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Original article

Influence of tick sex and geographic region on the microbiome of *Dermacentor variabilis* collected from dogs and cats across the United States

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ABSTRACT

As tick-borne diseases continue to increase across North America, current research strives to understand how the tick microbiome may affect pathogen acquisition, maintenance, and transmission. Prior high throughput amplicon-based microbial diversity surveys of the widespread tick *Dermacentor variabilis* have suggested that life stage, sex, and geographic region may influence the composition of the tick microbiome. Here, adult *D. variabilis* ticks ($n = 145$) were collected from dogs and cats from 32 states with specimens originating from all four regions of the United States (West, Midwest, South, and Northeast), and the tick microbiome was examined via V4-*16S* rRNA gene amplification and Illumina sequencing. A total of 481,246 bacterial sequences were obtained (median 2924 per sample, range 399–11,990). Fifty genera represented the majority (>80%) of the sequences detected, with the genera *Allofrancisella* and *Francisella* being the most abundant. Further, 97%, 23%, and 5.5% of the ticks contained sequences belonging to *Francisella* spp., *Rickettsia* spp., and *Coxiella* spp., respectively. No *Ehrlichia* spp. or *Anaplasma* spp. were identified. Co-occurrence analysis, by way of correlation coefficients, between the top 50 most abundant genera demonstrated five strong positive and no strong negative correlation relationships. Geographic region had a consistent effect on species richness with ticks from the Northeast having a significantly greater level of richness. Alpha diversity patterns were dependent on tick sex, with males exhibiting higher levels of diversity, and geographical region, with higher level of diversity observed in ticks obtained from the Northeast, but not on tick host. Community structure, or beta diversity, of tick microbiome was impacted by tick sex and geographic location, with microbiomes of ticks from the western US exhibiting a distinct community structure when compared to those from the other three regions (Northeast, South, and Midwest). In total, LEfSe (Linear discriminant analysis Effect Size) identified 18 specific genera driving these observed patterns of diversity and community structure. Collectively, these findings highlight the differences in bacterial diversity of *D. variabilis* across the US and supports the interpretation that tick sex and geographic region affects microbiome composition across a broad sampling distribution.

1. Introduction

Ticks and tick-borne diseases (TBD) have been studied and recognized as dangerous for many decades but expanding geographic ranges of ticks and identification of novel pathogens, along with other discoveries, have led to renewed research interest in ticks and the disease agents they transmit (Beard et al., 2021). For example, the American dog tick, *Dermacentor variabilis*, a common tick on animals and humans and vector of *Rickettsia rickettsii* (Rocky Mountain spotted fever) and other pathogens such as *Francisella tularensis*, has expanded its eastern and central United States (US) distribution to more northern and western regions due, in part, to changing landscape and host availability (Dergousoff et al., 2013; James et al., 2015; Minigan et al., 2018; Sonenshine, 2018; Lehane et al., 2020; Duncan et al., 2021). Additionally, the ability to detect TBD agents has improved leading to identification of novel, emerging pathogens. For instance, a newly identified novel spotted fever group *Rickettsia* has been implicated as the cause of fever and hematological abnormalities in dogs from the southcentral US; however, the tick vector, if any, and the ability to cause disease in humans has not yet been fully determined (Wilson et al., 2020).

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One expanding avenue of TBD research is the study of the tick microbiome. Previous studies have documented the influence of the resident microbiota on the introduction, presence, and persistence of pathogenic bacteria (Burgdorfer et al., 1981; Narasimhan and Fikrig, 2015). Next generation sequencing (NGS) technologies allow the characterization of tick microbiomes through shotgun or amplicon sequencing (Greay et al., 2018; Bonnet and Pollet, 2020). These technologies have demonstrated the complex and dynamic nature of the tick microbiome, and identified a strong pattern of tick species specificity (Hawlena et al., 2013; Bonnet et al., 2017; Chicana et al., 2019). Even though the tick microbiome also consists of viruses and eukaryotes, the most abundant microorganisms are bacteria, especially endosymbionts such as *Francisella* or *Rickettsia* spp. (Rynkiewicz et al., 2015; Varela-Stokes et al., 2017; Greay et al., 2018). Factors such as blood feeding, tick life stage, geographic origin, and vertebrate host appear to affect the tick microbial community to varying degrees as ticks may acquire agents vertically, horizontally, or through interactions with their host and environment (Narasimhan and Fikrig, 2015; Varela-Stokes et al., 2017; Bonnet and Pollet, 2020).

