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HOST MICROBE INTERACTIONS

Intestinal Microbial Community Dynamics of White-Tailed Deer (*Odocoileus virginianus*) in an Agroecosystem

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Abstract The intestinal microbiota has important functions that contribute to host health. The compositional dynamics of microbial communities are affected by many factors, including diet and presence of pathogens. In contrast to humans and domestic mammals, the composition and seasonal dynamics of intestinal microbiota of wildlife species remain comparatively understudied. White-tailed deer (*Odocoileus virginianus*) is an ecologically and economically important wildlife species that inhabits agricultural ecosystems and is known to be a reservoir of enteric pathogens. Nevertheless, there is a lack of knowledge of white-tailed deer intestinal microbiota diversity and taxonomic composition. This study's first objective was to characterize and compare the intestinal microbiota of 66 fecal samples from white-tailed deer collected during two sampling periods (March and June) using 16S rDNA pyrosequencing. Associations between community diversity and composition and factors including season, sex, host genetic relatedness, and spatial location were quantified. Results revealed that white-tailed deer intestinal microbiota was predominantly comprised of phyla Firmicutes and Proteobacteria, whose relative frequencies varied significantly between sampling periods. The second objective was to examine the associations between the presence of *Escherichia coli* and *Salmonella*, and microbiota composition and diversity. Results indicated that relative abundance of some microbial taxa varied when a pathogen was present. This study provides insights into microbial compositional dynamics of a wildlife species inhabiting coupled natural and agricultural landscapes. Data focus attention on the high prevalence of Proteobacteria particularly during the summer and highlight the need for future research regarding the role of white-tailed deer as a natural pathogen reservoir in agroecosystems.

Keywords Microbiota · White-tailed deer · 16S rRNA · Pyrosequencing · Agroecosystems · Enteric pathogens

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Introduction

Microbiota refers to the complex and dynamic community of microorganisms that lives within a host [1]. Different parts of an organism are characterized by distinct microbial taxonomic composition based on the source of acquisition, resource availability, and microbiota function [2]. For example, the intestinal microbiota of mammals has important functions in the host's metabolism, nutrient acquisition, and immune response [3], and is predominantly composed of phyla Firmicutes and Bacteroidetes [4]. Previous studies in humans and model organisms have shown that the intestinal microbiota is distinct from the microbiota of the mouth and stomach; however, intestinal microbiota has been shown to be more similar to fecal microbiota [5]. Consequently, studies in humans and animals have used fecal samples as a non-

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invasive sampling approach to gain insights into factors associated with intestinal microbiota diversity and composition [6, 7]. In our previous microbial studies of wildlife, we also used fecal material as a viable sampling source to characterize the presence of pathogens such as diarrheagenic *Escherichia coli* [8].

Given that the intestinal microbiota has been shown to be important for maintaining host health [5], there has recently been a marked increase in the number of studies that have characterized the intestinal microbiota of different species. With the advances in culture-independent sequencing technology, researchers have been able to overcome some of the difficulties associated with microbiological culture techniques, allowing a more comprehensive assessment of the diversity of microorganisms residing within different hosts, and an examination of factors and effects of the colonization of these microorganisms [1, 9, 10].

Previous studies have found that the composition of the intestinal microbial community is complex and dynamic, spatially, and temporally. In humans, for example, microbial communities of non-related individuals or individuals living far apart were found to be more distinct than individuals in close proximity or inhabiting similar environments [2]. Nevertheless, despite the large number of environmental and biotic forces affecting community composition and diversity, research has shown that the same core microbial species are maintained for long periods of time and are shared among individuals of the same host species [2, 11]. Although studies have shown that the same core microbial species exist within a species, there are certain factors that result in shifts in the microbial composition. Thus, questions have been raised about the factors that can contribute to changes in microbial communities. Previous studies have shown that major shifts in the microbial composition have been associated with illnesses [11] including infections caused by enteric pathogens [7]. Research in both humans and domestic animals have found that dietary changes as well as the presence of pathogens can affect the intestinal microbiota diversity and composition [5, 12, 13]. For instance, diet can affect the microbiota composition by modifying the gastrointestinal tract environmental conditions including pH, temperature, and oxygen levels [5]. Similarly, the presence of some pathogens has been linked to reductions in the microbiota diversity by displacing the niche of commensal microorganisms [8, 12, 13], while other pathogens contribute to an overgrowth of less beneficial phyla like Proteobacteria [7].

Unlike humans and domestic animals, few studies have been conducted to examine the intestinal microbiota composition in different wildlife species and to identify factors that impact compositional changes. Still, in few studies conducted on wildlife species, microbiota has been shown to include novel microbial species, increasing interest in investigating these wild hosts [14]. Because diets of wildlife species like white-tailed deer (*Odocoileus virginianus*) vary spatially and temporally (i.e., between seasons and due to landscape changes from anthropogenic activities like agriculture [15]) and are known reservoirs for many pathogens including diarrheagenic *E. coli* and *Salmonella* [8, 16–19], it is important to determine how these factors impact the intestinal microbial communities. Wildlife species such as white-tailed deer and the ecosystems they inhabit represent an interesting model to increase understanding of factors associated with inter-individual compositional differences and spatial and temporal dynamics of intestinal microbial community composition and diversity. Understanding of these effects on pathogen colonization in wildlife hosts can be important to identify new control points that may limit pathogen transmission to livestock and the likelihood of entering human food supplies.

