Characterizations of the Microbial Structures of the Gastrointestinal Tract Microbiome of Declining Moose Populations

Undergraduate Research Thesis

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by
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Abstract

Dramatic declines in the moose populations of the northern United States have prompted several ecological investigations into how the environment could potentially impact moose health. This study uses 16S rRNA gene sequencing to analyze the microbial community composition of the gastrointestinal tract (GIT) of moose from Minnesota and Columbus, Ohio. The objective was to better understand if the microbiome could serve as an indicator for wild animal health. Here we examine microbial community structure, membership, and diversity and correlate these with cause of death along the GIT from deceased moose. This is the first examination of the microbiome of the entire GIT of wild moose, as prior studies focused on rumen tissue and feces. My 16S rRNA gene analysis uses Illumina MiSeq sequencing of the V4 hyper-variable region. Raw reads were processed using QIIME 1.9.1 and were statistically analyzed using Primer v6 and R. Rumen fluid short-chain fatty acid (SCFA) concentrations were quantified using High Performance Liquid Chromatography. Statistical analyses showed that microbial community composition was structured based upon organ or excretion location within the GIT. Several intestinal locations were statistically differentiable based upon the health condition and the nutritional status of the moose. In particular, Shannon Diversity was much lower in the rumen fluid of moose classified as sick, which correlated to statistically lower concentrations of acetate and propionate. Higher enrichments of Bacteriodetes were observed in the microbiome of sick moose with the BS11 gut group OTU being the most prevalent enrichment. These findings show there is a correlation between moose health and their microbiota, suggesting that microbiome data could serve as a biological indicator for animal health status.

Introduction

In 1980, recreational visitors and outdoor enthusiasts exploring northwestern Minnesota frequently encountered one of the largest and most iconic animals inhabiting the wilderness, the North American moose. However, the probability of observing these animals is declining sharply with the moose population in the northwest consisting of only about 100 moose today (McDonald 2014). The northeastern moose population in Minnesota is following the same path with an estimated 50% decline of the moose population over the course of less than a decade (DelGiudice *et al.* 2014). Causes for these dramatic decreases in moose numbers are currently unknown, which has prompted considerable research into the issue. Disease agents have been characterized among the northeastern Minnesota moose populations (Carstensen *et al*. 2007) as one plausible explanation to the dramatic decline, but other factors related to climate change effects such as warmer winters, nutrient depletion in dietary forage, and habitat loss are being considered as well.

Climatic factors, such as increased UVB radiation, warmer temperatures, and increased CO_2, have been shown to negatively impact plant nutritional quality in the arctic (Lavola *et al*. 2013). This can impose a bottom-up constraint on herbivorous animals and may explain the current declining state of many moose populations (McArt *et al*. 2009). Declines in snow fall and fluctuations in other climatic factors increase the probability of over-browsing, which can prompt a cascading decline of nitrogen content and availability in the diet of arctic moose (Christensen *et al.* 2014). Studies done by our collaborators in Alaska have shown that nitrogen availability to the moose is negatively correlated to condensed tannin concentration (McArt *et al.* 2009). Condensed tannins bind plant macromolecules and can block microbial degradation in the rumen (Bhat *et al.* 1998). Condensed tannins can therefore cause a negative nitrogen balance preventing weight gain during the critical summer period that potentially is contributing to declines in Alaskan moose populations (McArt *et al.* 2009). Similar to these preceding concerns, the nutritional quality of moose diets in zoos and research facilities also limits the health and longevity of moose under human care (Shochat and Robbins 1997; Hofmann and Nygren 1992). Despite the importance of microbial communities in harvesting energy from plant material, few studies have explored the relationship between the microbiome and declining populations.

Energy derived from ruminant diets is primarily obtained through the microbial fermentation of complex polysaccharides to short-chain fatty acids (SCFA), which are absorbed by the rumen papillae and provide energy for the moose (Bergman 1990). While microbial fermentation of dietary plant nutrients in the rumen plays a critical role in host energy, our understanding of the moose gastrointestinal (GIT) microbiome is limited to a few 16S rRNA gene surveys of rumen tissue collected from hunter kills (Ishaq and Wright 2012, 2014). These studies examined healthy wild moose in different geographical regions but failed to compare their findings to declining populations, correlate microbial membership to rumen chemistry, or investigate the entire GIT.

This research opportunistically explores the GIT microbiome of a representative subset of wild moose from Minnesota and captive moose from the Columbus Zoo to identify changes in the microbial membership and fermentation end products in relation to measured health status. By examining the membership and structure of the GIT microbiome, we can ask the

questions: **(1)** Are microbial communities statistically different in moose that died of illness than moose that were healthy at the time of death? **(2)** Does gender, age, pregnancy or nutritional status impact the GIT microbiome? **(3)** Can feces serve as a proxy for assessing GIT health in wild moose populations? To begin answering these questions, my laboratory collaborated with the Minnesota Department of Natural Resources (MNDNR). MNDNR collected samples of rumen fluid, feces, and whole GIT tissue from wild Minnesota moose within 6 hours of death. We then extracted DNA, analyzed the microbial community members, and compared our findings to other ongoing laboratory moose microbiome studies in Alaska and at the Columbus Zoo.

Hypotheses

This study uses sequencing of the 16S rRNA gene, which is the phylogenetic and taxonomic marker for archaeal and bacterial communities, to explore the entire GIT microbiome (rumen, rumen fluid, reticulum, omasum, abomasum, small intestine, colon, and feces). We use this tool to characterize the structure and membership of the GIT microbiome in a wild, declining moose population. We analyzed moose that died from both anthropogenic causes (vehicle collision) and health-related illness in order to determine if the microbiome can act as a biosignature for the health status of wild herbivore populations. Through 16S rRNA analyses of the affected moose populations, this study is a novel exploration into the ecological impacts and possible foundations for reversing such detrimental declines in populations. In this study, three hypotheses were considered:

H1: The microbial community will be statistically differentiable in the rumen by cause of death and/or nutritional status.