Dermacentor variabilis is commonly encountered on pets and people, widely distributed across the US—including the western population known to some as *Dermacentor similis* sp. nov.—, and serves as vector for medically relevant pathogens (Eisen et al., 2017; Duncan et al., 2021; Lado et al., 2021). However, information on the *D. variabilis* microbiome is currently sparse. A few prior studies suggested a preponderance of *Francisella* spp., but the examined factors impacting patterns of diversity and community structure were inconclusive and even contradictory (Rynkiewicz et al., 2015; Chicana et al., 2019; Travanty et al., 2019; Lado et al., 2020). For instance, some data suggest the geographic origin of *D. variabilis* populations appears to have a significant effect on the microbial structure, while data from other populations show effects of geography on microbiome composition are relatively insignificant (Clow et al., 2018; Chicana et al., 2019; Lado et al., 2020). Further, since these studies examined field-collected ticks, the effects of a blood meal on *D. variabilis* have not been fully assessed or compared. Here, we report on the results of a national survey of *D. variabilis* using *16S* rRNA amplicon sequencing. The survey encompasses analysis of the individual microbiome of 145 ticks (male and female) obtained from cats and dogs from four distinct regions in the US (Northeast, South, Midwest, and West) with broad geographic representation for the current known distribution of this species (Duncan et al., 2021). Phylogenetic, co-occurrence, alpha diversity, community structure, and multiple LEfSe (Linear discriminant analysis Effect Size) analyses were employed to examine and correlate diversity and community structure patterns.

2. Materials and methods

2.1. Tick selection and sampling

A total of 145 adult ticks (102 females and 43 males) from 32 states and four geographic regions (West, $n = 12$ ticks; Midwest, $n = 50$ ticks; South, $n = 51$ ticks; and Northeast, $n = 32$ ticks) were included in the current study (Fig. 1); regions were defined as previously described (Blagburn et al., 1996). Ticks were collected from 114 pets (136 ticks from 106 dogs and 9 ticks from 8 cats) by various veterinary professionals in 2019 and 2020 (see Saleh et al., 2019 for details) (Table S1). Identification of *D. variabilis* from the West, where *D. andersoni* is also present, was confirmed by *ITS-2* sequence as previously described (Dergousoff and Chilton, 2007; Duncan et al., 2021). As mentioned earlier, the western population of *D. variabilis* may be considered by some to be the newly proposed species *D. similis* sp. nov. (Lado et al., 2021); however, the method for distinction is not accepted by all and the current study will utilize the historical name *D. variabilis* for that region. Because evaluated ticks were attached to and removed from pets, all ticks were considered to have taken a blood meal although the level of engorgement varied.

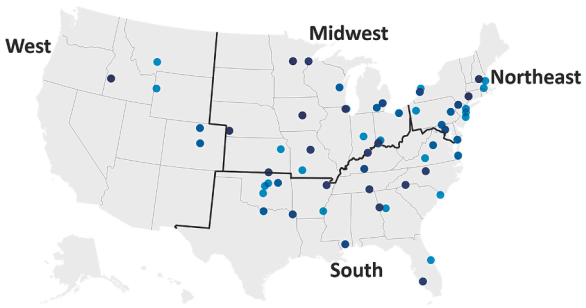

Fig. 1. Geographic distribution of sampled *Dermacentor variabilis* collected from dogs and cats as part of an ongoing national tick survey of dogs and cats (showusyourticks.org). Darker symbols indicate more than one tick was sampled from pets in that location (up to 5 ticks from each state were evaluated). The figure was created using datawrapper.de and Microsoft PowerPoint®.