White-tailed deer are a well-studied wildlife species that is widely distributed throughout North America. As ruminants, the white-tailed deer gastrointestinal system has a four-chambered stomach where fermentation of carbohydrates occurs, and intestines where absorption of water, electrolytes, and nutrients occurs [20]. Different microorganisms have been associated with the white-tailed deer gastrointestinal system. Some microorganisms in the rumen are producers of the enzymes required for the fermentation of cellulose, while other microorganisms are known pathogens that infect humans and domestic animals [8, 17, 21]. Nevertheless, white-tailed deer intestinal microbial communities and the factors that influence community diversity and composition are largely unknown. There are a few studies that have characterized microbial diversity in other cervids like roe deer (*Capreolus pygargus*) [22], sika deer (*Cervus nippon*) [23], and reindeer (*Rangifer tarandus tarandus*) [24], but to our knowledge, only one study by Gruninger et al. [21] has characterized the rumen microbiota of white-tailed deer using non-culture (16S rDNA next generation sequencing) methods. Thus, in this study, we sought to characterize microbial community diversity and composition of fecal samples collected from white-tailed deer using 16S rDNA pyrosequencing and to compare the microbial diversity and composition among samples as a function of sampling period (March/winter vs June/summer), sex, levels of host genetic relatedness, and spatial location. The second objective was to determine whether there was evidence of association between the presence of diarrheagenic *E. coli* and *Salmonella* on white-tailed deer intestinal microbial community diversity and composition.

Materials and Methods

Study Site and Sample Collection

This study was conducted at Michigan State University's Kellogg Biological Station (KBS), a field site for ecological and agricultural research located in Kalamazoo County, in southwest Michigan. The landscape at this site is a

characteristic of the upper Midwest regions of the USA. Different habitats within this location are highly fragmented and include agriculture fields consisting of corn, alfalfa, and soybeans as well as dairy pastures, hardwood forests, wetlands, streams, and lakes.

We used fecal samples as a non-invasive method to characterize the intestinal microbiota. Although samples had been exposed to the environment, studies have shown that samples exposed to room temperature (up to 3 days) do not show differences in microbiota composition compared to immediately frozen samples [25]. Nonetheless, different methods of DNA extraction have shown to affect the analyses' results [26]. Considering these limitations, we collected only fresh samples (as determined by dryness, texture, color, and firmness of fecal pellets), and all samples were stored and DNA was extracted equally to reduce biases.

Visually fresh samples of white-tailed deer, hereafter referred to as deer, feces were collected in March and June of 2012 as described previously [8]. 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 sterile plastic bags while walking transects and were assigned an individual identification number. Geographic locations were referenced for all samples using a handheld GPS unit (Fig. S1). All samples were stored at −80 °C after collection.

Deer DNA Isolation, Identification of Individual Deer, and Sex Determination

Extraction of deer genomic DNA was performed using a QIAamp DNA Stool isolation kit (Qiagen; Valencia, CA, USA) as described previously [8]. Briefly, eight fecal pellets from each individual sample were swabbed with a sterile swab on the pellet surface to obtain deer intestinal cells. Following suspension in ATL buffer, and DNA extraction, total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA, USA).

Eight microsatellite loci IGF1 [27], OBCAM [28], Cervid1, Cervid2 [29], Rt9, Rt23, Rt24, and Rt27 [30] were used to discriminate individual deer as described in a prior study [31]. Individual identification was determined by comparing the genotypes with the software Cervus 3.0 [32].

Deer sex was determined genetically based on gender-specific differences in the zinc-finger intron locus as described by Lindsay and Belant [33]. PCR conditions included an initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 35 s, annealing at 55 °C for 30 s, and an extension at 72 °C for 60 s. Positive controls for males and females and negative (no DNA) controls were used. Electrophoresis of the PCR products was carried out on a 1% agarose gel, where amplification of two bands indicated a male, and one band indicated a female.

Pathogen Detection

The methods for identifying diarrheagenic *E. coli* were described in our prior study [8]. Briefly, deer feces were added to 2X EC broth (Oxoid Ltd.; Waltham, MA, USA) supplemented with novobiocin (8 mg/l), rifampin (2 mg/l), and potassium tellurite (1 mg/l) for 24 h at 42 °C and subculture to CHROMoagar™ Shiga toxin-producing *E. coli* (STEC) (CHROMagar; Paris, France) and sorbitol MacConkey agar. Following overnight incubation at 37 °C, up to 20 single colonies were examined by PCR for the presence of one or more Shiga toxin genes (*stx1* or *stx2*) and the gene encoding the intimin (*eaeA*). Colonies (isolates) positive for at least one *stx* subtype were classified as STEC, while colonies containing *eae* and at least one *stx* subtype were classified as subset of STEC called enterohemorrhagic *E. coli* (EHEC). Isolates negative for any *stx* gene but positive for *eae* were classified as typical enteropathogenic *E. coli* (EPEC) if they contained the bundle forming pilus (*bfp*) gene; *eae*-positive isolates lacking *bfp* were classified as atypical EPEC [34].

