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Characterization of the Rumen Bacterial Communities of Bison Heifers Fed a Grass-Based Diet vs a Grain-Based Free-Choice Diet

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CHARACTERIZATION OF THE RUMEN BACTERIAL COMMUNITIES OF BISON HEIFERS FED A GRASS-BASED DIET VS A GRAIN-BASED FREE-CHOICE DIET

BY

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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
ARG	Antibiotic resistant genes
BCGrass	Animal from Blue Creek ranch fed a grass-based diet
BCGrain	Animal from Blue Creek ranch fed a grass-based diet
BCR	Blue Creek Ranch
bp	Base pair
DNA	Deoxyribonucleic acid
MCFAs	Medium chain fatty acids
NCBI	National center for biotechnology information
ng	Nanograms
OTU	Operational taxonomic unit
ORF	On ranch feeding
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis
p-RFI	Positive residual feed intake
RDP	Ribosomal database project
SARA	Subacute rumen acidosis
SBR	Standing Butte Ranch
SBGrass	Animals from Standing butte ranch fed a grass-based diet
SBGrain	Animals from Standing butte ranch fed a grain-based diet
SCFAs	Short chain fatty acids
TMR	Total mix ration

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ABSTRACT

CHARACTERIZATION OF THE RUMEN BACTERIAL COMMUNITIES OF BISON HEIFERS FED A GRASS-BASED DIET VS A GRAIN-BASED FREE-CHOICE DIET

ANLLY FRESNO RUEDA

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A century ago, the North American grasslands and prairie ecosystems were dominated by bison. At least 30 million bison roamed the Great Plains when the first explorers arrived. By 1900, there were little over a thousand bison remained in the United States and Canada. Recovery efforts has been made since the 20th century to reestablish the herds and increase the bison population. Today, over 500,000 bison are distributed across North America, with more than 90% of the existing bison population under commercial production. Modern conservation strategies are made via the collaborative efforts of conservationist, producers, and researchers, resulting in increased number of proposed research to better understand bison's biology. Given that the ruminal bacterial communities of North American bison are one of the most understudied areas of bison research, the aim of the current study was to determine and compare the diversity and composition of ruminal bacteria between bison heifers on two different diets at two different ranches. Stomach tubing was used to collect rumen fluid from lifetime grass-fed heifers between 25 and 30 months of age distributed between 2 ranches located in Standing-Butte (SBR; n=17), SD, and Blue-Creek (BCR; n=17), NE, respectively. A second set of samples was collected after the same individuals had been transitioned to a grain-based free-choice diet for 100 days. Bacterial composition was determined by

Illumina MiSeq (2×300) sequencing of PCR amplicons generated from the V1-V3 region of the 16S rRNA gene. Next-Generation Sequence data was analyzed using a combination of custom Perl scripts, and publicly available software (Mothur v.1.40, RDP classifier and NCBI Blast). Taxonomic analysis identified Bacteroidetes and Firmicutes as the dominant phyla across all samples analyzed. A total of 57,132 and 59,133 species-level Operational Taxonomic Units (OTUs) were identified in SBR and BCR grass-fed heifers, respectively, in contrast to 13,240 and 22,516 OTUs that were found in the same animals on a grain-based diet. A comparative analysis using the most abundant OTUs from each group was conducted using the Kruskal-Wallis sum-rank test. In the Standing Butte heifers, 28 abundant OTUs were found to be different between diets ($P < 0.05$), including Bb-00031 ($\overline{x}$grass = 0.04% vs $\overline{x}$grain = 1.45%) and Bb-00018 ($\overline{x}$grass = 0.58% vs $\overline{x}$grain = 0.06%). In the Blue Creek heifers, 17 of the most abundant OTUs were found to be different between diets ($P < 0.05$), including Bb-00046 ($\overline{x}$grass = 1.24% vs$\overline{x}$grain = 0.45%) and Bb-00058 ($\overline{x}$grass = 0.03% vs$\overline{x}$grain = 1.22%). Together these results indicate that the rumen of the North American bison harbors highly diverse bacterial communities that undergo dramatic changes in response to changes in diet, and they represent a starting point towards a better understanding of their rumen microbiome, leading to prospective practical applications to bison conservation and production.

CHAPTER 1

Literature Review

1. AMERICAN BISON: A BRIEF HISTORY

The North American bison (*Bison bison*) is one of the eight known species of bison (two extant and six extinct) that emerged around 5,000 to 10,000 years ago (Wilson et al., 2008). Since its appearance, the bison was hunted by humans mainly for food and clothing, and for political reasons to the point of near-extinction in the 19th century. Isenberg (2020) stated that the over harvest in the 1800s was partially driven by the intention of the U.S. government to restrict the range of bison and control Native Americans whose diet and culture relied on the bison herds. In addition, introduced bovine diseases and competition from domestic livestock (horses, cattle, sheep) also reduced bison numbers (Flores, 1991; Isenberg, 2020). Based on the accounts of Euro-American explorers, settlers, and hunters, the population of bison is estimated to have once been between 15 and 100 million (Dary, 1989; Demarais and Krausman, 2000), but by the late 19th Century, there were fewer than 1,000 remaining bison in North America (Homaday, 1887; Seton, 1927).

There was public concern as the large herds decreased, but few laws were passed to protect the bison (Danz, 1997). Most of the early attempts to conserve bison came from individual acts of private citizens, and their efforts preserved the founding stock for most contemporary bison herds (Gates et al., 2010). From the 1870s onwards, bison hunting was prohibited, and the number of these ruminants increased considerably, doubling between 1888 and 1902. In 1905, the American Bison Society (ABS) was

founded; this organization managed to develop many public herds of bison after lobbying the United States Congress (Coder, 1975; Danz, 1997). This initiative was also supported by Canada (Ogilivie, 1979). By 1909, the bison was no longer considered an endangered species (Coder, 1975). Then, by 1970, there were about 30,000 animals in North America, with approximately half of them in public herds and half in private herds (Demarais and Krausman, 2000). As of 2010, there were more than 20,500 bison in publicly owned conservation herds (Gates et al., 2010) and more than 400,000 raised privately, most for commercial purposes.

Nowadays, the bison herds are being restored via the combined efforts of conservationists, ranchers, and scientists. The National Bison Association (NBA) for example, is working towards achieving a goal of one million bison in the United States and Canada. The initiative is called “Bison 1 million”, and it claims that if herds could expand at a rate of around 10 % per year, the total North American populations would surpass one million animals by 2025 (Association, 2006). Other attempts include the reintroduction of free-ranging plains and wood bison at different places across their historical ranges to preserve wild bison's survival. The long-term goal of this initiative led by the Wildlife Conservation Society (WCS), is to reestablish large, free-ranging herds in extensive native habitats to promote interactions with other species and therefore support the ecological recovery of bison over their entire range. The future of bison restoration is also oriented towards increasing bison research and collecting more relevant data that will contribute to better understanding bison’s biology. South Dakota State University, the NBA, and the National Buffalo Association worked in collaboration to accomplish this objective. As a result, the Center of Excellence for Bison Studies was

established in 2020, and numerous bison research projects have been approved. From now on, this center for advance bison research will support restoration and production of bison herds in North America.

2. AMERICAN BISON: BIOLOGY

North American Bison possess unique physiological, anatomical, and behavioral characteristics. Such characteristics have enabled them to adapt and thrive under the broad range of North American climates and native habitats. Physiological adaptations, including lower metabolic activities during the winter, were observed by Christopherson et al. (1979), who showed major changes in seasonal energy metabolism compared to most ruminants. Furthermore, bison developed a thick pelage that acts as an insulating cape, helping them save energy during low-temperature periods (Peters and Slen, 1964). These biological characteristics allow them to survive in extremely cold conditions.

Bison also exhibit behavioral traits unique to the species. Grass availability brings together herds of cows, calves, and immature males with large bulls that naturally roam in solitary to start a new breeding season (Berger and Cunningham, 1994). A well-established behavior known as “wallowing”, often described as “dust bathing”, is commonly seen in bison of all ages and genders. Male wallowing is more commonly seen to stimulate females during the breeding season (Bowyer et al., 1998), but it can also help them lower body temperature during the hot seasons and alleviate skin discomfort due to insect bites (McMillan et al., 2000).

Bison distribution is very much dependent on the availability of food. When looking for food, bison herds maintain defined paths (Hornaday, 1889), preventing harm

to the prairie, and favoring the establishment of robust vegetation and other animal species inhabiting the area. For these ecological benefits, bison are often referred to in the literature as a keystone species.

3. AMERICAN BISON: ECOLOGICAL ROLE

Bison are considered an ecologically important species in the United States and Canada, as they help maintain native prairies and the biodiversity of grassland ecosystems. They contribute to their habitats through selective grazing (grazing patches), seed dispersal and trampling, nutrient redistribution (feces and urine deposition, as well as organic matter from the dead bodies of free-roaming bison), and by rubbing their bodies and horns on trees and shrubs (McHugh, 1958; Reynolds and Peden, 1987; Knapp et al., 1999). Habitat selection of free-roaming bison is made primarily based on nutritional requirements, forage quality, snow depth, and predator avoidance. Feeding behavior studies (Coppock et al., 1983; Hudson and Frank, 1987; Singer and Norland, 1994; Wallace et al., 1995) have previously reported that bison select more nutritious forages in a highly efficient manner that satisfies their nutritional needs. While such behavior seems very similar to cattle, bison tend to avoid grazing on previously grazed areas during the same growing season, thus minimizing overgrazing. In addition, bison spend less time near water bodies, making more use of steep slopes, softening them and reducing erosion (Reynolds et al., 1982; Miller et al., 2000).

Feeding patterns have also shown important symbiotic relationships with other animals. Bison, for example, are frequently seen surrounded by different species of birds. These birds benefit from the insects that fly or move around in response to bison

movements and equally favor the bison by removing the ones that can cause discomfort (Friedmann, 1929). Similarly, bison can also boost the habitat suitability for prairie dogs, pronghorns, and other mammals. When grazing, bison reduce the height of the vegetation, allowing the settlement and establishment of groups of these animals; together, they preserve the vegetation's heterogeneity and create areas with high-quality forage (Coppock et al., 1983; Coppock and Detling, 1986; Miller et al., 2000). During rainy seasons, bison wallows can collect water favoring wetland plant species that allow amphibians and invertebrates to reproduce (Polley and Wallace, 1986; Knapp et al., 1999), representing an important feature for semi-arid ecosystems (List et al., 2007). Other important ecological contributions of bison include being a food supply source for scavengers and modifying fire regimes by consuming woody vegetation (Sanderson et al., 2008).

4. AMERICAN BISON: NUTRITION

4.1. Feeding Preferences

4.1.1. Free-range bison

Historically, bison have been recognized as grass-eating species, but seasonal variations, geographic areas, and management (e.g., confinement) can strongly influence their diet. During the summer, autumn and spring, free-range bison eat a varied mix of grasses, sedges, woody plants, and forbs. At the same time, much of their diet (>90%) in the winter is composed of graminoids, including sedges and rushes (Reynolds et al., 1982; Reynolds and Peden, 1987). Bison consume high-quality grasses during seasons of abundance and, similar to cattle, avoid the use of woody plants and forbs (<10%) (Hartnett et al., 1997; Steuter and Hidinger, 1999). However, during shortage periods,

when the only feeding choice is low-quality forages such as rushes and sedges, bison show a superior adaptation and perform better compared to cattle (Feldhamer et al., 2003).

The type of plants eaten by bison also varies according to the region. Numerous studies have compared feeding preferences from different bison populations and seasons. The reports include bison from Northeastern and Southwestern Colorado (Peden, 1976), Southern Utah (Van Vuren and Bray, 1983), Yellowstone National Park, Wyoming and Northern Canada (Meagher, 1973; Reynolds et al., 1978), Wood Buffalo National Park (Soper, 1941) and Elk Island National Park (Holsworth, 1960). A preference for grasses and sedges was observed at most sites. More precisely, the most common plants consumed by bison included perennial grasses such as buffalo grass (*Bouteloua dactyloides*), gramma grass (*Bouteloua gracilis*), sand dropseed (*Sporobolus cryptandrus*), beard grass (*Polypogon monspeliensis*), windmill grass (*Chloris spp*.), wheatgrass (*Thinopyrum intermedium*), brome grass (*Bromus spp*.), June grass (*Koeleria macrantha*) as well as annual grasses such as wild oat grass (*Avena spp*.) and common barley (*Hordenum spp.*). As an exception to grass consumption, a plant generally referred to as four-wing saltbush (*Atriplex canescens*) had a higher intake in Arizona, accounting for about 71% of the diet (Peden et al., 1974).

There are many factors that can influence bison nutrition, in particular food intake, growth, and metabolism (Christopherson et al., 1979; Hawley, 1981). These variations are usually linked to seasonal changes, hormone fluctuations, and forage quality. For instance, the daily amount of food required by bison during the winter is comparatively lower (1.4-1.8% of body weight) than that required by cattle, which

normally ranges between 2.5-3% of body weight (Feist, 2000a). When bison metabolism slows down, so does consumption of feed. Even though the loss of weight is common, such circumstances do not represent a significant challenge for free-range bison, as they manage to cope with changes in seasons and survive. This physiology, however, can pose a challenge for bison producers, trying to market animals in lean seasons.

4.1.2. Diet selection for bison in confinement: Grass-fed vs Grain-fed

Bison are finished for market based on producer philosophies and consumer preferences. Although some ranches choose to keep their animals on grass until marketing, others use a free choice combination of forage and grain feeds, while some feed various total mixed ration formulas (TMR) in a confined setting.

The goal behind grass-fed or grass-finished bison is to raise animals under 'natural' conditions, reduce environmental effects, and produce leaner and healthier meat products (Carter et al., 2010). Nevertheless, these types of production systems face significant operational challenges, including the need for sufficient land availability, longer time to finish animals (between 26 and 36 months), lighter finishing weights, inconsistent delivery into marketplace, and reliance on local weather and environmental conditions (Carter et al., 2010). Finishing bison with grain can overcome some of these issues. A challenge for grain-feeding operations is the lack of knowledge on the effects of a high starch diet on bison rumen physiology and its microbial composition, and consequently on the animal's overall health (Carter et al., 2010).

For grass-fed operations, the type and extent of the naturally grown grass depend on the area in which the ranch is located. Rotational grazing systems, which are

traditionally used in livestock, are not extensively used in bison production (Gegner, 1999). However, Carter et al. (2010), suggest implementing these grazing systems as an economical and sustainable type of production, which also improves pasture quality. On the other hand, diets consisting of grain or concentrate are normally offered either in totally mixed or free choice rations. Corn (the basis of most high-energy diets), barley, oats, and field peas are among the ingredients widely used in grain-based diets (Feist, 2000b). The use of these diets increases during heat waves or harsh winters, where it becomes a struggle to bring bison to market weight.

