to dairy cattle, these studies have failed to identify what bacterial taxa are influenced by replacing alfalfa with coproducts and what and how methanogenic microbes are influenced to reduce methane production. Additionally, how microbial changes effect CH_4 production by changing the rumen microbial community composition is poorly understood. In this study, we investigated rumen bacterial and archaeal community changes when replacing alfalfa hay with coproducts and straw mixtures as implemented in Chapter 2, to identify how rumen microbial community changes can affect methanogenesis in dairy cattle.

MATERIALS AND METHODS

Animals and Dietary Treatments

Animal care and experimental procedures were conducted according to the guidelines of the University of Nebraska-Lincoln (UNL) Animal Care and Use Committee. This study utilized samples collected from the study in Chapter 2. Briefly, 12 multiparous lactating Jersey cows averaging 71 ± 11.0 DIM and 447.5 ± 43.7 kg of BW were utilized in this study. All cows were housed in a temperature-controlled barn at the Dairy Metabolism Facility at the Animal Science Complex at the University of Nebraska-Lincoln in a 4 × 4 triplicated Latin square design. Cows were blocked by milk production

and assigned randomly to 1 of the 4 dietary treatments. In the current study, treatment diets will be referred to as: CON (CON), LCOP (**LowDDGS**), MCOP (**MedDDGS**), and HCOP (**HighDDGS**). The study was conducted with a total of 4 experimental periods, each being 35 d in duration. The 4 diets were formulated with treatments containing different concentrations of dried distillers grains and solubles (DDGS) and wheat straw (Table 3.1). Manipulation of alfalfa was achieved by incrementally decreasing alfalfa and increasing DDGS and wheat straw. The mixture of straw and DDGS were added to all diets, except the control, at 6.0%, 12.1%, and 18.1% of diet DM as part of a concentrate mixture. Alfalfa hay was added to all diets, except the HCP, at 18.2% of CON diet DM, 12.1% of LCOP diet DM, and 6.1% of MCOP diet DM. Complete diet compositions and nutrient analysis for all treatments are presented in Table 1. All dietary treatments contained corn silage and a concentrate mixture that was combined as a TMR. The TMR was mixed in a Calan Data Ranger (American Calan Inc., Northwood, NH) and fed once daily at 1000 h to the cows.

Sampling Rumen Digesta

At the conclusion of each 35-d period, rumen microbial samples were collected using esophageal tubing as described previously Paz et al. (2016) prior to feeding. Briefly, one end of the esophageal tube contained a metal strainer that was connected to a Gast High-Capacity vacuum pump (model DOA-P704-AA; Cole-Palmer, Vernon Hills, IL). The other end of the tube remained open to capture rumen fluid and feed particles. The rumen digesta were collected by passing the opposite end of the tube through a Frick speculum into the rumen. The first 10 mL of rumen fluid was discarded to prevent cross

contamination. The next 50 mL was collected into a conical tube (Thermofisher Scientific Inc, Waltham, MA, USA). After removal of the tube from the esophagus, fibrous particles attached to the end of the metal strainer were recovered and added into the conical tube to obtain a more representative sample of rumen content. The samples were snap frozen in liquid nitrogen and stored at -20°C until further processing. Between sampling of animals, warm water was used to wash the speculum and the strainer, and the tubing to prevent cross contamination.

Nucleic Acid Extraction, 16S rRNA Library Preparation and Sequencing of the V4 Region of Bacteria and V5-V6 Region of Archaea

Nucleic acid extraction was performed using the Mag-Bind® Soil DNA 96 kit (Omega Bio-tek, Inc., Norcross, GA) according to the manufacturer's directions, with the following modifications. All samples were extracted using 1.5 mL tubes. Samples were mixed with lysis buffer and homogenized for 10 min using a Qiagen TissueLyser (Qiagen, Inc., Valencia, CA) for mechanical disruption of bacterial cell walls with boiling between two homogenization steps at 90°C for 10 min. The resulting tubes were centrifuged at 5000 × g for 2 minutes at room temperature. The supernatant was used to precipitate nucleic acids as described by Yu and Morrison (2004) and Paz et al. (2018) with the exception of not drying the samples under vacuum, prior to using KingFisher (ThermoFisher Scientific, Waltham, MA) for further purification of DNA. Amplicon libraries of the 16S rRNA gene were prepared as described by Kozich et al. (2013) to evaluate the bacterial populations. Both bacterial and archaeal 16S libraries were prepped using the same strategy as described in Kozich et al. (2013). However, to achieve

archaea-specific 16S amplification, following primers sequences that have been described to universally amplify archaea populations were utilized; A751F (CCGACGGTGAGRGRYGAA) and A976R (CCGGCGTTGAMTCCAATT) (Blais Lecours et al., 2012; Kozich et al., 2013). The archaea primers were designed to amplify hypervariable region V5-V6 of the 16S rRNA gene. These primers were synthesized by Integrated Technologies (IDT). Briefly, a 25 µL PCR reaction contained 1X Terra PCR Direct Buffer, 0.625 Units Terra PCR Direct Polymerase Mix, 1 µL indexed fusion primers (10 µM, specific for bacteria or archaea for a final concentration of 0.4nM), and 20-50 ng of DNA. Thermocycler conditions included initial denaturation at 98°C for 3 min, followed by 25 cycles of 98°C for 30 s, 55°C for 30 s, and 68°C for 45 s, and a final extension of 68°C for 4 min. For archaea, thermocycler conditions included, initial denaturation at 98°C for 3 min, followed by 25 cycles of 98°C for 30 s, 54°C for 30 s, and 68°C for 45 s, and a final extension of 68°C for 4 min. Following amplification, PCR products were normalized (1 to 2 ng/µL) using the Just-a-Plate™ 96 PCR Purification and Normalization Kit for bacteria (Charm Biotech, San Diego, CA) and NGS Normalization 96-Well Kit for archaea (Norgen Biotek Corp., Thorold, ON, Canada) according to manufacturer's instructions. The normalized samples were pooled and further size selection and purification was performed using the Pippin Prep (Sage Science, Inc., Beverly, MA) automated size selection instrument using 1.5% gel cassettes. The resulting libraries were quality controlled using the Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and quantified using the QFX Flurometer (DeNovix, Wilmington, DE) and sequenced using the Illumina MiSeq System (Illumina,

San Diego, CA) using the 2 × 250 bp paired-end sequencing strategy using V2 500 cycles kit according to the manufacturer's instructions.

Data Processing and Community Analysis

Detailed information about the bioinformatic pipeline containing an R Markdown (1703DA_microbe) is available at https://github.com/FernandoLab/Knoell_1703DA. The R Markdown file allows full reproduction of the analyses used in this study. The DADA2 pipeline (Callahan et al., 2016) was used for subsequent analysis and to identify amplicon sequence variants (ASVs). Briefly, analytical steps were preformed using R (R Core Team, 2020) within the phyloseq package (McMurdie and Holmes, 2013). Low quality reads were filtered and trimmed (Q score of ≥ 30) and error rates were estimated to evaluate read quality. Both forward and reverse reads were used to generate contigs for subsequent analysis. Sequences with ambiguous bases, incorrect contig length, incorrect assembly, and chimeric sequences were removed during quality filtering. SILVA alignment reference base v138 was utilized to assign taxonomy while MOTHUR (v.1.35.1) (Schloss et al., 2009) was used to generate a phylogenetic tree for both bacteria and archaea datasets. The cow mentioned above that was removed from the production, was subsequently removed from the microbiome analysis. The resulting phylip.tree(s), sequence table(s), mapping file(s), and taxonomy table(s) were used to generate a "phyloseq object" for subsequent analysis.

For the bacterial community, ASVs assigned outside of the phylum Bacteria were removed while the archaeal community had ASVs assigned outside of the phylum Archaea were removed. This also includes the removal of the phylum 'Cyanobacteria' as

this may be due to chloroplast contamination. The ASVs present in the negative controls were removed, as these ASVs could be a result of contamination in the reagents used for DNA extraction. Samples with low read depth (Archaea, n = 3) were removed from the respective data. Additionally, prevalence filtering was performed to remove singletons and any ASV that was present in less than 10% of total samples (n = 4 for both bacteria and archaea) and total abundance filtering was performed at 0.01% to remove any ASV that have less than 0.01% of total reads. Rarefaction curves (Figure 3.1) were generated to evaluate if the read depth obtained for both bacteria and archaea was adequate to evaluate microbial community composition changes. The rarefaction curves reached plateau suggesting that adequate read depths were achieved to evaluate the rumen bacterial and archaeal communities. For various subsequent analyses, a proportion file was created from raw count data. Bacterial community data were rarefied to 8,578 and archaeal community data were rarefied to 9,560 for alpha diversity analyses. The bacterial community dataset contained 1,729,022 total reads at a depth of 41,167 reads per sample that accounted for 1,166 ASVs after prevalence and abundance filtering. Similarly, the archaea community dataset contained 1,608,157 total reads with an average depth of 40,203 reads per sample and was composed of 100 ASVs.

Statistical Analysis

To identify factors affecting community composition differences, observed, Shannon, and Chao1 (Chao et al., 2016) measures were used to estimate species diversity and richness (alpha diversity) on rarefied data using the pairwise Wilcox rank sum test (Wilcoxon, 1945) to determine differences in treatment. Global community statistics for

both bacteria and archaea community were conducted using the "vegan" package (Dixon et al., 2003) using the adonis function. A permutational multivariate analysis of variance (PERMANOVA) was performed using the Bray-Curtis dissimilarity matrix with fixed effect factors of treatment, square, period within square, and a random effect of cow within square. Adonis does not accommodate random effects and thus cow within square was fitted as fixed.

A core for each dietary treatment was made containing at least 75% of samples. The dietary cores were merged together to obtain overall unique core ASVs for the data set. A Venn diagram was constructed using from overall unique core ASVs to determine shared ASVs among dietary treatments. To analyze for differential ASVs, DESeq2 (Love et al., 2014) was utilized within the phyloseq package (McMurdie and Holmes, 2013). Differential abundance analysis was performed for each dietary combination to determine abundance differences of ASVs for the six different dietary combinations. The results from the DESeq2 test were merged together providing the unique differentially abundant ASVs. Additionally, differential ASVs using DESeq2 was performed to evaluate differential abundance of the shared ASVs. Hierarchical clustering was performed using the merged differentially abundant ASVs.

Spearman rank correlation test was performed to identify associations between ASV abundance ranking and CH_4 production ranking. Statistical significance in all analyses were determined at $P < 0.05$. Plots of top five most negative and most positive correlation associations for ASVs and CH_4 production were generated with a Shapiro-Wilk test for normality and a Kruskal-Wallis test to determine significance for bacteria and archaea. To determine the influence of the microbial community on methane

production values, a Bayesian regression model (BayesC) (Kizilkaya et al., 2010) was implemented in the BGLR package (Pérez and de los Campos, 2014) of R using default values. Fixed effects included square, treatment, and period within square. The nested effect of cow within square was fitted as a random effect. A total of 15,000 iterations were performed with the first 5,000 discarded as burn-in. The ASVs with the highest absolute posterior mean effect were selected for further examination into influence on CH_4 production. This was determined by arranging the posterior means from negative to positive. The mean and standard deviation were calculated for both the positive and negative posterior means. The ASVs identified for the bacterial community were greater than three standard deviations away from the mean, and the archaea were greater than two standard deviations away from the mean.

RESULTS AND DISCUSSION

Production Responses

Production measures for this study have been reported in Chapter 2. Briefly, diets had similar chemical composition (reproduced from Chapter 2; Table 3.1) neutral detergent fiber, and starch. Crude fat and crude protein (% DM) increased across diets. No differences were observed for dry matter intake and milk yield averaging 19.5 ± 0.60 kg/d and 29.6 ± 1.03 kg/d, respectively (Figure 3.2). A linear decrease in CH_4 production was observed ($P = 0.002$) from 429.4 L/d on the CON to 345.0 L/d on the HighDDGS (Figure 3.2). Linear decreases in CH4/DMI and CH4/MY from the CON to the HighDDGS were also observed at 22.2 to 18.1 L/kg/d and 14.8 to 11.9 L/kg/d, respectively.

Bacterial and Archaeal Community Composition

The taxonomic composition and abundance for each dietary treatment indicates that the bacterial community was comprised of several phyla which included Verrucomicrobiota, Synergistota, Spirochaetota, Proteobacteria, Planctomycetota, Patescibacteria, Firmicutes, Fibrobacterota, Elusimicrobiota, Desulfobacterota, Chloroflexi, Bacteroidota, and Actinobacteriota (Figure 3.3). The top six most abundant genera identified include *Prevotella*, *Prevotellaceae_UCG-001* (Family: Prevotellaceae), *UCG-004* (Family: Erysipelatoclostridiaceae), *Rikenellaceae_RC9_gut_group* (Family: Rikenellaceae), *NK4A214_group* (Family: Oscillospiraceae), and *Butyrivibrio*. These taxa combined accounted for 52% of the taxa on the CON diet, 48% on the LowDDGS, 46.5% on the MedDDGS, and 50% on the HighDDGS diet. *Prevotella* was the most abundant genus at 25.8 ± 4.9% on the CON, 31.8 ± 2.1% on the LowDDGS, 28.6 ± 11.5% on the MedDDGS, and 31.8 ± 2.7% on the HighDDGS. The genus *Prevotella* have been identified as the most abundant genus in 16S rRNA data from the rumen (Whitford et al., 1998; Koike et al., 2003; Stevenson and Weimer, 2007). It has been associated with polysaccharide breakdown (Matsui et al., 2000) and protein degradation (Wallace, 1996). Danielsson et al. (2017) fed dairy cattle a diet of forage and concentrate and observed at the genus level, *Prevotella* was the most abundant at approximately 48% of the bacterial community. *Prevotella* members are known to be in higher abundances in animals that are fed concentrate-based diets (Henderson et al., 2015), while the abundance was greater in the HighDDGS, it was not statistically significant. Paz et al. (2018) observed approximately 29% of reads belonging to *Prevotella* for cattle fed both

high-forage and high-concentrate diets. The species in the genus Butyrivibrio are known to possess the capability for hemicellulolytic and proteolytic digestion (Cotta and Hespell, 1986; Kelly et al., 2010).

The archaeal community was dominated by two families, Methanobacteriaceae (79.94 ± 0.02 %) and Methanomethylophilaceae (20.02 ± 0.02 %) across all diets (Figure 3.4). On each dietary treatment in this study, Methanobacteriaceae was present at a minimum of 76.3 ± 0.05 %, with *Methanobrevibacter* the most abundant member of this family being the major organisms identified. This lineage is commonly identified and has been designated as one of the most common hydrogenotrophic archaea in the rumen (Morgavi et al., 2010; Nagaraja, 2016; Tapio et al., 2017). Methanomethylophilaceae was present in all diets at 20.02 ± 0.02 % abundance. *Candidatus_Methanomethylophilus* was present on all diets and are known to utilize methylamines and methanol (Noel et al., 2016). These families accounted for >99% of the methanogens present.

Effect of Coproduct and Straw Mixture on Estimates for Bacterial and Archaeal Communities

Replacing alfalfa hay with a mixture of straw and coproducts indicates a potential for a shift in the rumen microflora. The addition of DDGS to dairy rations is used primarily as a source of energy due to the higher fat content and protein including rumen undegradable protein (RUP; Belyea et al., 2010). Alfalfa, according to the NRC (2001), has nutrient composition values for CP, NDF, ADF, lignin, and fat of 20.2%, 39.65, 31.2%, 7.0%, and 2.1%, respectively. The coproduct, DDGS, typically has nutrient composition for CP, NDF, ADF, lignin, and fat of 29.7%, 38.8%, 19.7%, 4.3%, and

10.0%, respectively (NRC, 2001). With the nutrient composition characteristics of increased CP and fat and a decrease in ADF, proteolytic bacteria populations would be expected to increase. Additionally, protein digesters are known to be amylolytic bacteria (Cotta and Hespell, 1986). Alfalfa exhibits a moderate amount of pectin, ranging from 10-15% (Mertens, 2003). *Prevotella* species are part of the bacterial population that utilize pectin (Marounek and Duskova, 1999). Therefore, the increase in *Prevotella* may be a result of the increased CP and alfalfa in the diet. This observation has been reported previously, the concentrations of *Prevotella* was shown to increase when animals were fed a concentrate-based diet (Bekele et al., 2010). Additionally, Zhu et al. (2018), observed an increase in *Prevotella* postpartum on a high-grain diet from a low-grain diet. In the current study, dietary treatments did not reflect significant differences in observed ASVs, Chao1, and Shannon diversity index alpha diversity measures ($P = 1.0$; Figure 3.5; Chao and Chiu, 2016; Shannon, 1948). This is in contrast to previous research (Tajima et al., 2001; Pitta et al., 2014) who observed a decrease in diversity replacing forage with coproducts. While not significant, a trend of decreased diversity was observed for the dietary treatments. Variation in DDGS chemical composition can occur due to the technique used to capture the coproduct (Berger and Singh, 2010). As a result, the nutrient profile of the coproducts utilized by Tajima et al. (2001) may be slightly different than the nutrient profile of coproducts in the current study. In the current study, the alpha diversity metrics displayed no differences in richness and evenness, as such, the global community structure for the bacteria population did not appear to visually change by diet (Figure 3.6). Statistically, all factors of treatment, square, period within square, and cow within square were significant ($P \leq 0.01$; Table 3.2).

This further suggests that the dietary ingredients did not appear to change the community. A lack of change may be due to similar substrates being available for the microbial community. Conversely, Danielsson et al. (2017) fed the same diet of concentrate and forage to dairy cattle in mid-lactation and observed distinct changes in the microbial community in high and low methane emitting cattle.

The archaeal community also showed no difference in richness and evenness based on alpha diversity indexes ($P \geq 0.43$; Figure 3.7). The Chao1 diversity index did decrease from the CON to HighDDGS indicating that community diversity may decrease during coproduct feeding, yet it was not statistically significant. Belanche et al. (2012) also indicated a decrease in diversity when cattle consume concentrate diets. The archaeal community is much lower in abundance compared to the bacterial community in the rumen (Lin et al., 1997). Janssen and Kirs (2008) stated that diversity of the rumen archaeal community is limited to four orders that includes Methanomicrobiales, Methanosarcinales, Methanococcales, and Methanobacteriales. Methanobacteriales represent the largest methanogen population and the abundance in the rumen can be variable (30-99%). The next most abundant archaeal taxa in the rumen are Methanomicrobiales followed by Methanosarcinales. Methanosarcinales have been known to be rare in the rumen, often at less than 3% (Janssen and Kirs, 2008). *Methanobrevibacter* is the most common methanogenic genus identified from the bovine rumen (Leahy et al., 2010) and is often identified as the most abundant methanogen in the rumen (Leahy et al., 2010). However, Danielsson et al. (2017) found *Methanobrevibacter* to be in similar abundance between low and high CH_4 emitting cattle, suggesting that methane emission may not be directly associated with methanogen abundance. A similar

occurrence was observed in primiparous cows that were transitioned from a low-grain to high-grain diet (Zhu et al., 2018). With improved sequencing methods, new orders have been identified. In 2014, (Borrel et al.) discovered a seventh order of methanogenic archaea that inhabits the digestive tracts of animals as well as other environments, Methanomassiliicoccales. Members of this order are characterized as methylotrophic methanogens as they utilize methylamines for energy metabolism (Poulsen et al., 2013). Henderson et al. (2015) also stated that these lineages present in the rumen are highly conserved, which could account for the low diversity often observed in rumen archaeal populations. Overall, these results suggest that replacing alfalfa with coproducts and straw do not elicit a visual global shift in community structure (Figure 3.8) and the decrease in CH_4 may not be a result of methanogen abundance changes (Table 3.2). Unfortunately, classification down to a species level for a majority of bacteria and all of archaea was not possible with the short-read sequencing strategy.

To further evaluate bacterial community differences at lower taxonomic levels, a core measurable microbiota (CMM) was determined utilizing selection criteria of an ASV being present in 75% of the animals in each dietary treatment. This CMM was composed of 348 ASVs for the bacterial dataset. A Venn diagram (Figure 3.9) of shared core ASVs among diets for bacterial core ASVs demonstrated a majority of the core ASVs to be shared between diets, suggesting that the dietary treatments are not impacting microbial taxa groups. This indicates that while dietary differences may occur, such dietary differences are not impacting the core microbial community present in the animals. Further supporting this notion, only two core ASVs were found to be differentially abundant. The reason for the CMM to remain constant during the dietary

treatments in this study may be due to the broader metabolic capacity of the core microbial community to utilize multiple substrate allowing these microbes to be maintained under the changing dietary conditions. The study design allowed for 35 d periods to ensure no crossover effects of treatments are prevalent. This may indicate that increasing the feeding term of the diets may not serve to indicate dietary differences in CMM.

Using the same parameters for the archaeal community, 55 ASVs were identified that represented the CMM (Figure 3.10). Similar to the bacterial core, a large proportion of the ASVs belonging to the CMM were similar among diets. This similarity of the CMM may be due to decreased diversity of methanogens in the rumen and the broader metabolic capacity of these microbes in the rumen.

Differential ASV Abundance in Response to Coproduct and Straw Levels in Bacterial and Archaeal Communities

Differential abundance of the bacterial community due to coproduct and straw mixture was evaluated to identify bacterial ASVs that are influenced by DDGS. With no difference in the number of different species present, identifying the most differentially abundant taxa may provide insight into understanding the role of the diet in decreasing CH_4. Differential abundance was conducted for each dietary combination (Figure 3.11).

The linear decrease of CH_4 from the CON to HighDDGS diet prompted further examination into microbial community changes that may affect methane reduction. Clustering and differential abundance of ASVs are apparent between the CON and HighDDGS diets (Figure 3.12). Three ASVs belonging to Prevotellaceae (ASVs 209 and

427; Genus: *Prevotella*) and Ruminococcaceae (ASV 103; Genus: *Ruminococcus*) were identified as being more abundant in HighDDGS diet compared to the CON diet. *Prevotella* has been noted to increase in abundance in the rumen when animals are fed a concentrate-based diets (Bekele et al., 2010). Additionally, *Prevotella* is a known protein digester that produces propionate as one of the major VFAs produced (Deusch et al., 2017). As such, propionate production may have competed with methanogenesis leading to a decrease methane during DDGS feeding. Genus *Ruminococcus*, was highly abundant in cows on the HighDDGS. Typically, members of this genus are shown to decrease in abundance when animals are fed concentrate diets, as it is a fibrolytic bacteria. Several other ASVs belonging to family Prevotellaceae (ASVs 205, 293, 354, 397), Spirochaetaceae (ASVs 622 and 623), and the order Clostridia_UCG-014 (ASV 1014) were more abundant in the CON diet. The order Clostridales often has its members assigned as unclassified, but is known to be comprised of members with cellulytic (Vos et al., 2011) and fibrolytic capabilities (Prins et al., 1972; Van Gylswyk and Van der Toorn, 1985). The family Spirochaetaceae is known to have digestion of xylan, pectin, and arabinogalactans capabilities over cellulytic capabilities (Paster and Canale-Parola, 1982).

Only one differentially abundant archaeal ASV could be identified in MedDDGS and HighDDGS compared to the CON diet. The CON and MedDDGS combination identified ASV 93 (Family: Methanomethylophilaceae) as the only differentially abundant ASV, and ASV 94 (Family: Methanomethylophilaceae) was the only differentially abundant ASV identified in the HighDDGS combination. Both of these ASVs belonged to order Methanomassiliicoccales, which are known to utilize

methylamines and methanol as substrate for methane production (Borrel et al., 2014). The abundance of these ASVs were higher in the CON diet compared to the HighDDGS diet. This suggests that increased methanogenesis in the CON diet may have been a result of methylamine and methanol metabolism to methane. Methylamines and methanol are often the fermentation products of bacteria from pectin (Pol and Demeyer, 1988) and betain and choline (Neill et al., 1978; Mitchell et al., 1979). Pectin are one molecule that comprises DDGS, and is more easily digested that other plant component parts (Kim et al., 2008). Methylamines are present in the rumen (Hill and Mangan, 1964), and may get converted to formic acid (Hill and Mangan, 1964), which gets converted to CH_4 in the rumen by other hydrogenotrophic methanogens. The production of methylamines from digestion in ruminants is apparent with a variety of diets (Hill and Mangan, 1964). These observations further support the notion of methylamine metabolism to methane may have resulted in increased methanogenesis in the control diet.

Associations Between Bacterial ASVs and CH_4 Production

Since CH_4 production decreased from the CON to HighDDGS diet with no differences in community structure, investigating the impact of each ASV and its abundance on methane observed may provide insight into which ASVs may be impacting methane emission. Using a Bayesian regression model fitted in the BGLR package (Pérez and de los Campos, 2014) within R we were able to postulate the effect of each ASV on CH_4 production. Using the selection criteria discussed in the Methods section, 20 bacterial ASVs were identified to affect CH_4 (Figure 3.13; Table 3.3). The top two ASVs with the highest posterior mean for negative association (ASV 210 and ASV 301) belong

to the family Prevotellaceae. Similarly, the top two ASVs with the highest posterior means for a positive association are ASV 429 and ASV 434, and also belong to the Prevotellaceae family. Several ASVs identified by this approach indicate that the genus *Prevotella* is able to utilize various substrates for digestion as these members have been implicated in the breakdown of carbohydrates, pectin, and protein as previously discussed. Henderson et al. (2015) also indicated that due to Prevotella being a more dominant genus in the rumen, that is it rarely disturbed by dietary changes, with its abundance noted to increase in diets with higher concentrates. This could be why *Prevotella* was seen on all diets and observed little changes over the dietary treatments. Carbohydrate-digesting enzymes that produce VFAs originate from Prevotellaceae taxa (Deusch et al., 2017). Higher amounts of propionate may have been produced on the HighDDGS diet, which may account for lowered CH_4 levels on the HighDDGS diet, acting as a hydrogen sink.

Spearman rank correlation tests were also conducted on the two most extreme diets, CON and HighDDGS, to determine if any ASVs for either dietary treatment affected CH_4 production. The top three bacterial ASVS that were significantly negatively and positively correlated to CH4 production were further examined. The three negatively correlated ASVs (103, 427, and 430, Figures 3.14-19, respectively) were observed to be more abundant on HighDDGS diets compared to the CON ($P < 0.01$), indicating that the presence of these taxa at high abundance may lead to lower CH_4 production. The genera represented by these three ASVs included *Ruminococcus* (ASV 103) and *Prevotella* (ASVs 427 and 430). Additionally, three positively correlated ASVs (205, 293, 397) with CH_4 production ($P < 0.05$) were also observed in the CON diet (Figures 3.20-25). These

three ASVs belonged to the family Prevotellaceae. Jewell et al. (2015) also observed a negative correlation with Ruminococcaceae, while Prevotella species have been associated with both efficient and inefficient cows (Flint et al., 2008; Jami and Mizrahi, 2014; Henderson et al., 2015; Lima et al., 2015). This could be due to these lineages containing members that degrade starch and influence propionate production, shifting the VFA profile to more propionate than acetate (Bryant and Small, 1956; Strobel, 1992; Zhu et al., 2018). Additionally, it further provides evidence for the wide breadth of substrates this genus can use making it well adapted utilizing multiple substrates in the rumen (Zhu et al., 2018).

The archaeal community was also tested using a Bayesian regression model (Figure 3.26; Table 3.4) and correlation analysis. Using the selection methods described previously, eight ASVs were identified as having an association with CH_4 production based on the posterior means. Four of these ASVs had negative posterior means indicative of a negative association, and four were positively associated with positive posterior means. Seven ASVs, a mixture of the positive and negative posterior means, belonged to the genus *Methanobrevibacter*. This is not surprising at the majority of the composition belonged to this genus, and are well established in the rumen. Separately, ASV 94 belonging to Methanomethylophilaceae, was correlated with higher CH_4 emissions (Figure 3.27), as it was found primarily in animals on the CON diet (Figure 3.28).

