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Identification and Characterization of Three Novel Rumen Bacterial Species in Sheep Fed a Concentrate Diet

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IDENTIFICATION AND CHARACTERIZATION OF THREE NOVEL RUMEN BACTERIAL SPECIES IN SHEEP FED A CONCENTRATE DIET

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BCTRC	Boneless closely trimmed retail cuts
BLAST	Basic local alignment search tool
BW	Body weight
CAZ	Carbohydrate-active enzyme
DNA	Deoxyribonucleic acid
GH	Glycoside hydrolase
HCW	Hot carcass weight
KEGG	Kyoto encyclopedia of genes and genomes
NCBI	National center for biotechnology information
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
RAST	Rapid annotation using subsystem technology
RDP	Ribosomal database project
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acid
SDSU	South Dakota State University
VFA	Volatile fatty acid

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF THREE NOVEL RUMEN BACTERIAL SPECIES IN SHEEP FED A CONCENTRATE DIET

REID FREDERICK ANEMA

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Sheep production in the United States is a popular venture for both small farmers and large enterprise owners, as there is a market for end products such as wool and meat. One major factor of raising sheep is nutrition as an adequate diet will allow an animal to reach its full genetic potential for growth. With that in mind, the composition of the diet must be formulated to benefit the animal. Ruminants have the ability to use a variety of plants that are often inedible for humans to create products that humans can consume. These varying feedstuffs, whether they include forages like hay or include grains like corn, can have an impact on the composition of the rumen microbial community. In an effort to gain more insight into the effects of a concentrate-based diet or a forage-based diet difference on the composition and metabolic activities of key rumen microbial species, rumen bacterial composition was determined by PCR amplification of the 16S rRNA V1-V3 region, followed by Illumina MiSeq 2X300 sequencing. In terms of composition of the ruminal bacterial community, different OTUs were found to be present in a higher abundance depending on what diet was fed, with a prime example of OTU RA1-01223 averaging a relative abundance of 6.78% in concentrate-fed samples as compared to the forage-based average of 1.56%. To further unravel metabolic potential of select OTUs from the samples, metagenomic analysis was conducted. The rumen fluid samples with the highest abundance of OTUs RA1-01223, RA1-00032, and RA1-04216

were respectively used as representatives for metagenomic investigations to assemble the genomes of their corresponding bacterial species. Purified microbial genomic DNA was used as a template for ‘shotgun’ sequencing with an Illumina Miseq (2 x 250) platform. Key pathways for vitamin B5 production, as well as ethanol metabolism were found in contigs produced from these OTUs, hinting at possible metabolic potential of novel bacterial species. Together, composition and characterization of sheep rumen bacteria fed different diets could open the door for manipulation of the gut to further improve efficiency and growth of sheep.

CHAPTER 1: LITERATURE REVIEW

1.1. THE SHEEP INDUSTRY AND ITS CONTRIBUTION TO THE US AGRICULTURE SECTOR

1.1.1. The United States Agricultural Sector

Within the United States, the agriculture and food related industries account for $1.53 trillion of the country's gross domestic product. Of those industries, farms, forestry, fishing, food manufacturing, food services, and textile manufacturing make up the majority. Exports of agricultural goods have grown from 2001 to 2023, with total exports of items such as meat, grains, oilseeds, and sugar valued at $178.7 billion in 2023, whereas imports have also grown and are slightly higher. The United States exports a majority of its goods to East Asia and other countries in North America, while major import countries include those in the European Union, as well as Mexico, Canada, and some other countries in South America. With changing markets, production of different agricultural products varies with location. Crops are raised in a majority of midwestern states, as well as in California, Florida, and Washington for specialized crops such as apples and oranges. Livestock follows similar patterns, as cattle production is based in the southwestern and midwestern states such as Nebraska and Texas, whereas other livestock such as hogs and poultry are large commodities in Iowa and Georgia, respectively (USDA-ERS, 2023).

The livestock sector in the United States dominates the world production of grain-fed beef, as well as produces the largest amount of poultry meat. With such large impacts on domestic production of goods and international trade, the livestock industry is an invaluable piece of the agricultural sector of the United States. The livestock sector is

continuing to grow, and is shifting towards larger enterprises to decrease costs and increase efficiency. Advances in technology focused on production and specialization in a specific commodities also lend themselves to increasing production in the industry (MacDonald and McBride, 2009). But as farms are increasing in size, specialized operators still continue to function as they find specific niches that are a good fit for what is produced, allowing smaller operations to survive (Macdonald & McBride, 2009).

1.1.2. The Sheep Industry in the United States

Sheep in the United States are seen as a dual-purpose species of livestock. Not only do they provide wool, but are also raised to produce meat, which can be sold as lamb or mutton. The number of sheep peaked at approximately 51 million head in 1884, but has since declined to almost 5 million head as of 2016 (USDA, 2020). While generally enjoyed in other countries such as in Europe and the Middle East, the strong flavors and odors that lamb and mutton can produce are often a deterrent for North Americans and younger consumers. (Rubino et al., 1999). Even with that hurdle, small-scale operations in the United States have seen an increase in the industry from 2007 to 2017, with their numbers seeing an increase of almost 20,000 operations (USDA, 2020) that were able to be counted, as surveying operations became more precise and were able to find small-scale operations that were family operated. The United States does import lamb from other countries, but relies on exports to minimize mutton waste as countries like Mexico have a higher demand for mutton. Many lamb consumers in the United States prefer high quality cuts, such as the loin or the leg, and excess meat often is

exported to Middle Eastern countries or used in the pet food industry (USDA-ERS, 2023).

Although meat makes up the majority of a sheep carcass, wool is considered the major product of the animal. The need for wool for textile use increased sharply in the United States during the Civil War, with supply chains of cotton shut down by the armed conflicts. Since then, the need for wool for use in milling and textile production has fluctuated, decreasing after the demand from the Civil War dissipated, then again increasing during World War I and World War II from stimulated trade and need for wool (Carlson, 1996). As wool products continue to maintain desirable qualities such as temperature regulation, flame resistance, and odor resistance, wool remains a viable industry (ASI, 2019). Presently, a number of conditions are very favorable, such as more and more consumers looking for domestically produced products, and policies such as the 'Berry Amendment' that dictate that the United States military cannot purchase textiles that are not produced in the US.

1.1.3. The Sheep Industry Around the World

While not as large as in the United States, the sheep industry is quite impactful in other countries like Australia, China, and New Zealand. In Australia, production of sheep has shifted more towards lamb production for meat and export much like the United States due to competition with synthetic fibers (East & Foreman, 2011). As of 2006, sheep and wool industries have accounted for 13% of total Australian agricultural exports alone (Curtis and Dolling, 2006). China has had steady increases in livestock production since 1980, which has included an increase in the amount of meat produced from sheep to

help meet the demand of their growing population (Bai et al., 2018). New Zealand leads the world in the production and export of lamb and mutton, and is the third largest producer of wool. The main targets for export for meat is the European Union and for wool, China. Sheep production in New Zealand relies on grazing paddocks supplying forage for livestock, as sheep and beef production use 9.7 million hectares of agricultural land, or 66% of all agricultural or forestry land available in New Zealand. Additionally, sheep and beef are often raised together by producers, instead of having two different dedicated production systems for each species, which is more commonly referred to as multi-species grazing (Morris, 2013).

1.1.4. Management of Sheep Operations

In terms of management, production tends to be similar no matter where the sheep are located, as they are typically raised one of two ways: large-scale or small-scale operations. Large operations focus on extensive grazing practices, whereas smaller farms follow more industrial production systems (Thorne et al., 2021). In the US, large grazing operations typically reside in the western half of the country, utilizing both private and federal lands for grazing. As the passage of different laws has restricted the ability of ranchers to graze public lands, the majority of acres available for grazing are privately owned (Kolodney et al., 2021). Smaller farms focus more on targeted feeding strategies that are more common in other species, such as beef and pork, as they allow the rancher to have a little more control over the diets presented to the animals, in turn allowing for more focus on increased performance for either wool, meat, or both. Both styles of management have their advantages and disadvantages. Intensive feeding uses less space

as it does not require large pastures for the animals to graze, but it can require more manual labor, as the producer needs to mix rations, or it can potentially impair performance as a result of higher risk of illnesses due to diet (Bowen et al., 2006). Extensive grazing, on the other hand, lets animals have more options for selecting their own feed, but securing sufficient land for grazing and ensuring dedicated growth and nutrient acquisition can be challenging. Additionally, inefficiencies can be seen in cases of arid environments or predator attacks, which are less likely to affect intensive feeding systems (da Silva et al., 2016; Howery and DeLiberto, 2004; Mosley et al., 2020). Regardless of the advantages and disadvantages of each management practice, nutrition is still considered the most important aspect of livestock production to meet yield and product quality goals, as well as optimal animal health (Delgado-Pertíñez and Horcada, 2021).

1.2. Dietary Strategies for Sheep Production

1.2.1. Overview of Ruminant Diets

Diet is an integral part of raising livestock, not only in ensuring sustenance but also growth to meet production goals. Much like the old saying goes, “You can lead a horse to water, but you can’t make it drink”, ruminants can have a natural preference as to what they consume and when they consume, which can vary based on factors such as taste, nutritional needs, quality of feed, or time of day (Rutter, 2006; Charles et al., 2012). Parsons et al (1994) found that sheep could switch diet preferences between grass and white clover to possibly balance out any deficiencies in their diet. They also found that

sheep followed a diurnal pattern of ingesting forage, which is useful in determining length of rumination to avoid grazing at night.

1.2.2. Grass vs Grain Diets in Sheep Production

Producers have the ability to choose what feed source their animals receive on a daily basis in certain operations. Diets for ruminants can include different forms of starch, typically fed as a grain, but they require inclusion of sufficient fiber in the form of grasses and hay to promote positive rumen function. Grass diets are centered on providing material for the rumen of an animal to ferment, and they are high in structural polysaccharides such as cellulose, hemicellulose, and pectin, whereas grain diets provide carbohydrates that are more easily digestible, like starch and saccharides. Inclusion of grain into the diet has a positive impact on functions critical for ruminant production, such as milk production and growth rates (McGrath et al., 2018). However, downsides for high-energy diets with ingredients such as grain include a higher risk for ruminal disorders such as acidosis. Many of the diets that are high in concentrate-based ingredients such as corn, wheat, or oats are termed as “hot” diets. Both diet types can be used in feeding sheep, but each impacts the composition and function of the rumen microbiome differently.

1.2.3. Transitioning Diets

As a strategy to maximize feed efficiency and increases in weight, concentrate diets are often used in a feedlot setting. However, previous research has showed an alteration in rumen morphology, gene expression, and metabolism, as well as many

chemical changes, that result from the switch from forage to concentrate (Brown et al., 2006; Fernando et al., 2010; Kuzinski et al., 2011; Lyle et al., 1981, Sun et al., 2010, Xu et al., 2018, Mackie & Gilchrist, 1979). For example, in Angus heifers fed a forage diet before switching to a concentrate diet, Petri et al (2013) were able to identify specific species of bacteria that increased in abundance as the animals went through a period of acidosis due to the abrupt diet change. With these changes in the diet, different sources of substrates for the microorganisms in the rumen are made available, for which the animal has to adapt to. Consequently, many traditional feedlots or intensive feeding systems use a step-up program to acclimate cattle or sheep to a diet that is high in concentrate when animals have been recently weaned or used to a diet that consists mostly of roughage.

1.2.4. Structure of Starch in Grains and Corn

When bacteria ferment starch in the rumen, there is an increase in the ratio of propionic acid, or propionate, to other SCFAs as compared to when cellulose is fermented (Ørskov, 1986). While propionate is a substrate that can be utilized in the liver in the process of making glucose, excess propionate can lead to a decrease in ruminal pH and can also be deposited into the subcutaneous fat of sheep which leads to soft fat that is unattractive to both processors and consumers (Berthelot et al., 2001). High starch levels can also lead to an increase in lactate production from start-utilizing bacteria, which can result in severe acidosis if pH levels drop too low (Hua et al., 2022). Starch is synthesized as two types of glucose polymers, amylose and amylopectin. These polymers are composed of α linkages, and they are used by plants as energy storage. Glucose monomers are connected through α 1-4 bonds to form amylose. Branching chains, or α 1-

6 linkages, allow for the formation of amylopectin. The two polymers allow for the formation of a branch-like structure of starch. While starch is easily digested in the rumen, bacteria can face problems in trying to access the carbohydrates found in plants. Whole grains, such as corn kernels, have a higher resistance to bacterial degradation due to their intact pericarp which resists bacterial attachment (McAllister et al., 1994). To combat this problem, different grain processing practices have been researched, including dry rolling, steam flaking, steam rolling, grinding, or high moisture, which have all been found to alter the rumen digestibility of starch (Huntington, 1997). These processing changes allow for higher substrate availability to rumen microorganisms, as well as more absorption farther down in the digestive tract, such as in the small intestine. Although increasing digestibility and availability of starch in the rumen allows for more bacterial fermentation and increased animal performance, excess short chain fatty acids produced from more available starch can lead to problems, as previously discussed.

1.2.5. Plant Structural Polysaccharides

On the other hand, other carbohydrates that are used for plant cell structure rather than storage are found more in forage-based feeds such as alfalfa and hay, and they are digested by different bacteria than the ones that utilize starch. One major structural polysaccharide is cellulose, which accounts for 20-40% of dry matter for most plants (Van Soest, 1994). Unlike starch, cellulose is made of β linkages, which requires hydrolytic enzymes that are expressed by a certain group of rumen microorganisms. Cellulose is generally more difficult to degrade because it is insoluble and intricately embedded in the plant cell wall. In terms of volatile fatty acids production, diets that

feature a majority of forage result in a higher concentration of acetate as compared to propionate (Murphy et al., 1982). Thus, much like with grains, the processing of the feed can have an impact on what is ultimately degraded and produced within the rumen (Janssen, 2010).