H2: Certain microbes will be enriched in the rumen of moose that had a poor nutritional status at the time of death.

H3: Moose in a poor nutritional condition will have statistically lower SCFA detected in the rumen fluid.

This research identifies key microbial members that may be responding to GIT illness, which are the same bacteria enriched on a low nutrient, Alaskan winter diet. My analyses of the BS11 gut group across the GIT, a family of the Bacteroidetes, was included in a recent publication, earning me an authorship (Solden *et al.* 2016). My analyses corroborate other ongoing studies about how climate-driven changes in the arctic and boreal vegetative landscape impact moose energy via rumen microbial communities. My thesis work also provides insight into new diagnostic tools for assessing wild moose population health and creates a foundation for future studies into how various factors impact the rumen microbiome of moose and other herbivores in climate sensitive regions. Such characterizations could potentially lead to the development of dietary supplements that could sustain the maintenance of moose in zoos and research centers.

Methods

Sample Collection: To identify factors contributing to the decline in the wild Minnesota moose population, we participated in a study lead by the MNDNR that collared 173 wild adult moose from 2013-2015 in northeastern Minnesota (Carstensen et al. 2015). The reproductive status of

examined female moose was either denoted as pregnant or not pregnant. Nutritional status of moose was determined by body condition score and was recorded as either very thin, thin, or normal. Gender, cause of death, and an obvious presence of disease load were also provided by our collaborators. Additional disease testing, definitive nutrition parameters, and in-depth diet analyses are currently being explored but is beyond the scope of this study. To minimize changes in the microbiome, deceased moose were identified within 24 hours of death, as assessed by mortality notification from the satellite-linked GPS collar or mortality implant transmitter. From 2013-2014, MNDNR collected gastrointestinal tissues and fluids aseptically during field necropsy from 10 moose. Tissue samples (~5g) and fluids (10 ml) were removed and placed in 20 mL of RNA later Stabilization Solution to preserve the microbial genomic information. Samples were refrigerated until shipment to Columbus for further processing. In addition to the wild moose from Minnesota, we also sampled two deceased moose calves from the Columbus Zoo. All calves had experienced severe diarrhea and weight loss prior to death. Tissue samples were collected with sterile forceps and a scalpel at necropsy at the Ohio State University Veterinary Clinic and frozen immediately at -80°C until processing.

Sample Processing: Genomic DNA was extracted from rumen fluid, feces, and tissue samples (0.5g each) using the PowerSoil® DNA Isolation Kit according to manufacturer protocols (Mo Bio Laboratories, Inc.) with the following exceptions:

- Bead tubes were heated at 70°C for 10 mins after the addition of solution C1 (an SDS solution), with 5 second vortexing at 5 mins of the heat treatment.
- Bead tubes were centrifuged with the C1 solution for 2 mins rather than 30 seconds before removal of supernatant.

Concentrations of DNA extracted from samples were immediately assessed using Qubit analyses and viable samples were stored at -20°C for sequencing.

DNA Sequencing / Processing: Universal primers 515F and 806R were used for PCR amplification of the V4 hypervariable region of 16S rRNA genes. The 515F primer contained a unique sequence tag to barcode each sample. Both primers contained sequencer adapter regions. DNA was sequenced at Argonne National Laboratory at the Next Generation Sequencing Facility with a single lane of Illumina MiSeq using 2 x 251 bp paired end reads following established HMP protocols (Caporaso et al. 2010). Data processing was performed with QIIME 1.9.0 unless otherwise noted. The specific processing steps were as follows: Raw fastq data were demultiplexed and quality filtered to a phred score of 20. This filtering step reduced the 2,076,016 paired end reads to 835,014. OTUs were chosen in a two-step process. First, sequences were clustered into OTUs using UCLUST followed by de novo OTU picking. OTUs were checked for chimeras using RDP gold database and assigned taxonomy using the 97_SILVA_111 rep set (Quast *et al* 2013). Sequences were used for comparison of the relative abundance of bacterial taxa.

High Performance Liquid Chromatography (HPLC): Samples were prepped by centrifuging 1 mL of rumen fluid at 3,000 X g for 5 mins and then filtered through 0.2 um nylon filter. All short-chain fatty acid analyses were performed with a Shimadzu HPLC equipped with an Aminex HPX-87H Organic Acid column. Samples were injected into the column using a mobile phase of 0.008 M H_2SO_4, flow rate of 0.6 mL/min at 55°C. Standard curves were created each day for desired SCFAs including acetate, butyrate, isobutyric acid, isovaleric acid, and propionate at biologically relevant concentrations (20 mM, 10 mM, 5 mM, 2.5 mM, and 1.25 mM).

Statistical Analyses: Statistical analyses were conducted using Primer V6 and graphics created using R and Adobe Illustrator. Constructed OTU tables were uploaded into Primer. Unless otherwise noted, the standard pre-treatment of the data was a square-root treatment. The data sets were transformed using a Bray-Curtis similarity matrix from which standard statistical analyses were conducted, such as non-metric multidimensional scaling (nMDS), Shannon Diversity, Richness, PERMANOVA, ANOSIM, and SIMPER. SIMPER results were compared to t-test results of the OTU tables where $p < 0.05$. nMDS analyses allow for all of the microbiota of one sample to be condensed into one data point arranged in a 2D figure based upon multiple factors. Shannon Diversity analyses provide a linear designation of how many unique OTUs make up a given community (richness) and the relative abundances of each OTU (evenness). ANOSIM allows for standard univariate one- and two-way ANOVA (analysis of variance) tests for resemblance matrices through the use of permutation/randomization tests. This test can be used to examine the null hypothesis that there are no differences between groups of samples that are defined by a single factor (such as site or treatment). PERMANOVA allows the user to test the simultaneous response of one or more variables to one or more factors with an ANOVA based upon permutations of a resemblance measure. The test performs a partitioning of the total sum of squares according to the user specified design that can yield distance-based pseudo-F values for each term in the model. P-values are determined through permutations of raw data or residuals of either full or reduced models (Clarke and Warwick 2001).

