Impact of Taxonomic Database Choice on Fungal Community Analysis in Cow Rumen Using ITS Data

Victoria Palecek

Author Contributions

Victoria Palecek is a graduate student, and they contributed to data analysis, statistics, creating figures, reading literature, writing the manuscript, and reviewing the drafts for submission.

Keywords

Amplicon Sequence Variants, Fungal Community, Alpha Diversity, Beta Diversity, Relative Abundance

Abstract:

The microbiome is crucial for digestion in the rumen of cows. As part of the microbiome, fungi play a major role in the degradation of fiber within the rumen. This study will investigate the impact of taxonomic database choice on the interpretation of fungal community composition and diversity metric data using cow rumen ITS sequencing data evaluating fungal *Saccharomyces cerevisiae* treatment in cows consuming a high-fiber or high-grain, acidosis-inducing diet. Amplicon sequencing variants (ASVs) were created from the ITS sequencing data and taxonomy was assigned using two different ITS taxonomic databases. The alpha diversity, beta diversity and relative abundance were examined using the DAD2 and phyloseq packages in R. The results revealed that diet choice, yeast supplementation, and sample location collectively had a significant impact on microbial community composition, as reflected by the beta diversity data. However, these same factors did not significantly affect alpha diversity, as no significant differences in richness were observed across sample groups. Additionally, both alpha and beta diversity, being independent of taxonomic assignment, were not affected by the choice of taxonomic database. Notably, no differences were detected in relative abundance data between the two taxonomic database assignments. These results indicate that choice of taxonomic database does not significantly affect the interpretation of fungal community diversity when using ASV-based metrics. These findings also indicate that the LSU_RDP and Unite ITS taxonomic databases exhibited sufficient similarity to avoid differences in taxonomic assignment for the cow rumen ITS data in this study. It may be beneficial to compare the LSU_RDP and Unite ITS taxonomic databases using more diverse sample origins in future investigations to identify to what extent these databases provide the same taxonomic assignment results.

Introduction:

Fungi play a crucial role in a cow's digestion. In the initial stages of fiber degradation, anaerobic fungi make the biggest impact on the digestion of plant cell walls. Anaerobic fungi within the rumen will secrete a variety of enzymes into the extracellular space to degrade lignin, cellulose and hemicellulose within the plant cell walls (Theodorou et al., 1996). The secretion of carbohydrate-active enzymes (CAZymes) such as hemicellulase and cellulase (both glycoside hydrolases) from fungi within the rumen is regulated primarily by soluble sugars and begins once plant material enters the rumen (Hartinger et al., 2021). Once a significant portion of the plant cell wall has been degraded by fungi in the rumen, bacteria have better access to continue the digestion of remaining plant materials (Zhang et al., 2022). Analyzing the composition of fungi in the cow rumen under high-fiber and high-grain diets allows us to understand how fungal diversity contributes to digestion in different feeding conditions.

Active dry yeast supplementation has been shown to aid in the digestion of cows in many ways. One of such benefits is an increase in bacterial diversity in the rumen while also decreasing methane-promoting organisms (Kampanat et al., 2021). Additional benefits in adult cows include increased rumen fungal diversity as well as an increase in the ability to break down fiber (Ding et al., 2014). Supplementation with active dry yeast has also been shown to improve immune system function and growth rates in calves (Ma et al., 2021).

The Internal Transcribed (ITS) Regions located between ribosomal RNA (rRNA) regions are commonly used to identify fungal diversity. The effectiveness of taxonomic identification of ITS data is strongly dependent on the taxonomic database used. Different taxonomic databases may have differing features such as taxonomic coverage and completeness (Wright et al., 2023). While ITS regions are present in both eukaryotes and prokaryotes, they are mainly useful for taxonomic identification in specific eukaryotic groups, such as fungi. For this reason, the use of a eukaryote-focused ITS database is important for reliable and accurate taxonomic assignments in studies targeting organisms such as fungi (Schoch et al., 2012).

We know diversity metrics based on amplicon sequence variants (ASVs) are always consistent regardless of taxonomic database selection, because the calculations for these metrics do not reference taxonomy assignment. Any calculation that takes taxonomic assignment into account is at risk of being altered by the choice of taxonomic database. Relative abundance is one of such calculations as it provides information on the percentage of all ASVs assigned to a specific taxa in a sample. In this study, we examined the effects the use of two different ITS taxonomic databases, UNITE Fungi (version 10.0) and the Fungi RDP LSU fixed train set (version 2), have on taxonomy assignment and diversity metric interpretation of cow rumen ITS data.

Materials and Methods:

Sample Collection

The data being used in this study originates from a study investigating the effects of diet-induced sub-acute ruminal acidosis (SARA) and active dry yeast supplementation on fungal and protozoal diversity. In the study, cows were either fed a high fiber diet or a high grain diet with active dry yeast (*Saccharomyces cerevisiae*) supplementation. The samples were collected from both the fluid within the rumen and the rumen epimural. The DNA extraction and sequencing methods used for this data can be found in the paper for which the data was originally collected (Ishaq et al., 2017).

Data Processing and Quality Control:
Quality control for the raw sequence data was visualized using the Rqc package in R (v.4.4)(Souza et al., 2014). Raw sequence data was processed using the DADA2 pipeline (Callahan et al., 2016) in R, which includes quality filtering, trimming, chimera removal, and ultimate use of just the forward reads to produce amplicon sequence variants (ASVs). The SVs for the two different taxonomic assignments were independently rarefied using phyloseq (McMurdie et al., 2013). As there were no negative controls for this dataset, no decontamination step was performed.
The dataset for this study was made up of 34 samples and 1,853,959 raw reads, with each read sequenced to 300 bases in length. Based on quality assessment, the forward read sequences were trimmed at cycle 250 to maintain a Phred quality score above 25, while reverse read sequences, despite having a similar quality assessment, were not included due to the absence of 12 samples in the reverse reads. Following filtering there were still 1,248,310 high-quality reads. Chimera checking identified 259 bimeras among 11,750 SVs, which were subsequently removed, resulting in 11,491 unique SVs across 34 samples. The phyloseq objects for each taxonomy database assignment were rarified to a sample size of 18000. This resulted in the removal of 7430 SVs and 4 samples (E21, E31, F29, F58) each.

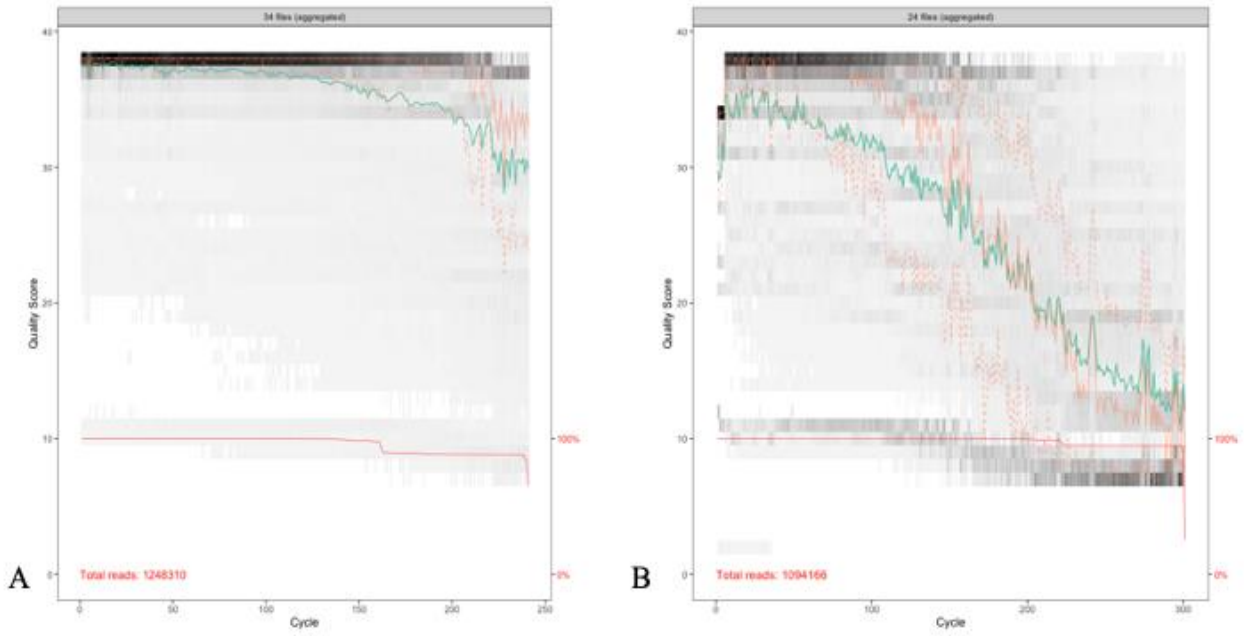

Figure 1: Aggregate Phred quality profile of forward (A) and reverse (B) sequence reads across all samples.