2.2. DNA extraction, PCR amplification, and Illumina sequencing

Prior to dissection, evaluated ticks ($n = 145$) were washed with 3% bleach, distilled water, and 95% ethanol twice as previously described (Lado et al., 2020). Subsequently, each tick was individually dissected and DNA from the composite internal tissues was extracted using a commercial blood and tissue kit (Qiagen®, Hilden, Germany) according to manufacturer's instructions. DNA obtained was quantified using Qubit® fluorometer (Life technologies®, Carlsbad, CA, USA) and used as template for amplifying the V4 hypervariable region of *16S* rRNA gene using the prokaryotic-specific primer pair 515-F and 805-R (Caporaso et al., 2011) with modifications to include sequencing adaptors. Amplification and lack of detectable contamination was confirmed using gel electrophoresis. Amplicons were purified using PureLink™ PCR purification kit (Invitrogen, Waltham, MA, USA) and barcoded using Nextera XT V2 Index kit (Illumina, Inc., San Diego, CA, USA). Products were again confirmed, purified, and quantified in the manner described above. Per manufacturer instructions, all individual concentrations were diluted to 20 nanomoles and once pooled, the final concentration was diluted to 100 picomoles immediately prior to sequencing (Illumina, Inc.). Pooled products were then sequenced using a pair-end Illumina iSeq-100 platform, as previously described (Caporaso et al., 2012; Colman et al., 2019). A total of two runs were performed to cover all samples ($n = 145$); the first run contained 73 samples and 3 negative controls while the second had 73 samples (one tick was re-sequenced due to low sequence reads) and 2 negative controls. Negative controls (i.e., reagents only) were started at the extraction process and carried through sequencing. Even though ticks were cleaned prior to dissection and DNA extraction, the work was not performed in a sterile environment so trace bacterial sequences were expected and found in these controls (Narasimhan et al., 2021). The genera identified were not consistent across all controls and the majority were considered minor taxa (i.e., not within the top 50 most abundant genera detected in tick specimens). Because of this, the sequences from tick samples were deemed of good quality and uncontaminated.

2.3. Sequence processing, alignment, and taxonomy

Mothur (Schloss et al., 2009) was used for sequence processing. The majority of the steps were performed according to the MiSeq SOP (standard operating procedure) (available at http://www.mothur.org/wiki/MiSeq_SOP) on Pete HPCC server housed at Oklahoma State University. Briefly, sequences were screened to eliminate those with an average quality score <25, containing ambiguous bases (i.e., no ambiguous bases allowed), with a homopolymer stretch greater than 8 bases, and sequences shorter than 250 bp. Default parameters for make.contigs in mothur were used to merge paired ends. Remaining

high-quality sequences were grouped in one fasta file for subsequent analysis. Sequences were aligned using the recreated Silva (Release 132) SEED alignment database (downloaded from the mothur website in June 2021). Subsequently, to remove sequences with potential sequencing errors (Huse et al., 2010), a pre-clustering and de-noising step was performed using pre.cluster command in mothur with default parameters selected (1 mismatch for every 100 base pairs allowed and ≤ 3 base differences to be considered the same cluster). Possible chimeric sequences were identified and removed using the chimera.vsearch command (Rognes et al., 2016). Remaining sequences were then clustered into operational taxonomic units (OTUs) at the putative genus level (0.06; 94% identity) using the classify.seqs command in mothur (Wang et al., 2013). Sequences were classified against the Silva taxonomic outline (Release 132, https://www.arb-silva.de/). Rarefaction and summary.single command in mothur was used for coverage analysis.