Identification of *Salmonella* from deer fecal samples was performed as described previously [35]. Feces were weighed and diluted 1:10 in Tetrathionate Broth (TTB) and incubated at 37 °C for 48 h. 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 h. 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 at the Diagnostic Center for Population and Animal Health at Michigan State University's College of Veterinary Medicine.

Extraction of Intestinal Microbial DNA and 16S rRNA Sequencing

Fecal pellets from each sample were homogenized. The extraction of the microbial DNA was performed using QIAamp DNA stool kit (Qiagen; Valencia, CA, USA) following the manufacturer's protocol with slight modifications of longer bead beating time and a 95 °C denaturation step. In brief, 0.3 g of the homogenized sample was added to tubes with beads to break open the bacterial cells and separate the DNA. Total genomic DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific Inc.; Waltham, MA, USA).

Amplification of the 16S V5-V3 regions was performed by PCR using universal primers 375F and 926R tagged with 454 universal primers A and B as was previously described for human fecal samples [7]. In brief, PCR amplification using the AccuPrime™ Taq DNA polymerase (Invitrogen™; Carlsbad, CA, USA) involved an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s, extension at

72 °C for 5 min, and a final extension at 72 °C for 5 min. PCR was carried out in triplicates to get adequate concentration and volume of PCR products for sequencing. The amplicon library was purified using the Agencourt AMPure XP (Beckman Coulter, Inc.; Brea, CA, USA) beads, quantified by pico green assays (Quant-iT™, PicoGreen ® dsDNA quantification kit, Invitrogen™; Carlsbad, CA, USA) after normalization and pooling. Sequencing was performed using the 454 GS Junior (Roche; Branford, CT, USA).

Data Analysis

Raw sequence data have been deposited online in the Sequence Read Archive (SRA – NCBI) under the accession number SRP078941. Bacterial sequences were analyzed using the software QIIME [36]. First, all sequences were subjected to a quality control screen that included a noise reduction step using the denoise_wrapper.py script, followed by removal of short sequences, and sequences with barcode mismatches. All chimeras were detected and removed using the Uchime program [37]. Following quality control checking, any sample with less than 1000 sequences was not included in the downstream analysis. A distance matrix using 97% of similarity between sequences was used to define operational taxonomic units (OTU), and unique sequences were aligned using the Greengenes reference database [38]. Analyses of alpha diversity were performed using rarefaction curves generated based on number of OTUs and the Shannon diversity index. Principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity index was used to visually compare microbial composition differences as a function of sampling period (March, June), sex (male, female), and presence of a pathogen (STEC, EHEC, EPEC, and *Salmonella*). Unweighted (based on Jaccard's distance) and weighted (based on Bray-Curtis) distances were used to create cladograms. Analysis of similarity (ANOSIM) tests were used to assess significant differences in community composition between samples collected during different periods, for different sexes, and between individuals with and without enteric pathogens present, using the compare_categories.py script available in QIIME. LEfSe software [39] was used to graph the relative frequencies of each phylum from each sample and identify taxa that significantly correlated to differences between variables. As a measure of deer relatedness, the intra-individual genetic distance based on the genotype of eight microsatellite loci was calculated using the codon-genotypic option in the software GenAlEx [40, 41]. This genetic distance measure was used to build a neighbor-joining phylogenetic tree (Fig. S2) with the software Mega [42]. Finally, Mantel test [43] in the ade4 library (*R*) was used to quantify relationships between inter-individual genetic relatedness or spatial distance and inter-sample beta diversity overall and within sampling period, permutations was set to 9999, and the matrices were transform to a Euclidean one if necessary.

Results

Identification of Deer and Intestinal Microbial Community Composition

Sixty-six fecal samples were collected and sequenced during two sampling periods. Thirty samples were collected in March, and 36 were collected in June. Individual identification analysis based on multi-locus microsatellite genotypes revealed that within a sampling period, all samples were from different individuals, but between sampling periods, eight individuals were sampled once in each sampling period. Of the 58 deer sampled, 24 were from males and 34 from females. Of the eight deer sampled at each sampling period, two were males and six females.

Nineteen microbial phyla were identified from all 66 deer samples collected at both time periods, and less than 1% of the sequences were unclassified at the phyla level (Table 1). Over both sampling periods, phylum Firmicutes was the most abundant (range 0.8–85.1%, mean 55.3%), followed by phyla Proteobacteria (range 0.02–83%, mean 20.3%), and Bacteroidetes (range 2.1–46.4, mean 17.5%) (Table 1). At the genus level, 304 distinct genera were identified, although many sequences were not classified at the family (14.7%) or genus level (46.4%). The predominant genus was unclassified *Ruminococcaceae* (range 0.02–53.3%, mean 24.8%). The four most abundant genera contributed to 49% of the total microbial abundance (Table 2).

