INVESTIGATION OF THE INTERRELATIONSHIP BETWEEN RUMINAL AND ORAL MICROBIOME OF ANGUS BULLS

by

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(Under the Direction of Jeferson M. Lourenco)

ABSTRACT

Cattle's gastrointestinal microbiome has been identified as an important component linked to animal performance, greenhouse gas production, and health. Specific to the rumen, while traditional methods like rumen cannulation and orogastric intubation are effective, they pose challenges in terms of animal welfare and practicality. Therefore, this study evaluated the feasibility of using buccal swabs as a non-invasive alternative for analysis of the microbes living in the rumen. Samples were collected from 541 Angus bulls across five feed testing centers in the United States, with DNA extracted from both ruminal and buccal samples. Advanced microbial genomics and metagenomics techniques were employed to analyze the samples. As distinct microbial communities were found between buccal and rumen samples, our findings indicate that the oral microbiome may not be a straightforward surrogate for rumen microbial communities. This realization underscores the necessity of directly examining the rumen microbiome for accurate insights into its composition and function.

INDEX WORDS: Rumen microbiome, Buccal swabs, Angus, Non-invasive sampling

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DEDICATION

I would like to dedicate this thesis to my family and our lab team. To my family, thank you for your unwavering love and support throughout my educational journey; none of this would have been possible without you. To my lab team, your collaboration and encouragement have been invaluable. This work is a testament to our collective passion for knowledge and discovery.

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CHAPTER 1

INTRODUCTION

Ruminant animals possess a unique digestive system that allows them to convert fibrous plant materials into energy (Stevens & Hume, 2004). This process is primarily facilitated by the rumen, an anaerobic fermentation chamber housing a complex microbiome (Mizrahi et al., 2021). This microbiome includes bacteria, archaea, protozoa, and fungi, each playing a specific role in breaking down cellulose, hemicellulose, and other fibrous components of plant cell walls. The end products of this microbial fermentation are volatile fatty acids (VFAs), methane, carbon dioxide, and microbial biomass (Newbold & Ramos-Morales, 2020). These VFAs, including acetate, propionate, and butyrate, are absorbed through the rumen wall and serve as major energy sources for the host animal (Cholewińska et al., 2020; The Rumen and Its Microbes - Robert E. Hungate - Google Books, 1996), significantly influencing their growth, health, and productivity.

Recent advancements in microbial genomics and metagenomics have provided unprecedented insights into the rumen microbiome. High-throughput sequencing technologies, such as next-generation sequencing (NGS), enable comprehensive analysis of microbial communities and their genetic material. NGS allows researchers to rapidly sequence and analyze vast amounts of DNA, offering a detailed view of the diversity and functional potential of the rumen microbiota. For instance, studies utilizing NGS have identified complex interactions among different microbial species and their roles in fermentation and nutrient synthesis. These insights are crucial for understanding how to

manipulate the microbiome to improve feed efficiency and animal health, which can be achieved by probiotic supplementation, fecal transfer, etc.

The study of the rumen microbiome is a topic with significant implications for ruminant health and agricultural sustainability. Historically, the study of rumen microbes was limited by the difficulty of sample collection (Castillo & Hernández, 2021) and culturing anaerobic microorganisms in laboratory. However, advances in molecular biology techniques have overcome these challenges, allowing for more detailed and accurate investigations. Researchers hypothesize that manipulating the rumen microbiome could lead to substantial improvements in feed efficiency, animal health, and environmental sustainability. For instance, enhancing the production of specific VFAs through microbiome manipulation could improve energy utilization and reduce greenhouse gas emissions from ruminant livestock.

Despite these advancements, a major challenge remains in the collection of representative rumen samples. Traditional methods such as rumen cannulation, rumenocentesis, and orogastric intubation, are invasive and pose significant welfare concerns for the animals (Castillo & Hernández, 2021). Rumen cannulation involves surgically creating a permanent fistula for sample collection, while orogastric intubation requires inserting a tube through the mouth into the rumen. Both methods are technically demanding, require skilled personnel, and can cause stress and discomfort to the animals. Furthermore, the invasive nature of these procedures can introduce variability in microbial composition, potentially affecting the accuracy of the research findings.

Given these challenges, this study explores the use of buccal swabs as a non-invasive alternative for rumen microbiome analysis. The primary objective is to evaluate the correlation between the microbial communities in buccal swabs and rumen samples from Angus bulls. By utilizing advanced microbial genomics and metagenomics techniques, the study aimed to determine whether buccal swabs can reliably reflect the rumen microbiome composition, thereby providing a less stressful and more practical method for microbiome sampling. The hypotheses being tested include the potential for buccal swabs to serve as proxies for rumen microbiota and the viability of this method for routine monitoring of ruminant animals.

LITERATURE CITED

Castillo, C., & Hernández, J. (2021). Ruminal Fistulation and Cannulation: A Necessary Procedure for the Advancement of Biotechnological Research in Ruminants. *Animals 2021, Vol. 11, Page 1870*, *11*(7), 1870. https://doi.org/10.3390/ANI11071870

Cholewińska, P., Czyz, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Animal Health Research Reviews*, *21*(1), 3–14. https://doi.org/10.1017/S1466252319000069

Hungate, R. E. (1996). *El rumen y sus microbios*. *1*, 533 pp. https://www.elsevier.com/books/the-rumen-and-its-microbes/hungate/978-1-4832-3308-6

Mizrahi, I., Wallace, R. J., & Moraïs, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nature Reviews Microbiology 2021 19:9*, *19*(9), 553–566. https://doi.org/10.1038/s41579-021-00543-6

Newbold, C. J., & Ramos-Morales, E. (2020). Review: Ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal*, *14*(S1), s78–s86. https://doi.org/10.1017/S1751731119003252

Stevens, C., & Hume, I. (2004). *Comparative physiology of the vertebrate digestive system*. https://books.google.com/books?hl=en&lr=&id=DZuAsci2apAC&oi=fnd&pg=PR15&dq=Comparative+Physiology+of+the+Vertebrate+Digestive+System+-+C.+Edward+Stevens,+Ian+D.+Hume+-+Google+Books&ots=rPiY0Kqv6Q&sig=W3vb5z0OyBCJRl0WLswG-fr6gdQ

CHAPTER 2

REVIEW OF THE LITERATURE

Rumen Microbiome Complexity and Functions

The Rumen Microbiome's Role in Fermentation and Digestion

The rumen microbiome plays a vital role in ruminants' unique digestive system. The microbiome helps these animals extract nutrients from plant-based diets that are largely inaccessible to other species (Antony 2019). The rumen is crucial in this process as an anaerobic fermentation chamber. In the rumen, a diverse and interconnected community of microorganisms collaboratively degrades cellulose, hemicellulose, and other fibrous elements in plant cell walls. The end products of this fermentation process are volatile fatty acids (VFAs), methane, carbon dioxide, and microbial biomass. This process provides energy and nutrients to the host (Stevens and Hume 2004; Mizrahi, Wallace, and Moraïs 2021). The ruminal intricate microbial ecosystem allows for the efficient utilization of fibrous feeds and significantly influences the animal's energy balance and nutrient metabolism, thereby impacting growth, health, and productivity.

Diversity of Microorganisms in the Rumen and Their Functions

The rumen hosts an incredibly diverse microbiota comprising bacteria, archaea, protozoa, and fungi (Li et al., 2019; Sanjorjo et al., 2019), each with specific roles that contribute to the overall efficiency of the rumen digestive process:

- **Bacteria:** The most populous microbial group in the rumen, bacteria are pivotal in hydrolyzing plant polysaccharides into simpler sugars (Inman 2011; Sichert and Cordero 2021). These sugars are then fermented to produce VFAs, the primary energy source for ruminants. Different bacterial species specialize in breaking down various plant cell wall components, demonstrating a highly specialized and diversified bacterial community (Dehority 1991).

- **Archaea:** Although less abundant than bacteria (Peng et al., 2022; Volmer, McRae, and Morrison 2023), archaea play a crucial role in the rumen ecosystem, primarily through methane production. This process is essential for maintaining a hydrogen balance within the rumen, facilitating bacteria's continued feed fermentation (Firkins 2021).

- **Protozoa:** Protozoa contribute to the mechanical breakdown of feed particles and the predation of bacterial cells (Charles J. Newbold et al., 2015; Williams et al., 2020), thereby influencing the population dynamics within the rumen. They ferment soluble carbohydrates and proteins (Williams et al., 2020), producing VFAs and ammonia.

- **Fungi:** Rumen fungi are adept at degrading fibrous plant materials, including cellulose and lignin (Orpin 1984), which are generally resistant to bacterial degradation. They physically penetrate feed particles (Gruninger et al., 2014;

Hess et al., 2020), making the substrates more accessible to other rumen microbes for further fermentation (Akin and Borneman 1990).

Biochemical Pathways in Fermentation and Production of VFAs

The fermentation of dietary fiber in the rumen involves a series of complex biochemical reactions that ultimately produce VFAs (Ahmad et al., 2020; Shi et al., 2023) The VFAs are absorbed through the rumen wall (Connery 2023) and serve as a vital energy source for the host animal. This process starts with microbial enzymes' enzymatic breakdown of polysaccharides into monosaccharides. These sugars are then fermented through several pathways, primarily leading to the production of acetate, propionate, and butyrate.

Acetate is primarily produced through the acetate kinase pathway and is a significant energy source for muscle and other tissues (Moffett et al., 2020a; 2020b). It also plays a role in milk fat synthesis in lactating animals.

Propionate is generated through the succinate or acrylate pathways and is the only VFA that undergoes gluconeogenesis (Lin et al., 2022). It provides a crucial glucose source for the animal. Propionate's efficient utilization is essential for energy production, particularly in high-producing animals.

Butyrate is produced via the butyrate kinase pathway (Guilloteau et al., 2010). It is a critical energy source for the epithelial cells lining the rumen and colon and promotes healthy gut development and function.

These fermentation pathways' delicate balance and efficiency are critical for optimizing feed conversion (Soltan et al., 2021), animal health, and productivity.

The Role of Rumen Microbiome in Synthesizing Essential Nutrients: Vitamins and Amino Acids

The rumen microbiome's ability to synthesize essential nutrients represents a cornerstone in the nutritional ecology of ruminants. The microbiome enables these animals to thrive on a diet primarily composed of plant materials by not only facilitating the breakdown of complex carbohydrates, but also by synthesizing a variety of essential nutrients, including vitamins and amino acids (Cammack et al., 2018). The rumen microorganisms synthesize vitamins such as B-complex (including B12, niacin, riboflavin, and thiamine) and vitamin K (Jiang et al., 2022). These vitamins directly contribute to the host's nutritional requirements (Hungate 1996). They play crucial roles in energy metabolism, red blood cell formation, and as coenzymes in various metabolic pathways (EFSA NDA Panel et al., 2009).

Amino acids, the building blocks of proteins, are another crucial nutrient rumen microbes synthesize. While ruminants consume proteins in their diet, a significant portion is degraded in the rumen. Rumen bacteria and protozoa utilize the ammonia released from this degradation to synthesize amino acids and proteins, which later become available for absorption in the small intestine (Cholewińska et al., 2020). This microbial protein synthesis is vital for supplying essential amino acids the host cannot synthesize.

Contribution of the Rumen Microbiota to Energy Metabolism

Ruminant energy metabolism is intricately linked to the activities of the rumen microbiome. The primary energy sources for ruminants are VFAs, specifically acetate, propionate, and butyrate, which are produced during the fermentation of dietary fiber by rumen microbes. These VFAs are absorbed through the rumen wall (Kelly et al., 2022), and can provide up to 70% of the host's total energy needs (Bergman 1990).

Acetate, the most abundantly produced VFA (Baldwin et al., 1971; Bergman 1990; Urrutia et al., 2019), is primarily utilized as a substrate for energy production and lipid synthesis (Moffett et al., 2020b). On the other hand, propionate is unique among the VFAs for its role in gluconeogenesis, the process by which glucose is synthesized from non-carbohydrate sources. This is particularly crucial in ruminants, where dietary carbohydrates are extensively fermented to VFAs, and direct sources of glucose are limited. Butyrate is metabolized in the rumen epithelium to ketones, which serve as an additional energy source, particularly during periods of high energy demand (C. J. Newbold and Ramos-Morales 2020).

VFA production and utilization efficiency are crucial determinants of ruminants' feed efficiency and energy balance. Factors such as the composition of the diet, the diversity and functionality of the rumen microbiota, and the animal's metabolic health can significantly influence these processes. Research into manipulating the rumen microbiome to enhance VFA production and utilization can improve energy efficiency (Soltan et al., 2021) and reduce feeding cost. The symbiotic relationship between ruminants and their rumen microbiome is thus fundamental to the animals' ability to

extract energy and synthesize vital nutrients from a diet primarily based on fibrous plant material. Advances in our understanding of this complex microbiome and its interactions with the host are essential for developing innovative strategies to improve the ruminant production system.

Advanced Research Methods in Microbial Genomics and Metagenomics

Current Methodologies in Microbial Genomics and Functional Metagenomics

Both genomics and metagenomics rely on high-throughput sequencing technologies, such as next-generation sequencing (NGS), which enable the rapid and cost-effective analysis (Miller et al., 2013; Jia et al. 2013) of vast amounts of DNA. Bioinformatic tools and databases are then employed to assemble sequences, annotate genes, and perform comparative analyses, linking genetic information to microbial identities and functions. These methodologies have opened new avenues for understanding the genetic basis of microbial traits and their contributions to the rumen ecosystem.

Exploring the rumen microbiome has been revolutionized by advances in microbial genomics (Sun et al., 2021) and metagenomics (Chai et al., 2024). These advances provide unprecedented insights into its complexity and function. Traditionally, culturing microorganisms was used as the primary method of studying microbiota. It is estimated that across Earth's microbiomes, uncultured genera and phyla might make up

approximately 81% and 25% of microbial cells, respectively (Lloyd et al., 2018). A culture-independent method like DNA sequencing is widely used in microbial genomics and metagenomics (Liu et al., 2022). Microbial genomics involves sequencing the DNA of individual microbial species, offering detailed information on their genetic makeup, capabilities, and functions (Hua et al., 2022). This approach has been instrumental in identifying novel microbial species (Friedersdorff et al., 2020) within the rumen and elucidating their specific roles in digestion and fermentation.

Functional metagenomics takes a broader view, sequencing all genetic material present in a sample from the rumen environment. This technique allows researchers to not only catalog the microbial diversity but also to infer the microbial community's metabolic pathways (Liu et al., 2022) and functional capabilities. The metabolic pathways can be predicted and reconstructed using tools like BlastKOLA (Kanehisa et al., 2016), KAAS (Moriya et al. 2007), RAST (Aziz et al., 2008), and GhostKOALA (Kanehisa et al., 2016. Functional metagenomics bypasses the need for culturing microbes in the laboratory. This has a significant advantage given that many rumen microbes are uncultivable under standard conditions (Liu et al., 2022). This approach provides a comprehensive snapshot of the genetic potential of the rumen microbiome, including genes involved in fiber degradation, fermentation, and nutrient synthesis.

The application of genomics and metagenomics has the potential to advance our rumen microbiome understanding significantly. By detailing the microbiome's genetic composition, researchers can identify specific genes (Xu et al., 2021) and pathways responsible for essential functions such as cellulose degradation (Li et al., 2021), methane

production, and VFA synthesis (Siciliano-Jones and Murphy 1989; France and Dijkstra 2005). This genetic insight is critical for identifying microbial strains with beneficial traits that can be targeted for manipulation to improve feed efficiency, reduce greenhouse gas emissions, and enhance animal health.