2.4. Data analysis

Percent abundance of bacterial genera were used to create a heatmap of the top 50 most abundant genera using the Phyloseq package (Release 4.1.1; R Core Team). Fisher's exact tests were used for comparisons of the abundance of genera of interest (i.e., *Coxiella, Francisella, Rickettsia*) to variables including tick sex, host, and geographical region. To determine the biological interactions within the microbial communities, FastSpar (PMID: **30169561**) was used to calculate Pearson correlation coefficient matrices between the abundances of all possible pairs of genera constituting the top 50 abundances, as well as the p-value for the significance of the correlations (Watts et al., 2019). Correlation plots were created using the corrplot package in R. For genera present in at least 2 samples, and with a minimum of 100 sequences, abundances were used to calculate alpha diversity (Shannon, Simpson, Fisher) and richness measures (Observed, Chao, Ace) using the microbiome package in R. Box plots of these measures were created using ggplot2 in R, and Student's t-tests were used to calculate the significance of difference in alpha diversity and richness based on the ticks' sex or host, while a single factor analysis of variance (ANOVA) was used to calculate the significance of difference in alpha diversity and richness based on the geographical region of origin. Beta diversity indices (Bray Curtis) were calculated using Vegan package in R and used to construct non-metric multidimensional scaling (NMDS) biplots using ggplot2. Analyses of molecular variance (AMOVAs) were performed in mothur to test for the effect of ticks' sex, host, and geographical region on beta diversity measures; to confirm our findings, perAMOVA (adonis) was also performed using Vegan package in R. Additionally, the abundances of the 50 most abundant genera were used to perform a canonical correspondence analysis (CCA) using the Vegan package in R, followed by an ANOVA to test for the effect of different variables on the community structure. CCA plots were created using ggplot2. To determine the taxa most likely to explain differences between ticks grouped by significant tick variables, Linear discriminant analysis Effect Size (LEfSe) plots were created with the Huttenhower Lab Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011). For all analyses, level of significance was set at alpha = 0.05, and Bonferroni correction was applied when multiple comparisons were performed.

3. Results

3.1. Sequencing overview

A total of 481,246 high-quality bacterial sequences were collectively obtained from 145 *D. variabilis* samples with a median of 2,924 sequences per tick sample (average 3,334; range 399–11,990) (Table S1). Median coverage value was 0.68 (average 0.62) suggesting that, overall, the majority of diversity within the samples were captured using the sequencing depth employed.

3.2. Phylogenetic diversity of the D. variabilis microbiome

Taxonomy was assigned to sequences based on comparison to the Silva database at the putative genus level (0.06; 94% identity). Using this approach, sequences were assigned to 1,110 genera, 391 families, 216 orders, 83 classes, and 32 bacterial phyla. The genera with the top fifty abundances constituted 82% (95% CI 81.4–81.6) of the total number of sequences, with two genera (*Allofrancisella* and *Francisella)* being the most abundant (Fig. 2). Sequences affiliated with these two genera represented 41% and 13% of the overall sequences in the top 50 genera, respectively. In some ticks, one or both of these genera represented nearly the entire (>98%) community (e.g. tick 3824) (Table S2). However, in a few cases, (e.g. tick 4049), these two seemingly ubiquitous genera were detected at a negligible level (<0.1%), suggesting their presence is not required for the survival of *D. variabilis* (Table S2). Of the top 50 most abundant genera, some have previously been detected in *D. variabilis* (e.g. *Arsenophonus* and *Francisella),* others have been identified in ticks in general (e.g. *Acinetobacter, Coxiella, Pseudomonas* and *Rickettsia),* while others have not, to our knowledge, been reported to the genus level in ticks (e.g. *Allofrancisella,* the soil-associated genera *Nitrobacter, Variibacter*, and *Qingshengfania*, the genus *Spongiispira* previously identified only in marine sponges, and genera in the family Thioglobaceae known to be endosymbionts of marine bivalves) (Sorokin et al., 1998; Kaesler et al., 2008; Kim et al., 2014; Rynkiewicz et al., 2015; Zhang et al., 2015; Qu et al., 2016; Varela-Stokes et al., 2017; Clow et al., 2018; Kaufman et al., 2018; Sperling et al., 2020; Morris and Spietz, 2021).