Taxonomic Assignment Using Multiple Databases:
To identify the effects of taxonomic database choice on interpretation of fungal community composition and relative abundance data, ASVs produced by the data were aligned to the UNITE Fungi (version 10.0)(Abarenkov et al.,2024) and the Fungi RDP LSU fixed train set (version

2)(Cole et al. 2014) taxonomy databases. ASVs were aligned to each taxonomic database using the assignTaxonomy function in DADA2.

Diversity Analysis:

Alpha Diversity: The alpha diversity metrics, Shannon, Chao1 and observed diversity were calculated for each sample. The phyloseq package in R was used to estimate diversity between sample groupings of sample location, diet and active dry yeast supplementation (figure 3). These diversity metrics were compared across taxonomy database assignments.

Relative Abundance: Taxonomic composition at the level of genus was determined by calculating the relative abundance of ASVs in each sample using a phyloseq object containing ASV and taxonomy data. Bar charts were created to visualize the composition of fungi across sample groups and taxonomic database assignment using ggplot2 in R (Wickham, 2022).

Beta Diversity: Beta diversity was assessed using Principal Coordinates Analysis (PCoA) based on unweighted Jaccard distances. PCoA was performed to visualize clustering patterns by sample group (diet, active dry yeast supplementation, and sample location). To test for significant differences in community composition, we performed permanova (9999 permutations). A p-value threshold of 0.05 was used for significance. We also evaluated beta dispersion to assess variability (spread) within groups using betadisper() with the same Jaccard distances. Differences in dispersion were tested with ANOVA as well as a permutation test (999 permutations) for homogeneity of multivariate dispersions using permutest() from the vegan package (Oksanen et al., 2001) in R, with significance set at $p < 0.05$ (Oksanen et al.,2024). These diversity metrics were compared across taxonomy database assignments.

Results:

Alpha Diversity

When comparing Chao1 and Observed diversity, there appear to be relatively few rare-taxa samples, as indicated by their similarity in figure 3. This suggests that the number of unseen species in the dataset was likely low. The kurtosis value for both the cleaned and rarified phyloseq objects was -0.450, indicating a relatively normal, if slightly negatively skewed, distribution of diversity measures. However, following Shapiro testing, the Shannon diversity data was found to be non-normally distributed ($p = 0.007891$), while the observed diversity data was normally distributed ($p = 0.9018$). The cleaned and rarified data also did not follow a normal distribution ($p = 6.19e-05$) for both phyloseq objects. We continued analysis of our data using non-parametric methods as a result of these tests. We used the Kruskal-Wallis test to determine if there was a statistically significant difference between medians of the rarified observed diversity (richness) data between sample groups. It was determined that there is not a statistically significant difference in median observed diversity between sample groups (chi-squared = 12.669, df = 7, $p = 0.806$). However, a linear model revealed that the group sampled from rumen fluid, fed a high grain diet without yeast supplementation, exhibited significantly lower observed richness compared to other sample groups ($p = 0.0272$) (figures 5A and 5B).

Alpha diversity metrics calculated from ASVs were consistent across both taxonomic databases. Richness (observed ASVs) and Shannon diversity results showed no significant differences (figures 4A and 4B). This was expected given that ASV-derived sample diversity is independent of taxonomy. This outcome reaffirms that alpha diversity metrics based on ASVs are not affected by the choice of taxonomic database.

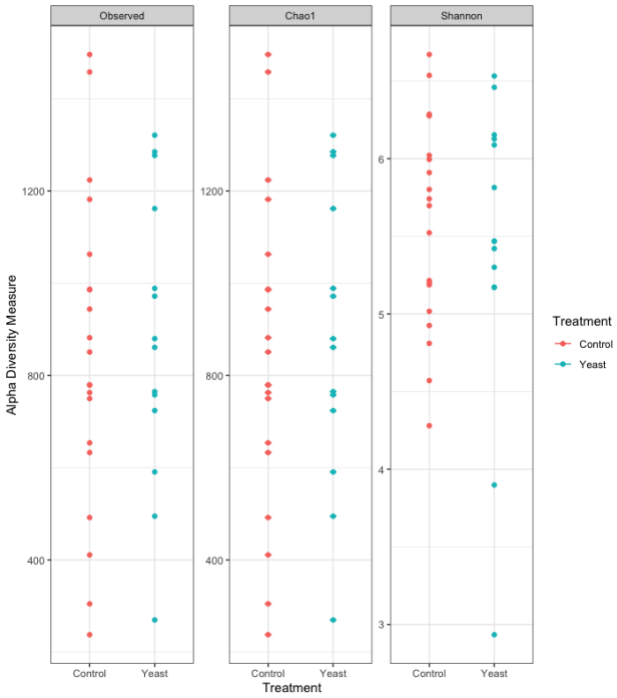

Figure 3: Alpha diversity measures (Observed, Chao1, and Shannon) for fungal communities in cow rumen samples with and without active dry yeast supplementation.

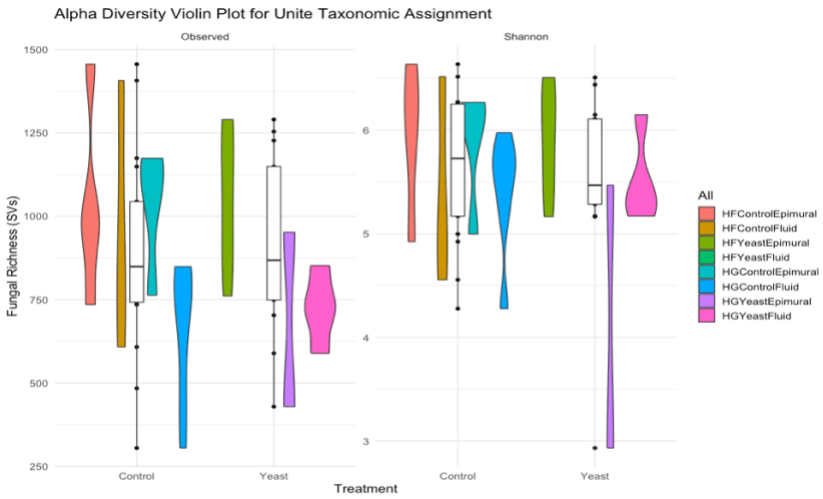

Figure 4A: Observed and Shannon diversity measures for fungal richness in cow rumen ITS data aligned to the Unite ITS taxonomic database and grouped by diet, yeast supplementation and sample location.

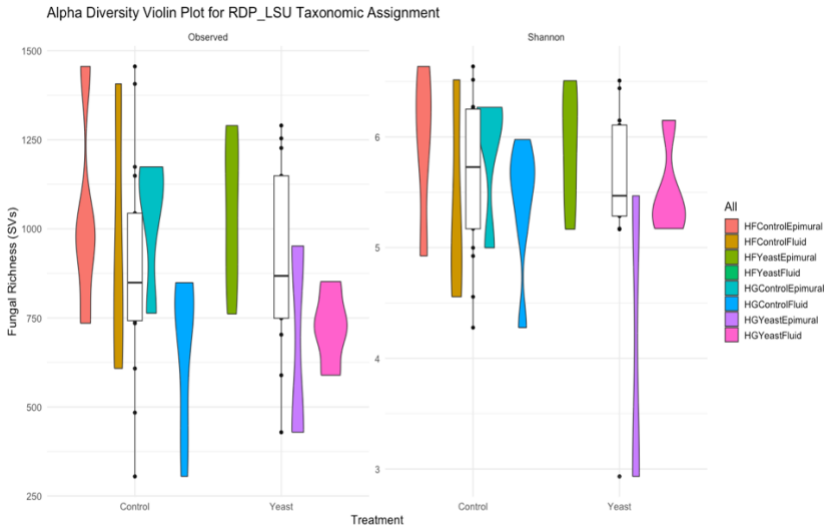

Figure 4B: Observed and Shannon diversity measures for fungal richness in cow rumen ITS data aligned to the RDP_LSU ITS taxonomic database and grouped by diet, yeast supplementation and sample location.