Both feeding methods bring positive aspects that contribute to current market strategies. Researchers at Colorado State University conducted a test panel of grass-fed versus grain-fed bison meat (Moseley, 2001). Results showed that consumers had no preference in terms of softness and juiciness between the meats in both diets, implying that both feeding systems produce an acceptable quality product for the consumer. However, taste preference was greater for meat from bison-fed concentrate compared to grass-fed meat. Similarly, a more recent research conducted at South Dakota State University (Janssen et al., 2021) evaluated the influence of Grain- and Grass-Finishing systems on carcass characteristics, meat quality, nutritional composition, and consumer sensory attributes of bison meat. They concluded that finishing system had an impact on nutrient content and fatty acid composition, more specifically, that grass-finished bison steaks had lower cholesterol content, percent fat, and omega 6:3 fatty acid ratios when compared to grain-finished bison steaks, however similar to Moseley (2001) study, differences in carcass and meat quality characteristics did not translate to differences in consumer preferences. Another study, conducted at Thompson Rivers University

(Canada), showed nutritional differences between grass-fed and grain-fed bison products. Bison fed with grass contained omega 6 and omega 3 ratios of 3:1 while, those fed with grain were 7:1 (Turner et al., 2014). Together, these studies suggest that grass-fed meat should be marketed to health-conscious customers interested in a more balanced ratio of Omega 6 to Omega 3.

4.2. General Digestion

Bison digestive physiology is similar to other ruminants such as cattle, sheep, goats, and deer, including a stomach, divided into four separate compartments, which allows efficient digestion of fibrous materials. After the feed is ingested, it is immediately stored in the second and largest compartment known as the rumen. There, food is partially digested into cud through microbial fermentation, then pushed back into the reticulum, the first compartment of the stomach, before being sent back to the mouth for additional mechanical grinding by mastication (rumination). When rumen content achieves a smaller particulate size, it is redirected to the stomach's third and smallest compartment, the omasum. There, excess water from the digesta is absorbed, and the solid materials are further broken down into smaller particles. Digesta is then sent to the last stomach compartment, the abomasum, also known as the true stomach, where acidity and host enzymatic processes further digest the original rumen contents, including microbial biomass. Digesta then reaches the small intestine, where absorption of nutrients occurs. Finally, materials that have not been digested reach the hindgut, where fermentation by resident microbial communities takes place.

While bison digestive processes have shown to produce similar results to those in cattle during periods of high feed abundance, higher digestive efficiencies have been reported for bison when food quality and quantity is limited (Feldhamer et al., 2003). Greater bison efficiency has been largely attributed to the microbial fermentation processes that occur in the rumen, as it provides nutrients that the animal directly uses as a source of energy.

4.3. Rumen physiology

Ruminants can obtain nutrients from plants that humans and other non-ruminant species cannot digest. They owe this ability to their digestive system structure and its symbiosis with the microorganisms inhabiting the rumen. Frequently referred to as a fermentation vat, the rumen enables ruminants like bison to break down structural carbohydrates (cellulose, hemicellulose, and pectin), which animal enzymes cannot efficiently digest. The rumen can also digest other dietary components, including starch, fat, and proteins.

The size of the rumen varies according to age and animal size. In most bovines, it is estimated to account for around 6% of the live weight of the animal (Membrive, 2016), however it only accounts for about 3% in bison (DeLiberto, 1995). The volumetric capacity of the cattle rumen varies from 100 to 300 liters (Membrive, 2016), while ruminal volumetric capacity in bison was estimated to be approximately 57 liters (DeLiberto, 1995).

Ideal rumen conditions enable the optimal growth of microorganisms responsible for fermentative digestion. Such conditions include optimal pH and buffering capacity,

temperature, osmotic pressure, as well as anaerobic (oxygen-free) conditions. Normal rumen pH ranges from 5.5 to 7.0 (Krause and Oetzel, 2006; Membrive, 2016), and it is strongly influenced by diet and buffering capacity. Bison rumen samples showed a more alkaline environment, with pH values between 7.19 and 7.25 (Ribeiro et al., 2017). However, feeding high-quality alfalfa hay and low-quality prairie hay (Towne et al., 1989), alfalfa corn based diets (Varel and Dehority, 1989), or prairie hay per se (Towne et al., 1988), resulted in similar pH responses to those observed in cattle. Maintaining a normal rumen pH also depends on the buffering capacity of saliva (Castillo-González et al., 2014). Longer rumination periods stimulate ruminants to produce larger amounts of saliva (Bailey, 1961); a constant supply of saliva (pH = 8.2) sent to the rumen helps to maintain a favorable environment for microbial growth (Grosskopf, 1965; Krause and Oetzel, 2006). Consumers of grass and roughage such as bison have been reported to spend more time grazing and less time ruminating, suggesting lower levels of saliva production (Asplund, 1994).

Microbial activity is also conditioned by a relatively constant rumen temperature. Hungate (2013) stated that ruminal temperature normally exceeds the ruminant's body temperature (38ºC). Factors such as food intake and fermentation processes (Gilchrist, 1957), cold water consumption (Cunningham et al., 1964), and ambient temperature (Boehmer et al., 2015) can influence ruminal temperature conditions (39ºC - 40ºC). Ruminal temperature above normal levels may affect the survival of rumen-populating microorganisms, as many of them cannot survive above 40 ºC (Hungate, 2013).

Ruminal content retains an osmotic pressure to prevent excessive water loss. This osmotic pressure is often subject to change with feeding regimens. For instance, rumen

osmotic pressure before feeding has been reported at < 200 mOsmol Kg^{-1} (Warner and Stacy, 1977), but this value increased to 350-400 mOsmol Kg^{-1} after a meal (Warner and Stacy, 1977; Bennink et al., 1978). Constant homeostatic conditions are necessary for ruminal microorganisms, as their survival can be compromised when high and sudden rumen osmolality variations occur (Grünberg and Constable, 2009).

Most rumen microorganisms are strictly anaerobic, meaning that they cannot use oxygen and will only survive in its absence. Anaerobic digestion of feed produces short-chain fatty acids (SCFA), mainly acetic, propionic, and butyric acids, as well as carbon dioxide (CO_2) and methane (CH_4). SCFAs are removed from the rumen through absorption by the epithelial cell wall and become the ruminant's main source of energy, as they conserve a large part (75%) of the energy from feed that is stored as glucose (Hungate, 2013). Other products of microbial metabolism, such as CO_2 and CH_4, are eliminated through eructation.

5. AMERICAN BISON: RUMEN MICROBIOLOGY

5.1. Importance of studying the rumen microbiome

It is important to study ruminal microorganisms (microbes) and their genomes (microbiome) because of their direct impact on the ruminant's productive efficiency and health. This direct relationship has been demonstrated in cattle by a number of studies conducted over the last decade (Jami et al., 2014; McCann et al., 2014a; Myer et al., 2015; Shabat et al., 2016; Li, 2017a; Schären et al., 2018). For example, Jami et al. (2014), found a connection between the physiological parameters of dairy cattle (milk yield and composition) and their resident rumen bacteria; in that study, *Prevotella* was

negatively associated with milk fat production. Other studies have also found members of the *Prevotella* genus that are associated with rumen acidosis (Golder et al., 2014), or with positive residual feed intake (p-RFI) in Brahman cattle (McCann et al., 2014b).

Diet is the main factor influencing the type of microorganisms in the rumen (Henderson et al., 2015). Inclusion of new ingredients, supplements, or additives to ruminant diets have led to improvements in feed utilization, mitigation of metabolic disorders, as well as the reduction in methane production, thus achieving higher efficiencies. Certain tannins, for instance, have been found to favorably modulate the rumen microbial ecosystem, improving body weight, milk yields, and reproductive performance, as well as decreasing methane production (Patra and Saxena, 2011). Furthermore, Lettat et al. (2012) suggested that the use of bacterial probiotics (*Lactobacilli* and *Propionibacterium*) is efficient in preventing subacute rumen acidosis (SARA). These types of approaches are not always effective in adult ruminants due to the resistance of the microbial environment to changes (Weimer et al., 2010). Hence, more research is directed towards interventions in pre-ruminant animals, which could theoretically give an animal good immune responses along with adulthood permanence (Yáñez-Ruiz et al., 2015).

One of the most controversial topics today is the emergence and spread of antibiotic-resistant genes (ARG) and the contribution of animal production to this problem. It has been recently discovered that diet has important effects on the representation of ARG in microbial rumen populations, with potential consequences for human and animal health (Auffret et al., 2017). In that study, high grain diets enhanced the abundance and diversity of ARG.

Development of new research techniques has led to important advances in microbiome knowledge in dairy and beef cattle. A better understanding of the rumen microbial composition has and will continue to lead to strategies that improve animal performance and well-being.

5.2. The rumen microbial ecosystem

The ruminal microbiota is a diverse and complex assortment of microbial groups that, in relation to its metabolic functions, constitute a crucial element for the development, health, and nutrition of the ruminant (Morgavi et al., 2010). These microorganisms can differ widely depending on several factors, including diet, host species, genetic background, age, geographic location, and season (Harrison and McAllan, 1980; Li et al., 2009; Romero-Pérez et al., 2011). The rumen microbiome is often described as a complex and dynamic ecosystem, given the large number of resident microorganisms and their taxonomic diversity. Differences in rumen microbial composition between ruminants of different species, between individuals of the same species, and even within individuals of the same herd, have already been documented (Jami and Mizrahi, 2012; Henderson et al., 2015; Indugu et al., 2017; Li et al., 2019). These variations stem primarily from differences in diet, environmental conditions, host genetics, and feeding behavior.

Regardless of the ruminant species, the rumen ecosystem is mainly populated by bacteria (10^{10} - 10^{13} cells/ml of ruminal content), followed by methanogenic archaea (10^{8} cells/ml), protozoa (10^{6} cells/ml), and fungi (10^{4} cells/ml). Beneficial, harmful, and competitive relationships are formed between these microorganisms for what is

considered a constantly changing system (Czerkawski, 2013). Rumen microorganisms are mainly classified on the basis of their function or the substrate they use (Hynd, 2019). The most common groups include cellulolytic (fiber utilizers), amylolytic (starch utilizers), proteolytic (protein utilizers), and lipolytic (lipid utilizers) microorganisms. Cellulolytic and amylolytic microorganisms hydrolyze polysaccharides, releasing oligosaccharides and simple sugars into the medium for their own use and that of other groups of microorganisms (Doré and Gouet, 1991), producing fermentation end products for use by the host. Microbial proteins and vitamins can be released by digestion of microbial cells in the abomasum. Methanogens (archaea) maintain low hydrogen gas (H_2) in the rumen by using it as a substrate for methane production.

Every microorganism in the rumen plays an important role in the breakdown and digestion of feed. However, the role of bacteria is more highly investigated since they account for more than 95% of the total microbial species and express a wide range of metabolic activities (Zhou et al., 2015). These characteristics give rumen bacteria a central role in ruminant nutrition and are consequently of interest for in-depth research.

5.3. Rumen Bacteria

Bacteroidetes and Firmicutes are generally the most common bacterial phyla found in ruminants, typically comprising at least 75% of the total bacterial populations (Deusch et al., 2017; Indugu et al., 2017; Liu et al., 2019; Min et al., 2019). In grain-finished and grass-finished bison, Bacteroidetes were found to be abundant in the rumen, while Firmicutes were mainly observed in the ileum (Bergmann, 2017). At the genus level, *Prevotella* is considered the most common genus, representing at least 42% of

ruminal bacteria (Stevenson and Weimer, 2007). In bison however, Bergmann (2017) found this bacterial genus to be more abundant in the hindgut (cecum, ascending colon, transverse colon, descending colon, and rectum) than in the rumen. It has been estimated that there are probably thousands of species of rumen bacteria, but only a limited number have been studied in detail. They are commonly referred to as the classic rumen bacteria and include cellulolytic bacteria, such as *Fibrobacter succinogenes*, a succinate, acetate and formate producer, *Ruminococcus albus,* whose main final products are acetate and formate, and *Butyrivibrio fibrisolvens,* which can produce acetate, formate, lactate and butyrate (Hungate, 2013). Classic ruminal amylolytic bacteria include *Ruminobacter amylophilus* and *Prevotella ruminicola*, which are both formate, acetate and succinate producers, as well as *Selenomonas ruminantium*, *Succinomonas amylolitica* and *Streptococcus bovis*, whose end products include acetate, propionate and lactate (Russell, 2002). Other bacterial species included in the list of classic rumen bacteria are *Bacteroides amylophilus* and *Anaerovibrio lipolytica*, which display proteolytic and lipolytic activities, respectively.

The composition and abundance of these bacterial species in the rumen of bison is not yet clear. Because of the lack of research in bison rumen microbiome, it is assumed that the digestive processes are carried out by strains of the classic rumen bacteria found in well-studied ruminants such as dairy and beef cattle. However, major nutritional differences in bison, such as feed preferences and higher efficiency in utilizing low-quality pastures, suggest that gut bacterial composition in bison may be very distinct from other ruminants.

5.4. Factors affecting bacterial diversity and composition in the rumen

Recent studies characterizing the ruminal microbial communities of 32 ruminant host species worldwide have shown that diet is the main factor influencing the rumen microbiome and that it is of higher importance than host species or geographical location (Henderson et al., 2015). Because of their greater abundance as well as phylogenetic and metabolic diversity in the rumen, bacteria tend to be more likely to respond to changes in dietary composition. In cattle studies, for example, rumen samples from individuals on grain-based diets showed less bacterial diversity, with entirely different bacterial populations compared to grass-based diets (Fernando et al., 2010; Plaizier et al., 2017; Liu et al., 2019).

In addition to diet, it has also been shown that ruminant host species-related factors, such as genetics and age, have an effect on rumen bacterial composition. For example, differences in rumen microbiota composition have been observed between Holstein and Jersey dairy cows who were given the same diet (Paz et al., 2016). Li et al. (2019) also suggested that certain microbial characteristics of the rumen are heritable, indicating that they can be affected by host genetics. To assess the effect of age, Jami et al. (2013) analyzed the dynamics of bacterial composition of the bovine rumen in five representative age groups ranging from birth to adulthood. They observed very distinct microbiota in each age group, with evidence of convergent bacterial communities as age increased. Furthermore, another study reported monthly temporal dynamics in the rumen bacterial composition of steers fed the same diet (Qiu et al., 2019). This study also found a positive correlation between *Succinivibrionaceae* and *Ruminobacter* abundances with ambient temperature, showing that geographical areas and subsequent seasonal variations

may also be reflected in changes in bacterial communities. (Resende et al., 2016). In that experiment, the predominant microbiota was very different between seasons, and a contrasting pattern of abundance of *Bacteroidetes* was also observed between the summer and winter.

6. METHODS TO ANALYZE THE RUMEN MICROBIOME

In order to investigate ruminal microbial communities, which form a complex and dynamic microbial ecosystem, several methods have been developed. Such methods can be divided into two categories: 1) culture-dependent (culture-based) and 2) culture-independent methods (DNA-based).