3.3. Identification of potentially pathogenic genera and relative abundance in D. variabilis

Of special interest is the identification of members of the genera *Anaplasma, Coxiella, Ehrlichia, Francisella*, and *Rickettsia*, some of which are pathogenic and can be transmitted by *Dermacentor* species (Saleh et al., 2021). While *Anaplasma* and *Ehrlichia* were not detected in any of the samples, 97% (141/145; 95% CI 92.9–99.2), 23% (33/145; 95% CI 16.7–30.3), and 5.5% (8/145; 95% CI 2.7–10.7) of the evaluated specimens contained sequences of *Francisella* spp., *Rickettsia* spp., and *Coxiella* spp., respectively. The relative abundances of these pathogens per sample ranged between 0.02–57.8 for *Francisella,* 0.02–40.8 for *Rickettsia*, and 0.02–44.8 for *Coxiella* (Table S2). Tick variables (sex, host, and region of origin) had no significant effect on the abundance of each of these genera (Fisher's exact tests p-value ≥ 0.1).

3.4. Co-occurrence patterns within D. variabilis microbiome

Examination of a large number ($n = 145$, in this case) of tick microbial communities allows for a statistically robust analysis of positive and negative correlation patterns between members of the community. Analysis of the correlation coefficients of the top 50 most abundant genera identified five significantly (p-value = 0.001) strong (cutoff > 0.5) positive correlations (co-infection) and no significantly strong (cutoff < -0.5) negative correlations (exclusion) (Fig. 3). The significantly strong co-infections (and their coefficient values) were between *Spongiispira* and *JL-ETNP-Z34* of the Family Thioglobaceae (0.76), *Spongiispira* and *Allofrancisella* (0.62), unclassified genera in Family Staphylococcaceae and unclassified genera in Family Aerococcaceae (0.56), unclassified genera in Family Rickettsiaceae and *Rickettsia* (0.54), and *Moellerella* and *Arsenophonus* (0.52) (Fig. 3). Additionally, 17 weakly positive correlations (0.3 to 0.5) were found significant while none were weakly negative (-0.5 to -0.3) and significant (Fig. 3). Correlations containing genera *Francisella* spp., *Rickettsia* spp., or *Coxiella* spp. were of particular interest as they contain some pathogenic species; numerous significant correlations (34 positive and 26 negative) were determined, but in only one instance (*Rickettsia* to unclassified genera in Family Rickettsiaceae) was the correlation coefficient deemed strong

