EFFECTS OF PATHOGENIC *ESCHERICHIA COLI* AND *SALMONELLA* ON THE INTESTINAL MICROBIOTA OF WHITE-TAILED DEER AND CATTLE SHARING AN AGROECOSYSTEM

By

Maria Lisette Delgado Aquije

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Fisheries and Wildlife - Master of Science

2015

ABSTRACT

EFFECTS OF PATHOGENIC *ESCHERICHIA COLI* AND *SALMONELLA* ON THE INTESTINAL MICROBIOTA OF WHITE-TAILED DEER AND CATTLE SHARING AN AGROECOSYSTEM

By

Maria Lisette Delgado Aquije

Intestinal microbiota fulfill important functions that contributed to their host´s health, and can be affected by the presence of pathogens. White-tailed deer and cattle share pastures in many agro-ecosystems, and have been reported to share strains of both pathogenic *E. coli* and *Salmonella*, suggesting direct interaction and interspecific transmission of microorganisms. However, there is a lack of knowledge of the composition of white-tailed deer intestinal microbiota, the effects of pathogenic *E. coli* and *Salmonella* to the microbiota, and the differences in composition and pathogen response between deer and cattle microbiota. This thesis first characterized the microbiota of 67 fecal samples of white-tailed deer using 16S rDNA pyrosequencing. White-tailed deer intestinal microbiota is composed mainly by Firmicutes and Proteobacteria. Results revealed the importance of seasonal variability in microbial composition, and suggested that enterohemorrhagic *E. coli* (EHEC) and *Salmonella* affect intestinal microbiota composition of white-tailed deer. The second chapter compared the microbiota of deer and cattle, and contrasted their response to EHEC. Results revealed that the main difference in microbiota composition between both species was the abundance of Proteobacteria (0.82% in cattle vs. 20.25% in deer). Comparison of microbial abundance in EHEC-positive vs. -negative in cattle did not show major differences; while, white-tailed deer microbiota differed in composition at both phyla and genera level. Results demonstrate a different core microbiota between cattle and deer that shared an environment, and a different response to the presence of the EHEC.

I dedicate this thesis to my family.
A special feeling to my parents José and María, for their love and continue encouragement to follow my goals.
My brothers José and Eduardo for always being by my side.
My grandparents Victoria, Estela, Jesús and José, for their love, supported and example of perseverance.

ACKNOWLEDGMENTS

First, I would like to acknowledge and thank my committee members: Dr. Kim Scribner, Dr. Shannon Manning, and Dr. Jean Tsao, for their collaborations and support. Special thank you and gratitude to Dr. Kim Scribner for giving the opportunity to work in his laboratory, and for his patient guidance, enthusiastic encouragement and useful critiques during the development of this work. And Dr. Shannon Manning for welcoming to her laboratory where the experimental procedures and analysis were developed, and for her valuable suggestions that allow the realization of this project.

I also acknowledge and thank Dr. Pallavi Singh for her time, guidance, and substantial contribution in pathogen detection, microbial DNA sequencing and data analysis.

I thank Drs. Julie Funk, Qiong Sha, David W. Lacher, Jacquelyn Del_Valle, Rebekah E. Mosci, Vilma Yuzbasiyan-Gurkanm, Daniel Grooms, Dr. Paul Barlett, Steven Rust, Cristina Venegas Vargas, Lindsey Ouellette, Scott Henderson, Akanksha Khare, Johnathan Lehnert for sample collection, preparations and pathogens detection; as well as, Dr. Jennifer Moore, for white-tailed deer relatedness analysis; and Emily Cannell and Dr. Jeannette Kanesfsy for white-tailed deer genotyping and sexing.

I would like to thank USDA (2011-67005-30004) and the W.K. Kellogg Foundation for their financial support that allow the realization of this project. Also, to the Fulbright commission from Peru and the Office for International Students and Scholars (OISS) that financed my master studies.

Finally I wish to thank my family, friends from Peru, and my new friends from Michigan for their advice, feedback and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

KEY OF ABBREVIATIONS

- A: adenine
- ANOSIM: analysis of similarities
- bfp: bundle forming pilus
- C: cytosine
- CFU: Colony Forming Unit
- DNA: deoxyribonucleic acid
- eae: intimin adhesion
- EHEC: enterohemorrhagic *Escherichia coli*
- EPEC: enteropathogenic *Escherichia coli*
- G: guanine
- GI: gastrointestinal
- GPS: Global Positioning System
- KBS: Kellogg Biological Station
- n: sample size
- ng: nanogram
- OTU: Operational Taxonomic Unit
- PCOA: principal coordinate analysis
- PCR: Polymerase Chain Reaction
- qPCR: quantitative PCR
- rDNA: ribosomal DNA
- RV: Rappaport-Vassilids broth
- STEC: Shiga toxin-producing *Escherichia coli*

- stx: shiga toxin gene
- T: thymine
- TTB: Tetrathionate broth
- ul: microliter

CHAPTER 1

Characterization of white-tailed deer fecal microbiota and comparisons between pathogenic *Escherichia coli* and *Salmonella* carriers and non-carriers

1.1 ABSTRACT

Enteric pathogens like Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* are an economic and public health burden. Recent studies have drawn attention to the importance of gastrointestinal (GI) microbiota to probabilities of acquisition and transmission of these enteric pathogens. Studies on humans and model animals have reported that pathogens can in turn affect the diversity and composition of GI microbiota. However dynamic interactions between hosts, microbiota, and pathogens are not well studied, particularly in wildlife hosts which are important pathogen reservoirs. White-tailed deer (*Odocoileus virginianus*) is one species known to transmit STEC and *Salmonella* through their feces. Little is known about this species´ GI microbial community or pertaining to factors that influence GI microbiota composition. Analyses of 67 fecal samples using 16S rDNA pyrosequencing revealed that 17 bacterial phyla were present in white-tailed deer feces. Firmicutes (55.26%) was the predominate phylum, followed by Proteobacteria (20.25%) and Bacteroidetes (17.45%). Comparative analysis showed significant differences (ANOSIM: R=0.211, p-value<0.001) in community composition between samples collected during different sampling periods (March vs. June). Temporal variation in the abundance of Proteobacteria was particularly notable (6% vs. 32%, respectively). Microbiota composition did not vary significantly between sexes or among deer of different levels of genetic relatedness, or as a function of presence of enteropathogenic *E. coli* (EPEC),

Shiga-toxin-producing *E.coli* (STEC), and enterohemorrhagic *E. coli* (EHEC). However intestinal community composition varied between EHEC-positive and EHEC-negative individuals. Microbial community Shannon alpha diversity indices were low in *Salmonella* infected individuals. Microbial composition also differed between samples with and without *Salmonella*. This study revealed the importance of seasonal variability in microbial composition which is likely attributed to changes in diet, and suggested that pathogens such as EHEC and *Salmonella* affect intestinal microbiota composition in white-tailed deer inhabiting agricultural ecosystems.

1.2 INTRODUCTION

Encroachment into wild habitats due to human activities like agriculture, has increased area cohabited by humans, domestic animals, and wildlife. Concurrent occupancy of agroecosystems promote interactions between pathogens, vectors and hosts, and the spread of infectious diseases (Patz *et al*. 2004; Shov *et al*. 2008; Ferens *et al*. 2011; Mentaberre *et al*. 2013; Jones *et al*. 2013). Among these diseases, foodborne diseases account for 76 million illnesses and 5200 deaths annually in the United States (CDC, 2003). Two of the most common foodborne pathogens are: Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* (Callaway *et al*. 2013; Hernandez-Reyes and Schikora 2013). In the United States, STEC causes 265000 infections, 31 deaths (Franklin *et al*. 2013), and cost $1 billion each year (Callaway *et al*. 2013); while *Salmonella* causes 1.2 million illness, approximately 400 deaths (CDC, 2010), and costs are estimated to be higher than $365 million per year (CDC, 2011).

Wildlife play an important role in the transmission of enteric pathogens and are considered amplifier hosts, because they may allow pathogens to evolve and spread to humans (Daniels *et al*. 2013; Lillehaug *et al*. 2005; Jones *et al*. 2013). Studies designed to understand spatial and temporal variation on pathogen prevalence have primarily focused on evolutionary, environmental, behavioral and social factors of the host. However, there are biological factors within the host, including the microbial communities of the gastrointestinal (GI) tract that may also impact the probability of acquisition and transmission of pathogens (Chambers and Gong, 2011). Due to this and other properties of the GI microbiota, there has been increased interest in studies of microbial communities among different species.

Advances in sequencing technology have helped to overcome some of the difficulties associated with microbiological culture techniques, allowing researchers to gain insight into the diversity of microorganisms that live within different hosts (Dahllof, 2002; Carroll *et al*. 2012; Aidy *et al.* 2013). Studies of the GI microbiota have established the important functions in host metabolism, nutrient acquisition, and immune response (Aidy *et al*. 2013). Changes in normal GI microbiota composition can lead to diseases such as colitis or ruminal acidosis (Costa *et al*. 2012; Lettat *et al*. 2012). Intestinal microbiota composition has also been correlated with shedding rates of pathogens like STEC in cattle (Zhao *et al*. 2013) and *Salmonella* in pigs (Bearson *et al*. 2013).

GI microbiota composition may be affected by factors such as diet, antibiotic consumption, and pathogens (Gu *et al*. 2013; Kamada *et al*. 2013). Diet affects the

microbiota by modifying GI tract environmental conditions including pH, temperature, motility, and oxygen level (Gu *et al.* 2013). Biotic factors including presence or absence of pathogens may also alter the composition and abundance of commensal microorganisms (Kamada *et al*. 2013). Although considerable attention has been focused on processes associated with acquisition and compositional change of microbiota in humans and some domestic animals, wildlife species are understudied. Furthermore, researchers lack understanding of the role of the microbiota in the shedding of pathogens, and the effect of the pathogen colonization on the microbiota composition.

White-tailed deer (*Odocoileus virginianus*) has been reported to be a reservoir for pathogenic *E. coli* and *Salmonella* (Renter *et al*. 2001; Renter *et al.* 2006; Braham *et al*. 2005; Singh *et al*. 2015). A previous study at Kellogg Biological Station (KBS) in Michigan (Singh *et al*. 2015) showed that the prevalence of pathogenic *E. coli* including Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) were 1%, 6%, and 22% in deer respectively. Prevalence of *Salmonella* (Typhimurium and Newport strains) was 13% (personal communication, J. Funk). Although low in prevalence, STEC and *Salmonella* outbreaks due to contact with deer feces have been reported and studies have shown that strains of both pathogens are shared with cattle (Branham *et al*. 2005; Renter *et al*. 2006; Singh *et al*. 2015). Furthermore in the case of STEC, quantitative PCR (qPCR) has demonstrated a high prevalence of phage-encoded Shiga toxin genes *stx*1 (10%) and *stx*2 (46%) in

deer in Pennsylvania, suggesting that white-tailed deer are also an important reservoir for *stx* genes that are carried on lambdoid bacteriophages (Kistler *et al*. 2011).

Given the importance of white-tailed deer in the transmission of pathogens like STEC and *Salmonella*, it is important to know the effect of these pathogens on the intestinal microbiota. White-tailed deer microbial communities and the factors that influence community composition are largely unknown. There are a few studies that have characterized microbial diversity in other cervids like roe deer (*Capreolus pygargus*) (Li *et al*. 2014), sika deer *(Cervus nippon*) (Li *et al*. 2013), and reindeer (*Rangifer tarandus tarandus*) (Sundset *et al.* 2009), but to my knowledge only one study by Gruninger *et al.* (2014) has characterized the rumen microbiota of white-tailed deer (n=3) using 16S rDNA sequencing methods. Results revealed genera that have not previously been described in domestic ruminants. No studies of the intestinal microbiota, however, have been conducted in white-tailed deer. Given that the diversity and abundance of the GI microbiota changes throughout the tract in other well studied organisms, and because fecal microbiota is more similar to the intestinal microbiota than stomach microbiota (Gu *et al*. 2013), we will described the microbiota as fecal or intestinal instead of gastrointestinal.