1.3. Rumen Physiology and Function

1.3.1. The Role of Rumen in Digestion

Ruminants are aptly named to describe their main digestive action, rumination. Although many species of animals start the digestion process with the presence of amylase in their saliva, ruminants are not afforded that luxury (McDougall, 1948). When a ruminant eats, it sends the feedstuffs to its stomach, which is comprised of four compartments, the reticulum, the rumen, the omasum, and finally the abomasum. The rumen is the main site of feed fermentation, while the reticulum is an intermediate compartment between the oesophagus and the rumen. During rumination, the 'cud', which consists of solid feed or plant biomass, is passed back from the rumen to the reticulum, then returned to the mouth for more mastication. This action reduces particle size in the cud, which is swallowed again for further fermentation in the rumen. Feed particles that have been sufficiently digested ultimately travel further downstream to the omasum, abomasum, and finally, the intestines. The omasum functions as an absorption center for fluid from the rumen, as well as moving digesta to the abomasum while also decreasing particle size (Backer et al., 1963). The abomasum is the location of enzymatic and acidic hydrolysis of proteins that have escaped the rumen and functions similar to that of a monogastric stomach. The intestines are split into the small intestine and large

intestine. The function of the small intestine in ruminants is to digest any remaining starch and lipids that were not fermented in the rumen, as well as serve as an absorption site for amino acids that result in the breakdown of protein or microbial species that left the previous compartments. Finally, the large intestine serves as a final fermentation location for structural carbohydrates, as well as absorption of water and other compounds that are important for animal function.

1.3.2. Rumen Development

The rumen functions as a fermentation center for material consumed by the animal. While the rumen is the main location for fermentation in mature animals, it is mostly undeveloped in the early postnatal stages. To avoid fermentation of milk during nursing, young ruminants form a structure called the ‘esophageal groove’ that bypasses both the rumen and reticulum. Thus, for the rumen to develop into a digestive organ, it requires stimulation by solid feed and its inoculation by microorganisms, which will allow an increase in cellular and tissue mass to occur (Diao et al., 2019). The continued ingestion of solid foods speeds up metabolic and physical growth of the rumen, leading to its maturation, as well as allowing for microorganisms to proliferate and the fermentation processes to be maintained. As the animal matures and continues to consume solid feed, the microbiome of the rumen also continues to flourish and diversify.

Short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are produced during the fermentation processes as end products. SCFAs are byproducts of many metabolic processes of organisms in the rumen and are used by the animal for energy and growth (Hungate, 1966). The rumen epithelium development occurs from two

different angles: increase in papillae and increase in cellular or tissue mass (Baldwin et al., 2004). Rumen papillae allow for higher absorption of nutrients, as they increase the surface area for the transport of SCFAs and other substances through the rumen epithelial lining for their eventual distribution to the host (Van Soest, 1994). Growth of the rumen epithelium can be augmented with an increase in butyrate, as it is a premium energy source for these cells (Bugaut, 1987; Scheppach, 1994; Shen et al., 2005). With increased papillae growth, there is more surface area to further enhance the absorption of nutrients as well as providing more space for fermentation to occur.

1.4. RUMEN MICROBIAL DEVELOPMENT AND COMPOSITION

1.4.1. Overview

The microbial composition of the rumen is in an ever-changing state, consisting of bacteria, archaea, protists, and fungi. The majority of rumen microorganisms are bacteria, as they make up 10^{11} cell/ml of rumen content, and members of that community are responsible for many different functional areas, with metabolic specialists that are more focused on amylolytic activity while others are more cellulolytic (Sirohi et al., 2012). Although there are many different members of the community, there is functional redundancy, with overlapping metabolic pathways carried about by multiple organisms; redundancy is more targeted towards members that can degrade central substrates such as cellulose or starch that are consumed in high quantities by the animal (Weimer, 2015). Even though there is functional redundancy in microbial functions, there is also individuality in rumen microbial composition between species of livestock and even individual animals of the same species. Previous studies have been conducted to

determine a “core” microbiome for different host species; examples include humans (Huse et al., 2012), cattle (Henderson et al., 2015), and swine (Holman et al., 2017). By definition, a core microbiome is a similar or common caste of microorganisms that are found predominantly in each host species. A concept laid out by Perlman et al (2022) characterized different definitions of a core microbiome, relating them to function of microorganisms, stage of development of the animal or just basing the definition off of abundance of taxa that were observed. While an interesting concept, defining a core microbiome is a tall order, as many studies that are done are either taken at one point in time and do not factor in environmental or developmental changes that could alter the composition of the microbiome, as well as technical differences amongst study designs. While acknowledging these limitations and challenges, research has found major bacterial phyla that are consistent in rumen microbial communities, regardless of any animal differences. They mainly consist of Bacillota (formerly Firmicutes), Bacteroidota (formerly Bacteroidetes), and Proteobacteria (Lopes et al., 2015).

1.4.2. Major Rumen Bacteria and their Functions

Certain genera from Bacillota, Bacteroidota, and Proteobacteria are also typically found to be more abundant in ruminants, including *Ruminococcus* and *Prevotella* (Kim et al., 2011b). In early weaned lambs, research has supported the idea that there are main taxa that are present (Wang et al., 2022), as has other research in other ruminant species (Morgavi et al., 2015; Zhang et al., 2021; McLoughlin et al., 2020; Langda et al., 2020; Petri et al., 2013). Prominent amylolytic genera, consisting of starch digesters, include *Ruminobacter, Butyrivibrio,* and many *Clostridium* ssp., whereas prominent cellulolytic

genera, consisting of fiber digesters, include *Fibrobacter* and *Ruminococcus* (Niwińska, 2012; Stiverson et al., 2011; Matthews et al., 2019). Various studies have identified *Prevotella ruminicola* as one of the most abundant bacterial species in rumen environments, attributing its predominance to its ability to digest many different forms of carbohydrates (Van Soest, 1994). Many other abundant *Prevotella* species have also been found in different ruminants, including both high and low concentrate fed animals (Ellison et al., 2017; Carberry et al., 2012). Depending on available substrates, many *Prevotella* members have the ability to directly form propionate or succinate, which are both essential parts of the gluconeogenesis pathway of the liver (Huntington, 1990) that supplies glucose for the animal. Similarly, members of the genus *Ruminococcus* have been found to be high in abundance in the rumen environment, highlighting fiber digesting activity, i.e. utilization of cellulose and hemicellulose (Dai et al, 2015). The difference in composition can have a confounding effect on the feed efficiency of the host animal. Zhou et al (2024) found that when comparing low-efficiency sheep vs high-efficiency sheep fed the same diet, genes encoding essential fermentation enzymes were altered in their relative abundance. In high feed-efficient sheep, a survey of genes and their encoded enzymes indicated a higher potential for the production of propionate, and for the production of butyrate from acetate. Main areas of recent study focus on finding carbohydrate-active enzymes, specifically glycoside hydrolases, as they have essential roles in the breakdown and metabolism of feed ingested by the animal (Neves et al., 2021).

1.4.3. Current Status of the Sheep Rumen Microbiome

Currently, interest specifically in the sheep rumen microbiome has increased over the last ten years. Characterization of relative abundance of the microbial population has been conducted over a variety of different alterations to diet and animal selection. The addition of feed additives such as tannins that are thought to inhibit many microbial processes in the rumen or essential oils that are used to try lower methane emissions caused small changes in composition, but the research still found Bacillota, Bacteroidota, and Proteobacteria at the forefront of populations. *Prevotella* was also found to be a prominent member of the community in both studies (Salami et al., 2018; Saro et al., 2018). Changes in diet also found Bacillota, Bacteroidota, and Proteobacteria to dominate the population with rapid shifts to grain diets (Seddik et al., 2018) or shifting from a non-grazing diet to a grazing diet (Belanche et al., 2019). Additional work has been conducted on the effects of abrupt weaning of lambs on the rumen microbiome (Li et al., 2022). Study of high versus low efficiency sheep has concluded similar findings, with Bacillota, Bacteroidota, and Proteobacteria as the main phyla present, with the most dominant genera being *Prevotella* in both solid and liquid phases of rumen material (McLoughlin et al., 2020). Furthermore, attempts to determine long-term effects of rumen fluid transplantation on microbial community composition and rumen epithelial morphology have found that, over time, the major phyla in the rumen fluctuate with the addition and establishment of new rumen fluid, but still represent the same big three phyla present in other studies (Liu et al., 2019).

1.4.4. Strategies used to Modulate Rumen Microbial Composition

While major phyla in the rumen have been found to be consistent, the more detailed composition of the microbial population in the rumen can be considered more dynamic and fluid. Several factors can play a role in altering the composition, including age (Jami et al., 2013), breed (Daghio et al., 2021; Li et al., 2019), sex (Sim et al., 2022), diet (Ellison et al., 2017), environmental location (Henderson et al., 2015), health status (Uyeno et al., 2010), and ruminal pH (Faniyi et al., 2019). Different approaches have been explored to alter the composition of the rumen microbiome. One strategy involves introducing new bacterial species from a rumen donor or by feeding probiotics. A simple approach consisting of switching rumen fluid from other ruminants to try alter the composition as a whole has been attempted. Ribeiro et al (2017), for instance, swapped rumen fluid from cattle with the rumen fluid of bison to study digestibility in the rumen. Bison are more efficient with digesting forage that is of low quality (Richmond et al., 1977), and the study found that the fluid swap caused the cattle to have increased protein and nitrogen digestibility, as well as an alteration in bacterial family composition and protozoa numbers in the rumen. The other strategy involves favoring the establishment and proliferation of particular bacterial groups with prebiotics or other plant-based compounds. For instance, essential oil-based products fed to dairy calves were found to increase the abundance of Bacteroidota as compared to calves not fed the essential oils (Poudel et al., 2019).

Many of these strategies, mainly through the use of feed additives, are focused on improving efficiency of the animal, whether it be enhancing fermentation, creating more opportunities to use up hydrogen produced in the rumen, or preventing health problems in

the animal. However, they require more insight to fight host resilience (Weimer, 2015). Indeed, although there continues to be many advancements in determining new ways to alter the composition of the ruminal microbiome, the resilience of recipient rumen communities has been shown to be strong, as it typically withstands any non-native competitors that may be introduced. Indeed, multiple studies found that dosed bacterial strains in the rumens of different animals were not detected later into the trial, or showed weak persistence (Krause et al., 2001; Chiquette et al., 2007; Varel et al., 1995).

1.5. METHODS TO ANALYZE MICROBIAL COMMUNITIES

1.5.1. Overview

Although there continues to be progress in understanding the complex relationships between the different microscopic symbionts and their ruminant host, the rest is a deep well of information that has not yet been discovered. The study of the diversity in composition of microbial ecosystems requires a process for the identification and discernment of different microbial species, ranging from eukaryotes and prokaryotes to archaea and fungi. To that end, strategies that have been developed and are used for the investigation of microbial communities can be split into two main categories: culture-dependent and culture-independent methods.

1.5.2. Culture-Dependent

Typical microbial communities are made up of an abundance of prokaryotes, mainly bacteria (Whitman et al., 1998; Bilen et al., 2018). As the name implies, culture-dependent methods rely on the process of culturing samples on plates or in broth to

identify different bacterial species. This technique was used extensively in the early days of microbiome research, and it allowed researchers to see differences in phenotypic characteristics (Hashsham, 2007; Zhou et al., 2015). Media used in culturing were produced with a few primary conditions that can influence bacterial growth, including nutrients, temperature, and time of incubation (Lagier et al., 2015). As methods have improved, a shift from non-selective media to media that can dictate what microorganism is selected to grow has allowed a quicker and more precise path to isolate pure colonies (Kerr, 2004). Even more specialized media has been produced to mimic the atmosphere of strict anaerobes or microaerobic organisms, in an attempt to capture the true range of microscopic organisms that were not previously recorded (Mauerhofer et al., 2019; Plugge, 2005; Kenters et al., 2011). Other advantages of using culture-dependent methods include a high sensitivity to select for specific organisms, as well as the ability to analyze the physiology of microorganisms (Figdor and Gulabivala, 2011).

While still used today, culturing does have limitations, mainly that it does not allow researchers to capture the more complex picture of the rumen microbiome. The phylogenetic differences between species, even with similar morphological and phenotypic characteristics, sometimes proved to be a roadblock in securing a concrete answer about the identity of different species, even when referencing standards, such as the Manual of Clinical Microbiology (Clarridge, 2004). Even with more specialized media, the exact nutrient needs for all microorganisms are unknown, so trying to culture them is nothing more than a dream at the moment (Stewart, 2012). Microscopic counts can also vary wildly from plate counts, as seen previously (Staley, 1985). Culturing may not reflect the composition of the sample that is grown, as some prokaryotes replicate

faster than others on certain media, and it can thus misrepresent the true community composition (Nocker et al., 2007). Although there are some limitations, culture-based methods have built the foundation that culture-independent methods can use to further the identification and characterization of different organisms.

1.5.3. Culture-Independent

One advantage of using culture-independent methods is that the process takes less time to perform than the culture-dependent methods. Some microorganisms can take less than 24 hours to grow in a culture and can be identified just as quickly using biochemical methods, but others take more time. Waiting upwards of six weeks just to culture a species can turn into a problem in certain scenarios. With culture-independent methods, the need for such an extended wait time is erased (Patel, 2001).