6.1. Culture-dependent methods

The rumen microbial ecosystem was traditionally investigated using techniques that were based on microbial cultures. Isolation and counting were among them, as were phenotypic or "observable" features including morphology, development, and metabolic and biochemical properties (Hashsham, 2007; Zhou et al., 2015). Although these methods are low-cost, reproducible and have allowed the identification of more than 200 microbial species (Russell and Hespell, 1981), they are limited in their ability to distinguish between different bacterial phylogenetic groups, and they greatly underestimate both microbial diversity and abundance of microorganisms that actually exist in the rumen. It has been estimated that approximately 95% of ruminal microorganisms have yet to be cultured as isolates (Creevey et al., 2014), with many currently considered “unculturable”. Limitations are primarily due to difficulties in mimicking environmental conditions, such as strict anaerobiosis, lack of information on chemical growth

requirements and the potential need for direct interactions with other microorganisms or the host (Zoetendal et al., 2004b).

6.2. Culture-independent methods

Considering the limitations of culture-dependent methods, rapidly developing molecular techniques have the advantage of providing a more comprehensive view of microbiota and their diversity. Indeed, molecular techniques allow the identification of a greater number of microorganisms (Pace, 1997; Zoetendal et al., 2004a). Because these methods focus on the analysis of nucleic acids, they can identify microorganisms without the need to cultivate or isolate them. Data generated from culture-independent approaches provide information on the richness (number of species) and regularity (relative abundance of each species) of different microorganisms in an environmental sample, which represent microbial diversity (Gerritsen et al., 2011). The emergence of next-generation technologies has provided greater resolution for this strategy. This approach has led to greater insights, allowing researchers to determine effects of and interactions among diet, age, species (genetic background) and environmental conditions with different bacterial communities.

6.2.1. The 16S rRNA gene

One of the most widely used culture-independent methods is based on the analysis of the 16S rRNA gene sequence (Clarridge, 2004). This gene is frequently used as a taxonomic marker because it possesses characteristics that make it a good tool for the identification of bacteria in the rumen: a highly conserved sequence that combines conserved and variable regions (Kataoka et al., 1997; Clarridge, 2004), and its essential

role in the synthesis of proteins (Dahlberg, 1989; Janda and Abbott, 2007; Wang et al., 2015). A number of 16S rRNA sequences have been targeted by universal primers for PCR amplification (McCabe et al., 1999; Baker et al., 2003), which is now combined with DNA sequencing, allowing the characterization of an increased number of bacterial species, including strains that are resistant to cultivation.

There are currently two types of binning or DNA sequence analysis methods: taxonomic dependent methods and OTU-based methods (Liu et al., 2008; Cai and Sun, 2011). Taxonomic dependent binning compares the DNA sequences of interest against a reference database to determine which taxon a sequence may belong to (Sedlar et al., 2017). Publicly available programs used to identify closely related organisms or taxonomic groups include RDP Classifier (Lan et al., 2012) and NCBI BLAST (Altschul et al., 1990). In contrast, OTU-based methods are intended to represent a taxonomic unit of a microbial species or genus depending on a sequence similarity threshold (Kim and Isaacson, 2015). 16S rRNA-based methods have limitations as they only allow taxonomic assignment but do not accurately evaluate functionality (Langille, 2018).

6.2.2. ‘Shotgun’ Metagenomics

To overcome the limitations of targeting single-gene markers, shotgun metagenomics can be used to identify microorganisms and reveal their biological functions through genome analysis. Shotgun metagenomics involves the extraction of microbial genomic DNA, but instead of amplification of a specific gene, it is directly used as a template for sequencing. The sequence reads that are randomly generated can then be assembled into contigs (longer contiguous sequences) or analyzed individually

(Sharpton, 2014). Using either contigs or individual reads, the functional diversity of a community can be determined by annotating coding sequences to known functions, such as enzymes and metabolic pathways. (Sharpton, 2014). The function of a coding sequence can be predicted based on its similarity to sequences in a database, and it can also be assigned to a specific taxon. The resulting information can then be used for diverse applications, including the identification and comparison of genomes of communities that are metabolically similar (Gevers et al., 2012), determination of how various treatments can influence the functional composition of a community (Looft and Allen, 2012), or revealing functions associated with specific environmental or host-physiological variables (i.e., biomarkers). Metagenomic sequences may also reveal the presence of novel genes (Nacke et al., 2012) or provide insight on the ecological conditions associated with those genes for which the function may be unknown (Buttigieg et al., 2013).

7. RESEARCH OBJECTIVE

Bison, as wild ruminants, have features or characteristics that set them apart from domesticated ruminants. Of these, their ability to maintain better on forage of poor quality than domestic grazers (Peden et al., 1974; Feldhamer et al., 2003) is of great interest for the bison industry, as it speaks to the environmental and economic sustainability of bison production. While pasture-based bison production is attractive to customers, as it favors leaner and healthier meat products with a lower overall impact on the environment, it poses significant challenges, including a longer time to finish animals. To overcome this challenge, many producers finish bison by feeding diets of

predominately grain or concentrate, as these are more easily digestible and have a higher energy content. However, finishing bison with starch-rich diets presents a dilemma, as it is inconsistent with the idea of 'natural' bison production.

Since ruminal microbial symbionts are directly responsible for providing nutrients to their host by digesting feed, their metabolic efficiency consequently impacts the efficiency with which bison can transform feed into animal products. In order to design effective strategies to modulate bison ruminal function to increase productivity and sustainability, there is a critical need for a deeper understanding of the metabolic capabilities of ruminal microorganisms. Indeed, our current knowledge gap about bison ruminal microbial communities is even more pronounced than for domesticated ruminants, with a comparatively very limited number of published reports on bison gut microbial communities (Towne et al., 1988; Rico et al., 2021). Most reported studies have so far been performed by sampling a limited number of animals, relying in some cases on opportunistic sampling from slaughter facilities rather than designed trials. Also, community composition analyses have focused on diversity metrics and/or have been based on higher-level taxonomic affiliations, which can only provide very limited insights on candidate bacterial species from the bison rumen.

In this context, the main objective of the research presented in this thesis was to characterize the rumen bacterial communities from grass-fed and grain-fed bison heifers using a DNA-based sequencing approach. Characterization of these bacteria will allow comparing bison ruminal bacterial communities under two different dietary treatments to better understand the impact of diet on bison rumen bacterial composition, potentially leading to new insights into bison feed efficiency strategies.

CHAPTER 2

CHARACTERIZATION OF THE RUMEN BACTERIAL COMMUNITIES OF BISON HEIFERS FED A GRASS-BASED DIET VS A GRAIN-BASED FREE-CHOICE DIET

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ABSTRACT

The ruminal bacterial communities of the North American bison have so far been largely unexplored. The current study aimed to determine and compare the diversity and composition of ruminal bacteria between bison heifers on two different diets at two different ranches. Stomach tubing was used to collect rumen fluid from lifetime grass-fed heifers between 25 and 30 months of age distributed between 2 ranches located in Standing-Butte (SBR; n=17), SD, and Blue-Creek (BCR; n=17), NE, respectively. A second set of samples was collected after the same individuals had been transitioned to a grain-based free-choice diet for 100 days. Bacterial composition was determined by Illumina MiSeq (2×300) sequencing of PCR amplicons generated from the V1-V3 region of the 16S rRNA gene. Next-Generation Sequence data was analyzed using a combination of custom Perl scripts, and publicly available software (Mothur v.1.40, RDP classifier and NCBI Blast). Taxonomic analysis identified Bacteroidetes and Firmicutes as the dominant phyla across all samples analyzed. A total of 57,132 and 59,133 species-level Operational Taxonomic Units (OTUs) were identified in SBR and BCR grass-fed heifers, respectively, in contrast to 13,240 and 22,516 OTUs that were found in the same animals on a grain-based diet. A comparative analysis using the most abundant OTUs

from each group was conducted using the Kruskal-Wallis sum-rank test. In the Standing Butte heifers, 28 abundant OTUs were found to be different between diets ($P < 0.05$), including Bb-00031 ($\bar{x}_{grass}$ = 0.04% vs $\bar{x}_{grain}$ = 1.45%) and Bb-00018 ($\bar{x}_{grass}$ = 0.58% vs $\bar{x}_{grain}$ = 0.06%). In the Blue Creek heifers, 17 of the most abundant OTUs were found to be different between diets ($P < 0.05$), including Bb-00046 ($\bar{x}_{grass}$ = 1.24% vs $\bar{x}_{grain}$ = 0.45%) and Bb-00058 ($\bar{x}_{grass}$ = 0.03% vs $\bar{x}_{grain}$ = 1.22%). Together, these results indicate that the rumen of the North American bison harbors highly diverse bacterial communities that undergo dramatic changes in response to changes in diet.

Key words: Bison, Rumen, Bacteria, 16S rRNA gene sequencing

1. INTRODUCTION

Over time, bison has evolved to efficiently extract nutrients from a wide variety of grasses native to North American prairie ecosystems. Due to this and other unique characteristics of the species, bison production started to be perceived as a sustainable economic activity. Bison ranching dates back to the early 1900s, but it wasn't until the 1970s that production began to increase, reliably producing bison on a commercial scale by the mid-1980s (Hawley, 1989). Although bison producers in general aim to maintain the animal's wild and natural heritage, the use of grain-based ingredients to finish bison in a confined setting, similar to what is used for beef cattle, is a common practice. Feeding grains and concentrate to bison has helped producers to mitigate reduced winter intake and other seasonal effects, increasing overall efficiency and get bison to market weight throughout the year. However, decades of research on cattle and other ruminant livestock have shown that a diet rich in fermentable carbohydrates can change the composition of rumen microorganisms, negatively impacting the rumen environment, affecting the physiology of the rumen as well as the health of the host animal.

In this context, one of the key issues faced by bison producers is how grain or concentrate diets can change the bison rumen microbiota and create potential adverse effects on animal health and efficiency. To gain further insight, the current study characterized and compared the rumen bacterial communities of bison heifers fed a grass-based versus a grain-based free-choice diet at two separate ranches.

2. MATERIALS AND METHODS

2.1. Animals and rumen fluid collection

The bison used in this study were sampled at two different ranches: the Standing Butte Ranch in central South Dakota and the Blue Creek Ranch on the southwestern edge of the Nebraska Sandhills. At each ranch, 20 bison heifers were selected for collection of rumen fluids. Sampling was carried out in two phases. The first phase consisted of collecting rumen fluid from grass-fed (diet composed only of grasses and forbs) bison heifers at 25-26 months of age immediately prior to placement on a higher energy diet. These animals had spent their entire life to this point on a pasture and forage-based diet. The second series of rumen fluid samples was collected from the same individual animals after exposure to a grain-based free-choice diet, which consisted of ad libitum access to corn, alfalfa hay and grass hay for 97 to 101 days prior to collection of rumen fluid. The bison were maintained in loose confinement (74.3-92.9 m^2 per animal) and consumed on average approximately 6.8 kg of corn, 4.5 kg of alfalfa hay and 1.8 kg of grass hay per day, but individual intake was not measure. Oral stomach tubing, previously described as a simpler, faster, and less invasive method of collecting rumen fluids (Geishauser, 1993; Duffield et al., 2004), was used for this study. In order to avoid saliva contamination, between 25 and 50 ml of the initial rumen fluid sample was discarded, then approximately 200 ml was collected. Samples were immediately frozen and sent to South Dakota State University facilities for further analysis.

Due to unexpected challenges during sample collection, we were unable to obtain rumen fluid from all heifers for both diets. Bacterial composition analysis was performed

using samples from heifers from which rumen fluid was successfully collected from both diets (n=17 / group).

2.2. Microbial DNA extraction and PCR amplification

Total microbial DNA was extracted from each rumen sample (n=68) using a bead beating and column approach as previously described (Yu and Morrison, 2004), which included the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed after extraction, where the bacterial V1-V3 regions of the 16S rRNA gene were amplified using the universal primers 27F-5'AGAGTTTGATCMTGCTCAG (Forward) and 519R-5'GWATTACCGCGCGCGCTG (Reverse). PCR was performed using a thermal cycling system, under the following conditions: an initial denaturing step at 98^{o}C (4 minutes), followed by 35 cycles of denaturation at 98^{o}C (10 seconds), annealing at 50^{o}C (30 seconds), extension at 72 ^{o}C (30 seconds). Amplification ended with an extension period at 72 ^{o}C (10 minutes). Agarose gel electrophoresis was then performed to check and confirm the quality (minimum 400 ng) and molecular weight (~500 bp) of the generated DNA fragments, which were recovered using a QiaexII Gel extraction kit (Qiagen, Hilden, Germany). Purified PCR samples were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq 2X300 platform to generate overlapping paired end reads.

2.3. Microbial Composition Analyses

Sequencing data were processed using a set of custom-written Perl scripts. Raw sequences of the 16S rRNA bacterial gene (V1-V3) were first screened on a full-length

basis and a quality score. Full-length sequences were first selected based on the existence of their respective forward (27F) and reverse (519R) primer sequences, while quality screening was carried out using a minimum quality threshold of no more than five nucleotides with a Phred quality score of less than 15. Following quality screens, sequence reads were aligned and clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 4% sequence dissimilarity, which is more suitable than 3% for this region (Kim et al., 2011). Following OTU clustering, three independent strategies were used to detect potential artifacts. First, OTUs were screened for chimeric sequences using the "chimera.slayer" (Haas et al., 2011) and "chimera.uchime" (Edgar et al., 2011) commands from the MOTHUR open source software package (Schloss et al., 2009). Secondly, the 5' and 3' ends were evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI "nt" database, as determined by BLAST (Altschul et al., 1997), OTUs with more than five nucleotides missing from the 5′ or 3′ end of their respective alignments were designated as artifacts. Finally, single-read OTUs (singletons) were subjected to an additional screen, where only sequences with a perfect or near-perfect match (maximum 1% of dissimilar nucleotides) to a sequence in the NCBI "nt" database were kept for analysis. After screenings, all flagged OTUs and their corresponding reads were subsequently removed from further analyses. Curated OTUs were subjected to taxonomic assignment as follows: two taxonomic level assignments (Phylum and Family) for all OTUs using RDP Classifier (Wang et al., 2007), and species-level assignments (≥97% of sequence similarity) for OTUs of interest (relative abundance ≥1% in at least one sample) using BLAST (Altschul et al., 1997). Alpha (Observed OTUs, Chao, Shannon and Ace)

and beta diversity (Bray-Curtis distance) indices were estimated using the "summary.single" and the "summary.shared" commands from the MOTHUR open source software package (Schloss et al., 2009), respectively. To account for uneven sampling, data was rarefied to the minimum sampling depth of 3500 sequences by subsampling of the original datasets. The Bray-Curtis distance analysis output was used as input for the MOTHUR command "pcoa" for Principal Coordinate Analysis (PCoA). Principal components 1 (PCo1) and 2 (PCo2), representing the highest level of variation, as well as alpha diversity indices were plotted using the Tableau Visualization Software (Version 2020.4, https://www.tableau.com/products/new-features).

2.4. Statistical Analyses

All statistical analyses were performed using the RStudio Statistical Software (Version 1.3.959 © 2009-2020 RStudio, PBC). First, a Shapiro-wilk test was conducted to check for normality assumption. Since normality assumption was not met, Kruskal-Wallis sum-rank tests for non-parametric data were conducted to determine statistical differences in the abundances of selected OTUs between experimental groups. Alpha diversity indices were tested using Analysis of variance (ANOVA), with a post-hoc 'HSD.test' pair-wise function. The 'adonis' function from the vegan package (Oksanen et al., 2013) was used for permutational multivariate analysis (PERMANOVA, 999 permutations) to detect statistical differences amongst sample sets, followed by the 'pair-wise.adonis' function from the 'devtools' package to identify pairs of sample groups that were different. For all analysis, $P \leq 0.05$ was considered significant.