CONCLUSION

Examining the microbial community when replacing alfalfa hay with a straw and coproducts mixture was evaluated using lactating dairy cattle. Global changes in the rumen microbial community were not observed for both bacteria and archaea. However, differential analysis and effect size analysis identified members of the genus *Prevotella* to be present in diets with low methane emission. This study demonstrates methanogenesis can be de decreased by changing the intermediate substrates produced by bacteria that are utilized by methanogens for methanogenesis without changing microbial community or archaeal community composition.

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CONFLICT OF INTEREST

Samodha C. Fernando, author of this publication has disclosed a significant financial interest in NUGUT, LLC. In accordance with its Conflict of Interest policy, the University of Nebraska-Lincoln's Conflict of Interest in Research Committee has determined that this must be disclosed. The rest of the authors have nothing to disclose.

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Table 3.1. Chemical composition and analysis of treatments formulated to contain increasing amounts of coproducts from Chapter 2

	Treatment[1]			
Item	Control	LCOP	MCOP	HCOP
Ingredient, % DM				
Corn silage	37.5	37.5	37.5	37.5
Alfalfa hay	18.2	12.1	6.1	-
Straw	-	2.1	4.2	6.2
Ground corn	14.7	13.7	12.8	12.0
Soybean meal	11.3	11.3	11.3	11.3
RFDDGS[2]	-	6.0	12.1	18.1
Nonenzymatically-browned soybean meal[3]	5.17	4.12	3.06	2.01
Molasses, dried	2.55	2.55	2.55	2.55
Whey	2.56	2.56	2.56	2.56
Lard	2.01	1.81	1.62	1.43
Soybean hulls	1.44	1.44	1.43	1.43
Calcium carbonate	1.33	1.48	1.63	1.77
Bloodmeal	0.89	0.89	0.89	0.89
Calcium soaps of fatty acids [4]	1.33	1.48	1.63	1.77
Sodium bicarbonate	0.57	0.56	0.56	0.55
Salt	0.33	0.33	0.33	0.33
Magnesium oxide	0.31	0.31	0.31	0.31
Calcium diphosphate	0.22	0.15	0.07	0.00
Rumen protected methionine[5]	0.07	0.07	0.07	0.07
Rumen protected lysine[6]	0.00	0.06	0.11	0.17
Vitamin premix[7]	0.05	0.05	0.05	0.05
Mineral premix[8]	0.04	0.04	0.04	0.04
Chemical Composition, % of diet DM[9]				
DM, %	60.2 (0.02)	59.9 (0.02)	60.1 (0.03)	60.0 (0.02)
CP	16.9 (0.48)	17.3 (0.43)	17.8 (0.38)	18.3 (0.35)
Crude fat	4.1 (0.32)	4.3 (0.29)	4.5 (0.28)	4.7 (0.28)
ADF	19.4 (1.11)	19.0 (0.87)	18.5 (0.67)	18.1 (0.55)
NDF	29.2 (1.41)	29.3 (1.07)	29.3 (0.76)	29.4 (0.50)
fNDF[10]	23.3 (1.49)	22.0 (1.28)	20.7 (1.28)	19.3 (1.02)
Lignin	3.8 (0.31)	3.6 (0.29)	3.5 (0.32)	3.3 (0.39)
Ash	8.4 (0.20)	8.1 (0.23)	7.9 (0.29)	7.6 (0.36)
Starch	24.3 (1.20)	24.3 (1.10)	24.2 (1.01)	24.1 (0.91)
Na	0.39 (0.02)	0.39 (0.01)	0.39 (0.01)	0.39 (0.01)
K	1.81 (0.04)	1.67 (0.04)	1.53 (0.03)	1.39 (0.04)
S	0.23 (0.02)	0.28 (0.02)	0.33 (0.02)	0.38 (0.02)
DCAD, % DM[11]	38.6 (1.71)	32.0 (1.44)	25.4 (1.28)	18.8 (1.25)

[1]Treatments: CON = 0% straw, 18.21% alfalfa hay, 44.27% CON concentrate; LCOP = 2.07% straw, 12.14% alfalfa hay, 29.51% CON concentrate, 18.76 BP concentrate; MCOP = 4.15% straw, 6.07% alfalfa hay, 14.75 CON concentrate, 37.51 BP concentrate; and HCOP = 0% alfalfa hay, 6.22% straw, 56.26% BP concentrate.

[2]RFDDGS -Dakota Gold reduced fat distillers grains plus solubles, POET Nutrition LLC, Sioux Falls, SD.
[3]Soypass, LignoTech, Overland Park, KS.
[4]Calcium salts of long-chain fatty acids marketed as Megalac by Church & Dwight Co. Inc. Princeton, NJ.
[5]Smartamine M, Adisseo Inc., Antony, France.
[6]AjiPro-L, Ajimomoto Heartland Inc., Chicago, IL.
[7]Contained 120,000 IU/d vitamin A, 24,000 IU/d of vitamin D, and 800 IU/d Vitamin E in total ration.
[8]Contained 13.9 % Ca, 0.03 % P, 0.42 % Mg, 0.20 % K, 4.20 % S, 0.08 % Na, 0.03 % Cl, 445 mg/kg Fe, 60,021 mg/kg Zn, 17,375 mg/kg Cu, 43,470 mh/kg Mn, 287 mg/kg Se, 527 mg/kg Co, and 870 mg/kg I in total ration.
[9]Values determined by Cumberland Valley Analytical Services, Hagerstown, MD, Mean (SD).
[10]fNDF, % DM – forage NDF fraction, as a percent of DM
[11]Dietary cation-anion difference (mEq/100g of DM = ((Na + K) – (Cl + S))/100 g of DM).

Table 3.2. Global effects of community differences in Jersey cattle fed diets that replaced alfalfa with a mixture of straw and DDGS

	P - value	
Item	Bacteria	Archaea
Square	0.001	0.097
Treatment	0.005	0.245
Period:Square	0.001	0.073
Cow:Square	0.001	0.001

Table 3.3. Bacterial ASVs identified using a Bayesian regression model (BGLR) to impact CH_4 production

ASV	Posterior Mean	Classification Level	Taxon
Negative			
210	-550.273	Species	*P. ruminicola*
301	-222.892	Species	*P. ruminicola*
54	-177.238	Family	Lachnospiraceae
177	-157.001	Family	Succinivibrionaceae_UCG-001
406	-147.699	Genus	*Prevotellaceae_NK3B31_group*
742	-141.130	Genus	*Rikenellaceae_RC9_gut_group*
238	-136.187	Genus	*Prevotella*
87	-132.976	Family	Ruminococcaceae
Positive			
429	299.127	Genus	*Prevotellaceae_UCG-001*
434	261.805	Genus	*Prevotella*
707	196.640	Family	Bacteroidales_RF16_group
961	177.373	Family	Erysipelatoclostridiaceae
300	142.057	Genus	*Prevotella*
242	141.187	Genus	*Prevotella*
237	139.877	Genus	*Prevotella*
693	135.809	Order	Bacteroidales
684	125.686	Order	Bacteroidales
1023	118.182	Order	Clostridia_UCG-014
384	112.871	Genus	*Prevotella*
847	108.890	Genus	*Succiniclasticum*

Table 3.4. Archaeal ASVs identified using a Bayesian regression model (BGLR) to impact CH4 production

ASV	Posterior Mean	Classification Level	Taxon
Negative			
4	-76.394	Family	Methanomethylophilaceae
76	-75.883	Genus	*Methanobrevibacter*
12	-72.704	Genus	*Methanobrevibacter*
19	-68.912	Genus	*Methanobrevibacter*
Positive			
20	143.288	Genus	*Methanobrevibacter*
78	87.311	Genus	*Methanobrevibacter*
87	83.091	Genus	*Methanobrevibacter*
11	80.093	Genus	*Methanobrevibacter*

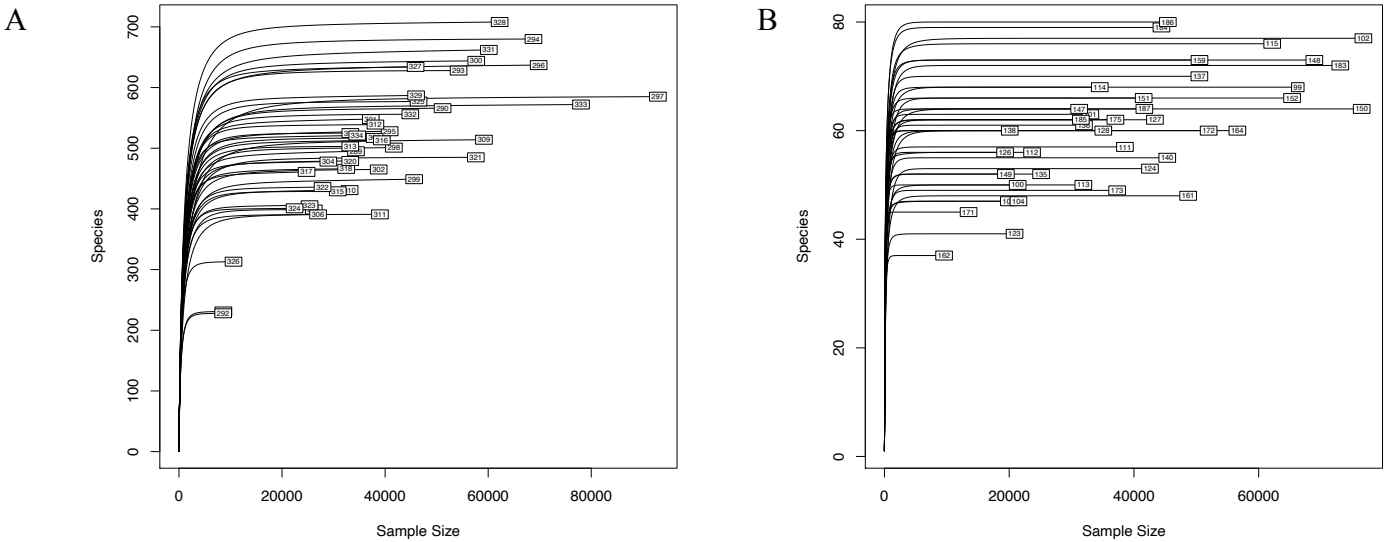

Figure 3.1. Rarefaction curves to evaluate sufficient sequencing depth. A – the rarefaction curve for the bacterial community. B – the rarefaction curve for the archaeal community.

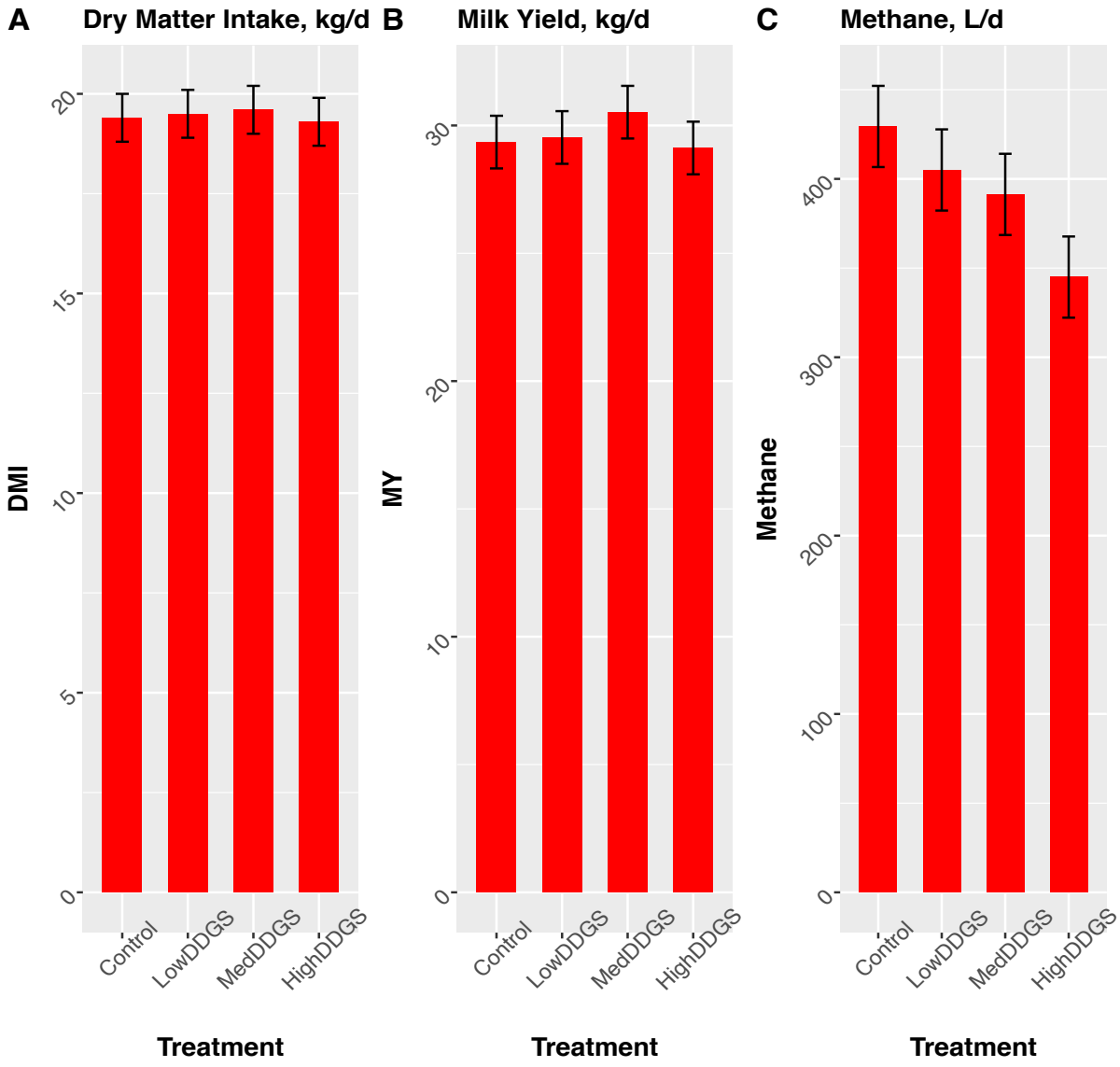

Figure 3.2. Production measures of dry matter intake, milk yield, and methane production for lactating Jersey cattle fed a mixture of straw and coproducts in replacement of alfalfa hay. Panel A shows no difference ($P = 0.96$) in intake among the dietary treatments and panel B shows no difference in milk yield ($P = 0.91$). Panel C shows a linear decrease ($P \leq 0.01$) in CH_4 with the lowest amount produced on the HighDDGS dietary treatment.

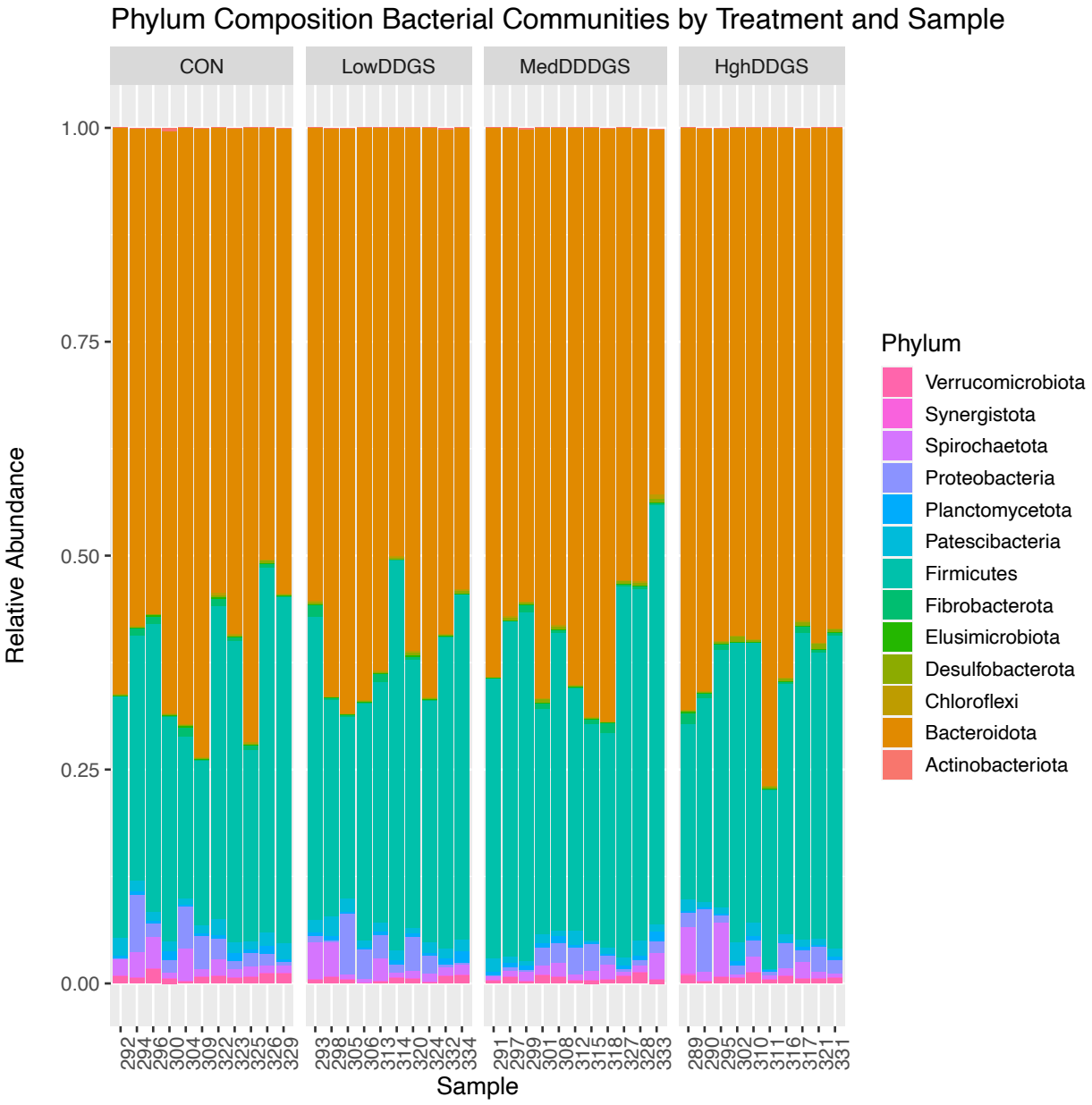

Figure 3.3. Phylum composition of the bacterial community by dietary treatment and sample.

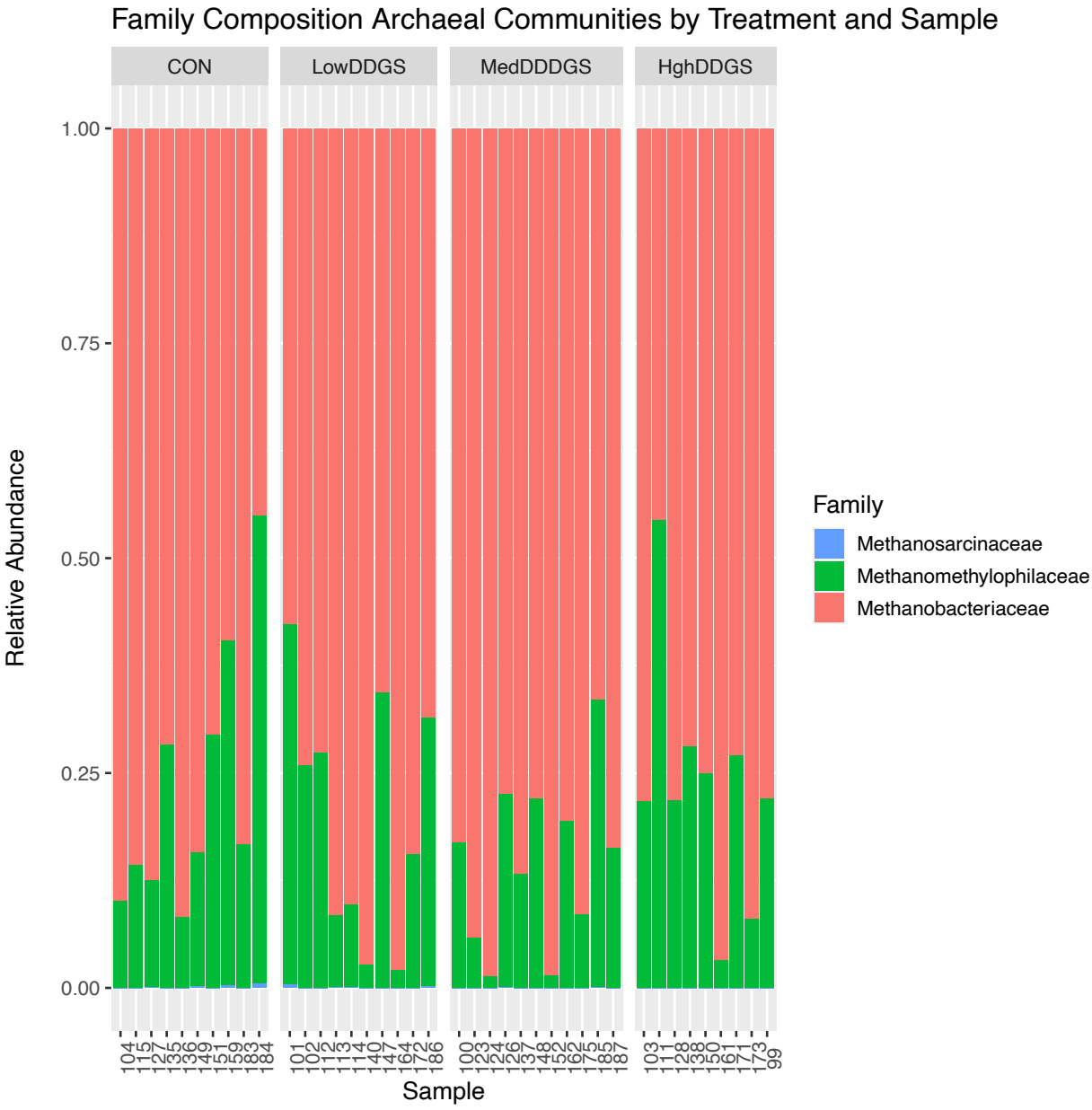

Figure 3.4. Family composition of the archaeal community by dietary treatment and sample.

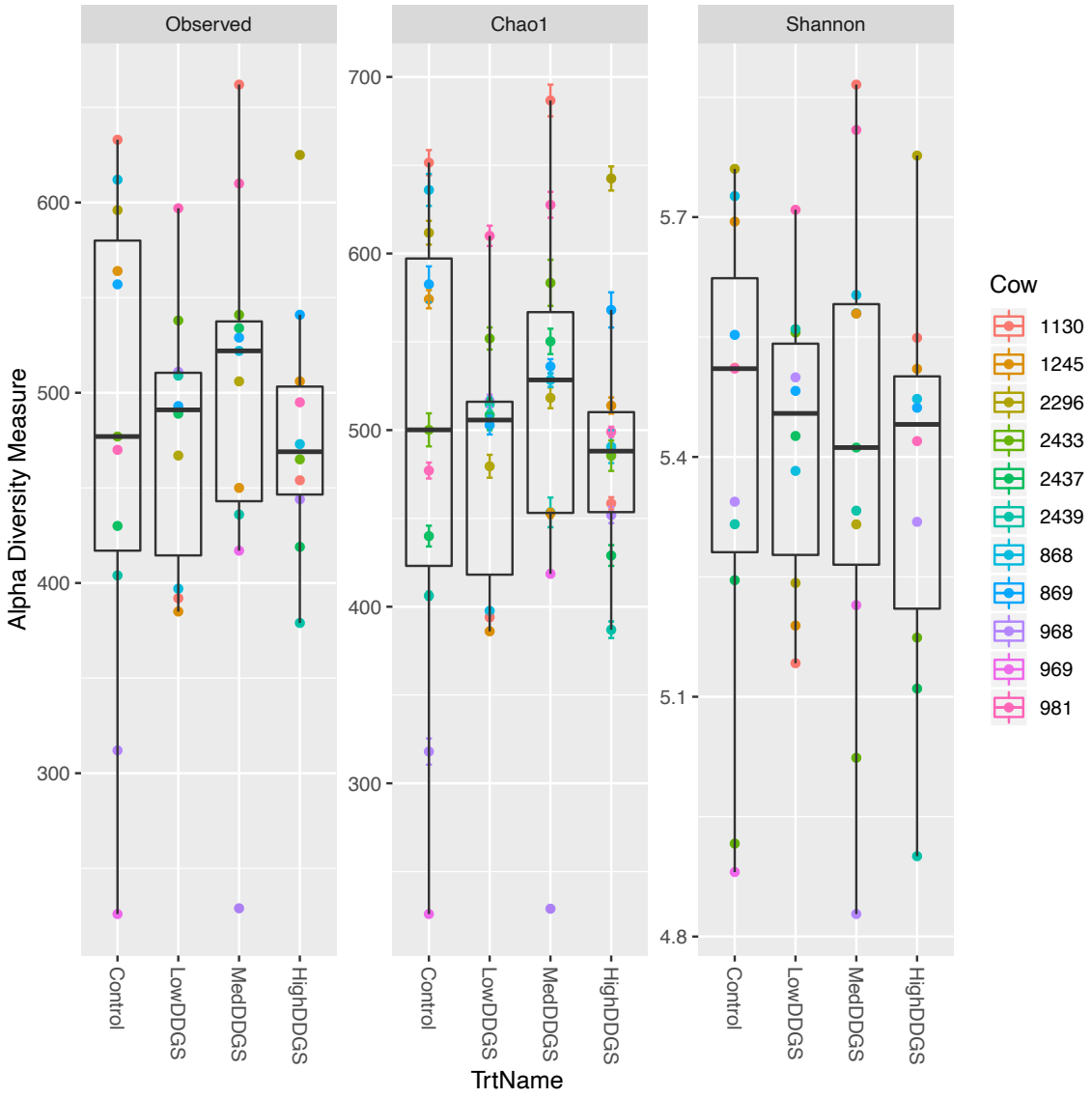

Figure 3.5. Alpha diversity of bacterial community for lactating Jersey cattle fed a mixture of straw and coproducts in replacement of alfalfa hay. No differences were observed ($P = 1.0$) for Observed ASVs, Chao1, and Shannon indexes for the community.