Results and Discussion

Based on ongoing diet studies in the lab with healthy Alaskan moose, rumen fluid microbial communities rapidly and reproducibly change with key microbial members responding to dietary changes (Solden *et al*. 2016). Here I examined if microbiomes in moose also respond to health status. To do this, GIT tissue and rumen fluid were opportunistically sampled from deceased moose. Rumen tissue and fluid microbial communities were statistically different in moose that died of illness and were cachectic (extremely thin) compared to moose that died of anthropogenic causes (e.g. vehicular collision). This is visualized with non-Metric Multidimensional Scaling (**Figure 1**) and is validated with a range of statistical tests used for discerning differences in treatment types for multivariate data (**Appendix C**). Figure 1 shows that when the community compositions of the rumen tissue and associated fluid are compared to one another, there is a clear, distinct grouping of communities based upon health status. Regardless of data input (presence/absence, relative abundance) or statistical analysis method (ANOSIM, PERMANOVA, mrpp), the same significant clustering of microbial communities was observed.

Additionally, statistical differences in the microbial community from healthy and sick moose were found in the feces, reticulum, and abomasum. While one mrpp did not provide for a significant correlation between fecal microbial communities and community health, there was a significant correlation in all ANOSIM and PERMANOVA analyses. This is key to note, as the use of PCR on non-invasively collected fecal samples could be a critical gauge to assess animal health in the wilderness.

Microbial community structure is based on membership (who is there) and diversity (evenness and richness). In rumen fluid, differences in the microbial community diversity are

measured by a Shannon Diversity index (**Figure 2a**). Here we show that diversity measurements from rumen fluid is higher in healthy moose and decreases in unhealthy moose, a trend not as readily observed in relation to community richness. Because the difference in richness (e.g. number of taxa detected) is not statistically different (**Figure 2b**), we infer that this decrease in diversity is largely driven by a decrease in the evenness of the community. Rank abundance curves of the rumen fluid show that sick moose possess more dominant bacterial members and are less even than that of healthy moose (**Figure 3**). Also, this analysis showed that specific bacteria affiliated with the Bacteroidetes phylum and BS11 genera(s) were most enriched in the rumen from sick moose.

Analyses of microbial membership demonstrated that on average, the Firmicutes phylum was lower in abundance in sick moose while the Bacteroidetes phylum was much higher. Although controversial, these results have also been observed in human feces on higher fiber diets (Marchesi *et al.* 2015, David *et al.* 2014, and Ley *et al.* 2005). Another investigation suggests that among elderly humans, Bacteroidetes were prevalent in higher proportions for those staying in long-stay care environments while Firmicutes were more common for those living in the community (Claesson *et al* 2011). Additionally, there was a positive correlation between Bacteroidetes-enriched humans and frailty (Claesson *et al* 2012). At an OTU level, we found in our study that this difference in phylum level changes is largely driven by one OTU from an uncultivated family of the Bacteroidetes, the BS11 gut group. This OTU was the most dominant member (13.6% ± 7.0) in 5 wild and captive moose that were extremely thin and/or died of illness (**Figure 4**). In contrast, this OTU was not enriched in 5 healthy wild Minnesota moose that died from vehicle collisions (1.7%± 1.8). BS11 enrichment was confined to rumen fluid and reticulum tissue, as tissues from the rumen, omasum, abomasum, small intestine, or colon, or in feces were not significantly higher than in healthy moose.

To explore this further, we looked at the abundance of BS11 across other ongoing moose studies in the lab and found that BS11 is abundant in sick Columbus Zoo moose and Alaskan moose consuming a more indigestible, high lignocellulosic diet (**Figure 4**). The relative abundance of BS11 in rumen fluids (**Figure 4**) was independent of host geography, season, captivity status, diet, age, and sex but was higher with more complex dietary carbon (Alaska winter) or disease (wild MN and Columbus Zoo). At this time, we cannot explain why BS11 is enriched in unhealthy animals but this could reflect the altered diet of these hosts or cellular resistance of BS11 to rumen acidosis or other factors caused by illness. However, given rapid declines in moose populations across North America, these findings suggest BS11 in rumen fluids could serve as a proxy for health status in wild moose and may be related to the effects of changing climate.

To determine what role BS11 may play in rumen metabolism, we explored other environments where BS11 was found. BS11 is almost always host associated or originating from rumen fluid. Our same dominant BS11 OTU was detected in rumen fluid from arctic reindeer, elk, white tailed deer, and muskoxen as well as yak, cow, camel, and sheep. A recent microbial consensus of rumen fluids from 32 different animal species revealed that a single BS11 OTU was the fifth most abundant microbe and prevalent in 94% of the ruminants (Henderson *et al.* 2015). Although prevalent and abundant in ruminants, these organisms are uncultured and lack genomic sampling. However, this research contributed to the first genomes sampled in this Bacteroidetes family, and we can now infer that BS11 ferment hemicellulose sugars in the

rumen and other mammalian gut. Together these findings show that moose with disease and/or poor body condition score (thin or very thin) are enriched in certain microbial taxa that are either responding or contributing to these stressors.