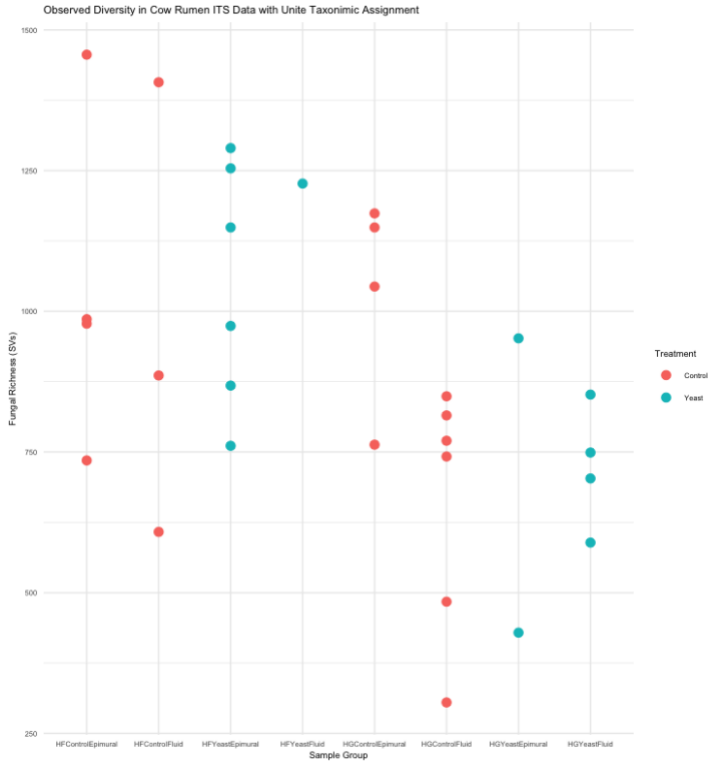

Figure 5A: Observed fungal richness across all treatment groups in cow rumen ITS data with Unite taxonomic assignment. Points represent individual samples colored by treatment group.

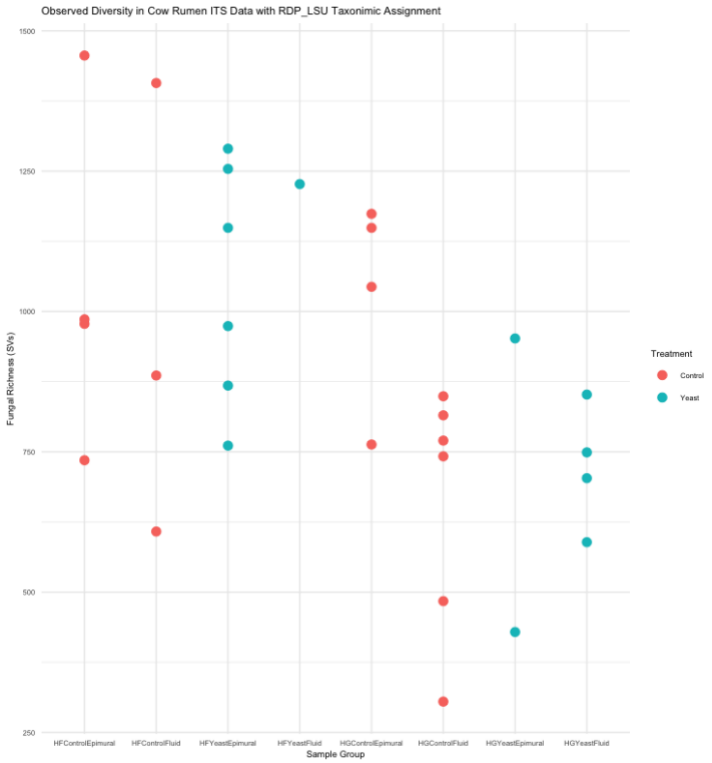

Figure 5B: Observed fungal richness across all treatment groups in cow rumen ITS data with RDP LSU taxonomic assignment. Points represent individual samples colored by treatment group.

Taxonomic Assignment Consistency

The two taxonomic databases produced identical taxonomic assignments across all ASVs. Taxa at higher ranks (ex. phylum, class, and order) were classified consistently, and there were no discrepancies in taxa names or hierarchical structure between the databases. Both databases identified the same number of ASVs to the same taxonomic levels, with no differences in taxa identified at genus or species levels. This high degree of correlation suggests the two databases are very strongly aligned for rumen-associated fungi.

Relative Abundance

Within this dataset there were many unidentifiable ASVs. Relative abundance analysis highlighted distinct phylum composition differences between diet groups, with Ascomycota making up the majority of fungi in cows fed a high-grain diet and Neocallimastigomycota making up the majority of fungi in those on a high-fiber diet.

Relative abundance created from taxonomic assignments was also identical between the two databases. Abundance distributions at the phylum, family, genus and species levels were consistent across samples, and there were no differences in the relative proportion of any taxa observed within sample groups.

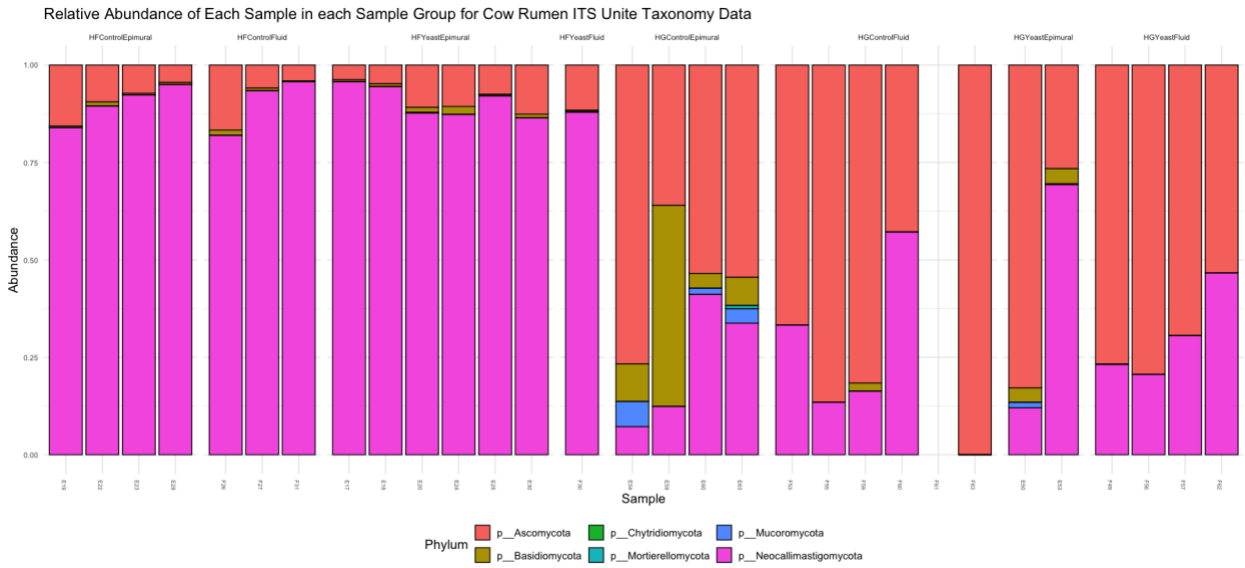

Figure 6A: Relative abundance of fungal phyla in cow rumen samples across all sample groups (diet, yeast supplementation and sample location) with taxonomic assignment from the Unite ITS taxonomic database.