Functional metagenomics can reveal how the rumen microbiome responds to different dietary interventions, environmental changes (Xu et al., 2023), and host genetic factors (Difford et al., 2018). By analyzing shifts in gene expression and pathway activity, researchers can understand the dynamic interactions between diet, microbiome, and host. This helps to make targeted and effective strategies for managing the ruminant production system. Integrating microbial genomics and metagenomics with other omics technologies, such as transcriptomics, proteomics, and metabolomics, offers a holistic view of the rumen microbiome's function (Denman, Morgavi, and Mcsweeney 2018). This multi-omics approach enables the correlation of genetic potential with actual microbial activities (Saraiva et al., 2021) and metabolite profiles.

Challenges in Rumen Microbiome Research

Difficulties of Collecting Rumen Samples: Technical and Ethical Considerations

Collecting accurate and representative samples from the rumen to study its microbiome involves a series of technical, ethical, and welfare-related challenges. These challenges are not trivial, as they directly impact the validity of research findings (Henderson et al., 2013a; Ramos-Morales et al., 2014; Hagey et al., 2022) and the animals' well-being.

While useful, the two primary traditional methods—rumen cannulation and orogastric intubation—present significant drawbacks that researchers must carefully consider. Rumen cannulation is invasive, requiring surgical intervention to create a permanent fistula through which samples can be repeatedly collected. While offering unparalleled access to the rumen environment, this procedure necessitates careful consideration of animal welfare, including the ethics of performing invasive surgery for research purposes. The procedure's invasiveness introduces risks such as infection and discomfort, necessitating stringent post-operative care and ongoing management to ensure the animal's well-being. Furthermore, the presence of a cannula can affect the animal's natural behavior (Olsson and Westlund 2007).

Orogastric intubation, though less invasive, is challenging. The procedure can be stressful for the animals (Izer, Dwyer, and Wilson 2023), potentially leading to resistance and distress during sample collection. Stress responses can alter physiological conditions, including the rumen's microbial composition, potentially biasing research outcomes. Moreover, the technique requires specific equipment, skilled personnel, and experience to ensure the animal's safety and the samples' reliability, limiting its applicability in settings with access to adequately trained personnel.

As the field advances, ethical and welfare considerations remain at the forefront of rumen microbiome research. Thus, developing less invasive sampling methods reflects a broader commitment within the scientific community to uphold the research standards. Ethical research practices ensure the well-being of research animals and enhance scientific findings' credibility and societal impact. Researchers must consider these factors

carefully, balancing the pursuit of knowledge with the imperative to minimize harm to animal subjects.

Innovations in Non-Invasive Sampling Techniques

Non-invasive Alternatives

The limitations inherent in traditional rumen sampling techniques have spurred interest in developing less invasive methods that minimize animal stress and discomfort. Innovations include using advanced molecular and computational techniques to analyze alternative biological samples, such as feces, urine, or saliva (Tapio et al., 2016). Compared to ruminal samples, buccal samples are easier to collect and less stressful for the animals. While these matrices do not directly represent the rumen microbiome, they can offer insights into the host-microbe interactions and the end-products of microbial fermentation. Advances in bioinformatics and machine learning offer powerful tools for interpreting complex datasets derived from indirect sampling methods (Bhaskar, Hoyle, and Singh 2006).

The quest for less invasive methods to study the complex ecosystem of the rumen microbiota has led researchers to explore innovative alternatives that promise to minimize animal stress. Buccal swabs have emerged as an up-and-coming technique (Amin et al., 2021). This approach involves collecting samples from ruminants' buccal cavity (the cheek area inside the mouth). The buccal swabbing is less invasive than traditional methods like rumen cannulation or orogastric intubation. The use of buccal swabs to study the rumen microbiome is supported by the natural behavior of ruminants,

specifically cud chewing. During this process, ruminants bring up partially digested food from the rumen back into the mouth to chew it further (Hofmann 1989), aiding in the breakdown of plant fibers. This action promotes mixing rumen contents with the buccal microbiota, creating a shared microbial community between the rumen and the buccal cavity. Consequently, buccal swabs can act as a practical and less invasive substitute, capturing a snapshot of the rumen's microbial landscape through the direct contact and exchange of microorganisms during cud chewing. This connection exploits the ruminants' natural feeding behaviors to provide a new and ethically sound method for studying the complex dynamics of the rumen microbiome.

Buccal swabbing offers several advantages, including ease of sample collection (Valinetz and Cangelosi 2021), minimal stress to the animal, and the potential for repeated sampling over time. This method aligns with the ethical imperative to reduce invasiveness in animal research, marking a significant step forward in the study of rumen microbiology. The simplicity of buccal swabbing also allows for more widespread monitoring of rumen health and microbiota dynamics across different environments and dietary regimes, offering a practical tool for farmers, veterinarians, and researchers.

Recent studies have begun to validate the use of buccal swabs as a proxy for assessing the rumen microbiome, with findings indicating a significant correlation between the microbial communities in the buccal cavity and those in the rumen. For instance, Tapio et al. (2016) conducted a pivotal study using oral samples as a proxy for accessing the composition of the rumen microbiome. Their findings revealed that the relative abundance of microbial taxa in buccal and rumen samples was similar, underscoring the feasibility of using buccal swabs to reflect the rumen microbial community. Further

reinforcing this correlation, Young et al. (2020) employed a machine-learning approach to demonstrate a significant correlation between the microbial profiles from buccal swabs and those from rumen samples. This methodological innovation provided a robust framework for predicting rumen microbiome composition based on less invasive buccal samples. Amin et al. (2021) highlighted the dynamic nature of the buccal and rumen microbiome and their interconnectedness. Their study added another layer of understanding to the ruminant's complex interactions between various microbial habitats. It emphasized the potential of buccal swabs for monitoring shifts in the rumen microbiome related to dietary changes, health status, and other factors. Such correlations are supported by the premise that both the buccal cavity and the rumen are part of the continuous oral gastrointestinal tract, where microbes can be transferred and shared. The presence of overlapping microbial communities suggests that buccal microbiota could serve as a valuable indicator of rumen health and function. Studies employing advanced sequencing technologies and bioinformatic analyses have further substantiated these relationships.

Impact of Extraction Methods on Microbiome Study

The impact of DNA extraction methods on microbiome studies in ruminants is a critical aspect of research that aims to accurately characterize the microbial communities within these animals. The choice of DNA extraction method can significantly affect the quantity and quality of the DNA obtained (Henderson et al., 2013a; Knudsen et al., 2016; Lim et

al., 2017; Stinson, Keelan, and Payne 2018; Vaidya et al., 2018; Teng et al., 2018; Mott et al., 2022), which in turn influences the observed microbial composition and diversity and, ultimately, the conclusions drawn from a study. The efficiency of DNA recovery and the purity of the extracted DNA vary across different extraction methods. Techniques that yield higher DNA concentrations and purer DNA samples without inhibitors are preferred for downstream applications like sequencing. The quantity of DNA obtained can especially impact metagenomic studies, where sufficient DNA is crucial for library preparation and sequencing (Knudsen et al., 2016).

Different extraction methods may preferentially lyse certain microbial groups over others, leading to biases in the detected microbial composition and diversity. For instance, some methods might efficiently extract DNA from Gram-negative bacteria but less from Gram-positive bacteria due to their tougher cell walls. This differential lysis can result in an underrepresentation or overrepresentation of certain taxa in the study findings (Knudsen et al., 2016; Lim et al., 2017). Due to the differences in cell wall composition and structure (such as presence of a thick layer of peptidoglycan) methods incorporating mechanical disruption (bead beating, heating) to enzymatic treatment may improve the lysis of tough-to-lyse cells, thus affecting the observed microbial composition (Knudsen et al., 2016).

The stability of extracted DNA and the reproducibility of microbial profiles across different extraction kits and protocols are essential for comparative studies and longitudinal analyses. Some methods may produce more consistent and stable results

over time, making them more suitable for studies that require sample storage and processing at different times (Lim et al., 2017). The effectiveness of DNA recovery and the removal of inhibitory substances (e.g., humic acids in soil or complex carbohydrates in plant material) also vary among extraction methods. These factors can influence the efficiency of subsequent PCR amplifications and sequencing, affecting the detectability of specific microbes (Knudsen et al., 2016).

Developing and adhering to standardized DNA extraction protocols for specific sample types can reduce the variability introduced by different extraction methods. This includes using standardized kits, reagents, and processing steps (Knudsen et al., 2016). Incorporating spike-in controls and mock communities with known microbial compositions can help assess the efficiency and bias of DNA extraction methods as well. Incorporating controls and mock communities also helps adjust and normalize data across different studies (Knudsen et al., 2016). Thoroughly validating DNA extraction methods for specific sample types and reporting the methods used in publications allow for better interpretation and comparison of results across studies.

Machine Learning Applications in Microbiome Research

The advent of machine learning (ML) in microbiome research has opened new frontiers in the analysis and interpretation of complex microbial datasets (Jiang et al., 2022). This computational approach is valuable in handling the vast amounts of data generated from

noninvasively collected samples, such as buccal swabs. It enables researchers to uncover nuanced correlations between microbiome profiles and host factors. Advanced machine learning techniques, including supervised and unsupervised learning models, have dissected the intricate relationships within microbiome datasets (Wu et al., 2021). Supervised learning algorithms, for example, can predict specific outcomes—such as disease states or phenotypic traits—based on microbial composition, offering insights into the functional roles of different microbial communities (Wu et al., 2021). Unsupervised learning helps identify natural groupings or patterns within the data, facilitating the discovery of previously unrecognized correlations between microbial communities and host characteristics. For example, Shi et al. (2022) systematically compared beta diversity and clustering methods in microbiome analyses, identifying critical scenarios where specific methods underperform and proposing a novel combined metric that improves clustering performance across diverse datasets.

One of the critical applications of machine learning in microbiome research is the development of predictive models that can accurately associate microbiome profiles with specific host conditions (Marcos-Zambrano et al., 2021). By analyzing microbiome data from noninvasively collected samples, ML algorithms can identify biomarkers of disease or health, predict responses to dietary interventions, and even forecast the potential impacts of environmental changes on microbial diversity and function. This capability is particularly beneficial in ruminant health, where machine learning models can analyze buccal swab data to predict rumen health and efficiency.

Furthermore, machine learning enhances the ability to perform longitudinal studies on the microbiome (Schüssler-Fiorenza Rose et al., 2019), tracking changes over time with minimal stress to the subjects. This aspect is crucial for understanding how temporal dynamics within the microbiome relate to shifts in health, productivity, or environmental interactions, providing a more comprehensive view of the microbiome's role in host physiology.

Integrating machine learning with microbiome research represents a transformative approach that significantly advances our understanding of microbial ecosystems. By efficiently analyzing complex datasets from noninvasively collected samples, ML algorithms streamline the research process and uncover deeper insights (Sudhakar et al., 2021) into the multifaceted interactions between microbes and their hosts. As machine learning techniques evolve, their application in microbiome research promises to unveil new dimensions of microbial life. This integration of machine learning into microbiome studies exemplifies collaborative approaches to leveraging computational power in biological research. It also highlights a future where data-driven insights can lead to more effective, sustainable animal health and agriculture solutions.

Comparative Analysis of Oral, Ruminal, and Fecal Microbiomes

The gastrointestinal tract of ruminants hosts a complex and dynamic microbial ecosystem critical for the animal's health, nutrition, and overall performance. Recent studies have increasingly focused on understanding the distinct microbial communities present in

different sections of the gastrointestinal tract, namely the oral, ruminal, and fecal microbiomes. Comparative analysis sheds light on how these diverse microbial habitats interact and influence one another, alongside their collective impact on the host. Research comparing these microbiomes highlights a fascinating gradient of microbial diversity and function along the gastrointestinal tract. The oral microbiome, primarily influenced by the external environment and the initial feed processing, sets the stage for the microbial fermentation processes that occur in the rumen. Studies have shown an overlap in microbial species between the oral and ruminal environments (Tapio et al., 2016; Young et al., 2020; Amin et al., 2021). The rumen microbiome is far more complex and specialized, reflecting its pivotal role in fiber degradation, volatile fatty acid production, and nutrient synthesis. This specialization is crucial for the efficient breakdown of plant-based diets, characteristic of ruminant feeding behavior.

The fecal microbiome provides a snapshot of the distal digestive processes and the microbes associated with waste formation and excretion. While distinct from the rumen microbiome (Liu et al., 2016; Lin et al., 2022; Mu et al., 2019), the fecal microbiome shares specific microbial populations with both the rumen and oral microbiomes, indicative of the continuous nature of the gastrointestinal tract. However, the fecal microbiome is influenced by different factors, including the absorption processes in the intestines and the animal's health status (Wang et al., 2017). The relationship between these microbiomes is not merely sequential but interactive, affecting animal health and performance in multifaceted ways. For example, alterations in the oral microbiome can influence rumen fermentation patterns and efficiency. Similarly, changes in rumen

microbial populations due to diet or health interventions can have downstream effects on fecal microbial communities, potentially impacting disease transmission and nutrient cycling in farm environments.

Understanding these microbiomes' comparative dynamics offers valuable insights into detecting and managing gastrointestinal diseases and optimizing feeding strategies. For instance, shifts in the microbial composition at any point along the gastrointestinal tract can serve as early indicators of health issues, allowing for timely interventions. Also, this knowledge assists in designing probiotics and other microbial-based interventions to promote beneficial microbial populations across the gastrointestinal tract, enhance feed efficiency (Bath et al., 2013), and reduce greenhouse gas emissions (Difford et al., 2018). By elucidating the intricate relationships between different microbiomes within the gastrointestinal tract, these studies contribute to a holistic understanding of the microbiome, paving the way for innovative strategies to enhance the productivity of ruminant farming practices.

Future Directions and Potential Applications

Emerging genetic and microbial engineering technologies present further opportunities to develop novel interventions to introduce or enhance beneficial microbial functions. For instance, engineering microbes that are more efficient at converting feed into energy or that can produce less methane could profoundly affect the sustainability of ruminant

agriculture. Understanding the interactions between diet, microbiome, and host genetics may also lead to personalized nutrition strategies that optimize the microbial composition for individual animals or specific breeds. In addition, developing less-invasive microbiome sampling techniques, such as buccal swabs, represents a significant leap forward for animal science research and industry practices. These methods align with the growing focus on animal welfare. Also, the less-invasive techniques offer tangible benefits that can be easily integrated for large-scale monitoring and assessment of livestock health and productivity. The ability to regularly and non-invasively sample the microbiome allows for more dynamic animal health monitoring. This proactive approach can enhance management practices, reduce the use of antibiotics through early intervention, and ultimately boost animal health and performance. The future of microbiome research and its application in animal science heralds a new era of precision agriculture, where data-driven insights and innovative technologies converge to optimize animal health, productivity, and environmental stewardship. The continued exploration of these frontiers will yield transformative benefits for the livestock industry.