Table 1 Frequencies of five of the most common bacteria phyla found in 66 fecal samples of white-tailed deer overall and by sampling period

Phyla	Total	Percentage by sampling period	
		March	June
Firmicutes	55.26	66.13	46.20
Proteobacteria	20.25	6.41	31.78
Bacteroidetes	17.45	17.03	17.79
Tenericutes	3.61	5.96	1.65
Actinobacteria	1.78	2.70	1.01
Others	0.712	0.83	0.61
Unassigned	0.92	0.89	0.94

Other phyla represented in frequencies <1% over all periods included Cyanobacteria, Verrucomicrobia, Spirochaetes, Fusobacteria, Planctomycetes, TM7, Lentisphaerae, Synergistetes, Acidobacteria, Elusimicrobia, Chloroflexi, Nitrospirae, Gemmatimonadetes, and Fibrobacteres

Table 2 Frequencies of the 10 most common bacteria genera found in 66 feces samples of white-tailed deer overall

Taxa	%
Phylum: Firmicutes; order: Clostridiales; family: Ruminococcaceae; genus: unclassified	24.8
Phylum: Firmicutes; order: Clostridiales; family: unclassified	11.4
Phylum: Proteobacteria; order: Pseudomonadales; family: Moraxellaceae; genus: Acinetobacter	8.6
Phylum: Proteobacteria; order: Enterobacteriales; family: Enterobacteriaceae; genus: unclassified	5.1
Phylum: Tenericutes; order: RF39; family: unclassified	3.5
Phylum: Bacteroidetes; order: Bacteroidales; family: Bacteroidaceae; genus: 5–7 N15	2.8
Phylum: Firmicutes; order: Clostridiales; family: Lachnospiraceae; genus: unclassified	2.8
Phylum: Bacteroidetes; order: Bacteroidales; family: 24–7; genus: unclassified	2.7
Phylum: Firmicutes; order: Bacillales; family: Planococcaceae; genus: Solibacillus	1.9
Phylum: Firmicutes; order: Clostridiales; family: unclassified	1.9

Seasonal Variation in Intestinal Microbial Communities of Deer

Shannon alpha diversity indices for all samples ranged from 3.2 to 7.7. Rarefaction curves comparing samples from March and June revealed that samples from March had an overall higher alpha diversity than samples from June (Fig. S3). The mean Shannon alpha diversity index from March samples was 6.79, and only three samples had a diversity index less than 6. The mean Shannon alpha diversity index for samples from June was 5.64.

PCoA also revealed that the microbial communities of samples collected in June were taxonomically more differentiated than samples collected in March, which tended to cluster close together (Fig. S4). The microbiota composition differed significantly between samples collected in March and June (ANOSIM test: $R = 0.211, p < 0.001$). As shown in the weighted cladogram based on Bray-Curtis distance, samples from June (bold black ID#) were more distinct from each other, while samples from March (light gray ID#) were compositionally more similar (Fig. 1). Examination of the microbial composition between sampling times demonstrated that the abundance of Firmicutes was higher in the March samples relative to the June samples (Fig. 2a). Although the abundance of Bacteroidetes was similar between sampling periods (Fig. 2b), and the relative abundance of Proteobacteria was significantly lower in March versus June samples (Fig. 2c). It is interesting to note that several phyla were only found in one sampling period. The phylum Gemmatimonadetes (0.001%), for example, was only found in March, whereas Fusobacteria (0.04%) was only found in June. Samples from June were generally characterized by a high relative abundance of Proteobacteria and lower relative abundance of Firmicutes. The samples from June also showed an association between low abundance of Proteobacteria (Firmicutes/Proteobacteria ratio >1) with high alpha diversity, and high abundance of Proteobacteria (Firmicutes/Proteobacteria ratio <1) with low alpha diversity (Fig. 3).

Eight individuals were sampled in both March and June. Fecal samples from these individuals were not found in the same spatial location. Phyla relative abundance in samples from all eight individuals changed from March to June, and the variation in relative abundance of the most abundant phyla between one sampling time to the next was different in each individual (Fig. 4).

Association Between Microbial Community Composition and Deer Sex, Relatedness, and Location

Rarefaction curves comparing samples between sexes did not show any difference in alpha diversity (Shannon diversity index male mean 6.39, female mean 6.01). Males and females were closely clustered when compared using both weighted and unweighted beta diversity measures (Figs. 1 and S5, respectively). Similarly, analyses comparing the composition and relative abundance of taxa between sexes were not significantly different (ANOSIM: $R = 0.001$, $p > 0.05$), and no significant association was observed between sexes by sampling periods (March and June) separately (data not shown).

Inter-individual genetic relatedness between deer hosts was not correlated with the microbiota composition (Mantel: $r = 0.099$ $p > 0.05$). By sampling period, results showed no significant association between microbiota taxonomic composition and inter-individual host relatedness in March (Mantel: $r = 0.084$, $p > 0.05$), and in June, the correlation was significant, but the r value was close to 0 (Mantel: $r = 0.013$, $p < 0.05$).

Significant correlation was observed between spatial location and microbial composition when analyses were conducted by sampling period (Mantel: March $r = 0.309$, $p < 0.05$; June $r = 0.126$, $p < 0.05$).