To generate data to support hypothesis three, we examined if the change in the microbial community resulted in less SCFA production (i.e. less inferred energy for the moose) by measuring the organic acid content in the rumen liquor. SCFA (which are organic acids of lactate, butyrate, and acetate) serve as good indicators of metabolic activity because they are the by-products of the microbial fermentation of complex plant polysaccharides that are consumed by moose. It is estimated that ruminants depend upon SFCA for up to 80% of the energy required for maintenance processes (Bergman 1990). There were statistical differences in the concentrations of acetate and propionate with healthy moose possessing higher concentrations than sick moose (**Figure 5**).

These significant differences in SCFA concentrations could be a result of the microbial community of sick moose having less metabolic potential to degrade complex polysaccharides; however, we only have a snap shot of the 16S rRNA gene of these microbial communities so it could contain the same metabolic potential with different members. Another possibility is that the sick moose are better equipped to absorb SCFA in a state of illness to address higher maintenance needs; however, this is unlikely because acidodic rumen tissue is likely damaged and is less able to absorb SCFA (Hofmann and Nygren 1992). Furthermore, this decrease in SCFA could reflect decreased nutritional quality in the plants consumed in Minnesota leading to increased susceptibility to disease. Future investigations into the metabolic linkages between the microbiome, plant nutrients, climate change, and ruminant health are required to identify the cause of declining moose populations.

There are a few limitations to this study that should be acknowledged. First, the n-size of the study is small; however, the limitation in regard to sample size is primarily constrained by the fact that these rare samples were opportunistically collected from wild animals and falls within the range of other large animal wildlife based studies (Grogan *et al.* 2014). Secondly, the distinction used by sample collectors to designate healthy and sick moose is not yet resolved, so we cannot yet correlate our findings to detailed analyses on specific health conditions. Finally, this biological survey of wild Minnesota moose and Columbus Zoo moose could not account for the behavior of the examined moose *ante mortem* although this study is one of the first of its kind to begin teasing apart the role the rumen microbiome plays in wild animal health.

Conclusion

Declines in moose populations across the northern United States have prompted researchers to consider any and all potential avenues in hopes of ascertaining a mitigation plan. This 16S rRNA gene study of the moose GIT, rumen fluid, and feces provides the first look into microbial signatures in the declining populations of wild moose. We have established that the microbial community is distinct in the rumen fluid and rumen tissue of moose that died of illness and that certain microbes (e.g. BS11 taxa) are enriched in the rumen of moose with poor nutritional status. These structural and taxonomic differences between the healthy and cachectic moose were correlated with significantly lower SCFA detected in the rumen liquor. BS11 was primarily confined to rumen fluid and not found in GIT tissues suggesting that members of the BS11 represent dormant metabolic potential that conditionally respond to nutritional, environmental, or health related stressors. Furthermore, the general consensus of statistical analyses for fecal communities suggests that there is a correlation between fecal community structure and host health index. This has important implications in the non-invasive use of feces to monitor animal health. These findings contributed significantly to a publication describing the hemicellulosic metabolism of BS11 taxa in the rumen fluid (Solden *et al.* 2016), which may be vital to wild herbivore adaptation in a rapidly changing world.

Acknowledgements

I would like to truly thank my research advisor, Dr. Kelly Wrighton, and my graduate student mentor, Lindsey Solden, for all of their assistance with my honors thesis. Without them, this project would not have been possible. A special thanks should be extended to Richard Wolfe and Rebecca Daly for their assistance and guidance on this project as well. Additionally, I would like to thank all of the research and sample collection collaborators our lab worked with both in Minnesota and at the Columbus zoo. In particular, I thank Drs. Barb Wolfe (Columbus Zoo) and Michelle Carstensen (MNDNR) for providing me access to samples.