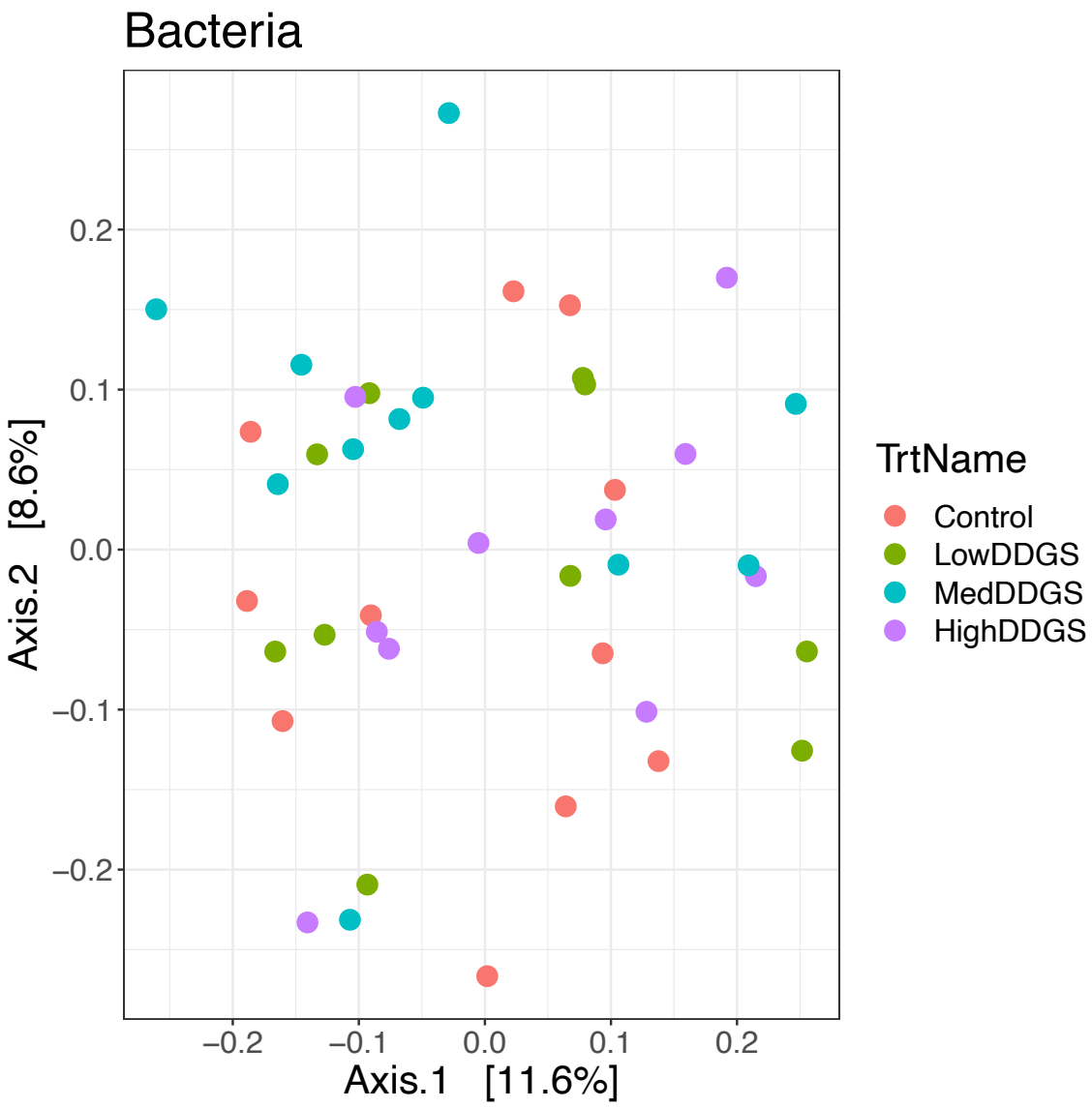

Figure 3.6. Global community structure for bacteria showing the effect of dietary treatment on community dynamics. No visual clustering to dietary treatment was observed.

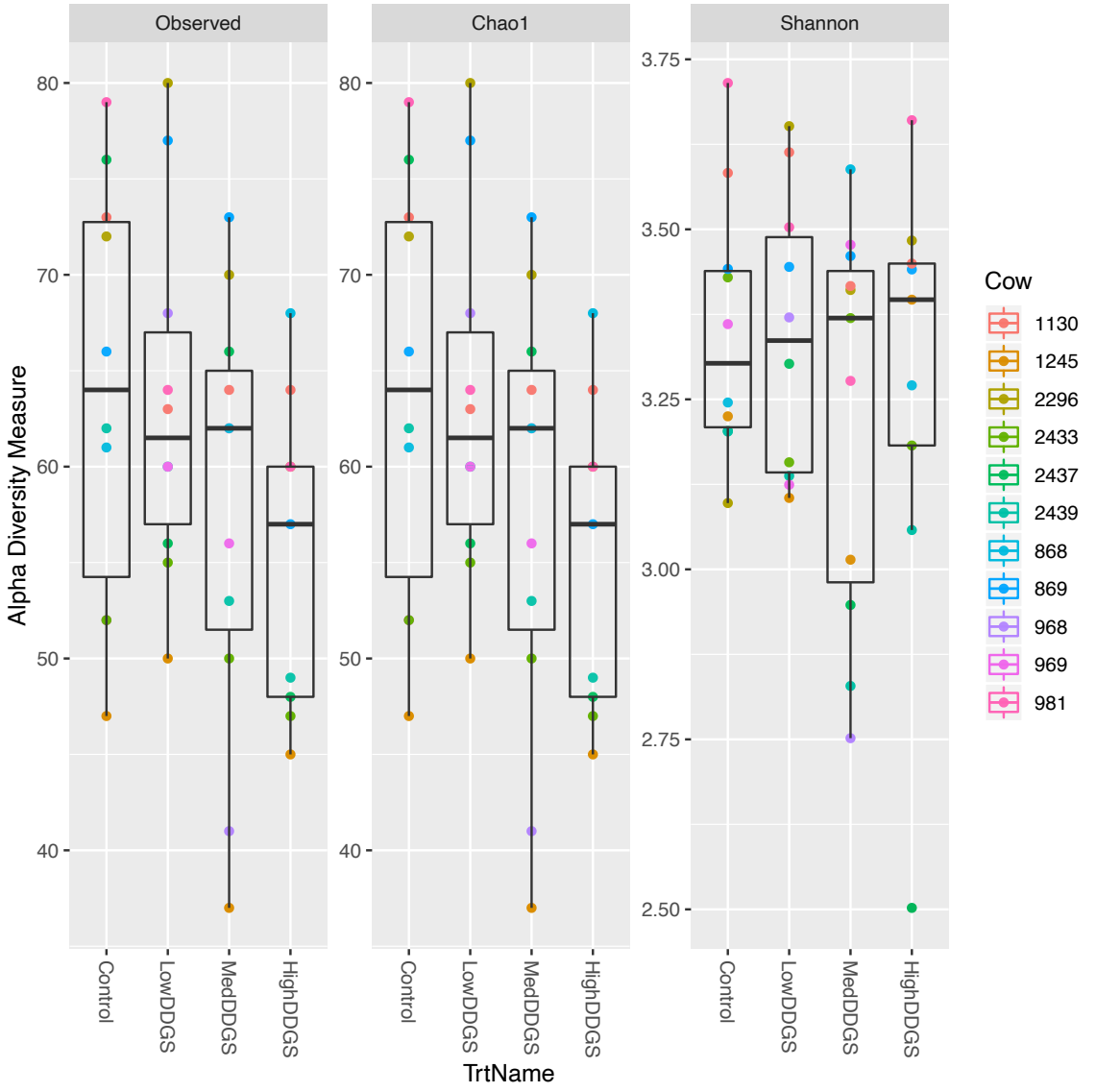

Figure 3.7. Alpha diversity of archaeal community for lactating Jersey cattle fed a mixture of straw and coproducts in replacement of alfalfa hay. The community observed no differences ($P \geq 0.43$) for Observed ASVs, Chao1, and Shannon index measures.

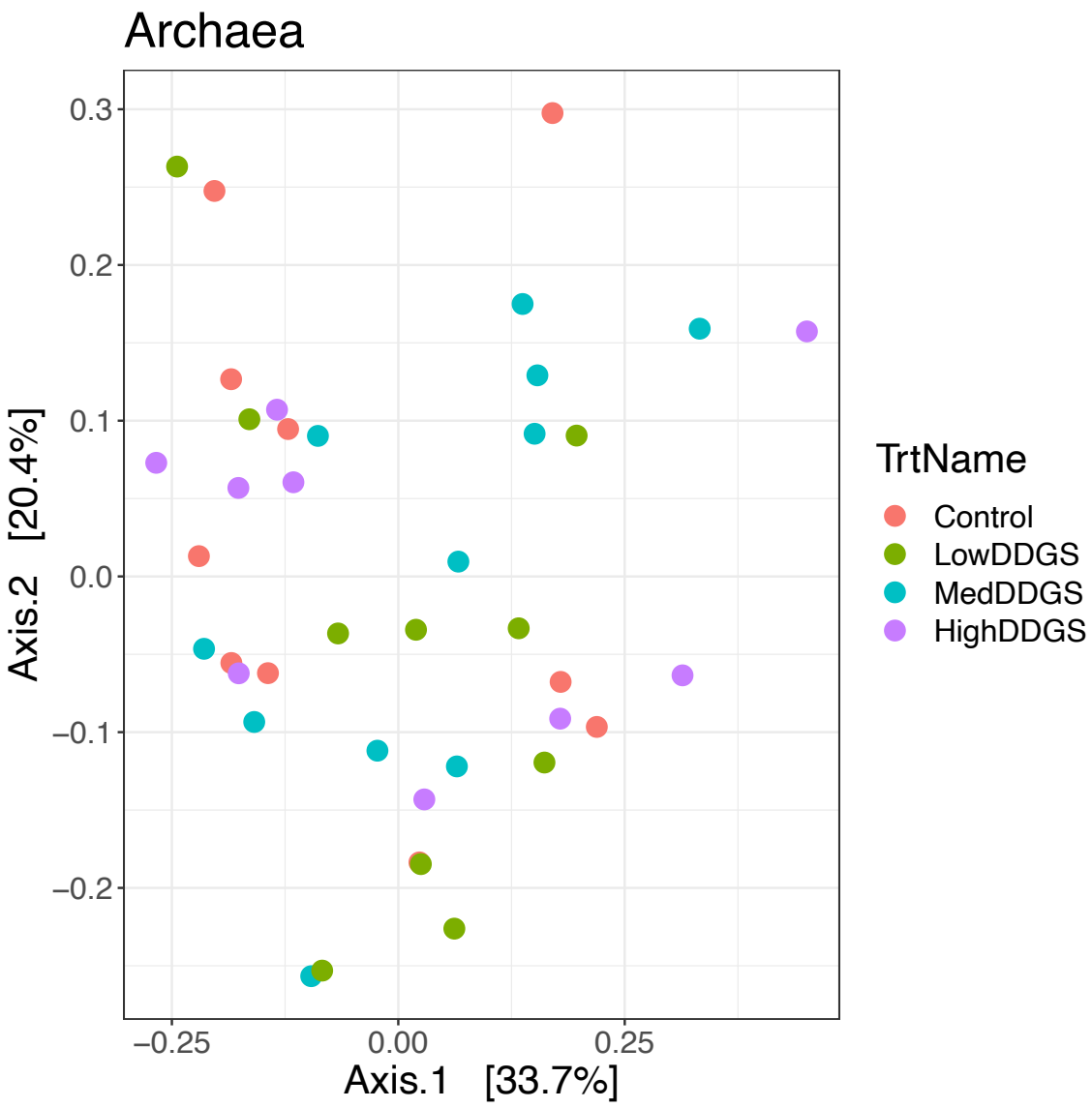

Figure 3.8. Global community structure for archaea showing the effect of dietary treatment on community dynamics. No visual clustering to dietary treatment was observed.

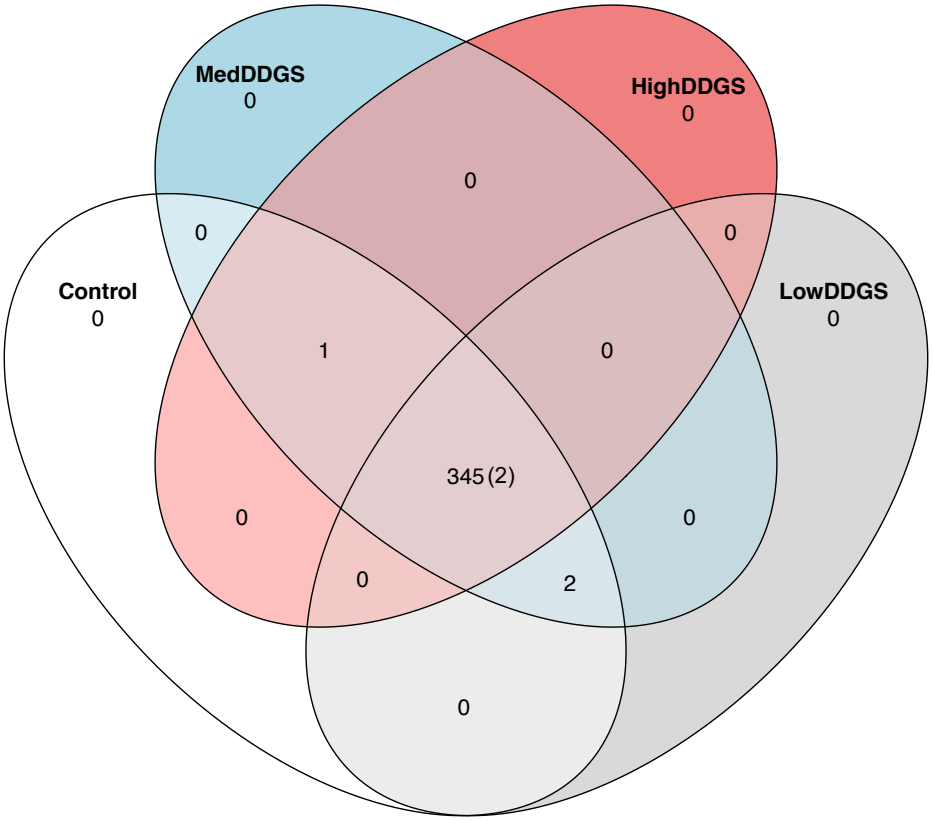

Figure 3.9. Venn diagram of bacteria shared core ASVs and shared core differential ASV in parentheses.

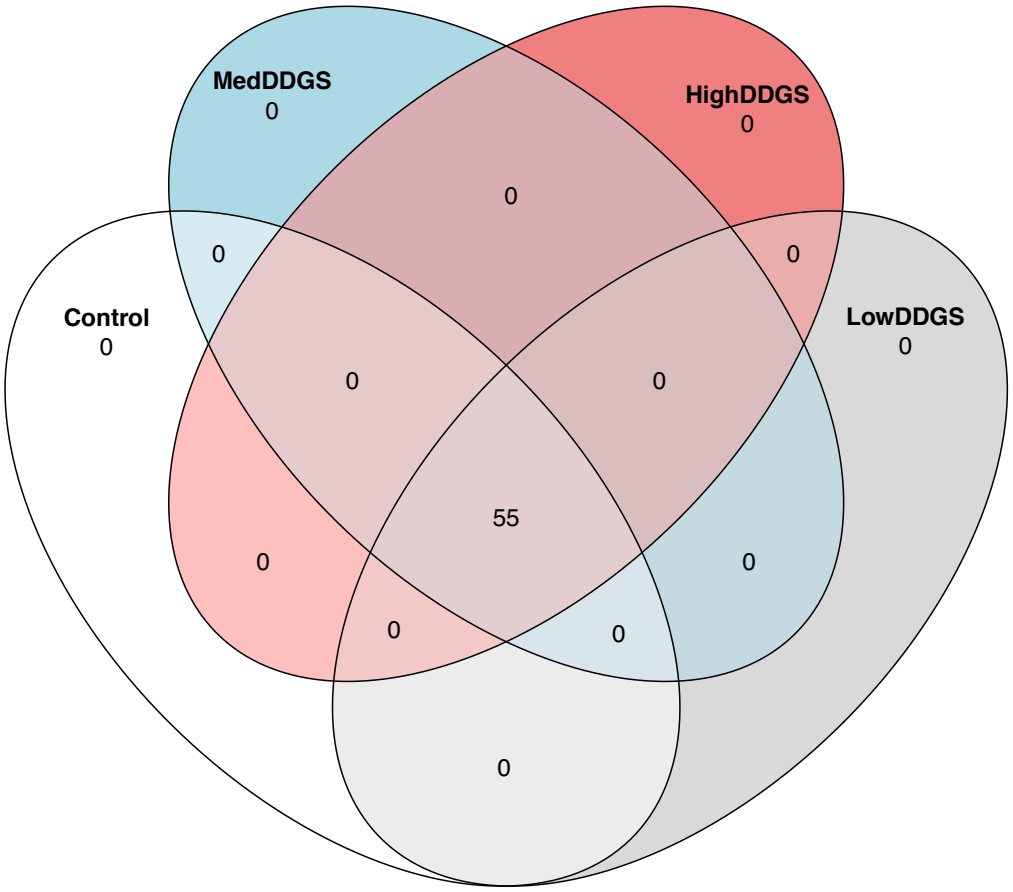

Figure 3.10. Venn diagram of archaea shared core ASVS indicating all shared ASV are shared among all dietary treatments.

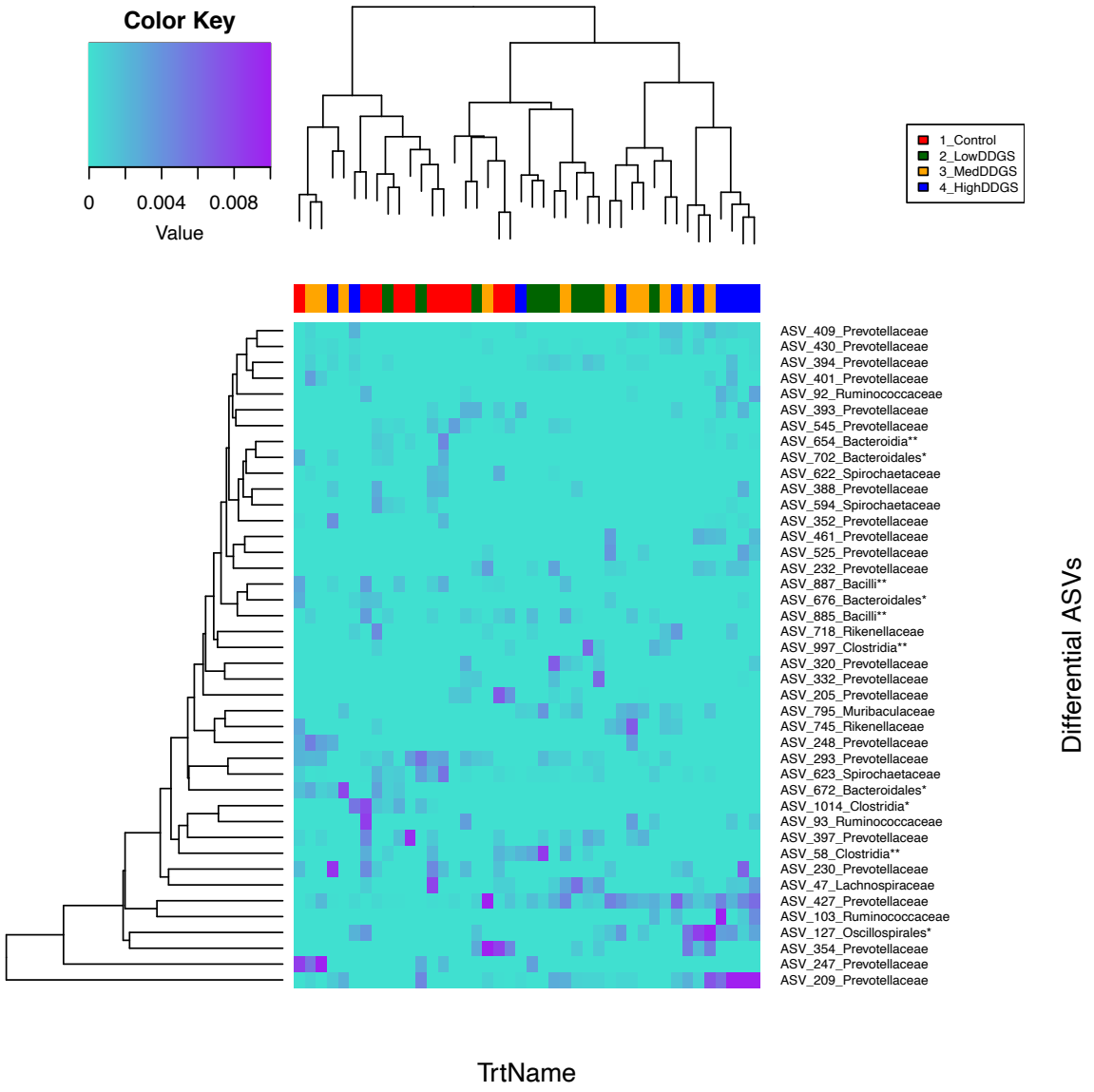

Figure 3.11. Bacterial hierarchical clustering of differentially abundant ASVs. One asterisk indicates order was used as family was not classified and two asterisks indicates that Class was used as higher phylogenetic classifications were not provided.

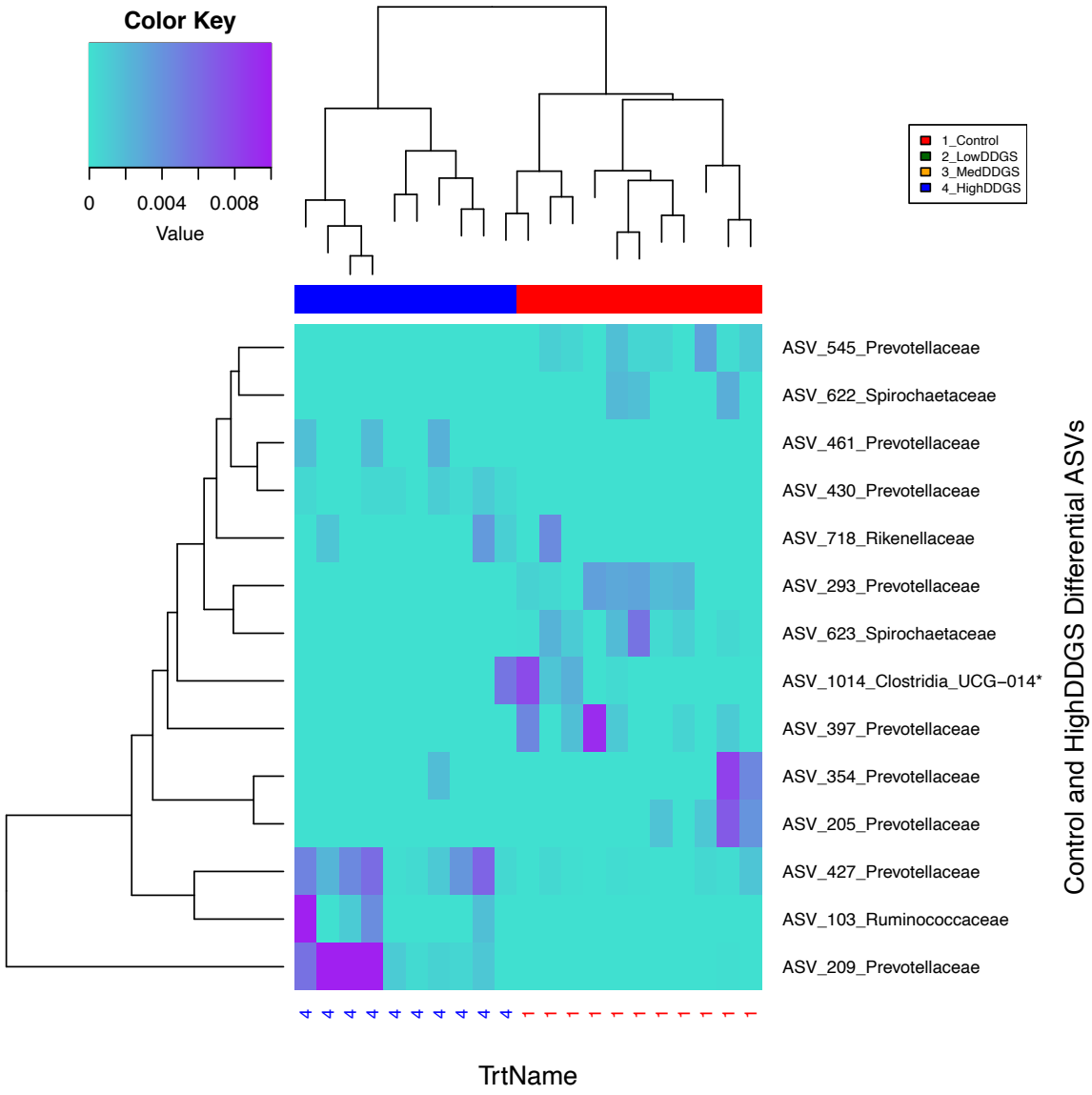

Figure 3.12. Bacterial hierarchical clustering of differentially abundant ASVs on the CON and HighDDGS diets. One asterisk indicates order was used as family was not classified and two asterisks indicates that Class was used as higher phylogenetic classifications were not provided.

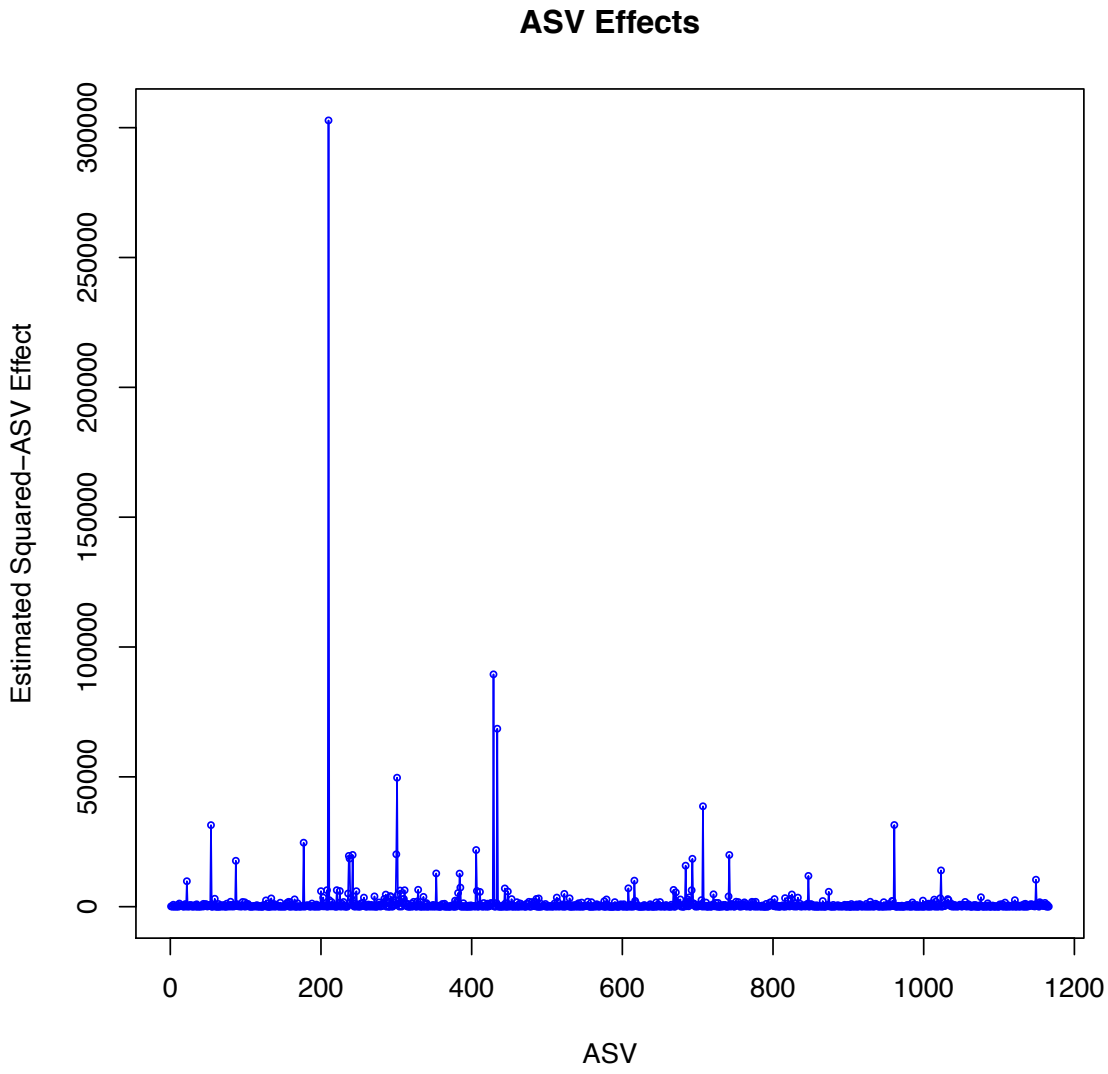

Figure 3.13. Squared effect of each ASV of the bacterial community on CH_4 phenotype. Effects were calculated from the posterior means obtained by fitting a Bayesian linear model (BayesC) in BGLR.