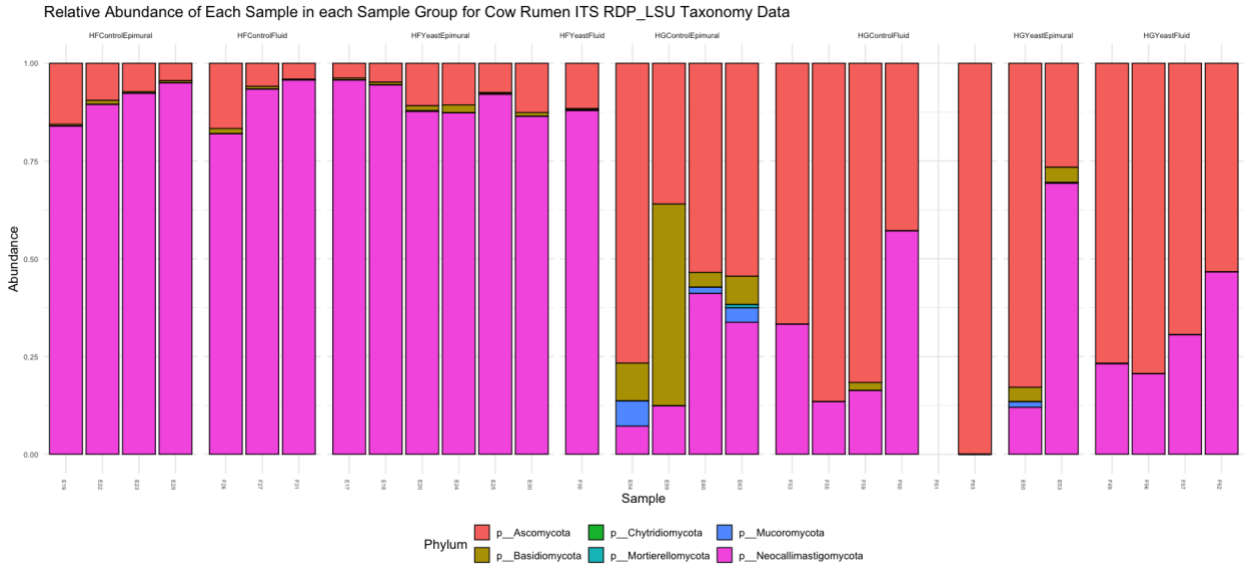

Figure 6B: Relative abundance of fungal phyla in cow rumen samples across all sample groups (diet, yeast supplementation and sample location) with taxonomic assignment from the RDP_LSU taxonomic database.

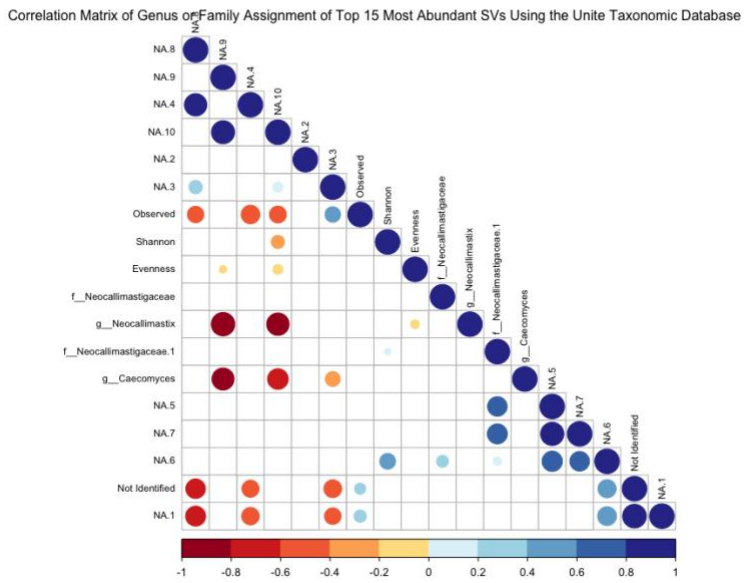

Figure 7A: Correlation matrix of genus or family assignments for the top 15 most abundant ASVs and alpha diversity measures using the Unite ITS taxonomic database. Only correlations with a p-value < 0.05 were plotted.

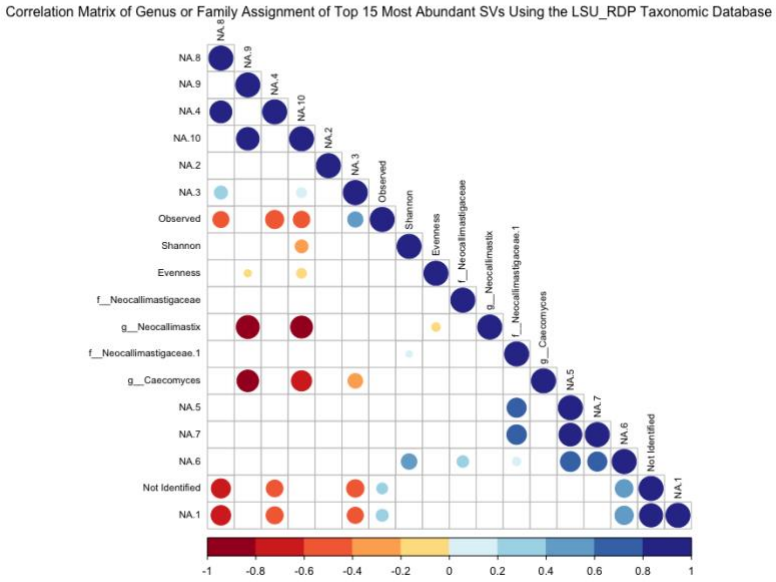

Figure 7B: Correlation matrix of genus or family assignments for the top 15 most abundant ASVs and alpha diversity measures using the RDP_LSU taxonomic database. Only correlations with a p-value < 0.05 were plotted.

Beta Diversity and Community Structure

We initially assessed beta diversity in the cow rumen ITS data by creating a Principal Coordinates Analysis (PCoA) plot using the Jaccard distance measure (figures 8A and 8B). The PCoA plot allowed us to visualize the dissimilarity between samples based on community composition. In our study, PCoA revealed distinct clustering patterns among the respective sample groups, which could indicate variability in microbial communities based on factors such as diet, yeast supplementation, and sample location. We then evaluated the statistical significance of these patterns using a permanova test. The model testing the effects of diet, yeast supplementation, and sample location was highly significant ($p < 0.0001$), which suggests that these factors contribute to the observed differences in microbial community composition across the samples.
To assess homogeneity of multivariate dispersions, we performed a beta dispersion analysis. The results indicated similar dispersions across most groups, with an exception for the HFYeastFluid group, which had zero variance due to only containing a single sample. However, the ANOVA test of beta dispersion indicated significant differences in group dispersion ($p = 5.86e\text{-}12$). This shows that while microbial community compositions are distinct between groups, the degree of variation within groups also differs significantly. These results are also supported by the permutation test for homogeneity of dispersions, which produced a significant p-value of 0.001. This suggests that the variability within groups is not equal across the different treatment groups.

There were no differences in beta diversity between the data aligned to the Unite taxonomic database or the RDP_LSU taxonomic database, as expected. The PCoA, permanova and ANOVA test results for both taxonomic assignments were identical. This is because beta diversity does not rely on taxonomic assignment. These results are based on community structure within sample groups and cannot be changed by taxonomic identification of its ASVs.