Correlation Between Pathogen Presence and Deer Microbiota Profiles

Twenty of the 66 samples (30.3%) were positive for at least one of the four pathogens examined, two samples were positive for EHEC, 14 were positive for EPEC (8 atypical and 6

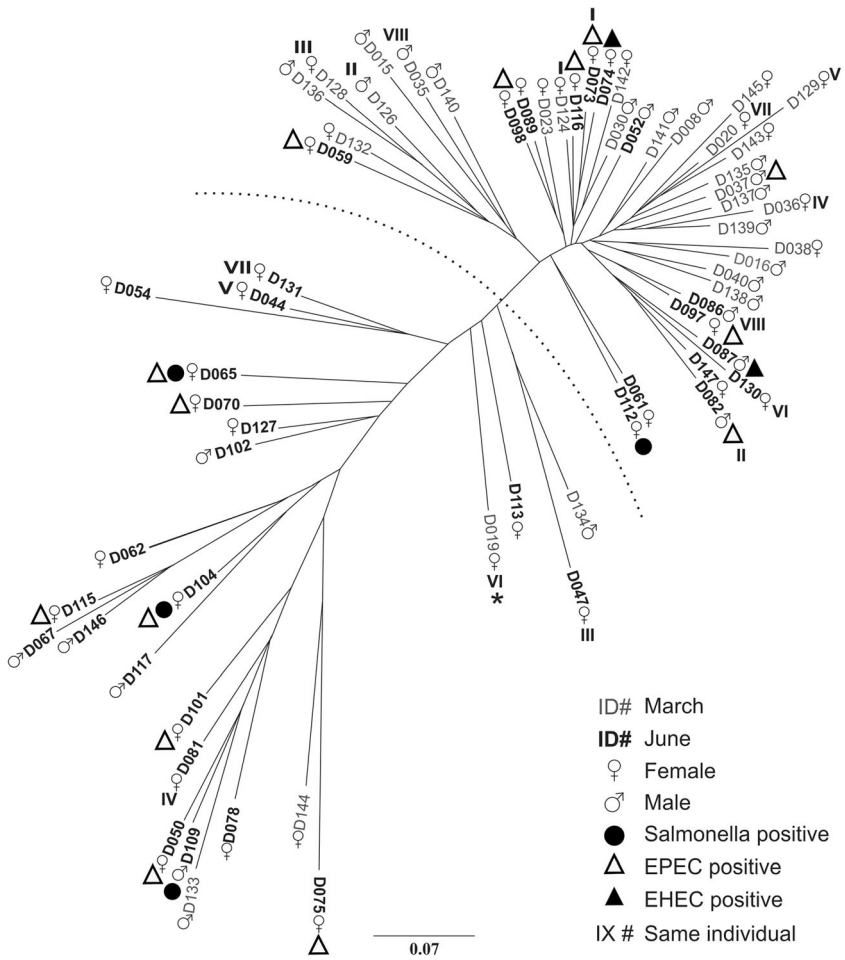

Fig. 1 Cladogram based on the weighted beta diversity distances (Bray-Curtis) showing differences in microbial community composition among the fecal samples. Samples *left and below the dotted line* have Shannon diversity indices lower than 6 except for one sample D019 (*). Sampling period is indicated by *different colors* (March-*light gray*, June-*bold black*). The presence of pathogens is indicated by symbols (*white triangle*: EPEC, *dark triangle*: EHEC, *dark circle*: *Salmonella*). *Roman numbers* indicate individuals that were collected in both sampling periods (see Fig. 4 for additional details)