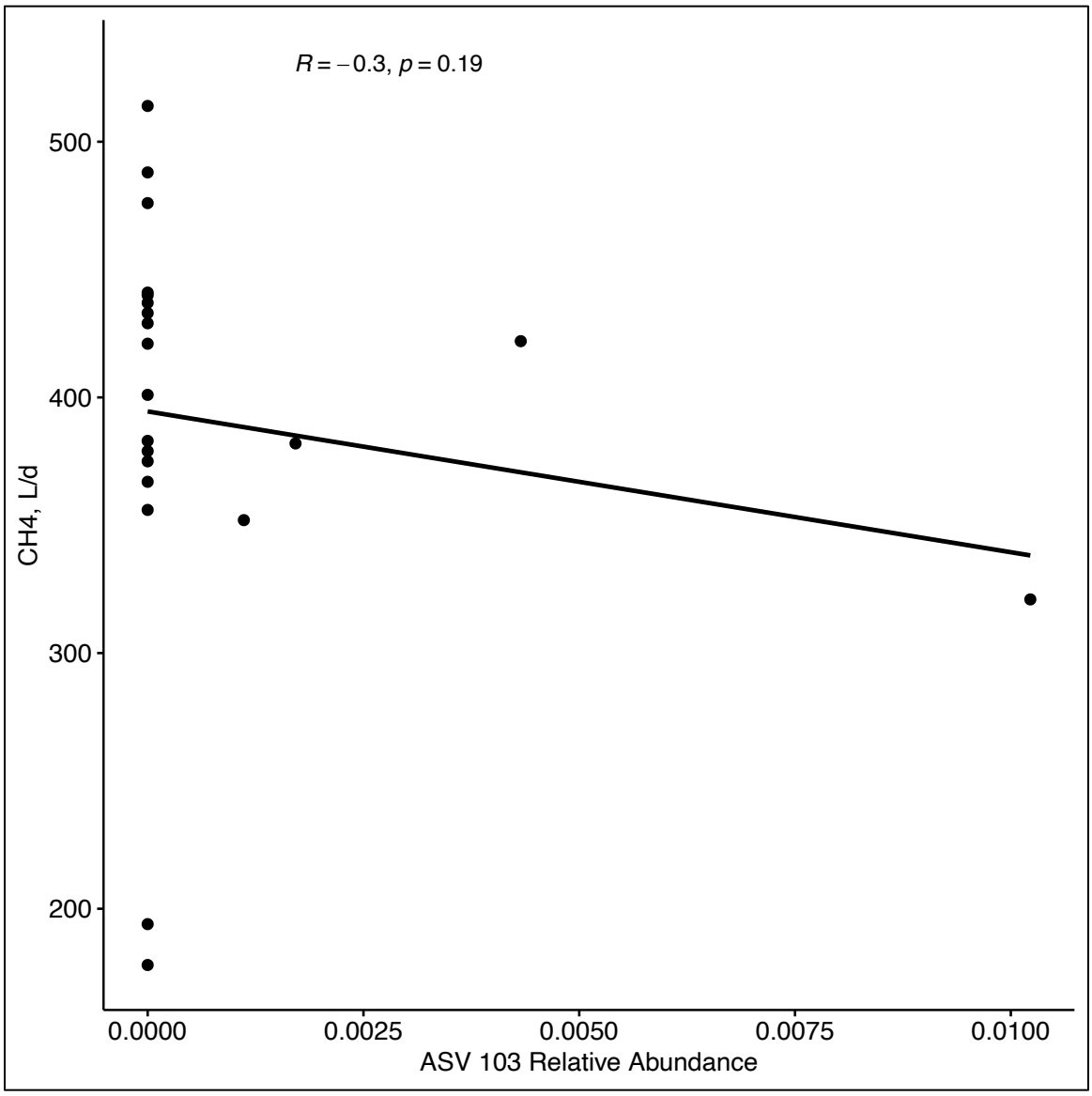

Figure 3.14. Correlation Abundance of the bacterial ASV 103 with methane production levels.

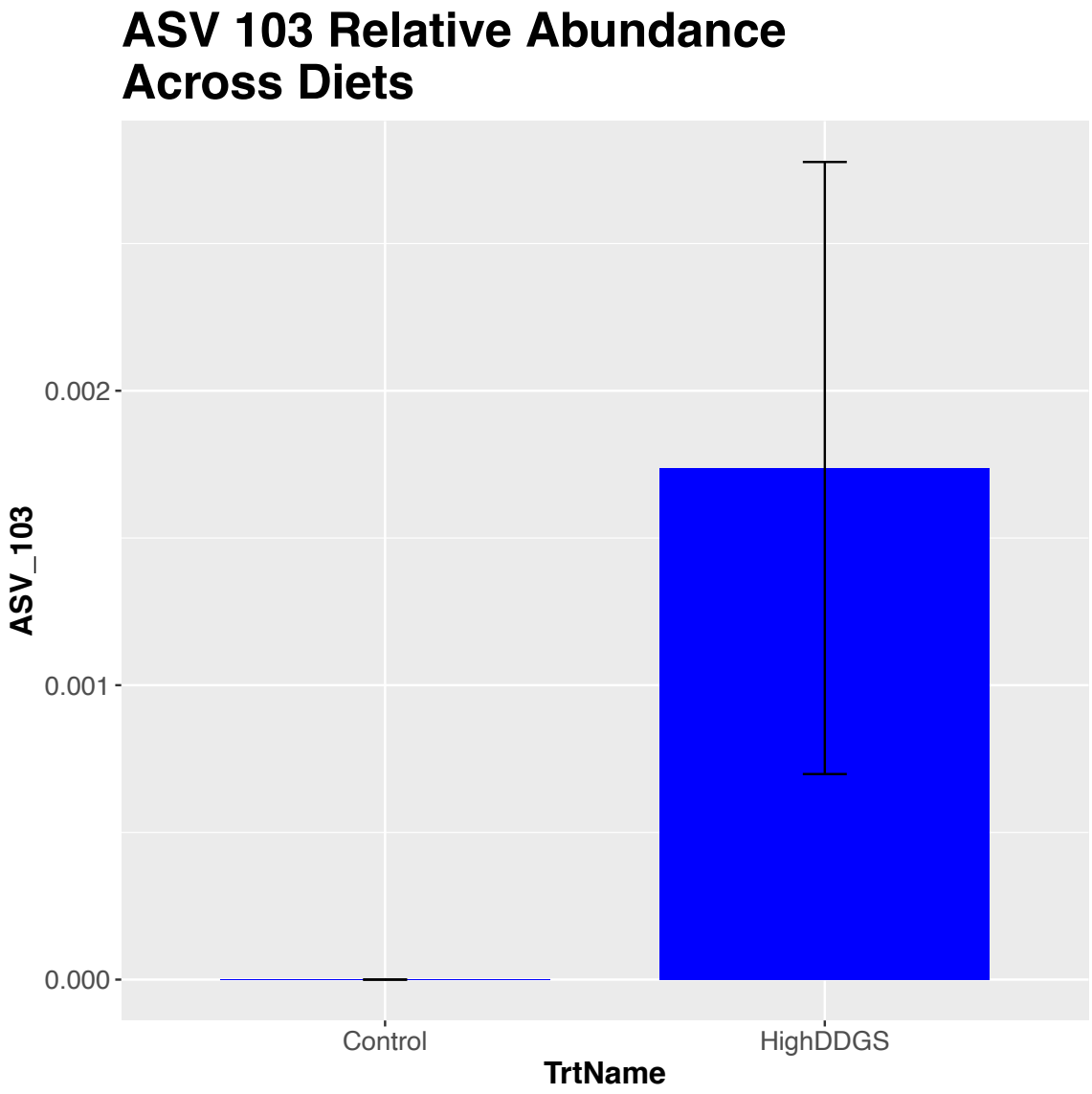

Figure 3.15. The relative abundance of ASV 103 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P < 0.01$) indicate there is a significant difference in abundance of ASV 103 on the HighDDGS diet.

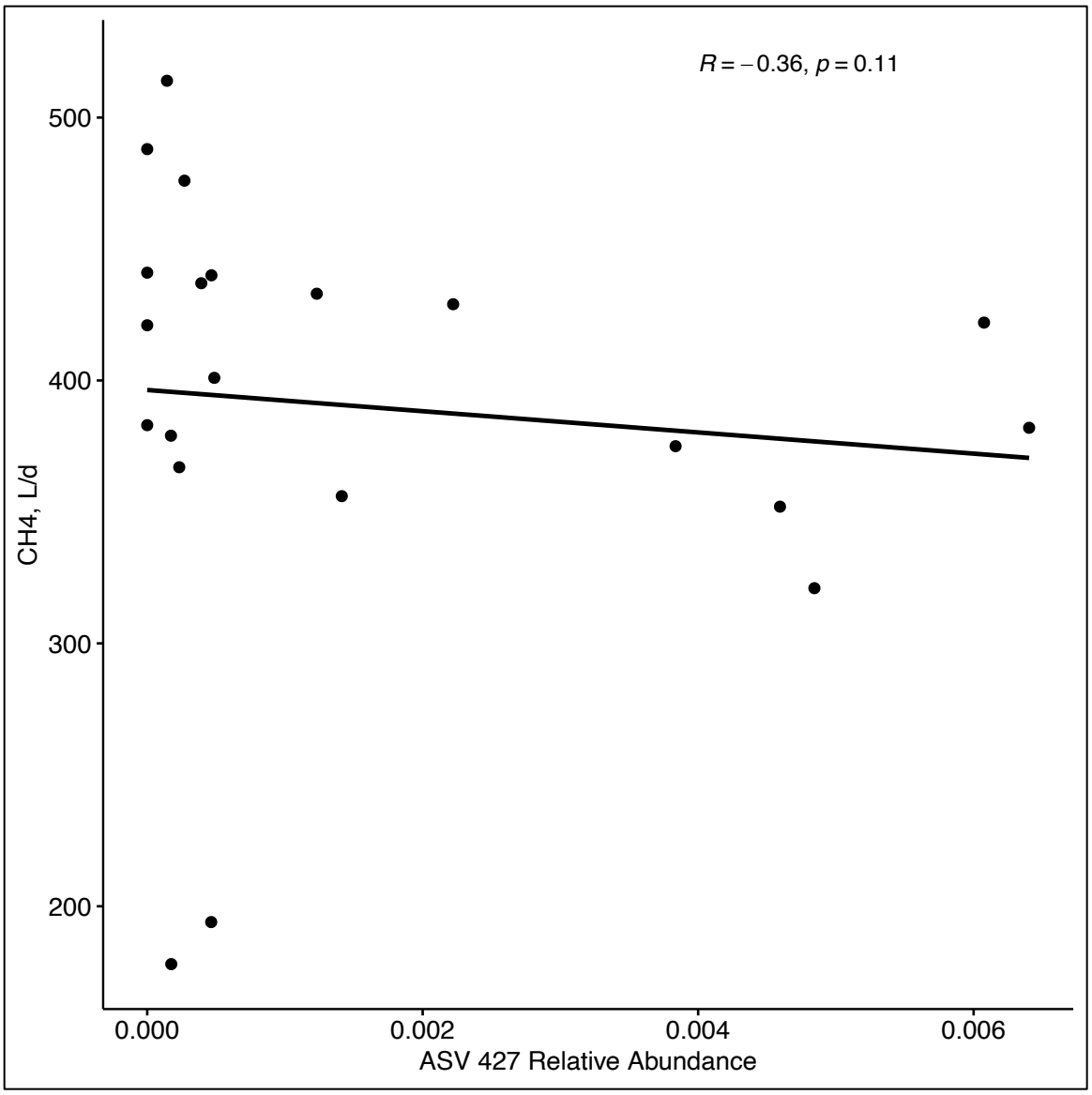

Figure 3.16. Abundance of the bacterial ASV 427 with methane production levels.

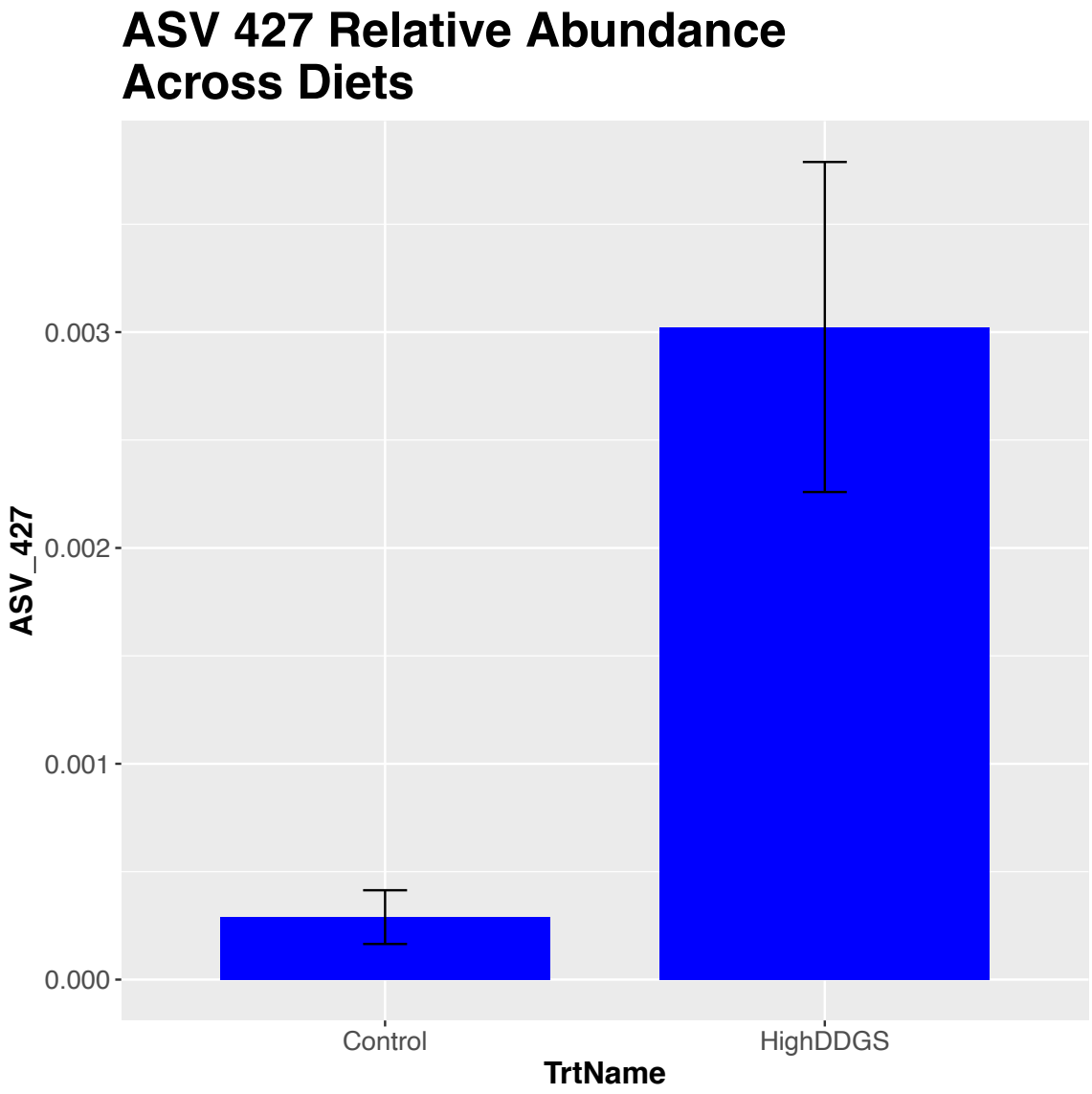

Figure 3.17. The relative abundance of ASV 427 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P < 0.01$) indicate there is a significant difference in abundance of ASV 427 on the HighDDGS diet.

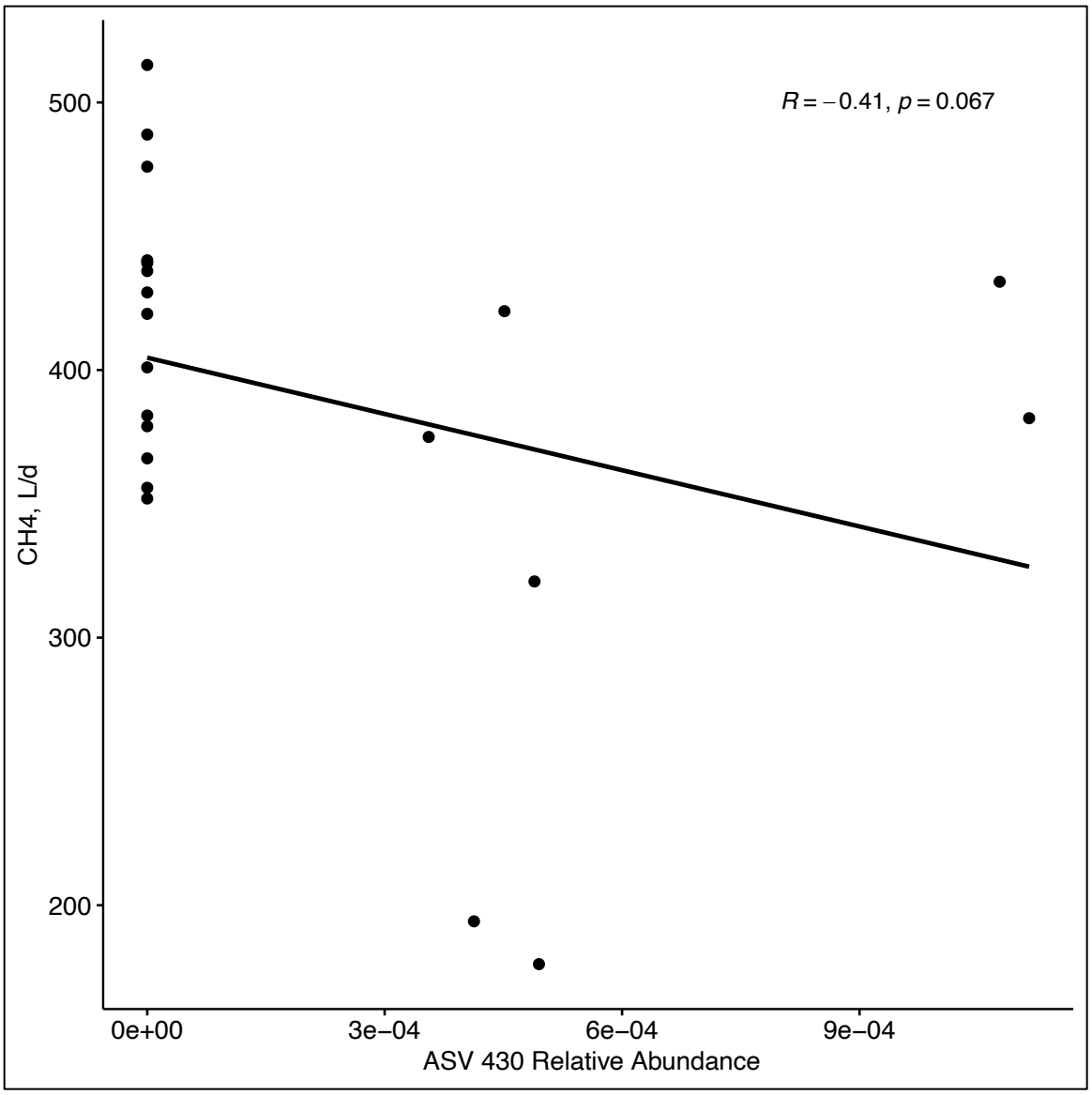

Figure 3.18. Abundance of the bacterial ASV 430 with methane production levels.

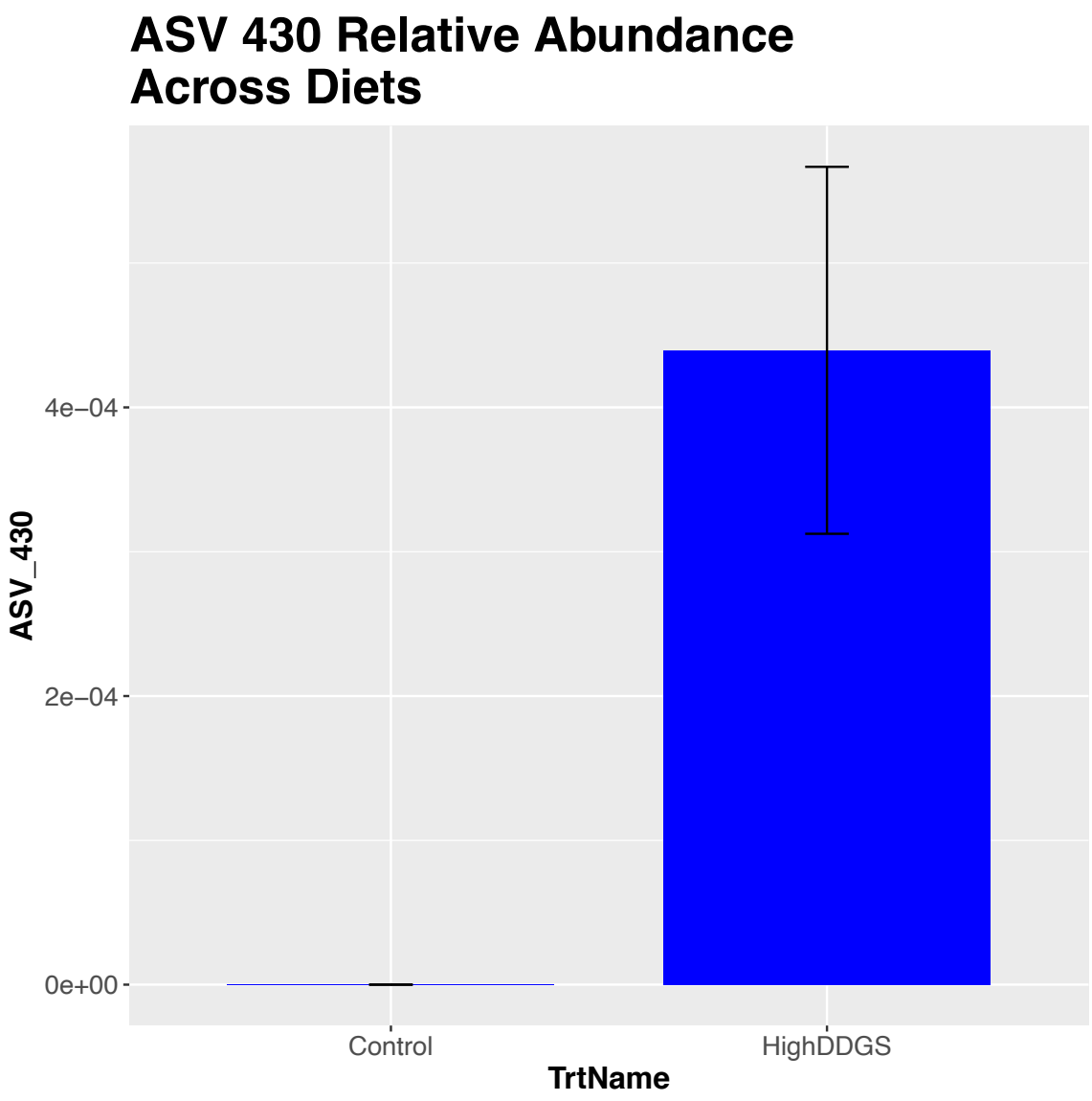

Figure 3.19. The relative abundance of ASV 430 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P < 0.01$) indicate there is a significant difference in abundance of ASV 430 with a higher abundance observed in the HighDDGS diet.

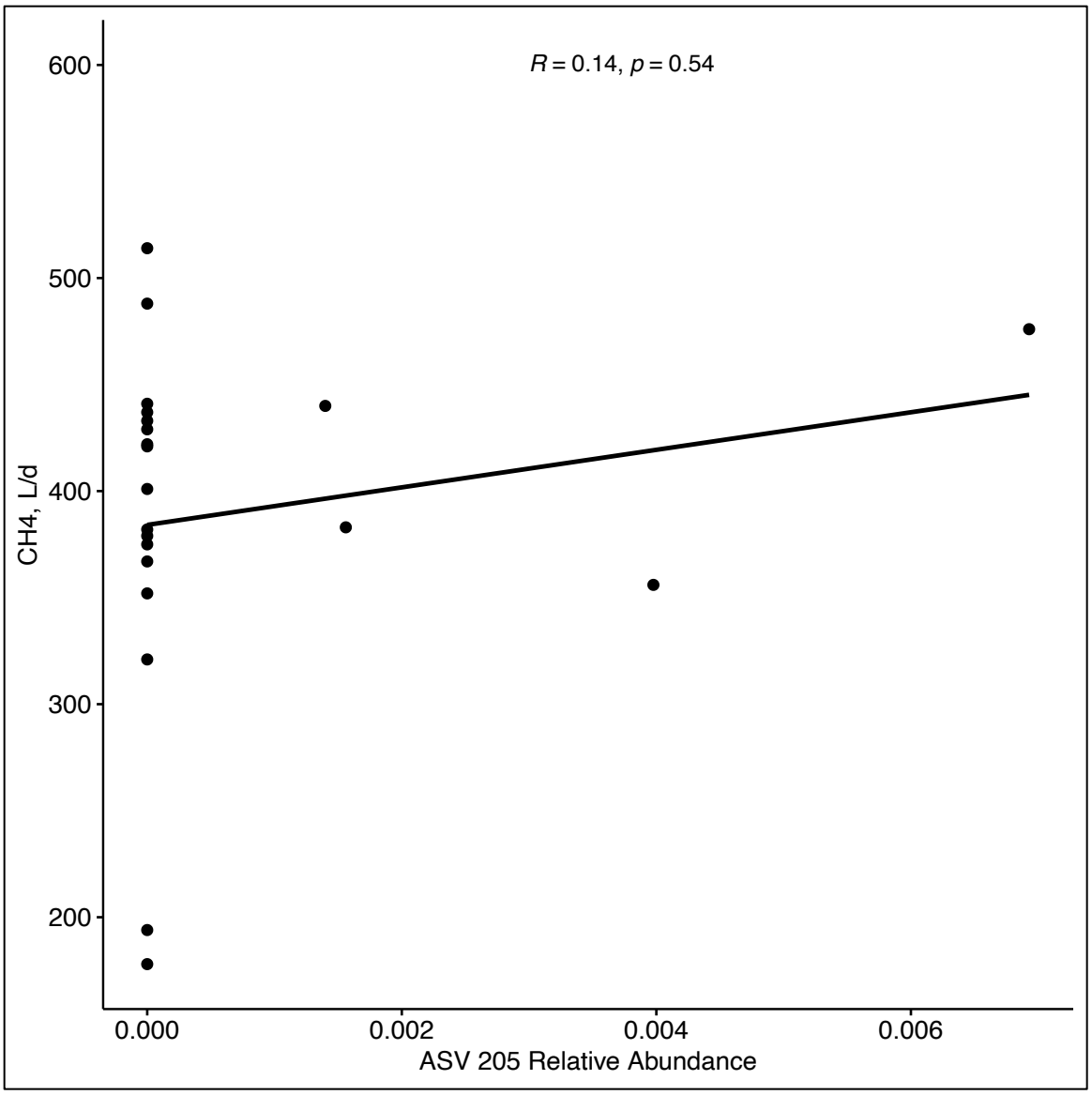

Figure 3.20. Abundance of the bacterial ASV 205 with methane production levels.