The first objective of this study was to characterize the microbial diversity and composition of fecal samples collected from white-tailed deer at the Kellogg Biological Station (KBS), using 16S rDNA pyrosequencing, and compare the microbial diversity and composition between samples as a function of sampling periods (March vs June),

gender, and levels of host genetic relatedness. The second objective was to determine the impact that the presence of pathogenic *E. coli* (STEC, EHEC, and EPEC) and *Salmonella* have on the intestinal communities of white-tailed.

1.3 METHODOLOGY

1.3.1 Study site

The study was conducted at Michigan State University's KBS, a field site for ecological and agricultural research located in Barry county, south central Michigan. The landscape at this site is characteristic of the upper Midwest regions of the United States. Different habitats within this location are highly interspersed and include agriculture fields consisting of corn, alfalfa and soybeans as well as dairy pastures, hardwood forests, wetlands, streams, and lakes.

1.3.2 Sample collection

Visually fresh samples of white-tailed deer (*Odocoileus virginianus*) feces were collected in March and June of 2012. A stratified random sample of transects were selected from forest and pasture locations, near water sources and near the pasture dairy center. Samples were collected in plastic bags while walking transects and were assigned an individual identification number. Geographic locations were referenced for all samples using a handheld GPS unit (Appendix 1). All samples were stored at -80° C after collection. Studies have shown that fresh feces samples can be kept at "room" temperature for up to 14 days without reducing the ability to quantify microbiota composition (Lauber *et al*., 2011; Caroll *et al*, 2012).

1.3.3 Extraction of white-tailed deer DNA

Extraction of genomic DNA from each feces sample was performed by using a QIAamp DNA Stool isolation kit (Qiagen; Valencia, CA). Eight fecal pellets from each individual sample were swabbed with a sterile swab on the pellet surface to obtain deer intestinal cells. The swab was submerged in ATL buffer, and then extraction steps were performed following the manufacturer´s instructions. Total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific).

1.3.4 Discrimination of deer individuals

Eight microsatellite loci IGFl (Kirkpatrick, 1992), OBCAM (Moore *et al*. 1992), Cervid1, Cervid2 (DeWoody *et al*. 1995), Rt7, RT9, Rt24, and Rt27 (Wilson *et al*. 1997) were used for the discrimination of individual deer (Grear *et al*. 2010). PCR conditions for each locus are shown in Appendix 2. Individual identification was determined by comparing the genotypes with the software Cervus 3.0 (Kalinwoski *et al*. 2007). Measures of inter-individual relatedness were determined as described by Goodnight and Queller (1999) using GeneAlex software (Peakall and Smouse 2006, 2012).

1.3.5 Determination of gender

Deer sex was determined genetically using the protocol described by Lindsay and Belant (2008). The primers used were: CerZFXYf: 5´-GCTGACCCTGGAGAAGATGACTTA and CerZFXYr: 5´TCATTCTCAGGCTCACTCTCCACA. The PCR conditions included an initial

denaturation step at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 35 seconds, annealing at 55°C for 30 seconds, and an extension at 72°C for 60 seconds. Positive and negative controls for male and female were used. Electrophoresis of the PCR products was carried out on a 1% agarose gel to determine gender as amplification of two bands indicated male, and one band indicated a female.

1.3.6 Determination of samples disease status

Pathogenic *Escherichia coli*: All deer fecal samples were cultivated at the MSU Microbial Evolution and Epidemiology Laboratory. A loop of deer feces was cultivated following enrichment in EC broth overnight at 37°C and subculture to CHROMoagar (CHROMagar; Paris). Single colonies were confirmed to be EHEC, STEC or EPEC by multiplex PCR targeting the intimin adhesion (*eae*), *stx1* and *stx2* was performed as described in Manning *et al.* (2008) followed by amplification of the bundle forming pilus (*bfp*), a common EPEC marker, was performed as described in Trabulsi *et al.* (2002). Isolates were classified as atypical EPEC if they were *eae*-positive and *stx*-negative, typical EPEC if they were *eae*- and *bfp*-positive, STEC if they were *stx*1 and/or *stx*2 positive, and EHEC if were *eae* and *stx*1 and/or *stx*2 positive. Confirmed isolates were stored at -80°C in glycerol stock.

Salmonella: White-tailed deer fecal samples were cultivated at the MSU Diagnostic Center for Population and Animal Health using standard protocols. Ten g of feces were diluted 1:10 in Tetrathionate Broth (TTB) and incubated at 37°C for 48 hours. An aliquot (100 µl) of the fecal-TTB solution was inoculated into 9.9 ml of Rappaport-Vassiliadis broth (RV) and incubated at 42°C for 24 hours. The RV broth was then

plated onto XLT4 agar and incubated at 37°C overnight. Colonies with typical morphology of *Salmonella* were biochemically confirmed and serotyped. All samples and *Salmonella* isolates were stored at –80°C.

1.3.7 Quantification of stx1 and stx2

Quantification of *stx*1 and *stx*2 in samples was determined following a qPCR protocol by Sharma and Dean-Nystrom (2003). PCR conditions were modified from the original protocol, and consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 20sec; and a melting curve. The standard Sakai was used and the standard curve included dilutions from 10^2 to 10^6 CFU/ml.

1.3.8 Extraction of intestinal microbial community DNA

Fecal pellets from each sample were mashed and homogenized. The extraction of the microbial communities was performed using QIAamp DNA stool kit (Qiagen; Valencia, CA) according to the manufacturer's protocol with slight modification of bead beating and denaturation at 95°C. In brief, 0.3g of the homogenized sample was added to tubes with beads to break open the bacterial cells initially. Total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific).

1.3.9 DNA quality verification

The amount of DNA degradation and the DNA quality was verified prior to sequencing by the amplification of the 16S ribosomal DNA (*rDNA*) gene for each sample. The

primers used were: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Lane, 1991). The PCR conditions consisted on an initial denaturation step at 95° C for 2 min, 30 cycles of denaturation at 15°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. Electrophoresis was performed on a 1% agarose gel to confirm amplification.

1.3.10 16S rDNA sequencing

Sixty seven samples out of 163 were selected based on research objectives and prepared for sequencing using specific 16S gene specific primers (sequence: CCGTCAATTCMTTTRAGT) linked to barcodes for multiplexing. A 3 ng/µl aliquot of each DNA sample was used for the PCR reaction, and each sample was amplified in triplicate along with a negative control. An AccuPrime taq kit (Invitrogen^{TM}) was used to amplify the 16S rDNA genes. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 5minutes; and a final extension at 72°C for 5 minutes. PCR reactions were carried out in triplicates so as to get adequate concentration and volume of the PCR product to further downstream. The triplicates of each sample were mixed together, and verified by electrophoresis. PCR products were quantified using a picogreen assay by Qubit© (Invitrogen) before and after the purification process. Finally, all samples were pooled in equimolar ratios based on DNA concentration. Pyrosequencing was performed using a 454 titanium flex sequencing kit on a Roche Junior sequencer.

1.3.11 Sequencing analysis

Sequences were analyzed using the software QIIME (Caporaso *et al*. 2010). First, all sequences were subjected to a quality control that included a noise reduction using the denoise_wrapper.py script, removal of short sequences and sequences with barcode mismatches. Then, unique sequences were used to align against the Greengenes reference database. All chimeras were detected and removed using Uchime (Edgar, 2011). Following quality control checking, any sample with less than 1000 sequences was not included in the downstream analysis. A distance matrix using 0.03% phylogenetic distances was performed to define the operational taxonomic units (OTU). Rarefaction curves were generated based on Shannon diversity index. Principal coordinate analysis (PCOA) analysis based on the Bray-Curtis dissimilarity index was used to visually compare microbial composition as a function of sampling period (March, June), gender (male, female), and presence of pathogen (STEC, EHEC, EPEC, and *Salmonella*). After the ANOSIM test was used to assess significant differences between variables with the compare_categories.py script. A non-parametric t-test was used to determine differences in OTU abundance between variables using the group_significance.py script. Finally, a pairwise matrix was used to determine the correlation of beta-diversity index and levels of genetic relatedness using a Mantel test (Mantel, 1967).

1.4 RESULTS

Thirty white-tailed deer fecal samples were collected in March, and 37 samples were collected in June of 2012. Of the 67 samples 26 were recovered from males and 41

were from females. White-tailed deer identification via microsatellite loci typing found that seven deer had samples collected in both March and June. The number of pathogen-positives feces evaluated in this study for EPEC, STEC and EHEC were six, three and 11, respectively. The 3 STEC positive were assigned as supper-shedders (cfu/gm > 1E+04) by qPCR. Four *Salmonella* positive samples were also included in this study.

1.4.1 Characterization of the white-tailed deer fecal microbiota

Among the 67 white-tailed deer fecal samples evaluated, a total of 17 microbial phyla were identified. The phylum Firmicutes was the most abundant (55.3%), followed by phyla Proteobacteria with 20.3%, and Bacteroidetes with 17.5% (Figure 1). Other phyla included: Acidobacteria, Chlorofexi, Cyanobacteria, Elusimicrobia, Fibrobacteria, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Planctomycetes, Spirochaetes, Synergistetes, Tenericutes, and Verrucomicrobia. Less than 1% of the sequences were unclassified at the phyla level.

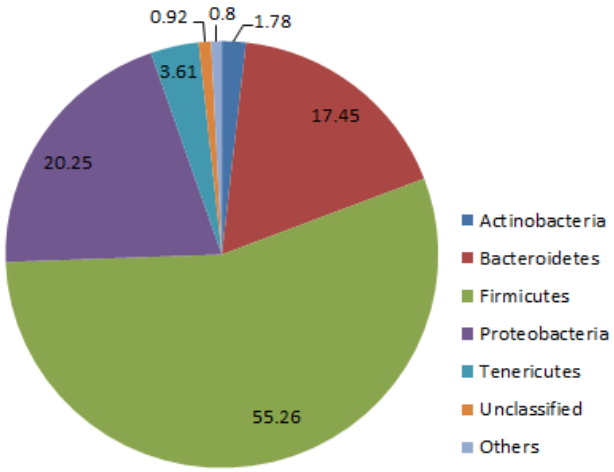

Figure 1. Taxonomic composition of fecal microbiota by phyla from 67 samples of white-tailed deer. Numbers indicate percent composition.

At the genus level, 307 genera were identified. Although the predominant (24.8%) genus could not be classified, it belonged to the Ruminococcaceae family (Figure 2). The four most abundant genera constituted 49% of the all genera.