References

Ahmad, A. A., Yang, C., Zhang, J., Kalwar, Q., Liang, Z., Li, C., Du, M., Yan, P., Long, R., Han, J., & Ding, X. (2020). Effects of Dietary Energy Levels on Rumen Fermentation, Microbial Diversity, and Feed Efficiency of Yaks (Bos grunniens). *Frontiers in Microbiology, 11*, 496651. https://doi.org/10.3389/FMICB.2020.00625/BIBTEX

Akin, D. E., & Borneman, W. S. (1990). Role of Rumen Fungi in Fiber Degradation. *Journal of Dairy Science*, *73*(10), 3023–3032. https://doi.org/10.3168/JDS.S0022-0302(90)78989-8

Amin, N., Schwarzkopf, S., Kinoshita, A., Tröscher-Mußotter, J., Dänicke, S., Camarinha-Silva, A., Huber, K., Frahm, J., & Seifert, J. (2021). Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning. *Animal Microbiome*, *3*(1), 1–15. https://doi.org/10.1186/S42523-021-00095-3/FIGURES/7

Antony, M. (2019). *Rumen Microbial Culture Library and in Vitro Analysis of Selected Bacterial Species on Colonization Resistance Against Bovine Enteric Pathogens*. https://openprairie.sdstate.edu/etd/3636/

Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O. (2008). The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics*, *9*(1), 1–15. https://doi.org/10.1186/1471-2164-9-75/TABLES/3

Baldwin, R., Science, N. S.-J. of D., & 1971, undefined. (n.d.). Intermediary aspects and tissue interactions of ruminant fat metabolism. *ElsevierRL Baldwin, NE SmithJournal of Dairy Science, 1971•Elsevier*. Retrieved March 31, 2024, from https://www.sciencedirect.com/science/article/pii/S0022030271858897

Bath, C., Morrison, M., Ross, E. M., Hayes, B. J., & Cocks, B. G. (2013). The symbiotic rumen microbiome and cattle performance: a brief review. *Animal Production Science*, *53*(9), 876–881. https://doi.org/10.1071/AN12369

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, *70*(2), 567–590. https://doi.org/10.1152/PHYSREV.1990.70.2.567

Bhaskar, H., Hoyle, D. C., & Singh, S. (2006). Machine learning in bioinformatics: A brief survey and recommendations for practitioners. *Computers in Biology and Medicine*, *36*(10), 1104–1125. https://doi.org/10.1016/J.COMPBIOMED.2005.09.002

Cammack, K. M., Austin, K. J., Lamberson, W. R., Conant, G. C., & Cunningham, H. C. (2018). RUMINNAT NUTRITION SYMPOSIUM: Tiny but mighty: the role of the rumen microbes in livestock production. *Journal of Animal Science*, *96*(2), 752–770. https://doi.org/10.1093/JAS/SKX053

Chai, J., Zhuang, Y., Cui, K., Bi, Y., & Zhang, N. (2024). Metagenomics reveals the temporal dynamics of the rumen resistome and microbiome in goat kids. *Microbiome*, *12*(1), 1–18. https://doi.org/10.1186/S40168-023-01733-5/FIGURES/7

Cholewińska, P., Czyz, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Animal Health Research Reviews*, *21*(1), 3–14. https://doi.org/10.1017/S1466252319000069

Connery, A. (2023). The Intricate Machinery of Digestion: Unveiling the Digestive System of a Cow. *Journal of Veterinary Medicine and Surgery*, *7*(2), 1–1. https://doi.org/10.36648/2574-2868.7.2.12

Dehority, B. A. (1991). Effects of microbial synergism on fibre digestion in the rumen. *Proceedings of the Nutrition Society*, *50*(2), 149–159. https://doi.org/10.1079/PNS19910026

Denman, S. E., Morgavi, D. P., & Mcsweeney, C. S. (2018). Review: The application of omics to rumen microbiota function. *Animal*, *12*(s2), s233–s245. https://doi.org/10.1017/S175173111800229X

Difford, G. F., Plichta, D. R., Løvendahl, P., Lassen, J., Noel, S. J., Højberg, O., Wright, A. D. G., Zhu, Z., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B., & Sahana, G. (2018). Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLOS Genetics*, *14*(10), e1007580. https://doi.org/10.1371/JOURNAL.PGEN.1007580

Firkins, J. L. (2021). Invited Review: Advances in rumen efficiency. *Applied Animal Science*, *37*(4), 388–403. https://doi.org/10.15232/AAS.2021-02163
France, J., & Dijkstra, J. (2005). Volatile fatty acid production. *Quantitative Aspects of Ruminant Digestion and Metabolism*, 157–175. https://doi.org/10.1079/9780851998145.0157

Friedersdorff, J. C. A., Kingston-Smith, A. H., Pachebat, J. A., Cookson, A. R., Rooke, D., & Creevey, C. J. (2020). The Isolation and Genome Sequencing of Five Novel Bacteriophages From the Rumen Active Against Butyrivibrio fibrisolvens. *Frontiers in Microbiology*, *11*, 522243. https://doi.org/10.3389/FMICB.2020.01588/BIBTEX

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S. S., Fliegerova, K., Griffith, G. W., Forster, R., Tsang, A., Mcallister, T., & Elshahed, M. S. (2014). Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential.

Academic.Oup.ComRJ Gruninger, AK Puniya, TM Callaghan, JE Edwards, N Youssef, SS Dagar, K FliegerovaFEMS Microbiology Ecology, 2014•academic.Oup.Com, *90*(1), 1–17. https://doi.org/10.1111/1574-6941.12383

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., & van Immerseel, F. (2010). From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutrition Research Reviews*, *23*(2), 366–384. https://doi.org/10.1017/S0954422410000247

Hagey, J. v., Laabs, M., Maga, E. A., & DePeters, E. J. (2022). Rumen sampling methods bias bacterial communities observed. *PLOS ONE*, *17*(5), e0258176. https://doi.org/10.1371/JOURNAL.PONE.0258176

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., Waghorn, G. C., & Janssen, P. H. (2013). Effect of DNA Extraction Methods and Sampling Techniques on the Apparent Structure of Cow and Sheep Rumen Microbial Communities. *PLOS ONE*, *8*(9), e74787. https://doi.org/10.1371/JOURNAL.PONE.0074787

Hofmann, R. R. (1989). Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia*, *78*(4), 443–457. https://doi.org/10.1007/BF00378733/METRICS

Hua, D., Hendriks, W. H., Xiong, B., & Pellikaan, W. F. (2022). Starch and Cellulose Degradation in the Rumen and Applications of Metagenomics on Ruminal Microorganisms. *Animals 2022, Vol. 12, Page 3020*, *12*(21), 3020. https://doi.org/10.3390/ANI12213020

Inman, M. (2011). How Bacteria Turn Fiber into Food. *PLOS Biology*, *9*(12), e1001227. https://doi.org/10.1371/JOURNAL.PBIO.1001227

Izer, J., Dwyer, C., & Wilson, R. P. (2023). Anesthesia and analgesia in ruminants. *Anesthesia and Analgesia in Laboratory Animals*, 515–541. https://doi.org/10.1016/B978-0-12-822215-7.00025-1

Jia, B., Xuan, L., Cai, K., Hu, Z., Ma, L., & Wei, C. (2013). NeSSM: A Next-Generation Sequencing Simulator for Metagenomics. *PLOS ONE*, *8*(10), e75448. https://doi.org/10.1371/JOURNAL.PONE.0075448

Jiang, Q., Lin, L., Xie, F., Jin, W., Zhu, W., Wang, M., Qiu, Q., Li, Z., Liu, J., & Mao, S. (2022). Metagenomic insights into the microbe-mediated B and K2 vitamin biosynthesis in the gastrointestinal microbiome of ruminants. *Microbiome*, *10*(1), 1–16. https://doi.org/10.1186/S40168-022-01298-9/FIGURES/5

Jiang, Y., Luo, J., Huang, D., Liu, Y., & Li, D. D. (2022). Machine Learning Advances in Microbiology: A Review of Methods and Applications. *Frontiers in Microbiology*, *13*, 925454. https://doi.org/10.3389/FMICB.2022.925454/BIBTEX

Kanehisa, M., Sato, Y., & Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome

Sequences. *Journal of Molecular Biology*, *428*(4), 726–731. https://doi.org/10.1016/J.JMB.2015.11.006

Kelly, W. J., Mackie, R. I., Attwood, G. T., Janssen, P. H., McAllister, T. A., & Leahy, S. C. (2022). Hydrogen and formate production and utilisation in the rumen and the human colon. *Animal Microbiome*, *4*(1), 1–8. https://doi.org/10.1186/S42523-022-00174-Z/TABLES/1

Knudsen, B. E., Bergmark, L., Munk, P., Lukjancenko, O., Priemé, A., Aarestrup, F. M., & Pamp, S. J. (2016). Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. *MSystems*, *1*(5), 95–111. https://doi.org/10.1128/MSYSTEMS.00095-16/FORMAT/EPUB

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G., & Guan, L. L. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome*, *7*(1). https://doi.org/10.1186/S40168-019-0699-1

Li, M. M., White, R. R., Guan, L. L., Harthan, L., & Hanigan, M. D. (2021). Metatranscriptomic analyses reveal ruminal pH regulates fiber degradation and fermentation by shifting the microbial community and gene expression of carbohydrate-active enzymes. *Animal Microbiome*, *3*(1), 1–17. https://doi.org/10.1186/S42523-021-00092-6/FIGURES/7

Lim, Y., Totsika, M., Morrison, M., & Punyadeera, C. (2017). The saliva microbiome profiles are minimally affected by collection method or DNA extraction protocols. *Scientific Reports 2017 7:1*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-07885-3

Lin, M., Jiang, M., Yang, T., Zhao, G., & Zhan, K. (2022). Overexpression of GPR41 attenuated glucose production in propionate-induced bovine hepatocytes. *Frontiers in Veterinary Science*, *9*, 981640. https://doi.org/10.3389/FVETS.2022.981640/BIBTEX

Liu, S., Moon, C. D., Zheng, N., Huws, S., Zhao, S., & Wang, J. (2022). Opportunities and challenges of using metagenomic data to bring uncultured microbes into cultivation. *Microbiome 2022 10:1*, *10*(1), 1–14. https://doi.org/10.1186/S40168-022-01272-5

Lloyd, K. G., Steen, A. D., Ladau, J., Yin, J., & Crosby, L. (2018). *Phylogenetically novel uncultured microbial cells dominate earth microbiomes. mSystems 3: e00055-18*. https://doi.org/10.1128/mSystems.00055-18

Marcos-Zambrano, L. J., Karaduzovic-Hadziabdic, K., Loncar Turukalo, T., Przymus, P., Trajkovik, V., Aasmets, O., Berland, M., Gruca, A., Hasic, J., Hron, K., Klammsteiner, T., Kolev, M., Lahti, L., Lopes, M. B., Moreno, V., Naskinova,

I., Org, E., Paciência, I., Papoutsoglou, G., … Truu, J. (2021). Applications of Machine Learning in Human Microbiome Studies: A Review on Feature Selection, Biomarker Identification, Disease Prediction and Treatment. *Frontiers in Microbiology*, *12*, 634511. https://doi.org/10.3389/FMICB.2021.634511/BIBTEX

Miller, R. R., Montoya, V., Gardy, J. L., Patrick, D. M., & Tang, P. (2013). Metagenomics for pathogen detection in public health. *Genome Medicine*, *5*(9), 1–14. https://doi.org/10.1186/GM485/TABLES/4

Mizrahi, I., Wallace, R. J., & Moraïs, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nature Reviews Microbiology 2021 19:9*, *19*(9), 553–566. https://doi.org/10.1038/s41579-021-00543-6

Moffett, J. R., Puthillathu, N., Vengilote, R., Jaworski, D. M., & Namboodiri, A. M. (2020). Acetate Revisited: A Key Biomolecule at the Nexus of Metabolism, Epigenetics, and Oncogenesis – Part 2: Acetate and ACSS2 in Health and Disease. *Frontiers in Physiology*, *11*, 580171. https://doi.org/10.3389/FPHYS.2020.580171/BIBTEX

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. C., & Kanehisa, M. (2007). KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Research*, *35*(suppl_2), W182–W185. https://doi.org/10.1093/NAR/GKM321

Mott, A. C., Schneider, D., Hünerberg, M., Hummel, J., & Tetens, J. (2022). Bovine Rumen Microbiome: Impact of DNA Extraction Methods and Comparison of Non-Invasive Sampling Sites. *Ruminants 2022, Vol. 2, Pages 112-132*, *2*(1), 112–132. https://doi.org/10.3390/RUMINANTS2010007

Mu, Y., Lin, X., Wang, Z., Hou, Q., Wang, Y., & Hu, Z. (2019). High-production dairy cattle exhibit different rumen and fecal bacterial community and rumen metabolite profile than low-production cattle. *MicrobiologyOpen*, *8*(4), e00673. https://doi.org/10.1002/MBO3.673

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Frontiers in Microbiology*, *6*(NOV), 164310. https://doi.org/10.3389/FMICB.2015.01313/BIBTEX

Newbold, C. J., & Ramos-Morales, E. (2020). Review: Ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal*, *14*(S1), s78–s86. https://doi.org/10.1017/S1751731119003252

Olsson, I. A. S., & Westlund, K. (2007). More than numbers matter: The effect of social factors on behaviour and welfare of laboratory rodents and non-human primates. *Applied Animal Behaviour Science*, *103*(3–4), 229–254. https://doi.org/10.1016/J.APPLANIM.2006.05.022

Orpin, C. G. (1984). Microbial attack on lignocellulose in the rumen. *Applied Biochemistry and Biotechnology*, *9*(4), 327–328. https://doi.org/10.1007/BF02798959/METRICS

Peng, Y., Xie, T., Wu, Z., Zheng, W., Zhang, T., Howe, S., Chai, J., Deng, F., Li, Y., & Zhao, J. (2022). Archaea: An under-estimated kingdom in livestock animals. *Frontiers in Veterinary Science*, *9*, 973508. https://doi.org/10.3389/FVETS.2022.973508/BIBTEX

Ramos-Morales, E., Arco-Pérez, A., Martín-García, A. I., Yáñez-Ruiz, D. R., Frutos, P., & Hervás, G. (2014). Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Animal Feed Science and Technology*, *198*, 57–66. https://doi.org/10.1016/J.ANIFEEDSCI.2014.09.016

Sanjorjo, R., Tseten, T., Kang, M., Kwon, M., Fermentation, S. K.-, & 2023, undefined. (n.d.). In Pursuit of Understanding the Rumen Microbiome. *Mdpi.ComRA Sanjorjo, T Tseten, MK Kang, M Kwon, SW KimFermentation, 2023•mdpi.Com*. Retrieved March 31, 2024, from https://www.mdpi.com/2311-5637/9/2/114

Saraiva, J. P., Worrich, A., Karakoç, C., Kallies, R., Chatzinotas, A., Centler, F., & da Rocha, U. N. (2021). Mining Synergistic Microbial Interactions: A Roadmap on How to Integrate Multi-Omics Data. *Microorganisms 2021, Vol. 9, Page 840*, *9*(4), 840. https://doi.org/10.3390/MICROORGANISMS9040840

Schüssler-Fiorenza Rose, S. M., Contrepois, K., Moneghetti, K. J., Zhou, W., Mishra, T., Mataraso, S., Dagan-Rosenfeld, O., Ganz, A. B., Dunn, J., Hornburg, D., Rego, S., Perelman, D., Ahadi, S., Sailani, M. R., Zhou, Y., Leopold, S. R., Chen, J., Ashland, M., Christle, J. W., … Snyder, M. P. (2019). A longitudinal big data approach for precision health. *Nature Medicine 2019 25:5*, *25*(5), 792–804. https://doi.org/10.1038/s41591-019-0414-6

Scientific Opinion on the substantiation of health claims related to vitamin B12 and red blood cell formation (ID 92, 101), cell division (ID 93), energy-yielding metabolism (ID 99, 190) and function of the immune system (ID 107) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. (2009). *EFSA Journal*, *7*(10). https://doi.org/10.2903/J.EFSA.2009.1223

Shi, R., Dong, S., Mao, J., Wang, J., Cao, Z., Wang, Y., Li, S., & Zhao, G. (2023). Dietary Neutral Detergent Fiber Levels Impacting Dairy Cows' Feeding Behavior, Rumen Fermentation, and Production Performance during the Period of Peak-Lactation. *Animals 2023, Vol. 13, Page 2876*, *13*(18), 2876. https://doi.org/10.3390/ANI13182876