1.5.4. 16S rRNA Gene

Prokaryotes, like eukaryotes, possess ribosomes. Ribosomes in prokaryotic cells are comprised of two subunits: the 50S and 30S subunits. Inside of the 30S subunit, the 16S rRNA gene lays the foundation for the initiation of protein synthesis (Wimberly et al., 2000). Due to the fact that the 16S rRNA gene is found in all bacteria, it has proved to be an important biological marker for identification of bacterial species. The gene acts as a unique label for each bacterial species due to the structure of the rRNA. The structure of the 16S rRNA gene consists of conserved regions that alternate with nine variable regions (V1-V9). The variable regions allow for the phylogenetic differentiation of different bacteria and archaea (Weinroth et al., 2022; Clarridge, 2004). Another advantage of using

16S rRNA is that the sequence of the gene is diverse enough to be able to distinguish most differences at least at the genus level (Patel, 2001). Polymerase chain reaction amplification of DNA coupled with sequencing of DNA has provided a route to characterize both cultured and currently "unculturable" strains. Furthermore, due to the wide use of the 16S rRNA gene as a marker, researchers have the ability to draw upon a large library of sequence data from previous research. 16S rRNA analysis can be used in tandem with culturing techniques to determine the identity of isolates when trying new culturing methods as well.

Limitations of using the 16S rRNA marker include the varied preferences amongst different research groups in the sub-regions that are targeted for sequencing and analysis. As stated before, the 16S rRNA has nine variable regions, but due to both considerations in cost and sequencing capability, different regions of the gene are preferably used for analysis. Many researchers opt for using sub regions like V1-V3, V3-V4, or V4-V5, while other researchers sequence the entire 16S rRNA gene (Abellan-Schneyder et al., 2021). While there is a great deal of variability within those regions, previous research has found that the V1-V3 region is a more accurate representation of the whole V1-V9 region when compared to other sub region selections. Additionally, selecting more regions (V1-V3 vs V4) supports more phylogenetic differences that can lead to pinpointing new species as opposed to being limited to genus level identifications (Kim et al., 2011a; Johnson et al., 2019). Finally, the metabolic functions of individual microorganisms or their antimicrobial resistance capability cannot be inferred from 16S analysis alone. Objectively, there are many different methods that are used to perform 16S rRNA analysis, which can lead to bias in what organisms are grown, or the decision

to further remove sequences from analysis to achieve a more accurate result (Mignard and Flandrois, 2006). However, despite these differences, there is a common sequence of events that take place when running 16S rRNA analysis. After extracting microbial DNA and sequencing, the first step is assessing the quality of the reads. The error probability in base calling can vary depending on the type of equipment used, the age of the programs, as well as the sequencing platform used (e.g. NGS from Illumina or TGS from Oxford Nanopore. With quality checking, non-specific reads and reads with sequencing errors can be eliminated (Regueira-Iglesias et al., 2023). Next, reads are clustered into Operational Taxonomic Units (OTUs) that can be interpreted as representing individual bacterial species. Researchers typically use a cutoff of sequence identity to cluster sequences into OTUs, i.e. groups of sequences that are very closely related. Kim et al (2011a) reported that a distance level of 0.04, or 96% identity, resulted in a similar number of OTUs produced from the V1-V3 region as if sequencing the entire V1-V9 region. OTUs are then analyzed for other artifacts, such as chimeras produced during PCR, and then finally binning for further analysis.

To further analyze the sequence data of the 16S rRNA gene, two distinct methods have been developed: OTU-based methods and taxonomy-based methods (Schloss and Westcott, 2011). OTU-based methods focus on the action of grouping similar sequences together in a particular dataset. Sequences are representative of a “taxonomic unit” based on threshold values of sequence identity, oftentimes making use of open access software such as MOTHUR (Schloss et al., 2009) to estimate species richness in a microbial community. On the other hand, taxonomy-dependent methods use different tools to group sequences into separate taxonomic groups, either based on reference sequences or

alignments. Resources such as BLAST (Altschul et al., 1997) and RDP Classifier (Wang et al., 2007) use different algorithms to assign sequences to different taxonomic groups (Sedlar et al., 2017).

1.5.5. Metagenomics

Although the 16S rRNA marker is a useful tool in deciphering the identity of microorganisms in a sample, function and metabolism cannot be inferred if there is not a perfect match to a previously known organism, characterized by culturing or genomics. The 16S rRNA approach makes use of a conserved region of nucleotides, whereas metagenomics, also known as 'shotgun sequencing', allows for the assembly or capture of complete or partial genomes. The term "shotgun" refers conceptually to the ability of sequencing platforms to generate sequence data from individual DNA molecules that are in the same pool or solution, an analogy to a group of pellets fired from a shotgun that hit a target at random locations.

A common method for analyzing metagenomic sequences is to assemble them into longer sequences, referred to as contigs, in an effort to 'reconstruct' the original chromosome DNA from which the sequence reads were generated. Because they are longer, contigs are more likely to contain full length coding sequences, which can be used to assign function or identity if their encoded proteins are a close match to previously characterized proteins from a database. Applications such as Rapid Annotation for Subsystem Technology (RAST) (Aziz et al., 2008) can allocate sequence reads to different families of enzymes or proteins, and one can infer metabolic function based on the metabolic products produced or consumed by those enzymes through the use of other

tools like the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000). Applications of metagenomic analysis include comparisons to different microbial communities found in different locations or hosts (Tringe et al., 2005; Dinsdale et al., 2008). Additionally, modeling of host-microbe interaction, such as drug targets, is also becoming a more popular area of research due to the influence of metagenomic analysis (Zaneveld et al., 2008).

However, metagenomics also has disadvantages or weaknesses. Due to the complexity of many microbial environments, randomly generating sequences from a small aliquot or 'swab' to identify species of interest by pairing up shared enzymes can be a challenge. Metagenomic profiling can also often be impacted by the presence of sequencing artifacts, and can possibly lead to an inaccurate estimation of diversity within a sample or community (Wooley and Ye, 2009). Another concern regarding the efficacy of metagenomic analyses is that the chance for a large number of similar sequence reads to be generated at the same position is extremely low, unless there was specific targeting of that location for sequencing.

1.6. Rationale and Objectives of Research

Likely due to the differences in size between the cattle and sheep industries, a greater amount of research has been performed on cattle to elucidate rumen bacterial function as it pertains to qualities such as feed efficiency, disease resistance, overall animal development, and metabolism. While sheep and cattle are both ruminants, they vary in various aspects, such as size, behavior and nutrient requirements. Therefore, there are limits to how knowledge generated from cattle can be applicable to sheep. Indeed, work to date has shown that separate host species show differences in their ruminal bacterial composition (Xin et al., 2019). Due to these differences, optimal efficiency in terms of VFA production and microbial degradation of carbohydrates and proteins needs to be detailed through further research of sheep as a whole. Limited numbers of studies have focused on the transition between diets (Seddik et al., 2018; Belanche et al., 2019), while many studies have studied separate diets at different points in time. To further understand the basis of ruminal bacterial composition in sheep, more research is needed in the field of changes in the rumen microbiome during diet transitions, as diet is an integral part of an animal's lifecycle.

Furthermore, sheep management can vary greatly, with extensive grazing to intensive feedlot operations, so it is difficult to blanket the whole industry with a few studies that only look at one side of things. Additionally, a significant number of higher-level taxonomic analyses have been conducted without deeper investigation performed, leaving information about candidate bacterial species out of the picture. As metagenomics allows for a better understanding of function rather than identity, it opens the door for further deconstruction of the overall function of the rumen and its inhabitants. With so

many species of microorganisms that are currently undiscovered in the rumen, filling in those gaps of knowledge will allow producers to make more informed feeding decisions that can increase the health and productivity of the animal.

In this context, the microbiome research presented in this thesis took advantage of an animal trial that was performed in sheep, where lambs were transitioned from a forage-based diet to a concentrate-based diet. Rumen samples were collected from lambs under each diet regiment. The objectives of the microbiome project were to:

1) characterize and compare the diversity and composition of the rumen bacterial communities between lambs fed a forage-based diet and lambs fed a concentrate-based diet

2) use a metagenomics approach to further investigate the metabolic functions of novel candidate rumen bacterial species that were identified from objective 1.

Answering these questions has the potential for discoveries pertaining to upgrades in efficiency as well as developing an understanding of the complex environment that is the rumen. A switch in composition of bacterial communities was found when lambs were transitioned to concentrate feed from forage, as there were alterations in the abundance of specific OTUs in each sample. As uncharacterized species of ruminal bacteria were present in both feeding systems, metagenomics allowed for identification of enzymes from select OTUs that function in metabolic pathways critical for the rumen, such as chorismite and vitamin B5 production.

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CHAPTER 2: CHARACTERIZATION OF THE RUMEN BACTERIAL COMMUNITIES OF LAMBS THAT TRANSITIONED FROM FORAGE TO A CONCENTRATE-BASED DIET

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ABSTRACT

Forage and concentrate-based diets are distinct strategies used in ruminant production. The transition from a forage-based diet to a concentrate-based diet has been shown to have a great impact on the rumen microbiome composition due to vast differences in substrates provided by each diet type. To gain further insight on the nature of the changes that occur in the rumen microbiome as a result of a switch in diet, ruminal bacterial composition was compared between lambs initially fed a forage-based diet and lambs that were switched to a concentrate-based diet for approximately 60 days. From rumen fluid collected after slaughter, data was generated by Illumina MiSeq 2X300 sequencing of PCR amplicons targeting the V1-V3 regions of the 16S rRNA gene. Next-Generation Sequence data was analyzed using a combination of custom Perl scripts, and publicly available software (Mothur (v.1.44.3), RDP classifier and NCBI Blast). A total of 10,693 species-level operational taxonomic units (OTUs) were identified across all samples, with 86 OTUs shared between diets. A comparative analysis using the non-parametric Kruskal-Wallis test identified 36 highly represented species-level OTUs that differed in abundance across dietary treatments ($P < 0.05$). Of these, three OTUs, RA3-082995, RA1-35340, and RA1-04216, were found to be of particular interest. OTU RA3-

082995 was at its highest representation in samples from individuals given a forage-based diet, with an average abundance of 1.30% compared to 0.04% in samples from animals fed a concentrate-based diet. RA3-082995 was predicted to be a novel species of the family Prevotellaceae based on its low 16S rRNA gene sequence identity (91.7%) to its closest valid relative, *Prevotella ruminicola.* OTU RA1-35340, predicted to be a strain of *Sharpea azabuensis,* was also in much higher abundance in the concentrate-based diet as compared to the forage-based diet (2.83% and 0.05%, respectively). RA1-04216, which was predicted to be a strain of *Succinivibrio dextrinosolvens* (96.9%), was at its highest abundance in samples from individuals given a concentrate-based diet, with an average representation of 6.77% compared to 0.05% in samples from animals fed a forage-based diet. These, and other specific ruminal OTUs whose abundance are affected by changes in diet, can then be studied further to gain more insight on the effects of the transition from forage to concentrate on rumen physiology.

Keywords: rumen, 16S rRNA, microbiome, sheep, OTU

2.1. Introduction

While not as popular as beef production, sheep production is the United States is a major player in the agricultural industry, providing meat, wool, and milk for both domestic use and export opportunities (Council, 2008). The American sheep industry, even though it is not as big as other countries' production, still has an economic impact of over $5 billion (Association, 2017). In a wider scope, many other countries, including Greece, Italy, Australia, and Spain, are leaders in growing sheep production, mainly for dairy sheep, but can include wool and meat (Pulina et al., 2018). One of the major inputs to the sheep industry is feed, as with any livestock species. It can account for up to 70% of total direct costs (Kenny et al., 2018). As diet is what allows an animal to reach its genetic growth potential, the composition of the diet to meet nutrient requirements must be predicted. The composition of diets can change depending on availability of ingredients, price fluctuations, and ultimately, the end goal for the animals. When finishing ruminants, a diet high in grains such as corn or corn silage is often used (Hilscher et al., 2022; Schrage et al., 1991) to provide more easily accessible nutrients to the animals. On the other hand, some operations focus on forage-based diets that consist mainly of hay or other roughage to promote healthier rumen activity and at a lower cost, but achieve slower growth rates and weights (Wand, 2014). Concentrate diets are more widely found in the United States as compared to European countries, who opt for the forage-based diets. Ultimately, composition of the diet will affect the end product, which in many cases, is meat for consumption. It is important to consider how the animal is using the diet it is given, and in ruminants, the usage of feedstuffs is centered in the rumen.

Microorganisms in the rumen are responsible for the fermentation of feed that is given to the animal, and produce essential substrates that are used for energy production by the host. The composition of the rumen consists of a wide range of residents, including bacteria, protozoa, fungi, archaea, and viral particles, of which bacteria represent the majority (McSweeney & Mackie, 2012). Due to the high representation of bacteria in the rumen, they are the target of intense research to determine identity and function as to understand the role of each individual species that is present. Many of these bacteria can utilize carbohydrates that are present in plant matter, ranging from structural polysaccharides like cellulose to energy storage glucose polymers like starch. Depending on the diet that the animal consumes, the proportions of end products such as volatile fatty acids (VFAs) can be altered, resulting in differing growth rates and maximum output from the animal. These VFAs are produced through fermentation and are used by the host for energy production. The most prominent are acetate, propionate, and butyrate. Other end-products have also been seen to affect the animal in terms of quality, as higher levels of forage in sheep diets have been found to increase the levels of linoleic acid, a fatty acid that is more prone to oxidation and off-flavors, thus less favorable to the majority of consumers (Díaz et al., 2005; Rousset-Akrim et al., 1997; Priolo et al., 2002). Differing diets then ultimately have an effect on the composition of the rumen, which can alter fermentation end-product proportions.

In light of these findings, the use of 16S rRNA analysis to characterize bacterial community composition in the rumen has served as an invaluable tool to determine variations due to differences in animal breeds (Daghio et al., 2021), feed efficiency (Ellison et al., 2017), age (Jamie et al., 2013), as well as change in diet. While some

research has been conducted on the effect of transitioning diets on the rumen microbiome in sheep, additional investigations are needed to gain a more in-depth understanding. In this context, this report presents the characterization of rumen bacterial communities in sheep that were transitioned from a forage-based diet to a concentrate-based diet.