3. RESULTS

3.1. Taxonomic composition of rumen bacterial communities in grass and grain-fed bison

Characterization of the bacterial communities in the rumen of bison from two different ranches and on two different diets was performed by generating different datasets for each of the four experimental groups: Standing Butte grass (SBGrass), Standing Butte grain (SBGrain), Blue Creek grass (BCGrass) and Blue Creek grain (BCGrain). A total of 657,096 quality-filtered sequences with an average of 164,274 (standard deviation: 13,113) reads per dataset and 9,664 (standard deviation: 771) sequences per sample were obtained from the 68 samples. Collectively, these sequences were classified into 20 main phyla and 83 major family level bacterial groups; 0.20-10% of sequences could not be assigned to any known phylum (unclassified bacteria). Overall, the proportion of *Bacteroidetes* and *Firmicutes* (two dominant bacterial phyla in gut environments) varied greatly amongst samples (Figures 1-2). In three of the four groups (SBGrass, SBGrain and BCGrass), *Bacteroidetes* was the most predominant phylum, ranging between 50.69-56.29% of total sequences, while *Firmicutes* was the second most predominant phylum, accounting for 36.57-42.74% of total sequences (Table 1). On the other hand, in BCGrain the abundance of *Bacteroidetes* was lower compared to SBGrass, SBGrain and BCGrass, whereas *Firmicutes* had between 1.4 (SBGrass) and 1.7 (BCGrass) - fold increase in response to grain supplementation (Table 1). Together these two phyla groups represented more than 90% of total sequences across all experimental groups. *Planctomycetes* and *Proteobacteria* were the third and fourth most highly represented phyla, accounting for 0.37- 1.14% and 0.50-5.21% of total sequences, respectively. *Proteobacteria* was most abundant in SBGrain, representing up to 27.16 and

40.46% of total sequences in heifer 10 and heifer 16, respectively (Figure 2a). *Prevotellaceae* was the most predominant *Bacteroidetes* family in SBGrass, SBGrain and BCGrass, with abundances between 32.44-42.71% (Table 1). While a single Firmicutes family, *Ruminococcaceae*, was most abundant (SBR: 24.32% ; BCR: 25.68%) in grain-based diets (Table 1, Figure 2a-2b), a more diverse set of Firmicutes families were observed in grass-based diets, including *Lachnospiraceae, Unclassified Clostridiales, and other Firmicutes*, which ranged from 1.98 to 24.13% (Table 1, Figure 1a-1b).

3.2. OTU Composition analysis of rumen bacterial communities in grass and grain-fed bison

A total of 139,805 distinct OTUs were identified across all experimental groups, with 12,216 OTUs occurring in more than one experimental group (Shared OTUs). Approximately, 57.26 and 47.95% OTUs were assigned to *Bacteroidetes* or *Firmicutes*, respectively. ANOVA and Tukey's HSD showed differences in alpha diversity across groups (Table 2, Figure 3). These results were supported by Principal Coordinate Analysis (PCoA), PERMANOVA and pairwise adonis tests, which showed four different clusters, each representing one experimental group ($p = 0.001$) (Figure 4). Since PCoA indicated differences in bacterial composition between experimental groups, further analyses were performed on the most abundant OTUs. Seventy-four OTUs (SBGrass = 18, SBGrain = 20, BCGrass = 16, BCGrain = 20) were each represented by ≥1% of total sequences in at least one sample, and considered as most abundant (Tables 3-6). From these, only five OTUs (**SBGrass =** Bb-00004, Bb-00013; **SBGrain =** Bb-00022, Bb-00025; **BCGrain =** Bb-00064) showed a high degree of sequence identity (≥97%) to known bacterial species, and were assigned to *Fibrobacteres*, *Bacteroidetes*,

Proteobacteria, and *Firmicutes,* respectively. For the remaining OTUs, 21 showed sequence identities between 90-97%, and 48 showed sequence identities lower than 90% to their respective closest relative; OTUs from these categories were designated as unknown bacterial species.

3.2.1. OTU analysis for Grass-fed bison heifers

To gain further insight into the ruminal bacterial communities of grass-fed bison, OTU composition was compared between the two locations (Standing Butte, SD – Blue Creek, NE). From the 57,132 and 59,133 OTUs identified in SBGrass and BCGrass, respectively, 34 OTUs were identified as most abundant, as they were found to be present at a minimum of 1% in at least one sample (Tables 3 and 5). From the most abundant, 18 OTUs were assigned to *Bacteroidetes* compared to 10 affiliated to *Firmicutes*. Furthermore, SBGrass and BCGrass shared 33 of the most abundant OTUs, including OTUs Bb-00002 and Bb-00003, both found to be abundant in both locations; while one OTU (Bb-00039) whose closest relative was *Prevotella brevis* (89.96%), was only found in BCGrass (Figure 5a). From the 33 shared OTUs, 21 were found to be statistically different between locations (Table 7). Only two OTUs (Bb-00004 and Bb-00013), both from Standing Butte, were predicted to be strains of known bacterial species, with 98.41% and 98.95% sequence identity to *Fibrobacter succinogenes and Ruminococcus flavefaciens,* respectively.

3.2.2. OTU analysis for Grain-fed bison heifers

The number of OTUs identified during the grain-based free-choice phase was 22,516 in SBGrain and 13,240 in BCGrain, from which 40 OTUs were selected as the

most abundant (Tables 4 and 6). Twenty OTUs were assigned to *Bacteroidetes*, compared to 17 for *Firmicutes*. From the 40 most abundant OTUs, 32 were shared between SBGrain and BCGrain, including 5 OTUs (Bb-00020, Bb-00029, Bb-00056, Bb-00058, and Bb-00068) that were found in high abundance in both locations. Five OTUs (Bb-00027, Bb-00028, Bb-00033, Bb-00036, Bb-00037) were identified only in Standing Butte, while three (Bb-00071, Bb-00059, Bb-00073) were found only in Blue Creek (Figure 5b). From the 32 shared OTUs, 18 were statistically different ($p<0.05$) between ranches (Table 8), of which 3 were predicted to be strains of known bacterial species: Bb-00022 (*Prevotella ruminicola*, 97.90%), Bb-00025 (*Succinivibrio dextrinosolvens*, 97.11%) and Bb-00064 (*Streptococcus lutetiensis*, 100%).

3.2.3. OTU comparison: Effect of diet change on the diversity and composition of ruminal bacteria in bison heifers

One of the main objectives of this study was to compare the microbial communities in the rumen of bison fed two different diets. Results showed that the transition from a grass to a grain-based diet significantly changed the composition of bison ruminal bacterial groups. The change was observed in both: the type of bacterial OTUs, and their respective abundances. The number of OTUs decreased by 2.5x [57,132 to 22,516] in Standing Butte and by 4.47x [59,133 to 13,240] in Blue Creek, following the change in diet. When comparing the number of shared and unique OTUs, SBGrass and SBGrain were found to have the highest number of OTUs in common, while BCGrain had the most unique OTUs (11) when compared to the same individuals under a grass-based diet (Figure 6). In the Standing Butte heifers, the abundance of 28 well-represented OTUs significantly varied between diets ($P<0.05$), including Bb-00022, Bb-

00013, and Bb-00004, which were predicted to be strains of known bacterial species (Table 9). Two OTUs were not affected by diet change, while the abundance of eight OTUs either decreased to a not detectable level or were not present (Figure 7a), which included OTU Bb-00025, closely related to *Succinivibrio dextrinosolvens.* In Blue Creek, 17 of the most abundant OTUs were different between diets ($p<0.05$), including OTU Bb-00064, predicted as *Streptococcus lutetiensis* (Table 10), while eighteen OTUs were either not present or below detection and only one abundant OTU was not affected by the transition to grain (Figure 7b). At both locations, the most abundant OTUs tended to exhibit significantly higher levels in grain-based diets, with OTUs Bb-00022, Bb-00031, Bb-00029 and Bb-00024 from Standing Butte (Figure 8a), and Bb-00055, Bb-00066, Bb-00070, Bb-00063 and Bb-00058 from Blue Creek (Figure 8b) showing the highest abundance difference between grass and grain-based diets ($P<0.001$-$P<0.02$).

4. DISCUSSION

A number of studies on the roles played by rumen bacteria in animal production and health has led to important insights, such as a functional link between the rumen microbiome and feed efficiency (Li, 2017b), as well as defining a “core rumen microbiota” in beef (Petri et al., 2013), dairy cows (Jami and Mizrahi, 2012; Lettat and Benchaar, 2013) and for other ruminants (Henderson et al., 2015). Research has also shown that diet composition has a great impact on the type and abundance of bacteria present in the rumen. Knowledge regarding the rumen microbiome in bison, especially during a diet transition is limited, thus the motivation for this study. Elucidating the bacterial composition and diversity in the rumen of bison under different conditions will begin to provide insights into the effects of different feeding protocols and feed

transitions in the rumen microbiota that benefits both the animal and the producer. We compared the bacterial composition in the rumen of bison heifers fed two different diets (grass and free-choice grain-based) at two separate locations (Standing Butte, SD – Blue Creek, NE). General taxonomic findings from this research showed that *Bacteroidetes* and *Firmicutes* were predominant irrespective of the diet. However, similar to previous reports (Kala et al., 2017), the respective proportions of *Proteobacteria* and *Fibrobacteres* were found to be dependent on the diet consumed. At the family level, *Prevotellaceae*, a family that includes some of the common rumen bacteria such as *Prevotella ruminicola*, *Prevotella brevis*, and *Prevotella albensis*, was the most abundant in the rumen of bison fed either diet, consistent with previous reports (Bergmann, 2017). As members of this family are known to have different metabolic capabilities, including the ability to use a wide variety of substrates such as xylans (Miyazaki et al., 1997), proteins and peptides (Wallace et al., 1997) or other complex carbohydrates (Flint et al., 2012), changes in diet composition may not have a major impact on this group as a whole. Surprisingly *Ruminococcaceae*, typically recognized as active plant fiber utilizers, were more abundant in animals fed grain-based diets, which is in contrast to other findings (Henderson et al., 2015). However, since the free-choice diet included grass and alfalfa hay during the grain-based phase, consumption of these feedstocks may have maintained the abundance of members of the *Ruminococcaceae* family in the rumen of grain-fed bison heifers.

An evaluation of the OTU composition as a function of diet and location was also performed. The high number of low abundance OTUs observed in the rumen of grass-fed bison at both locations suggests a highly diverse microbial ecosystem that offers the

possibility of identifying rare and previously unknown microbial species that could explain bison's ability to perform better than cattle, when consuming low-quality grasses. Indeed, only OTUs Bb-00004 and Bb-00013 were found to be closely related to *Fibrobacter succinogenes* and *Ruminococcus flavefaciens,* respectively. Since bison are primarily grazers, the presence of these bacterial species in the rumen would be expected because their ability to digest cellulose has been reported to be greater than that of other cellulolytic bacteria (Koike and Kobayashi, 2009). Indeed, *F. succinogenes* is able to efficiently adhere to and breakdown plant cell walls due to the production of a variety of fibrolytic enzymes (Béra-Maillet et al., 2004), generating succinate a main end product, followed by acetate and formate. *R. flavefaciens* is also a predominant ruminal cellulolytic bacterial species, however its attachment mode and site to plant fiber is different from *F. succinogenes* (Cheng et al., 1984; Gaudet and Gaillard, 1987), suggesting that a reciprocal rather than a competitive relationship between both species may allow an accelerated rate of digestion. Indeed, past reports have demonstrated that both bacterial species coexist in equal proportions and that there is not apparent growth inhibition of either species when the substrate is available in sufficient amounts (Shi and Weimer, 1997).

The rumen of bison fed a free choice grain-based diet showed lower diversity as a lower number of OTUs were identified, however their relative abundances were higher. Bb-00022 was closely related to *Prevotella ruminicola*, which is capable of using pectin as an energy source to generate acetate (Marounek and Kalachnyuk, 1995; Dušková and Marounek, 2001), an essential product of rumen metabolism. The presence of this bacterial species represents a beneficial feature for these animals, as pectin is commonly

added to ruminant feeds through incorporation of different agricultural by products- to avoid the use of highly fermentable grains and the proliferation of ruminal acidosis-associated bacteria (Santos et al., 2014). The second most abundant OTU (Bb-00025) identified in the concentrate diet was closely related to *Succinivibrio dextrinosolvens*. Members of the *Succinivibrio* genus are beneficial for performance in cattle, through the formation of propionate, a SCFA that can be readily absorbed through the rumen wall and used by the liver to generate glucose. (Hernandez-Sanabria et al., 2012). These bacteria have been found in greater numbers when ruminants are fed grain-based diets (Bryant, 1959), suggesting a role in the rumen as utilizers of starch fermentation products such as succinate (Leaver et al., 1956). The presence of these bacteria in the rumen could help decreasing the acetate: propionate ratio, which could potentially be more favorable for the animal's performance.

Among *Streptococci*, *Streptococcus bovis* is the most studied species in the rumen of cattle and sheep. Because of its high capacity for lactic acid production (Hungate, 2013), this bacteria has been associated with rumen metabolic diseases such as rumen acidosis (Russell and Hino, 1985; Asanuma and Hino, 2002). In this study, OTU Bb-00064 was found to be 100% identical to *Streptococcus lutetiensis*, a bacterial species identified in humans but with an undefined function in the rumen. Certain physical and biochemical characteristics previously described for this species include the presence of Beta-glucosidase, an enzyme that catalyzes cellulose hydrolysis, and esculin, a sugar molecule that releases glucose from its hydrolysis (Schlegel et al., 2000; Poyart et al., 2002). Unlike other members of the *Streptococcus* group, this bacterial species does not

appear to be associated with starch fermentation, but it is listed as a potential pathogen as it was associated with sudden death in calves (Clarke et al., 2016).

Because we observed a significant decrease in the number of OTUs after animals had transitioned from grass to grain, diet appears to be a primary determinant of microbial composition in the rumen of bison. Grass-fed animals displayed more diverse bacterial compositions, and a reduction was observed with the inclusion of starch in the diet. From the most abundant OTUs identified, a low percentage (3-5%) were not affected by the change in diet, suggesting that their preferred substrate was still available (Grass/alfalfa hay) or that their function was required in the rumen regarding of the substrate present. In contrast, 47-74% of OTUs in grass-fed animals were significantly reduced after the diet transition, which included 21-50% of OTUs that were reduced to non-detectable levels.

The majority of OTUs identified in this study were phylogenetically too distant from their closest relatives to reliably infer their function based on 16S rRNA gene sequence comparisons alone. OTU Bb-00019, for instance, was observed in high abundance in grain-fed bison heifers, and it was only 83.58%, similar to its closest relative. These types of scenarios indicate that much additional research is needed to determine the metabolic capabilities of these bacteria and their potential functions in the rumen of bison. Shotgun metagenomics is a tool that could provide further insight into taxonomic affiliation and functional profiles. This information could, in turn, contribute to practical applications for bison producers, which may benefit bison health and performance in the future.