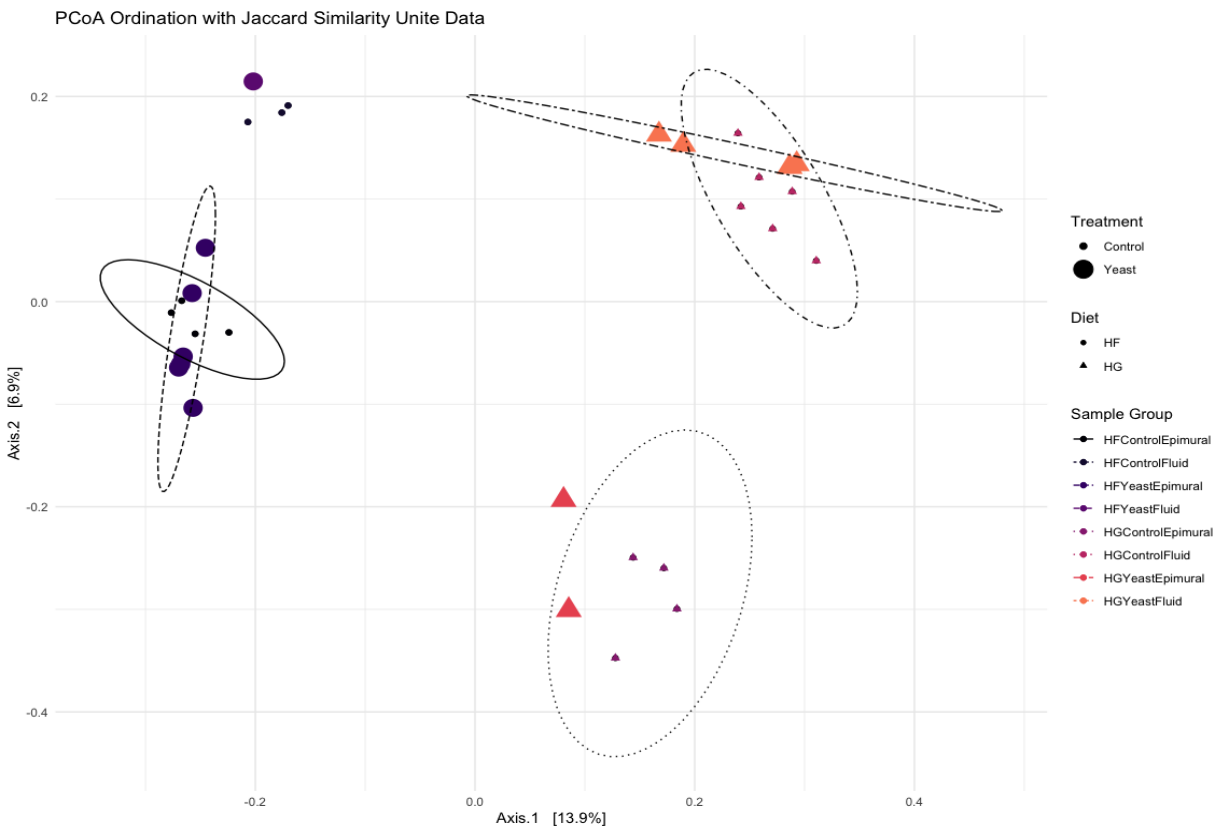

Figure 8A: Principal Coordinates Analysis (PCoA) ordination plot based on Jaccard similarity of fungal community composition in cow rumen samples assigned taxonomy using the Unite ITS taxonomic database.

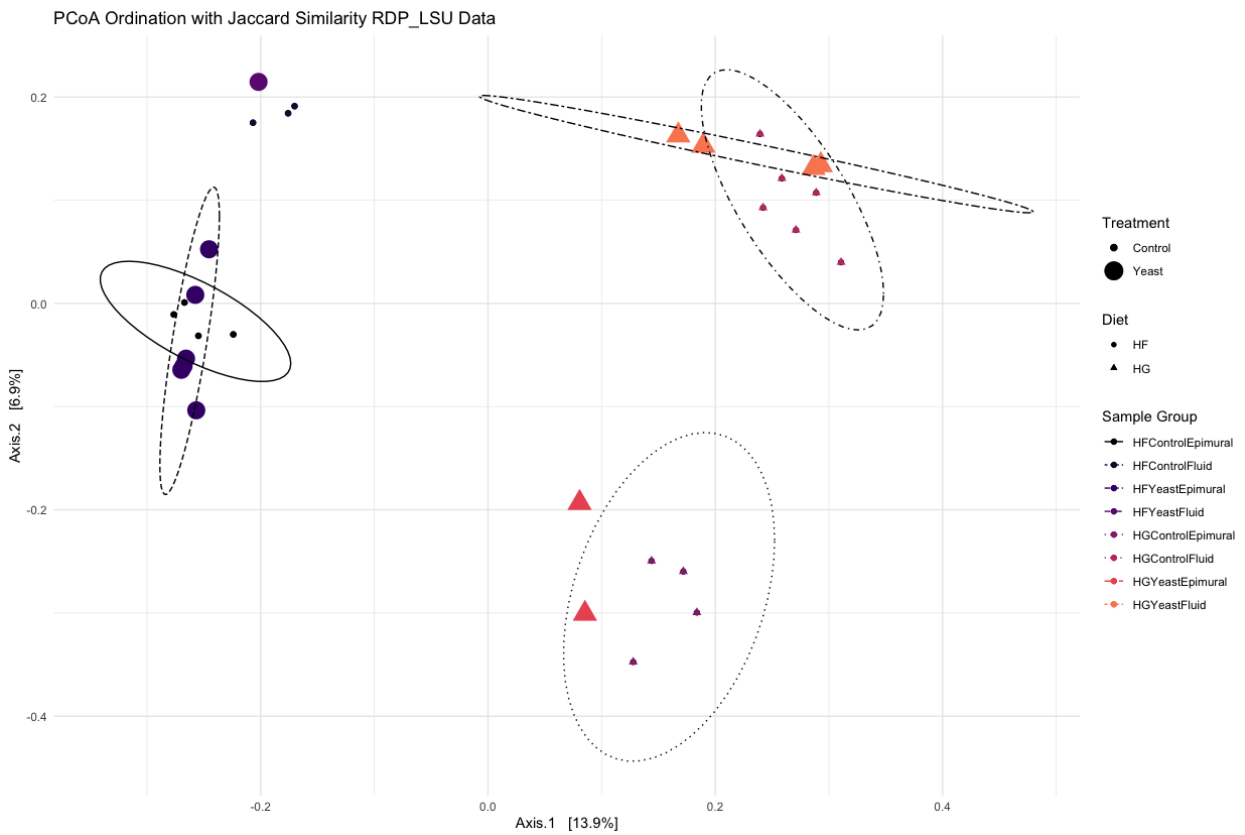

Figure 8B: Principal Coordinates Analysis (PCoA) ordination plot based on Jaccard similarity of fungal community composition in cow rumen samples assigned taxonomy using the RDP_LSU taxonomic database.

Discussion:

In this study, we aimed to examine the effects the use of two different ITS taxonomic databases have on taxonomic assignment and diversity metric interpretation of cow rumen ITS data. Our results indicate that the impact of taxonomic database selection on alpha and beta diversity metrics is inconsequential. This reinforces that diversity metrics based solely on ASVs are independent of taxonomic assignment.

For alpha diversity, both the Shannon and observed diversity metrics produced consistent results between the two taxonomic databases. This result is not surprising, as ASVs are used to define these diversity metrics. ASV-based calculations are not influenced by the choice of taxonomic database. As determined by Kruskal-Wallis and linear modeling, the non-significant differences in observed richness across sample groups indicate that fungal richness was relatively stable across the sample groupings.

The consistency of taxonomic assignments between the UNITE and RDP_LSU databases supports the equivalent performance of both taxonomic databases with rumen-associated fungi. Both databases produced identical taxonomic classifications across all samples, with no discrepancies in taxa identified at any level, from phylum to species. This high level of agreement between databases suggests that, for this dataset, both databases are reliable for assigning taxonomy to rumen-associated fungi. Additionally, there were no differences in

relative abundance distributions between the two databases, which indicates that relative abundance estimates were not impacted by the choice of taxonomic database for this dataset.

Beta diversity analysis confirmed that community composition patterns were influenced by sample group factors (diet, yeast supplementation and sample location). There were significant differences in microbial communities between these groupings. This emphasizes the significant role these environmental factors play in shaping the fungal community structure of the rumen. As expected, beta diversity was not affected by the choice of taxonomic database. The analysis of beta diversity in this study relied on examining overall community structure and not the taxonomic identity of individual ASVs.

This study demonstrates that choice of taxonomic database does not significantly affect the interpretation of fungal community diversity in cow rumens when using ASV-based metrics. Although it was not the case in this study, choice of taxonomic database does have the opportunity to alter relative abundance results (Yunlong et al., 2024). The choice of taxonomic database may change the coverage at different taxonomic levels leading to differences in how ASVs are assigned at those levels. Based on our data in this study we can say that the LSU_RDP and Unite ITS taxonomic databases are sufficiently similar to not cause discrepancies in taxonomic assignment for cow rumen ITS data in this experiment. This conclusion is limited to the fungi found in the rumen of the cows used in this experiment. Future comparisons of these taxonomic databases on different samples and sampling environments may lead to greater assurance of their similarities.

Acknowledgements:

I would like to thank Dr. Sue Ishaq, The University of Maine, for access to her data and assistance in analysis for this study.