2.2. Materials and Methods

2.2.1. Animals and Sample Collection

All procedures involving the use of animals in this study were approved by the South Dakota State University (SDSU) Institutional Animal Care and Use Committee (IACUC protocol #2101-004E). The animal trial was conducted at the SDSU Sheep Research and Teaching Unit, and harvest of animals was performed at the SDSU Meat Laboratory.

The lambs used in the study were Polypay wethers sourced from the sheep herd maintained at SDSU. After being fed a forage-based diet for 60 days following weaning, lambs [n = 84; initial shrunk body weight (BW) = 38.8 kg ± 4.8 kg] were transitioned to a concentrate-based diet for a period of 9 weeks (Table 2.1). Prior to the change in diet (D0), four lambs with BW closest to the study group mean (n = 4; BW = 39.1 kg ± 0.4 kg) were harvested to determine the bacterial composition in the rumen prior to the change in diet. The remaining wethers (n = 80) were blocked by BW into separate pens (n = 16; n = 5 wethers/pen) during the concentrate diet phase. Lambs were weighed on D7, D14, D21, D56, and D63, with single representatives from different pens randomly selected for harvesting and collection of rumen samples on D56 (n=7) and D63 (n=8); whole digestive tracts were first removed from the carcasses, then the rumen was

separated for collection of unfiltered rumen contents. Samples were stored at -80°C until DNA extractions were performed.

2.2.2. DNA Isolation and Amplification of 16S rRNA Gene

Microbial DNA was isolated and purified from 250 µl of rumen contents using the repeated bead beating plus column method, as previously described (Yu & Morrison, 2004). Briefly, samples were first lysed by bead beating with 0.4 g of zirconium beads in lysis buffer (0.5 M NaCl, 50 mM Tris. HCL, 50 Mm EDTA, 4% SDS), then SDS was removed with ammonium acetate (10 M, 20% volume), followed by DNA precipitation with isopropanol. After its recovery by centrifugation, DNA was purified with the QIAamp DNA Stool Kit (QIAGEN, Hilden, Germany) according to the standard instructions of the manufacturer. DNA concentration and quality (260/280 ratio) were determined using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). Purified microbial genomic DNA samples were subsequently submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for V1-V3 amplification of the 16S rRNA gene and amplicon sequencing using an Illumina MiSeq (2 × 300) platform to generate overlapping paired-end reads. The universal forward 27F-5’AGAGTTTGATCMTGCTCAG (Edwards et al., 1989) and reverse 519R-5’GWATTACCGCGCGCGCTG (Lane et al., 1985) primers were used for targeted amplification of the V1-V3 region of the bacterial 16S rRNA gene.

2.2.3. 16S rRNA Analysis

Unless specified, bioinformatic analyses were performed using custom written Perl scripts. Using the 'make.contigs' command from the MOTHUR open-source software package (version 1.44.3) (Schloss et al., 2009), raw overlapping sequence reads generated from the same flow cell cluster were assembled to create contigs corresponding to the V1-V3 region of the 16S rRNA gene. Contig sequences were then screened to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 bp, and an average Phred quality score of at least Q33. After quality screening, sequenced reads were aligned, then clustered into operational taxonomic units (OTUs) at a 4% sequence dissimilarity cutoff. While 3% is the most commonly used clustering threshold, 4% is more suitable for the V1-V3 region based on previously published reports (Kim et al., 2011; Johnson et al., 2019).

OTUs were screened for artifacts using three independent approaches (Poudel et al., 2020). Chimeric sequences were first identified with the 'chimera.uchime' (Edgar et al., 2011) and 'chimera.slayer' (Haas et al., 2011) commands in MOTHUR (v. 1.44.3) (Schloss et al., 2009). Next, the quality of the 5' and 3' ends of OTUs was assessed using a database alignment search-based approach; OTUs were designated as artifacts if they were missing more than 5 nucleotides from the 5' or 3' end of their respective alignments when compared to their closest match of equal or longer length from the NCBI 'nt' database, as determined by blastn (Altschul et al., 1997). Finally, additional screening was applied to OTUs with only one or two assigned reads, where only sequences with a perfect or near perfect match (maximum of 1% dissimilarity) to a sequence in the NCBI

‘nt’ database were kept for analysis. All OTUs and their assigned reads that were flagged during the artifact screens were removed from further analysis.

Taxonomic assignment of curated OTUs was performed using a combination of RDP Classifier (Ribosomal Database Project) (Wang et al., 2007) and blastn (Altschul et al., 1997), while MOTHUR (v. 1.44.3) (Schloss et al., 2009) was used for alpha and beta diversity analyses. The alpha diversity indices ‘Observed OTUs’, ‘Chao’, ‘Ace’, ‘Shannon’ and ‘Simpson’ were determined with the ‘summary.single’ command. For beta diversity analysis, Bray–Curtis distances were first calculated with ‘summary.shared’, then the command ‘pcoa’ was run for principal coordinate analysis (PCoA). Curated OTU datasets were rarefied to 6,466 sequences with custom Perl scripts prior to performing alpha and beta diversity analyses.

2.2.4. Statistical Analysis

Statistical analyses were performed in R (version R-3.6.2). Normal distribution of data was first assessed with the Shapiro Wilk test (command ‘shapiro.test’) to determine if parametric or non-parametric testing should be applied. Alpha diversity indices were analyzed by ANOVA and by Tukey’s range test for multiple comparisons (command ‘aov’, command ‘HSD.test’). For statistical analysis of taxonomic groups and of most abundant OTUs, the non-parametric Kruskal-Wallis test (command ‘kruskal.test’) and the Wilcoxon test for multiple pairwise comparisons (command ‘pairwise.wilcox.test’) were used. A threshold of $p \leq 0.05$ was considered significant.

2.3. Results

2.3.1. Taxonomic Composition Analysis

From 19 samples analyzed (D0 n=4, D56 n=7, D63 n=8), a combined total of 607,716 high quality sequence reads were generated, ranging between 6466 and 48681 sequence reads per sample. Taxonomic analysis identified Bacillota (formerly Firmicutes) as being the most highly represented phylum in each sample (42.76 - 94.00% of reads per sample). Average D0 and D56 sample concentrations of Bacillota (65.75% and 59.12%) were not statistically different, but both were different than D63 at 78.9% of the sample. Sequences assigned to Lachnospiraceae, Lactobacillaceae, Veillonellaceae, and Ruminococcaceae represented 51.22-94.94% of Bacillota sequences and mostly followed a trend that showed D0 being statistically different than the D56 and D63 groups (Table 2.3). The second most abundant phylum was Bacteroidota (formerly Bacteroidetes) ranging from 1.07 - 35.91%. Similar to Bacillota, the relative abundance of Bacteroidota was statistically different in the D63 group as compared to the D0 and D56 groups (12.49% vs 22.85% and 21.26%). Prevotellaceae were identified as the highest represented family, with 49.60-99.13% of Bacteroidota sequences. Finally, Proteobacteria represented 0.10-23.88% of sequences, with Succinivibrionaceae composing 1.89-94.70% of Proteobacteria sequences. Proteobacteria were found to be statistically different between all three groups, with mean relative abundances of 0.63% for D0, 9.60% for D56, and 2.06% for D63.

2.3.2. Alpha and Beta Diversity

Considering the impact of diet on the ruminal bacterial composition of lambs at the taxonomic level, analyses for alpha and beta diversity were next performed to evaluate the extent of these effects at the Operational Taxonomic Unit (OTU) level. All alpha indices analyzed (Observed OTUs, Ace, Chao, Shannon and Simpson) were found to differ between D0 and D56/D63 diets ($p < 0.05$), while no significant differences were observed between the D56 and D63 groups (Table 2.4). Accordingly, principal coordinate analysis (PCoA) clearly showed that D0 samples clustered as a distinct group from the D56 and D63 samples, further indicating major differences in OTU composition as a result of feeding different diets (Figure 2.1).

2.3.3. OTU Composition Analysis

In light of the results from the diversity analyses, the nature of the differences in OTU composition were further explored. Overall, a total of 10,693 OTUs were identified amongst all samples analyzed, with eighty-seven OTUs shared by all three sample groups (DO, D56, D63). A comparative analysis of the 36 most abundant OTUs, defined as having an average abundance of at least 1% in at least one group, revealed different composition patterns. Overall, shared OTUs followed the same pattern, with a higher representation of Bacillota-associated OTUs than any other phylum. For instance, seven OTUs [RA1-55937, RA11-38187, RA1-14031, RA14-043428, RA5-48363, RA3-082995, and RA1-43608] were found to be in greater abundance in the forage group ($P<0.05$) compared to the two concentrate groups, while no statistical differences in representation were found between the D56 and D63 groups for these OTUs. Notably, all

seven OTUs in this category were predicted to be novel species, as the highest level of nucleotide sequence identity to their respective closest valid taxon was 95.4%. Amongst OTUs of this category, differences in abundance between the forage group and concentrate groups ranged between 120X and 333X.

Thirteen other OTUs [RA1-00172, RA1_03436, RA1_09153, RA1_00065, RA1_00707, RA1_00941, RA1_01491, RA1_03396, RA1_04211, RA1_07845, RA1_26218, RA1_48142, RA2_01986] displayed an opposite pattern, with greater abundance in concentrate samples compared to the forage group ($P<0.05$), while similarly no significant differences in abundance were observed between samples from the D56 and D63 timepoints; differences in abundance between the concentrate groups and the forage group ranged between 3X and 114X.

Only 10 of the 36 OTUs displayed a sequence identity of 95% or above to their closest valid relative (Table 2.5-2.6), suggesting that 26 OTUs likely correspond to uncharacterized ruminal bacterial species. Three OTUs had 100% sequence identity matches for their closest known relative: OTU RA1-00941 (*Anaerolactibacter massiliensis*), RA1-26218 (*Limosilactobacillus mucosae*), and RA1-35340 (*Sharpea azabuensis*). The other seven OTUs were above 95% in their nucleotide sequence identity match to their closest valid relative (Table 2.5-2.6). OTU RA1-00065 was matched to *Hallella mizrahii* (99.95%), RA2-68837 to *Selenomonas bovis (*99.61%), RA1-05338 to *Psuedoruminococcus massiliensis (*98.73%), RA1-11230 to *Pseudoramibacter porci* (97.72%), RA1-04216 to *Succinivibrio dextrinosolvens* (96.9%), RA2-01968 to *Mobilibacterium massiliense* (96.89%), and RA1-43608 to *Aristaeella lactis* (95.35%).

2.4. Discussion

As different diets offer different substrates for microorganisms to utilize, it is important to realize the potential outputs of those microorganisms, and how they can affect the host. In both diets, the composition of the rumen microbiome was dominated by three main phyla, Bacillota, Bacteroidota, and Proteobacteria. Similar to previous studies in sheep and other ruminants, including cattle and goats, these phyla all hold high percentages of the bacterial rumen microbiome composition (Jami et al., 2013; Callaway et al., 2010; Brulc et al., 2009; Lee et al., 2012; Petri et al, 2013).

Bacillota was the predominant phylum present in all samples, averaging 69.01% of total phyla present in concentrate samples, and 65.75% in forage-based samples. Bacillota, previously referred to as Firmicutes, play a central role in the fermentation action of the rumen. Many species within families, including Lachnospiraceae, Lactobacillaceae, and Ruminococcaceae, produce lactate as a major end product of fermentation (Molinero et al, 2021). Focusing in on the Bacteroidota phylum, the family Prevotellaceae was the dominant family for both forage and concentrate diets, especially for the concentrate diets, where it comprised of over 90% of the microorganisms detected. A prominent group within this family is the *Prevotella* genus, represented by OUT RA3-082995, with many species having been found to reside in not only the rumen (Dao et al., 2021), but in other intestinal tracts and body systems as well (Könönen, 1993; Bik et al., 2006). *Prevotella* is an important genus for the gluconeogenesis pathway, as one of its main products is propionate, which the host uses in the liver to create glucose to supply tissues that require simple sugar as an energy source (Armstrong & Blaxter, 1957).

While propionate is a major factor in energy production in the rumen, other VFAs also act as sources of energy and can be produced by other microorganisms in the rumen. Looking at specific OTUs present, *Succinivibrio dextrinosolvens*, which is the closest valid relative to RA1_04216 (96.9%) uses different carbohydrates to produce acetate, a main VFA, and succinate, a precursor for propionate production (Bryant & Small, 1956; O'Herrin & Kenealy, 1993). Acetate has been found to be crucial for milk fat synthesis (Aschenbach et al., 2010) and overall energy production. Other microbial products of interest include lactate and formate. Formate is much less studied, but may play a role as an electron carrier between bacterial species to facilitate methane production in the rumen (Hungate et al., 1970; Kelly et al., 2022; He et al., 2019). OTU RA1-35340 had the highest percent identity match (100%) to *Sharpea azabuensis*, which has been found in sheep (Ellison et al., 2017; McLoughlin et al., 2023; Kamke et al., 2016), and horses (Morita et al., 2008). It is interesting to note that several studies have found a higher abundance of *S. azabuensis* in sheep that produce less methane (Wu et al., 2022), in conjunction with a higher ruminal abundance of *Megasphaera elsedenii*, which is a well-known lactate metabolizer (Chen et al., 2019). While the *Sharpea* species were in low abundance with the forage diet (0.04-0.05%), that range increased in the concentrate samples (0.05-36.59%), which could be indicative of the increased need to metabolize lactate in order to combat ruminal pH changes due to a high concentrate-based diet.