5. CONCLUSION

Microbial communities of bison rumen were found to undergo substantial changes in composition in response to a change in diet. Similar community compositions were, in contrast, observed for animals on the same diet for a given location, confirming once again the impact of diet on rumen bacterial communities. Although more research is required to establish the genetic and metabolic capabilities of the identified bacteria, as well as their contribution to host efficiency and health, this study contributes to a better understanding of the impact of grain as a substrate to a rumen ecosystem that has evolved to metabolize native grasses over the course of millennia.

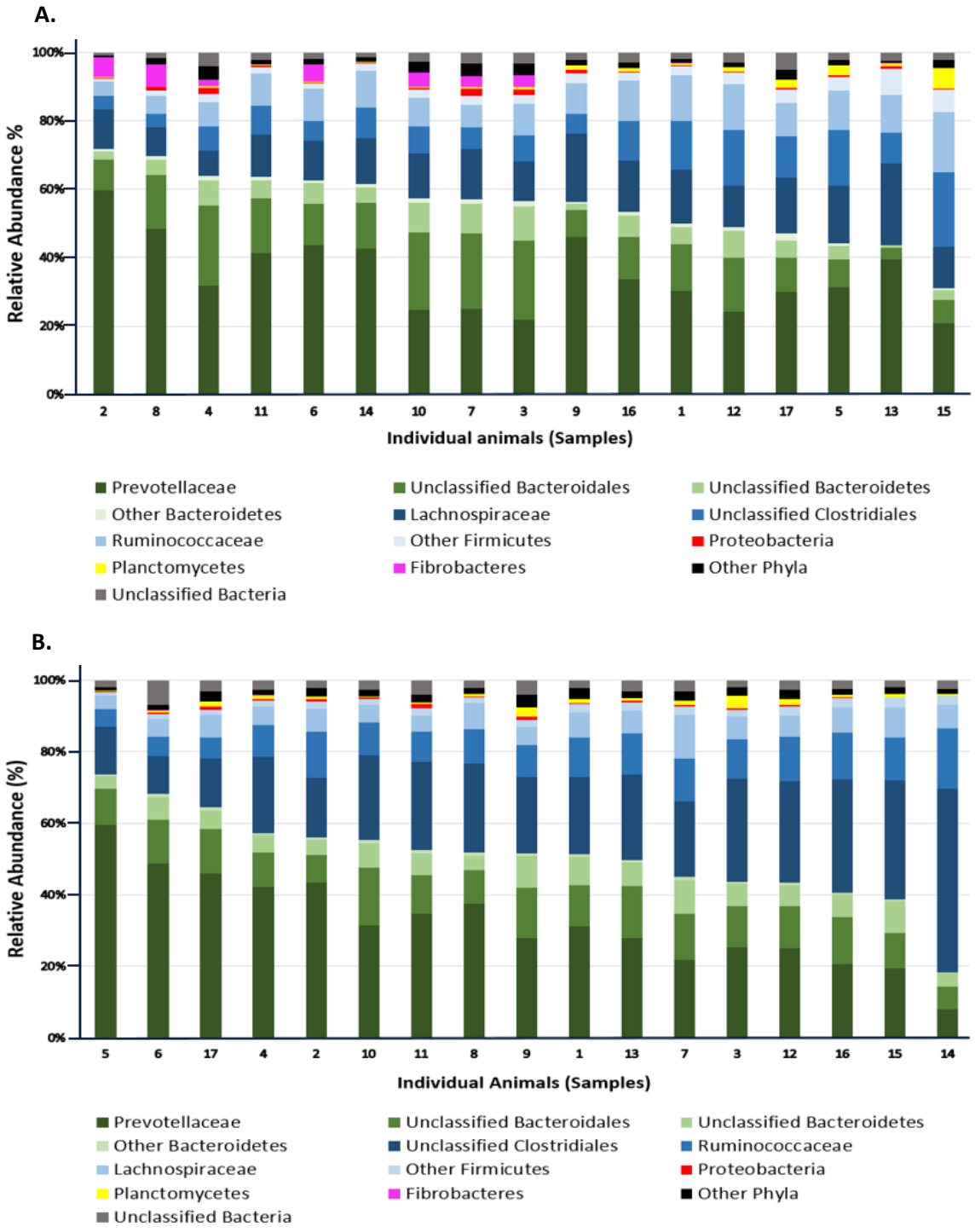

Figure 1. Phylum and Family level taxonomic composition of rumen bacterial communities in grass-fed bison. **(A)** Standing Butte grass **(B)** Blue Creek grass. Families belonging to the same phylum are represented by different shades of the same color: Bacteroidetes (green) and Firmicutes (blue).

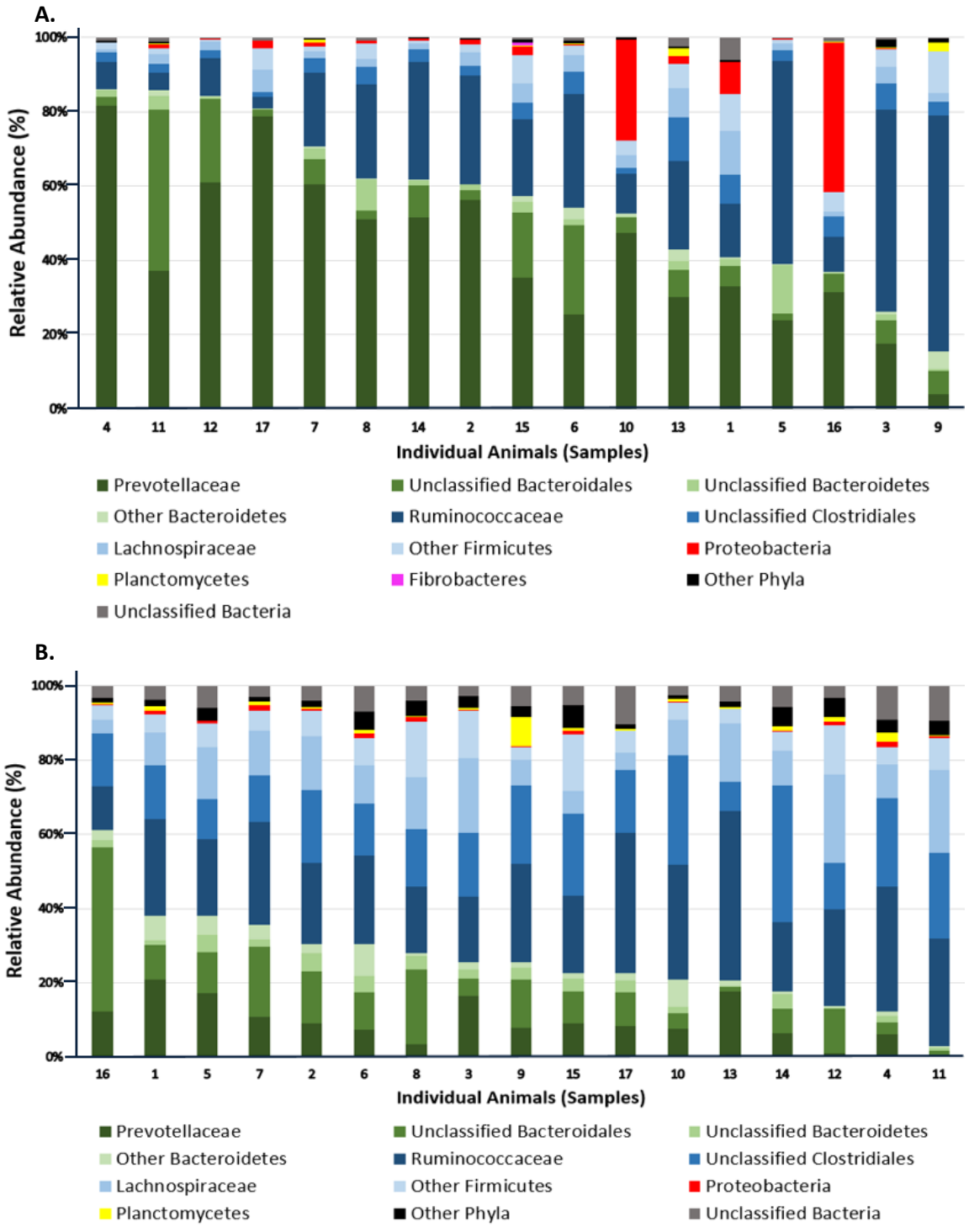

Figure 2. Phylum and Family level taxonomic composition of rumen bacterial communities in grain-fed bison. **(A)** Standing Butte grain **(B)** Blue Creek grain. Families belonging to the same phylum are represented by different shades of the same color: Bacteroidetes (green) and Firmicutes (blue).

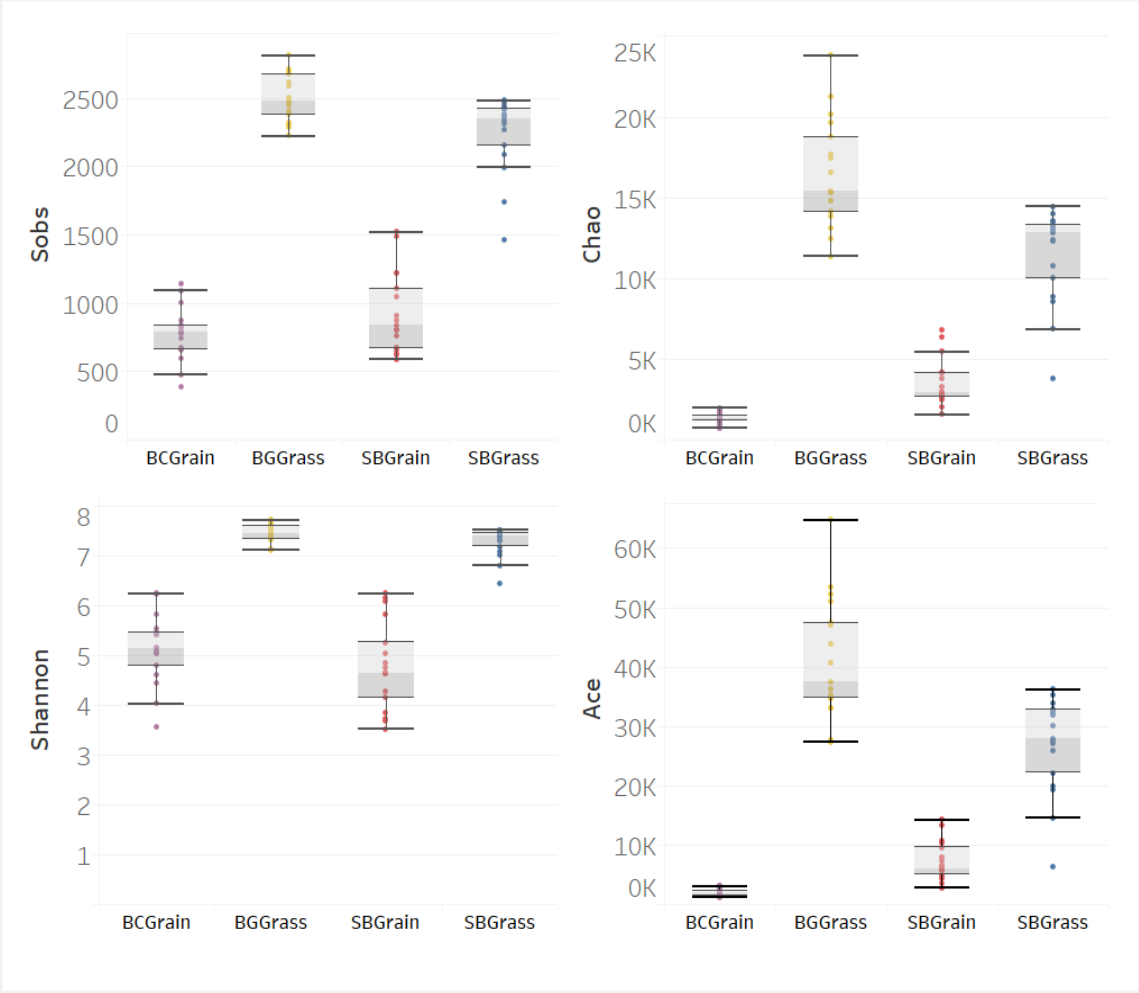

Figure 3. Boxplots showing alpha diversity variations across experimental groups

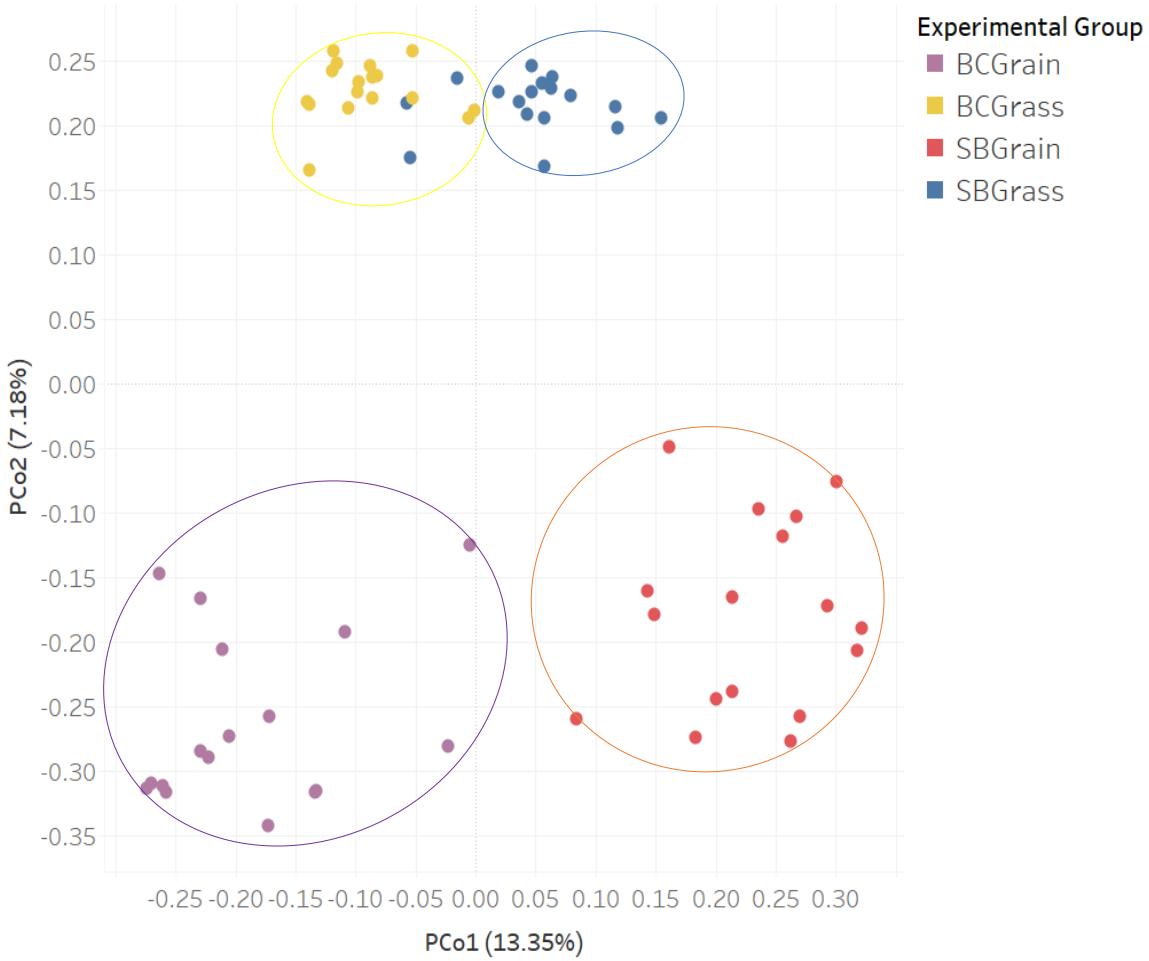

Figure 4. Comparison of rumen bacterial communities from grass and grain-fed bison heifers from two locations using Principal Coordinate Analysis (PCoA). The x and y axes correspond to Principal Component 1 (PCo1) and Principal Component 2 (PCo2), which explain the highest (20.53%) level of variation. Ellipses represent differences between experimental groups resolved by PERMANOVA and adonis tests ($p = 0.001$).