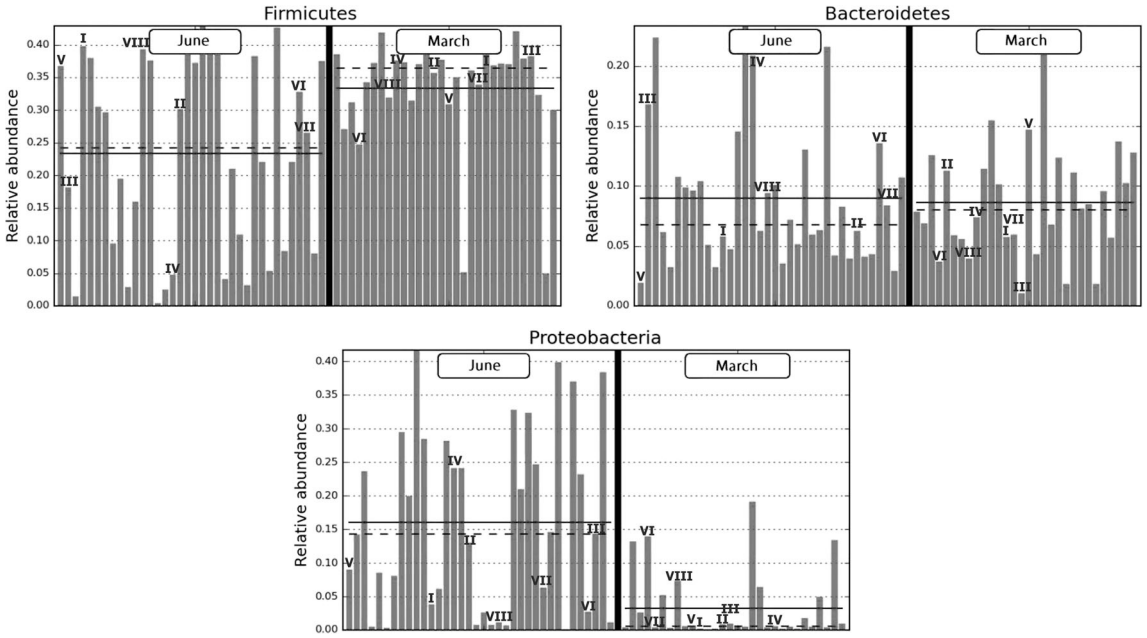

Fig. 2 Histograms describing the relative frequencies of selected phyla in white-tailed deer fecal samples collected during June and March. Each *bar* represents one fecal sample. **a** Phylum Firmicutes. **b** Phylum Bacteroidetes. **c** Phylum Proteobacteria. Means are shown in *straight lines* and medians in *dashed lines*. *Roman numbers* indicate the eight individuals that were collected in both sampling periods (as also shown associated with the entire communities in Fig. 1)

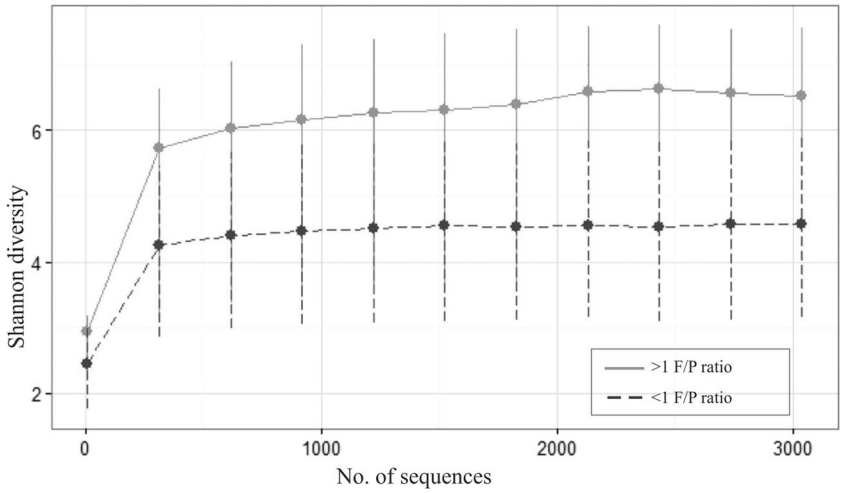

Fig. 3 Rarefaction curve of Shannon diversity index of samples of white-tailed deer collected in June. The *dark gray line* indicates the average diversity of samples with a Firmicutes:Proteobacteria ratio of >1, while the *light gray line* indicates the average diversity of samples with a Firmicutes/Proteobacteria ratio of <1

typical), and four samples had *Salmonella*. Shannon diversity rarefaction plots revealed no significant difference in microbial community diversity between EPEC and EHEC positive and negative samples (data not shown). EPEC-positive samples had variable Shannon diversity indices ranging from 3.2 to 7.6 (average = 5.76), while EHEC-positive samples average Shannon diversity ranged from 6.3 to 6.4 (average = 6.38). EPEC- and EHEC-positive samples were mainly from June, except for one EPEC-positive found in March. The increased abundance of representative taxa from the *Enterobacteriaceae* family was more prevalent in June samples compared to March samples (Fig. S6). However, as shown in the cladogram, no significant association was observed between the presence of diarrheagenic *E. coli* and overall microbial community composition (Figs. 1 and S5). When considering only samples collected in June, samples positive for EPEC and EHEC were characterized by higher abundance of members of the order Clostridia including from the *Ruminococcaceae* family compare to negative samples (average: positive = 23%, negative = 17%), and a higher abundance of Gammaproteobacteria including the genus *Providencia*, member of the *Enterobacteriaceae* family (average: positive = 0.5%, negative = 0.05%), while samples negative for EPEC and EHEC were characterized by a higher abundance of the genera *Acinetobacter* (average: positive = 12%, negative = 17%) and *Solibacillus* (average: positive = 1.3%, negative = 4.9%).