Shi, Y., Zhang, L., Peterson, C. B., Do, K. A., & Jenq, R. R. (2022). Performance determinants of unsupervised clustering methods for microbiome data. *Microbiome*, *10*(1). https://doi.org/10.1186/S40168-021-01199-3

Sichert, A., & Cordero, O. X. (2021). Polysaccharide-Bacteria Interactions From the Lens of Evolutionary Ecology. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/FMICB.2021.705082

Siciliano-Jones, J., & Murphy, M. R. (1989). Production of Volatile Fatty Acids in the Rumen and Cecum-Colon of Steers as Affected by Forage: Concentrate and Forage Physical Form. *Journal of Dairy Science*, *72*(2), 485–492. https://doi.org/10.3168/JDS.S0022-0302(89)79130-X

Soltan, Y. A., Patra, A. K., Soltan, Y. A., & Patra, A. K. (2021). *Ruminal Microbiome Manipulation to Improve Fermentation Efficiency in Ruminants*. https://doi.org/10.5772/INTECHOPEN.101582

Stevens, C., & Hume, I. (2004). *Comparative physiology of the vertebrate digestive system*. https://books.google.com/books?hl=en&lr=&id=DZuAsci2apAC&oi=fnd&pg=PR15&dq=Comparative+Physiology+of+the+Vertebrate+Digestive+System+-+C.+Edward+Stevens,+Ian+D.+Hume+-+Google+Books&ots=rPiY0Kqv6Q&sig=W3vb5z0OyBCJRl0WLswG-fr6gdQ

Stinson, L. F., Keelan, J. A., & Payne, M. S. (2018). Comparison of Meconium DNA extraction methods for use in microbiome studies. *Frontiers in Microbiology*, *9*(FEB), 306524. https://doi.org/10.3389/FMICB.2018.00270/BIBTEX

Sudhakar, P., Machiels, K., Verstockt, B., Korcsmaros, T., & Vermeire, S. (2021). Computational Biology and Machine Learning Approaches to Understand Mechanistic Microbiome-Host Interactions. *Frontiers in Microbiology*, *12*, 618856. https://doi.org/10.3389/FMICB.2021.618856/BIBTEX

Sun, H. Z., Peng, K. L., Xue, M. Y., & Liu, J. X. (2021). Metagenomics analysis revealed the distinctive ruminal microbiome and resistive profiles in dairy buffaloes. *Animal Microbiome*, *3*(1), 1–13. https://doi.org/10.1186/S42523-021-00103-6/FIGURES/5

Tapio, I., Shingfield, K. J., McKain, N., Bonin, A., Fischer, D., Bayat, A. R., Vilkki, J., Taberlet, P., Snelling, T. J., & Wallace, R. J. (2016). Oral Samples as Non-Invasive Proxies for Assessing the Composition of the Rumen Microbial Community. *PLOS ONE*, *11*(3), e0151220. https://doi.org/10.1371/JOURNAL.PONE.0151220

Teng, F., Darveekaran Nair, S. S., Zhu, P., Li, S., Huang, S., Li, X., Xu, J., & Yang, F. (2018). Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. *Scientific Reports 2018 8:1*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-34294-x

Urrutia, N., Bomberger, R., Matamoros, C., & Harvatine, K. J. (2019). Effect of dietary supplementation of sodium acetate and calcium butyrate on milk fat synthesis in lactating dairy cows. *Journal of Dairy Science*, *102*(6), 5172–5181. https://doi.org/10.3168/JDS.2018-16024

Vaidya, J. D., van den Bogert, B., Edwards, J. E., Boekhorst, J., van Gastelen, S., Saccenti, E., Plugge, C. M., & Smidt, H. (2018). The effect of DNA extraction methods on observed microbial communities from fibrous and liquid rumen fractions of dairy cows. *Frontiers in Microbiology*, *9*(JAN), 298167. https://doi.org/10.3389/FMICB.2018.00092/BIBTEX

Valinetz, E. D., & Cangelosi, G. A. (2021). A Look Inside: Oral Sampling for Detection of Non-oral Infectious Diseases. *Journal of Clinical Microbiology*, *59*(10). https://doi.org/10.1128/JCM.02360-20

Volmer, J. G., McRae, H., & Morrison, M. (2023). The evolving role of methanogenic archaea in mammalian microbiomes. *Frontiers in Microbiology*, *14*, 1268451. https://doi.org/10.3389/FMICB.2023.1268451/BIBTEX

Wang, J., Fan, H., Han, Y., Zhao, J., & Zhou, Z. (2017). Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. *Asian-Australasian Journal of Animal Sciences*, *30*(1), 100. https://doi.org/10.5713/AJAS.16.0166

Williams, C. L., Thomas, B. J., McEwan, N. R., Rees Stevens, P., Creevey, C. J., & Huws, S. A. (2020). Rumen Protozoa Play a Significant Role in Fungal Predation and Plant Carbohydrate Breakdown. *Frontiers in Microbiology*, *11*, 521741. https://doi.org/10.3389/FMICB.2020.00720/BIBTEX

Wu, S., Chen, Y., Li, Z., Li, J., Zhao, F., & Su, X. (2021). Towards multi-label classification: Next step of machine learning for microbiome research. *Computational and Structural Biotechnology Journal*, *19*, 2742–2749. https://doi.org/10.1016/J.CSBJ.2021.04.054

Xu, Q., Qiao, Q., Gao, Y., Hou, J., Hu, M., Du, Y., Zhao, K., & Li, X. (2021). Gut Microbiota and Their Role in Health and Metabolic Disease of Dairy Cow. *Frontiers in Nutrition*, *8*, 701511. https://doi.org/10.3389/FNUT.2021.701511/BIBTEX

Xu, Q., Ungerfeld, E. M., Morgavi, D. P., Waters, S. M., Liu, J., Du, W., & Zhao, S. (2023). Editorial: Rumen microbiome: interacting with host genetics, dietary nutrients metabolism, animal production, and environment. *Frontiers in Microbiology*, *14*, 1267149. https://doi.org/10.3389/FMICB.2023.1267149/BIBTEX

Young, J., Skarlupka, J. H., Cox, M. S., Resende, R. T., Fischer, A., Kalscheur, K. F., Mcclure, J. C., Cole, J. B., Suen, G., & Bickhart, D. M. (2020). Validating the use of bovine buccal sampling as a proxy for the rumen microbiota by using a time course and random forest classification approach. *Am Soc Microbiol, MS Cox, RT Resende, A Fischer, KF Kalscheur, JC McClure, JB Cole, G Suen, DM BickhartApplied and Environmental Microbiology, 2020•Am Soc Microbiol*, *86*(17). https://doi.org/10.1128/AEM.00861-20

CHAPTER 3

INVESTIGATION OF THE INTERRELATIONSHIP BETWEEN RUMINAL AND ORAL MICROBIOME OF ANGUS BULLS

Lamichhane, Utsav. To be submitted to *Animals*.

Abstract

The gut microbiome of ruminant animals plays an essential role in their digestion. Therefore, methodologies that facilitate such investigations are valuable. This study investigated the interrelationship between the rumen and buccal microbiomes of Angus bulls, aiming to explore non-invasive sampling methods for studying the rumen's complex microbial ecosystem. Both samples were collected from 541 registered Angus bulls from five farms in three states Of the United States, with microbial DNA extracted and analyzed using 16S rRNA gene sequencing. Microbiome composition, richness, and diversity were assessed. Ruminal samples had more significant number ($P < 0.001$) of amplicon sequence variants (ASV), greater microbial diversity (expressed as Shannon diversity index; $P < 0.001$), and greater microbial evenness ($P < 0.001$). Beta-diversity (unweighted UniFrac distance) was also significantly different ($P < 0.001$) between the samples. A total of 4323 microbial species were observed in the rumen and oral cavity combined, but only one-third (33.1%) of those species occurred in both the rumen and oral compartments. Moreover, the relative abundance of only 30.7% of the species significantly differed between the rumen and the mouth of the bulls. Contrary to expectations of a significant interrelationship, findings show a complex, limited overlap, emphasizing the uniqueness of each microbial ecosystem.

Lay Summary

When we think about how animals like cows digest their food, it is important to know they have a special stomach part called the rumen, which is a fermentation tank full of microorganisms that help them by breaking down grass and other feedstuffs. Scientists are very interested in understanding the role of those microorganisms because they are

crucial for the animal's nutrition, health, and the quality of meat and milk we get from them. Usually, to study those microorganisms, scientists need to use methods that are invasive and consequently uncomfortable for the animal. Our study tested a less invasive approach. Since ruminants remasticate their digesta several times throughout the day, we used swabs from the animals' mouths as a more straight-forward, kinder way to glimpse the microbial composition found in the rumen. Our research involved analyzing the mouth and rumen samples from 541 Angus bulls, looking for similarities and differences in the microorganisms. Our research found that the microorganisms in the mouth differ from those in the rumen, surprising us since we thought they would be more alike. This shows us that these groups of microbes are specially adapted to live in their specific ecosystem.

Introduction

Ruminants are distinguished by their unique gastrointestinal anatomy, particularly the presence of a highly specialized fermentation chamber – the rumen (Stevens and Hume 2004). This organ houses a diverse and complex microbiome, essential for digesting plant-based carbohydrates such as cellulose and hemicellulose (Mizrahi, Wallace, and Moraïs 2021; Javad Gharechahi et al., 2021) These fibrous compounds, generally indigestible to monogastric animals, are efficiently broken down by ruminants due to the synergistic activities of the rumen microorganisms (Dehority 1991; Firkins 2021). The ruminal microbial consortium, consisting of bacteria, archaea, protozoa, and fungi, coordinates the fermentation process, transforming these fibrous substrates into volatile fatty acids (VFAs) such as acetate, propionate, and butyrate (C. J. Newbold and Ramos-Morales 2020). In addition to energy metabolism, the rumen microbiome plays a crucial role in synthesizing essential nutrients, including certain vitamins and amino acids ("The Rumen and Its Microbes - Robert E. Hungate - Google Books," 2013; Cholewińska et al., 2020), further underscoring its importance in the overall nutritional status and health of ruminants. Moreover, the fermentation's efficiency that occurs in the rumen microbiome directly impacts the animal's growth rate and meat quality, linking it intrinsically to the economic and sustainable aspects of ruminant farming. Given these critical roles, there is an increasing emphasis on the need to understand the intricate dynamics of the rumen microbiome. Advanced research in this area, particularly in the realms of microbial genomics and functional metagenomics, aims to unravel the complex interactions within the rumen microbiota. Such insights are pivotal in developing strategies to manipulate and optimize the rumen microbial population, thereby enhancing the productivity, health, and environmental sustainability of ruminant livestock production systems.

The comprehensive study of the rumen microbiome, while invaluable, presents significant challenges, particularly in the collection of rumen samples. Rumen cannulation and stomach tubing are the two main techniques used to study ruminal microbial community composition (Ramos-Morales et al., 2014). Current procedures such as rumen cannulation and orogastric intubation are not only technically demanding but also invasive, necessitating skilled personnel for their execution (Castillo and Hernández 2021). Orogastric intubation involves the insertion of a tube through the mouth until it reaches the rumen, allowing for the sampling of rumen contents; however, it carries the risk of stress and discomfort to the animal, in addition to the need for specialized equipment. Rumen cannulation and fistulation, though providing direct and repeated access to the rumen contents, are surgical procedures that create a permanent opening in the animal's rumen. Therefore, while effective, it raises concerns regarding animal welfare and require meticulous post-operative care and management. The complexity of those procedures underscores the need for developing less invasive, yet equally reliable, techniques for rumen sampling. This advancement is crucial for enhancing our understanding of the rumen microbiome while ensuring animal welfare.

Relative to traditional rumen sampling methods in ruminants, emerging research has identified the buccal microbiome as a promising, non-invasive alternative for studying the rumen microbiota (Lindström and Redbo 2000). This approach, involving the simple and less time-consuming process of swabbing the oral cavity, significantly reduces the stress and discomfort experienced by animals (Tapio et al., 2016). Positive correlations between the microbial communities found in the mouth and in the rumen qualify the buccal swab as a viable candidate for a proxy for investigation of the rumen microbiota (Beauchemin 2018). For instance, Tapio et al. (2016) showed that the relative abundance of various microbial taxa in buccal and rumen samples were comparably similar. Moreover, Young et al. (2020) employed a machine learning approach to demonstrate a significant correlation between the microbial profiles from buccal swabs and rumen samples, and Amin et al. (2021) highlighted the dynamic nature of the buccal and rumen microbiome and their interconnectedness.

Given the importance of achieving a better understanding of ruminants' gastrointestinal microbiome and the need for less-invasive techniques, this study investigated the relationship between the oral and ruminal microbiomes of Angus bulls. We hypothesized that the microbial profile found in the buccal swabs would have a significant correlation with the profile found in the rumen of those animals, allowing the use of a less-invasive method like the oral swab for the investigation of the ruminal microbiome.

Materials and Methods

Farms and Animals

The study utilized registered Angus bulls (N = 541) -registered to the American Angus Association (AAA)- produced by four commercial feed testing centers in Georgia, Iowa,

and Montana. Each feed testing center had an average of 140 bulls (568.1 ± 32 kg; 19 ± 6 months of age), and all sample collections took place from November 2022 through January 2023. The bulls were adapted to their feed ration for at least four weeks before the sample collection.

Sample Collection

The collection of ruminal samples was performed by orogastric intubation as previously described (Lourenco et al. 2020). This method consists of inserting a sterile tube into the animal's rumen through the oral cavity, and then employing negative pressure with an electric pump to draw the rumen fluid into a sterile bottle. To reduce contamination from saliva, the initial 200 mL of fluid collected immediately after the tube's insertion into the rumen was discarded. Following this precautionary step, the rumen fluid was homogenized, transferred into a 15 mL tube, and immediately flash-frozen using liquid nitrogen to preserve sample integrity. Samples were transported to the laboratory in dry ice and stored at -80 degrees Celsius until further processing.

The oral microbiota was accessed by collecting two buccal swabs as described by Young et al. (2020). Briefly, two swabs were gently scraped into the inner side of the cheeks and the tongue's surface for approximately 10 seconds. After the swabbing process, the top part of each swab was broken, and the remainder was placed into a sterile 15 mL conical tube containing 1 mL of phosphate-buffered saline (PBS). The 15 mL conical tubes were immediately flash-frozen in liquid nitrogen to preserve the samples. The tubes were then transported to our laboratory in dry ice and stored at -80 degrees Celsius until the DNA extraction process was performed.

DNA Extractions

Rumen samples were first thawed and homogenized at room temperature, and 350 microliters were placed in a Lysing Matrix E (MP Biomedicals, LLC, Irvine, CA). The genomic DNA was extracted through a combination of mechanical and enzymatic methods as previously described (Williamson et al., 2022). The mechanical procedures included homogenization of samples at 6m/s for two sets of 40 seconds with a gap of 20 seconds between the sets in the FastPrep-24 5G instrument (MP Biomedicals, LLC, Irvine, CA), followed by a hot water bath (95 degrees Celsius). The enzymatic portion of the protocol followed the recommendations established by the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germantown, MD). The purified DNA was eluted in solution (50 microliters) made of Tris-HCl, EDTA, and NaN3. Eluted DNA was quantified using the Qubit Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA), and then stored at 20 degrees Celsius.