Supplementary Material:

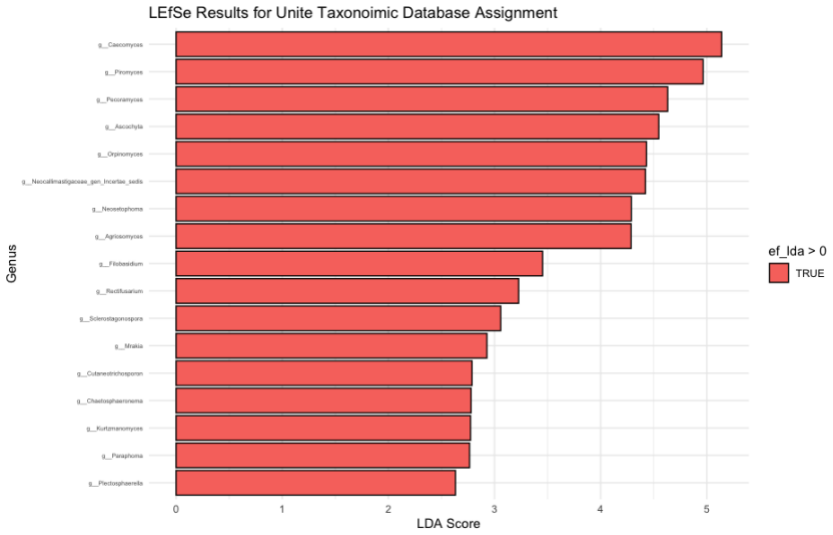

Figure 9A: The LEfSe results bar plot displays the genera, assigned taxonomy using the RDP LSU taxonomic database, with significant LDA scores (LDA > 2), indicating their discriminative

ability across dietary groups (high grain and high fiber). Each bar represents a genus, ordered by its LDA score, with the color indicating whether the genus was enriched in the positive class (red) or not (blue). The x-axis shows the genus names, while the y-axis represents the corresponding LDA score. Genera with higher LDA scores contribute more strongly to the differences between groups.

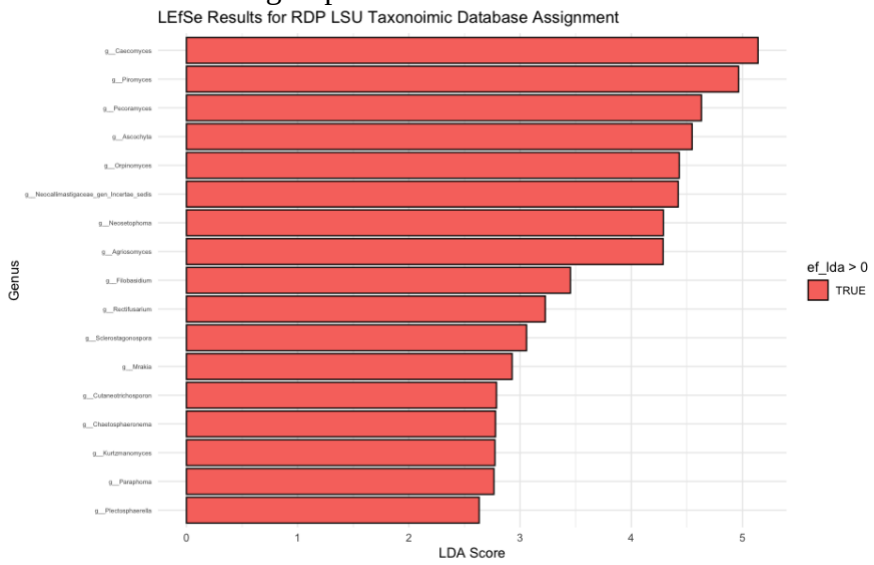

Figure 9B: The LEfSe results bar plot displays the genera, assigned taxonomy using the RDP LSU taxonomic database, with significant LDA scores (LDA > 2), indicating their discriminative ability across dietary groups (high grain and high fiber). Each bar represents a genus, ordered by its LDA score, with the color indicating whether the genus was enriched in the positive class (red) or not (blue). The x-axis shows the genus names, while the y-axis represents the corresponding LDA score. Genera with higher LDA scores contribute more strongly to the differences between groups.

LEfSe Results: LEfSe analysis was conducted to identify taxa with differential abundance between dietary groups (high grain (HG) and high fiber (HF)). Seventeen genera were identified as significant microbiome markers, with LDA scores above 2, indicating the level of discriminance of these genera between the diet groups. Of these, 16 genera were enriched in the high-fiber (HF) diet group, while one genus, *Rectifusarium*, was enriched in the high-grain (HG) diet group. The genera *Caecomyces* and *Piromyceshad* the highest LDA scores of 5.14 and 4.97, respectively, suggesting they are the most strongly associated with the HF group. All identified genera showed statistically significant differences in abundance, with p-values ranging from 0.0001 to 0.0485. These results suggest that the HF diet supports a unique fungal community composition.

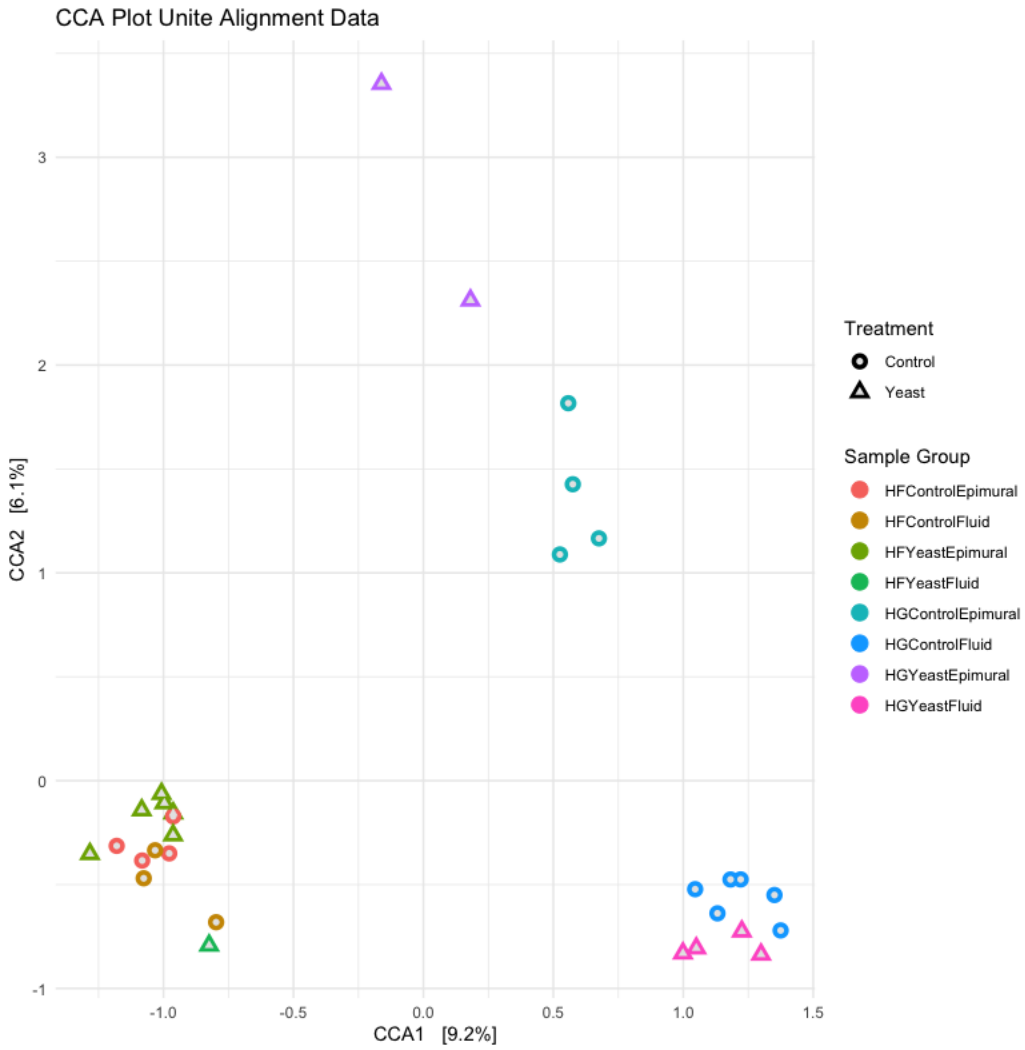

Figure 10A: CCA was performed to assess the relationship between fungi community composition and experimental factors (diet, yeast supplementation, sample location) with taxonomy assignment from the UNITE taxonomic database. Samples were ordinated based on Bray-Curtis dissimilarity of microbial communities.