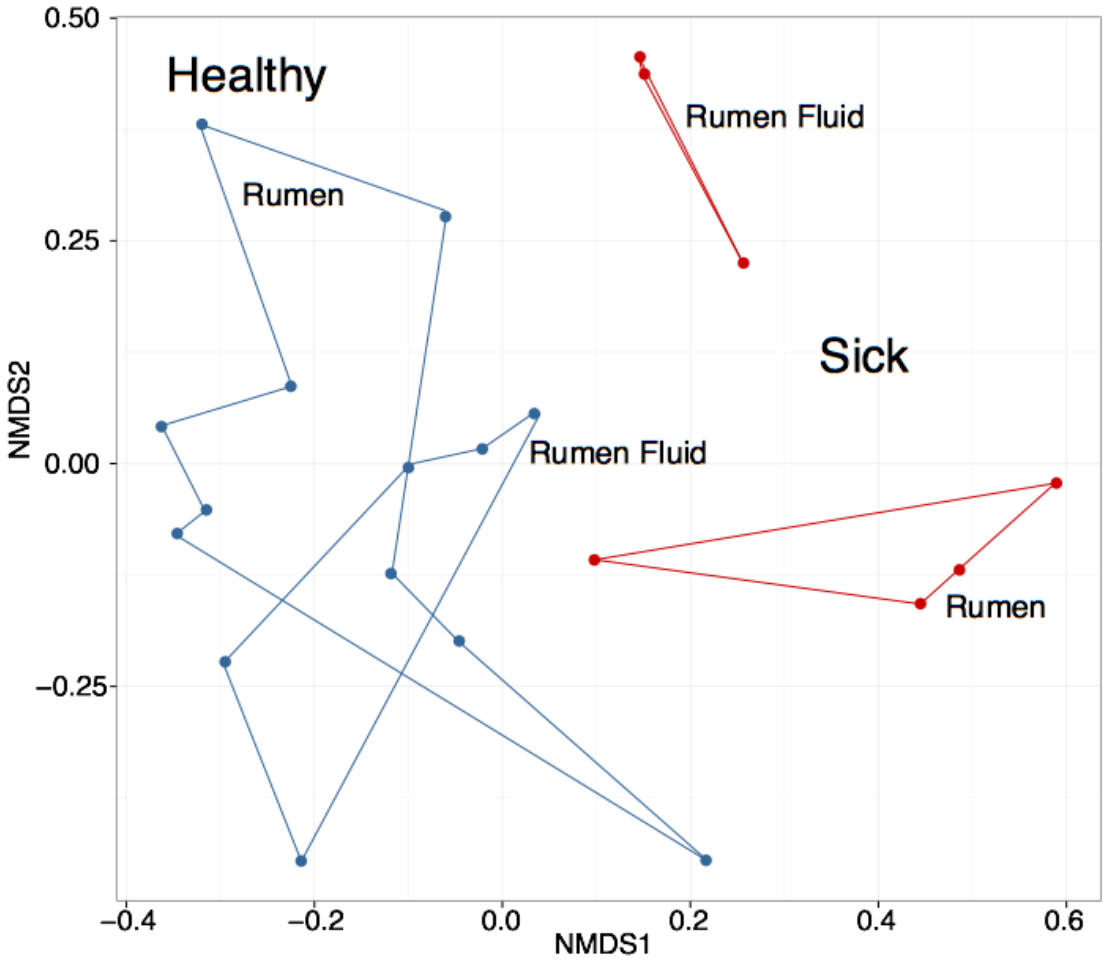


Figure 1. Rumen and Rumen Fluid Microbial Communities are both statistically discernable by health status at time of death. Community structures are also differentiable by tissue sample versus fluid from the rumen. Statistical significances were calculated using PERMANOVA with the following generation of p-values: $p > 0.011$ for health condition factor in rumen fluid, $p > 0.006$ for health condition factor in rumen, and $p > 0.001$ for differentiability based upon location of sample in G.I. tract. An mrpp through R additionally yielded a delta of 0.001.

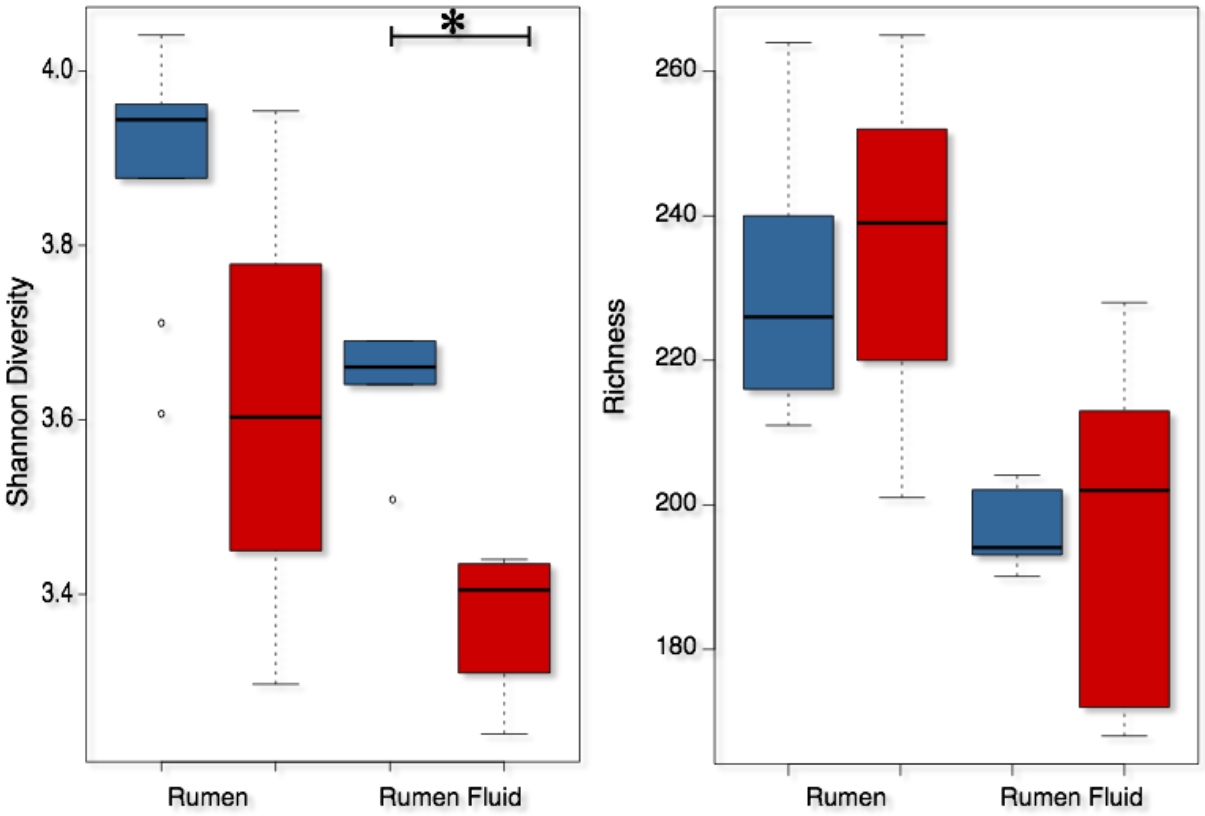


Figure 2. Shannon Diversity in Rumen Fluid is statistically higher in moose that died from vehicle collisions (blue) than moose that died of health related illness (red). The rumen fluid of sick moose was statistically less diverse than that of healthy moose, which was consistent with the observed trend: healthy systems appear to be more diverse in the sense of evenness but are less rich than sick systems.