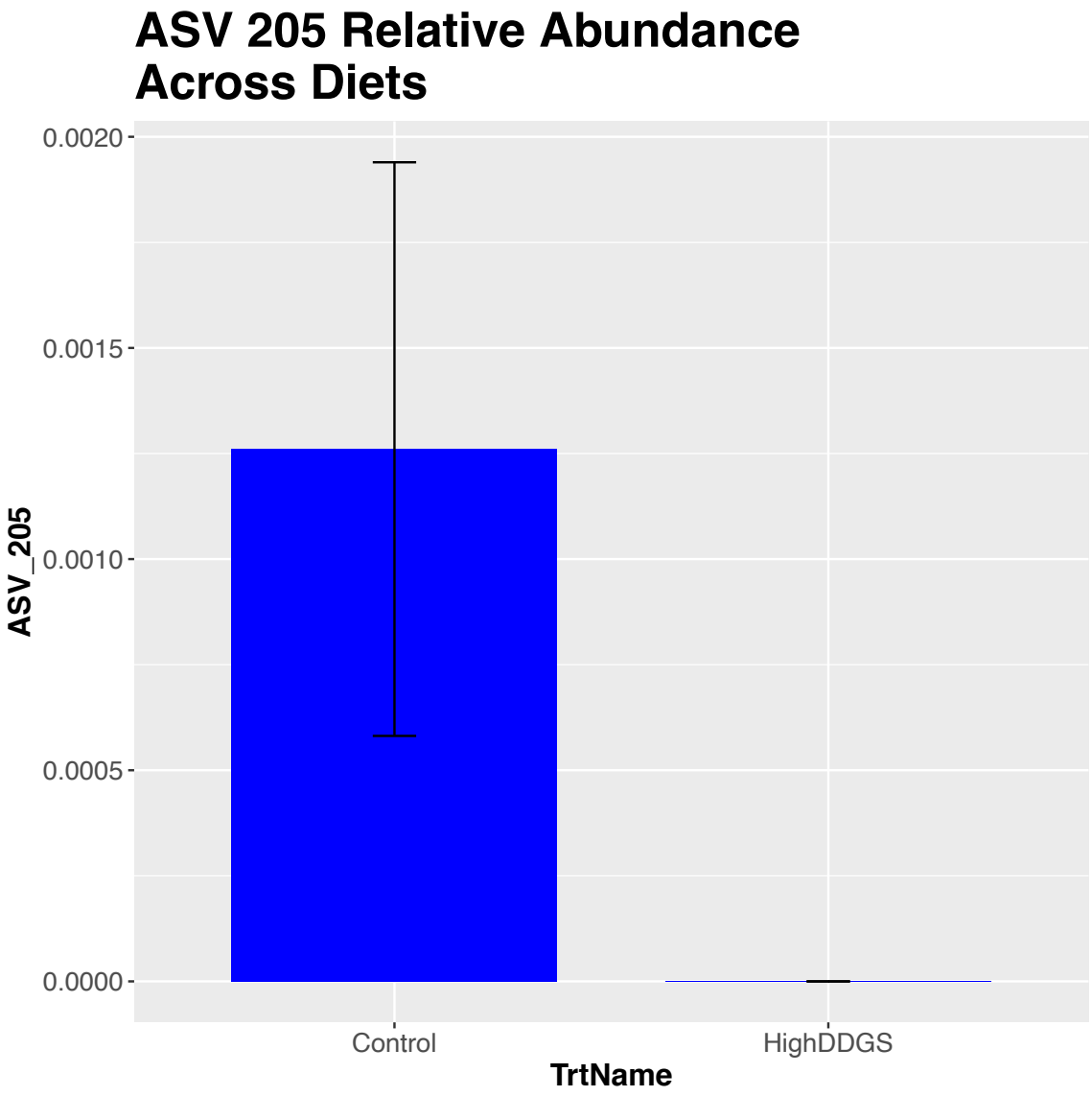

Figure 3.21. The relative abundance of ASV 205 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P = 0.04$) indicate there is a significant difference in abundance of ASV 205 with the abundance being higher in the CON diet.

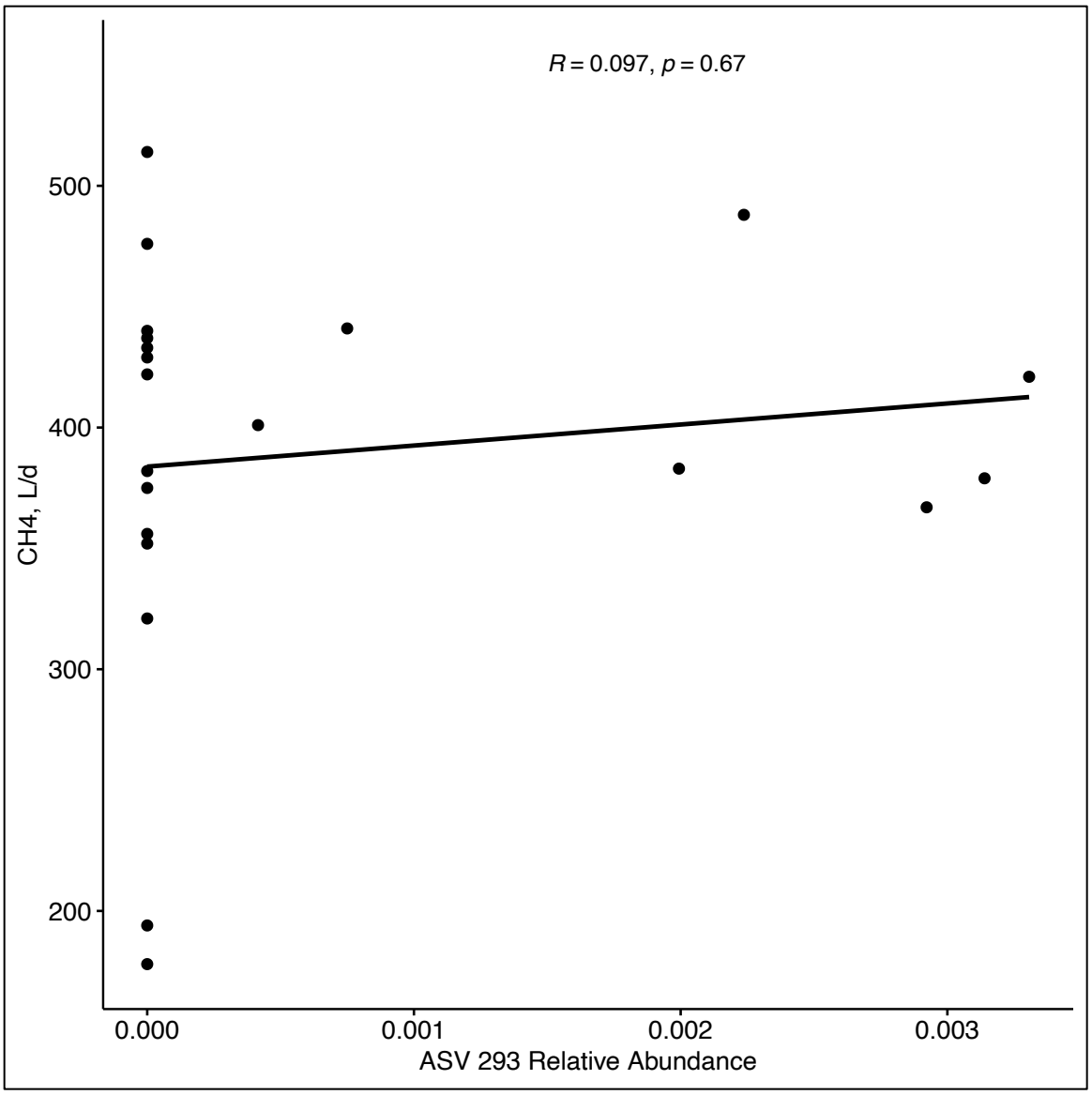

Figure 3.22. Abundance of the bacterial ASV 293 with methane production levels.

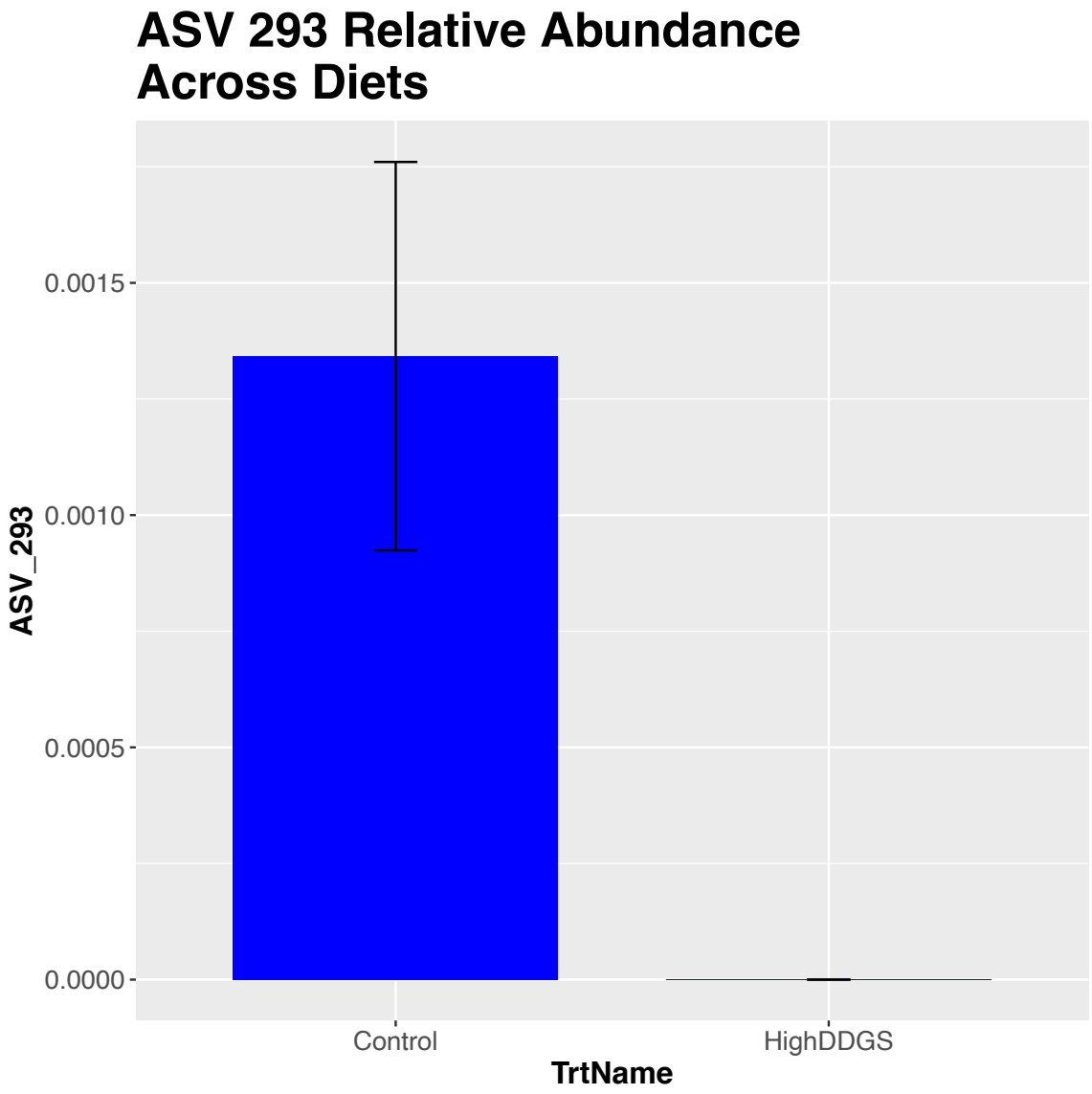

Figure 3.23. The relative abundance of ASV 293 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P < 0.01$) indicate there is a significant difference in abundance of ASV 293 with the abundance being higher in the CON diet.

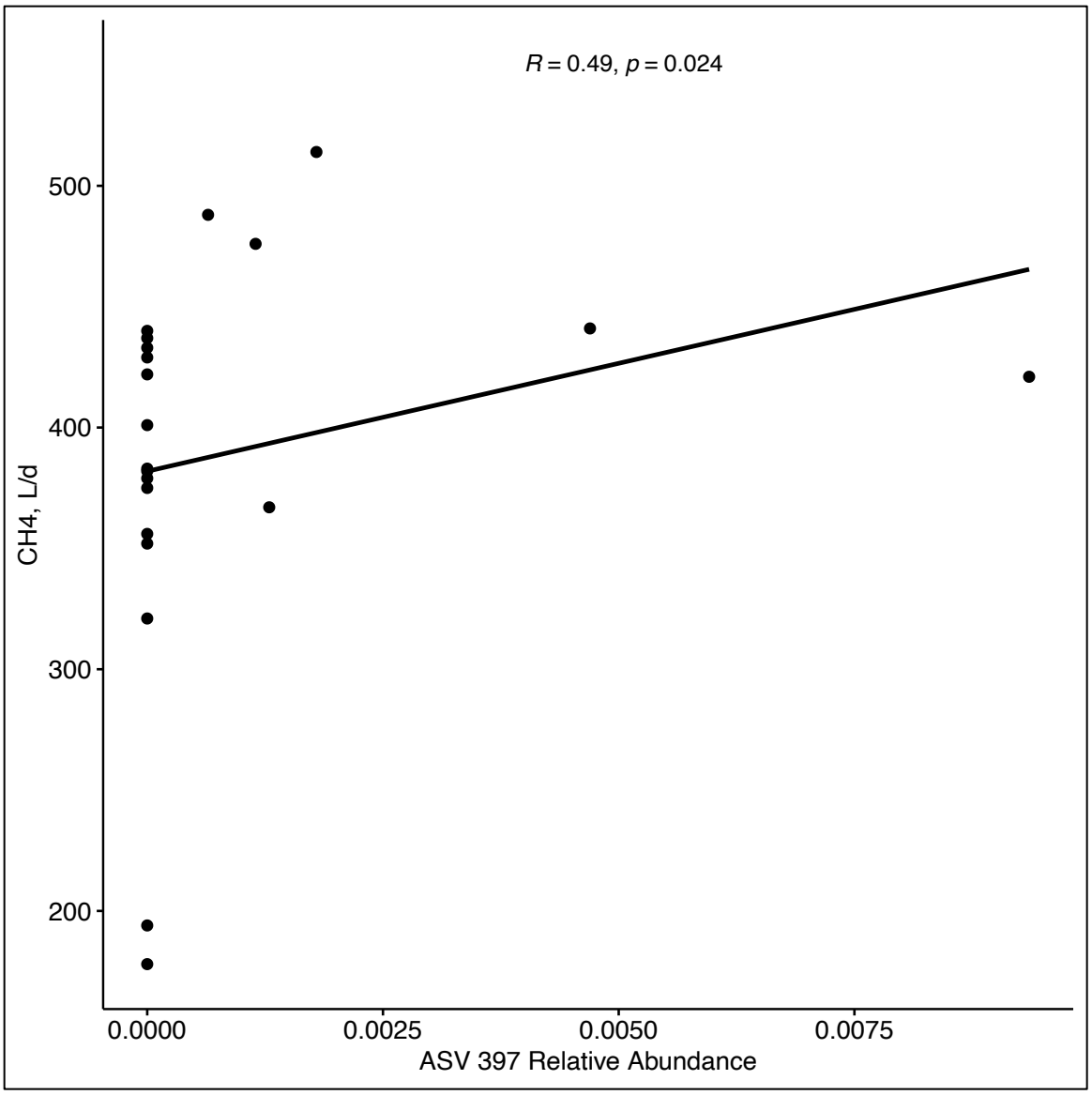

Figure 3.24. Abundance of the bacterial ASV 397 with methane production levels.

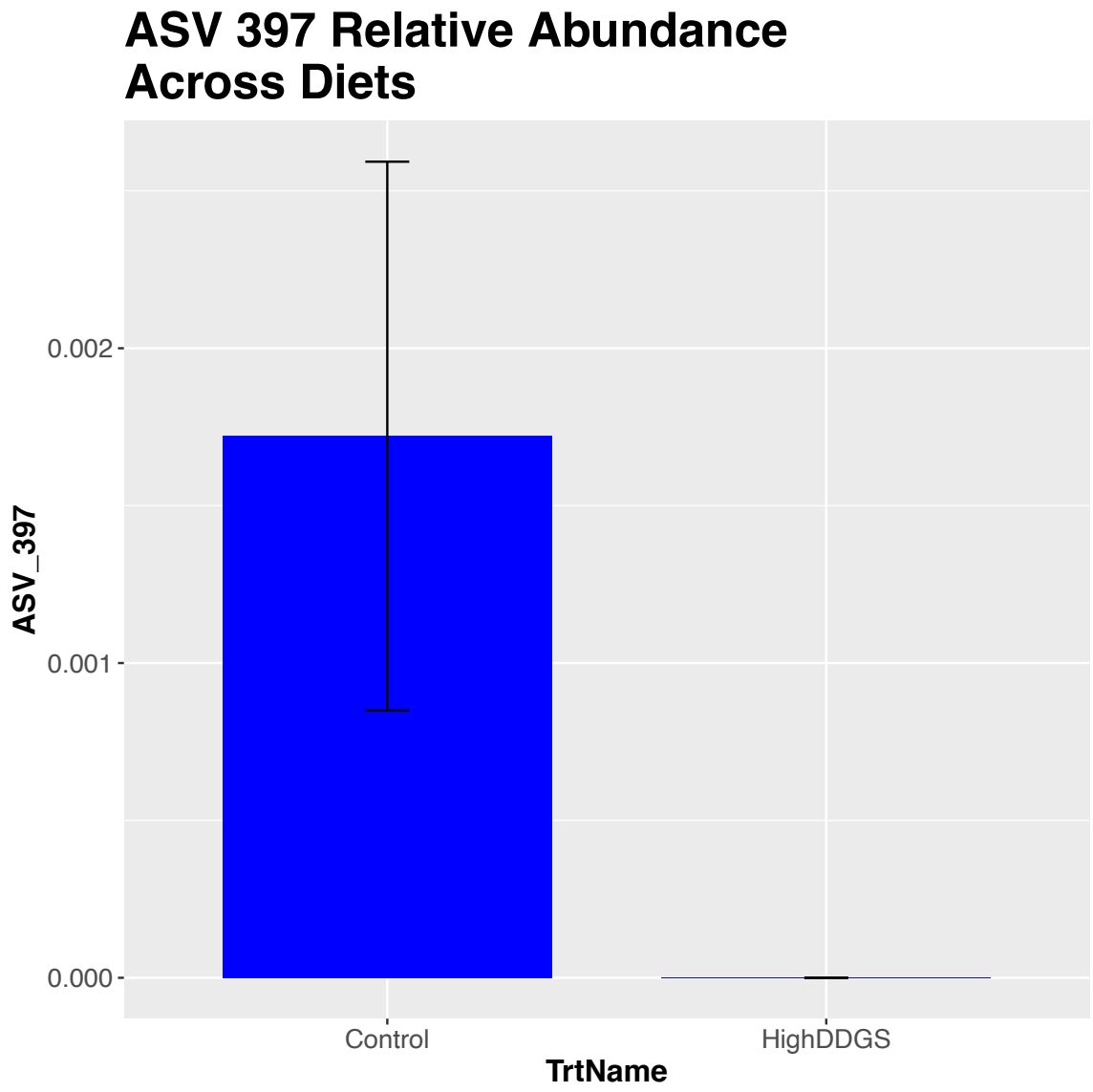

Figure 3.25. The relative abundance of ASV 397 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P < 0.01$) indicate there is a significant difference in abundance of ASV 397 with the abundance being higher in the CON diet.

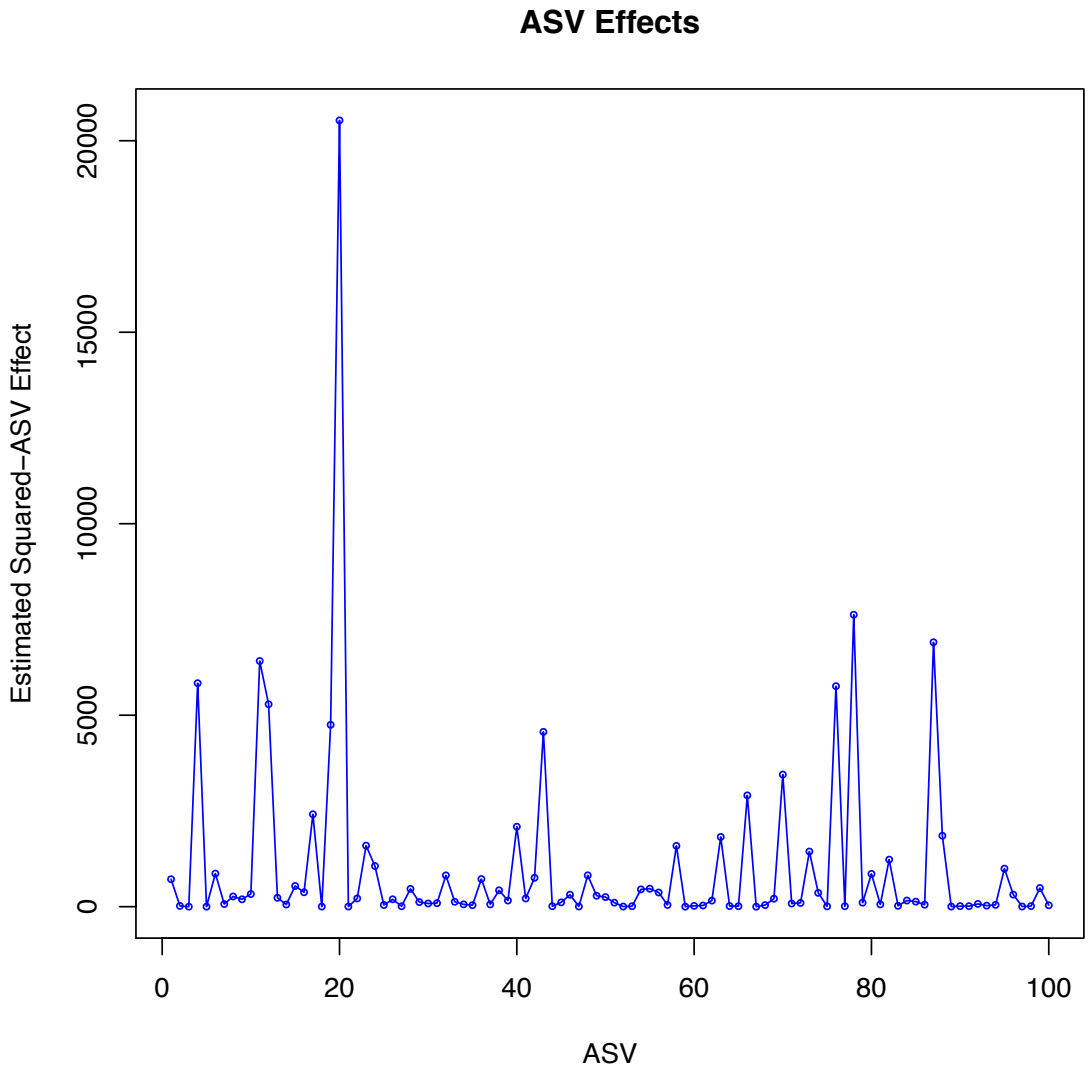

Figure 3.26. Squared effect of each ASV of the archaeal community on CH_4 phenotype. Effects were calculated from the posterior means obtained by fitting a Bayesian linear model (BayesC) in BGLR.

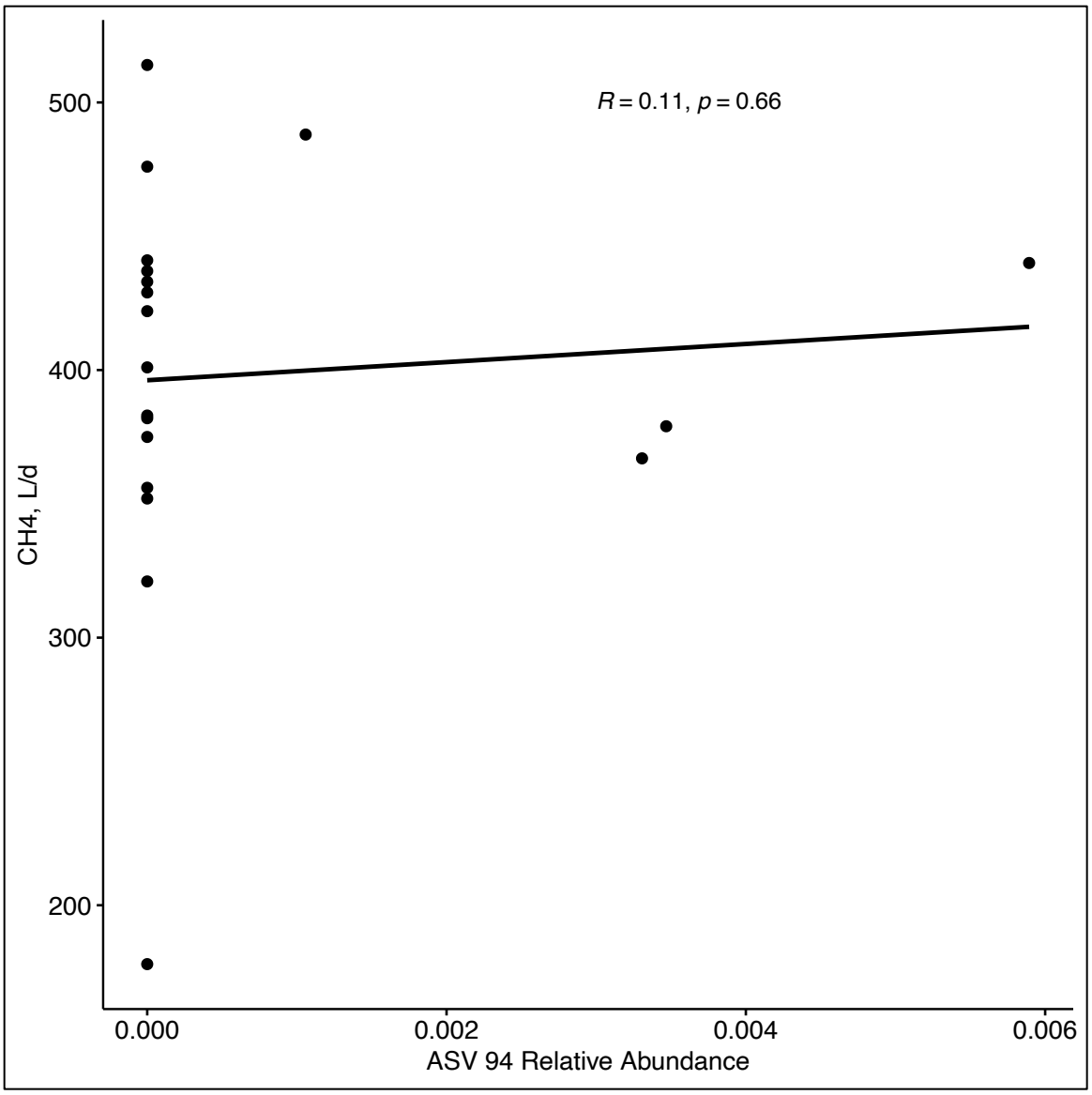

Figure 3.27. Abundance of the archaeal ASV 94 with methane production levels.