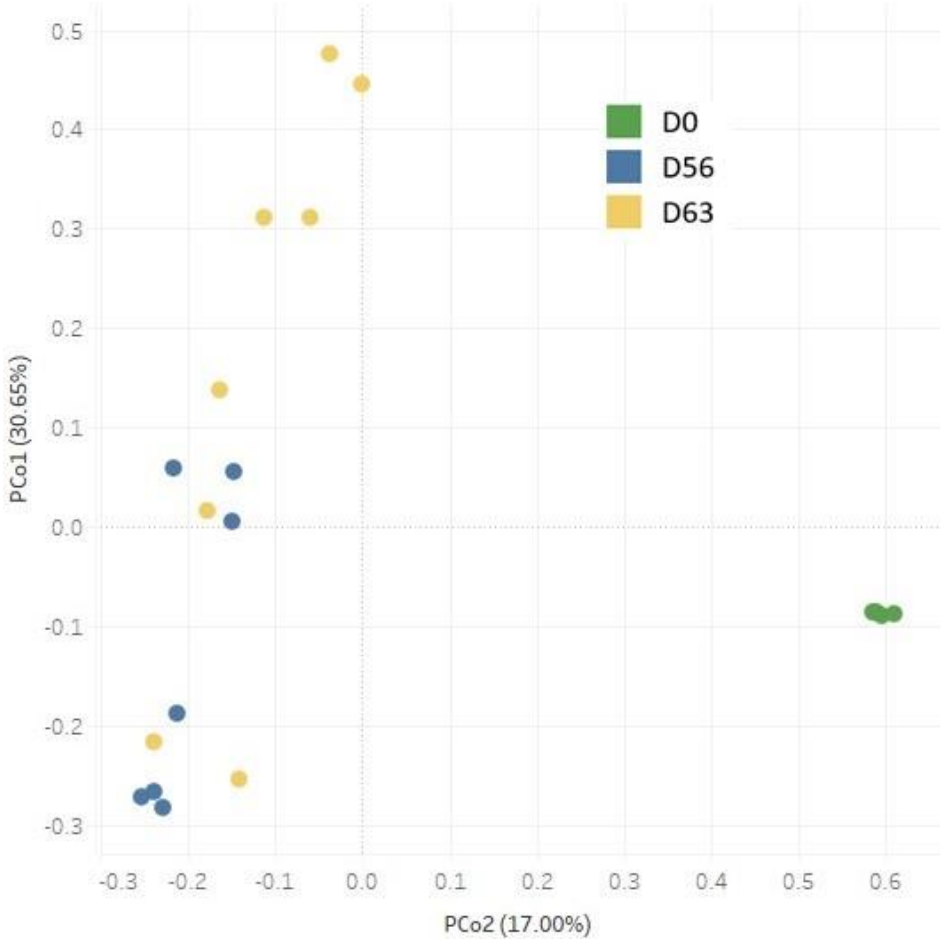

Figure 2.1. Principal Coordinate Analysis (PCoA) using Bray-Curtis distance matrix. The x and y axes correspond to Principal components 2 (PCo2) and 1 (PCo1) respectively, and represent 47.65% of the variation

Table 2.1. Composition of diets

Ingredient, % DM[1]	**Concentrate (D56, D63)**	**Forage (D0)**
Whole Corn, %	65.5	-
Pelleted Soy Hulls, %	5	-
Grower Pellet, %	28.6	27.5
Soy Oil, %	0.9	-
Oat Hay, %	-	57.5
DDGS[2], %	-	15
Analyzed Nutrient Composition		
DM, %	88.7	87.4
CP[3], %	14.1	13.7
NE_m[4], Mcal/kg	91.5	74.5
NE_g[5], Mcal/kg	60.4	42.9

[1] DM = Dry matter content of feed, [2] DDGS = Dried Distillers with solubles, [3] CP = Crude protein, [4] NE_m = Net energy for maintenance, [5] NE_g = Net energy for gain

Table 2.2. Mean carcass traits for each slaughter timepoint of D0 (forage) and D56/D63 (concentrate). Values shown represent mean and standard error, respectively.

Trait	D0	D56	D63
HCW, lbs	39.5 ± 0.87	62.7 ± 6.16	63.38 ± 6.98
REA, sq in	1.58 ± 0.13	2.21 ± 0.18	2.46 ± 0.19
BCTRC, %	48.9 ± 0.42	45.9 ± 0.74	46.8 ± 0.89

Carcass Characteristics

Lambs in each harvest timepoint demonstrated normal growth, with the later timepoint lambs, D56 and D63, displaying heavier hot carcass weights (HWC), larger rib eye areas (REA), but all three possessed similar percentages of boneless, closely trimmed retail cuts (BCTRC), as shown in Table 2. One possible explanation for the similarities in boneless, closely trimmed retail cuts is that the older lambs gained more fat, which then had to be removed at the time of processing, as compared to the D0 lambs.

Table 2.3. Mean relative abundance[1] (%) of main bacterial phyla and families identified in D0, D56, and D63 groups. Values shown represent mean and standard error, respectively.

Taxonomic Affiliation	D0	D56	D63
Bacillota	**65.75 ± 2.10^{a}**	**59.12 ± 4.68^{a}**	**78.90 ± 5.28^{b}**
Lachnospiraceae	21.39 ± 0.33	16.62 ± 3.04	18.21 ± 2.67
Lactobacillaceae	0.10 ± 0.01^{a}	3.74 ± 1.14^{b}	8.15 ± 2.73^{b}
Clostridiales Incertae Sedis XIII	5.49 ± 0.45^{a}	0.44 ± 0.09^{b}	4.32 ± 2.02^{ab}
Veillonellaceae	0.10 ± 0.01^{a}	9.10 ± 1.94^{b}	4.40 ± 1.73^{b}
Erysipelotrichaceae	0.67 ± 0.05^{a}	8.02 ± 2.75^{b}	14.03 ± 4.45^{b}
Ruminococcaceae	15.82 ± 0.52	13.66 ± 5.97	23.47 ± 6.10
Other Bacillota$^{\#}$	22.18 ± 2.11	7.54 ± 1.38	6.31 ± 0.99
Bacteroidota	**22.85 ± 1.44^{a}**	**21.26 ± 2.32^{a}**	**12.49 ± 3.63^{b}**
Prevotellaceae	12.66 ± 0.97^{a}	20.65 ± 2.22^{b}	11.64 ± 3.62^{a}
Other Bacteroidetes$^{\#}$	10.19 ± 0.95	0.61 ± 0.12	0.85 ± 0.29
Proteobacteria	**0.63 ± 0.11^{a}**	**9.60 ± 3.00^{b}**	**2.06 ± 1.73^{c}**
Succinivibrionaceae	0.06 ± 0.003^{ab}	7.08 ± 3.03^{a}	0.22 ± 0.12^{b}
Other Proteobacteria$^{\#}$	0.57 ± 0.11	2.53 ± 0.91	1.84 ± 1.61
Other Bacteria$^{\#\$}$	10.78 ± 0.61	10.02 ± 4.88	6.55 ± 1.14

[1]Mean relative abundance of taxonomic groups is presented as a percentage (%) of the total number of analyzed reads per sample. Phylum-level groups are highlighted in bold, followed by their respective family-level groups. Different superscripts in the same row indicate that groups are statistically different ($P < 0.05$). $^{\#}$Statistical test not performed because of group heterogeneity. $^{\$}$Other bacteria included sequences associated to the phyla Tenericutes, Fibrobacteres, Lentisphaerae, Chloroflexi, Planctomycetes, Spirochaetes, Actinobacteria, Armatimonadetes, Elusimicrobia, Candidatus Saccharibacteria, SR1, Synergistetes, Campilobacterota, and Verrucomicrobia, in addition to unclassified bacteria.

Table 2.4. Alpha diversity indices[1] from ruminal bacterial communities of lambs at different kill days. Values are presented as means and standard error of the mean, respectively.

Index	D0	D56	D63	*P*-value
Ace	6345.44 ± 228.33[a]	1713.53 ± 309.26[b]	1899.56 ± 760.47[b]	<0.001
Chao	4190.86 ± 175.95[a]	976.91 ± 129.66[b]	1023.72 ± 261.42[b]	<0.001
Shannon	6.52 ± 0.09[a]	3.49 ± 0.33[b]	3.26 ± 0.35[b]	<0.001
Simpson	0.008 ± 0.003[a]	0.09 ± 0.04[b]	0.12 ± 0.05[b]	<0.001
Sobs	2007.25 ± 55.89[a]	426.57 ± 44.97[b]	405.25 ± 65.79[b]	<0.001

[1]Different superscripts in the same row indicate that groups are statistically different ($P < 0.05$).

Table 2.5. Mean relative abundance[1] (%) of shared operational taxonomic units (OTUs) assigned to Bacillota in rumen samples collected from lambs in D0, D56, or D63 groups. Values shown represent mean and standard error, respectively.

OTU	D0	D56	D63	Closest Valid Taxon (id% *)
RA1-00044	0.06 ± 0.03	5.07 ± 4.60	1.43 ± 1.27	*Acetivibrio alkalicellulosi* (79.19%)
RA1-00941	0.03 ± 0.01^{a}	0.44 ± 0.12^{b}	1.95 ± 0.75^{b}	*Anaerolactibacter massiliensis* (100%)
RA1-01491	0.03 ± <0.01 ^{a}	1.19 ± 0.31^{b}	1.30 ± 0.50^{b}	*Butyrivibrio proteoclasticus* (87.62%)
RA1-03396	0.04 ± 0.01^{a}	1.22 ± 0.21^{b}	1.89 ± 0.74^{b}	*Lachnospira multipara* (88.45%)
RA1-04211	0.02 ± <0.01^{a}	1.24 ± 0.77^{b}	0.31 ± 0.12^{b}	*Saccharofermentans acetigens* (81.74%)
RA1-05338	0.01 ± 0.01	1.08 ± 0.79	0.17 ± 0.11	*Pseudoruminococcus massiliensis* (98.73%)
RA1-07845	0.03 ± 0.01^{a}	0.61 ± 0.09^{b}	2.60 ± 1.01^{b}	*Massiliimalia timonesis* (85.28%)
RA1-11230	0.02 ± <0.01^{a}	0.45 ± 0.09^{b}	1.74 ± 0.42^{c}	*Pseudoramibacter porci* (97.72%)
RA1-26218	0.03 ± <0.01^{a}	1.10 ± 0.32^{b}	2.48 ± 1.05^{b}	*Limosilactobacillus mucosae* (100%)
RA1-34458	0.04 ± 0.01^{a}	1.37 ± 0.49^{ab}	3.49 ± 1.44^{b}	*Ligilactobacillus ruminis* (94.36%)
RA1-43608	1.03 ± 0.09^{a}	<0.01 ± <0.01^{b}	<0.01 ± <0.01^{b}	*Aristaeella lactis* (95.35%)
RA1-45621	0.19 ± 0.03^{a}	0.03 ± 0.01^{b}	1.68 ± 1.31^{ab}	*Congobacterium massiliense* (90.74%)
RA1-48142	0.02 ± <0.01^{a}	0.91 ± 0.21^{b}	1.11 ± 0.51^{b}	*Lactobacillus rogosae* (92.36%)
RA2-01986	0.01 ± <0.01^{a}	0.04 ± 0.01^{b}	1.10 ± 0.41^{b}	*Mobilibacterium massiliense* (96.89%)
RA2-68837	0.02 ± <0.01	1.37 ± 0.89	0.79 ± 0.43	*Selenomonas bovis* (99.61%)
RA1-00172	0.08 ± 0.01^{a}	8.17 ± 1.81^{b}	3.48 ± 1.34^{b}	*Dialister succinatiphilus* (94.4%)
RA1-01494	0.04 ± 0.01^{a}	5.59 ± 2.40^{b}	0.09 ± 0.02^{c}	*Intestinibaculum porci* (89.9%)
RA1-00105$^{\#}$	0.08 ± 0.01^{a}	5.30 ± 3.00^{b}	1.47 ± 1.12	*Coprococcus eutactus* (91.7%)
RA1-55937	4.91 ± 2.41^{a}	0.03 ± <0.01^{b}	0.03 ± <0.01^{b}	*Pectinatus haikarae* (79.8%)
RA11-38187	1.56 ± 0.26^{a}	0.01 ± <0.01^{b}	0.01 ± <0.01^{b}	*Saccharofermentans acetigenes* (87.9%)
RA1-14031	1.14 ± 0.11^{a}	0.01 ± <0.01^{b}	0.01 ± <0.01^{b}	*Eubacterium ruminantium* (90.0%)
RA14-043428	1.06 ± 0.15^{a}	<0.01 ± <0.01^{b}	<0.01 ± <0.01^{b}	*Acetivibrio thermocellus* (83.7%)
RA1-01223	0.12 ± 0.01^{a}	2.34 ± 1.45^{ab}	11.21 ± 5.11^{b}	*Ruminococcoides bili* (94.8%)
RA1-00032	0.15 ± 0.02	7.92 ± 4.23	6.99 ± 4.01	*Ruminococcoides bili* (86.8%)

RA1-03436	0.08 ± 0.01[a]	1.59 ± 0.42[b]	6.92 ± 3.13[b]	*Blautia hominis* (86.5%)
RA1-09153	0.05 ± <0.01[a]	0.22 ± 0.05[b]	4.93 ± 2.11[b]	*Solobacterium moorei* (89.8%)
RA1-35340	0.05 ± <0.01[a]	0.18 ± 0.07[b]	4.79 ± 4.25[ab]	*Sharpea azabuensis* (100%)

[1] Mean relative abundance of taxonomic groups is presented as a percentage (%) of the total number of analyzed reads per sample. Different letter superscripts in the same row indicate that groups were significantly different ($P < 0.05$). [#] OTUs between the groups were not found to be significantly different by the Wilcoxon rank sum test. [*] Nucleotide sequence identity (%) between each OTU and its corresponding closest valid relative.