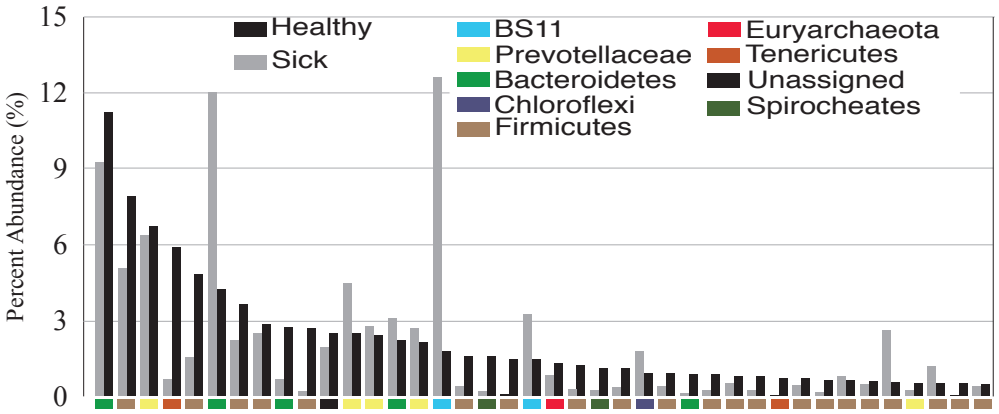


Figure 3. Key taxa are enriched in sick moose. Rank abundance of the top 40 OTUs in rumen fluid from healthy moose (black) and the corresponding abundance of the same OTUs in sick moose (gray). Colored boxes on the x-axis indicate taxonomic assignments. While this figure is only a snap shot of the top 40 OTUS and does not reflect the holistic diversity of Bacteroidetes and Firmicutes in samples, it does show the predominance of BS11 in sick moose.

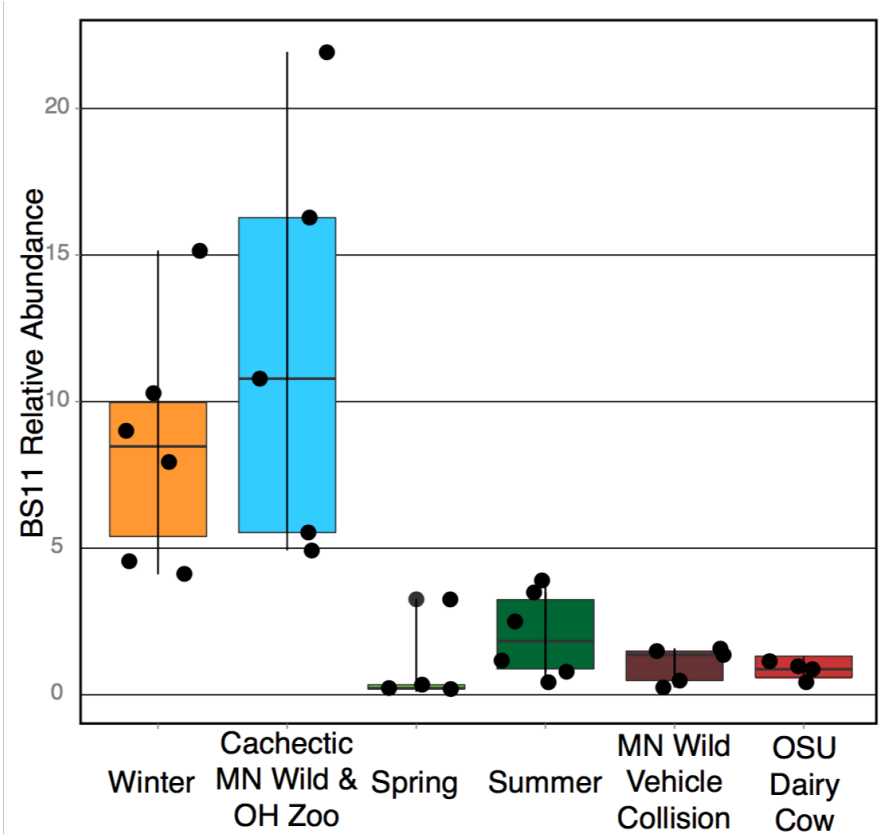


Figure 4. BS11 relative abundances are considerably higher in Cachectic moose than moose that died due to anthropogenic reasons. An mrpp through R resulted in p = 0.001 when comparing the relative abundances of BS11 in cachectic Minnesota wild and Ohio zoo moose with Minnesota wild that were involved in vehicle collisions potentially alluding to an indicator organism.