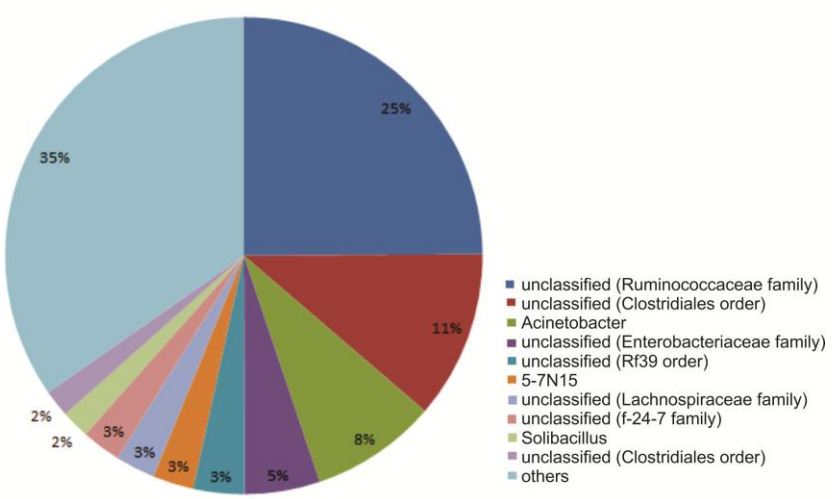

Figure 2. Taxonomic composition of fecal microbiota by genus from 67 samples of white-tailed deer. Numbers indicate the percent composition.

The Shannon alpha diversity indices for all samples ranged between 3.2 and 7.7. The alpha diversity rarefaction curve for both month and gender plateau, indicating that the sample size is sufficient to accurately estimate alpha diversity. Furthermore, the plots indicate that there is a difference in diversity between sampling times but not between genders (Figure 3). Samples collected in March show a higher diversity (Shannon average = 6.8) than samples collected in June (Shannon average = 5.7).

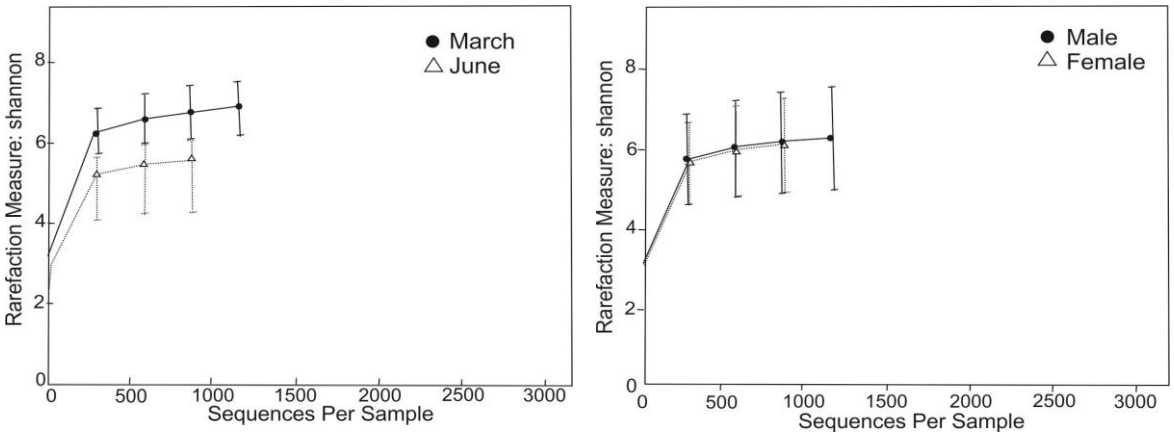

Figure 3. Rarefaction plots of Shannon alpha diversity indices of fecal microbiota by month of collection and gender of 67 white-tailed deer samples.

In addition, a principal coordinate analysis (PCOA) shows that microbial communities in samples collected in June are more disperse than samples collected in March (Figure 4). PCOA plots based on to gender did not show clustering (data not shown).

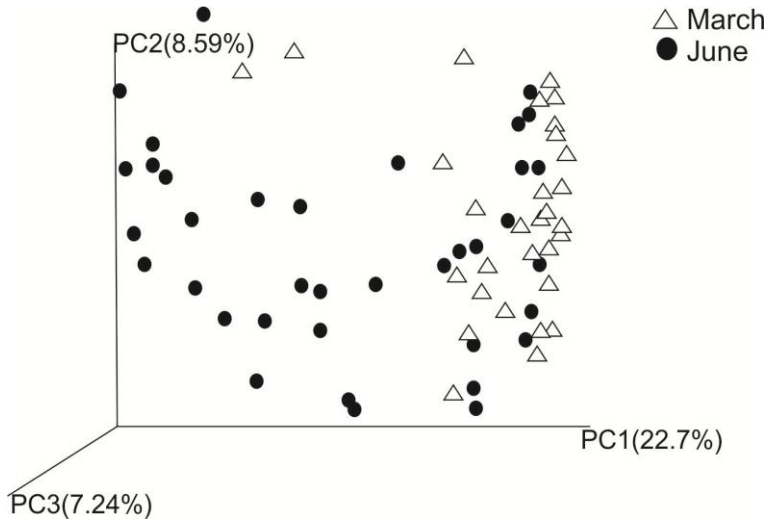

Figure 4. Principal coordinate analysis plot based on Bray-Curtis index of 67 fecal microbiota samples. Triangles are samples from March and black dots represent samples from June

Analysis of similarities (ANOSIM) also revealed significant differences in microbiota composition between the March and June samples (R= 0.211, p<0.001). To better understand which microbiota may be responsible for the differences observed in these analyses, we examined the composition and abundance of microbes at both time points. Notably, the March samples included higher percentages of Firmicutes (66% in March vs. 46% in June), while the June samples were characterized by higher percentages of Proteobacteria (6% in March vs 32% in June) (Figure 5). Bacteroidetes did not differ by more than 1% over the two time points, and some phyla were only found in one sampling point. The phylum Gemmatimonadetes, for instance, was only found in March, whereas the phylum Fusobacteria was only found in June, though the abundance of each was less than 1%. A non-parametric test was used to compare OTU abundance between sampling periods and 179 OTU differ significantly between March and June (p-value < 0.05).

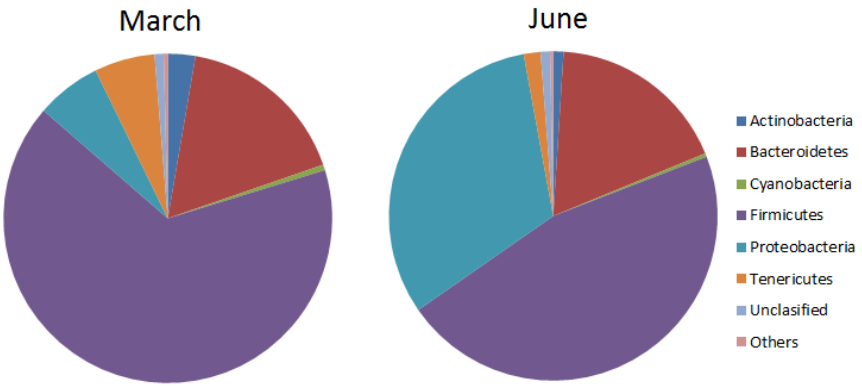

Figure 5. Taxonomic composition of fecal microbiota by phyla in samples from two sampling periods (March and June).

By contrast, theANOSIM analysis comparing microbial communities across genders was not significant (R= 0.001, p>0.05). A Mantel test showed no significant correlation between beta-diversity (Bray-Curtis index) between samples and inter-individual

genetic relatedness ($p>0.05$). These analyses were also performed separately by sampling point, but no association was found in either month ($p>0.05$, data not shown).

1.4.2 Effect of pathogenic E. coli and Salmonella in deer's microbiota

A total of 20 fecal samples examined through this study were also culture positive for pathogenic *E.coli*: EPEC (n=6), STEC (n=3), and EHEC (n=11). qPCR analysis targeting the *stx1* and *stx2* genes in the 15 STEC- and –EHEC positive samples confirmed positivity. From the 67 samples collected, 34.3% were positive for *stx1* and *stx2* genes by qPCR, where 23.9% were classified as super-shedders (CFU/g > 10^3) and 10.4% as moderate-shedders (CFU/g >10^2).

Shannon diversity rarefaction plots show no difference in microbial community diversity between STEC, super-shedders STEC, and EHEC carriers and non-carriers (Figure 6). However, EPEC appear to have an effect on microbial diversity, where EPEC positive samples were less diverse (Figure 6).

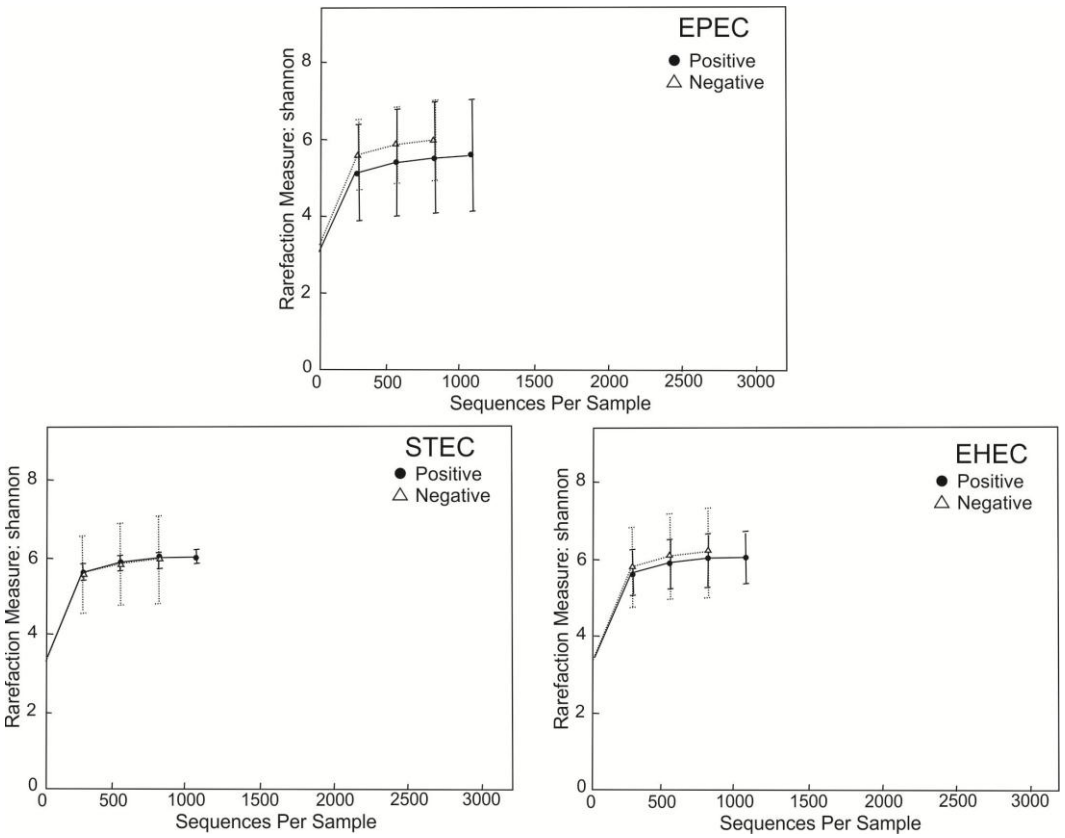

Figure 6. Rarefaction plots of Shannon alpha diversity index by white-tailed deer samples that were culture-positive for EPEC, STEC, and EHEC relative to culture-negative samples.

In order to better understand the microbiota differences between deer with EPEC relative to deer that lacked the presence of a pathogen, the microbial composition was examined at the phyla and genera levels from one sampling time (June). A modest difference in the abundance of phyla Firmicutes and Bacteroidetes was observed in the EPEC-negative animals relative to the positive animals (Figure 7). Nonetheless, the ANOSIM test revealed no significance difference in community composition between EPEC positive and negative deer ($p>0.05$).