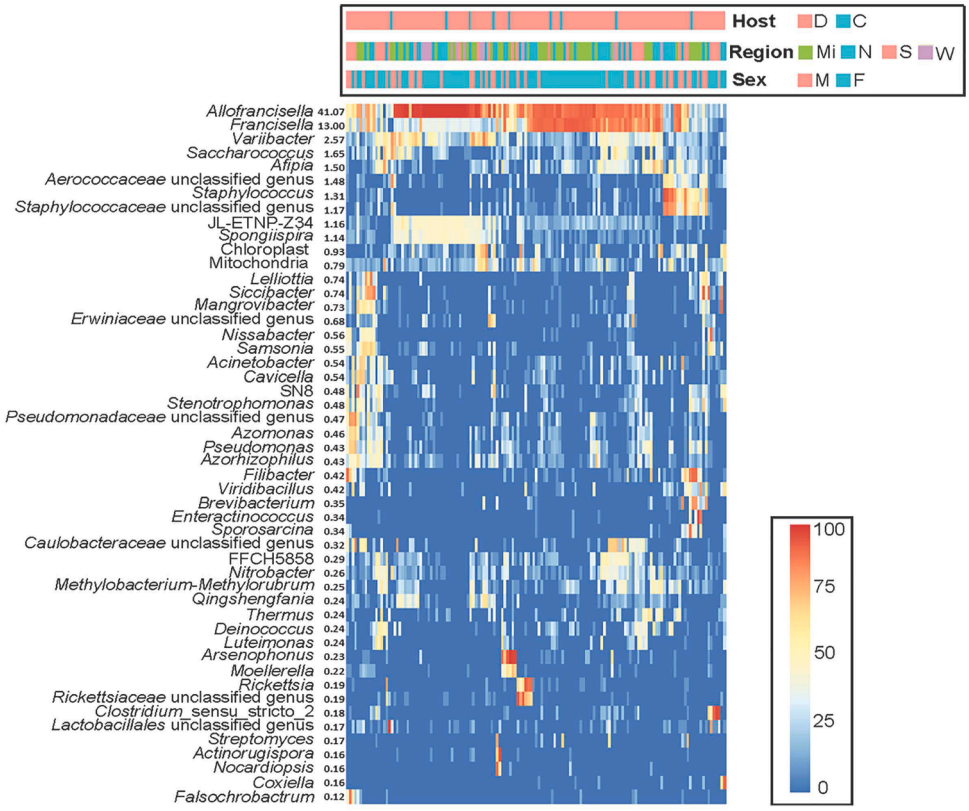

Fig. 2. Top 50 most abundant bacterial genera detected in *Dermacentor variabilis*. Percent abundance is shown next to each genus. Each column of data represents a single tick evaluated for a total of 145 ticks. The warmer the color, the more abundant the genus for that sample. Tick variables (host, region of origin, and sex) are shown at the top of the graph. D = dog, C = cat, Mi = Midwest, N = Northeast, S = South, W = West, M = male, F = female.

(0.54). Otherwise, no other pairs had correlation coefficients of substance (i.e. < -0.3 or > 0.3).

3.5. Diversity patterns in D. variabilis microbiome

Alpha diversity analysis was conducted on a set of 204 genera having at least 100 total sequences detected across all samples and that were present in at least 2 samples of *D. variabilis*. Observed species richness ranged between 13 and 101 genera that co-exist per sample (average 42 ± 20), with Chao estimator predicting a species richness ranging between 15 and 160 genera. The tick's sex and host had no significant effect on the tick microbiome species richness based on Student's t-test of Chao and Ace indices (p-values > 0.05). However, when the same analysis was performed on the observed values, tick sex, but not host, significantly impacted the species richness, with a greater richness identified in male ticks (p-value = 0.005, t-value 1.98) (Fig. 4). The variable with a consistently significant effect on all species richness indices was geographic region of origin based on single-factor ANOVAs (p-values < 0.0001). More specifically, ticks from the Northeast and West were significantly different in comparison to ticks from the other regions based on Student's t-tests (p-values < 0.01) with the northeastern ticks having the greatest species richness and the western ticks having the lowest.

On the other hand, comparison of alpha diversity measures (i.e. Shannon, Simpson, and Fisher indices) against tick variables revealed both tick sex (Student's t-test p-values < 0.01) and geographic region of origin (single factor ANOVA p-values < 0.01) had a significant impact on the tick microbiome alpha diversity, whereas the tick host did not (Student's t-test p-values > 0.5). In particular, male *D. variabilis* microbiome consistently had a significantly higher average alpha diversity than females (Student's t-test p-values < 0.01). Also, ticks from the Northeast harbored a significantly more diverse microbiome in all diversity indices (Student's t-test p-values ≤ 0.01), while ticks from the West region harbored a significantly less diverse microbiome in the Fisher index only (p-value = 0.003, t-value 2.09) (Fig. 4).