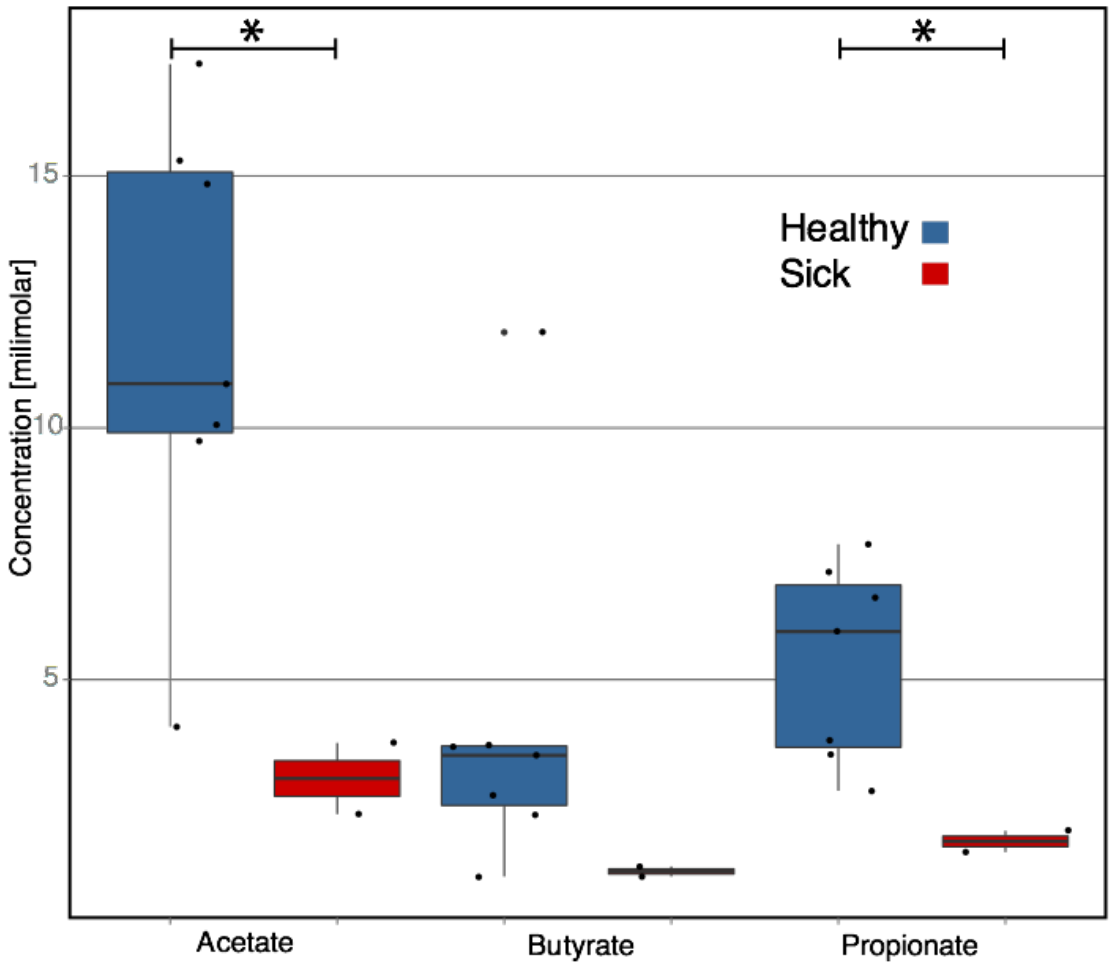
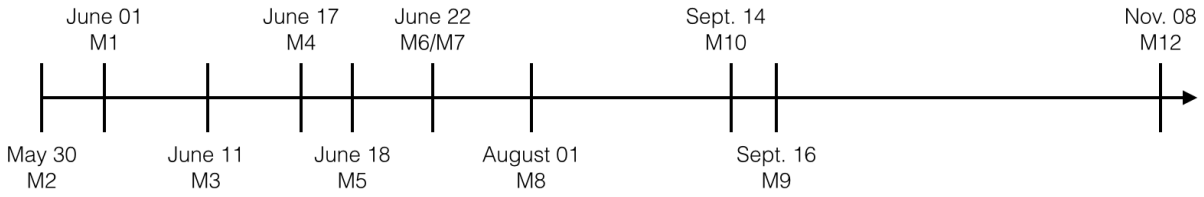


Figure 5. SCFA analyses of all Rumen Fluid Samples reveals a statistical difference between the concentrations of acetate and propionate detected in the rumen fluid from healthy and sick wild moose. Concentrations of SCFA were determined with HPLC, and statistical significances were derived from two-tailed t-tests of all concentrations based upon health condition: $p = 0.034$ for acetate, $p = 0.044$ for propionate, and $p = 0.303$ for butyrate.

Appendix A. Metadata for Moose Subjects

Moose Lab ID	Moose Collection ID	Location	Gender	Age	Cause of Death	Presence of Flukes	Body Condition
M1	015DEAD	Minnesota	Female	Adult	Sickness		Very Thin
M2	053114MC	Minnesota	Female	Yearling	Vehicle		Normal
M3	061114DCD	Minnesota	Female	Adult	Vehicle		Normal
M4	061814MD	Minnesota	Male	Adult	Vehicle	Major Liver Fluke	Normal
M5	061814LO	Minnesota	Male	Adult	Vehicle	Minor Liver Fluke	
M6	062214DP1	Minnesota	Female	Adult	Vehicle		Normal
M7	062214DP2	Minnesota	Male	Yearling	Vehicle		Thin
M8	080114DCP	Minnesota	Male	Yearling	Vehicle		Normal
M9	0151DEAD	Minnesota	Male	Adult	Sickness		Thin
M10	091414BL	Minnesota	Male	Adult	Vehicle		Normal
M11	112014EH	Minnesota			Vehicle		
M12	076DEAD	Minnesota	Female	Adult	Sickness		Normal

Appendix B: Timeline for Recorded Moose Deaths in 2014



Appendix C: Table of Statistical Analyses and Results

Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
ANOSIM - ALL Wild	0.001	0.311	Origin of sample	6-10-15	Communities cluster by location in GI tract	
ANOSIM - ALL Wild	0.001	0.458	Intestinal Region	6-10-15	Communities are more similar among closer organs than further ones	
ANOSIM - Wild Rumen	0.994	-0.272	Age	6-10-15	Communities do not cluster by age	
ANOSIM - Wild Rumen	0.907	-0.104	Gender	6-10-15	Communities do not cluster by gender	
ANOSIM - Wild Rumen	0.078	0.439	Body Condition	6-10-15	Communities do not cluster by body type	