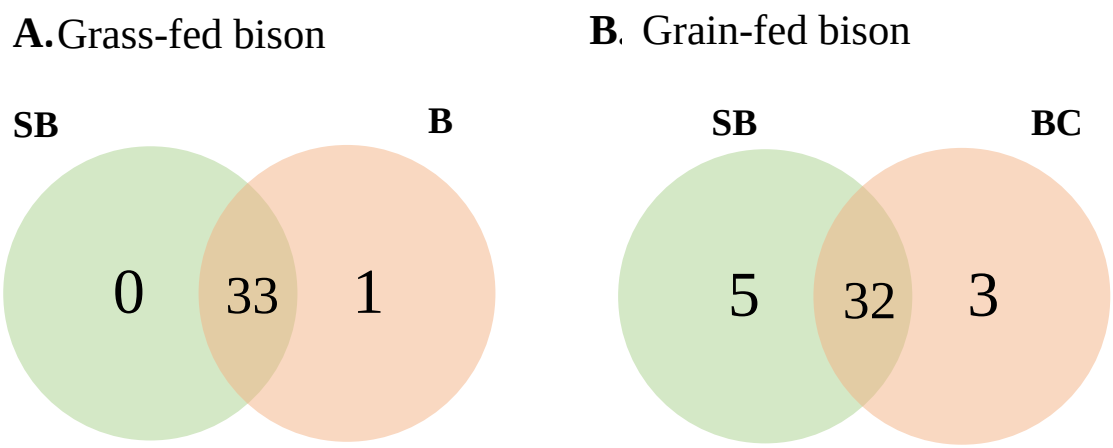

Figure 5. Venn diagrams showing the number of most abundant shared and distinct OTUs between locations. **(A)** OTUs shared between Standing Butte Grass and Blue Creek Grass **(B)** OTUs shared between Standing Butte Grain and Blue Creek grain

Figure 6. Venn diagrams showing the number of most abundant shared and distinct OTUs between experimental groups. **SBGrass:** Standing Butte grass, **SBGrain:** Standing Butte grain, **BCGrass:** Blue Creek grass, **BCGrain:** Blue Creek grain.

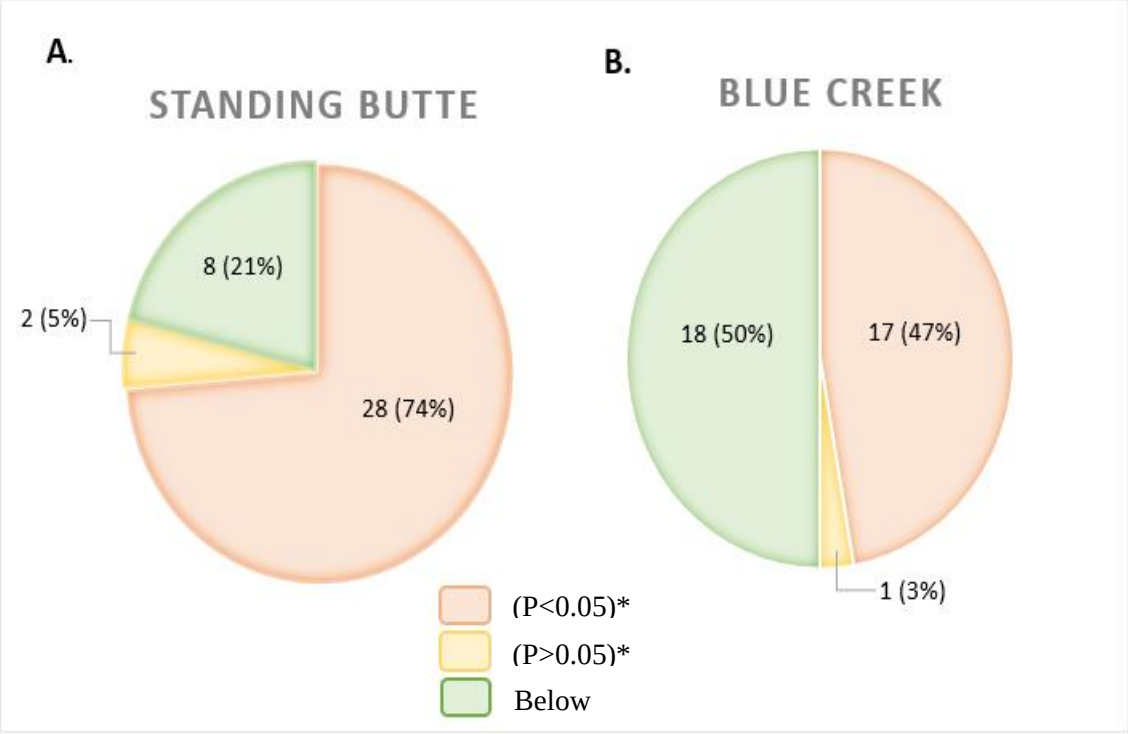

Figure 7. Diagram showing the percent and number of OTUs whose relative abundance was statistically different during the transition from grass-based to grain-based free-choice diets. *Kruskal-Wallis sum-rank test (P<0.05).

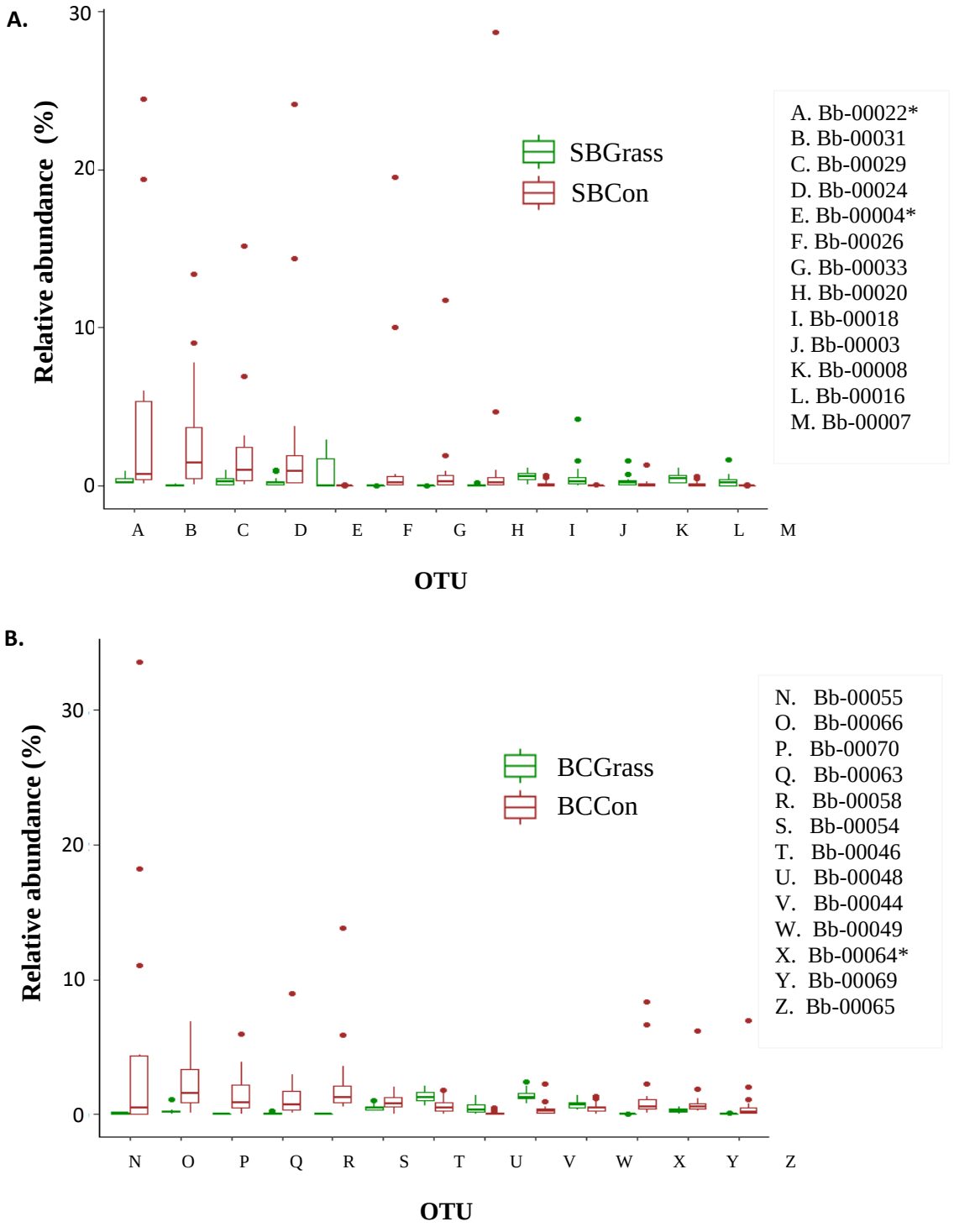

Figure 8. Impact of diet in the abundance of selected OTUs. The relative abundance differences were determined using Kruskal-Wallis sum rank tests ($P<0.05$). All OTUs (A-Z) were statistically different between diets. **(A)** Abundant OTUs from Standing Butte heifers **(B)** Abundant OTUs from Blue Creek heifers. *Strain of known bacterial species.

Table 1. Major Taxonomic groups identified in the rumen of bison heifers

Taxon	Relative Abundance (%)			
	SBGrass	**SBGrain**	**BCGrass**	**BCGrain**
Bacteroidetes	55.22	56.29	50.69	26.22
Prevotellaceae	35.02	42.71	32.44	9.53
Unclassified Bacteroidales	13.81	9.88	11.40	11.30
Unclassified Bacteroidetes	5.35	2.60	6.18	2.52
Other Bacteroidetes	1.03	1.10	0.67	2.88
Firmicutes	36.57	36.57	42.74	63.58
Ruminococcaceae	9.85	24.32	10.25	25.68
Unclassified Clostridiales	9.82	4.34	24.13	18.34
Lachnospiraceae	13.89	3.76	6.38	12.07
Other Firmicutes	3.01	4.15	1.98	7.49
Planctomycetes	1.13	0.37	1.02	1.14
Proteobacteria	0.76	5.21	0.50	0.71
Other Phyla	1.94	0.45	2.09	3.06

Taxonomic affiliation greater than 80%. **SBGrass:** Standing Butte Grass; **SBGrain:** Standing Butte Grain; **BCGrass:** Blue Creek Grass; **BCGrain:** Blue Creek Grain

Table 2. Estimation of observed OTUs and alpha-diversity indices of bison rumen microbiome

Index	Experimental Group				P-value
	SBGrass	SBGrain	BCGrass	BCGrain	
Observed OTUs	2241[a]	925[b]	2505[c]	772[b]	<0.001
Chao	11402[a]	3565[b]	16525[c]	1348[d]	<0.001
Ace	26780[a]	7252[b]	41155[c]	1989[b]	<0.001
Shannon	7.26[a]	4.78[b]	7.43[a]	5.13[b]	<0.001

Note: Values are presented as mean of 68 rarefied samples containing 3500 sequences. Pair-wise differences of alpha diversity indices were calculated using an HSD.test. Significance level was set to
$p < 0.05$.

Table 3. Most abundant OTUs identified in the rumen of Standing Butte heifers fed a grass-based diet (SBGrass)

OTU	Median (%)	Range (%)	Closest Valid Taxon (% Identity)
Bb-00001	0.04	0.01 - 8.63	*B; Prevotella brevis* (89.16)
Bb-00002	0.03	0.00 - 5.41	*B; Prevotella ruminicola* (90.32)
Bb-00005	0.04	0.00 - 2.22	*B; Prevotella ruminicola* (90.38)
Bb-00006	0.02	0.00 - 1.92	*B; Prevotella ruminicola* (91.87)
Bb-00008	0.19	0.00 - 1.56	*B; Prevotella brevis* (90.32)
Bb-00010	0.24	0.02 - 1.49	*B; Prevotella shahii* (89.94)
Bb-00011	0.22	0.04 - 1.33	*B; Prevotella ruminicola* (89.87)
Bb-00012	0.02	0.00 - 1.30	*B; Alistipes finegoldii* (84)
Bb-00014	0.31	0.08 - 1.26	*B; Prevotella ruminicola* (91.84)
Bb-00018	0.58	0.08 - 1.10	*B; Prevotella brevis* (91.86)
Bb-00007	0.19	0.01 - 1.69	*F; Pseudobutyrivibrio ruminis* (96.5)
Bb-00009	0.14	0.00 - 1.49	*F; Clostridium bolteae* (91.92)
Bb-00013	0.06	0.02 - 1.29	*F; Ruminococcus flavefaciens* (98.95)
Bb-00015	0.09	0.01 - 1.16	*F; Ileibacterium massiliense* (89.16)
Bb-00016	0.46	0.13 - 1.13	*F; Saccharofermentans acetigenes* (89.02)
Bb-00003	0.26	0.01 - 4.26	*Pl; Thermostilla marina* (82.05)
Bb-00004	0.02	0.00 - 2.93	*Fb; Fibrobacter succinogenes* (98.41)
Bb-00017	0.24	0.09 - 1.12	*C; Flexilinea flocculi* (91.3)

Taxonomic Affiliations: B: Bacteroidetes, **F:** Firmicutes, **Pl:** Planctomycetes, **Fb:** Fibrobacteres, **C:** Chloroflexi

Table 4. Most abundant OTUs identified in the rumen of Standing Butte heifers fed a grain-based free-choice diet (SBGrain)