3.6. D. variabilis microbiome community structure (aka beta diversity)

Canonical correspondence analysis (CCA) based on the abundance of the top 50 most abundant genera, as well as non-metric multidimensional scaling (NMDS) based on all possible pairwise Bray Curtis indices were used to examine *D. variabilis* bacterial community structure. CCA was included in our analysis to show what structure differences, if any, were impacted by abundance. Our analysis demonstrated that tick sex (NMDS: p-value < 0.001, F value of 4.08; CCA: p-value = 0.05, F value of 1.32), and region of origin (NMDS: p-value = 0.001, F value of 2.28; CCA: p-value = 0.001, F value of 1.60) both had a significant effect on community structure, but the tick host did not (NMDS: p-value = 0.84, F value of 0.59; CCA: p-value = 0.8, F value of 0.58) (Fig. 5). For the NMDS data, a second test (perMANOVA) confirmed our findings that tick sex (p-value < 0.001; F value of 4.71) and region of origin (p-value < 0.001; F value of 2.12) had a significant effect. In particular, microbiome community structure for ticks originating in the West was significantly different from ticks originating in the Midwest (p-value = 0.003, F value of 4.10), Northeast (p-value < 0.001, F value of 3.51), and South (p-value = 0.005, F value of 3.42) (Fig. 5B).

LEfSe analysis was used to identify the community specific genera that are driving these differences. In particular, 11 genera (*Nitrobacter, Varribacter, FFCH5858* of the Family Beijerinckiaceae, unclassified genera in the Family Aerococcaceae, unclassified genera in the Family Staphylococcaceae, *Qingshengfania, Pseudomonas, Deinococcus, Afipia, Methylobacterium_Methylorubrum*, and unclassified genera in the Order Rickettsiales) were found to be more consistently abundant in males than in females, whereas *Francisella* and *Allofrancisella* were more