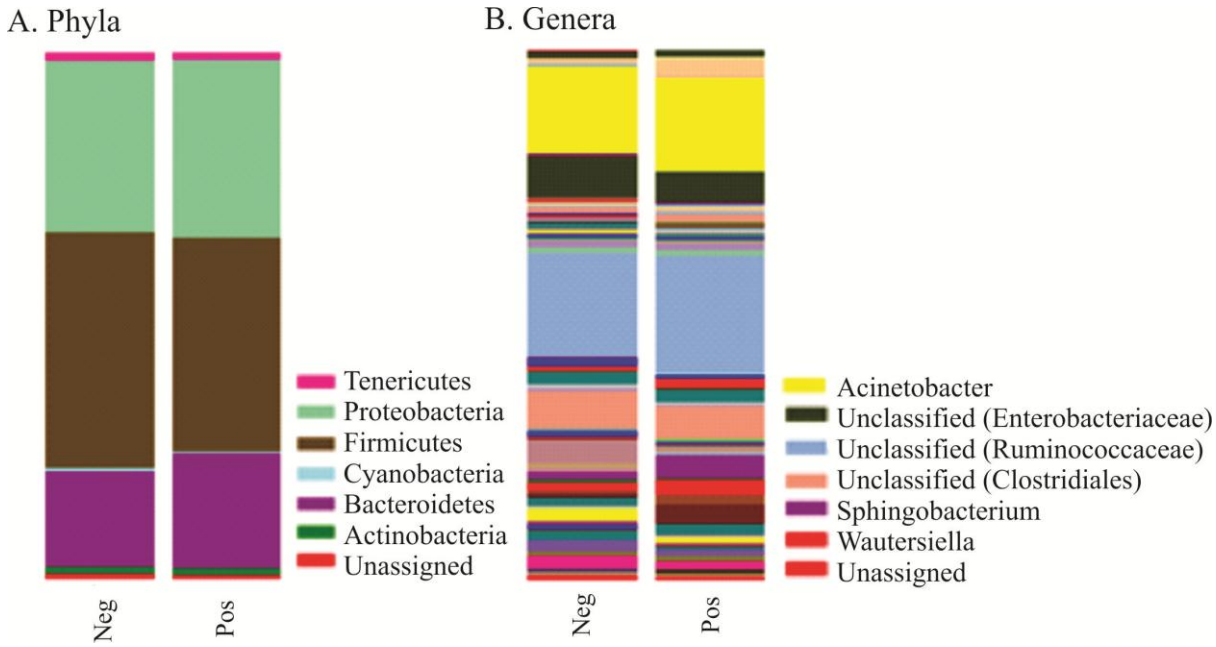

Figure 7. Taxonomic compositional differences in white-tailed deer fecal microbiota between negative and EPEC-positive samples at the (A) phyla, and (B) genera levels.

Although no difference was identified in the rarefaction cures, further analysis of the microbiota composition among EHEC positive (n=11) and negative (n=26) animals shows differences at both the phylum and genus level (Figure 8). OTU significance tests revealed that the abundance of 90 OTUs were significant differently between negative and EHEC positive samples, specifically, genus Acinetobacter.

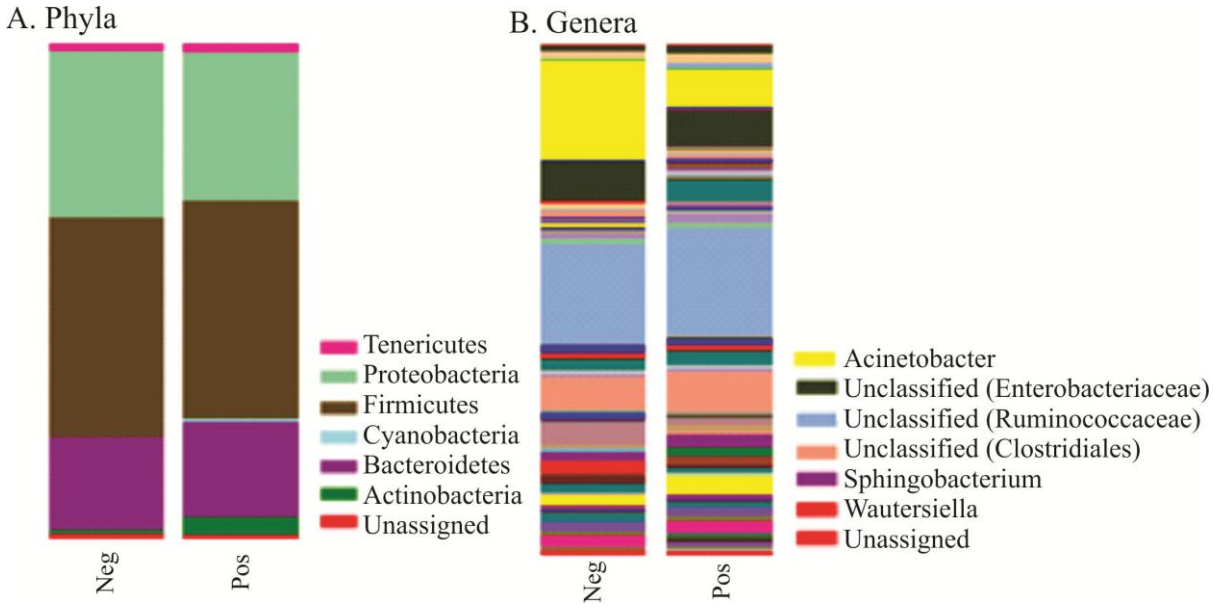

Figure 8. Taxonomic compositional differences in white-tailed deer fecal microbiota between EHEC negative and positive samples at the (A) phyla, and (B) genera levels.

Because a subset of deer was also found to acquire STEC or EPEC over the sampling period, we evaluated the microbial communities in two deer sampled at both time points. Phylum Tenericutes phylum were more abundant in the five individuals that did not acquire pathogenic *E. coli* relative to the two individuals that had acquired one of the two pathogens.

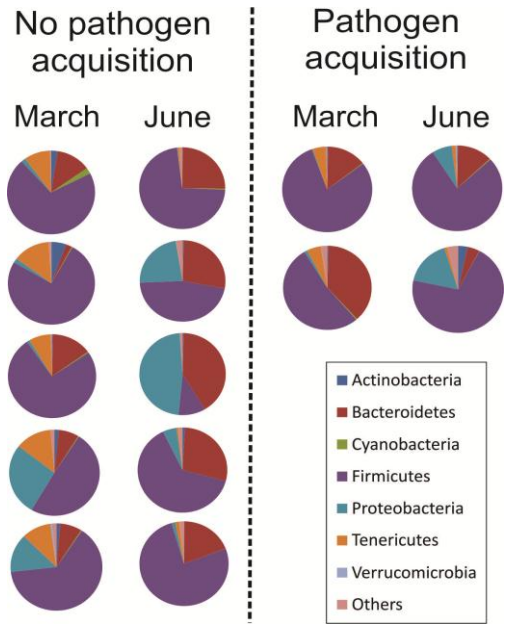

Figure 9. Taxonomic compositional of white-tailed deer microbiota for individuals sampled in March and June. Pathogen acquisition indicates that individuals were STEC or EPEC culture positive in June.

In the case of *Salmonella* only four animals were culture positive. The diversity was lower in samples with the pathogen (Shannon average=5.04) relative to negative samples from June (Shannon average=5.76) (Figure 10).

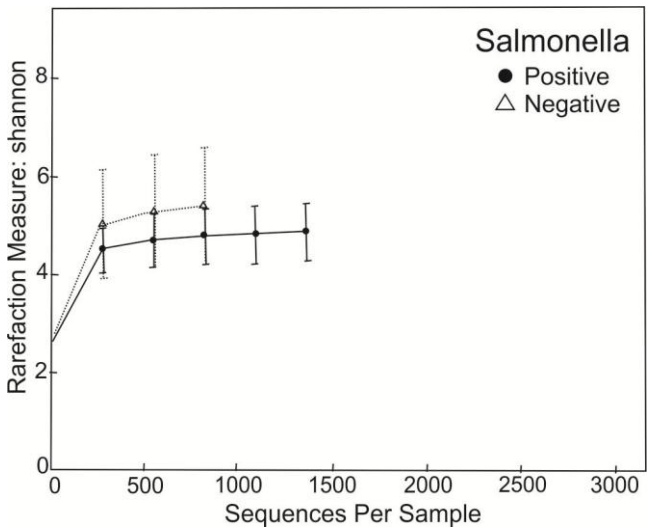

Figure 10. Rarefaction plot of Shannon alpha diversity with and without *Salmonella* present. Only negative samples from June were considered

When comparing the composition of the 10 most abundant genera in *Salmonella*-negative and -positive samples, the predominate genus belong to the Ruminococcaceae family, though the abundance was reduced from 33% to 26% in samples with *Salmonella* (Figure 11). In contrast, the genus Acinetobacter increased in abundance from 20% to 34%. Also the genus S24-7, a member of the Bacteroidetes phylum, also increased in abundance from 3% to 10%. Non-parametric t-test detected significant differences in OTU abundance for 22 genera including those mentioned above ($p<0.05$) when comparing *Salmonella* positive and negative animals examined in June.

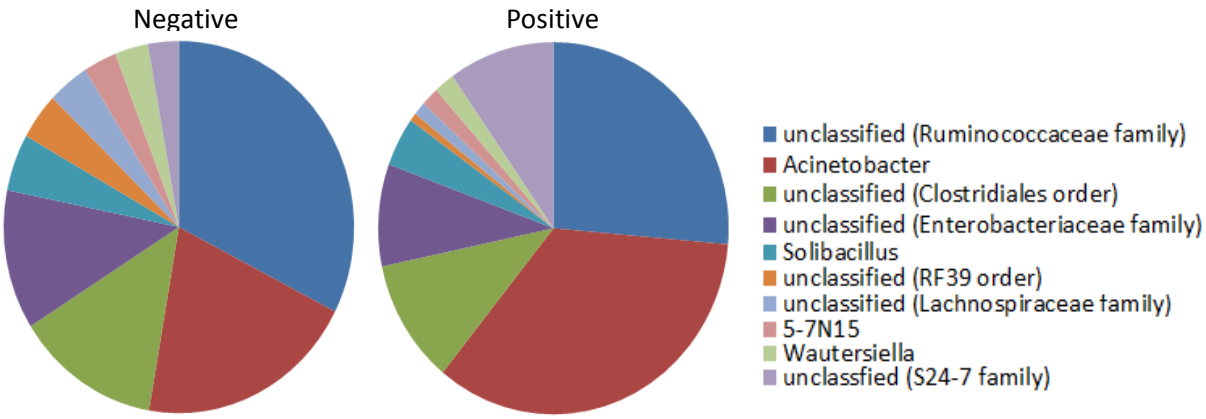

Figure 11. Differences in taxonomic composition between genera of samples with or without *Salmonella* present

1.5 DISCUSSION

Feces samples are increasingly used to characterize the microbiota in wildlife species, as feces allow for non-invasive sampling. Although it has been established that different parts of the digestive tract have a distinguished microbiota composition; fecal samples can provided a good representation of the composition of the intestinal microbiota (Avershina *et al*, 2014).