In DNA extraction from buccal samples, samples were thawed to room temperature. Once thawed, the samples were vortexed for 3 minutes and centrifuged at 4,000 x g for 2 minutes for sedimentation of the particulate matter. The swabs were then removed, and the content of the 15 mL tubes was transferred to 2 mL centrifuge tubes, which were subjected to further centrifugation at 21,000 x g for 2 minutes. After this second centrifugation, the supernatant was discarded, leaving only the pellet. The DNA extraction procedure continued using the QIAamp BiOstic Bacteremia DNA Kit (QIAGEN, Germantown, MD) following manufacturer's instructions. The purified DNA was eluted in solution (35 μL) made of 10 mM Tris-HCl, pH 8.5, and quantified using the

Qubit Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA). Samples were then stored at -20 degrees Celsius until further analysis.

DNA Amplification and Sequencing

Library preparation began by amplifying the V3-V4 hypervariable regions of the 16S rRNA gene using the S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pair (Klindworth et al. 2013). This amplification was performed for each sample using a mix of 12.5 μL 2×KAPA HiFi HotStart Ready Mix (Roche Molecular Systems, Inc., California), 5 μL of forward and 5 μL of reverse primers, and 2.5 μL of template DNA at a concentration of 5 ng/μL. The PCR conditions were set as follows: an initial denaturation at 95 °C for 3 minutes, followed by 25 cycles, each consisting of 30 seconds at 95 °C, 30 seconds at 55 °C, 30 seconds at 72 °C, and a final step of 5 minutes at 72 °C. Post-PCR, the amplicons were purified using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN), following the manufacturer's instructions. A second round of PCR was performed for incorporation of dual-indexed barcodes using the multiplex Nextera XT kit (Illumina, Inc., San Diego, CA). This second PCR was carried out with 5 μL of the amplicons from the first PCR, 25 μL of 2× KAPA HiFi HotStart Ready Mix, 5 μL of each index (i7 and i5), and 10 μL of PCR grade water. The PCR conditions were set to an initial denaturation at 95 °C for 3 minutes, followed by 8 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C, concluding with a 5-minute extension at 72 °C. Following this, the libraries from the second PCR were purified again using AMPure

XP beads. Libraries were quantified using the Qubit Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA).

The microbial DNA isolated from the buccal samples was processed using the same procedures described for the ruminal samples, with some modifications due to some samples having lower concentration of DNA. The volume of template DNA in the first PCR reaction was adjusted to 15 μL for samples that had a concentration of less than 1 ng/μL; and to 5 μL for samples that had a concentration between 1 and 5 ng/μL. The number of cycles in the first round of PCR was set to 30, and in the second round it was set to 10.

Before sequencing, all the libraries were normalized to 4 nM using 10 mM Tris pH 8.5, and 5 μL of each library was aliquoted and mixed for pooling. Pooled samples were then denatured by dilution with fresh 0.2 N NaOH and normalized to 7 pM. A PhiX control library (Illumina, Inc., San Diego, CA) was also denatured and diluted to 7 pM and included at a rate of 20%. Sequencing was carried out on an Illumina MiSeq platform, employing the standard MiSeq v3 reagent kit protocol (Illumina, Inc., San Diego, CA). Samples were sequenced as paired-end reads with read lengths of 251 nucleotides each run.

Bioinformatics Pipeline

Sequenced data was demultiplexed and downloaded from the Illumina BaseSpace Sequence Hub (Illumina, Inc., San Diego, CA). The demultiplexed sequence files were imported into a QIIME2 artifact (Bolyen et al., 2019). Samples were denoised, filtered for chimeric sequences, and merged using the dada2 plugin (Callahan et al., 2016) by

applying forward and reversed truncations at 245 and 228 nucleotide positions, respectively. For taxonomic classification, a pre-trained classifier (Bokulich et al., 2018)based on the Greengenes2 reference database (McDonald et al., 2023) was employed. For diversity analyses, the sequencing depth was set at 10,000 sequences per sample, ensuring that the average Good's coverage was above 99.5%. The outputs of the QIIME2 pipeline were further cleaned to remove outliers based on Pielou's evenness index. Samples not found within the 1.5 IQR (interquartile range) were considered outliers and were excluded from further analysis.

Statistical Analysis

Relative abundances of taxa at the family, genus, species, and amplicon sequence variants (ASV) levels were evaluated. Various alpha diversity metrics were evaluated using an approach similar to the one utilized for the evaluation of individual taxa. Kruskal-Wallis tests were used to assess differences in alpha diversity between groups - oral and rumen samples. Differences in beta diversity were assessed by permutational multivariate analysis of variance (PERMANOVA) on the Unweighted UniFrac distances. Subsequently, beta diversity was visualized employing the qiime emperor plugin (Vázquez-Baeza et al., 2013) and plotting them against the three main axes, providing a multi-dimensional perspective of microbial diversity. Additionally, visualizations of the predicted metabolic pathways were achieved by employing Bray-Curtis dissimilarity and projecting the results onto a 3-D plot, and differences were accessed by PERMANOVA.

Results

Overview

The geographical distribution map of sample collection across various states depicts a notable variation in the number of animals sampled. Most samples were collected in the state of Montana: 298 animals (586 samples). Iowa followed with 227 animals (454 samples), representing another major data source (Figure 1). In contrast, Georgia had the least number of animals sampled, with a count of 16 (32 samples).

Given the greater number of animals available for collection, farm E had the highest count of rumen (n= 298) and oral samples (n= 298) in our dataset. In contrast, farm A had the lowest number of animals sampled rumen (n= 16) and oral samples (n= 16). The counts for farms B, C, and D displayed a relatively balanced distribution regarding the number of samples. Overall, the average number of samples collected in each farm was 108 ruminal and 108 oral samples.

Alpha Diversity

Observed Features

The alpha diversity analysis of microbial communities within buccal and rumen samples, using the "Observed Features" metric, indicates the total number of individual ASV found in each sample. A significant divergence in this metric ($P < 0.001$), which indicates species richness, was observed between the rumen and oral samples (Figure 2). The alpha

diversity boxplots demonstrate a lower median value and narrower interquartile range for the buccal samples, suggesting a less rich microbial community. In contrast, the ruminal samples displayed a higher median and a broader interquartile range, indicative of a richer microbial community. Regarding the different farms, the number of ASV in oral samples varied significantly ($P < 0.001$), and the same was observed for ruminal samples ($P < 0.001$).

Shannon Diversity Index

Shannon Diversity index was computed to assess the microbial diversity within the oral and rumen sample types. The diversity within each sample type was visualized through boxplots, which displayed a spread of that diversity index (Figure 2). In oral samples, the diversity was lower ($P < 0.001$) than in rumen samples. The Kruskal-Wallis H test yielded an H value of 220.5, indicating the strong variability between the rumen and oral samples. Further analysis of Shannon Diversity showed significant ($P < 0.001$) differences between farms, both for ruminal and oral samples.

Pielou’s Evenness Index

The evenness of species distribution within the sample types was assessed using Pielou's Evenness index. The boxplots in Figure 2 present the spread of evenness values for oral and rumen samples. The rumen samples exhibited a higher ($P < 0.001$) Pielou's Evenness than the oral samples, with the latter showing a wider spread of values as evidenced by the range of data points and outliers. These outliers, particularly in the oral sample set, may indicate a notable deviation in community evenness in some instances.

As observed for microbial richness and diversity, species evenness also varied by farm in the current study, both in the oral and ruminal samples ($P < 0.001$).

Beta Diversity

A detailed statistical examination of microbial community composition across a combined sample type and farm was conducted using unweighted UniFrac distances. The PERMANOVA approach was utilized to discern whether significant differences exist between the groups. The compositional differences between the microbial communities of oral and rumen samples were investigated using a Principal Coordinate Analysis (PCoA) based on unweighted UniFrac distances. The PCoA plot, illustrated in Figure 4, reveals a distinct clustering pattern corresponding to the two sample types: rumen and oral.

Rumen samples are predominantly clustered along the lower end of Axis 1 (which explains 28.47% of the variation), while oral samples are dispersed more broadly along the same axis. This separation along Axis 1 suggests a fundamental difference in community composition between oral and rumen microbiomes. Axes 2 and 3, accounting for 5.838% and 4.049% of the variation, respectively, show additional, albeit less pronounced, distinctions between the two sample types. Further comparisons by farm revealed that beta diversity was significantly distinct ($P = 0.001$) both for oral and ruminal samples.

Relative Abundances

Family and Genus Level

The family Bacteroidaceae was the most abundant (Figure 5) in the rumen samples, constituting approximately 36.70% of the microbial population. This was followed by Lachnospiraceae, with an abundance of 10.18%, and Paludibacteraceae, with an abundance of 7.36%. Other notably abundant families included UBA932 at 5.03% and Acutalibacteraceae at 4.30%. The family Acidaminococcaceae also had a significant presence with 4.06%. Less abundant but still notable were Muribaculaceae and Oscillospiraceae_88309, with 2.97% and 2.73%, respectively.

The oral samples were dominated by the family Pasteurellaceae, which represented 13.58% of the microbial community, closely followed by Streptococcaceae at 13.35%. The Moraxellaceae family was also prevalent, making up 10.09% of the community. Lachnospiraceae and Bacteroidaceae were also significant, with relative abundances of 6.11% and 5.15%, respectively. Neisseriaceae_563222 and Mycobacteriaceae were present at 4.32% and 4.25%, indicating their considerable contribution to the oral microbiota.

The analysis of relative abundances of genera in buccal and rumen samples revealed distinct microbial profiles between the two types of samples, underscoring the specificity of microbial communities to their respective environments within the host. In buccal samples, the genus Streptococcus was found to be the most abundant, accounting for 13.35% of the microbial composition. This was followed by Bibersteinia (7.71%). Moraxella and Psychrobacter also showed notable abundances at 4.30% and 4.01%,

respectively. This profile suggests a diverse bacterial community with a dominance of genera known for their presence in the oral cavity, reflecting the unique ecological niche of the buccal environment.

Contrastingly, rumen samples exhibited a markedly different microbial composition, with Prevotella being the most abundant genus, constituting 31.07% of the microbial population. This was followed by a significantly lower presence of genus RF16 (7.34%), Cryptobacteroides (4.69%), and Succiniclasticum (4.03%).

Presence of Common Species

In the comparative analysis of microbial genera present in rumen and oral samples of ruminants, we identified a total of 54 unique genera among the top 30 genera in each sample type, based on their average relative abundance. The rumen samples were notably abundant in the genus Prevotella, which holds a crucial role in the degradation of plant-derived polysaccharides within the rumen ecosystem. In contrast, oral samples were predominantly colonized by the genus Streptococcus, which includes species known for their various roles in the oral microbiota, ranging from commensalism to pathogenicity.

In the comparative analysis of microbial genera present in rumen and oral samples, of the top 30 genera in each sample type, only 5.3% (3 genera) were found in both locations (Figure 6), which suggests a distinct partitioning of the microbial community composition between these two environments. The shared genera were Prevotella, Ruminococuss, and an unidentified genus from the family Lachnospiraceae. Each environment harbored a significant number of exclusive genera, with 27 (47.4%)

unique to the rumen and 27 (47.4%) unique to the oral cavity, reflecting the specialized functions and selective pressures exerted by the respective environments.

The genera exclusive to the rumen are predominantly associated with fermentation, vital for the host's energy supply. In contrast, the oral cavity's unique genera exhibit various functions, including colonization, symbiosis, and pathogenicity.

The limited genera overlap between the two microbial communities underscores the specificity and adaptation of the microbiota to their local environments. The discrete nature of these communities reveals the presence of distinct microbial ecosystems within the same host, shaped by the vastly different conditions of the rumen and oral cavity.

PICRUSt Analysis

Principal Coordinates Analysis (PCoA) for the metabolic pathways.

A comprehensive microbial function analysis of buccal and ruminal samples was conducted using the MetaCyc database of metabolic pathways and enzymes. Principal Coordinate Analysis of Bray-Curtis dissimilarities was employed and highlighted the uniqueness in microbial function between the two environments (Figure 7). The buccal samples and rumen samples were distinctly separated along Axis 1, which accounts for a substantial 78.09% of the total variation. This pronounced separation underscores a fundamental difference in the functional capabilities of the microbial communities within the buccal and rumen environments. Axes 2 and 3 contribute 3.65% and 2.74% to the overall variation, respectively, indicating further, though more subtle, distinctions in microbial function between the sample types.

The distinct clustering patterns observed in the PCoA plot suggest significant disparities in enzyme activity and metabolic functions, which could reflect the specialized adaptations of microbial communities to their respective environments in the rumen and oral cavity. However, the distinct profiles also indicate the limitations of this approach for specific enzymatic or metabolic insights.

The enzymatic profiles derived from buccal and rumen samples were analyzed to assess functional differences between the microbial communities. The top 15 enzymes shown in Table 3 reveals distinct enzymatic activities across both sample types. While several enzymes are found to be common between the two environments, it is noted that these shared enzymes are primarily related to DNA processing rather than metabolic functions. This distinction underscores the specialized adaptations of the microbial communities to their respective environments, with metabolic enzymes having different expressions between buccal and rumen samples. DNA-related enzymes in both environments reflect the fundamental cellular processes essential for microbial survival and replication, which are conserved across different microbial niches. The violin plots (Figure 8) show the expression profiles of the top five enzymes within each sample type.

Distribution of Top Enzymes in Buccal and Rumen Microbial Communities.

The violin plots as in Figure 8 distinctly demonstrate the expression profiles of top enzymes within the buccal and rumen samples. These visual representations reveal that, while both sample types share the presence of several DNA-related enzymes, there is a notable divergence in the expression of metabolic enzymes. This observation

underscores a fundamental distinction between the metabolic processes occurring in the rumen and buccal environments when the universal, DNA-related enzymes are excluded from the analysis.

The metabolic enzymes, which are directly involved in specific biochemical pathways crucial to their respective environments' unique dietary and physiological conditions, exhibit distinct patterns of variability and expression. In rumen samples, enzymes pivotal to fermentation and nutrient absorption show a broad range of expression, reflecting the rumen's complex and variable dietary intake. Conversely, the buccal samples display more stable enzyme expression, indicating a consistent role in the less variable oral environment.

This selective analysis of metabolic enzymes highlights their critical role in adapting to and functioning within the specific conditions of each microbial habitat. By focusing on these enzymes, it becomes evident that the enzymatic landscapes of the rumen and buccal microbiomes are fundamentally distinct, driven by their differing roles in animal physiology and health. This distinction is crucial for understanding these communities' microbial ecology and functional capacities, particularly when considering the potential for targeting specific metabolic pathways in health and disease interventions.

Discussion

In our study, we compared the rumen and oral microbiomes across 541 Angus bulls, markedly expanding upon the cohort sizes of previous studies. In early studies, Lodge-Ivey (Lodge-Ivey et al., 2009) conducted pioneering work by studying the microbiomes of 2 cattle and 3 sheep, laying foundational knowledge for subsequent research. Building

on this, Kittelmann et al. (2015)further explored this topic through their study of 24 sheep. Tapio et al. (2016)provided insights into the microbiomes of 5 dairy cattle, emphasizing the diversity present even in smaller cohorts. Young et al. (2020)recently undertook a study involving 21 dairy cattle, contributing to a growing understanding of these complex microbial ecosystems. Thus, the significant increase in sample size in our current study marks a considerable advance in the field and contributes to substantiate our findings, particularly in beef cattle.

Based on our results, it becomes evident that there is a significant dissimilarity in the microbiome composition between rumen and oral samples. This variation was demonstrated by alpha-diversity indexes, which included the number of observed ASVs, Shannon diversity index, and Pielou's evenness index, alongside beta diversity, computed using Unweighted UniFrac distances. Such analysis allowed a comprehensive understanding of microbial diversity and distribution and revealed distinct microbial communities within the two environments. Further, the relative abundance of microorganisms at various taxonomic levels underscored the specificity of microbiome composition inherent to the rumen and oral niches.