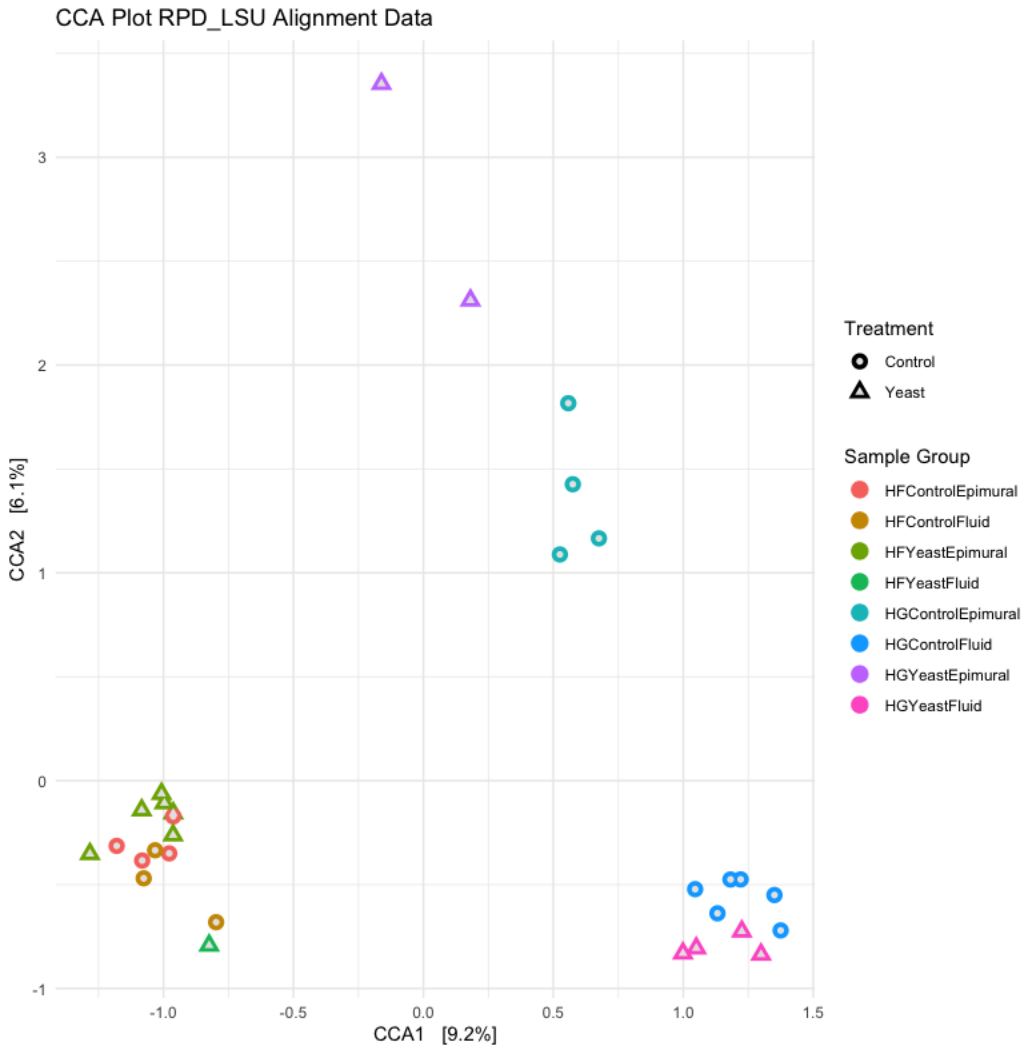

Figure 10B: CCA was performed to assess the relationship between fungi community composition and experimental factors (diet, yeast supplementation, sample location) with taxonomy assignment from the RDP LSU taxonomic database. Samples were ordinated based on Bray-Curtis dissimilarity of microbial communities.

CCA Results: Several patterns are made apparent through the interpretation of our Canonical Correspondence Analysis (CCA). The clustering of all samples from cows fed a high fiber diet indicates that a high fiber diet in cows may be a major influence on overall fungi community composition within the rumen. The large separation of the high grain diet samples and the high fiber diet samples along the CCA1 axis indicates choice of diet is an important driving factor for the differences in fungi community composition in our test samples. The distance between the clusters of samples fed a high grain diet taken from the epimural and the samples fed a high grain diet taken from the rumen fluid may indicate the rumen epimural is more susceptible to changes in fungi community composition while being fed a high grain diet. It does not appear, based on these CCA results, that supplementation with active dry yeast plays as big of a role in changes in fungi community composition as choice of diet. A permutation test was performed using the CCA data comparing sample groups using all factors (diet, sample location and yeast supplementation) and as a result, we can conclude that these factors combined significantly ($p <$

0.001) influence fungi community composition in the cow rumen. By performing a permutation test for the CCA results of just the diet factor we also found that choice of diet alone is a significant factor ($p < 0.001$) in fungi community composition.

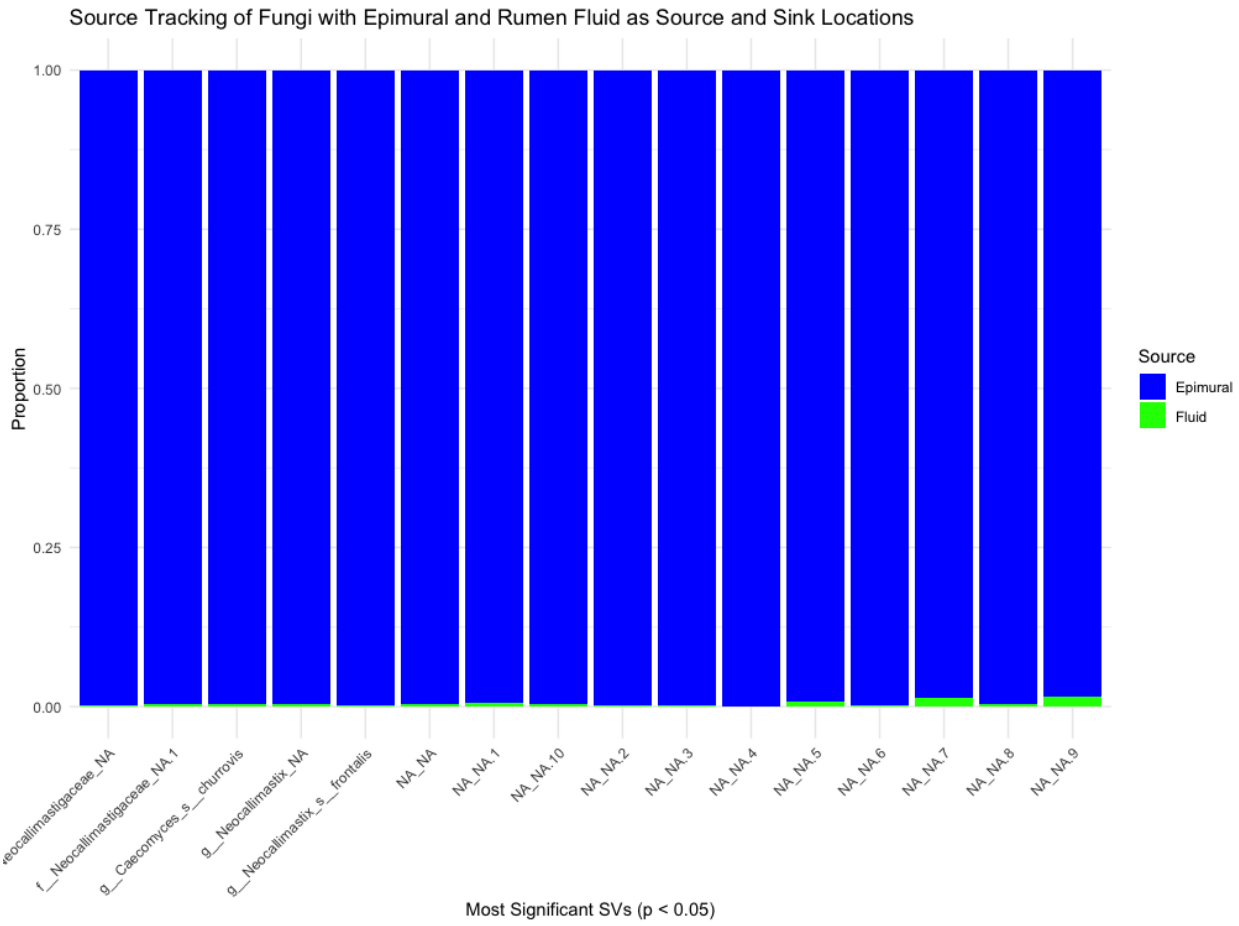

Figure 11: This figure displays the proportions of source contributions (Epimural and Fluid) to each sample based on source tracker predictions.