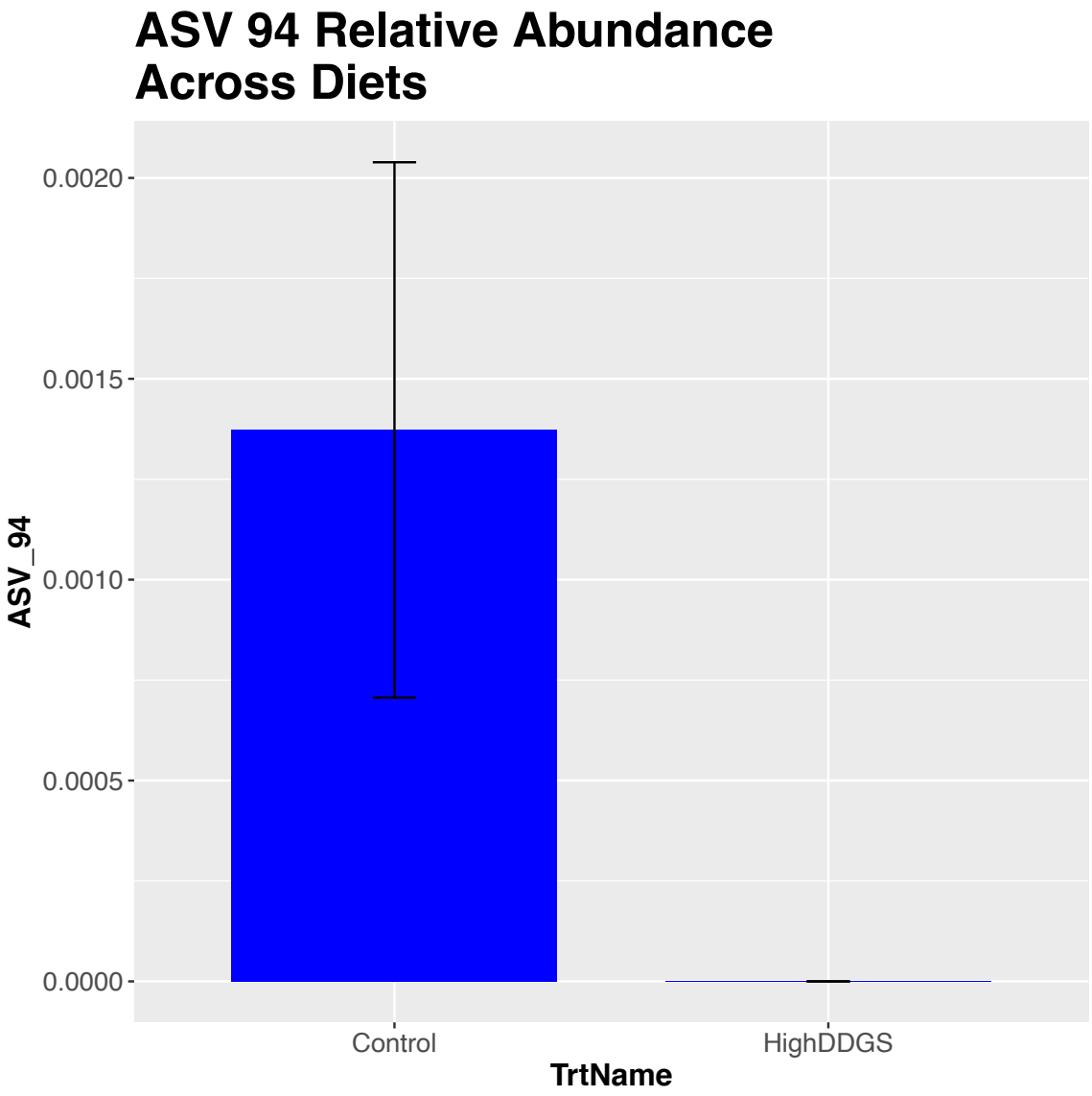

Figure 3.28. The relative abundance of the archaeal ASV 94 on the CON and HighDDGS diets. Results from a Kruskal-Wallis test ($P = 0.04$) indicate there is a significant difference in abundance of ASV 94 with the abundance being higher in the CON diet.

CHAPTER 4

METAGENOMIC ANALYSIS OF THE CATTLE RUMEN ON HIGH AND LOW METHANE PRODUCING FORAGE DIETS

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ABSTRACT

Background

The anaerobic conversion of complex organic matter to methane in ruminants is an essential link in the global carbon cycle. The microscale processes of this microbial community greatly influence methane production. In the current study we investigated the microbial processes that contribute towards methanogenesis in the rumen using high- and low-quality fiber diets.

Results

The high-quality (HQ) and the low quality (LQ) forage diets produced different levels of methane, where the HQ diet produced lower levels of methane compared to the LQ diet. Shotgun metagenomic analyses of the rumen samples detected methanogenic pathways in all samples and were syntrophically linked to propionate, acetate, and butyrate. Genes that are directly involved in methanogenesis were not differentially abundant in the two diets. However, methane emission levels were negatively correlated with propionate metabolism, especially in betaproteobacteria. This was congruent with the 16S rRNA gene sequence analyses results, where the high-quality diet and methane levels were correlated to OTUs of betaproteobacteria.

Conclusions

Results from this study suggest syntrophism among rumen microbes influence methanogenesis, and methane mitigation strategies could be aimed at eubacteria,

especially targeting hydrogen-scavenging pathways such as propionate production and bio-hydrogenation in the rumen instead of archaea and their ecological niches.

Keywords

Bacteria - Metagenomics – cattle – rumen - methane

Introduction

Microbial metabolism plays a pivotal role in all major element cycles on earth including carbon recycling (Morris et al., 2013). Among the products of carbon cycle, methane holds a key position due to its role in global warming (Moss et al., 2000). The rapid increase in methane in the atmosphere is partly due to human related activities such as agriculture and farming. Of these anthropological sources, about 37% of methane are produced by ruminant livestock - making them the single largest source of methane worldwide (Cottle et al., 2011). With the predicted increase in world population by 73% by 2050 (McLeod, 2011), ruminant agriculture is expected to increase leading to increased greenhouse gas (GHG) emissions from ruminant livestock. Thus, there is an immediate need to minimize ruminant methanogenesis.

The demand for meat and milk is well poised to increase in the future. This emphasizes the need for ruminant agriculture practices to adopt methods to mitigate GHG (Havlik et al., 2014). As such, understanding methanogenesis in ruminants is critical towards sustainable agriculture. Anaerobic methane production occupies a critical position in the microbial food chain within ruminants. The microscale processes of this

microbial community greatly influence methane production. Previous studies investigating the rumen microbial community has demonstrated diet to be a significant driver of microbial community structure and in turn metabolic end products produced by the microbial community (Poulsen et al., 2013). When plant materials in the diet are metabolized in the rumen, the catabolism produces hydrogen, which in turn is accepted by methanogenic archaea as its major energy source (Nallathambi, 1997). Thus, dietary factors that determine the microbial assemblages within the rumen (Tajima et al., 2001) will directly affect methanogenesis and in-turn methane emissions (Johnson and Johnson, 1995). Hence, opportunities are present to develop dietary intervention strategies towards methane mitigation. To this end, understanding the interactions between diet, methane emission and the rumen associated microbial community structure and function is critical to develop novel dietary intervention strategies towards methane mitigation.

To date, most studies investigating the rumen microbial community structure and function using molecular community and metagenomic studies have targeted archaeal and eubacterial taxa and functions (Janssen and Kirs, 2008), yet have failed to simultaneously measure methane emission. As such, how the rumen microbial population and its complex microbial interaction effects methanogenesis is poorly understood. Literature on methanogenesis from natural ecosystems point to a syntrophic cooperative metabolism between bacteria and archaea (Schink, 2016). Hence, the study presented here was designed to identify prokaryotic community composition and functions that lead to increased methane emission on high and low methane-producing forage diets. To this end, we carried out 16S rRNA based community analyses and shotgun metagenomic analysis and measured methane emission in beef cattle to identify interactions within the

rumen microbial community that effect methanogenesis. The results presented in this study demonstrates syntrophism among rumen microbes influence methanogenesis.

Results

Evaluation of low and high methane producing forage diets

To obtain a similar microbial baseline population and limit carryover effects, before the start of the study all animals (n=16) were maintained on a common basal diet for 5 days. This baseline feeding demonstrated similar microbial community composition among all animals (Figure 4.1), thus suggesting that the microbial community and functional differences observed during feeding of high- and low-quality forage is in response to diet than carry over effects. Subsequently, after adaptation to the common diet the test animals were assigned randomly to two groups (n=8) where one group was fed a high-quality fiber diet (HQ) and other group was fed a low-quality fiber diet (LQ) (Table 4.1). Methane emissions were measured in both the common diet and during feeding of the test diets (high-quality and low-quality fiber diets). The CH_4/CO_2 ratio was used to evaluate methane emissions during the study as this ratio will help account for differences in intake that would lead to more CO_2 and CH_4 emission with increased intake. Cattle fed the HQ diet produced significantly lower methane at day 63 when compared to both LQ and common basal diet on a per gain basis (Pesta, 2015). In contrast, the animals fed the low-quality fiber diet displayed significant increase in emissions on a per gain basis (Pesta, 2015).

Microbial communities are different in high and low methane emitting diets:

The eubacterial and archaeal community structure was examined using 16S rRNA amplicon sequencing using Ion Torrent sequencing platform (Ion Torrent PGM, ThermoScientific). The average read counts were 17.1 k (SEM ± 2.6 k) for the archaeal V6 region and 20.1 k (SEM ± 2.2 k) for the eubacterial V3 region, with minimum of 3055 and 7374 reads for archaea and bacteria, respectively. The taxonomic attribution was cut-off at the class level for reliability. In all bacterial samples (common diet and high- and low-quality forage diets) were predominated by Operational taxonomic units (OTUs) assigned to genus *Prevotella* (27 OTUs – 33.6 %). The archaeal OTUs were predominantly assigned to classes Thermoplasmata and Methanobacteria (89.1 – 91.5 %), where most of the Methanobacteria belong to genus *Methanobrevibacter*. However, there were no significant differences in abundances of these dominant archaea OTUs between the high-quality and low-quality forage diets. A total of 15 archaeal genera (*Methanoregula, Nitrososphaera, Methanimicrococcus, Methanobacterium, Methanobrevibacter, Methanocella, Methanocorpusculum, Methanoculleus, Methanolinea, Methanolobus, Methanomethylovorans, Methanosaeta, Methanosarcina, Methanosphaera* and *Methanospirillum*) were observed in addition to reads that were close to vadinCA11 OTU type.

As denoted in the ternary plot in Figure 4.1A, six classes (Betaproteobacteria, Mollicutes, Candidatus Saccharibacteria, Clostridia, Elusimicrobia and Synergistica) were significantly different from the basal common diet compared to the two growing diets (HQ and LQ). Two of the classes, Betaproteobacteria and Elusimicrobia were also significantly different between the LQ and HQ growing diets. Betaproteobacteria was

significantly higher in the HQ diet whereas Elusimicrobia were significantly higher in the LQ. Microbial community structuring based on diet were visualized using principle coordinate analyses (using ape extension in R-package suit (Paradis et al., 2004)) on the 75% core measurable microbiome (CMM). The archaeal communities looked similar between the 3 diets (Figure 4.1C). Regardless, bacterial communities from the basal common diet and the two forage quality diets clustered separately (Figure 4.1B) suggesting structuring of bacterial communities by diet. To further identify how differential OTUs may influence methane emission, we performed correlation analyses on each of the significantly different OTUs with level of methane released. Spearman correlation analysis revealed only one OTU to be correlated with methane ($P < 0.05$). This OTU taxonomically belonged to class Betaproteobacteria. Correlation between OTU abundance and methane emission levels are shown in Figure 4.2.

Functional analysis of the rumen metagenome

A total of 43.7 gigabases of sequencing data (pertaining to 173.68 million reads) were obtained with Illumina HiSeq sequencing. A quality threshold of Q20 with at least 50% of the read length higher than Q30 was used as a threshold to remove low quality reads. FLASH (Magoč and Salzberg, 2011) was used to pair reads. Sequential trimming was performed, where sequences were trimmed from the end of the read to 125, 100, and 80 bases. Longest read lengths that met the quality threshold were retained. This allowed 73.1% of the sequences that equated to 67.05% of the base information (when corrected to trimming reduction in read lengths) to be assembled by Megahit (Li et al., 2015) into contigs. Open reading frames (ORFs) were predicted using PRODIGAL (Hyatt et al.,

2010). The largest contig was 72,882,228 bases. The ORFs predicted were annotated using BLAT searches against a local KEGG database (give KEGG version). Reads from each sample was aligned back to the assembled total dataset to obtain read count hits (abundances) for each the functional gene. A total of 1956 enzyme genes were detected (as EC codes).

Genes involved in diet-associated methane emissions were shortlisted using the following criteria. Firstly, by associating the genes in each diet, and secondly using the correlations between gene abundance and methane emission levels. Of the 1956 genes, 1208 were higher in HQ diet when compared to LQ diet. Among these genes, 492 and 317 enzyme genes were significantly higher in HQ and LQ diets, respectively. Positive and negative correlations between gene abundances and CH_4 emission were estimated using Spearman coefficient using a cut-off of 0.5. A total of 220 and 169 genes (Tables S4.1A and S4.1B) were found to be positively and negatively correlated with methane, respectively. All genes that displayed positive correlation with methane were more abundant in the LQ diet, with 124 genes ($P < 0.05$), and those that were negatively correlated were higher in HQ diet, with 106 ($P < 0.05$).

Metagenomic analyses reveals methanogenic and syntrophic methanogenic gene networks

A methanogenesis network was built using the ORF identified in the rumen metagenome. As described above, the ORF were mapped to the KEGG database and the annotated reads were used to build the methanogenic gene network (Figure 4.3). No methanotrophic genes were detected in the dataset; however, methylotrophic genes involved in the breakdown of methanol to formaldehyde were found in all tested samples.

The analysis of methanogenic genes did not show any significant difference in methanogenesis gene abundance (using mcrA) between diets in pairwise-ANOVA. However, acetate and propionate syntrophic pathway associated genes were differentially abundant where HQ diet that was involved in lower methane emission rates had a significantly greater number of hits to contigs that contained genes involved in propionate production from fumarate. However, the low-quality diet had significantly higher acetate pathway genes compared to those of HQ forage diets. Most of the reads that mapped to propionate pathway were taxonomically assigned to Betaproteobacterium (>30%), whereas the other syntrophic pathways were mostly from Bacteroidetes (>22%).

Functional gene abundance in the rumen is correlated with CH4 emission

Positive and negative correlations between gene abundances and CH_4 emission were estimated using Spearman coefficient using a cut-off of $P < 0.05$. A total of 564 and 249 genes (Tables S4.1A and S4.1B) were found to be positively and negatively correlated with methane, respectively, and also demonstrated a statistical significance in pairwise-ANOVA. The genes that were within the syntrophic methanogenic network are denoted in Figure 4.3. Syntrophic propionate pathway genes were negatively correlated with methane emission levels, whereas butyrate and acetate pathway genes were positively correlated with methane.

Metabolic pathways that are not directly related to methanogenesis

Since it was difficult to discern patterns in pathways that are not known to be associated with methanogenesis, a less stringent approach (compared to the criteria used

for syntrophic methanogenic pathways) was taken to increase resolution. To this end, genes that were significantly higher in HQ diet, and those that were negatively correlated with methane levels were accounted as genes involved in low methane emission. This resulted in a total of 555 genes, where, 106 genes were found in both categories (Supplementary Figure S4.1). Conversely, genes that were significantly higher in LQ diet, and those that were positively correlated with methane levels were accounted as genes associated with high methane emission. This resulted in 413 genes, where, 124 genes were in both categories (Supplementary Figure S4.1).

Amino acid metabolism: A major proportion of the pathways that were associated with either low or high methane producing diets were involved in amino acid metabolisms (Supplementary Figure S4.2). However, differences in pathways were identified. For example, ORFs from high methane producing diet (LQ) indicated higher potential for L-glutamate synthesis from L-histidinol and carnosine (Supplementary Figure S4.2-A), while in low methane diet (HQ) L-glutamine and L-glutamate were sequentially metabolized to produce ammonia, propanyl-CoA and succinyl-CoA (Supplementary Figure S4.2-L). Additionally, L-glutamate was potentially converted to oxaloacetate in low methane emitting diet (HQ) (Supplementary Figure S4.2-G), whereas in to fumarate in high methane diet (LQ) (Supplementary Figure S4.2-H). High methane emissions were also correlated with L-isoleucine synthesis from homoserine. L-isoleucine degradation genes were also significantly higher in this diet (LQ), potentially leading to the production of propanyl-CoA and acetyl-CoA (Supplementary Figure S4.2-B). Furthermore, genes that synthesize L-valine from pyruvate were also associated with high methane (Supplementary Figure S4.2-C). Two pathways leading to lysine

biosynthesis was associated with low methane diet (HQ) (Supplementary Figure S4.2-D and E). Low methane emitting high-quality diet also seem to encourage peptide degradation to proline (Supplementary Figure S4.2-I).

Other pathways: Degradation of cellulose to produce D-glucose was negatively correlated with methane levels (Supplementary Figure S4.3A) and was significantly less in high methane producing diet. In the sulfur cycle pathways, methane was negatively associated with sulfite and sulfide production genes (Supplementary Figure S4.3B). Additionally, low methane diet was associated with genes responsible for nitrous oxide emission (Supplementary Figure S4.3C). Genes associated with ammonia, another greenhouse gas, was positively correlated with methane emission levels.

Potential contribution of methanogenic archaea and betaproteobacteria in methanogenesis: To assess the potential roles played by methanogenic archaea, contigs containing methanogenic genes of archaeal origins (EC 4.4.1.19, 3.1.3.71, 4.1.1.79, 1.8.98.1, 2.1.1.86 and 2.8.4.1) were analyzed. This allowed characterizing other genes within the same contig and further identifying archaeal functions. The annotated genes were mapped on the syntrophic methanogenic network (Figure 4.4A). Apart from the core methanogenesis enzymes, genes involved in propionate and acetate syntrophic methanogenesis were identified among the methanogen contigs. Taxonomic information of the archaeal genes was obtained using an arbitrary cut off of detecting at least five genes from taxa and having abundance of at least in five different samples. This resulted in a total 81 genera (Figure 4.4B). Of these, 13 were also noted in PCR based 16S rRNA community analyses. The three that were not observed in the shotgun libraries were

Methanimicrococcus, *Methanoliena* and vadinCA11. A total of 68 genera (*Acidianus, Acidilobus, Aciduliprofundum, Aeropyrum, Archaeoglobus, Caldisphaera, Caldivirga, Caldiarchaeum, Korarchaeum, Methanomassiliicoccus, Methanomethylophilus, Methanosphaerula, Nitrosopumilus, Cenarchaeum, Desulfurococcus, Ferroglobus, Ferroplasma, Fervidicoccus, Halalkalicoccus, Haloarcula, Halobacterium, Haloferax, Halogeometricum, Halomicrobium, Halophilic archaeon DL31, Halopiger, Haloquadratum, Halorhabdus, Halorubrum, Haloterrigena, Halovivax, Hyperthermus, Ignicoccus, Ignisphaera, Metallosphaera, Methanocaldococcus, Methanococcoides, Methanococcus, Methanohalobium, Methanohalophilus, Methanolacinia, Methanopyrus, Methanosalsum, Methanothermobacter, Methanothermococcus, Methanothermus, Methanotorris, Nanoarchaeum, Natrialba, Natrinema, Natronobacterium, Natronococcus, Natronomonas, Picrophilus, Pyrobaculum, Pyrococcus, Pyrolobus, Salinarchaeum, Staphylothermus, Sulfolobus, Thermococcus, Thermofilum, Thermogladius, Thermoplasma, Thermoplasmatales, Thermoproteus, Thermosphaera* and *Vulcanisaeta*) were only seen in the metagenomic libraries. Although 16S rRNA gene sequence analyses had not shown distinctive patterns in archaeal diversity between the diets (Figure 4.4A), distribution of archaeal genera using shotgun sequencing revealed that the high methane emitting LQ diet had more diverse archaeal population (Figure 4.4B).

We observed that betaproteobacteria seem to play a key role in reducing methane emissions in syntrophic methanogenic network: 1) OTUs from this class were significantly high in low methane emitting HQ diet (Figure 4.4B), 2) OTU abundance of betaproteobacteria were negatively correlated with methane emission levels, and 3) most

of the propionate pathway genes that were negatively correlated to methane production (Figure 4.3) and high in HQ diet were attributed to betaproteobacteria. Hence, we attempted to investigate the potential other roles of bacteria from this taxon in methanogenesis. When annotated genes from contigs that showed more than 80% ORFs to betaproteobacterium were mapped to the syntrophic methanogenesis network, the enzymes mapped to propionate and butyrate pathways. The genes mapped from methanogenic archaeal and betaproteobacterium contigs are shown in Figure 4.3.

Discussion

This study was designed to identify overall microbial processes that govern methanogenesis in the rumen utilizing high- and low-forage diets. The two diets used in this study differed considerably in their forage quality. The low-quality forage diet (LQ diet) lead to high methane emissions on a per gain basis whereas the high-quality forage diet (HQ) significantly reduced emission levels on a per gain basis. Thus, the HQ diet treatment provides a unique opportunity to investigate shifts in the microbial community and function to develop dietary intervention strategies towards methane mitigation.

As a first step towards understanding microbial processes, 16S rRNA gene sequence community analyses were carried out both on eubacteria and archaea. Operational taxonomic units belonging to the class Betaproteobacteria were found to be significantly associated with low methane producing diet (HQ). This taxon was also the only OTU that was correlated with methane emission levels. Members of this family have been shown to utilize cellulose, hemicellulose, lignin and xylose (Kameshwar and Qin, 2016), therefore the presence of this taxa is expected in the rumen. However, to the best

of our knowledge the importance of betaproteobacteria in rumen methanogenesis has not been documented.

Interesting enough, despite methanogenesis being an archaeal process, no differential archaeal OTUs were observed from targeted 16S rRNA analyses of archaea. Taxonomic data obtained from functional genes, in contrast, showed higher archaeal diversity compared to those obtained by 16S rRNA alone. This may be due to inherent PCR biases and primer mismatches as many of the archaea in the rumen are uncharacterized and the homology to currently used universal archaea primers are unknown. For instance, *Nanoarcheaum*, a taxa observed only in the metagenomic libraries are well known to have low amplification with current universal primers (Baker et al., 2003), and generally require specific primers (Casanueva et al., 2008). Within the context of *Nanoarchaeum*, it is intriguing to note that genes of their obligate symbiont, *Ignicoccus* (Forterre et al., 2009), was also demonstrated. The three genera *Methanimicrococcus*, *Methanoliena* and vadinCA11 were only observed in 16S rRNA community analyses but not in the shotgun sequences. These three taxa groups do not have a representative genome sequenced and hence it is understandable on why we did not detect their genes. This underscores the urgent need to sequence more genomes from rumen environment, especially those of archaea.

More than taxonomic diversity elucidation, metagenomic functional libraries are useful in identifying metabolic processes to particular functional traits. In this study, we were interested in identifying the rumen metabolism that is associated with methane emission and genes that are associated with diet. Within the syntrophic methanogenic network there appears to be competitive dynamics between propionate metabolism and

acetate and butyrate metabolisms. Within this network, there was a significant clustering of genes by diet and genes correlated with methane emission. Propionate metabolism genes were high when methane emission was low and is in agreement with previous reports as propionate production in the rumen is a hydrogen sink that competes for hydrogen (Moss et al., 2000; Susanti et al., 2014; Janssen, 2010) with methanogenesis.

The enzymes in the propionate pathway indicate fumarate as the possible substrate for propionate production. Interestingly, *in vitro* study by Asanuma et al. has shown that fumarate metabolism can reduce methane levels (Asanuma et al., 1999). Direct feeding of fumarate seems to have no effect on reducing methane levels, most likely as the substrate may be affected by rumen pH before it could be metabolized to propionate (McGinn et al., 2004). However, encapsulating fumarate decreases methane, which supports that fumarate when available for rumen flora can be used to mitigate methane (Wood et al., 2009). We believe that a similar metabolism occurs during fermentation of high-quality fiber diet where fumarate is synthesized and readily metabolized to propionate. This reiterates the importance of fumarate to propionate has an important pathway to reduce methane from cattle. However, unlike with intrinsically low methane yielding sheep ruminants (Kamke et al., 2016), lactate to propionate synthesis was not observed in this study.

High methane producing diet (LQ diet) was associated more with aceticlastic methanogenesis. It also displayed potential increase in butyrate synthesis. This is in agreement with contemporary understanding that acetate and butyrate promote methanogenesis (Moss et al., 2000). Furthermore, propionate is inversely related to butyrate in methanogenesis (Moss et al., 2000). The high levels of pyruvate metabolizing

genes are similar to that identified in metagenomes of high methane emitting cattle (Wallace et al., 2016).

The metagenomics analyses performed in the current study demonstrates the role of propionate metabolism in methane mitigation. We tested the taxa that are most dominant within this pathway and found that betaproteobacteria was the largest contributor of these genes. The OTUs from 16S rRNA of this class of bacteria were significantly higher in low methane diet and also was negatively correlated with methane levels. These data agree with the fact that propionate synthesis genes were also negatively correlated with methane concentration. Thus, there is a reasonable suggestive evidence that betaproteobacteria are playing a key role in syntrophic methanogenesis and are a good target for methane mitigation.

In order to understand the interactions between betaproteobacteria and methanogenic archaea within the methanogenic network, we mapped the genes from their respective contigs to syntrophic methanogenesis. Although the main methanogenic pathway was solely archaeal, it was interesting to note that both archaea and betaproteobacteria are potentially involved in propionate and butyrate synthesis pathways, both key pathways that seem to influence methane emission in the rumen. *Sharpea* sp. which are shown to be key player in low methane sheep displayed a similar profile of betaproteobacteria identified in this study by the presence of both propionate and butyrate synthesis genes (Kamke et al., 2016; Suvorova et al., 2012)

Apart from the major syntrophic methanogenesis pathways, amino acid syntheses and cellulose breakdown were predominant pathways identified. Of the amino acid syntheses pathways identified, two leading to lysine biosynthesis were observed. In light

of methane production, one of the byproducts of lysine biosynthesis is 2-oxoadipate, which is required for coenzyme B synthesis in methanogenesis (Drevland, 2009).

Conclusions

- Despite ruminant methanogenesis being solely an archaeal process, 16S rRNA community analyses did not show differential patterns between low- and high-quality forage diets that produced different methane levels. However, taxonomic information obtained from shotgun metagenomics displayed a clear difference in diversity between the diets. PCR based community analyses of archaea is grossly underestimated archaeal diversity in rumen.
- Propionate synthesis pathway is enriched in low methane emitting diet, which competes with methanogenesis for hydrogen. In HQ diet that produced less methane, propionate is likely synthesized by using fumarate as the substrate.
- Of the eubacteria, Betaproteobacteria seems to play a pivotal role in syntrophic methanogenesis in low methane emitting diet (HQ). OTUs from this taxon was significantly higher in HQ diet and negatively correlated with methane emission levels. Genes from this class were the most dominant in propionate pathway which supports the above hypothesis.
- More archaeal genomes need to be characterized from the rumen. Shotgun metagenomics is an excellent tool to map taxonomic identities to functions, but it requires deeper sequencing of metagenomic data due to low abundance of archaea in the rumen.

Declarations

Competing interests: The authors declare that they have no competing interests.

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Supplementary methods

Animals and diets

The study included 16 animals that were initially fed a basal common diet. The common diet encompassed 47.5% sweet bran, 47.5% alfalfa hay, and 5% supplement at 2.0% projected body weight for 5 days prior to trial initiation to equalize-gut fill and maintain a similar microbial population. After the basal diet period 8 animals were fed a high-quality forage consisting of 60:40 alfalfa hay:sorghum silage blend, and low-quality forage consisted of ground corn stalks. All diets contained 20% modified distillers grains plus soluble (see Table 4.1 for detailed dietary composition).

Methane emission measurement and estimation

In order to measure methane and carbon dioxide emissions, respired air from the steers were collected from individual animals using the Calan gate bunks that were partially enclosed and outfitted with small air pump (Aqua Lifter, MO). Gas was collected during feeding which included a mixture of ambient air and respired breath at constant rate of 1 litter/minute. Additional gas samples were collected from empty bunks to correct for ambient air methane and carbon dioxide levels. Gas collections were semi-automated by initiating collection only when the animal was in the bunk feeding. Methane and carbon dioxide collected over a 10 min feeding period was measured using gas chromatograph (SRI instruments, CA). Gas samples were collected by weekly over the full growing period. Daily methane production values were calculated using the equation developed by Madsen et al. 2010 (Madsen et al., 2016). This method uses CO2

as an internal marker, as an alternative to using SF6 as a tracer gas. The following is an outline of the calculations and assumptions made in the Madsen equation:

ME Intake = feed units × (7.8/0.7)

Feed unit = kg TDN intake × 0.78

Assumes 7.8 MJ NE/feed unit and 70% utilization of ME

Heat production = ME Intake – (kg ADG × 20)

Assumes 1 kg weight gain contains 20 MJ

Daily CO2 production (L/d) = (heat production × 1000)/21.75

Daily CH4 production (L/d) = Daily CO2 production × measured CH4:CO2

Collection of rumen samples and DNA extraction

Rumen samples were collected on the common diet and on days 21 and 63, prior to feeding. The samples were collected via esophageal tubing as described previously (Paz et al., 2016). The rumen samples were snap frozen in liquid nitrogen and stored at -80°C until used for microbial community and metagenome analysis. Whole community genomic DNA were extracted from the rumen samples 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 the raw sample was aliquoted to a sterile 2.0 mL Safe-Lock tube 125 (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 heat lysed samples were centrifuged (4,500 x G) and then the supernatant was transferred into sterile 1.5 mL tubes

(Fisherbrand, Fisher Scientific, USA). Finally, 130 µL of elution buffer was used to elute the DNA. Quality of the extracted DNA was evaluated using gel electrophoresis.