We found that microbial richness, diversity, and evenness were all greater in the rumen. The observed disparities in the microbiome composition between rumen and oral samples underline these environments' distinct physiological and ecological roles (Henderson et al., 2013a; Mizrahi, Wallace, and Moraïs 2021) within the host. The rumen, an anaerobic fermentation chamber (Hook et al., 2010), is optimized for breaking down complex plant materials (Gharechahi et al., 2021), a process facilitated by a highly diverse and specialized microbial community (Mizrahi, Wallace, and Moraïs 2021). This diversity is

essential for efficiently converting fibrous plant material into useful products like volatile fatty acids (Malheiros et al., 2021; Li et al., 2022), critical for the host's energy supply. The unique environmental conditions of the rumen, such as its pH (Mizrahi, Wallace, and Morais 2021), temperature, and anoxic atmosphere, create a habitat conducive to a wide array of microbial taxa, each contributing to the rumen's metabolic functions (Gharechahi et al., 2021).

Conversely, the oral cavity serves as the entry point to the digestive tract, encountering a variety of substrates and being subjected to fluctuations in conditions (Parish et al., 2023) such as oxygen levels, salivary flow, and exposure to external microorganisms from the environment. These factors contribute to a microbial community that, while less diverse than the rumen (Amin et al., 2021; Kodithuwakku et al., 2022; Monteiro et al., 2022) is adapted to rapidly colonize and utilize the substrates available in the oral environment. Moreover, these microorganisms can play roles in nutrient preprocessing, protection against pathogens, and even influencing rumen microbiome composition through ingested feed and saliva.

The distinct microbial profiles between the mouth and rumen observed in our study reflect their adaptation to these specific roles (Amin et al., 2021) and conditions (Li et al., 2022). The rumen's high abundance of Bacteroidaceae, known for their capability to degrade complex polysaccharides (Lapébie et al., 2019; Mckee et al., 2021), reflects the rumen's critical function in fiber digestion and nutrient assimilation. The presence of Lachnospiraceae and Paludibacteraceae, along with other cellulolytic bacteria like UBA932 and Acutalibacteraceae, further supports the rumen's role in breaking down plant material into absorbable nutrients (Kaminsky et al., 2023), thus underlining the

symbiotic relationship between these microbes and their bovine host (Mizrahi, Wallace, and Moraïs 2021).

In contrast, the oral microbiome's composition, with Pasteurellaceae and Streptococcaceae as the most abundant families illustrates a microbial community adapted to the oral cavity's unique environment. These families are known for their roles in oral health, contributing to mucosal immunity and maintaining the balance against pathogenic invasion (Wu et al., 2014). The presence of Moraxellaceae and Neisseriaceae underscores the oral cavity's dynamic interface with the external environment (Vigors et al., 2023), reflecting its exposure to diverse microbial inputs.

At the genus level, the genera exclusive to the rumen were predominantly associated with fermentation, which is the vital process for the host's energy supply. However, the oral cavity's unique genera were composed of bacteria that exhibit various functions, including colonization, symbiosis, and pathogenicity. The limited genera overlap between the two microbial communities underscores the specificity and adaptation of the microbiota to their local environments.

Concerning microbial function, the enzymes which are directly involved in specific biochemical pathways exhibited distinct patterns of variability and expression, crucial to their respective environments' unique conditions. In rumen samples, enzymes pivotal to fermentation and nutrient absorption showed a broad range of expression. Conversely, the buccal samples displayed more stable enzyme expression, indicating a consistent role in the less variable oral environment. Furthermore, while both sample types shared the presence of several DNA-related enzymes, there is a notable divergence in the expression of enzymes that participate in the general metabolism. For instance, Beta-glucosidase,

which is an enzyme that participates in the final step of cellulose biodegradation (Zhang et al., 2011), was one of the top 10 enzymes most produced by the microbiome in the rumen; however, it was simply not produced by the oral microbiome. This exemplifies the distinction between the metabolic processes occurring in the rumen and buccal environments when the universal, DNA-related enzymes are excluded from the analysis. Lastly, the distinct clustering patterns observed in the PCoA plot summarizing Bray-Curtis distances for the metabolic pathways further indicates pronounced disparities in metabolic functions of the microbiomes, which are specialized and adapted to their respective environments. Thus, it seems evident that the enzymatic/metabolic landscapes of the rumen and buccal microbiomes are fundamentally distinct, driven by their differing roles in animal physiology.

Our investigation reveals a distinct microbial profile between oral and rumen samples, challenging the consensus of several preceding studies. Notably, Kittelmann et al. (2015)posited that buccal samples could serve as accurate proxies for rumen microbiome characterization in sheep, suggesting a level of similarity between these microbiomes that our findings do not support. Similarly, Tapio et al. (2016)reported congruence in microbiome profiles between buccal and rumen samples in dairy cattle, indicating that oral samples could reliably represent the rumen's microbial community. Furthermore, Young et al. (2020)observed comparable diversity and evenness in the microbiomes of oral and rumen samples collected from lactating Holstein cows before morning feeding, reinforcing the notion of microbiome similarity across these niches. Amin et al. (2021)further supported this view by finding that rumen-specific bacterial taxa were equally present in buccal swabs of Holstein's calves. The obvious divergence between our

study and previous findings from the literature Click or tap here to enter text.may originate from several key methodological and contextual factors. Firstly, our study's unique focus was on Angus bulls, whereas others investigated different species and genders, suggesting that the microbiome may exhibit considerable variability across different ruminant populations. This species and gender-specific distinction could account for some of the observed discrepancies in microbial community structure and function between our findings and those of prior research. Another factor contributing to the differences observed may be the varied environmental conditions (Wu et al., 2020; Mizrahi, Wallace, and Morais 2021) and management practices across the multiple farms and geographical locations from which our samples were collected. Unlike previous studies that benefited from consistent sampling times and conditions within a single farm, the diversity of our sampling context may have introduced additional variability in the microbiome profiles.

Furthermore, our adaptation of DNA extraction and amplification protocols to accommodate the lower DNA load in buccal swabs, as detailed in our methods section, might have influenced the composition and detectability of microbial DNA (Henderson et al., 2013b; Mott et al., 2022), potentially contributing to the observed differences. Lastly, utilizing the Greengenes2 reference database, released in March 2023, for microbial identification places our study at the forefront of microbiome research by employing the most current genetic information available. However, the reference database used could significantly impact the specificity and accuracy of microbial identification (Park et al., 2018; Smith et al., 2020), possibly explaining discrepancies with previous studies conducted using earlier databases.

In light of these considerations, our findings underscore the significance of recognizing the unique ecological niches that the rumen and oral cavities represent. The differences in microbial community structures and functions we observed suggest that while specific bacterial taxa may overlap between the two environments, significant distinctions exist. Our study contributes to the ongoing dialogue in microbial ecology by highlighting the importance of methodological approaches, sample population characteristics, and the use of updated genetic databases in shaping our understanding of microbial diversity and its implications.

Conflict of Interest Statement

The authors declare no conflict of interest.

Tables and Figures

Table 1. Top 30 Genera identified in the oral sample.

Rank	Genera	Average%	SD
1st	Streptococcus	13.35	9.32
2nd	Bibersteinia	7.71	7.01
3rd	Unidentified genus from family Pasteurellaceae	4.40	4.27
4th	Moraxella_C_651924	4.30	5.39
5th	Psychrobacter	4.01	3.90
6th	Corynebacterium	3.92	2.81
7th	Unidentified genus from family Lachnospiraceae	3.20	3.02
8th	Alysiella	2.90	3.71
9th	Prevotella	2.86	4.70
10th	Jeotgalicoccus_A_310962	2.29	1.83
11th	Rothia	1.90	3.10
12th	Planococcus	1.81	1.64
13th	Ralstonia	1.59	3.32
14th	Romboutsia_B	1.39	0.90
15th	Faecousia	1.17	1.37
16th	Ruminococcus_E	1.13	1.20
17th	Turicibacter	1.12	0.76

18th	Alkanindiges	1.09	2.82
19th	Clostridium_T	0.96	0.82
20th	Fundicoccus	0.94	0.74
21st	Atopostipes	0.90	1.00
22nd	Unidentified genus from family Peptostreptococcaceae	0.89	0.55
23rd	Fusobacterium_C	0.71	1.88
24th	Staphylococcus	0.70	2.60
25th	Facklamia_A_322620	0.68	0.62
26th	Acinetobacter	0.64	1.63
27th	Bacteroides_H	0.62	1.93
28th	Mannheimia	0.61	1.86
29th	Phocaeicola_A_858004	0.60	0.82
30th	Unidentified genus from family Neisseriaceae	0.60	1.26

Table 2. Top 30 Genera identified in the ruminal sample.

Rank	**Genera**	**Average%**	**SD**
1st	Prevotella	31.07	8.57
2nd	RF16	7.34	4.06
3rd	Cryptobacteroides	4.69	2.54
4th	Succiniclasticum	4.03	2.03
5th	Unidentified genus from family Lachnospiraceae	3.59	1.91
6th	Paraprevotella	3.41	1.77
7th	UBA1711	2.35	1.96
8th	UBA2810	2.18	5.28
9th	Sodaliphilus	2.09	1.97
10th	Ruminococcus_E	1.86	2.02
11th	SFMI01	1.56	1.03
12th	Limivicinus	1.44	0.74
13th	RUG420	1.42	1.47
14th	UBA4334	1.09	1.05
15th	Oribacterium	1.07	0.74
16th	Methanobrevibacter_A	1.01	0.95
17th	Fibrobacter	0.73	0.80
18th	Unidentified genus from family Bacteroidaceae	0.73	0.44
19th	Unidentified genus from class Gammaproteobacteria	0.64	1.03
20th	UBA3207	0.64	1.00
21st	UBA2450	0.63	1.28
22nd	Treponema_D	0.59	0.49
23rd	Butyrivibrio_A_168226	0.57	0.47
24th	Unidentified genus from class Gammaproteobacteria1	0.54	0.95

25th	Pseudobutyrivibrio	0.51	0.41
26th	Saccharofermentans	0.51	0.36
27th	Limimorpha	0.48	1.15
28th	CAG-791	0.48	0.70
29th	UBA1067	0.47	0.42
30th	Unidentified genus from family Selenomonadaceae	0.47	0.46

Table 3. Top 15 Enzymes Expressed in Rumen and Buccal Sample.

Enzyme	**Rumen Frequency**	**Buccal Frequency**	**Metabolic Function**
DNA polymerase I (EC:2.7.7.7)	120459410	112631813	No
Holliday junction DNA helicase (EC:3.6.4.12)	116701843	103632881	No
Peptidylprolyl isomerase (EC:5.2.1.8)	80554161	55552641	No
Histidine kinase (EC:2.7.13.3)	43871348	69963752	No
DNA-directed RNA polymerase (EC:2.7.7.6)	43632514	47334327	No
Oxidoreductases (EC:1.6.5.3)	85499402	0	Yes
Site-specific DNA-methyltransferase (EC:2.1.1.72)	41106026	37143714	No
2-oxoglutarate synthase (EC:1.2.7.3)	53779748	0	Yes
Beta-glucosidase (EC:3.2.1.21)	46359255	0	Yes
Electron transfer complex I (EC:1.6.5.3)	0	44933028	Yes
Beta-galactosidase (EC:3.2.1.23)	44577909	0	Yes
L-sorbose PTS permease (EC:2.7.1.69)	0	41750992	Yes
Beta-ketoacyl-ACP reductase (EC:1.1.1.100)	0	32172969	Yes
Acetyl coenzyme A carboxylase (EC:6.4.1.2)	0	31720923	Yes
DD-peptidase (EC:3.4.16.4)	25629129	25629129	No

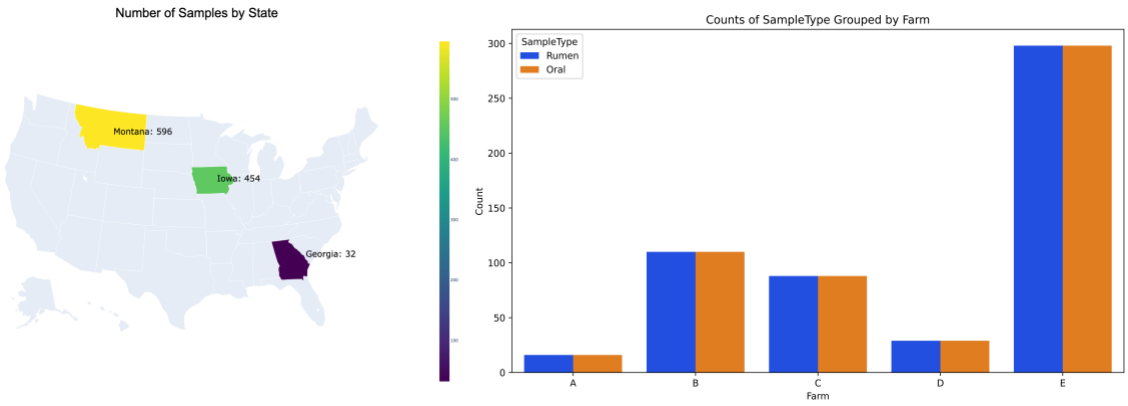

Figure 1. Comparative Analysis of Sample Collections by State and Farm.

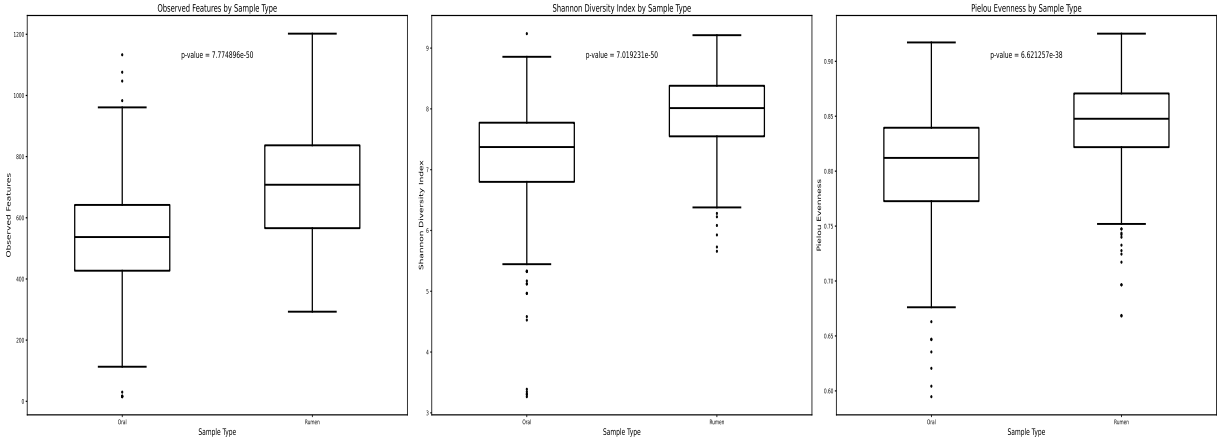

Figure 2 . Shannon Diversity Index, Pielou's Evenness Index, and Pielou's Evenness Index for Oral and Rumen Samples.