Our results show that the fecal microbiota of white-tailed deer is composed primarily of 3 phyla: Firmicutes, Bacteroidetes and Proteobacteria. When compared to another wild ruminant (roe deer) fecal microbiota and microbiota from a domestic ruminant (cattle) the high percentage of Firmicutes is a consistent across taxa (Li *et al*, 2014; Durso *et al*, 2010). However, the high proportion (20.25%) of Proteobacteria distinguishes white-tailed deer fecal microbiota from roe deer (~2%) and cattle (4.4%). The phylum Proteobacteria is a diverse phylum of pathogenic importance because included medically important pathogens (Mukhopadhya *et al.* 2012), thus greater attention to the

role of white-tailed deer in the transmission of other diseases in this phylum would be useful. Furthermore, the increase of Proteobacteria in the gut had been associated to severe illnesses in mouse (Reeves *et al*. 2011) and humans (Mukhopadhya *et al.* 2012), questioning whether healthy white-tailed deer core microbiota included this high abundance of Proteobacteria or if individuals sampled were in ill health.

At the genus level, members of the family *Ruminococcaceae* were most abundant. This is consistent with earlier studies in roe deer (Li *et al*. 2014). However, roe deer and cattle shared a relative high abundance of the genus *Prevotella* (Li *et al*, 2014; Dowd *et al*, 2008, Tremaroli and Backhed, 2012), which has been associated with fiber rich diets and degradation of cellulose, while the abundance of this genus in KBS was only 0.3%.

Diversity measured by the Shannon index was ranged between 3.19 and 7.69. Microbial community diversity of white-tailed deer was lower compared with roe deer (Shannon index = 8.44) (Li *et al*, 2014). Analyses comparing sampling periods showed that March samples had higher alpha diversity (Shannon index) than samples from June. PCOA analyses revealed significant difference of microbiota composition between seasons. Analysis excluding the individuals, whose samples were collected in March and June, show the same result (data not shown). These results are consistent with previous research showing that white-tailed deer modify their feeding habits according to season and that diet is an important factor shaping the intestinal microbial communities. Deer behavioral studies demonstrate that during winter the protein

consumption of deer decreases as food availability and quality decreases. In agricultural settings, deer prefer to feed on plants with high protein content (Dostaler *et al.* 2011).

Gender was not a significant factor influencing the taxonomic richness or composition of white-tailed deer fecal microbiota. Jenks *et al* (1994) compared the digesta and ruminal content between white-tailed deer males and females. This study found differences only in lactating females, which had longer intestinal tract and greater ruminal content. However, although the composition of digesta may be different, the diet quality is similar for both genders, thus the microbial community does not show significant differences. Nevertheless, it is noteworthy that differences in microbiota composition has been documented between sexes in other species like humans (Mueller *et al*, 2006), and rats (Bernhom *et al*. 2006).

We hypothesized that closely related individuals were more likely to exhibit a similar behaviors and would forage in similar locations; however Mantel tests did not reveal significant correlation between inter-individual microbiota beta-diversity and levels of genetic relatedness. Other studies have shown this correlation, as closely related individuals that inhabit the same location shared the same diet and probabilities of acquisition of microorganisms (Banks *et al*. 2009).

Analyses of pathogen included only 3 animals that were culture positive for STEC, however, the qPCR analysis of all samples show a higher percentage of samples that have the *stx1* and *stx2* genes. This result is similar to findings by Kristler *et al*. (2011)

in Pennsylvania were qPCR analyses determine prevalence of *stx1* (10%) and *stx2* (46%) phage genes in deer. The contrast between prevalence of lower positive *stx E. coli* by culture samples may be related to the stability of phage interactions with bacteria. Other study in healthy humans proved that free *stx* phage collected from feces, were able to infect *E. coli* and propagate in a laboratory (Martinez-Castillo *et al.* 2013). Thus, these results highlight the importance to considering wildlife and environmental reservoirs of phage, given that the prevalence of phage shedding appears to be high.

Studies about Shiga toxin-producing *E. coli* in cattle and other ruminants suggest that these species lack the toxin receptor, thus this pathogen does not produce major effects on these hosts (Callaway et al, 2013). However, pathogenic *E. coli* have been reported to cause diarrhea in calves (Abu-li *et al*, 2009). Comparisons of microbial diversity show no significant differences between carriers and non-carriers. Nevertheless presence of EHEC appeared to have a significant effect on the abundance of 90 OTUs, including members of the family *Ruminococcaea*, which were higher in EHEC positive samples. *Ruminococcaea* family species are involved in metabolic activities and the production of short-chain fatty acids (SCFA). These SCFA have also been associated with colonization of *Salmonella* before (Bearson *et al*, 2013). Contrary, the abundance of Proteobacteria Acinetobacter decreased significantly in EHEC positive samples. When comparing the microbial composition in individuals sampled in both sampling times (Figure 9), results show that there is each individual have a distinct microbial composition. Results also showed that the microbiota of individuals that did not acquire strains of pathogenic *E. coli* have in common a higher abundance of Tenericutes phyla

than individuals that acquire the pathogen. No direct association between high abundance of Tenericutes and a protective function can be concluded, however, further studies of the role of Tenericutes in the intestine may be valuable.

In samples with *Salmonella*, alpha diversity results showed a reduced diversity, suggesting that this pathogen may alter the microbiota. Understanding the mechanism that *Salmonella* uses for colonization is expected this reduction on diversity. *Salmonella* usually colonize the ileum or colon, and takes advantage of host inflammatory response to grow and outcompete commensal microorganisms (Thiennimtr *et al*, 2012). Contrary to samples with EHEC, the presence of *Salmonella* was associated with a significant increase in abundance of the Proteobacteria Acinetobacter, and Bacteriodetes. Unfortunately, the sample size is too small to make strong conclusions.

In conclusion, the white-tailed deer microbiota is a complex and plastic community that respond to environmental changes like season, and that the microbial diversity is affected by pathogens like *Salmonella*, and its composition may be alter by pathogens like EHEC. These results suggested that white-tailed deer is not an asymptomatic carrier of these pathogens but its health may be affected.

CHAPTER 2

Comparative analysis of fecal microbiota between white-tailed deer and cattle in a shared agroecosystem and differences in response to enterohemorrhagic *Escherichia coli* (EHEC)

2.1. ABSTRACT

Agro-ecosystems are environments that facilitate interactions between domestic animals and wildlife species, creating a favorable setting for inter-specific disease transmission. Cattle and white-tailed deer are ruminants that share pastures in many agro-ecosystems and have been reported to share strains of pathogens such as pathogenic *Escherichia coli,* suggesting direct interaction and interspecific transmission of microorganisms. In this study we used next generation sequencing to compare the microbiota between cattle and white-tailed deer that cohabitated the same agroecosystem at the Kellogg Biological Station (KBS), and contrast the microbiota response by examining microbiota from fecal samples of enterohemorrhagic *E. coli* (EHEC) carriers and non-carriers. Results revealed that cattle feces had higher microbiota diversity than white-tailed deer. The main difference in microbiota composition was the abundance of Proteobacteria, 0.82% in cattle vs. 20.25% in white-tailed deer. Comparison of microbial composition in cattle EHEC carriers and non-carriers did not show differences; however, the microbiota of white-tailed deer EHEC carriers vs non-carriers differed in composition at both phyla and genera level. This study demonstrates different core microbiota between cattle and white-tailed deer that shared an environment, and showed a different response to the presence of the EHEC.

2.2. INTRODUCTION

Gastrointestinal (GI) microbiota are essential to host health, and are an important factor associated with disease transmission (Kamada *et al*. 2013). Domestic and wild animals including cattle (*Bos taurus*) and white-tailed deer (*Odocoileus virginianus*) are known reservoirs of foodborne diseases like pathogenic *Escherichia coli* (Branham *et al*. 2005). Pathogenic *E. coli* and production of Shiga toxins (*stx*) cause several human illnesses, where Shiga toxin-producing *E. coli* (STEC) alone is responsible of 265,000 infections and 31 deaths per year (Franklin *et al*. 2013). As reservoirs of pathogenic *E. coli*, white-tailed deer and 30% of cattle are consider asymptomatic carriers and that are not clinically affected. Yet, in the case of cattle, enterohemorrhagic *E. coli* (EHEC) have been reported to cause diarrhea in calves (Callaway *et al*, 2009; Abu-Ali *et al* 2008).

Studies have found associations between intestinal microbiota and pathogenic *E. coli*. Zhao and collaborators (2013) found associations between high shedding level of STEC (high-shedders) and low microbiota diversity; however results from Aluthge *et al* (2014) were conflicting. These authors found that high-shedders had higher bacteria diversity but lower abundance of the genus *Prevotella* than low-shedders. A previous study conducted by Delgado (2015) in white-tailed deer found that EHEC did not affect microbiota diversity, though there were significant differences in the abundance of certain taxa.

Previous research has shown that there are differences in the diversity and taxonomic composition of microbiota in different host species (Lee *et al*. 2011). Gruninger *et al* (2014) studied the rumen microbiota of elk (*Cervus elephus*) and white-tailed deer. The authors found microbial genera not previously described in bovine rumens. However, there is a lack of information on differences in microbiota between domestic and wild ruminants. No studies have investigated the effects of pathogenic *E. coli* presence on intestinal microbiota diversity and taxonomic composition in cattle and deer simultaneously.

A recent study reported that cattle and white-tailed deer in a common agroecosystem exchange pathogens such as STEC (Singh *et al*. 2015). Transmission of other pathogens like *Salmonella* has also been documented (Braham *et al*. 2005). These studies suggest that cattle and white-tailed deer that coinhabit the same environment might share other microorganisms, and thus have a similar core microbiota that could respond similarly to colonization of pathogens like pathogenic *E. coli*.

To test this hypothesis, our first objective was to evaluate the levels of interspecific similarities in microbiota communities of white-tailed deer and cattle collected in the same agroecosystem. The second objective was to assess the similarities in response of the microbiota to enterohemorrhagic *Escherichia coli* (EHEC) presence in both species.

2.3. METHODOLOGY

2.3.1 Sample collection

Fresh samples of white-tailed deer feces were collected in March and June of 2012 from Kellogg Biological Station (KBS), located in Barry county, south central Michigan. Stratified random transects were selected from forest and pasture locations, near water sources and near the pasture dairy center. The samples were collected in plastic bags while walking transects and were assigned an individual identification. Geographic locations were referenced for all samples using a handheld GPS unit (Appendix 1). All samples were stored at -80° C.

2.3.2 Extraction of white-tailed deer DNA

The extraction of genomic DNA from each fecal sample was performed by using a QIAamp DNA Stool isolation kit (Qiagen; Valencia, CA). Eight fecal pellets from each individual sample were swabbed with a sterile swab on the pellet surface to obtain deer intestinal cells. The swab was submerged in ATL buffer, and then extraction steps were performed following the manufacturer´s instructions. Total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific).

2.3.3 Discrimination of deer individuals

Eight microsatellites loci including IGFl (Kirkpatrick, 1992), OBCAM (Moore *et al*. 1992), Cervid1, Cervid2 (DeWoody *et al*. 1995), Rt7, RT9, Rt24, and Rt27 (Wilson *et al*. 1997) were used for the discrimination of individual deer (Grear *et al*. 2010). PCR conditions for each locus are shown in Appendix 2. Individual identification was determined by comparing the genotypes from fecal-derived DNA using the software Cervus 3.0 (Kalinwoski *et al*. 2007).

2.3.4 Identification of pathogenic *E. coli*

All fecal samples were cultivated at the MSU Microbial Evolution and Epidemiology Laboratory. A loop of deer feces was cultivated following enrichment in EC broth overnight at 37°C and subculture to CHROMoagar (CHROMagar; Paris). Single colonies were confirmed to be EHEC, STEC or EPEC by multiplex PCR targeting the intimin adhesion (*eae*), *stx1* and *stx2* was performed as described in Manning *et al.* (2008) followed by amplification of the bundle forming pilus (*bfp*), a common EPEC marker, was performed as described in Trabulsi *et al.* (2002). Isolates were classified as atypical EPEC if they were *eae*-positive and *stx*- negative, typical EPEC if they were *eae*- and *bfp*-positive, STEC if they were *stx*1 and/or *stx*2 positive, and EHEC if were *eae* and *stx*1 and/or *stx*2 positive. Confirmed isolates were stored at -80°C in glycerol stock.