SSU-rRNA amplicon library preparation and analyses

The V3 region of the eubacterial 16S rRNA gene in the rumen DNA samples were amplified using universal 16S primers 341F and 518R as described by Whiteley et al. 2012 (Whiteley et al., 2012). The V3 region of the 16S rRNA gene was amplified in a 15 µL reaction volume consisting of 1X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life TechnologiesTM, Massachusetts, USA), 1.7 µM of 341F and 0.2 µM of 518R primer, approx. 50 ng of extracted total DNA. PCR 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 were 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.

The V6 region of the archaeal 16S rRNA gene was amplified using extracted total rumen DNA using universal archaeal specific primers 751F and 934R Whiteley et al., 2012 (Whiteley et al., 2012). The PCR amplification was carried out as a 20µL reaction contained 1X of Power SYBR® Green PCR Master Mix (Applied Biosystems by Life TechnologiesTM, Massachusetts, USA) along with 1.25 µM 751F and 0.15 µM 934R primer, approximately 50 ng of rumen DNA. Thermoamplifications were carried out as 95℃ for 10 min for initial denaturation; followed by 30 cycles of 95℃ for 30 s, 52°C for 30 s, and 72℃ for 45 s. Following the PCR, 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.

Statistical analyses

The sequences were analyzed for OTU clustering statistics and alpha diversity on MOTHUR suit (Schloss et al., 2009). Statistical analyses were carried out on OTU abundances adjusted to median rarefication (Cárcer et al., 2011) to avoid wasting data. The taxonomy of each OTU was inferred by comparison to SILVA ribosomal RNA gene database (Quast et al., 2013). Due to the short size PCR fragments and coverage of only one variable region, taxonomic assignments were limited to "class". The OTU abundance matrices were adjust to 75% core, where an OTU was considered to be true only if it was present in 75% of the samples regardless of the sample source (diet). Pairwise oneway-ANOVA was carried out between each taxa group abundance values using relevant module in R-package. Ternary plot on the tripartite distribution of each class was visualized using GGtern extension of GGplot in R. Principle component analyses on the OTU abundances were carried out and the statistical significance of the clustering was obtained by pvCLUST on R (Suzuki and Shimodaira, 2006). Correlation coefficients of Spearman were calculated in R suit using "cor" function and p-values for the correlations were parsed from cor.test function.

Metagenomic library preparation and analyses

The metagenomic libraries were prepared using the Nexterra XT kit according to the manufacturers protocol. The resulting libraries were size selected using the pippin prep (Sage Science) automated gel extraction system to select for fragments between 250 bp and 1200 bp. The resulting fragments were assessed for fragment size distribution using Bioanalyser (Agilent Technologies, USA). The fragments were then quantified using the Qubit 2.0 (Life technologies) and were pooled to obtain equal concentration of each library. The pooled libraries were sequenced using the Illumina 150 bp paired-end sequencing strategy as described by the manufacturer. The resulting reads were demultiplexed and were paired using FLASH pairer. The unpaired-reads were screened for phred quality threshold of 100% >Q20 and 50% >Q30. In order to reduce excessive sequence discarding the reads that did not meet the above phred scores were trimmed to 125, 100 and 80 nts sequentially and checking for quality after each trimming.

Trimming and quality checks were carried out using the tools available on FASTx toolbox (Gordon and Hannon, 2010). As the FASTx tools do not produce a bin with unused sequences a bash script was used to acquire these reads (https://bitbucket.org/SanjayAB/processing_raw_fastq_files/src/900677e33372c29a131b132c9ddecf4ca6219c0a/LoopQualityTrimming.sh?at=master&fileviewer=file-view-default). The paired and quality checked reads were assembled using a single node assembler MEGAHIT (Li et al., 2015). ORFs of final assemblies were called using PRODIGAL (Hyatt et al., 2010). Amino acid sequences from the ORFs were annotated by BLAT alignment (Kent, 2002) with sequences on KEGG database (Kanehisa et al.,

2008). Each of the read match were assigned with their respective Enzyme Commission number. Pairwise ANOVA was carried out on the log-normalized abundance data for each enzyme. The taxonomic information from the KEGG was also collected and the hit statistics for taxonomy placed at the genera level hierarchy.

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Table 4.1. Composition of high- and low-quality forage diets used in this study

Ingredients	High-quality diet	Low-quality diet
Alfalfa hay	45	-
Sorghum silage	30	-
Ground corn stalks	-	75
Modified distillers grains plus solubles	20	20
Supplement		
Fine ground corn	4.501	1.654
Urea	-	1.650
Limestone	-	1.194
Salt	0.300	0.300
Tallow	0.125	0.125
Trace mineral	0.050	0.050
Vitamin A-D-E	0.015	0.015
Rumensin-90	0.009	0.012
Nutrient composition		
Crude protein	14.8	13.9
Neutral detergent fiber	50.5	68.5
Acid detergent fiber	37.1	48.3

Table 4.2. Propionate genes impacting methane production

Code used in figure	Enzyme commission (EC) code	Enzyme name
E-01	6.2.1.17	propionate---CoA ligase
E-02	2.8.3.1	propionate CoA-transferase
E-03	6.2.1.13	acetate---CoA ligase (ADP-forming)
E-04	4.1.1.41	(S)-methylmalonyl-CoA decarboxylase
E-05	5.1.99.1	methylmalonyl-CoA epimerase
E-06	5.4.99.2	methylmalonyl-CoA mutase
E-07	6.2.1.4	succinate---CoA ligase (GDP-forming)
E-08	6.2.1.5	succinate---CoA ligase (ADP-forming)
E-09	1.3.5.1	succinate dehydrogenase
E-10	4.2.1.2	fumarate hydratase
E-11	1.1.1.37	malate dehydrogenase
E-12	1.1.1.82	malate dehydrogenase (NADP+)
E-13	6.4.1.1	pyruvate carboxylase
E-14	4.1.1.32	phosphoenolpyruvate carboxykinase (GTP)
E-15	2.7.1.40	pyruvate kinase
E-16	1.1.1.27	L-lactate dehydrogenase
E-17	4.2.1.54	lactoyl-CoA dehydratase
E-18	2.1.3.1	methylmalonyl-CoA carboxytransferase
E-19	1.3.8.7	medium-chain acyl-CoA dehydrogenase
E-20	1.13.12.4	lactate 2-monooxygenase
E-21	6.2.1.1	acetate---CoA ligase
E-22	2.3.1.54	formate C-acetyltransferase
E-23	2.3.3.10	hydroxymethylglutaryl-CoA synthase
E-24	1.1.1.35	3-hydroxyacyl-CoA dehydrogenase
E-25	1.1.1.157	3-hydroxybutyryl-CoA dehydrogenase
E-26	4.2.1.17	enoyl-CoA hydratase
E-27	1.3.1.44	trans-2-enoyl-CoA reductase (NAD+)
E-28	2.8.3.8	acetate CoA-transferase
E-29	1.2.7.4	anaerobic carbon-monoxide dehydrogenase
E-31	1.2.1.43	formate dehydrogenase (NADP+)
E-32	1.2.1.2	formate dehydrogenase
E-33	4.4.1.19	phosphosulfolactate synthase
E-34	3.1.3.71	2-phosphosulfolactate phosphatase
E-35	4.1.1.79	sulfopyruvate decarboxylase
E-36	2.7.7.68	2-phospho-L-lactate guanylyltransferase
E-37	2.7.8.28	2-phospho-L-lactate transferase
E-38	2.5.1.77	7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase
E-39	6.3.2.31	coenzyme F420-0
E-40	1.12.98.1	coenzyme F420

E-41	1.12.98.2	5,10-methenyltetrahydromethanopterin hydrogenase
E-42	3.5.4.27	methenyltetrahydromethanopterin cyclohydrolase
E-43	1.2.99.5	formylmethanofuran dehydrogenase
E-44	2.3.1.101	formylmethanofuran---tetrahydromethanopterin N-formyltransferase
E-45	1.8.98.1	CoB---CoM heterodisulfide reductase
E-46	2.1.1.86	tetrahydromethanopterin S-methyltransferase
E-47	2.8.4.1	coenzyme-B sulfoethylthiotransferase

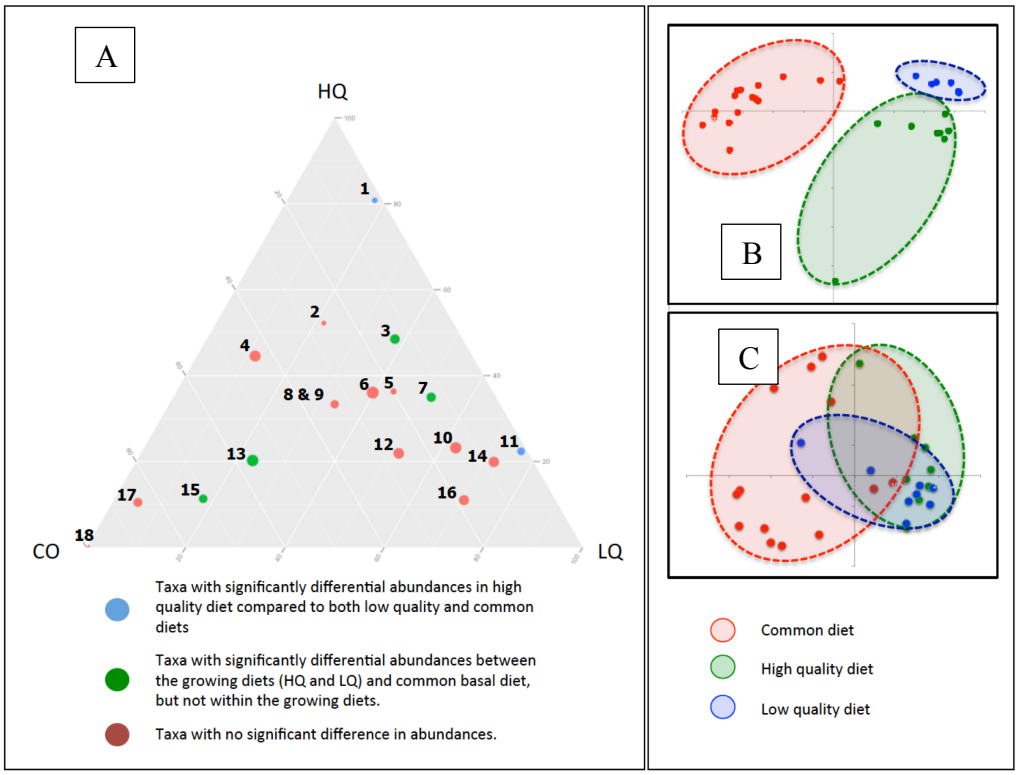


Figure 4.1. Microbial community patterns between the diets. 1A: Ternary plot distribution pattern of each eubacterial class from each of the three diets used. CO- common based diet, HQ – high-quality forage diet (low methane), LQ – low-quality forage diet (high methane). Position of each point denotes the contribution of each diet towards total abundance of each class. 1) Betaproteobacteria, 2) Epsilonproteobacteria, 3) Mollicutes, 4) Gammaproteobacteria, 5) Spirochaetes, 6) Bacteroidia, 7) Candidatus Saccharibacteria , 8) Fibrobacteria, 9) Deltaproteobacteria, 10) Verrucomicrobia, 11) Elusimicrobia, 12) Erysipelotrichi, 13) Clostridia, 14) Alphaproteobacteria, 15) Synergistia, 16) Lentisphaeria, 17) Coriobacteriia and 18) Actinobacteria. 1B and C: Ordination plots of first two coordinates on PCoA. PCoA based on OTU abundances values from IonTorrent sequencing of 16S rRNA gene sequence reads. 1B is based on bacterial V3 region diversity (PC1=42.3%, PC2=11.1%) and 1C shows archaeal V6 (PC1=24.6, PC2=12.2).

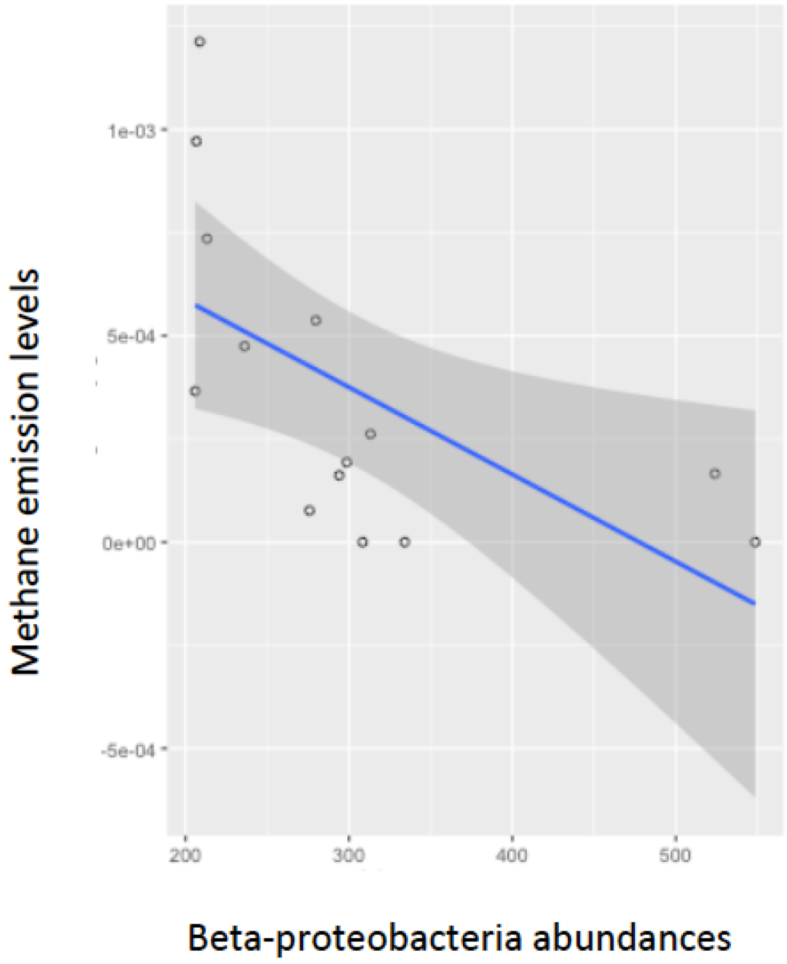


Figure 4.2. Correlation scatter plot visualizing a negative relationship between Betaproteobacteria abundances and methane emission levels.

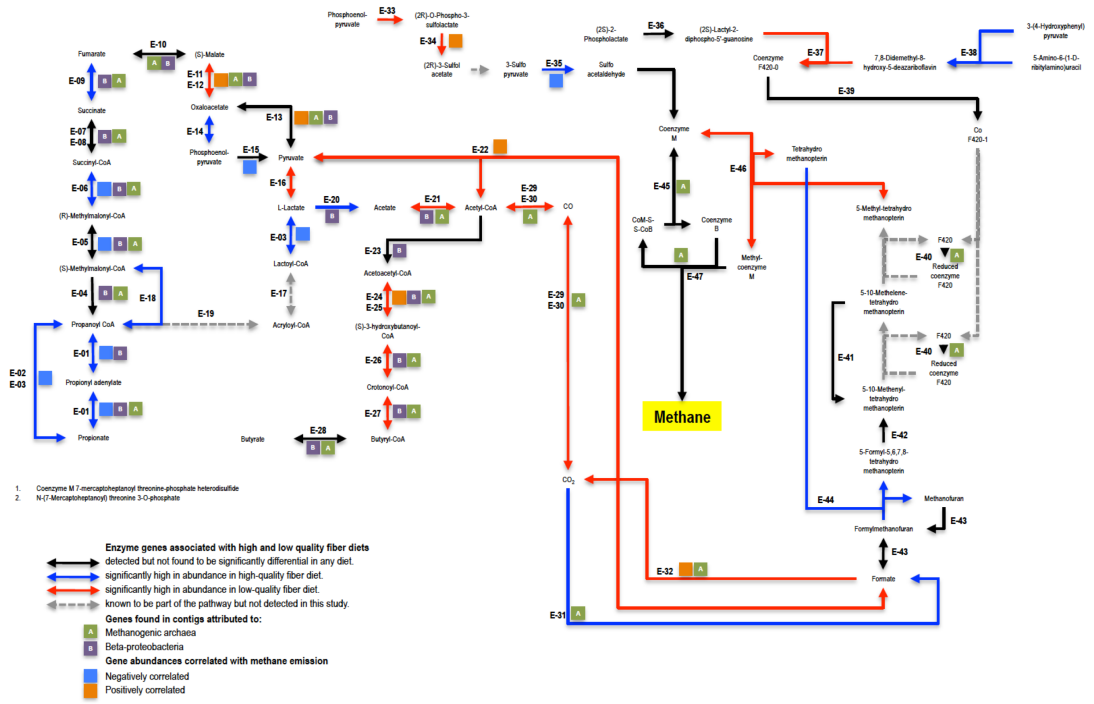


Figure 4.3. Overall syntrophic methanogenesis network in rumen gut environment. The pathways were mapped depending on the detection of genes involved in the pathways from metagenomic reads. Each arrow represents an enzyme gene. Official enzyme commission numbers of the enzymes and their functions are listed in Supplementary Tables S4.1A and 4.1B. 1) Substrates associated genes: Functions that are associated with each of the diets are denoted by color of the arrows. Red: significantly higher in high quality diets; blue: significantly higher in low quality diets; black: detected but did not show any statistically significant difference between diets; and grey dotted line: not detected.

2) Key microbial component associated genes: Genes that are found on contigs from major microbial players in the network. A : from methanogenic archaeal contig, and B : Betaproteobacteria contig.

3) Metabolite associated genes: Enzyme gene abundances that correlated with methane emission levels.

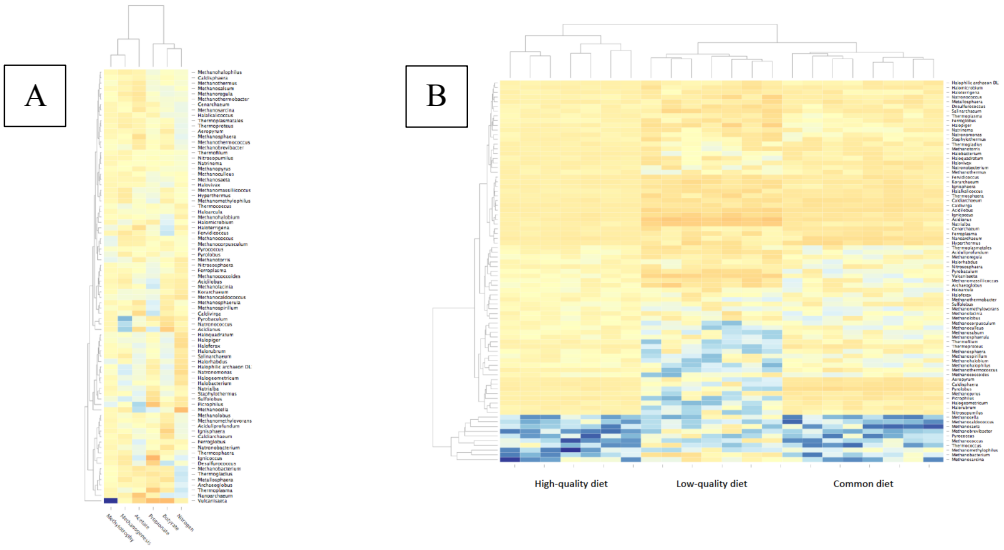


Figure 4.4. Genes found in methanogenic archaeal contigs. Methanogenic contigs were recognized based on the presence of EC 4.4.1.19, 3.1.3.71, 4.1.1.79, 1.8.98.1, 2.1.1.86 and 2.8.4.1 genes. A: Heatmap of gene abundances from each methanogenic archaea that are potentially syntrophically linked to methanogenesis. B: Heatmap of total archaeal taxa gene abundances from each of the sample from the three diets used in this study.

Supplementary Table S4.1A. Gene codes found to be positively correlated to methane

Enzyme commission (EC) code	Pearson correlation coefficient	Pearson correlation p-value
1.-.-.-	0.663438481	0.009689095
1.1.1.1	0.620429177	0.017916518
1.1.1.103	0.571986065	0.032578839
1.1.1.125	0.558251249	0.038011898
1.1.1.141	0.649655375	0.011914975
1.1.1.170	0.566978592	0.034488112
1.1.1.189	0.658785767	0.010401146
1.1.1.261	0.58137417	0.029212144
1.1.1.272	0.565452841	0.03508598
1.1.1.276	0.573720451	0.031936199
1.1.1.281	0.569737104	0.03342637
1.1.1.284	0.547260205	0.042818644
1.1.1.35	0.536936658	0.047725541
1.1.1.37	0.555561974	0.039149322
1.1.1.42	0.568516319	0.033893214
1.1.1.44	0.544435296	0.044122842
1.1.1.5	0.616646704	0.018835182
1.1.1.57	0.577586074	0.030537796
1.1.1.65	0.537945566	0.047228751
1.1.1.77	0.559129576	0.037645742
1.1.1.85	0.558155317	0.038052048
1.1.1.95	0.638111338	0.014064584
1.11.1.12	0.598094867	0.02386713
1.12.99.-	0.569882779	0.033370982
1.13.-.-	0.589604163	0.026480351
1.14.11.27	0.547326046	0.042788587
1.14.13.149	0.658785767	0.010401146
1.17.4.1	0.5391677	0.046632028
1.18.6.1	0.556598364	0.038708057
1.2.1.18	0.584051639	0.028301372
1.2.1.2	0.563153911	0.03600125
1.2.1.21	0.567999482	0.034092307
1.2.1.5	0.54660701	0.043117667
1.2.4.4	0.593232132	0.025338825
1.2.99.-	0.588438526	0.026855154
1.3.1.2	0.61152486	0.020135459
1.3.1.83	0.550438312	0.041385393
1.3.3.3	0.604636465	0.021989777
1.3.98.1	0.649681013	0.011910501

1.3.99.15	0.576346169	0.030981272
1.4.1.9	0.579759347	0.0297719
1.4.3.3	0.537838072	0.047281501
1.4.3.6	0.585087987	0.027954598
1.5.1.20	0.579457449	0.029877429
1.5.3.1	0.53607006	0.048155287
1.5.99.2	0.593764326	0.025174527
1.5.99.8	0.563953223	0.035681044
1.6.1.2	0.542335588	0.045110885
1.6.5.2	0.534206579	0.049088921
1.6.99.3	0.57158246	0.032729753
1.7.-.-	0.628851671	0.015993229
1.7.1.4	0.571505184	0.032758706
1.8.1.9	0.584681492	0.028090236
1.8.4.1	0.545884922	0.043450004
1.8.4.12	0.547716383	0.042610716
1.8.98.-	0.589628557	0.026472549
1.97.-.-	0.572171512	0.032509672
2.-.-.-	0.566174003	0.034802444
2.1.1.10	0.570623129	0.03309054
2.1.1.107	0.608457877	0.020945867
2.1.1.131	0.629317617	0.015891623
2.1.1.141	0.532822393	0.049790891
2.1.1.148	0.698620612	0.005443786
2.1.1.183	0.565419722	0.035099043
2.1.1.192	0.536425164	0.047978852
2.1.1.205	0.550386129	0.041408638
2.1.1.221	0.581646971	0.029118361
2.1.1.43	0.577603525	0.030531588
2.1.1.45	0.583810989	0.028382354
2.1.1.64	0.5559668	0.038976521
2.1.1.79	0.56726184	0.034377956
2.1.3.6	0.56554817	0.035048403
2.2.1.6	0.662361987	0.009850393
2.2.1.7	0.590471013	0.026204169
2.3.1.12	0.572453821	0.032404586
2.3.1.158	0.538225957	0.047091357
2.3.1.183	0.562354437	0.036323638
2.3.1.5	0.55416139	0.039751518
2.3.1.54	0.567377375	0.034333099
2.3.1.88	0.78385827	0.000907404
2.3.1.97	0.591276562	0.025949457
2.4.1.211	0.599532794	0.023444509

2.4.1.226	0.542528531	0.045019425
2.4.2.-	0.539575908	0.046433946
2.4.2.1	0.534933137	0.048723351
2.4.2.11	0.556780047	0.038631081
2.5.1.42	0.620736306	0.017843445
2.5.1.54	0.623725913	0.017143905
2.5.1.56	0.561281668	0.036759576
2.5.1.72	0.557014776	0.038531796
2.5.1.90	0.557189188	0.038458146
2.6.1.11	0.604103858	0.022138326
2.6.1.16	0.586756882	0.027402847
2.6.1.45	0.535261782	0.048558645
2.6.1.50	0.56761064	0.034242665
2.7.1.113	0.629136195	0.015931126
2.7.1.157	0.600623538	0.023127699
2.7.1.16	0.573107254	0.032162321
2.7.1.20	0.560657679	0.037014914
2.7.1.25	0.575284824	0.031364672
2.7.1.28	0.568390348	0.03394166
2.7.1.39	0.591639356	0.025835349
2.7.1.40	0.626730419	0.016462063
2.7.1.48	0.549018539	0.042021257
2.7.1.68	0.583632544	0.028442514
2.7.10.1	0.594847186	0.024842694
2.7.11.11	0.585825607	0.027709721
2.7.3.9	0.600045743	0.023295118
2.7.4.10	0.579440746	0.029883275
2.7.4.7	0.58222013	0.028922054
2.7.4.9	0.587583853	0.027132479
2.7.7.1	0.73279339	0.002870939
2.7.7.23	0.633643017	0.014971689
2.7.7.56	0.545437319	0.04365695
2.7.7.65	0.557560564	0.038301668
2.7.7.n1	0.55695974	0.038555058
2.8.1.-	0.537334462	0.047529209
2.8.1.6	0.550092783	0.041539488
3.1.1.4	0.62152305	0.017657292
3.1.2.6	0.582840049	0.028710844
3.1.21.3	0.552803891	0.040341662
3.1.21.4	0.589740432	0.026436792
3.1.25.-	0.554681815	0.039526967
3.1.25.1	0.647308579	0.012330059
3.1.3.1	0.578350611	0.030266709

3.1.3.18	0.615606145	0.01909405
3.1.3.41	0.53404196	0.049172026
3.1.3.71	0.598644731	0.02370485
3.1.7.-	0.598608614	0.023715483
3.1.7.2	0.589276769	0.026585225
3.2.-.-	0.54834385	0.042325923
3.2.1.14	0.572870996	0.032249759
3.2.1.3	0.6182906	0.018431643
3.2.1.68	0.562205091	0.036384098
3.2.1.7	0.606627059	0.021441223
3.2.1.75	0.548158347	0.042409973
3.2.1.86	0.543508497	0.044556987
3.2.1.94	0.560860609	0.036931732
3.2.1.98	0.56798266	0.034098802
3.3.2.6	0.739233572	0.002518403
3.4.11.4	0.534733846	0.048823427
3.4.14.12	0.540102968	0.0461791
3.4.14.4	0.566645711	0.034617904
3.4.16.2	0.599532794	0.023444509
3.4.17.13	0.549049699	0.042007225
3.4.21.107	0.601342144	0.022920744
3.4.21.108	0.564801046	0.035343711
3.4.21.43	0.534972319	0.048703693
3.4.22.36	0.579264565	0.029944997
3.4.23.28	0.658785767	0.010401146
3.4.23.3	0.593764326	0.025174527
3.4.24.15	0.591668549	0.025826184
3.4.24.29	0.598668924	0.023697729
3.4.99.-	0.533192318	0.049602579
3.5.1.10	0.566568736	0.034647969
3.5.1.108	0.584378709	0.028191586
3.5.1.14	0.561036902	0.03685958
3.5.1.2	0.534328575	0.049027399
3.5.1.4	0.605735977	0.02168549
3.5.2.17	0.627562443	0.016276942
3.5.2.6	0.542742548	0.044918134
3.5.2.7	0.539880249	0.046286665
3.5.3.1	0.587998774	0.026997579
3.5.3.18	0.608261256	0.020998648
3.5.4.5	0.56837737	0.033946655
3.5.4.6	0.593764326	0.025174527
3.6.1.27	0.534086257	0.049149653
3.6.1.6	0.554832374	0.039462178