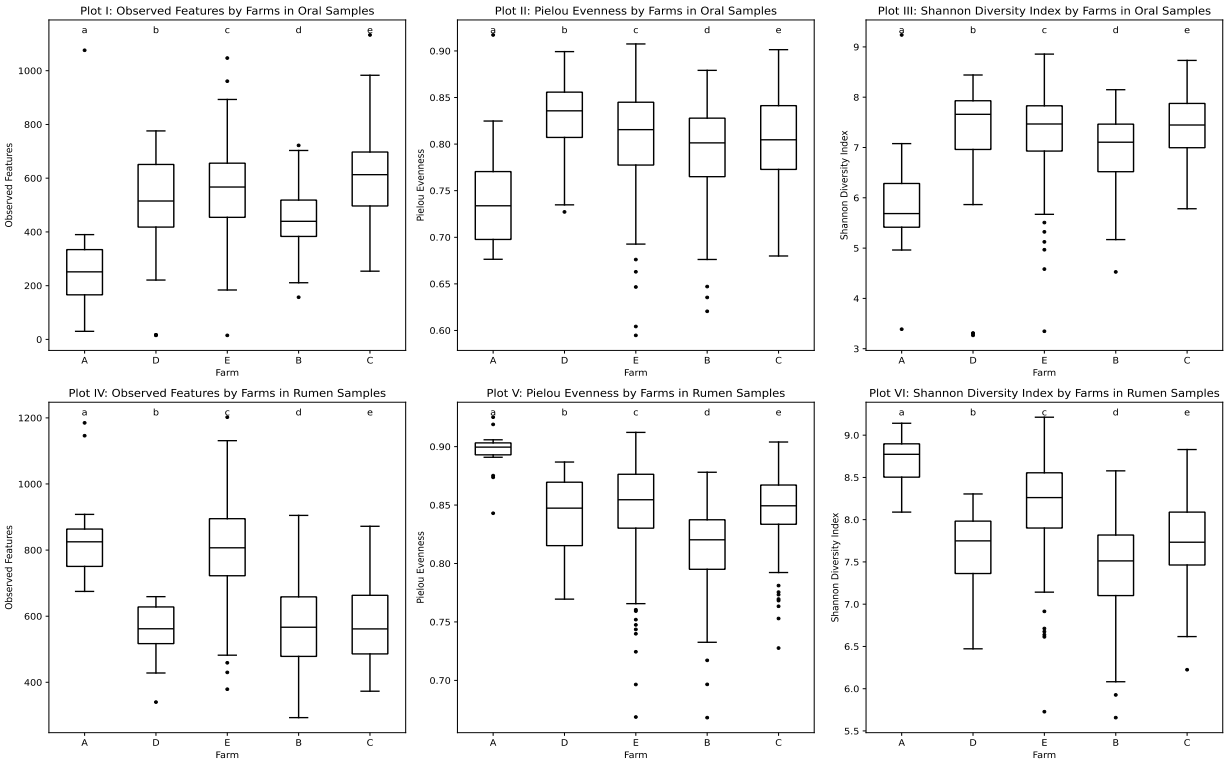

Figure 3 . Observed Features, Pielou's Evenness Index, and Shannon Diversity Index for Oral and Rumen Samples in different Farms. The p-value for the Plot I, II, III, IV, V, and VI are 1.560e-19, 1.588e-06, 6.078e-11, 2.637e-52, 8.521e-23, and 2.047e-37 respectively.

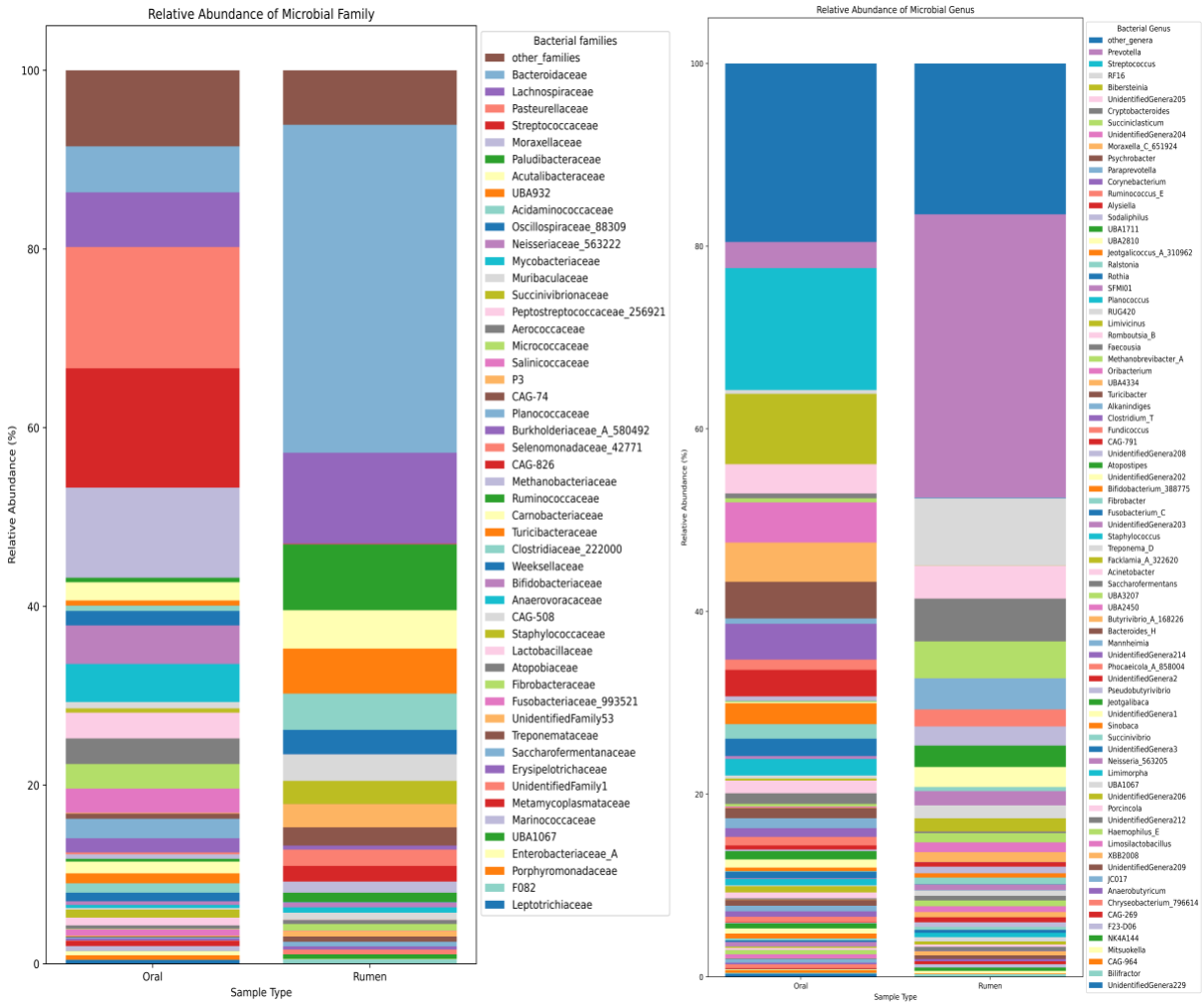

Figure 4 . Relative Abundance of Microbial Families and Genera for Oral and Rumen Samples.

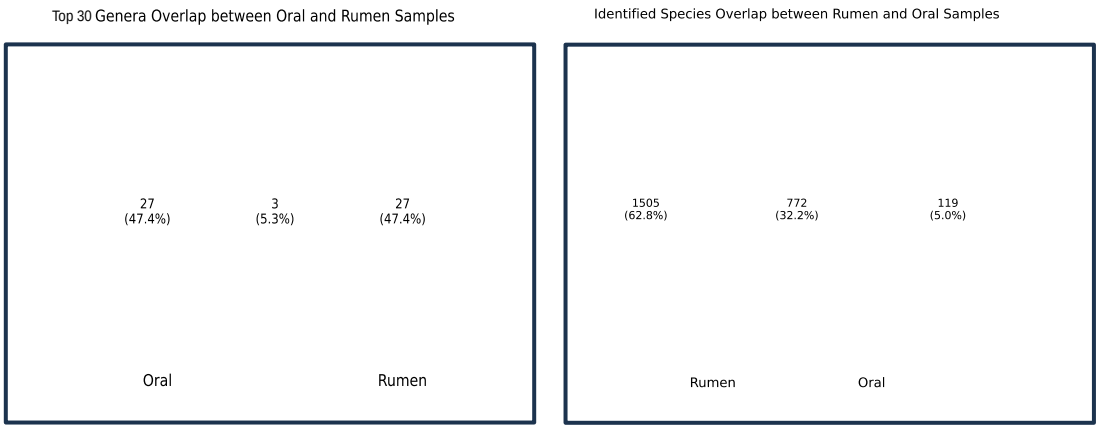

Figure 5. Venn Diagram Showing Genera Overlap among Top 30 Genera in Rumen and Oral Samples and Identified Species Overlap in Rumen and Oral Samples.

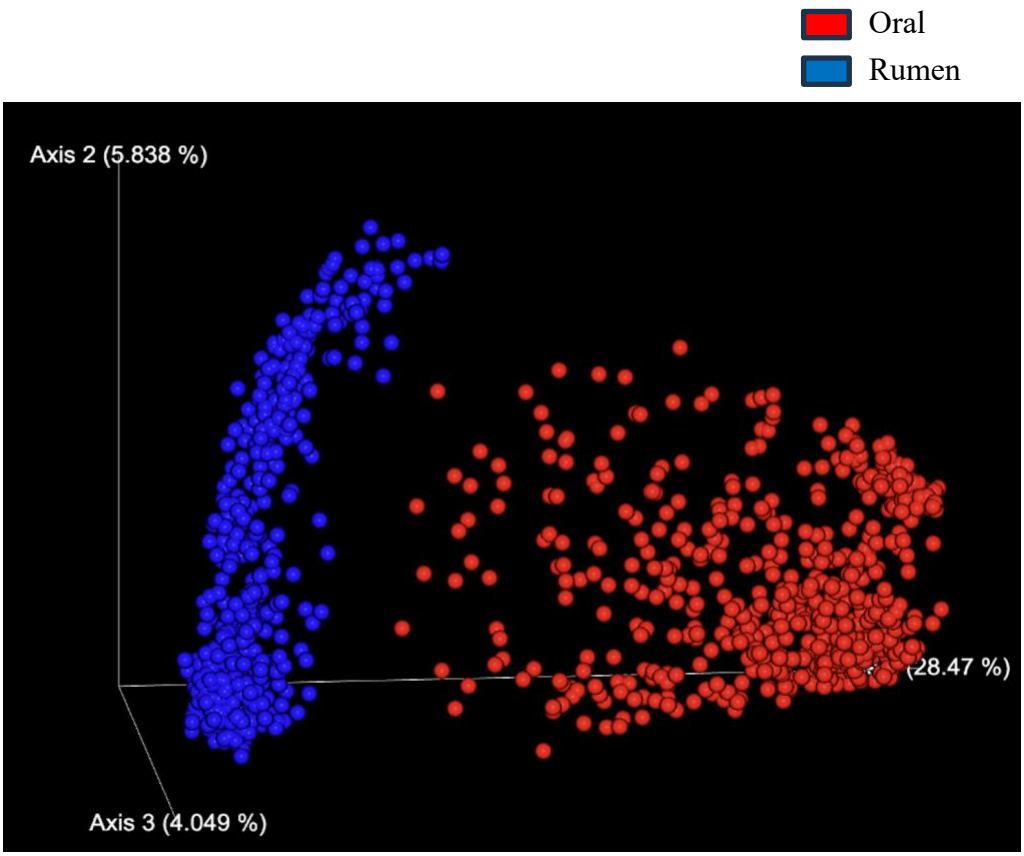

Figure 6. Principal Coordinates Analysis (PCoA) of Buccal and Rumen Microbiomes Based on Bray-Curtis Distances.

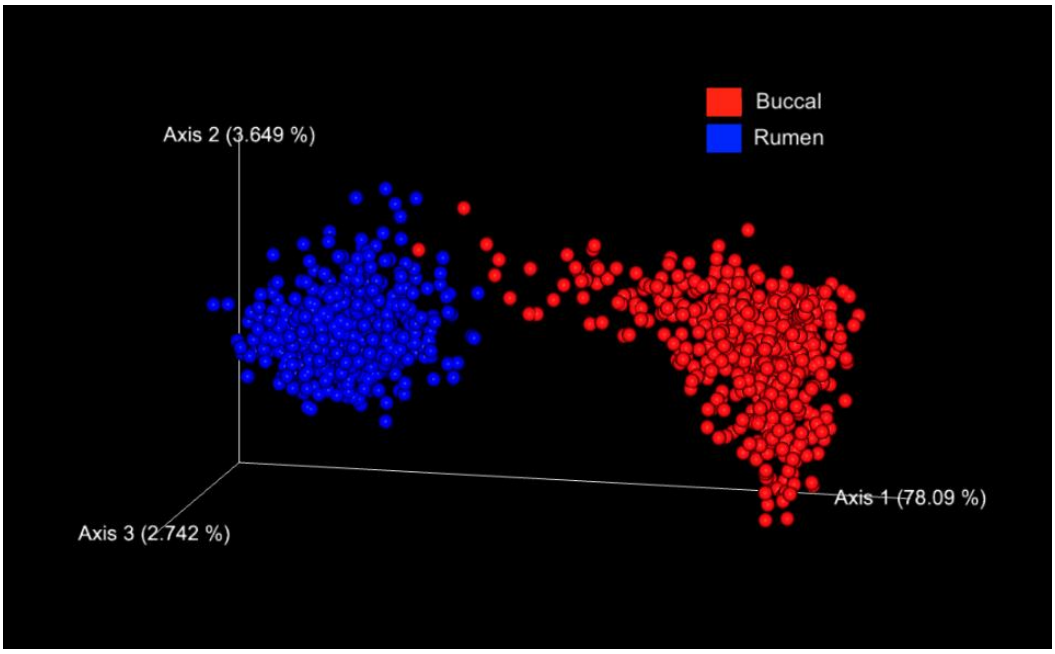

Figure 7. Principal Coordinates Analysis (PCoA) for Metabolic Pathways of Buccal and Rumen Microbiomes Based on Bray-Curtis Distances.