Appendix C: Table of Statistical Analyses and Results

Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
ANOSIM - Wild Rumen	0.004	0.705	Cause of Death	6-10-15	Communities do cluster by cause of death	
ANOSIM - ALL Columbus	0.001	0.863	Origin of sample	6-10-15	Communities cluster by location in GI tract	Most samples are feces or rumen fluid.
ANOSIM - ALL Columbus	0.001	0.534	Intestinal Region	6-10-15	Communities are more similar among closer organs than further ones	
ANOSIM - ALL Moose	0.001	0.350	Origin of sample	6-17-15	Communities cluster by location in GI tract	
ANOSIM - ALL Moose	0.001	0.142	Cause of Death	6-17-15	Communities do cluster by cause of death	Train kill and road kill were considered separate factor categories
ANOSIM - ALL Moose	0.001	0.155	Cause of Death	6-17-15	Communities do cluster by cause of death	Analysis of Death by Vehicle (DBV) vs. Sick
ANOSIM Two-Way Nested - ALL Moose	0.001	0.385	Cause of Death across all Origins	6-17-15	Communities do cluster by cause of death when effect of Origin is considered	

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Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
ANOSIM Two-Way Nested - ALL Moose	0.005	0.456	Origin of sample using Cause of Death groups as samples	6-17-15	Communities do cluster by location in the GI tract among both sick and DBV groups	
ANOSIM - Wild Rumen Fluid	0.400	0.111	Season of Death	6-17-15	Communities do not cluster based upon season of death	
ANOSIM - ALL Moose	0.001	0.137	Moose identity	6-17-15	Communities do cluster by moose	The R-value is incredibly low.
ANOSIM - ALL Feces	0.003	0.301	Cause of Death	6-17-15	Communities do cluster by cause of death	
ANOSIM - ALL Rumen	0.012	0.720	Cause of Death	6-17-15	Communities do cluster by cause of death	
ANOSIM - ALL Omassum	0.012	0.720	Cause of Death	6-17-15	Communities do cluster by cause of death	
ANOSIM - ALL Abomasum	0.017	0.595	Cause of Death	6-17-15	Communities do cluster by cause of death	
ANOSIM - ALL Rumen Fluid	0.008	0.563	Cause of Death	6-17-15	Communities do cluster by cause of death	
ANOSIM - ALL Feces	0.001	0.301	Cause of Death	6-17-15	Communities do cluster by cause of death	

Appendix C: Table of Statistical Analyses and Results

Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
PERMANOVA - ALL Feces	0.003	3.4102	Moose identity	6-18-15	Communities do cluster by moose	
PERMANOVA - ALL Feces	0.016	4.3327	Cause of Death	6-18-15	Communities do cluster by cause of death	
PERMANOVA - ALL Rumen Fluid	0.008	3.1479	Cause of Death	6-18-15	Communities do cluster by cause of death	
PERMANOVA - ALL Moose	0.001	11.984	Cause of Death	6-18-15	Communities do cluster by cause of death	This test was ran concurrently with Moose identity and Origin of sample
PERMANOVA - ALL Moose	0.001	12.624	Origin of sample	6-18-15	Communities cluster by location in GI tract	This test was ran concurrently with Cause of Death and Moose identity
PERMANOVA - ALL Moose	0.001	4.8429	Moose identity	6-18-15	Communities do cluster by moose	This test was ran concurrently with Cause of Death and Origin of sample
PERMDISP - ALL Moose	0.016	4.5493	Origin of sample	6-18-15	Communities are distinct by location in GI tract	
PERMDISP - ALL Moose	0.083	4.2757	Cause of Death	6-18-15	Communities are distinct by cause of death	

Appendix C: Table of Statistical Analyses and Results

Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
PERMDISP - ALL Moose	0.941	0.74847	Moose identity	6-18-15	Communities are not distinct by moose	
PERMANOVA Nested - ALL Moose	0.034	2.552	Cause of Death nested in Moose Identity	6-18-15	Communities do cluster by cause of death	
PERMANOVA Nested - ALL Moose	0.001	3.1286	Cause of Death nested in Origin of Sample	6-18-15	Communities do cluster by cause of death	
PERMANOVA Nested - ALL Rumen Fluid	0.004	3.2814	Cause of Death	6-24-15	Communities do cluster by cause of death	
PERMANOVA Two-Way Pair-Wise, ALL Moose	A: 0.005	A: 1.5847	Cause of Death vs. Origin of Sample	6-25-15	Communities do cluster by cause of death in abomasum	
PERMANOVA Two-Way Pair-Wise, ALL Moose	F: 0.001	F: 2.4496	Cause of Death vs. Origin of Sample	6-26-15	Communities do cluster by cause of death in feces	
PERMANOVA Two-Way Pair-Wise, ALL Moose	O: 0.087	O: 1.2949	Cause of Death vs. Origin of Sample	6-27-15	Communities do not cluster by cause of death in omasum	
PERMANOVA Two-Way Pair-Wise, ALL Moose	C: 0.057	C: 1.6378	Cause of Death vs. Origin of Sample	6-28-15	Communities do not cluster by cause of death in colon	

Appendix C: Table of Statistical Analyses and Results

Test	P-Value	F/T/R Value	Factor	Date	Interpretation	Notes
PERMANOVA Two-Way Pair-Wise, ALL Moose	RE: 0.019	RE: 1.5029	Cause of Death vs. Origin of Sample	6-29-15	Communities do cluster by cause of death in reticulum	
PERMANOVA Two-Way Pair-Wise, ALL Moose	RF: 0.011	RF: 1.6813	Cause of Death vs. Origin of Sample	6-30-15	Communities do cluster by cause of death in rumen fluid	
PERMANOVA Two-Way Pair-Wise, ALL Moose	R: 0.006	R: 1.6911	Cause of Death vs. Origin of Sample	7-1-15	Communities do cluster by cause of death in rumen	
PERMANOVA Two-Way Pair-Wise, ALL Moose	SI: 0.565	SI: 0.94939	Cause of Death vs. Origin of Sample	7-2-15	Communities do not cluster by cause of death in small intestines	

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