Table 2.6. Mean relative abundance[1] (%) of shared operational taxonomic units (OTUs) assigned to Bacteroidota and Proteobacteria in rumen samples collected from lambs in D0, D56, or D63 groups. Values shown represent mean and standard error, respectively.

OTU	D0	D56	D63	Closest Valid Taxon (id%*)
Bacteroidota				
RA1-00065	0.04 ± 0.01^{a}	4.66 ± 1.40^{b}	1.83 ± 0.56^{b}	*Hallella mizrahii (99.95%)*
RA1-00707	0.06 ± 0.01^{a}	3.96 ± 1.23^{b}	1.51 ± 0.69^{b}	*Xylanibacter ruminicola (90.89%)*
RA1-08049	$0.05 \pm <0.01^{a}$	2.03 ± 0.71^{b}	1.11 ± 0.45^{ab}	*Xylanibacter ruminicola (91.11%)*
RA1-00081	0.08 ± 0.01^{a}	6.58 ± 1.34^{b}	1.58 ± 0.67^{c}	*Prevotella ruminicola* (90.7%)
RA5-48363	1.95 ± 0.65^{a}	$0.01 \pm <0.01^{b}$	$0.01 \pm <0.01^{b}$	*Butyricimonas paravirosa* (81.8%)
RA3-082995	1.30 ± 0.36^{a}	$0.01 \pm <0.01^{b}$	$0.01 \pm <0.01^{b}$	*Prevotella ruminicola* (91.7%)
RA1-29009	$0.02 \pm <0.01^{a}$	0.09 ± 0.03^{b}	3.62 ± 3.25^{ab}	*Prevotella multisaccharivorax* (90.9%)
Proteobacteria				
RA1-36189	$0.02 \pm <0.01^{a}$	1.97 ± 0.85^{b}	1.59 ± 1.44^{ab}	*Haemophilus influenzae* (83.03%)
RA1-04216[#]	0.05 ± 0.01	6.97 ± 3.00	0.21 ± 0.11	*Succinivibrio dextrinosolvens* (96.9%)

[1] Mean relative abundance of taxonomic groups is presented as a percentage (%) of the total number of analyzed reads per sample. Different letter superscripts in the same row indicate that groups were significantly different ($P < 0.05$). [#] OTUs between the groups were not found to be significantly different by the Wilcoxon rank sum test. [*] Nucleotide sequence identity (%) between each OTU and its corresponding closest valid relative.

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CHAPTER 3: METABOLIC POTENTIAL OF THREE NOVEL RUMEN BACTERIAL SPECIES FROM SHEEP CHARACTERIZED BY UTILIZING A METAGENOMIC-BASED APPROACH

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ABSTRACT

The sheep rumen microbiome contains a vast wealth of known and unknown members, whose metabolic processes impact nutrient acquisition, as well as gut and overall health of the animal. Based on previous research from our group on investigating the impact of a change in diet on the composition of the rumen microbiome in lambs, further insights into the metabolic functions of novel bacterial species represented by three Operational Taxonomic Units (OTUs) were pursued. The rumen fluid samples with the highest abundance of OTUs RA1-01223, RA1-00032, and RA1-04216 were respectively used as representatives for metagenomic investigations to assemble the genomes of their corresponding bacterial species. Purified microbial genomic DNA was used as a template for 'shotgun' sequencing with an Illumina Miseq (2 x 250) platform. Raw datasets were filtered for quality then used for contig assembly with an in-house developed set of Perl scripts, generating 64 contigs for RA1-01223 (combined total length: 2,046,349 base pairs (bp)), 118 contigs for RA1-00032 (combined total length: 3,704,255 bp) and 119 contigs for RA1-04216 (combined total length: 8,884,743 bp). After contig building, coding sequences were identified and annotated using RAST, with

metabolic pathways of predicted enzymes constructed for each OTU of interest using KEGG pathways as a model reference. Annotated coding sequences were also screened by blastp in order to assign contig sets to each OTU based on their respective taxonomic affiliation; both OTU RA1-01223 and OTU RA1-00032 were found to be most closely related to *Ruminococcus bili* (94.8% and 86.8%, respectively), while RA1-04216 was affiliated to *Succinivibrio dextrinsolvens* (96.9%). As RA1-01223 and RA1-00032 were found to possess enzymes responsible for lactate metabolism, they could potentially affect susceptibility to acidosis by acting as modulators of ruminal pH or by producing lactate from starch degradation. Distinguishing features between these two OTUS included the pathway for vitamin B5 synthesis, using uracil or L-aspartate as precursors (RA1-01223), and the ability to produce ethanol as an end product of ruminal fermentation (RA1-00032). Distinguishing metabolic activities of interest for RA1-04216 included synthesis of chorismate and prephenate, which are precursors for tryptophan and phenylalanine, respectively. Together, these results contribute to a better understanding of the metabolic roles of individual microorganisms in the rumen, providing information that is crucial for creating a healthy and proactive environment that benefits the host.

Keywords: metagenomics, rumen, sheep, OTUs, metabolic potential

3.1. Introduction

Cellulose and starch are major substrates for the fermentation process that microorganisms in the rumen employ to provide energy to the host animal. They are broken down into volatile fatty acids (VFAs) like butyrate, acetate, and propionate to be used in further metabolic processes. While the general function of bacteria in the rumen is known to be fermentation of these carbohydrates and other substances, the individuality of each microorganism in the rumen and what they can digest is an important line of research to investigate. The bacteria in the rumen are largely undiscovered, both due to the sheer number of bacteria and the difficulty of culturing unknown species (Creevey et al., 2014; Stewart, 2012). Consequently, the metabolic potential of the majority of microbial species in the rumen is still undiscovered.

Previous research has focused on the identification of new species of bacteria in the rumen by utilizing 16S rRNA analysis. While 16S analysis does answer the question of "who", it does not answer the question of "how". Microbial species that are not found to be 97% similar to their closest taxonomic relative can be considered novel species, and their function is therefore unknown. The closest valid relative does give a clue as to their metabolic potential, but it is not definite proof of function. Presently, more research has been conducted in the field of metagenomics. With metagenomics, the construction of long nucleotide sequences that correspond to microbial chromosomes allows the identification of enzymes that can be expressed and used by a bacterial species in either a catabolic or anabolic setting. Together, 16S rRNA and metagenomic analysis can elucidate the potential function of novel bacteria that are found in the rumen.

Knowing the function of each species in the rumen can further the understanding of how that complex ecosystem works to provide for the host and how different microorganisms interact. To that end, the present study aimed to determine the metabolic potential of three novel rumen bacterial species that were first identified through 16S rRNA analysis.

3.2. Materials and Methods

3.2.1. Metagenomic Analysis

Select samples with the highest abundance of OTUs of interest (RA1-01223, RA1-00032, and RA1-04216) were further analyzed by metagenomics (Table 3.1). Purified microbial genomic DNA was sequenced using a 'shotgun' approach with an Illumina MiSeq (2 x 250) platform (Molecular Research DNA, Shallowater, TX, USA). Raw datasets were filtered to keep reads with a length of at least 200 bp, which were then used for metagenomic contig assembly with an in-house-developed set of Perl scripts. Considering the complexity of ruminal bacterial communities in this study, as assessed by 16S rRNA gene composition analysis, it was assumed that not all contigs assembled from a given sample would necessarily belong to its most abundant OTU. To this end, an amino acid sequence comparison-based approach was developed to identify contigs most likely to belong to each OTU of interest. More specifically, the analysis consisted of using blastp (Altschul et al., 1997) to compare the translated coding sequences from the contig set of a sample with the genome-translated proteome of a close valid relative of its corresponding OTU. For RA1-04216, genomes from *Succinivibrio dextrinosolvens* were readily available to use as reference (Genbank Accession: GCF_016747875.1). However,

in the absence of available genome sequence information for *Ruminococcoides bili*, *Ruminococcus bromii* (Genbank Accession: GCF_002834225.1) and *Ruminococcus bovis* (Genbank Accession: GCF_005601135.1) were used as representative valid taxa for both RA1-00032 and RA1-01223. Based on prior research, thresholds of 50% amino acid sequence identity and 70% alignment length were used as cutoffs for assigning contigs to an OTU (Bandarupalli & St-Pierre, 2023).

The metabolic potential of each OTU was then assessed from the translated proteome of its corresponding contig set. Pathways were predicted through a combination of gene annotation by RAST (Rapid Annotation using Subsystems) and model pathway references from KEGG (Kyoto Encyclopedia of Genes and Genomes) (Aziz et al., 2008; Kanehisa & Goto, 2000). Coding sequences were also annotated using dbCAN3 (automated carbohydrate-active enzyme & substrate annotation) (Zheng et al., 2023), which is based on the CAZy (Carbohydrate-Active Enzymes) database (Drula et al., 2022). CAZy provides extensive information on the different families of enzymes that degrade, modify, or create glycosidic bonds. A contig coding sequence was considered to be a positive match to a CAZy enzyme when all three tools from dbCAN3 (HMMER: dbCAN, DIAMOND: CAZy, and HMMER: dbCAN_sub) identified the same GH family member.

3.3. Results

3.3.1. Overview of Approach

To further understand the metabolic capabilities of selected OTUs, a metagenomics approach was used. It is reasonable to assume that sequence datasets

generated from samples with a high abundance of a specific OTU would possess a higher abundance of sequence reads from that OTU, which in turn would be more favorable for the assembly of contigs that would yield sufficient coverage of the genome for that OTU, as well as its metabolic potential. Considering that the OTUs of interest correspond to uncharacterized species for which chromosome length is unknown, coding sequences for ribosomal proteins were used to assess genome completion, since all bacterial species require a full set of ribosomal protein sequences.

3.3.2. Metabolic Potential of RA-01223

With that in mind, 64 contigs were generated for a combined length of 2,046,349 bp from 25,859,525 reads obtained from the culture that possessed the highest abundance of OTU RA1-01223 (37.2%). Contig size ranged from 4 bp to 286,948 bp, with a N50 of 75,026 bp. A blastp-based approach was then used to identify contigs most likely to correspond to Ruminococcus bromii and Ruminococcus bovis as they are closely related to Ruminococcoides bili, as well as RA1-01223 (94.8% identity). From that, a subset of 11 contigs were identified, ranging from 8,747 bp to 286,948 bp and a total length of 913,345 bp (N50 = 107,062). Assessment of the ribosomal proteins disclosed that 6 of the 21 small subunit proteins were present (S6p, S7p, S10p, S12p, S16p, S18p) and 7 of the 33 large subunit proteins were found (L1P, L9P, L10P, L11P, L7/L12P, L31P, L33P). A total of 971 coding sequences were annotated, with 472 coding sequences belonging to hypothetical proteins and 499 belonging to proteins of known function.

A complete pathway for the synthesis of (R)-pantothenate (vitamin B5) from uracil was found in the selected contigs, which indicates the capability for production of

vitamin B5 in the rumen by OTU RA-01223 (Figure 3.1). Uracil is predicted to be a major substrate for the formation of vitamin B5 and other pathways due to the presence of an uracil permease, in addition to an uracil phosphoribosyltransferase (EC 2.4.2.9). The coding sequence of the enzyme required to change L-aspartate to beta-alanine was also discovered, which allows another substrate to be utilized for the production of either vitamin B5 or beta-alanine, which is essential for cell wall synthesis. Oligosaccharides, were predicted to be utilized by RA1-01223 which was supported by the presence of coding sequences for both an alpha-glucosidase (EC 3.2.1.20) and a beta-galactosidase (EC 3.2.1.23). Both enzymes belong to families of glycoside hydrolases (GH2 and GH13) and play a role in cleaving alpha and beta linkages, respectively, to release substrates such as glucose and galactose that can then be used for fermentation by the bacterial cell. In terms of acid metabolism, the presence of lactate dehydrogenase also indicated that RA-01223 could produce or utilize lactate.

3.3.3. Metabolic Potential of RA1-00032

A total of 118 contigs with a combined length of 3,704,255 bp (N50 = 68,718) were generated from 26,554,126 reads from the culture that had the highest abundance of RA1-00032 (30.9%). Those 118 contigs ranged from 478 bp to 214,179 bp. From those contigs, 11 were assigned to the OTU through a blastp-based approach which matched contigs that belonged to *R. bovis* and *R. bromii* as RA1-00032's closest valid relative was the same as RA1-01223 but had a lower identity percentage match at 86.8%. Those contigs ranged from 29,311 bp to 214,179 bp, had a total length of 864,996, and a N50 of 86,048 bp, from which 897 coding sequences were annotated;470 coding sequences were

designated as hypothetical proteins, while 427 coding sequences were matched to enzymes of known function. Thirteen of the 21 small ribosomal subunit proteins were identified (missing: S1p, S6p, S9p, S15p, S16p, S18p, S20p, and S21p) and 20 of the 33 large ribosomal subunits were present in the contig set (missing: L1P, L9P, L10P, L11P, L7/L12P, L13P, L21P, L27P, L28P, L31P, L33P, L34P, and L36P). Using the USearch-based approach to target large ribosomal subunit proteins that were missing or had not been built yet, five additional proteins were found: L1P, L10P, L11P, L7/L12P, and L33P, which is a total of 25 large subunit proteins found overall. While incomplete, the results indicated that the contig set could provide a partial assessment of the metabolic potential of RA1-00032.