2.3.5 Extraction of intestinal microbial community DNA

Fecal pellets from each sample were mashed and homogenized. The extraction of the microbial communities was performed using QIAamp DNA stool kit (Qiagen; Valencia, CA) according to the manufacturer's protocol with slight modification of bead beating and denaturation at 95°C. In brief, 0.3g of the homogenized sample was added to tubes with beads to break open the bacterial cells initially. Total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific).

2.3.6 DNA quality verification

The amount of DNA degradation and the DNA quality was verified prior to sequencing by the amplification of the 16S ribosomal RNA (*rRNA*) gene for each sample. The primers used were: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Lane, 1991). The PCR conditions consisted on an initial denaturation step at 95° C for 2 min, 30 cycles of denaturation at 15°C for 15 sec, annealing at 57°C for 15 seconds, extension at 72°C for 30 sec; and a final extension at 72°C for 10 min. Electrophoresis was performed on a 1% agarose gel to confirm amplification.

2.3.7 16S rDNA sequencing

Sixty-seven samples from white-tailed deer were selected was based on research objectives and prepared for sequencing using specific 16S gene specific primers linked (sequence: CCGTCAATTCMTTTRAGT) to barcodes for multiplexing. A 3 ng/µl aliquot of each DNA sample was used for the PCR reaction, and each sample was amplified in triplicate along with a negative control. An AccuPrime taq kit (Invitrogen^{TM}) was used to amplify the 16S rDNA genes. The PCR conditions included an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 5minutes; and a final extension at 72°C for 5 minutes. PCR reaction was carried out in triplicates so as to get adequate concentration and volume of the PCR product to further downstream. The triplicates of each sample were mixed together, and verified by electrophoresis. PCR products were quantified using a picogreen assay by Qubit© (Invitrogen) before and after the purification process. Finally, all samples were pooled in equimolar ratios

based on DNA concentration. Pyrosequencing was performed using a 454 titanium flex sequencing kit on a Roche Junior sequencer.

2.3.8 Sequencing analysis

Sequences from 67 white-tailed deer samples, and existing sequences from 48 cattle samples were analyzed using the software QIIME (Caporaso *et al*. 2010). Existing sequences from cattle were collected from lactating dairy individuals in KBS herd two weeks after June sampling of deer feces, individuals did not receive antibiotics and 80% of their diet was pasture (Singh *et al*. 2012). First, all sequences were subjected to a quality control that included a noise reduction using the denoise_wrapper.py script, removal of short sequences and sequences with barcode mismatches. Then, unique sequences were used to align against the Greengenes reference database. All chimeras were detected and removed using Uchime (Edgar, 2011). Following quality control checking, any sample with less than 1000 sequences were not included in the downstream analysis. A distance matrix using 0.03% phylogenetic distances was performed to define the operational taxonomic units (OTU). Rarefaction curves were generated based on Shannon diversity index. Principal coordinate analysis (PCOA) analysis based on the Bray-Curtis dissimilarity index was used to visually compare microbial composition as a function of species (deer-March, deer-June, cattle), and presence of pathogen (STEC, EHEC, EPEC). After the ANOSIM test was used to assess significant differences between microbiota and presence of pathogen using the compare_categories.py script. Finally a non-parametric t-test was used to determine

differences in OTU abundance between variables using the group_significance.py script.

2.4. RESULTS

After quality control analyses sequences, 13 samples from cattle were eliminated because of low sequence number. A total of 35 cattle samples and 67 deer (March=30, June=37) samples are used in the following analysis. Sample size for STEC and EPEC positives were too small (Cattle: 6 STEC, 1 EPEC; Deer: 3 STEC, 6 EPEC) thus these variables are not being tested. However, sample sizes for EHEC positive for cattle were 18 and in white-tailed deer 11, and samples were analyzed.

Previous analyses have shown that microbiota composition differed significantly between sampling periods (March and June) (Delgado, 2015), so white-tailed deer samples were analyzed according to sampling period. Shannon rarefaction curves (Figure 12) revealed that cattle have higher diversity than white-tailed deer. OTUs classification shows that cattle microbiota was composed of microorganism in 12 phyla, while white-tailed deer microbiota was composed of 17 phyla.

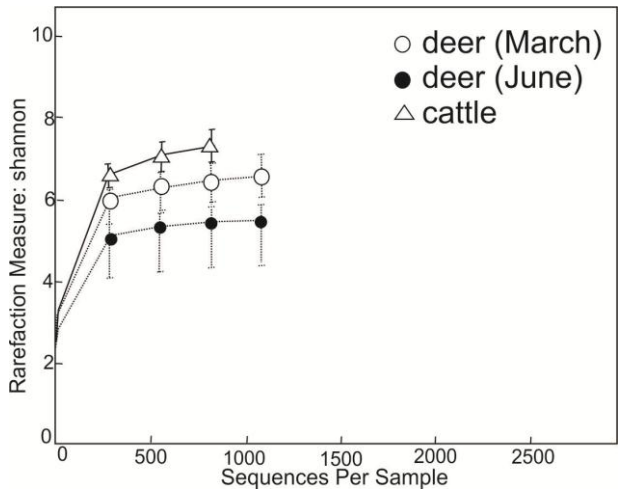

Figure 12. Rarefaction plot of Shannon alpha diversity index of cattle and white-tailed deer fecal microbiota (by sampling period

Characterization of the microbiota composition by species (Figure 13) shows that Proteobacteria is the phyla that differed most in frequency between species (cattle: 0.82%, deer March: 7.9%, deer June: 32.6%). Actinobacteria is a second phyla that exhibited pronounced differences in frequency between species (0.1% abundance in cattle vs. 3.5% in deer in March and 1.4% in deer in June).

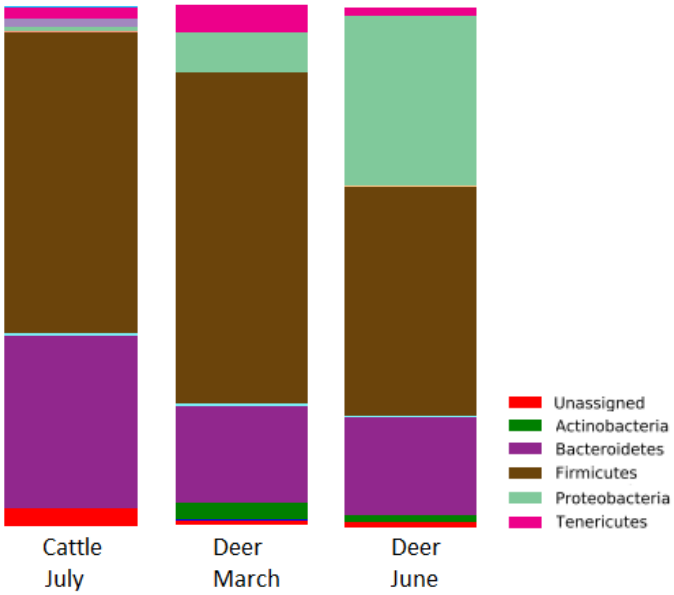

Figure 13. Microbiota taxonomic composition at the phyla level in cattle and deer

Principal coordinate analysis (POCA) show a strong difference in microbiota taxonomic composition between cattle samples communities and white-tailed deer communities (Figure 13). Cattle samples microbiota cluster and are more similar to each other than they are to white-tailed deer sample communities, which are more scattered on the plot. An ANOSIM test revealed significant differences between the species ($R = 0.64$, $p < 0.001$).

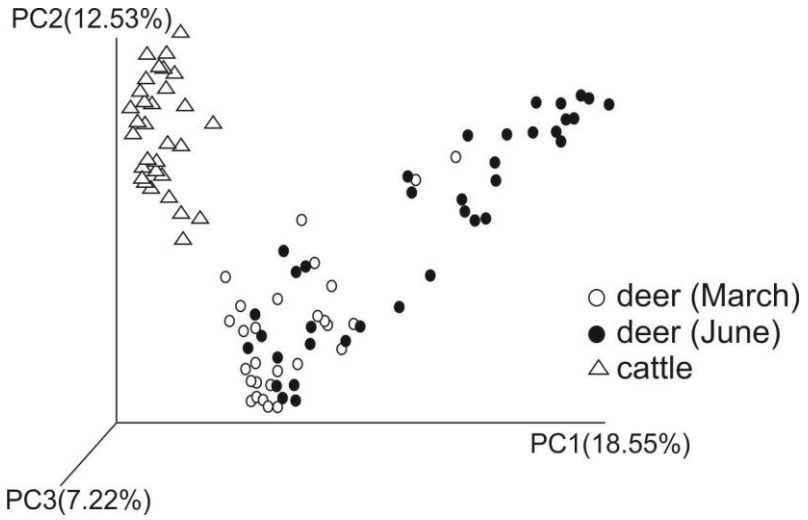

Figure 14. Principal component analysis (PCOA) plot characterizing fecal microbiota differences of cattle (triangle), deer collected in June (dark circle), and deer collected in March (white circle)

Notably, white-tailed deer samples collected in March are more tightly clustered than deer samples collected in June (Figure 14). Analysis of microbiota composition at the phyla level revealed that white-tailed deer samples collected in March were more similar to cattle than deer samples collected in June (Figure 13).

Figure 15 describes and contrasts microbiota composition of EHEC carriers and non-carriers in both species. When comparing EHEC carriers and non-carriers in cattle there

is no differentiation between samples (ANOSIM R=-0,01, p-value > 0.05). However, relative abundance of 63 OTUs show to differ significantly (p-value < 0.05) between positive and negative samples. The genus *Prevotella* differed most significantly. In the case of white-tailed deer differences in the total microbial composition at both phyla and genus levels between carriers and non-carriers (only June samples) were modest (but not significant). However, a total of 90 OTUs including genus from *Ruminococcaceae* family showed to be significant between EHEC positive and negative (p<005).