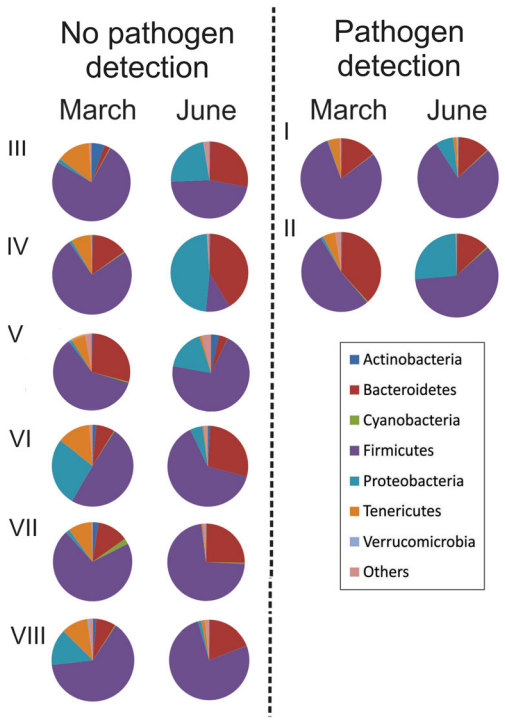

Fig. 4 Microbiota composition by phyla of eight individuals (*roman number*) sampled in March and June. No pathogen detection indicates that in both sampling time were pathogen negative by culture. Pathogen detection indicates that the sample was EPEC negative in March but positive in June

Two individuals (I and II) out of the eight sampled at both time periods shed the EPEC pathogen (Fig. 4). Microbial composition between individuals free of enteric pathogens and individuals that EPEC was detected showed differences including an increase of the relative abundance of Bacteroidetes (average 9%) in non-EPEC carriers and increase of the relative abundance of Proteobacteria (mean 15.7%) in EPEC carriers (Fig. 4).

In the case of *Salmonella*, four samples collected from June were found to be positive. The rarefaction plot of the Shannon alpha diversity showed that microbial communities of *Salmonella*-positive samples were less diverse than negative samples from June (mean diversity positive = 5.04, negative = 5.56). When comparing the composition of the 10 most abundant bacteria genera in *Salmonella* infected and non-infected samples, the prevalence of the most predominant genus (*Ruminococcaceae* family) was lower (average: negative = 33%, positive = 26%) in samples with *Salmonella*. Seven out of the 10 most abundant genera were decreased in abundance when *Salmonella* was present (Fig. S7). In contrast, the prevalence of genus *Acinetobacter* was higher in

Salmonella-positive samples (average: negative = 20%, positive = 34%; Fig. S7), as well as the unclassified genus of the family S24–7 (average: negative = 1.8%, positive = 6.1%; Fig. S7).

Discussion

Studies of intestinal microbiota have shown the importance of these commensal organisms to host health. Still, studies of microbiota of wildlife species, particularly focusing on how ecological factors influence the spatial and temporal dynamics of these communities, are limited. We described the microbiota of 66 fecal samples of deer from an agroecosystem and how ecological and biological factors influenced host community diversity and composition. This study's main findings revealed that deer intestinal microbial diversity and composition appear to be influenced by season and vary with the presence of diarrheagenic *E. coli* and *Salmonella*.

Our results showed that the intestinal microbiota of deer is composed primarily of three phyla: Firmicutes, Proteobacteria, and Bacteroidetes. The higher prevalence of Firmicutes (mean 55.3%) and Bacteroidetes (mean 17.5%), compared to other phyla, is consistent with the studies of other domestic and wild ruminants such as cattle (Firmicutes 62.8%, Bacteroidetes 29.5%) and roe deer (Firmicutes 56.4%, Bacteroidetes 37%) where Firmicutes and Bacteroidetes were the dominant phyla [6, 22]. Conversely, the high abundance of Proteobacteria (range 0.002–83%, mean 20.3%) distinguishes white-tailed deer intestinal microbiota from the microbiota reported from roe deer (~2%) and cattle (4.4%). Proteobacteria is a diverse phylum that includes important pathogens [44]. The microbial diversity of white-tailed deer (mean Shannon index = 6.16) was lower than reported in other wild ruminants like roe deer (mean Shannon index = 8.4), although the sample size in roe deer study was lower ($n = 3$) [22]. The high abundance of Proteobacteria and low abundance of Firmicutes (Firmicutes/Proteobacteria ratio <1) may be responsible for low microbial diversity (Fig. 3). This observed microbial compositional shift to a Proteobacteria dominant community has been seen before in murine model individuals treated with antibiotics [45], and in calves antibiotics have shown to impact the composition of fecal microbiota [46]; however, livestock in KBS were not reported to be under antibiotic treatment.

Analyses comparing microbial communities in March and June showed that microbiota diversity and composition changed between seasons. March samples had higher alpha diversity (Shannon index) than samples from June, and the high abundance of Proteobacteria was found to distinguish June samples (Fig. 2). Studies have shown that diet is an important factor shaping the intestinal microbial communities in many species such as humans and domestic animals like cattle [47]. Diet is expected to change with season, and behavioral studies of deer have demonstrated that during winter, the protein consumption of deer decreases as food availability and quality decreases [15]. Nevertheless, in agricultural settings such as KBS, it is likely deer shift their diet to plants of high nutritional quality in summer. Thus, the changes in vegetation available associated with crops planted and the modification of deer feeding habits according to season may explain the differences in microbiota diversity and composition. Furthermore, summer months have been associated to peak levels of fecal shedding in many pathogens such as pathogenic *E. coli* [48]. Thus, we hypothesize that seasonal increases in temperature coupled with increasing prevalence of pathogens can lead to higher prevalence of Proteobacteria. Further studies evaluating the microbiota in other seasons would be important to evaluate the seasonal dynamics and to determine deer intestinal microbiota stability which can enable detection of when the community is in dysbiosis.