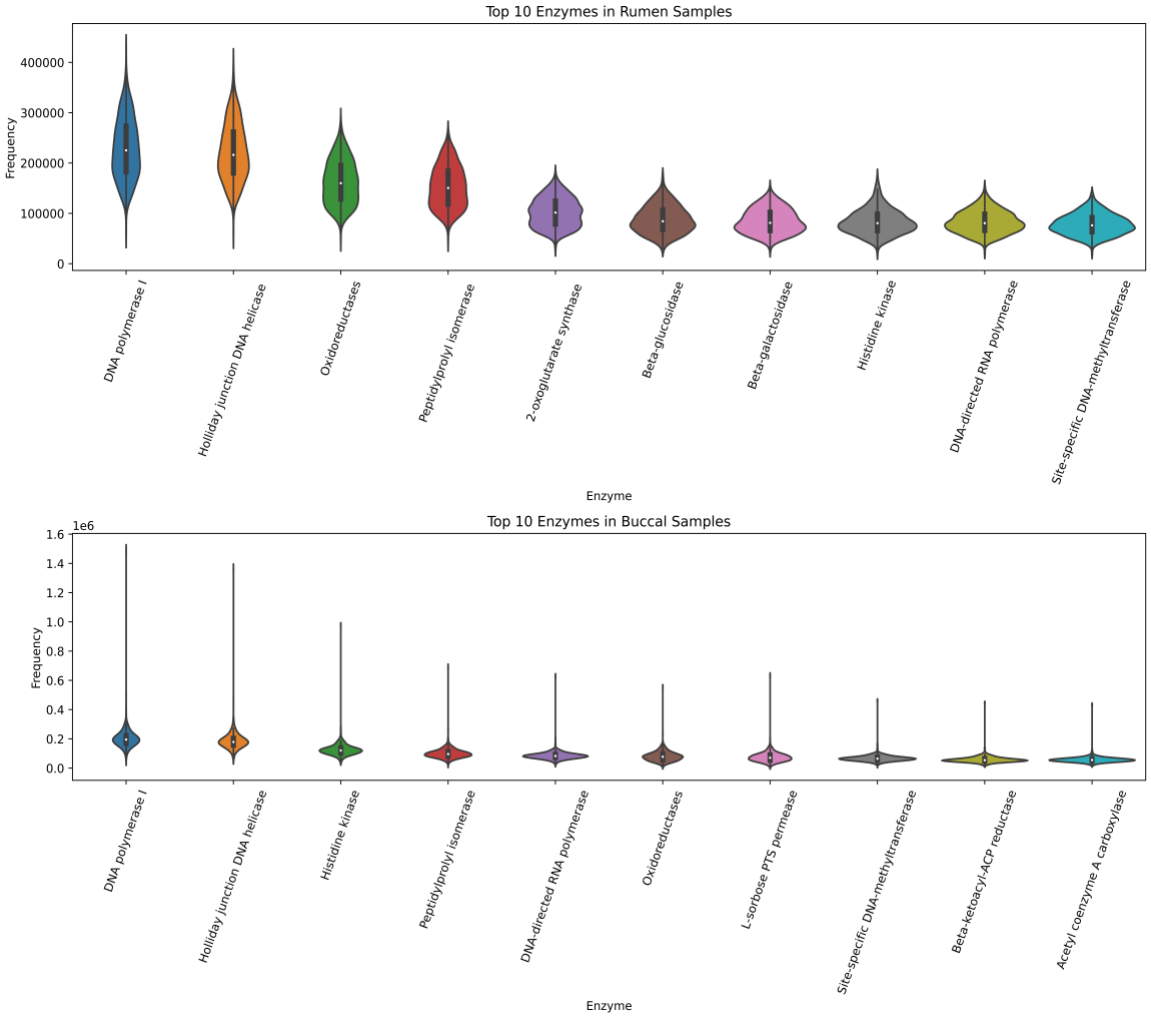

Figure 8. Violin Plots Illustrating the Distribution of Top 10 Enzymes in Rumen and Buccal Samples.

LITERATURE CITED

Amin, N., Schwarzkopf, S., Kinoshita, A., Tröscher-Mußotter, J., Dänicke, S., Camarinha-Silva, A., Huber, K., Frahm, J., & Seifert, J. (2021). Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning. *Animal Microbiome*, *3*(1), 1–15. https://doi.org/10.1186/S42523-021-00095-3/FIGURES/7

Beauchemin, K. A. (2018). Invited review: Current perspectives on eating and rumination activity in dairy cows. *Journal of Dairy Science*, *101*(6), 4762–4784. https://doi.org/10.3168/JDS.2017-13706

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of

marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 1–17. https://doi.org/10.1186/S40168-018-0470-Z/TABLES/3

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology 2019 37:8*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods 2016 13:7*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Castillo, C., & Hernández, J. (2021). Ruminal Fistulation and Cannulation: A Necessary Procedure for the Advancement of Biotechnological Research in Ruminants. *Animals 2021, Vol. 11, Page 1870*, *11*(7), 1870. https://doi.org/10.3390/ANI11071870

Cholewińska, P., Czyz, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Animal Health Research Reviews*, *21*(1), 3–14. https://doi.org/10.1017/S1466252319000069

Dehority, B. A. (1991). Effects of microbial synergism on fibre digestion in the rumen. *Proceedings of the Nutrition Society*, *50*(2), 149–159. https://doi.org/10.1079/PNS19910026

Gharechahi, J., Vahidi, M. F., Bahram, M., Han, J. L., Ding, X. Z., & Salekdeh, G. H. (2021). Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen. *The ISME Journal*, *15*(4), 1108–1120. https://doi.org/10.1038/S41396-020-00837-2

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., Waghorn, G. C., & Janssen, P. H. (2013). Effect of DNA Extraction Methods and Sampling Techniques on the Apparent Structure of Cow and Sheep Rumen Microbial Communities. *PLOS ONE*, *8*(9), e74787. https://doi.org/10.1371/JOURNAL.PONE.0074787

Hook, S., Wright, A., Archaea, B. M.-, & 2010, undefined. (n.d.). Methanogens: methane producers of the rumen and mitigation strategies. *Hindawi.ComSE Hook, ADG Wright, BW McBrideArchaea, 2010•hindawi.Com*. Retrieved March 26, 2024, from https://www.hindawi.com/journals/archive/2010/945785/

Hungate, R. E. (1996). *El rumen y sus microbios*. *1*, 533 pp. https://www.elsevier.com/books/the-rumen-and-its-microbes/hungate/978-1-4832-3308-6

Kaminsky, R. A., Reid, P. M., Altermann, E., Kenters, N., Kelly, W. J., Noel, S. J., Attwood, G. T., & Janssen, P. H. (2023). Rumen Lachnospiraceae isolate NK3A20 exhibits metabolic flexibility in response to substrate and coculture with a methanogen. *Applied and Environmental Microbiology*, *89*(10). https://doi.org/10.1128/AEM.00634-23

Kittelmann, S., Kirk, M. R., Jonker, A., Mcculloch, A., & Janssen, P. H. (2015). Buccal swabbing as a noninvasive method to determine bacterial, archaeal, and eukaryotic microbial community structures in the rumen. *Am Soc MicrobiolS Kittelmann, MR Kirk, A Jonker, A McCulloch, PH JanssenApplied and Environmental Microbiology, 2015•Am Soc Microbiol*, *81*(21), 7470–7483. https://doi.org/10.1128/AEM.02385-15

Kodithuwakku, H., Maruyama, D., Owada, H., reports, Y. W.-S., & 2022, undefined. (n.d.). Alterations in rumen microbiota via oral fiber administration during early life in dairy cows. *Nature.ComH Kodithuwakku, D Maruyama, H Owada, Y Watabe, H Miura, Y Suzuki, K HiranoScientific Reports, 2022•nature.Com*. Retrieved March 26, 2024, from https://www.nature.com/articles/s41598-022-15155-0

Lapébie, P., Lombard, V., Drula, E., … N. T.-N., & 2019, undefined. (n.d.). Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nature.ComP Lapébie, V Lombard, E Drula, N Terrapon, B HenrissatNature Communications, 2019•nature.Com*. Retrieved March 26, 2024, from https://www.nature.com/articles/s41467-019-10068-5

Li, B., Jia, G., Wen, D., Zhao, X., Zhang, J., Xu, Q., Zhao, X., Jiang, N., Liu, Z., & Wang, Y. (2022). Rumen microbiota of indigenous and introduced ruminants and their adaptation to the Qinghai–Tibetan plateau. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/FMICB.2022.1027138

Lindström, T., & Redbo, I. (2000). Effect of feeding duration and rumen fill on behaviour in dairy cows. *Applied Animal Behaviour Science*, *70*(2), 83–97. https://doi.org/10.1016/S0168-1591(00)00148-9

Lodge-Ivey, S., … J. B.-S.-J. of A., & 2009, undefined. (n.d.). Bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. *Academic.Oup.ComSL Lodge-Ivey, J Browne-Silva, MB HorvathJournal of Animal Science, 2009•academic.Oup.Com*. Retrieved March 26, 2024, from https://academic.oup.com/jas/article-abstract/87/7/2333/4731215

Lourenco, J. M., Kieran, T. J., Seidel, D. S., Glenn, T. C., da Silveira, M. F., Callaway, T. R., & Stewart, R. L. (2020). Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. *PLOS ONE*, *15*(4), e0231533. https://doi.org/10.1371/JOURNAL.PONE.0231533

Malheiros, J., Correia, B., Ceribeli, C., Reports, D. C.-S., & 2021, undefined. (n.d.). Comparative untargeted metabolome analysis of ruminal fluid and feces of Nelore steers

(Bos indicus). *Nature.ComJM Malheiros, BSB Correia, C Ceribeli, DR Cardoso, LA Colnago, SB Junior, JM ReecyScientific Reports, 2021•nature.Com*. Retrieved March 26, 2024, from https://www.nature.com/articles/s41598-021-92179-y

McDonald, D., Jiang, Y., Balaban, M., Cantrell, K., Zhu, Q., Gonzalez, A., Morton, J. T., Nicolaou, G., Parks, D. H., Karst, S. M., Albertsen, M., Hugenholtz, P., DeSantis, T., Song, S. J., Bartko, A., Havulinna, A. S., Jousilahti, P., Cheng, S., Inouye, M., … Knight, R. (2023). Greengenes2 unifies microbial data in a single reference tree. *Nature Biotechnology 2023*, *14*, 1–4. https://doi.org/10.1038/s41587-023-01845-1

Mckee, L. S., Leanti, S., Rosa, L., Westereng, B., Eijsink, V. G., Pope, P. B., & Larsbrink, J. (2021). Polysaccharide degradation by the Bacteroidetes: mechanisms and nomenclature. *Wiley Online LibraryLS McKee, SL La Rosa, B Westereng, VG Eijsink, PB Pope, J LarsbrinkEnvironmental Microbiology Reports, 2021•Wiley Online Library*, *13*(5), 559–581. https://doi.org/10.1111/1758-2229.12980

Mizrahi, I., Wallace, R. J., & Moraïs, S. (2021a). The rumen microbiome: balancing food security and environmental impacts. *Nature Reviews Microbiology 2021 19:9*, *19*(9), 553–566. https://doi.org/10.1038/s41579-021-00543-6

Mizrahi, I., Wallace, R. J., & Moraïs, S. (2021b). The rumen microbiome: balancing food security and environmental impacts. *Nature Reviews Microbiology 2021 19:9*, *19*(9), 553–566. https://doi.org/10.1038/s41579-021-00543-6

Monteiro, H., Zhou, Z., Gomes, M., reports, P. P.-S., & 2022, undefined. (n.d.). Rumen and lower gut microbiomes relationship with feed efficiency and production traits throughout the lactation of Holstein dairy cows. *Nature.ComHF Monteiro, Z Zhou, MS Gomes, PMG Peixoto, ECR Bonsaglia, IF Canisso, BC WeimerScientific Reports, 2022•nature.Com*. Retrieved March 26, 2024, from https://www.nature.com/articles/s41598-022-08761-5

Mott, A. C., Schneider, D., Hünerberg, M., Hummel, J., & Tetens, J. (2022). Bovine Rumen Microbiome: Impact of DNA Extraction Methods and Comparison of Non-Invasive Sampling Sites. *Ruminants 2022, Vol. 2, Pages 112-132*, *2*(1), 112–132. https://doi.org/10.3390/RUMINANTS2010007

Park, S., informatics, S. W.-G. &, & 2018, undefined. (n.d.). Evaluation of 16S rRNA databases for taxonomic assignments using a mock community. *Ncbi.Nlm.Nih.GovSC Park, S WonGenomics & Informatics, 2018•ncbi.Nlm.Nih.Gov*. Retrieved March 26, 2024, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6440677/

Ramos-Morales, E., Arco-Pérez, A., Martín-García, A. I., Yáñez-Ruiz, D. R., Frutos, P., & Hervás, G. (2014). Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Animal Feed Science and Technology*, *198*, 57–66. https://doi.org/10.1016/J.ANIFEEDSCI.2014.09.016

Smith, P. E., Waters, S. M., Gómez Expósito, R., Smidt, H., Carberry, C. A., & McCabe, M. S. (2020). Synthetic Sequencing Standards: A Guide to Database Choice for Rumen Microbiota Amplicon Sequencing Analysis. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/FMICB.2020.606825

Stevens, C., & Hume, I. (2004). *Comparative physiology of the vertebrate digestive system*. https://books.google.com/books?hl=en&lr=&id=DZuAsci2apAC&oi=fnd&pg=PR15&dq=Comparative+Physiology+of+the+Vertebrate+Digestive+System+-+C.+Edward+Stevens,+Ian+D.+Hume+-+Google+Books&ots=rPiY0Kqv6Q&sig=W3vb5z0OyBCJRl0WLswG-fr6gdQ

Tapio, I., Shingfield, K. J., McKain, N., Bonin, A., Fischer, D., Bayat, A. R., Vilkki, J., Taberlet, P., Snelling, T. J., & Wallace, R. J. (2016). Oral Samples as Non-Invasive Proxies for Assessing the Composition of the Rumen Microbial Community. *PLOS ONE*, *11*(3), e0151220. https://doi.org/10.1371/JOURNAL.PONE.0151220

Vázquez-Baeza, Y., Pirrung, M., Gigascience, A. G.-, & 2013, undefined. (n.d.). EMPeror: a tool for visualizing high-throughput microbial community data. *Academic.Oup.ComY Vázquez-Baeza, M Pirrung, A Gonzalez, R KnightGigascience, 2013•academic.Oup.Com*. Retrieved March 26, 2024, from https://academic.oup.com/gigascience/article-abstract/2/1/2047-217X-2-16/2656132

Vigors, S., Flores-Villalva, S., Reports, K. M.-S., & 2023, undefined. (n.d.). The impact of vitamin D3 supplementation on the faecal and oral microbiome of dairy calves indoors or at pasture. *Nature.ComS Vigors, S Flores-Villalva, KG MeadeScientific Reports, 2023•nature.Com*. Retrieved March 26, 2024, from https://www.nature.com/articles/s41598-023-34840-2

Williamson, J., Callaway, T., … J. L.-F. in, & 2022, undefined. (n.d.). Characterization of rumen, fecal, and milk microbiota in lactating dairy cows. *Frontiersin.OrgJR Williamson, TR Callaway, JM Lourenco, VE RymanFrontiers in Microbiology, 2022•frontiersin.Org*. Retrieved March 26, 2024, from https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2022.984119

Wu, S., Chen, Y., Li, Z., Li, J., Zhao, F., & Su, X. (2021). Towards multi-label classification: Next step of machine learning for microbiome research. *Computational and Structural Biotechnology Journal*, *19*, 2742–2749. https://doi.org/10.1016/J.CSBJ.2021.04.054

Young, J., Skarlupka, J. H., Cox, M. S., Resende, R. T., Fischer, A., Kalscheur, K. F., Mcclure, J. C., Cole, J. B., Suen, G., & Bickhart, D. M. (2020). Validating the use of bovine buccal sampling as a proxy for the rumen microbiota by using a time course and random forest classification approach. *Am Soc Microbiol, MS Cox, RT Resende, A Fischer, KF Kalscheur, JC McClure, JB Cole, G Suen, DM BickhartApplied and*

Environmental Microbiology, 2020•Am Soc Microbiol, *86*(17). https://doi.org/10.1128/AEM.00861-20

Zhang, Y., Chen, L., Wu, Z., & Sun, C. (2011). Kinetic parameters of soil β-glucosidase response to environmental temperature and moisture regimes. *Revista Brasileira de Ciência Do Solo*, *35*(4), 1285–1291. https://doi.org/10.1590/S0100-06832011000400022

CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

A comprehensive investigation of the ruminal environment is crucial for improving animal production and for guiding potential interventions to enhance feed efficiency and reduce methane emissions. Our investigation of non-invasive buccal swabs as proxy for obtaining the ruminal microbial composition revealed very distinct microbial profiles for the rumen and oral cavity, contrary to some previous literature findings. This divergence from previous findings highlights the complexity of the rumen microbiome and suggests that the oral microbiome may not serve as a straightforward surrogate for studying rumen microbial communities. This realization carries implications for microbiome sampling strategies and microbial data interpretation in ruminant health and nutrition studies. It emphasizes the need to directly examine the rumen microbiome for accurate insights into its composition and function. Moreover, the divergence from previous studies invites further research to explore the underlying mechanisms driving the differences observed between these two microbiomes and to reconcile these findings with previous studies.