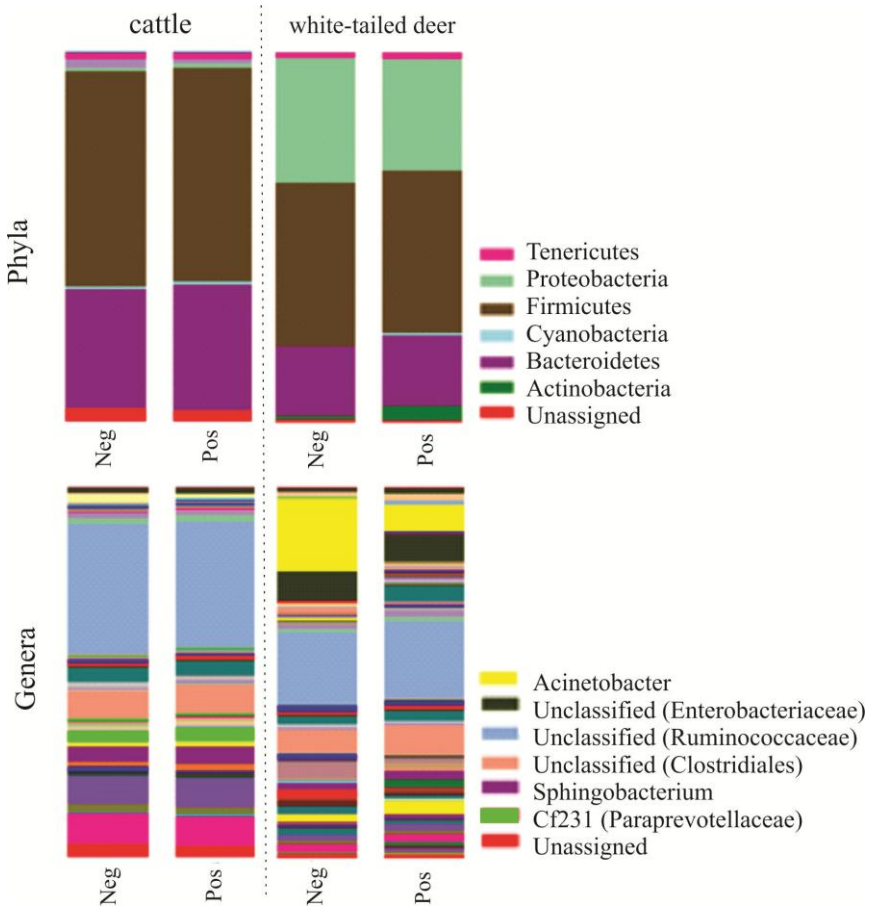

Figure 15. Fecal microbial taxonomic composition between cattle and white-tailed deer EHEC positive and negative samples at the phyla and genera level.

2.5. DISCUSSION

Interspecific analysis of white-tailed deer and cattle microbiota from individuals that cohabitat a common agricultural landscape revealed that the microbiota diversity of cattle was higher than deer. Cattle received a dietary supplement that consisted of alfalfa and ground corn, which constituted 20% of their diet, while white-tailed deer feed in pastures and on natural forest vegetation. Cattle diet may be a factor for higher microbial diversity than white-tailed deer. Diet has been previously documented to be associated with difference in bacteria diversity between species (Dougal *et al*. 2014; Wu *et al*. 2011). Furthermore, previous studies in humans and mice suggested that high abundance of Bacteroidetes (as in cattle samples) is associated to a balanced diet rich in fiber and proteins (Candela *et al*. 2012; Tremaroli and Backhed, 2012).

Principal coordinate analysis (Figure 14) revealed that microbiota composition of cattle samples were similar and clustered together, while deer samples were more dissimilar and scattered. Cattle samples were collected two weeks after the deer samples from June; however, white-tailed deer samples from March are more similar to cattle samples than deer samples from June. Deer behavior studies have shown that during winter (March) deer that inhabited agroecosystems prefer to forage on agricultural vegetation, because of higher protein content (Dostaler *et al.* 2011). During summer months, forest vegetation is more abundant and deer may choose to feed in the forest. Dietary differences between seasons likely explain why deer microbiota from March is more similar to cattle microbiota.

Cattle microbiota composition is primarily composed of Firmicutes and Bacteroidetes, as shown in previous studies (Xu *et al*. 2014). The main difference with white-tailed deer microbiota is the high percentage of Proteobacteria. During June, abundance of Proteobacteria in deer increased to 32%, which is higher than reported in other species, including in a previous survey of white-tailed deer's rumen (Gruninger *et al*. 2014). Increase of Proteobacteria is associated with severe illnesses, as Proteobacteria phylum is consider including several pathogenic species (Mukhopadhya *et al*. 2012).

Because of the small sample size of culture positive STEC and EPEC samples, we focused our analyses in EHEC positive samples. Figure 15 show the composition at phyla and genera level of EHEC negative and positive cattle and white-tailed deer. Cattle microbiota composition did not differ between negative and positive samples. Studies have reported that calves are affected by EHEC, however, adults are considered just asymptomatic carriers (Callaway *et al*, 2009; Abu-Ali *et al* 2008). Our analysis shows that 63 microbial taxa differed significantly between EHEC positive and negative samples. The genus *Prevotella* was the most significant different reduced in presence of EHEC. Decrease of *Prevotella* abundance has been reported on other species as a response to the presence of pathogens (Bearson *et al*, 2013). Unfortunately, we cannot make further inferences concerning whether EHEC alone was responsible or consequences of differences in taxonomic composition to host health.

White-tailed deer microbial communities show differences in presence of EHEC although statistical analysis of the overall community is not significant. Analysis by

taxa reveals that 90 taxa differ significantly, and among these taxa are several members of the Bacteroidetes phyla which are absence when EHEC is present. Members of the Bacteroidetes phylum included species which function is to protect the intestine against infection by the activation of the immune system. Thus, the host may be more susceptible to colonization of pathogens when Bacteroidetes are reduced in frequency (Buffie *et al*, 2013).

Results suggest that although cattle and white-tailed deer have indirect interaction and transmit pathogens, their microbiota are distinct and responded differently to the presence of EHEC. The microbiota of adult dairy cattle seems unaffected by the presence of EHEC, while the microbiota white-tailed deer differ in composition with and without presence of EHEC, suggesting the deer microbiota are affected by EHEC. This result suggests that white-tailed deer may be more than an intermediary carrier for the transmission of this pathogen.

APPENDICES

Appendix 1 Kellogg Biological Station Map

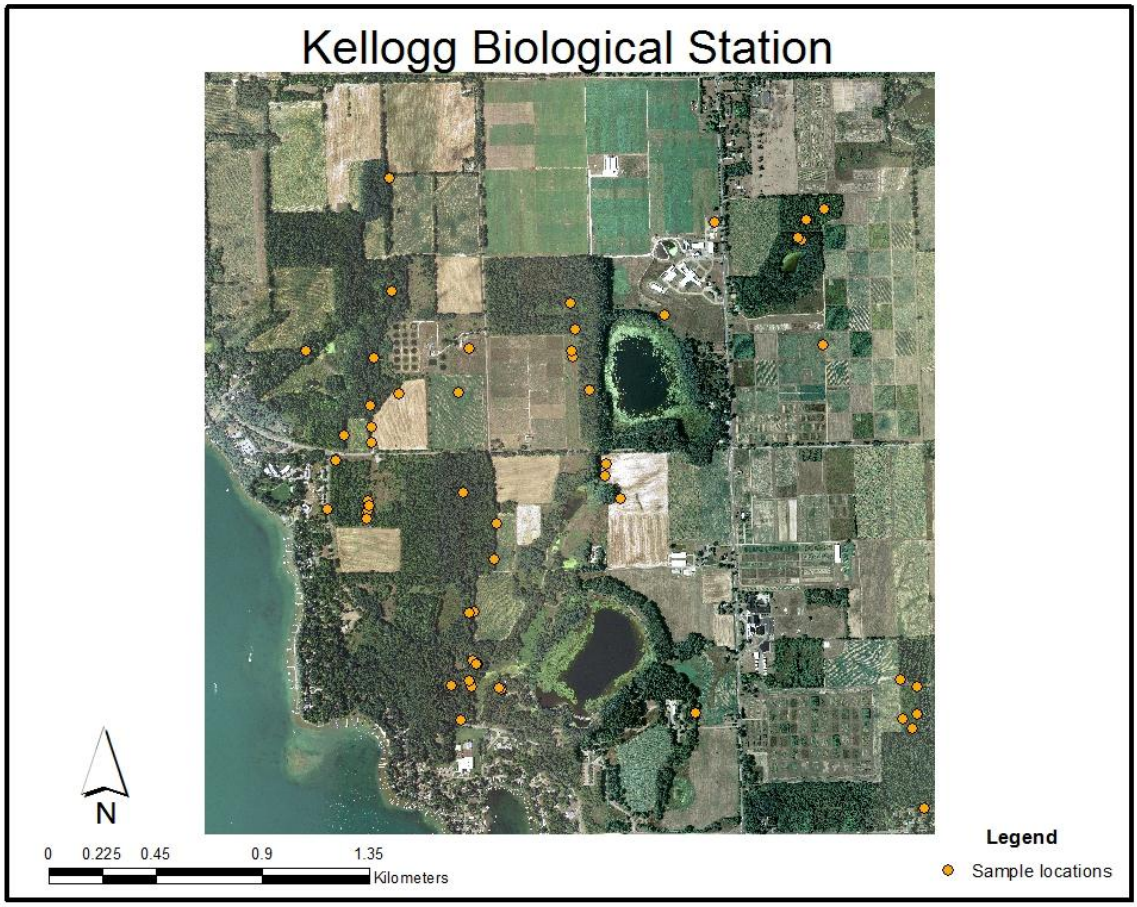

Figure 16. Kellogg Biological Station map and sample locations

Appendix 2 Microsatellite loci table

Table 1.- PCR conditions for the microsatellite used for discrimination of deer individuals

	Repeat sequence	Annelling Temp (°C)	# alleles	Reference
IGF1	CA	58	10	Kirkpatric, 1992
OBCAM		50	10	Moore *et al*. 1992
Cervid1	$(CA)_{17}TA(CA)_5$	60	11	DeWoddy *et al*. 1995
Cervid2	$(AC)_{12}AA(AC)_7$	60	8	DeWoddy *et al*. 1995
Rt7	$(GT)_{21}$	54	11	Wilson *et al*. 1997
Rt9	$(GT)_{18}$	54	9	Wilson *et al*. 1997
Rt24	$(GT)_3A(GT)_{17}$	54	10	Wilson *et al*. 1997
Rt27	$(GT)_{16}$	54	11	Wilson *et al*. 1997

BIBLIOGRAPHY

BIBLIOGRAPHY

- Abu-Ali, G. S., Lacher, D. W., Wick, L. M., Qi, W. & Whittam, T. S. Genomic diversity of pathogenic *Escherichia coli* of the EHEC 2 clonal complex. *BMC Genomics* 10, 296 (2009).

- Aidy, S., Van den Abbeele, P., Van de Wiele, T., Louis P., Kleerebezem, M. Intestinal colonization: How key microbial players became established in this dynamic process. *Bioessays*. 35: 913-923 (2013).

- Aluthge, Nirosh D, *et al.* Differences in Fecal Bacterial Community Composition Between Beef Steers which are HighShedders and Low-Shedders of Shiga Toxin-Producing *Escherichia coli* (STEC) (2014). Nebraska Beef Cattle Reports. Paper 797.http://digitalcommons.unl.edu/animalscinbcr/797

- Avershina, E. *et al*. Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children. *FEMS Microbiol*. Ecol. 87, 280–90 (2014).

- Banks, J. C., Cary, S. C. & Hogg, I. D. The phylogeography of Adelie penguin faecal flora. *Environ. Microbiol*. 11, 577–88 (2009).

- Bearson, SMD., Allen, HK., Berason, BL., Looft, T., Brunelle, BW., Kich, JD., Tuggle, CK., Bayles, DO., Alt, D., Levine, UY., Stanton, TB. Profiling the gastrointestinal microbiota in response to *Salmonella*: Low versus high Salmonella shedding in the natural porcine host. *Infection, Genetics and Evolution*. 16:330-340 (2013).

- Braham, LA., Carr, MA., Scott, CB., Callaway, TR. *Escherichia coli* O157 and Salmonella spp. in white-tailed deer and livestock. *Curr Iss Intest Microbiol*. 6: 25-29 (2005).

- Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat. Rev. Immunol*. 13, 790–801 (2013).

- Callaway, TR., Edrington, TS., Loneragan, GH., Carr, JK., Nisbet, DJ. Shiga toxin producing *Escherichia coli* (STEC) ecology in cattle and management based options for reducing fecal shedding. *Agriculture Food Analytical Bacteriol*. 3: 39-69 (2013).