We hypothesized that demographic factors such as sex, levels of host genetic relatedness, and spatial locations would have an effect on deer intestinal microbiota diversity and composition; however, results showed no significant difference of the microbiota associated with sex and host genetic relatedness. Sex was thought to be important due to differences in movements and feeding behavior between males and females. The lack of influence of sex on the taxonomic richness and composition of deer intestinal microbiota may be due to the fact that deer are behaviorally plastic, specifically under different habitat conditions [49]. Also, deer behavior has been shown to be altered in an agroecosystem due to anthropogenic influences, and they inhabit larger home ranges that overlap between social groups [49]. Furthermore, a study that compared the digesta and ruminal content between male and female deer found differences only in lactating females, which had longer intestinal tracts and greater ruminal content [50]. Our data did not distinguish between lactating and non-lactating females, yet even then, although the composition of digesta may be different, the diet quality is likely to be similar leading to a similar microbial community composition.

Microsatellite analyses allowed us to identify samples from the same individual collected during different periods and levels of genetic relatedness between individuals. We hypothesized that closely related individuals would possess taxonomically similar microbial communities because they were more likely to exhibit similar behaviors and would forage in similar locations. 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 [51]. However, Mantel tests did not reveal significant correlations between inter-individual microbiota beta diversity and levels of genetic relatedness. This result may be due to the fact that the study was conducted in an agricultural landscape. In agricultural settings, deer have been shown to increase home range size [49], and there was substantial overlap of individuals within social/kin groups. Thus, individuals

from the same or different social groups would be expected to share the same habitat and have similar diets, and therefore, intestinal microbiota should be similar as well. Nevertheless, a significant correlation was found between geographic proximity and fecal microbiota composition, indicating that deer in close spatial proximity likely have similar diets.

Eight individuals were sampled at both sampling time points. Results from these eight individuals confirm the dynamic nature of microbial communities, as all eight individuals showed temporal shifts in their microbial community composition (Fig. 4). Six of eight individuals did not acquire diarrheagenic *E. coli* or *Salmonella*. Comparing the difference in community composition, results showed that individuals that had not acquired pathogens were characterized by a higher relative abundance of Bacteroidetes. These data are consistent with findings from humans, where a study found an inverse correlation between infection with *Clostridium* pathogen and Bacteroidetes abundance [52]. Nevertheless, a larger sample size and further analyses are necessary to conclude that higher abundance of Bacteroidetes has a protective function against pathogens.

Diarrheagenic *E. coli* have been shown to reduce microbiota diversity in different hosts such as humans [7]. However, studies of Shiga toxin-producing *E. coli* in cattle and other ruminants suggest that these species lack the toxin receptor, inferring that this pathogen does not produce major effects on these hosts [53]. A recent study found no significant difference in alpha microbiota diversity between STEC-positive and STEC-negative cattle [54]. Nevertheless, diarrheagenic *E. coli* have been reported to cause diarrhea in calves suggesting they have an effect in the host [55]. Comparisons of overall microbial diversity revealed no differences between EPEC and EHEC carriers and non-carriers (Fig. 1). Although the relative abundance of some taxa showed to be higher or lower when considering the presence of EPEC and EHEC; however, it is difficult to determine if these observed differences are due to the presence of diarrheagenic *E. coli*. Given the limited sample size of positive EPEC and EHEC samples, further studies of the role of diarrheagenic *E. coli* in deer health are warranted, especially because earlier studies have shown a direct transmission of diarrheagenic *E. coli* between deer and domestic animals like cattle [8].

Microbial communities of *Salmonella* carriers were characterized by a lower alpha diversity compared to non-carriers, suggesting that this pathogen may alter the microbiota. This result was expected given the mechanism that *Salmonella* uses for colonization. *Salmonella* usually colonizes the ileum or colon and takes advantage of host inflammatory response to grow and outcompete commensal microorganisms [56]. Moreover, the presence of *Salmonella* was associated with a significant increase in abundance of the Proteobacteria such as the genus *Acinetobacter* (Fig. S7). Unfortunately, the small sample size of *Salmonella*-positive individuals does not allow strong conclusions. Further studies are warranted to further examine the effects of *Salmonella* on deer microbiota.

In conclusion, our results have revealed that deer intestinal microbiota is a complex and taxonomically plastic community. Microbiota composition shifted by season, likely due to changes in the environment including diet modifications. Furthermore, deer microbiota in the warmer season was found to have an unexpectedly high abundance of Proteobacteria, with 15 of 36 samples having more sequences assigned to Proteobacteria than Firmicutes. Although sample sizes were small, positive carriers of *Salmonella* and diarrheagenic *E. coli* appeared to be associated with the presence and/or abundance of certain taxa. Results showed that white-tailed deer are reservoirs of pathogens that may contribute to infections in domestic animals and humans. Given that agroecosystems are places of complex and frequent interactions between wildlife, livestock, and humans, further research is necessary to determine the role of wildlife species such as deer in the transmission of different pathogens and the effect of these pathogens on the health of wildlife.

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