Similar to RA1-01223, RA1-00032 could have the potential to utilize lactate by the presence of D-lactate dehydrogenase (EC 1.1.1.28), although whether the OTU produces or utilizes lactate is currently unknown. Producing lactate can become a problem in the rumen, as its accumulation lowers pH and causes or exacerbates the onset of acidosis (Slyter, 1976). On the other hand, utilization of lactate suggests a possible defense against the buildup of lactic acid, and the production of substrates to be used for ATP synthesis. In terms of substrate production, a coding sequence was found for glycogen phosphorylase (EC 2.4.1.1) which suggests the use of glycogen to supply glucose to the microorganism. An incomplete pathway for the production of ethanol from glucose was discovered using the USearch-based approach by targeting common kmers found in glycolysis enzymes (Figure 3.2) Additionally, a glycoside hydrolase from the GH77 family was also identified (Table 3.2). As a monospecific family with amylomaltase (EC 2.4.1.25), that enzyme is responsible for branching and transferring a

segment of a (1->4)-alpha-D-glucan to a new position. Other potential substrates include spermidine and putrescine (polyamides) as indicated by the presence of the polyamine ABC transporter-type system components potA, potB, potC, and potD. Finally, chitin could also be a substrate of choice for RA1-00032, with the presence of coding sequences for chitinase (EC 3.2.1.14), which was confirmed through dbCAN3 by its identification as a member of GH families GH18 and GH19 (Table 3.2).

Intriguingly, nitrilase (EC 3.5.5.7) was also encoded in the contig set for RA1-00032. Nitrilase is an important enzyme in the degradation of aliphatic nitriles, which are toxic to the host (Ramteke et al., 2013). Nitriles can be produced during the breakdown of glucosinolates, which are plant compounds that can be present in certain feeds.

3.3.4. Metabolic Potential of RA1-04216

The rumen culture with the highest abundance of OTU RA1-04216 (22.23%) was selected for metagenomics analysis. From a total of 27,551,188 reads generated from that sample, 119 contigs were assembled for a combined length of 8,884,743 bp, with contigs ranging from 420 bp to 403,325 bp, and an N50 of 156,216 bp. A blastp-based approach was utilized to identify contigs closely related to *Succinivibrio dextrinosolvens*, due to its close match to the OTU. Using this approach, 14 contigs were selected, ranging from 420 bp to 403,325 bp, with a length of 1,667,353 (N50 = 170,841 bp). The extent of coverage of the 14 contig dataset for RA1-04216 was assessed by surveying the encoded ribosomal proteins with analysis revealing that 12 of the 21 small subunit ribosomal proteins were found (missing: S4p, S6p, S7p, S9p, S12p, S15p, S18p, S20p, and S21p) and 19 of the 33 large subunit ribosomal proteins were found (missing: L1P, L10P, L11P, L7/L12P, L13P,

L19P, L20P, L25P, L28P, L32P, L33P, L34P, L35P, and L36P). These results indicated that the 7 contig dataset, while incomplete, would offer sufficient coverage to assess the metabolic potential of RA1-04216.

A total of 619 coding sequences were annotated, with 282 coding sequences assigned to proteins of known function, and 337 coding sequences that were categorized as “hypothetical” proteins. In terms of end-products, lactate and formate could be generated from pyruvate due to the presence of L-lactate dehydrogenase (EC 1.1.1.27) and pyruvate formate lyase (EC 2.3.1.54), which can also produce acetyl-CoA to be further metabolized into acetate, an end product that can be utilized by the host for energy. Shikimate, as well as 3-dehydroquinate, were predicted to act as substrates due to the presence of coding sequences for most of the enzymes required to produce chorismate and prephenate (Figure 3.3).

3.4. Discussion

Understanding the metabolism of microorganisms in the rumen is essential when determining what feed to provide to the animal, as well as any future applications of additives such as pre- or probiotics. The observed OTUs that were selected for further metagenomic analysis were the highest in abundance in each respective sample that they were in, and all three shared the characteristic of a high abundance in sheep fed a concentrate-based diet.

Consistent with the utilization or production of lactate by starch digesters, all three OTUs included coding sequences for L- and D-lactate dehydrogenase (EC 1.1.1.27,

EC 1.1.1.28). Previous research has found that a sudden switch from a forage-based diet to a concentrate-based diet can result in acute acidosis (Seddik et al., 2018; Petri et al., 2013). Due to the ability of amylolytic bacteria to degrade starch into glucose, which can then be fermented to produce ATP and lactate, the presence of lactate dehydrogenases is consistent. The question of whether or not the bacterial species produce or utilize lactate for further use still remains to be determined. In low methane yielding sheep, the production and utilization of lactate by bacterial species such as *Sharpea azabuensis* and different species of *Megasphaera* has been quantified (Kamke et al., 2016). The OTUs explored in this study could potentially play a role in generating substrates for these bacterial species found in low methane producing sheep. Little is known about *Ruminococcoides bili*, but it has been found to be metabolically similar to other species in the *Ruminococcaceae* family, such as *R. bromii*. Molinero et al (2021) determined that an isolate of *R. bili* had the ability to produce lactate, further supporting the present study.

RA1-01223 and RA1-00032 shared a closest valid relative in *Ruminococcoides bili* at 94.8% and 86.8% similarity respectively. As previously stated, little has been discovered about *R. bili*, but similarities to *R. bromii* and *R. bovis* were seen in enzymes present. *R. bovis* has been found to ferment glucose, fructose, and galactose (Gaffney et al., 2021), which is consistent with coding sequences found for glycosidic hydrolases that are pivotal in the breakdown of glucose and galactose in both OTUs.

RA1-01223 contained coding sequences for all enzymes present for the production of pantothenic acid, or vitamin B5. Vitamin B5 has been shown to be an important component of sterol formation and the formation of fatty acids (Tahiliani et al., 1991; Vijayalakshmy et al., 2018). Furthermore, beta-alanine was predicted to be a

substrate that is used in the formation of vitamin B5, and can play a role in improving nutrient digestibility, as well as promoting nitrogen utilization (Hu et al., 2024). In terms of energy production, contigs for RA1-01223 had coding sequences for almost all V-type ATP synthase subunits, only missing subunits G and H. These subunits assemble in a complex that generates ATP from a transmembrane gradient, which provides an important stream of cellular energy that the cell can use (Jonckheere et al., 2012).

RA1-04126 was closely related to *Succinivibrio dextrinsolvens* (96.9% similarity). In prior cases, *S. dextrinosolvens* was predicted to produce succinate as well as formate (Bryant & Small, 1955), which is supported by the identification of coding sequences for pyruvate formate lyase (EC 2.3.1.54) and succinate dehydrogenase (EC 1.3.5.1) in this study. Formate has been observed to be a byproduct of the reaction of pyruvate to acetyl-CoA through pyruvate formate lyase, which contributes to ATP production (Crain & Broderick, 2014). Additionally, succinate in the rumen is an essential component of increase propionate production, which ultimately is used for gluconeogenesis by the host (Abbas et al., 2020). While contigs for RA1-04216 did not contain as many coding sequences for enzymes involved in nitrogen metabolism as currently known strains of *S. dextrinosolvens* (Hailemariam et al., 2020), building additional contigs for this OTU could provide more insight on this aspect of RA1-04216 metabolism.

RA1-04216 contained the coding sequences of all enzymes for the metabolism of shikimate. As ruminants consume a large variety of plant material, the ingestion of quinate or other phenolic acids is very likely. The breakdown of both starch and cellulose gives rise to various short chain fatty acids (SCFAs), and it has been shown that the

proportion of those SCFAs is not only impacted by the specific carbohydrate that is fermented, but also by other compounds. For instance, butyrate has been found to be produced in a higher concentration when shikimate is digested, as opposed to acetate or propionate (Russell & Van Soest, 1984). The potential expression of enzymes that lead to the formation of all three SCFAs displays the range that RA1-04216 has on metabolizing different substrates in the rumen, and also a link between carbohydrate breakdown and aromatic biosynthesis, as the shikimate pathway can lead to the formation of amino acids such as phenylalanine or tryptophan.

Overall, the metagenomic analysis of three OTUs has revealed the potential functionality of novel bacterial species. However, similar to any other research, analyses such as these is faced with limitations. For example, computing power can affect the speed of building contigs, which can lead to delays in generating results. All sequences are compared to a reference database, so without a reliable reference, many enzymes might remain unidentified during the analysis. In addition, much like the case for RA1-01223 and RA1-00032, genome sequences were not available for the closest valid taxonomic relative, so bacterial genomes of related but more distant species had to be used. These issues could be solved through genome sequencing of these novel species once they are isolated, as this would avoid contamination from other microorganisms, allowing a better comparison to genomes of related species.

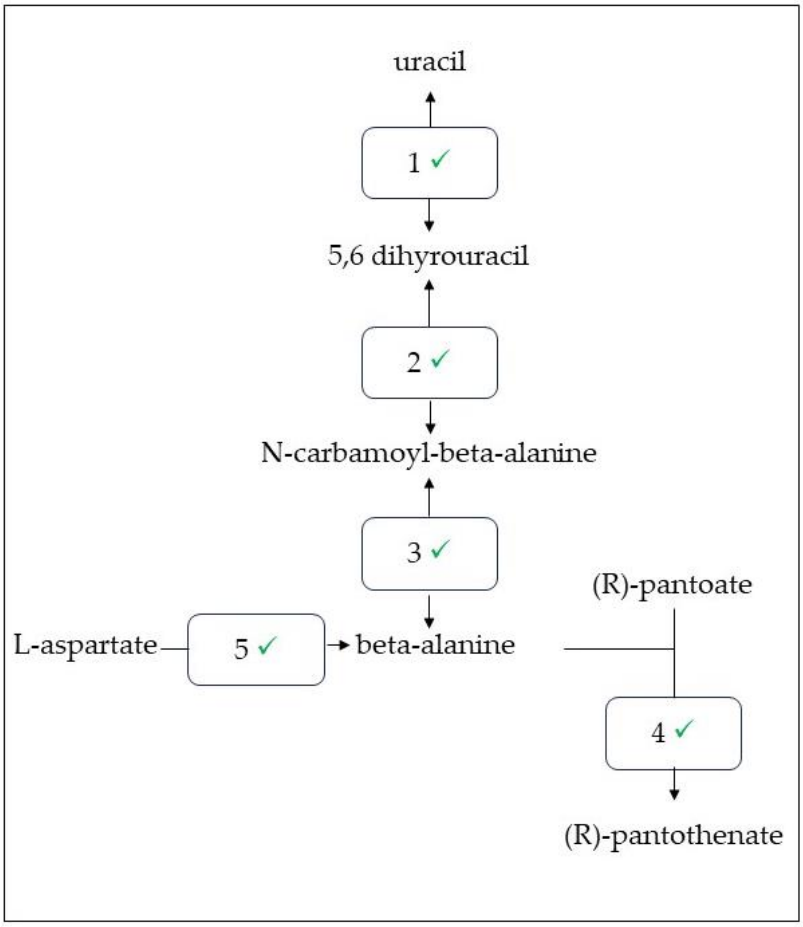

Figure 3.1. Proposed (R)-pantothenate synthesis pathway for RA1-01223. Key precursors for the pathway are uracil (pyrimidines), spermine (polyamine catabolism), or pyruvate (glycolysis). Enzymes labelled with a red x were not found, while enzymes labelled with a green checkmark were encoded in the contig set for RA1-012233. Key for enzyme legend: (1) dihydropyrimidine dehy-drogenase (EC 1.3.1.2); (2) dihydropyrimidinase (EC 3.5.2.2); (3) beta-ureidopropionase (EC 3.5.1.6); (4) pantoate synthetase (EC 6.3.2.1); (5) aspartate 1-decarboxylase (EC 4.1.1.11).