OTU	Median (%)	Range (%)	Closest Valid Taxon (% Identity)
Bb-00022	0.77	0.12 - 24.45	*B; Prevotella ruminicola* (97.90)
Bb-00024	0.93	0.13 - 24.12	*B; Prevotella brevis* (89.96)
Bb-00020	0.20	0.02 - 28.69	*B; Mediterranea massiliensis* (84.28)
Bb-00027	0.07	0.00 - 19.01	*B; Alistipes finegoldii* (84)
Bb-00023	0.18	0.03 - 24.22	*B; Prevotella ruminicola* (92.63)
Bb-00028	0.10	0.00 - 17.73	*B; Prevotella copri DSM 18205* (88.16)
Bb-00021	0.11	0.01 - 25.62	*B; Prevotella buccalis* (88.78)
Bb-00033	0.26	0.00 - 11.77	*B; Alistipes finegoldii* (85.01)
Bb-00032	0.02	0.00 - 12.46	*B; Prevotella copri DSM 18205* (89.47)
Bb-00034	0.04	0.00 - 7.63	*B; Prevotella veroralis* (91.15)
Bb-00038	0.07	0.00 - 2.27	*B; Alistipes finegoldii* (84.79)
Bb-00035	0.03	0.00 - 7.17	*B; Alistipes finegoldii* (84.66)
Bb-00037	0.00	0.00 - 5.69	*B; Bacteroides coprophilus* (84.77)
Bb-00058*	1.45	0.11 - 13.40	*F; Negativibacillus massiliensis* (87.55)
Bb-00068*	0.22	0.02 - 19.54	*F; Anaeromasilibacillus senegalensis* (88.97)
Bb-00056*	0.41	0.03 - 14.05	*F; Ruminococcus bromii* (91.24)
Bb-00029	0.99	0.06 - 15.17	*F; Anaeromasilibacillus senegalensis* (85.10)
Bb-00036	0.14	0.00 - 6.18	*F; Ruthenibacterium lactatiformans* (85.66)
Bb-00025	0.17	0.00 - 20.67	*P; Succinivibrio dextrinosolvens* (97.11)
Bb-00019	0.06	0.01 - 39.49	*P; Ruminobacter amylophilus* (83.58)

Taxonomic Affiliations: B: *Bacteroidetes*, **F:** *Firmicutes*, **P:** *Proteobacteria.* * OTUs that were found to be present in high abundance in grain-fed Blue Creek heifers (Shared OTUs)

Table 5. Most abundant OTUs identified in the rumen of Blue Creek heifers fed a grass-based diet (BCGrass)

OTU	Median (%)	Range (%)	Closest Valid Taxon (% Identity)
Bb-00002*	0.15	0.00 - 3.47	*B; Prevotella ruminicola* (90.32)
Bb-00040	0.17	0.00 - 5.42	*B; Prevotella ruminicola* (90.46)
Bb-00039	0.03	0.00 - 6.46	*B; Prevotella brevis* (89.96)
Bb-00043	0.03	0.00 - 2.64	*B; Prevotella shahii* (88.32)
Bb-00053	0.15	0.02 - 1.05	*B; Prevotella ruminicola* (92.79)
Bb-00042	0.02	0.00 - 2.75	*B; Prevotella brevis* (88.85)
Bb-00052	0.04	0.00 - 1.11	*B; Prevotella paludivivens* (90.49)
Bb-00050	0.01	0.00 - 1.36	*B; Prevotella shahii* (90.13)
Bb-00044	1.25	0.81 - 2.37	*F; Hungateiclostridium thermocellum* (84.88)
Bb-00046	1.24	0.00 - 1.36	*F; Christensenella massiliensis* (85.53)
Bb-00049	0.72	0.29 - 1.39	*F; Hungateiclostridium thermocellum* (84.56)
Bb-00054	0.48	0.21 - 1.03	*F; Neglecta timonensis* (84.94)
Bb-00045	0.23	0.00 - 2.19	*F; Clostridium amylolyticum* (80.84)
Bb-00003*	0.32	0.02 - 1.43	*Pl; Thermostilla marina* (82.05)
Bb-00047	0.10	0.00 - 1.46	*Pl; Rhodopirellula lusitana* (82.91)
Bb-00051	0.16	0.04 - 1.22	*S; Thermodesulfobium narugense* (77.82)

Taxonomic Affiliations: B: *Bacteroidetes*, **F:** *Firmicutes*, **P:** *Proteobacteria.*
*OTUs that were found to be present in high abundance in grass-fed Standing Butte heifers (Shared OTUs)

Table 6. Most abundant OTUs identified in the rumen of Blue Creek heifers fed a grain-based free-choice diet (BCGrain)

OTU	Median (%)	Range (%)	Closest Valid Taxon (% Identity)
Bb-00020*	0.48	0.00 - 33.59	*B; Mediterranea massiliensis* (84.28)
Bb-00057	0.12	0.02 - 16.46	*B; Prevotella ruminicola* (91.12)
Bb-00059	0.07	0.00 - 13.31	*B; Barnesiella viscericola* (85.74)
Bb-00062	0.14	0.00 - 9.47	*B; Prevotella loescheii* (87.95)
Bb-00067	0.11	0.00 - 6.65	*B; Coprobacter fastidiosus* (83.55)
Bb-00071	0.27	0.00 - 5.09	*B; Parabacteroides chongii* (83.58)
Bb-00072	0.00	0.00 - 4.93	*B; Prevotella brevis* (89.75)
Bb-00056	3.77	0.26 - 23.93	*F; Ruminococcus bromii* (91.24)
Bb-00058	1.22	0.57 - 13.80	*F; Negativibacillus massiliensis* (87.55)
Bb-00060	0.66	0.19 - 12.40	*F; Anaerocolumna xylanovorans* (89.73)
Bb-00061	0.61	0.00 - 10.18	*F; Colidextribacter massiliensis* (89.73)
Bb-00063	0.67	0.12 - 8.95	*F; Oscillibacter valericigenes* (87.93)
Bb-00064	0.56	0.11 - 8.31	*F; Streptococcus lutetiensis* (100)
Bb-00029*	1.56	0.13 - 6.91	*F; Anaeromasilibacillus senegalensis* (85.10)
Bb-00068	0.35	0.01 - 6.37	*F; Anaeromasilibacillus senegalensis* (88.97)
Bb-00069	0.56	0.22 - 6.18	*F; Anaeromasilibacillus senegalensis* (86.30)
Bb-00070	0.84	0.05 - 5.96	*F; Anaerocolumna xylanovorans* (90.11)
Bb-00073	0.23	0.01 - 4.74	*F; Anaeromasilibacillus senegalensis* (89.72)
Bb-00074	0.00	0.00 - 4.58	*F; Eisenbergiella massiliensis* (92.44)
Bb-00065	0.17	0.00 - 6.95	*Pl; Rhodopirellula baltica SH 1 16S* (80.36)

Taxonomic Affiliations: B: *Bacteroidetes*, **F:** *Firmicutes*, **Pl:** *Planctomycetes.* * OTUs that were found to be present in high abundance in grain-fed Standing butte heifers (Shared OTUs)

Table 7. Relative abundance of main OTUs shared between Standing Butte and Blue Creek heifers fed a grass-based diet

OTU	Relative Abundance Median (%)		*P*-value	Closest Valid Taxon (%Identity)
	SBGrass	BCGrass		
Bb-00018#	0.58	0.38	0.0036	*B; Prevotella brevis* (91.86)
Bb-00001#	0.04	0.00	<0.001	*B; Prevotella brevis* (89.16)
Bb-00014	0.31	0.24	0.0760	*B; Prevotella ruminicola* (91.84)
Bb-00011	0.22	0.40	0.0819	*B; Prevotella ruminicola* (89.87)
Bb-00002#	0.03	0.15	0.0004	*B; Prevotella ruminicola* (89.18)
Bb-00010#	0.24	0.01	<0.001	*B; Prevotella shahii* (89.94)
Bb-00008#	0.19	0.00	<0.001	*B; Prevotella brevis* (90.32)
Bb-00005	0.04	0.08	0.8094	*B; Prevotella ruminicola* (90.38)
Bb-00006#	0.02	0.01	0.0354	*B; Prevotella ruminicola* (91.87)
Bb-00012#	0.02	0.00	0.0001	*B; Alistipes finegoldii* (84)
Bb-00040#	0.00	0.17	<0.001	*B; Prevotella ruminicola* (90.46)
Bb-00042#	0.00	0.02	0.0006	*B; Prevotella brevis* (88.85)
Bb-00043#	0.00	0.03	0.0037	*B; Prevotella shahii* (88.32)
Bb-00050	0.00	0.01	0.2442	*B; Prevotella shahii* (90.13)
Bb-00052	0.04	0.04	0.7958	*B; Prevotella paludivivens* (90.49)
Bb-00053	0.13	0.15	0.4589	*B; Prevotella ruminicola* (92.79)
Bb-00039	0.00*	0.03	-	*B; Prevotella brevis* (89.96)
Bb-00016#	0.46	0.16	0.0010	*F; Saccharofermentans acetigenes* (89.02)
Bb-00007#	0.19	0.04	0.0151	*F; Pseudobutyrivibrio ruminis* (96.5)
Bb-00009#	0.14	0.08	0.0219	*F; Clostridium bolteae* (91.92)
Bb-00015#	0.09	0.03	0.0040	*F; Ileibacterium massiliense* (89.16)
Bb-00013#	0.06	0.01	<0.001	*F; Ruminococcus flavefaciens* (98.95)
Bb-00044#	0.27	1.25	<0.001	*F; Hungateiclostridium thermocellum* (84.88)
Bb-00045	0.19	0.23	0.5581	*F; Clostridium amylolyticum* (80.84)
Bb-00046#	0.35	1.24	<0.001	*F; Christensenella massiliensis* (85.53)
Bb-00049#	0.19	0.72	<0.001	*F; Hungateiclostridium thermocellum* (84.56)
Bb-00054#	0.19	0.48	0.0040	*F; Neglecta timonensis* (84.94)
Bb-00003	0.26	0.32	0.5239	*Pl; Thermostilla marina* (82.05)
Bb-00047	0.08	0.10	0.5465	*Pl; Rhodopirellula lusitana* (82.91)
Bb-00004#	0.02	0.00	0.0051	*Fb; Fibrobacter succinogenes* (98.41)
Bb-00017#	0.24	0.57	0.0011	*C; Flexilinea flocculi* (91.3)
Bb-00051	0.09	0.16	0.2213	*S; Thermodesulfobium narugense* (77.82)

SBGrass: Standing Butte Grass; **BCGrass:** Blue Creek Grass; **Taxonomic Affiliations: B**: Bacteroidetes, **F:** Firmicutes, **Pl:** Planctomycetes, **Fb:** Fibrobacteres, **C:** Chloroflexi, **S:** SR1.

* Below Detection

Taxa showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p < 0.05$)

Table 8. Relative abundance of main OTUs shared between Standing Butte and Blue Creek heifers fed a grain-based free-choice diet

OTU	Relative Abundance Median (%)		*P*-value	Closest Valid Taxon (% Identity)
	SBGrain	BCGrain		
Bb-00020	0.20	0.48	0.5931	*B; Mediterranea massiliensis* (84.28)
Bb-00021	0.11	0.40	0.1964	*B; Prevotella buccalis* (88.78)
Bb-00022#	0.77	0.05	<0.001	*B; Prevotella ruminicola* (97.90)
Bb-00023#	0.18	0.00	<0.001	*B; Prevotella ruminicola* (92.63)
Bb-00024#	0.93	0.00	<0.001	*B; Prevotella brevis* (89.96)
Bb-00038#	0.07	0.00	0.0001	*B; Alistipes finegoldii* (84.79)
Bb-00032#	0.02	0.00	0.0116	*B; Prevotella copri* (89.47)
Bb-00035	0.03	0.02	0.3165	*B; Alistipes finegoldii* (84.66)
Bb-00034#	0.04	0.00	<0.001	*B; Prevotella veroralis* (91.15)
Bb-00027	0.07	0.00*	-	*B; Alistipes finegoldii* (84)
Bb-00028	0.10	0.00*	-	*B; Prevotella copri* (88.16)
Bb-00033	0.26	0.00*	-	*B; Alistipes finegoldii* (85.01)
Bb-00037	0.00	0.00*	-	*B; Bacteroides coprophilous* (84.77)
Bb-00057#	0.06	0.12	0.0200	*B; Prevotella ruminicola* (91.12)
Bb-00062	0.04	0.14	0.3261	*B; Prevotella loescheii* (87.95)
Bb-00067#	0.02	0.11	0.0051	*B; Coprobacter fastidiosus* (83.55)
Bb-00072	0.03	0.00	0.0561	*B; Prevotella brevis* (89.75)
Bb-00071	0.00*	0.27	-	*B; Parabacteroides chongii* (83.58)
Bb-00059	0.00*	0.07	-	*B; Barnesiella viscericola* (85.74)
Bb-00029	0.99	1.56	0.2485	*F; Anaeromasilibacillus senegalensis* (85.10)
Bb-00036	0.14	0.00*	-	*F; Ruthenibacterium lactatiformans* (85.66)
Bb-00056#	0.44	3.77	0.0044	*F; Ruminococcus bromii* (91.24)
Bb-00058	1.50	1.22	0.9040	*F; Negativibacillus massiliensis* (87.55)
Bb-00060#	0.13	0.66	<0.001	*F; Anaerocolumna xylanovorans* (89.73)
Bb-00061#	0.00	0.61	<0.001	*F; Colidextribacter massiliensis* (89.73)
Bb-00063#	0.26	0.67	0.0102	*F; Oscillibacter valericigenes* (87.93)
Bb-00064#	0.05	0.56	<0.001	*F; Streptococcus lutetiensis* (100)
Bb-00068	0.34	0.35	0.9862	*F; Anaeromasilibacillus senegalensis* (88.97)
Bb-00069#	0.22	0.56	0.0055	*F; Anaeromasilibacillus senegalensis* (86.30)
Bb-00070#	0.14	0.84	0.0007	*F; Anaeromasilibacillus senegalensis* (90.11)
Bb-00074	0.01	0.00	0.2049	*F; Eisenbergiella massiliensis* (92.44)
Bb-00073	0.00*	0.23	-	*F; Anaeromasilibacillus senegalensis* (89.72)
Bb-00019#	0.06	0.00	<0.001	*P; Ruminobacter amylophilus* (83.58)
Bb-00025#	0.17	0.00	<0.001	*P; Succinivibrio dextrinosolvens* (97.11)
Bb-00065#	0.02	0.17	0.0166	*S; Rhodopirellula baltica* (80.36)

SBGrain: Standing Butte Grain; **BCGrain:** Blue Creek Grain. **Taxonomic Affiliations:**
B: Bacteroidetes, **F:** Firmicutes, **P:** Proteobacteria, **S:** SR1.