- Candela, M., Biagi, E., Maccaferri, S., Turroni, S. & Brigidi, P. Intestinal microbiota is a plastic factor responding to environmental changes. *Trends Microbiol*. 20, 385–391 (2012).

- Caporaso, JG., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, FD., et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335–336 (2010).

- Carroll, IM., Ringel-Kulka, T., Siddle, JP., Klaenhammer, TR., Ringel, Y. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLoS ONE*. 7(10) (2012).

- CDC. Centers of Disease Control and Prevention. Url: http://www.cdc.gov/.

- Chambers, J. R. & Gong, J. The intestinal microbiota and its modulation for *Salmonella* control in chickens. *Food Res. Int*. 44: 3149–3159 (2011).

- Costa, MC., Arroyo, LG., Allen-Vercoe, E., Stampfli, HR., Kim, PT., et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. *PlOS ONE*. 7(7). (2012).

- Dahllöf, I. Molecular community analysis of microbial diversity. *Curr. Opin. Biotechnol*. 13: 213–217 (2002).

- Daniels, MJ., Hutchings, MR., Greig, A. The risk of disease transmission to livestock posed by contamination of farm stored feed by wildlife excreta. *Epidemiol infect*. 130: 561-568 (2003).

- Delgado, ML. Characterization of white-tailed deer fecal microbiota and comparisons between pathogenic *Escherichia coli* and *Salmonella* carriers and non-carriers. Thesis, chapter1 (2015).

- DeWoody, JA., Honeycutt, RL., Skow, LC. Microsatellite markers in white-tailed deer. *Journal of Heredity*. 86: 317–319 (1995).

- Dostaler, S., Ouellet, J. P., Therrien, J. F. & Côté, S. D. Are feeding preferences of white-tailed deer related to plant constituents? J. *Wildl. Manage*. 75, 913–918 (2011).

- Dougal, K. et al. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. *PLoS One* 9, e87424 (2014).

- Dowd, S. E. *et al*. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol*. 8, 125 (2008).

- Durso, L. M. *et al*. Animal-to-animal variation in fecal microbial diversity among beef cattle. *Appl. Environ. Microbiol*. 76, 4858–62 (2010).

- Edgar,RC, Haas,BJ, Clemente,JC, Quince,C, Knight,R. UCHIME improves sensitivity and speed of chimera detection, *Bioinformatics* doi: 10.1093/bioinformatics/btr381. (2011).

- Ferens, WA., Hovde, CJ. Escherichia coli O157:H7: animal reservoir and sources of human infection. *Foodborne Pathog* Dis.8:465-487 (2011).

- Franklin, AB., VerCauteren, KC., Maguire, H., Cichon, MK., Fischer, JW., Lavelle, MJ., Powell, A., Root, JJ., Scallan, E. Wild ungulates as disseminators of Shiga toxin-producing *Escherichia coli* in urban areas. *PLoS ONE*. 8(12). (2013).

- Goodnight, KF and Queller, DC. Computer software for performing likelihood tests of pedigree relationship using genetic markers. *Molecular Ecology* 8(7): 1231–1234 (1999).

- Grear, DA., Samuel, MD., Scribner, KT., Weckworth, BV., Langenberg, JA. Influence of genetic relatedness and spatial proximity on chronic wasting disease infection among female white-tailed deer. *J Appl Ecol*. 47: 532-540 (2010).

- Gruninger, RJ., Sense, CW., McAllister, T., Forster. Diversity of rumen bacteria in Canadian cervids. *PLoS ONE*. 9(2) (2014).

- Gu, S., Che, D., Zhang, JN., Lv, X., Wang, K., Duan, LP., Nie, Y., Wu, XL. Bacterial community mapping of the mouse gastrointestinal tract. *PLOS ONE*, 8 (10) (2013).
- Hernandez-Reyes, C., Schikora, A. Salmonella, a cross-kingdom pathogen infecting humans and plants. *FEMS Microbiol Lett*. 343: 1-7 (2013).

- Jenks, J. a, Leslie, D. M., Lochmiller, R. L. & Melchiors, M. a. Variation in gastrointestinal characteristics of male and female white-tailed deer: implications for resource partitioning. *J. Mammal.* 75, 1045–1053 (1994).

- Jones. BA., Grace, D., Kock, R., Alonso, S., Rushton, J., Said, MY., McKeever, D., Mutua, F., Young, J., McDermott, J., Pfeiffer, DU. Zoonosis emergence linked to agricultural intensifications and environmental change. *Proc Acadof Sci USA*. 110: 8399-8404 (2013).

- Kalinowski, ST., Taper, ML., Marshall, TC. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*. 16: 1099-1006 (2007).

- Kamada, N., Chen, GY., Inohara, N., Nuñez, G. Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*. 14(7):685-690 (2013).

- Kistler, W. M., Mulugeta, S. & Mauro, S. a. Detection of *stx*1 and *stx*2 Genes in Pennsylvanian White-Tailed Deer. *Toxins* (Basel). 3: 640–646 (2011).

- Lane, D. J. 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. E. Stackebrandt and M. Goodfellow, eds. New York, NY, *John Wiley and Sons*: 115-175. (1991).

- Lauber, C. L., Zhou, N., Gordon, J. I. & Knight, R. NIH Public Access. 307, 80–86 (2011).

- Lee, J. E., Lee, S., Sung, J. & Ko, G. Analysis of human and animal fecal microbiota for microbial source tracking. *ISME J*. 5, 362–365 (2011).

- Lettat, A. et al. Rumen microbial and fermentation characteristics are affected differently by bacterial probiotic supplementation during induced lactic and subacute acidosis in sheep. *BMC Microbiol*. 12, 142 (2012).

- Li, Z. et al. Bacteria and Methanogens Differ along the Gastrointestinal Tract of Chinese Roe Deer (Capreolus pygargus). *PLoS One* 9, e114513 (2014).

- Li, ZP., Liu, HL., Li, GY., Bao, K., Wang, KY., Yang, YF., Yang, FH., Wright, ADG. Molecular diversity of rumen bacterial communities from tannin-rich and fiber-rich forage fed domestic Sika deer (*Cervus nippon*) in China. *BMC Microbiology*. 13:151 (2013).

- Lillehaug, A., Bergsjo, B., Schau, J., Bruheim, T., Vikoren, T., Handeland, K. *Campylobacter* spp., *Salmonella* spp., Verocytotoxic *Escherichia coli*, and antibiotic resistance in indicator organism in wild cervids. *Act vet scand*. 46:23-32 (2005).

- Lindsay, AR., Belant, JL. A simple and improved PCR-based technique for white-tailed deer (*Odocoileus virginianus*) sex identification. *Conserv Genet*. 9: 443-447 (2008).

- Manning, S.D., *et al*. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease 699 outbreaks. *Proc Natl Acad Sci* USA 105: 4868-4873 (2008).
- Mantel N. The detection of disease clustering and a generalized regression approach. *Cancer Res*.;27:209–220 (1967).

- Martinez-Castillo, A., Quirós, P., Navarro, F., Miró, E. & Muniesa, M. Shiga toxin 2-encoding bacteriophages in human fecal samples from healthy individuals. *Appl. Environ. Microbiol*. 79, 4862–8 (2013).

- Mentaberre, G., Porreero, MC., Navarro-Gonzales, N., Serrano, E., Dominguez, L., Lavin, S. Cattle drive Salmonella infection in the wildlife-livestock interface. *Zoonoses Public Health*. 60: 510-518 (2013).

- Moore, SS., W Barendse, KTt. Berger, SM. Armitage, DJ. .S hetzel. Bovine and ovine DNA microsatellites from the EMBL and Genbank databases. *Animal genetics*. 23:463-467 (1992).

- Mueller, S. *et al*. Differences in Fecal Microbiota in Different European Study Populations in Relation to Age, Gender, and Country : a Cross-Sectional Study. *Appl. Environ. Microbiol*. 72, 1027–1033 (2006).

- Mukhopadhya, I., Hansen, R., El-Omar, E. M. & Hold, G. L. IBD—what role do Proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230 (2012).

- Patz, JA., Daszak, P., Tabor, GM., Aguirre, AA., Pearl, M., Epstein, J., Wolfe, ND., Kilpatrick, AM., Foufopoulos, J., Molyneux, D., Bradley, DJ. Unhealthy landscapes: policy recommendations on land use change and infectious disease emergence. *Environmental health perspectives*. 12(10): 1092-1098 (2004).

- Peakall, R. and Smouse P.E. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537-2539 (2012).

- Renter, DG., Gnad, DP., Sargeant, JM., Hygnstrom, SE. Prevalence and serovars of *Salmonella* in the feces of free-ranging white-tailed deer (*Odocoileus virginianus*) in Nebraska. *J Wild Dis*. 4d2: 699-703 (2006).

- Renter, DG., Sargeant, JM., Hygnstorm, SE., Hoffman, JD., Gillspie, JR. Escherichia coli O157:H7 in free-ranging deer in Nebraska. *J Wild Dis*. 37: 755-760 (2001).
- Sekirov, I and Finlay, BB. The role of the intestinal microbiota in enteric infection. *J Physiol* 587(17): 4159-4167 (2009).

- Sharma, VK., Dean-Nystrom, EA. Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. *Veterinary Microbiology*. 93: 247-260 (2003).

- Shov, MN., Madsen, JJ., Rahbek, C., Lodal, J., Jerpersen, JB., Jorgensen, JC., Dietz, HH., Chriel, M., Baggesen, DL. Transmission of Salmonella between wildlife and meat-production animals in Denmark. *J Appl Microbiol*. 105: 1558-1568 (2008).

- Singh, P. et al. Characterization of enteropathogenic and Shiga toxin-producing Escherichia coli in cattle and deer in a shared agroecosystem. *Front. Cell. Infect. Microbiol*. 5: 29 (2015).

- Singh, P., Jerningan, K., Radek, C., Britton, R., Venegas, C., Rust, S., Bartlett, P., Grooms, D., Manning, SD. Variation in microbiota diversity between beef and dairy cattle. American Society for Microbiology Annual Meeting, San Francisco, CA. June 2012.

- Sundset, Ma., Edwards, JE., Cheng YF, Senosiain, RD., Fraile, MN., Northwood, KS., Praesteng, KE., Glad, T., Mathiesen, SD., Wright, ADG. Molecular diversity of the rumen Microbiome of Norwegian reindeer on natural summer pasture. *Microb Ecol*. 57: 335-348 (2009).

- Thiennimitr, P., Winter, SE., Baumler, A. *Salmonella*, the host and its microbiota. *Curr Opin Microbiol*. 15(1): 108-114 (2012).

- Trabulsi, L.R., Keller, R., and Gomes, T. Typical and atypical enteropathogenic Escherichia coli. *Emerg Infect Dis* 8: 508-513. (2002).

- Tremaroli, V. & Bäckhed, F. Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–249 (2012).

- Wilson, GA., Stroneck, C., Wu, L., Coffin, JW. Characterization of microsatellite loci in caribou, *Rangifer tarandus*, and their use in other artiodactyls. *Molecular Ecology*. 6: 697–699 (1997).

- Wu GD, Chen J, Hoffman C, Bittinger K, Chen Y-Y, et al. Linking long- term dietary patterns with gut microbial enterotypes. *Science* 334: 105–108 (2011).

- Zhao, L. *et al*. Correlation analysis of Shiga toxin-producing *Escherichia coli* shedding and faecal bacterial composition in beef cattle. *J. Appl. Microbiol*. 115, 591–603 (2013).