Significant SV Taxonomic Assignment	Fluid	Epimural
f__Neocallimastigaceae_NA	0.00254778	0.99745222
f__Neocallimastigaceae_NA.1	0.00359218	0.99640782
g__Caecomyces_s__churrovis	0.00312834	0.99687166
g__Neocallimastix_NA	0.00343617	0.99656383
g__Neocallimastix_s__frontalis	0.0016659	0.9983341
NA_NA	0.00450698	0.99549302
NA_NA.1	0.00689178	0.99310822
NA_NA.10	0.00421519	0.99578481
NA_NA.2	0.00168051	0.99831949
NA_NA.3	0.00235294	0.99764706
NA_NA.4	0.00050139	0.99949861
NA_NA.5	0.00768895	0.99231105
NA_NA.6	0.00191287	0.99808713
NA_NA.7	0.0141071	0.9858929
NA_NA.8	0.0032121	0.9967879
NA_NA.9	0.01590803	0.98409197

Table 1: Proportion of significant Amplicon Sequence Variants (ASVs) across source locations. The table shows the proportion of each significant SV ($p < 0.05$) for the epimural and rumen fluid sample locations. Each row represents a unique SV taxonomic assignment, with proportions found in the epimural and rumen fluid in separate columns to indicate the relative contribution of each location.

Source Tracking Results: The source tracking results displayed in figure 11 and table 1 show the proportion of significant SVs ($p < 0.05$) originating from the two different sample locations (epimural and rumen fluid). The data indicates that only a very small proportion of each significant SV is associated with the rumen fluid, in contrast to the larger proportions found in the epimural. This suggests that the epimural serves as the primary source of microbiome components, with the rumen fluid acting as the sink. These findings imply a directional flow of microbial taxa from the epimural to the rumen fluid, suggesting that the epimural environment is likely the donor of microbiome components to the rumen fluid.

References:

Abarenkov, Kessy, R. Henrik Nilsson, Karl-Henrik Larsson, Andy F. S. Taylor, Tom W. May, Tobias Guldberg Frøslev, Julia Pawlowska, et al. “The UNITE Database for Molecular Identification and Taxonomic Communication of Fungi and Other Eukaryotes: Sequences, Taxa and Classifications Reconsidered.” *Nucleic Acids Research* 52, no. D1 (January 5, 2024): D791–97. https://doi.org/10.1093/nar/gkad1039.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. “DADA2: High-Resolution Sample Inference from Illumina Amplicon Data.” *Nature Methods* 13, no. 7 (July 2016): 581–83. https://doi.org/10.1038/nmeth.3869.

Cole, James R., Qiong Wang, Jordan A. Fish, Benli Chai, Donna M. McGarrell, Yanni Sun, C. Titus Brown, Andrea Porras-Alfaro, Cheryl R. Kuske, and James M. Tiedje. "Ribosomal Database Project: Data and Tools for High Throughput rRNA Analysis." *Nucleic Acids Research* 42, no. D1 (January 2014): D633–42. https://doi.org/10.1093/nar/gkt1244.

Ding, Gengzhi, Ying Chang, Liping Zhao, Zhenming Zhou, Liping Ren, and Qingxiang Meng. "Effect of Saccharomyces Cerevisiae on Alfalfa Nutrient Degradation Characteristics and Rumen Microbial Populations of Steers Fed Diets with Different Concentrate-to-Forage Ratios." *Journal of Animal Science and Biotechnology* 5, no. 1 (May 1, 2014): 24. https://doi.org/10.1186/2049-1891-5-24.

Hartinger, Thomas, and Qendrim Zebeli. "The Present Role and New Potentials of Anaerobic Fungi in Ruminant Nutrition." *Journal of Fungi* 7, no. 3 (March 2021): 200. https://doi.org/10.3390/jof7030200.

Ishaq, Suzanne L., Ousama AlZahal, Nicola Walker, and Brian McBride. "An Investigation into Rumen Fungal and Protozoal Diversity in Three Rumen Fractions, during High-Fiber or Grain-Induced Sub-Acute Ruminal Acidosis Conditions, with or without Active Dry Yeast Supplementation." *Frontiers in Microbiology* 8 (October 10, 2017). https://doi.org/10.3389/fmicb.2017.01943.

Liu, Yunlong, Morteza H. Ghaffari, Tao Ma, and Yan Tu. "Impact of Database Choice and Confidence Score on the Performance of Taxonomic Classification Using Kraken2." *aBIOTECH*, July 31, 2024. https://doi.org/10.1007/s42994-024-00178-0.

Ma, Jian, Cheng Wang, Zhisheng Wang, Guang Cao, Rui Hu, Xueying Wang, Huawei Zou, et al. "Active Dry Yeast Supplementation Improves the Growth Performance, Rumen Fermentation, and Immune Response of Weaned Beef Calves." *Animal Nutrition* 7, no. 4 (December 1, 2021): 1352–59. https://doi.org/10.1016/j.aninu.2021.06.006.

McMurdie, Paul J., and Susan Holmes. "Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." *PLOS ONE* 8, no. 4 (April 22, 2013): e61217. https://doi.org/10.1371/journal.pone.0061217.

Oksanen, Jari, Gavin L. Simpson, F. Guillaume Blanchet, Roeland Kindt, Pierre Legendre, Peter R. Minchin, R.B. O'Hara, et al. "Vegan: Community Ecology Package," September 6, 2001. https://doi.org/10.32614/CRAN.package.vegan.

Phesatcha, Kampanat, Krittika Chunwijitra, Burarat Phesatcha, Metha Wanapat, and Anusorn Cherdthong. "Addition of Active Dry Yeast Could Enhance Feed Intake and Rumen Bacterial Population While Reducing Protozoa and Methanogen Population in Beef Cattle." *Fermentation* 7, no. 3 (September 2021): 172. https://doi.org/10.3390/fermentation7030172.

Schoch, Conrad L., Keith A. Seifert, Sabine Huhndorf, Vincent Robert, John L. Spouge, C. André Levesque, Wen Chen, et al. "Nuclear Ribosomal Internal Transcribed Spacer (ITS)

Region as a Universal DNA Barcode Marker for Fungi." *Proceedings of the National Academy of Sciences* 109, no. 16 (April 17, 2012): 6241–46. https://doi.org/10.1073/pnas.1117018109.

Souza, Wélliton de, Benilton de Sá Carvalho, and Iscia Lopes-Cendes. "Rqc: A Bioconductor Package for Quality Control of High-Throughput Sequencing Data." *Journal of Statistical Software* 87 (November 12, 2018): 1–14. https://doi.org/10.18637/jss.v087.c02.

Theodorou, Michael K., Georgina Mennim, David R. Davies, Wei-Yun Zhu, Anthony P. J. Trinci, and Jayne L. Brookman. "Anaerobic Fungi in the Digestive Tract of Mammalian Herbivores and Their Potential for Exploitation." *Proceedings of the Nutrition Society* 55, no. 3 (November 1996): 913–26. https://doi.org/10.1079/PNS19960088.

Wickham, Hadley. *Ggplot2: Elegant Graphics for Data Analysis*. Springer, 2016.

Wright, Robyn J., André M. Comeau, and Morgan G. I. Langille. "From Defaults to Databases: Parameter and Database Choice Dramatically Impact the Performance of Metagenomic Taxonomic Classification Tools." *Microbial Genomics* 9, no. 3 (2023): 000949. https://doi.org/10.1099/mgen.0.000949.

Zhang, Zhenwei, Xu Gao, Wanting Dong, Bingjian Huang, Yonghui Wang, and Mingxia Zhu. "Plant Cell Wall Breakdown by Hindgut Microorganisms: Can We Get Scientific Insights From Rumen Microorganisms?" *Journal of Equine Veterinary Science* 115 (August 1, 2022): 104027. https://doi.org/10.1016/j.jevs.2022.104027.