3.6.3.17	0.585638579	0.027771659
3.6.3.2	0.586065552	0.027630411
3.6.3.24	0.59111077	0.026001727
3.6.3.28	0.627656204	0.01625618
3.6.3.33	0.572007438	0.032570862
3.6.3.43	0.545353568	0.043695752
3.6.4.12	0.596551028	0.024327223
4.1.1.18	0.550480224	0.04136673
4.1.1.21	0.567708188	0.034204899
4.1.1.36	0.584626957	0.02810847
4.1.1.48	0.580325878	0.029574618
4.1.2.22	0.544719135	0.043990503
4.1.2.5	0.570834446	0.033010816
4.1.2.9	0.564263286	0.035557401
4.1.3.38	0.557638437	0.038268916
4.1.3.39	0.616082932	0.018975104
4.1.99.-	0.594772422	0.024865499
4.1.99.11	0.658785767	0.010401146
4.2.1.109	0.658785767	0.010401146
4.2.1.22	0.593677208	0.025201367
4.2.1.46	0.545314754	0.043713743
4.2.1.76	0.658785767	0.010401146
4.2.1.79	0.573053554	0.032182179
4.2.3.12	0.565346904	0.035127775
4.2.99.20	0.608864929	0.020836916
4.3.1.1	0.641005082	0.01349994
4.3.1.14	0.592678957	0.025510451
4.3.1.19	0.582390538	0.02886388
4.3.1.4	0.571443164	0.032781957
4.4.1.8	0.574655627	0.031593621
5.1.1.1	0.583642681	0.028439094
5.1.3.-	0.582191774	0.028931742
5.1.3.6	0.692226436	0.006079474
5.1.3.n2	0.650833673	0.011710654
5.3.1.26	0.576165367	0.031046338
5.3.99.6	0.543147754	0.044726813
5.4.2.1	0.615230563	0.019188145
5.4.2.4	0.563755865	0.03575991
5.4.2.7	0.604821857	0.021938246
5.4.2.9	0.544865166	0.04392253
5.4.99.23	0.554503907	0.039603624
5.4.99.45	0.614100114	0.019473476
6.-.-.-	0.613678692	0.019580662

6.1.1.2	0.680238478	0.00742585
6.3.2.19	0.576750849	0.030836008
6.3.2.25	0.535038254	0.048670626
6.3.2.30	0.578312398	0.030280216
6.3.4.21	0.625029886	0.016845397
6.3.4.3	0.575558423	0.031265502
6.3.4.4	0.603806305	0.022221644
6.4.1.1	0.575367055	0.031334841
6.5.1.-	0.542782954	0.044899029

Supplementary Table S4.1B. Gene codes found to be negatively correlated to methane

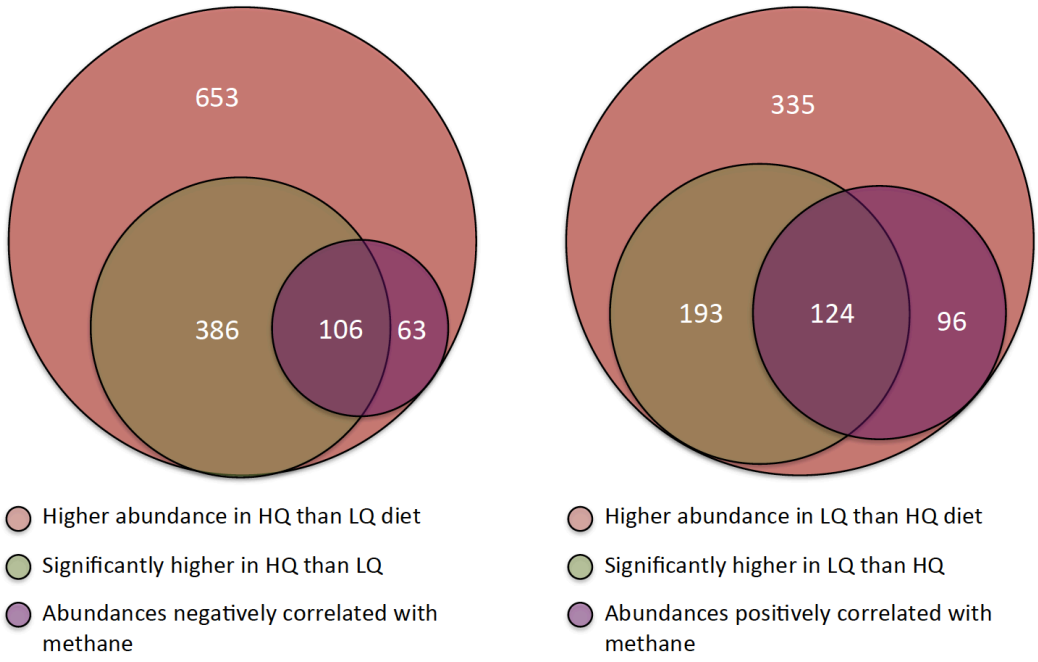
Enzyme commission (EC) code	Pearson correlation coefficient	Pearson correlation p-value
1.1.1.14	-0.540195725	0.046134356
1.1.1.154	-0.660508741	0.010132919
1.1.1.169	-0.630248032	0.015690203
1.1.1.18	-0.539859369	0.046296759
1.1.1.244	-0.598332019	0.023797038
1.1.1.251	-0.539574473	0.046434641
1.1.1.271	-0.542778179	0.044901286
1.1.1.274	-0.553574088	0.04000605
1.1.1.56	-0.558164174	0.03804834
1.1.1.69	-0.579648961	0.029810453
1.1.1.81	-0.545975436	0.043408243
1.1.2.4	-0.559153724	0.037635712
1.1.3.41	-0.626274605	0.016564155
1.1.5.2	-0.585321794	0.027876804
1.11.1.9	-0.560677367	0.037006838
1.13.11.-	-0.590060702	0.026334626
1.14.-.-	-0.585540117	0.027804308
1.18.1.6	-0.567558732	0.034262774
1.2.1.4	-0.544792278	0.043956448
1.2.1.41	-0.552464574	0.040490173
1.2.4.2	-0.538413361	0.04699969
1.3.1.14	-0.565047068	0.035246266
1.3.1.38	-0.537315885	0.047538364
1.3.1.6	-0.593686737	0.02519843
1.4.1.-	-0.550767953	0.041238774
1.4.1.2	-0.546212639	0.043298942
1.4.3.19	-0.587174051	0.027266208
1.4.3.5	-0.544968901	0.043874291
1.5.1.2	-0.536374124	0.048004182
1.5.1.34	-0.54210122	0.045222163
1.5.1.7	-0.548561312	0.042227548
1.7.1.1	-0.636789503	0.01432843
1.7.1.7	-0.625373038	0.016767503
1.7.2.2	-0.550260123	0.041464807
1.7.2.5	-0.572308804	0.032458535
1.7.99.4	-0.562890279	0.036107325
1.8.4.9	-0.536614524	0.04788496
2.1.1.132	-0.546369232	0.043226896

2.1.1.51	-0.600949451	0.023033665
2.1.3.9	-0.534124302	0.049130444
2.3.1.117	-0.602704987	0.022532082
2.3.1.179	-0.551587121	0.040876075
2.3.1.28	-0.600524479	0.023156338
2.3.1.79	-0.584131248	0.028274621
2.3.1.87	-0.565057162	0.035242272
2.3.2.-	-0.591285127	0.025946758
2.3.2.2	-0.579394529	0.029899457
2.3.3.-	-0.565587691	0.035032833
2.4.1.134	-0.566072173	0.034842377
2.4.1.141	-0.560550935	0.037058725
2.4.1.144	-0.593751397	0.025178508
2.4.1.207	-0.579728909	0.029782527
2.4.1.212	-0.610401489	0.020429494
2.4.1.25	-0.539656765	0.046394783
2.4.2.10	-0.542643759	0.044964868
2.4.2.8	-0.640872061	0.013525509
2.5.1.15	-0.535631051	0.048374064
2.5.1.17	-0.600294356	0.02322297
2.5.1.29	-0.568962871	0.03372189
2.5.1.3	-0.562742861	0.036166741
2.5.1.48	-0.542962257	0.044814322
2.5.1.64	-0.533796477	0.049296143
2.5.1.78	-0.550216357	0.041484329
2.6.1.21	-0.562969349	0.036075486
2.6.1.44	-0.607717604	0.021145108
2.6.1.82	-0.568755589	0.033801336
2.7.1.145	-0.63390511	0.014917282
2.7.1.2	-0.621005973	0.017779472
2.7.1.21	-0.540548856	0.045964301
2.7.1.35	-0.551934827	0.040722833
2.7.1.52	-0.577809563	0.030458366
2.7.10.-	-0.624141396	0.017048359
2.7.11.-	-0.599674394	0.023403198
2.7.11.14	-0.64826199	0.012160111
2.7.2.7	-0.558671982	0.037836177
2.7.4.22	-0.53919264	0.046619909
2.7.4.25	-0.556028014	0.03895044
2.7.4.6	-0.546585067	0.043127738
2.7.6.2	-0.535726532	0.04832642
2.7.7.2	-0.57935394	0.029913674
2.7.7.46	-0.56358029	0.035830178

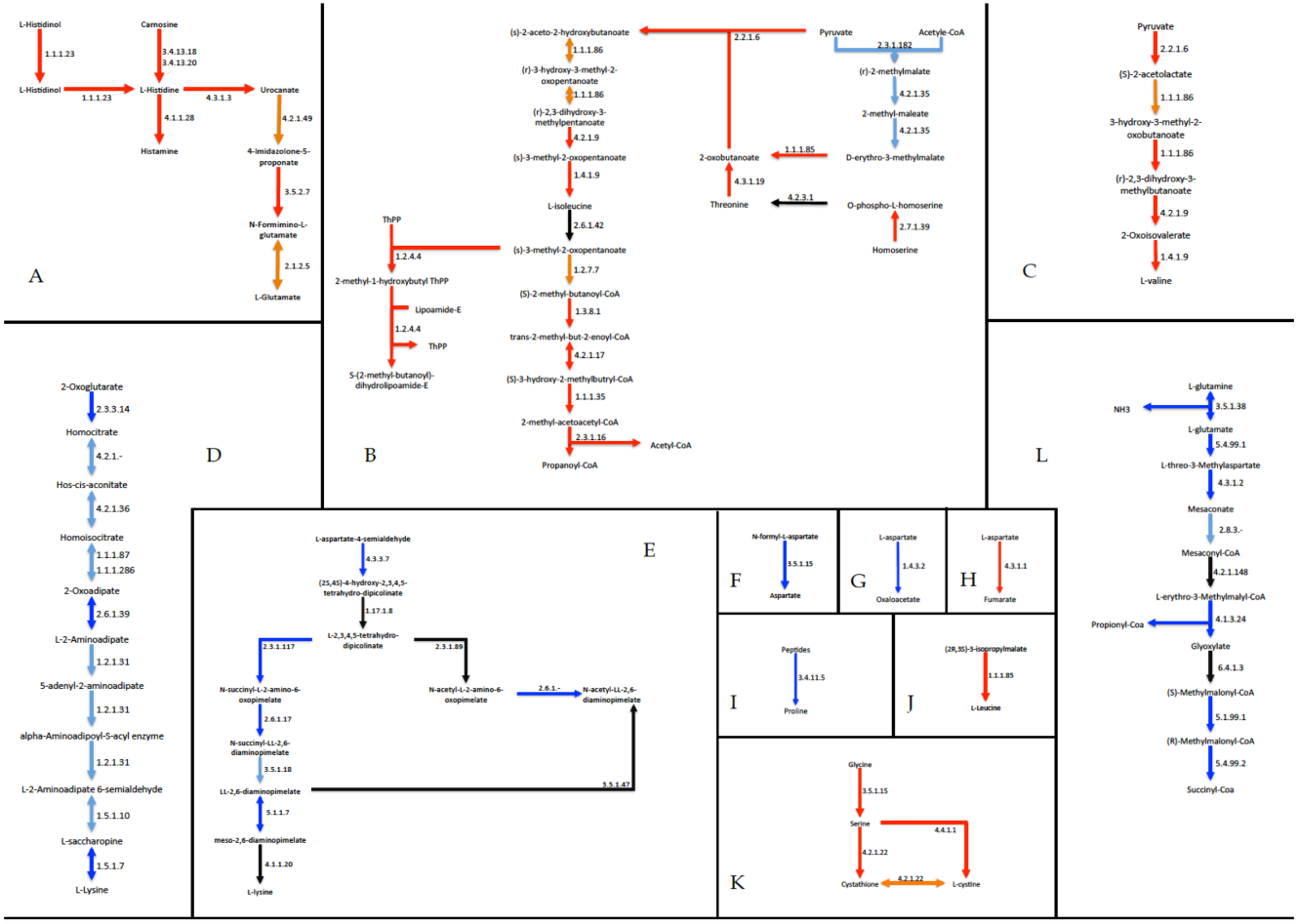
2.7.7.48	-0.547665831	0.042633721
2.7.8.15	-0.583086143	0.028627318
2.7.8.25	-0.579332869	0.029921057
2.7.8.5	-0.575884624	0.03114757
2.7.8.8	-0.582755973	0.028739421
2.7.9.3	-0.573689555	0.031947564
2.8.1.1	-0.535764558	0.048307455
2.8.1.5	-0.55697193	0.038549905
2.8.3.1	-0.72835561	0.003135708
2.8.3.10	-0.623566338	0.017180709
2.8.3.12	-0.624790746	0.016899844
3.1.1.20	-0.54514093	0.04379438
3.1.1.3	-0.538178796	0.047114446
3.1.2.14	-0.590120498	0.026315584
3.1.26.12	-0.533316848	0.049539303
3.1.3.25	-0.5532454	0.040149023
3.1.3.3	-0.556372645	0.038803848
3.1.31.1	-0.551202276	0.041046178
3.1.4.4	-0.559915428	0.037320347
3.2.1.136	-0.568288783	0.033980758
3.2.1.39	-0.541285096	0.045611228
3.2.1.89	-0.641526203	0.013400126
3.2.2.-	-0.554053436	0.039798215
3.2.2.24	-0.534589711	0.048895899
3.4.11.5	-0.602862273	0.022487548
3.4.19.14	-0.623785454	0.017130188
3.4.21.26	-0.594462528	0.024960191
3.4.21.4	-0.645995075	0.012567169
3.4.21.62	-0.556793377	0.038625438
3.4.24.28	-0.599289567	0.023515598
3.5.1.11	-0.683542693	0.007033698
3.5.1.15	-0.593113252	0.025375635
3.5.1.47	-0.651726322	0.011557664
3.5.1.83	-0.627532043	0.016283678
3.5.1.89	-0.539566442	0.046438533
3.5.1.91	-0.550419752	0.041393659
3.5.1.98	-0.538082735	0.047161501
3.5.3.6	-0.5992369	0.023531012
3.5.3.9	-0.545570542	0.04359528
3.5.4.10	-0.607596618	0.021177806
3.5.99.3	-0.553311863	0.040120083
3.6.1.29	-0.608473199	0.020941758
3.6.5.1	-0.588664821	0.026782082

3.6.5.3	-0.542135139	0.045206046
3.7.1.9	-0.54603008	0.043383045
4.1.1.1	-0.552533235	0.040460089
4.1.1.39	-0.53352056	0.04943592
4.1.1.5	-0.649019823	0.012026312
4.1.1.79	-0.644697625	0.012804796
4.1.1.81	-0.568600938	0.0338607
4.1.2.17	-0.571916602	0.032604775
4.2.-.-	-0.658104584	0.010508684
4.2.1.1	-0.567786516	0.034174597
4.2.1.44	-0.723800678	0.003427097
4.2.1.80	-0.602990998	0.02245115
4.2.1.91	-0.587431488	0.027182142
4.2.2.-	-0.56146198	0.036686035
4.2.3.3	-0.611401835	0.020167503
4.3.1.2	-0.562020855	0.036458784
4.4.1.5	-0.56877469	0.033794009
4.99.1.-	-0.581431562	0.029192395
5.1.1.-	-0.600095686	0.023280611
5.1.1.11	-0.549602064	0.041759056
5.1.1.7	-0.578896208	0.030074351
5.1.3.9	-0.552753795	0.040363562
5.1.99.1	-0.588438771	0.026855075
5.3.3.1	-0.593954144	0.02511612
5.3.99.-	-0.600762089	0.023087688
5.4.1.-	-0.545631901	0.043566898
5.4.1.2	-0.610332677	0.02044761
5.4.99.18	-0.592555341	0.025548923
5.4.99.2	-0.58531149	0.027880229
5.4.99.4	-0.56685463	0.034536404
5.5.1.1	-0.540734986	0.045874852
5.5.1.7	-0.535771375	0.048304056
5.99.1.4	-0.553123835	0.040201997
6.1.1.-	-0.619672919	0.018097415
6.1.1.14	-0.620047309	0.018007689
6.1.1.18	-0.636285305	0.014430061
6.2.1.13	-0.579035737	0.030025304
6.2.1.17	-0.578862469	0.03008622
6.2.1.22	-0.56346989	0.035874415
6.2.1.27	-0.540044825	0.046207164
6.3.1.10	-0.566270658	0.034764572
6.3.2.12	-0.62421322	0.017031883
6.3.3.-	-0.588514621	0.026830566

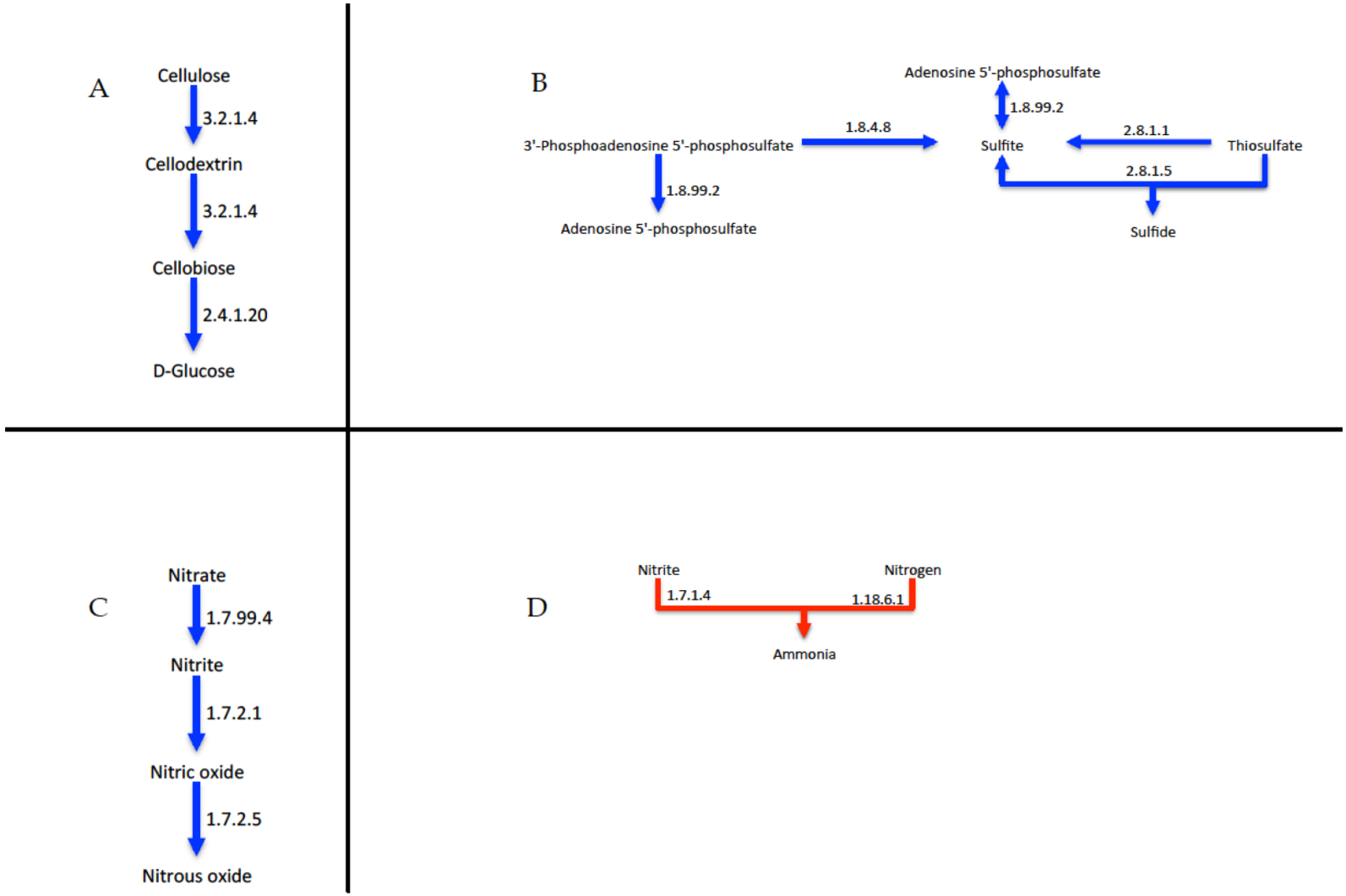
6.3.5.11	-0.628911021	0.01598026
6.4.-.-	-0.584507281	0.028148516



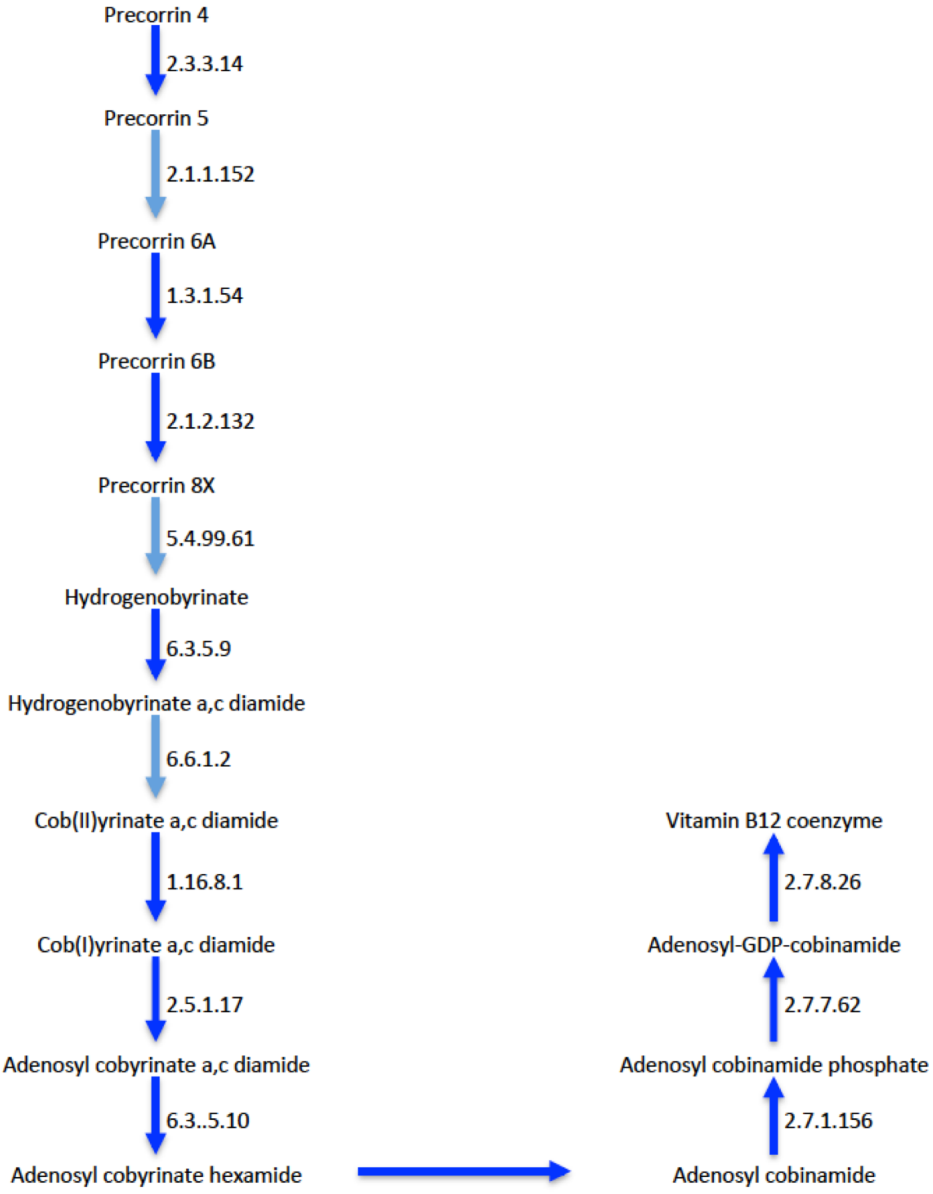
Supplemental Figure S4.1. Venn diagrams indicating abundance levels between HQ and LQ diets in negatively and positively correlated with methane.



Supplemental Figure S4.2. Pathways that were associated with low or high methane-producing diets that are involved in amino acid metabolism.



Supplemental Figure S4.3. Other pathways that are that either negatively or positively associated in methane-producing diets.



Supplemental Figure S4.4. Pathway indicating the route of conversion of precorrin 4 to vitamin B12 coenzyme.

DISSERTATION CONCLUSION

Resources for livestock production are becoming increasingly limited. Producing meat and milk products to sustain a growing population is a current challenge for the beef and dairy cow industries. One strategy to investigate to increase the supply of meat and milk is the rumen microbiome. The functional gene content within the rumen microbiome still remains elusive; however, measures are being taken to identify genes to improve energy harvest. Feed efficiency is one measure that predicts how the animal converts consumed feed to tissue or milk effectively. Traditional measures for increasing animal efficiency, such as intake or gain, while effective, do not account for the heritability of the microbiome. Understanding the functionality of the rumen microbiome and the impact on efficiency and measure may provide avenues to pursue to improve overall animal health and performance.

Replacing forages with nonforage fiber sources, maintained production measures such as milk yield and intake with an increase in milk protein was observed. A decrease in CH_4 output was also observed. In areas of the world where water as a resource is scarce, including nonforage fiber sources can serve to maintain performance without compromising body reserves and water sources.

Observing a decrease in methane production indicates a shift in the microbial community from less fibrolytic to more amylolytic microbes. However, community shifts may be more focused on abundances and substrates produced. Rumen methanogens are low abundance microbes with little diversity present. Methanogens, can be impervious to dietary changes, utilize bacterial fermentation products, thereby being controlled by the bacterial population. However, these dietary changes can impart changes in fermentation

products, providing mitigation substrates such as hydrogen sinks or competition for the reduced number of substrates available.

The mechanisms of methane production in the rumen are complex and remain vague. Dietary factors such as forage quality elicit changes in methane production. Rumen methanogens are known to be the last group to use substrates for metabolic processes, making this group dependent on a syntrophic digestion pyramid. In the diet high-quality, the propionate pathway in enhanced, diverting hydrogen from methanogenesis. Betaproteobacteria are implicated in reduction of methanogenesis through syntrophy in the high-quality diet, with genes found in the propionate pathway.

Inhibiting methane production in ruminants may impact performance leading towards more energy to go to tissue gain or milk production. Methane reduction can occur by using diet to provide competition of substrates, diversion into other energy favoring molecules, targeting specific taxa to increase in abundance, and to overall decrease substrates available for methanogenesis. The rumen microbiome is still virtually a black box of genomic capability, and understanding how it is affected by fermentation substrates may provide insight into improving animal health and performance.