***** Below Detection

Taxa showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p < 0.05$)

Table 9. Relative abundance of main OTUs shared between grass and grain-fed Standing Butte heifers

OTU	Relative Abundance Median (%)		p-value	Closest Valid Taxon (%Identity)
	Grass	Grain		
Bb-00018#	0.58	0.06	<0.001	*B; Prevotella brevis* (91.86)
Bb-00014	0.31	0.38	0.5934	*B; Prevotella ruminicola* (91.84)
Bb-00011#	0.22	0.00	<0.001	*B; Prevotella ruminicola* (89.87)
Bb-00002#	0.03	0.00	0.0074	*B; Prevotella ruminicola* (89.18)
Bb-00010#	0.24	0.06	0.0200	*B; Prevotella shahii* (89.94)
Bb-00008#	0.19	0.02	0.0494	*B; Prevotella brevis* (90.32)
Bb-00005#	0.04	0.01	0.0073	*B; Prevotella ruminicola* (90.38)
Bb-00006#	0.02	0.00	<0.001	*B; Prevotella ruminicola* (91.87)
Bb-00020#	0.03	0.20	<0.001	*B; Mediterranea massiliensis* (84.28)
Bb-00021#	0.00	0.11	<0.001	*B; Prevotella buccalis* (88.78)
Bb-00022#	0.24	0.77	0.0032	*B; Prevotella ruminicola* (97.90)
Bb-00023	0.15	0.18	0.3435	*B; Prevotella ruminicola* (92.63)
Bb-00024#	0.23	0.93	0.0036	*B; Prevotella brevis* (89.96)
Bb-00027#	0.02	0.07	0.0022	*B; Alistipes finegoldii* (84%)
Bb-00038#	0.02	0.07	0.0037	*B; Alistipes finegoldii* (84.79)
Bb-00033#	0.00	0.26	<0.001	*B; Alistipes finegoldii* (85.01)
Bb-00034#	0.00	0.04	<0.001	*B; Prevotella veroralis* (91.15)
Bb-00035#	0.01	0.03	0.0041	*B; Alistipes finegoldii* (84.66)
Bb-00037#	0.16	0.00	<0.001	*B; Bacteroides coprophilus* (84.77)
Bb-00001	0.04	0.00*	-	*B; Prevotella brevis* (89.16)
Bb-00012	0.02	0.00*	-	*B; Alistipes finegoldii* (84)
Bb-00028	0.00*	0.10	-	*B; Prevotella copri* (88.16)
Bb-00032	0.00*	0.02	-	*B; Prevotella copri* (89.47)
Bb-00016#	0.46	0.06	<0.001	*F; Saccharofermentans acetigenes* (89.02)
Bb-00007#	0.19	0.02	<0.001	*F; Pseudobutyrivibrio ruminis* (96.5)
Bb-00009#	0.14	0.00	<0.001	*F; Clostridium bolteae* (91.92)
Bb-00015#	0.09	0.00	<0.001	*F; Ileibacterium massiliense* (89.16)
Bb-00013#	0.06	0.01	0.0011	*F; Ruminococcus flavefaciens* (98.95)
Bb-00026#	0.00	0.22	<0.001	*F; Anaeromasilibacillus senegalensis* (88.14)
Bb-00029#	0.25	0.99	0.0084	*F; Anaeromasilibacillus senegalensis* (85.10)
Bb-00031#	0.04	1.45	<0.001	*F; Negativibacillus massiliensis* (86.92)
Bb-00030	0.00*	0.41	-	*F; Colidextribacter massiliensis* (87.50)
Bb-00036	0.00*	0.14	-	*F; Ruthenibacterium lactatiformans* (85.66)
Bb-00019#	0.00	0.06	<0.001	*P; Ruminobacter amylophilus* (83.58)
Bb-00025	0.00*	0.17	-	*P; Succinivibrio dextrinosolvens* (97.11)
Bb-00004#	0.02	0.00	<0.001	*Fb; Fibrobacter succinogenes* (98.41)
Bb-00003#	0.26	0.00	<0.001	*Pl; Thermostilla marina* (82.05)
Bb-00017	0.24	0.00*	-	*C; Flexilinea flocculi* (91.3)

Taxonomic Affiliations: B: Bacteroidetes, **F:** Firmicutes, **P:** Proteobacteria **Fb:** Fibrobacteres, **Pl:** Planctomycetes**, C:** Chloroflexi.

* Below Detection

Taxa showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p < 0.05$)

Table 10. Relative abundance of main OTUs shared between grass and grain-fed Blue Creek heifers

OTU	Relative Abundance Median (%)		*P*-value	Closest Valid Taxon (%Identity)
	Grass	Grain		
Bb-00041#	0.13	0.00	<0.001	*B; Prevotella ruminicola* (90.3)
Bb-00052#	0.04	0.00	<0.001	*B; Prevotella paludivivens* (90.49)
Bb-00053#	0.15	0.00	<0.001	*B; Prevotella ruminicola* (92.79)
Bb-00039	0.03	0.00*	-	*B; Prevotella brevis* (89.96)
Bb-00040	0.17	0.00*	-	*B; Prevotella ruminicola* (90.46)
Bb-00042	0.02	0.00*	-	*B; Prevotella brevis* (88.85)
Bb-00043	0.03	0.00*	-	*B; Prevotella shahii* (88.32)
Bb-00050	0.01	0.00*	-	*B; Prevotella shahii* (90.13)
Bb-00055#	0.08	0.48	0.0236	*B; Mediterranea massiliensis* (83.71)
Bb-00072	0.00	0.00	0.5317	*B; Prevotella brevis* (89.75)
Bb-00057	0.00*	0.12	-	*B; Prevotella ruminicola* (91.12)
Bb-00059	0.00*	0.07	-	*B; Barnesiella viscericola* (85.74)
Bb-00062	0.00*	0.14	-	*B; Prevotella loescheii* (87.95)
Bb-00067	0.00*	0.11	-	*B; Coprobacter fastidiosus* (83.55)
Bb-00071	0.00*	0.27	-	*B; Parabacteroides chongii* (83.58)
Bb-00045#	0.23	0.00	<0.001	*F; Clostridium amylolyticum* (80.8)
Bb-00046#	1.24	0.45	0.0003	*F; Christensenella massiliensis* (85.53)
Bb-00049#	0.72	0.45	0.0313	*F; Hungateiclostridium thermocellum* (84.56)
Bb-00044#	1.25	0.23	<0.001	*F; Hungateiclostridium thermocellum* (84.8)
Bb-00054#	0.48	0.79	0.0263	*F; Neglecta timonensis* (84.94)
Bb-00058#	0.03	1.22	<0.001	*F; Negativibacillus massiliensis* (87.55)
Bb-00063#	0.05	0.67	<0.001	*F; Oscillibacter valericigenes* (87.93)
Bb-00064#	0.00	0.56	<0.001	*F; Streptococcus lutetiensis* (100)
Bb-00066#	0.16	1.56	<0.001	*F; Saccharofermentans acetigenes* (87.08)
Bb-00069#	0.27	0.56	0.0002	*F; Anaeromasilibacillus senegalensis* (86.30)
Bb-00070#	0.02	0.84	<0.001	*F; Anaerocolumna xylanovorans* (90.11)
Bb-00056	0.00*	3.77	-	*F; Ruminococcus bromii* (91.24)
Bb-00060	0.00*	0.66	-	*F; Anaerocolumna xylanovorans* (89.73)
Bb-00061	0.00*	0.61	-	*F; Colidextribacter massiliensis* (89.73)
Bb-00068	0.00*	0.35	-	*F; Anaeromasilibacillus senegalensis* (88.97)
Bb-00073	0.00*	0.23	-	*F; Anaeromasilibacillus senegalensis* (89.72)
Bb-00074	0.00*	0.00	-	*F; Eisenbergiella massiliensis* (92.44)
Bb-00047	0.10	0.00*	-	*Pl; Rhodopirellula lusitana* (82.91)
Bb-00051	0.16	0.00*	-	*Pl; Thermodesulfobium narugense* (77.82)
Bb-00048#	0.32	0.02	<0.001	*Pl; Pirellula staleyi* (82.26)
Bb-00065#	0.01	0.17	<0.001	*S; Rhodopirellula baltica* (80.36)

Taxonomic Affiliations: B: Bacteroidetes, **F:** Firmicutes, **Pl:** Planctomycetes, **S:** SR1.

***** Below Detection

Taxa showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p < 0.05$)

CHAPTER 3

Future Directions and Impact of Research

1. Introduction

The bison industry is contributing to the return of the bison on the Great Plains. As bison producers build the herds to meet growing consumer demands, efforts are also made to protect the integrity of the species and to gain a deeper understanding of their biological, ecological, physiological, and behavioral characteristics. Decades of research on cattle and other ruminant livestock species have shown that the gut microbiome plays a very important role in the ruminant's health by digesting plant biomass and benefiting their immune system. An imbalance between beneficial and harmful microorganisms in the gut can lead to disease and gastrointestinal disorders. Due to their complexity and major roles in the fermentation of feeds, the microbial communities inhabiting the rumen have remained a point of great interest. Research efforts have revealed several connections between rumen microorganisms and host physiological functions, revealing important insights on the central role of the rumen for ruminants' productivity. However, as far as the bison rumen microbiome is concerned, the rate of applicable research information has not grown at the same pace as the growth of the industry. In the absence of bison rumen microbiome research, many commercial bison producers have been implementing practices without knowing potential effects that may be harmful to bison, relying on studies that have been done in cattle to infer physiological responses in bison. In an effort to offer more reliable information to the bison industry, the current study aimed to compare the bacterial communities residing in the rumen of the North American

bison under two distinct diets: grass and free-choice grain-based. These results have contributed to the budding research performed on the bison gut microbiome.

2. Experimental findings and future outlook

The results described in this thesis showed an important change in the diversity and composition of bison rumen bacteria with the incorporation of grains into the diet. While these easily fermentable ingredients and their by-products can improve productivity, maximize host genetic potential, and enhance cost-efficiency in the short term, they may have undesirable consequences for animal health. We showed that the rumen microbiome of bison has more diverse bacterial communities when bison are fed grass compared to grain rations. These microbial communities were replaced during the grain diet phase by a less complex microbiota, including increased representation of members of the Proteobacteria phylum. While the potential health impact of this loss in diversity is still unclear, increases in the relative abundance of Proteobacteria may increase the risk of host disease, as this phylum includes bacterial species associated with antimicrobial resistance genes and pathogenicity (Durso et al., 2012), imbalances in the gut microbial community (dysbiosis), and inflammation (Winter and Bäumler, 2014),.

As expected, a change in diet was an important factor associated with observed rumen microbiota alterations. However, it was also observed that distinct bacterial compositions were found in subpopulations of animals belonging to the same experimental group. Effects associated with management, such as the feeding system used (free choice), could be a potential factor, however, varying selectivity of ingredients amongst animals of the same group and even a wide variety of chemical compounds

present in the grasses consumed may explain the differences in microbial communities observed within the same experimental group. Distinct bacterial communities amongst and within groups would indicate differences in metabolic potential, likely in response to differences in substrate composition amongst different dietary ingredients. Since all bison in this study were under free-choice regimens, investigating bison microbial communities under a more strict grain-finishing system, such as daily feeding of a totally-mixed ration (TRM), would be needed to compare and evaluate the effects of high-inclusion of grain on gut microbial communities.

In bison production, research priorities may vary among segments of the industry, but in order to generate more practical research, a basic understanding of the bacterial communities responsible for rumen fermentation is needed. The use of a 16S rRNA gene diversity approach combined with the availability of a high number of 16S rRNA reference sequences only allowed us to infer metabolic function from taxonomy data generated in OTUs closely related to a valid taxon. While this approach allowed to identify a broad spectrum of bacterial species from a wide range of sample sources, further investigations are needed to increase the accuracy of functional profiling for OTUs that were phylogenetically too distant from a given known bacterial species.

3. Future directions and potential applications

Culturing microbial isolates is a technique that has so far proven very challenging for rumen microorganisms. Thus, shotgun metagenomic sequencing may provide a more comprehensive understanding with increased genome coverage that would offer greater insight about microbiome functional potential. This approach, for instance, could assist in

the understanding of mechanisms responsible for the higher efficiency of bison on low-quality feed, including potential microbial metabolic functions such as cellulose/hemicellulose hydrolysis, lignin breakdown, and *de novo* amino acid synthesis. In addition to revealing the presence or absence of these functions in bison ruminal bacteria, metagenomics can also uncover other metabolic capabilities, such as the potential to utilize other substrates provided in feed or show the absence of specific metabolic pathways that could indicate a requirement for certain nutrients. It would also provide crucial insights into other findings; for instance, higher production of medium-chain fatty acids (MCFAs) by bison rumen bacteria using cellulose was recently reported (Rico et al., 2021). MCFAs with an odd number of carbons could be used by the host as precursors for propionate, an important substrate for gluconeogenesis, and thus very beneficial for ruminant performance. Overall, this knowledge would then allow the identification of desirable ruminal bacterial species, permitting the development of applications that would benefit pasture-based bison production. Potential innovations could include the selection of bison with higher levels of desirable symbionts as a marker of efficiency or the development of natural pre- or probiotics that would increase the abundance or activity of desirable symbionts in the rumen of bison in a herd. While it would require a longer timeline to implement, a prebiotic or probiotic approach could be developed based on the results from the metagenomic analyses, aiming to increase the abundance of beneficial bacteria in bison on pasture without needing selection. This strategy would have the benefit of being flexible in optimizing herd performance. Ultimately, ‘probiotic cocktails’ could be developed for inoculating calves at weaning in conjunction with vaccination and other prevention treatments during processing.

Meat products from grass-fed ruminants have been shown to provide additional nutritional benefits. Since rumen microorganisms can metabolize a vast array of plant-based substrates, including fatty acids and tannins, further improvements to the nutritive quality of bison products could be achieved by selection of particular rumen bacteria that could potentially produce various metabolic processes correlated with carcass characteristics.

The effective contribution of bison ruminal bacteria identified as beneficial or favorable based on their metabolic potential could be assessed by correlating their abundance with animal performance and other physiological parameters. Since such future studies would require a much larger scale, optimization of qPCR-based assays would greatly facilitate screening high numbers of animals. If successful, this strategy could allow the use of bacteria as indicators or markers of higher performance.

4. Impact of Research

In bison production, one major ranch management decision to be made is the proportion of grass and grain that should be used when it comes to finishing rations. So far, such decisions have been made based on consumer demand for healthier meat of better quality and operation maintenance costs. The principles of feeding bison are similar to feeding other ruminants, but the social structure, seasonal change effects, and other factors have a greater impact on bison management compared to domesticated ruminants (Carter et al., 2010). While bison producers understand that gradual incorporation of grain in rations is critical for maintaining a healthy microbial ecosystem, they may be less familiar with how much these populations are susceptible to changes in

diet and to what extent this could potentially create unintended challenges to their operations. This study represents a starting point towards a better understanding of the rumen microbiome in bison. Future possible directions could include investigating the potential effects of the bacterial diversity loss observed during the grain-feeding phase or the changes in rumen metabolites and metabolic pathways through exploring the genomes of the candidate bacterial species identified in this study.

As demonstrated in this thesis, nutritional changes can cause major alterations in the diversity and abundance of rumen bacterial communities. Although no major negative effects on animal health and productivity have been reported, bison producers may need to take into consideration the impact that grain-based diets have on the diversity of rumen bacterial species. To this end, identifying the genetic potential of these species, as discussed earlier in this chapter, would provide greater insight into their metabolic potential. Further research would provide necessary insights and potentially lead to the development of improved strategies for higher feed efficiency and improved management.

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