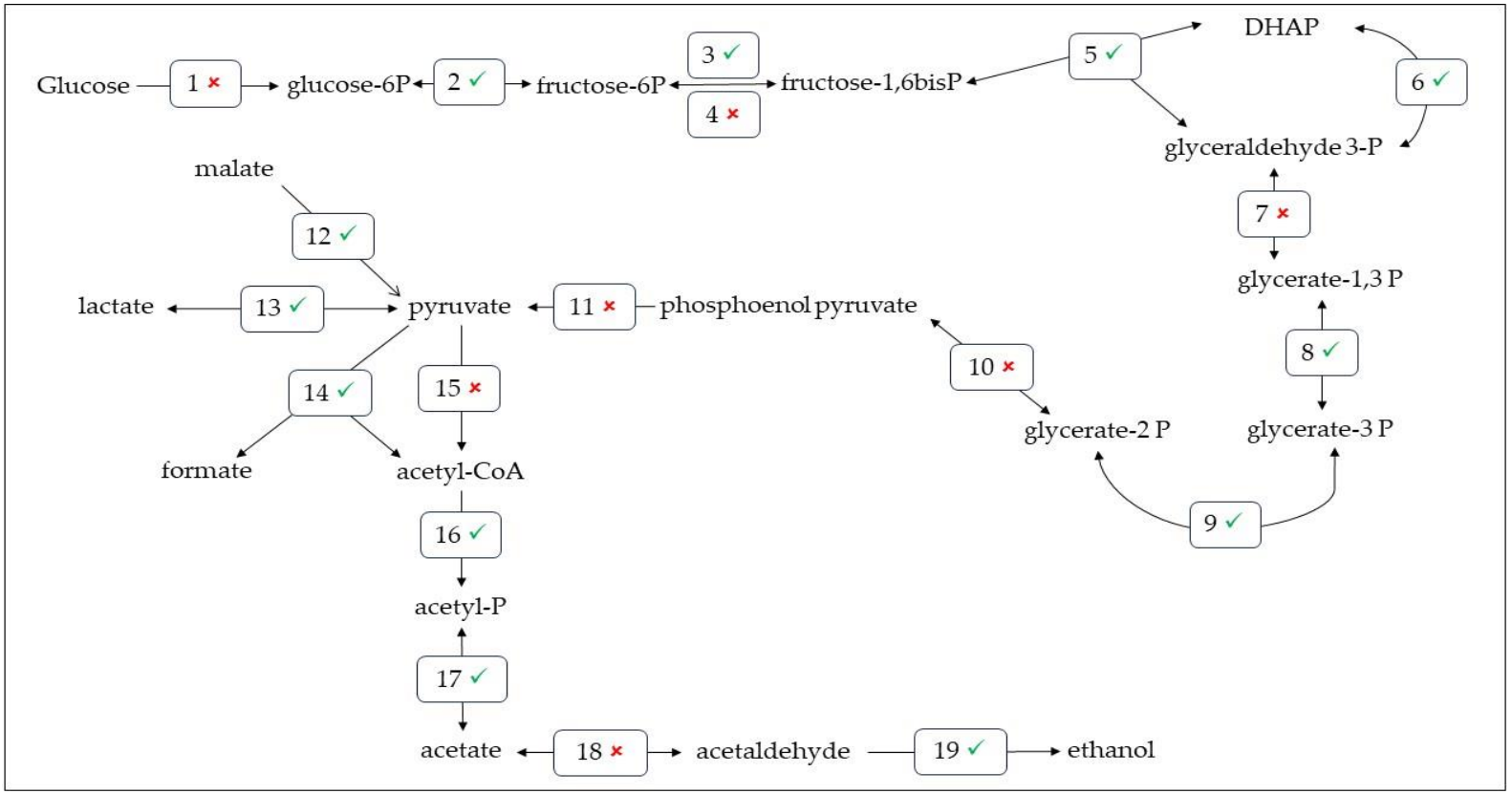

Figure 3.2. Proposed ethanol synthesis pathway for RA1-00032. Key precursors for the pathway are glucose, malate, and potentially lactate. Enzymes labelled with a red x were not found, while enzymes labelled with a green checkmark were encoded in the contig set for RA1-00032. Key for enzyme legend: (1) hexokinase (EC 2.7.1.2); (2) glucose-6 phosphate isomerase (EC 5.3.1.9); (3) 6-phosphofructokinase (EC 2.7.1.11); (4) fructose-1,6-bisphosphatase (EC 3.1.3.11); (5) aldolase (EC 4.1.2.13); (6) triosephosphate isomerase (EC 5.3.1.1); (7) glyceraldehyde-3-phosphate dehydro-genase (EC 1.2.1.12); (8) phosphoglycerate kinase (EC 2.7.2.3); (9) 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1); (10) enolase (EC 4.2.1.11); (11) pyruvate kinase (EC 2.7.1.40); (12) NADP-dependent malic enzyme (EC 1.1.1.40); (13) D-lactate dehydrogenase (EC 1.1.1.28); (14) pyruvate formate lyase (EC 2.3.1.54); (15) pyruvate-flavodoxin oxidoreductase (EC 1.2.7.-); (16) phosphate acetyltransferase (EC 2.3.1.8); (17) acetate kinase (EC 2.7.2.1); (18) aldehyde dehydrogenase (EC 1.2.1.5); (19) alcohol dehydrogenase (EC 1.1.1.1).

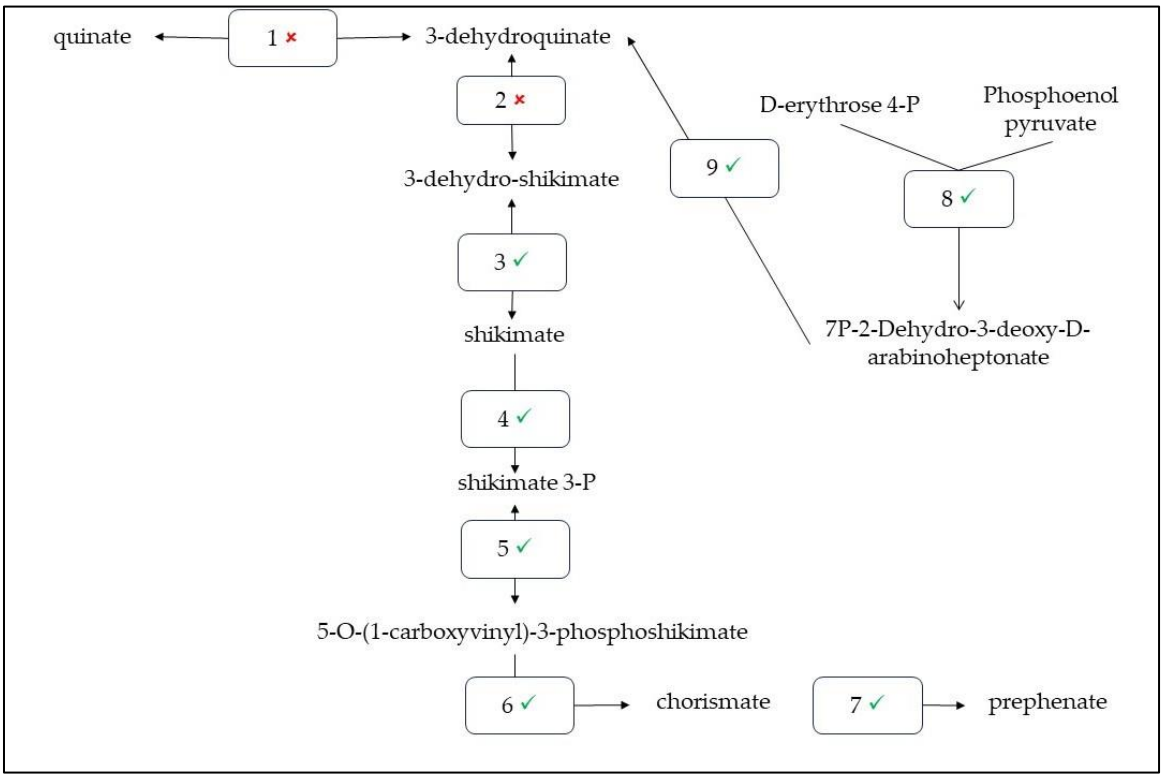

Figure 3.3. Proposed chorismate and prephenate synthesis pathway for RA1-04216. Key pre-cursors for the pathway are quinate or shikimate (plant compounds), or phosphoenol pyruvate (glycolysis) and-erythrose 4-P (pentose phosphate pathway). Enzymes labelled with a red x were not found, while enzymes labelled with a green checkmark were encoded in the contig set for RA1-04216. Key for enzyme legend: (1) quinate/shikimate dehydrogenase (EC 1.1.1.24); (2) 3-dehydroquinate dehydrase II (EC 4.2.1.10); (3) shikimate 5-dehydrogenase I alpha (EC 1.1.1.25); (4) shikimate kinase I (EC 2.7.1.71); (5) 5-Enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19); (6) chorismate synthase (EC 4.2.3.5); (7) chorismate mutase I (EC 5.4.99.5); (8) 3-deoxy-7-phosphoheptulonate synthase (EC 2.5.1.54); (9) 3-dehydroquinate synthase (EC 4.2.3.4).

Table 3.1. OTUs selected for metagenomic analysis. OTUs were identified in the previous experiment.

OTU	Phylum	Closest Valid Taxon (id%*)
RA1-01223	Bacillota	*Ruminococcoides bili* (94.8%)
RA1-00032	Bacillota	*Ruminococcoides bili* (86.8%)
RA1-04216	Pseudomonadota	*Succinivibrio dextrinosolvens* (96.9%)

* Nucleotide sequence identity (%) between each OTU and its corresponding closest valid relative.

Table 3.2. Presence of different glycoside hydrolase (GH) families as described by dbCAN3 analysis* in each OTU

GH Family	RA1-04216	RA1-01223	RA1-00032
2		**x**	**x**
3	**x**		
5		**x**	**x**
13	**x**	**x**	
18		**x**	**x**
19			**x**
20			**x**
23	**x**		
25		**x**	
42		**x**	
43		**x**	**x**
77			**x**
78		**x**	
94			**x**
130	**x**		

*Each family was counted only if all three dbCAN3 tools returned a result.

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CHAPTER 4: FUTURE RESEARCH AND IMPLICATIONS

4.1. Introduction

Although considered a small sector of the United States livestock sector, the sheep industry is providing essential outputs in the form of wool, meat, and live animals for both domestic and international use. More and more people are creating small sheep herds that help fill local and national demand. Due to the increase in operations, management style is an important factor in raising sheep to meet their genetic potential, and a key component of management is the diet that is fed to the animal.

Diet is an essential factor in the development and growth of an animal. In ruminants, feed is utilized by both the host and the microbial ecosystems that reside within the digestive tract. Supplying those communities within the animal with the correct nutrients is crucial for forming substrates that the animal can use for energy and metabolism. Ecological niches are formed within the rumen itself by different microbial species that have a specific function. Due to the composition of diets by most ruminants, major parties of carbohydrate-degrading bacteria are formed, which can vary depending on the type of carbohydrate that is being digested, such as starch, cellulose, hemicellulose, or pectin. The VFAs that are produced by bacteria in the rumen from the fermentation of feed are used by the animal as energy.

As diet is such an integral part of an animal's lifestyle, it is important to understand how altering the diets of ruminants, such as sheep, can affect their overall performance. The composition of the bacterial community in the rumen is in a constant state of flux as the animal ingests combinations of feed. The number of starch degrading

bacteria might be higher when the animal is fed a majority of concentrate, and the same is true for cellulolytic bacteria when the animal is fed forage. Knowing how the rumen microbiome reacts as the diet changes can be a factor for producers to make key decisions about what feed to buy or preventing digestive disorders by providing the animal feed that could throw the rumen equilibrium out of order.

To fully flesh out how transitioning to different diets ultimately affects ruminal bacteria composition in sheep, the first study outlined in this thesis aimed to compare a transition from forage-based diets to concentrate-based diets. To further elucidate unknown members of the rumen microbiome, the second study presented in this thesis offered a metagenomic analysis of the metabolic potential of highly abundant bacterial species that were found in sheep.

4.2. Research Findings and Implications

The results from the first study support previous research in ruminant microbiomes by identifying taxonomic changes when diets were altered. Lambs that were fed concentrate-based diets had a higher abundance of bacteria affiliated with the phylum Proteobacteria, whereas the abundance of Bacillota and Bacteroidota were relatively similar. These three phyla can be seen time and time again in sheep and other ruminants as the mainstays of the rumen microbiome. Additionally, as species-level OTUs were identified through data analysis, close relatives of key bacteria were found to be in a higher abundance in concentrate diets. Relatives of *Ruminococcoides bili, Prevotella* spp., *and Succinivibrio dextrinosolvens* were in much higher abundance in concentrate diets. *R. bili* has been shown to degrade different starches (Molinero et al., 2022), as have

many *Prevotella* species (Betancur-Murillo et al., 2023), which is essential for the catabolism of plant energy storage in concentrate-based diets. The difference in abundance between the two diets suggests that the diets did play a role in what population of bacterial thrived in the rumen.

Due to the rumen bacterial microbiome being largely uncharacterized (Creevey et al., 2014), further metagenomic analysis of metabolic potential is needed to uncover specific function of all bacterial members of the rumen community. The analysis of the second study found enzymes that were a part of essential pathways for substrate production. Glycogen synthesis, vitamin B5 synthesis, lactate production, and chorismate utilization were all pathways predicted to be used by the OTUs of interest due to the presence of all or a majority of enzymes needed. To determine if the genes for the enzymes found were actually active, more investigations using metatranscriptomics could reveal active genes and uncover the full potential of bacterial species in the rumen.

4.3. Limitations and Future Research

Performing reproducible experiments is a major requirement in any scientific setting. As the rumen presents a mostly unexplored microbial environment that is constantly changing, reliable reproducible research can be challenge, considering how microbiome research is dependent on methods that can vary amongst research groups for sampling, DNA isolation techniques or data analysis. One option is to utilize metagenomic analysis in conjunction with 16S rRNA analysis to offset some of the limitations presented when using one or the other. Metagenomics can offer a more comprehensive picture of metabolic potential of the rumen as a whole, while 16S rRNA

allows a more accurate assessment of composition and can better pinpoint previously uncharacterized species. Together, this approach could potentially be used to characterize the metabolic function of the ovine hindgut, as, while the composition has been previously studied, little is known about the true metabolic potential of all members of that microbial community (Xu et al., 2023).

The microbial communities of the hindgut have been even less investigated in ruminants, as the contributions of hindgut fermentation activities to the nutrition of the host are not nearly as impactful compared to the rumen. In beef cattle, they have been characterized as a potential source of dysbiosis, or an imbalance in the composition of microbial species within a community, that could lead to disease and unhealthy animals (Gomez et al., 2017). Much like cattle, sheep also ferment substrates in their hindgut. As they can suffer from losses due to conditions like scours, elucidating the functions of hindgut microbial symbionts in sheep could lead to the design of pre- or probiotics that could have beneficial health applications in the livestock industry.

Large operations in the livestock industry target maximized performance and output of their animals while reducing risk and stress to the animal. Further research into disease prevention or key players of the microbiome that help with resistance can be accomplished through metagenomic analyses to see the potential role of bacteria in more specific functional settings. One example is the interactions with the host epithelium. For instance, prior studies of the sheep microbiome and its association with the epithelial response to infestation by breech flies has demonstrated that the composition and metabolic potential of the microbiome associated with the sheep epithelium is mostly unknown (Greeff et al., 2021).

While many studies have evaluated the relationship between feed efficiency and microbiome composition or methane production, there are few that have investigated the relationship between the composition of the rumen microbiome and the composition of the hindgut. Although they have different functions, both areas of the digestive tract focus on the fermentation of substrates into products usable by the host. To that end, there have been no comparable studies in sheep that have characterized any patterns in changes to composition of the microbiome of both the rumen and hindgut of animals transitioned to one diet or another. Most research focuses on changes in the composition of one area of the digestive tract. To combat this gap in knowledge, performing a comparative study where changes in both the rumen and hindgut microbiome could be illustrated is essential in understanding the role of microbes in all corners of the animal's digestive tract.

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