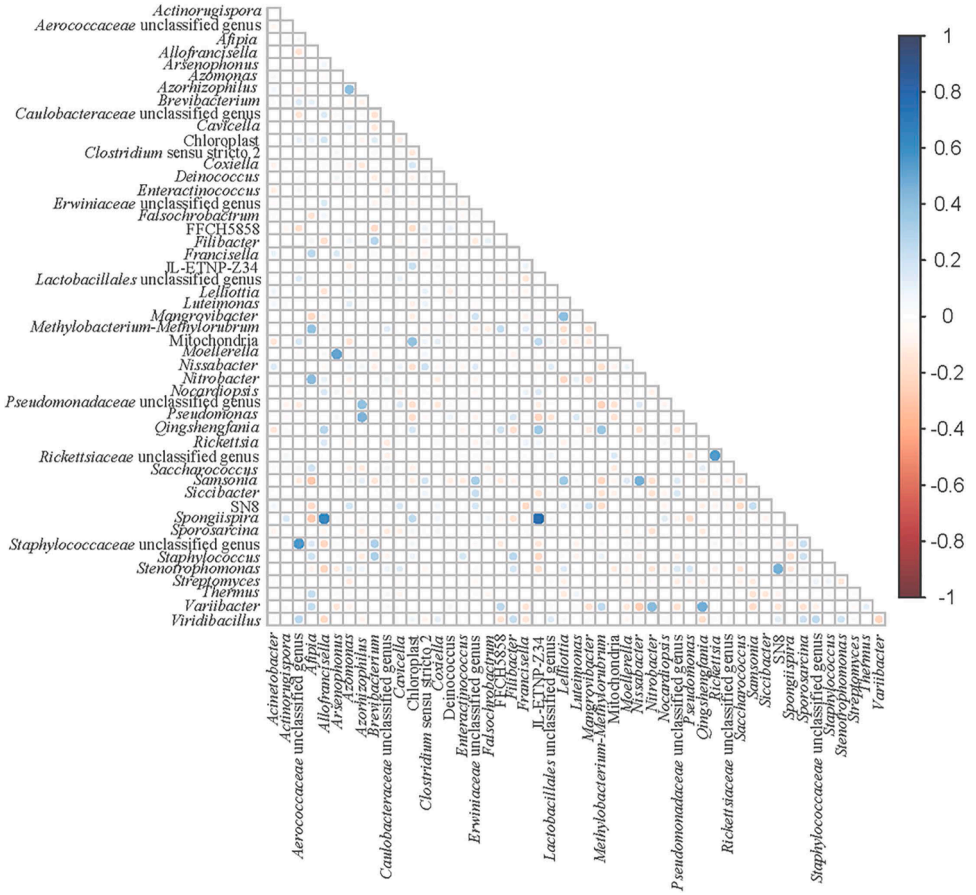

Fig. 3. Co-occurrence correlation coefficients for all possible pairs of genera within the top 50 most abundant taxa in *Dermacentor variabilis*. Only statistically significant correlations are depicted by a circle, with the size relating to the magnitude of the correlation coefficient (greater the circle, higher the correlation coefficient) and the color depicting the positive (blue) or negative (red) correlation.

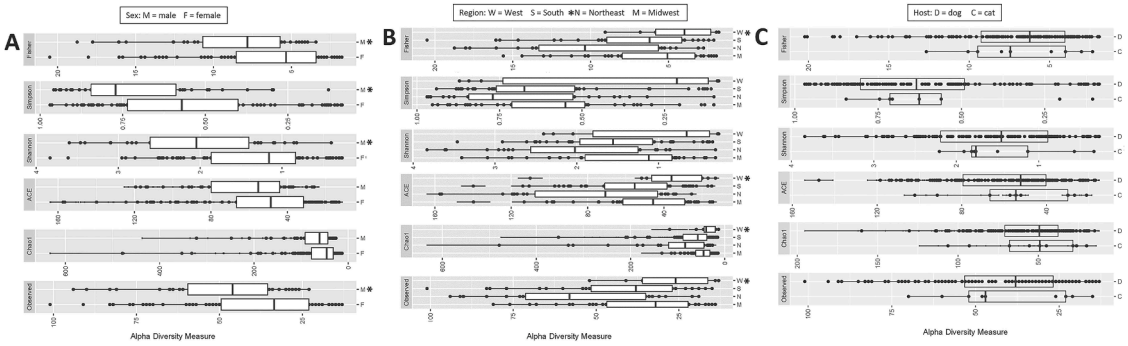

Fig. 4. Box plots of alpha diversity and richness measures for bacterial genera ($n = 204$) with at least 100 sequences detected and present in at least 2 specimens of *Dermacentor variabilis* by sex of tick (A), geographic region of origin (B), and tick host (C). Within the plots, an asterisk (*) denotes significant difference based on Student's t-tests.

consistently detected in females than in males (Fig. 6A). Geographically, ticks from the Northeast had five genera (*Clostridium, Deinococcus, Pseudomonas, Luteimonas*, and unclassified genera in the Family Caulobacteraceae) responsible for the significantly different microbial community when compared to other three regions, while ticks from the West were more significantly enriched in *Allofrancisella, Spongiispira*, and *JL-ENTP-Z34* of the Family Thioglobaceae (Fig. 6B).

4. Discussion

The current study sought to explore the effects of tick characteristics on their microbiome, and our results strongly suggest tick sex and geographic region of origin in the US (West, Midwest, South, or Northeast) influence the microbiome diversity and community structure patterns of adult *D. variabilis*. In particular, males had significantly higher alpha diversity measures than females, indicating a greater distribution of taxa across the male specimens. Other *D. variabilis* microbiome studies performed in North America either reported similar findings (Travanty et al., 2019) or insignificantly lower alpha diversity in males than females (Clow et al., 2018). It is also interesting to note that microbiome studies performed on other *Dermacentor* species demonstrated a similar sex difference to the current study; with males

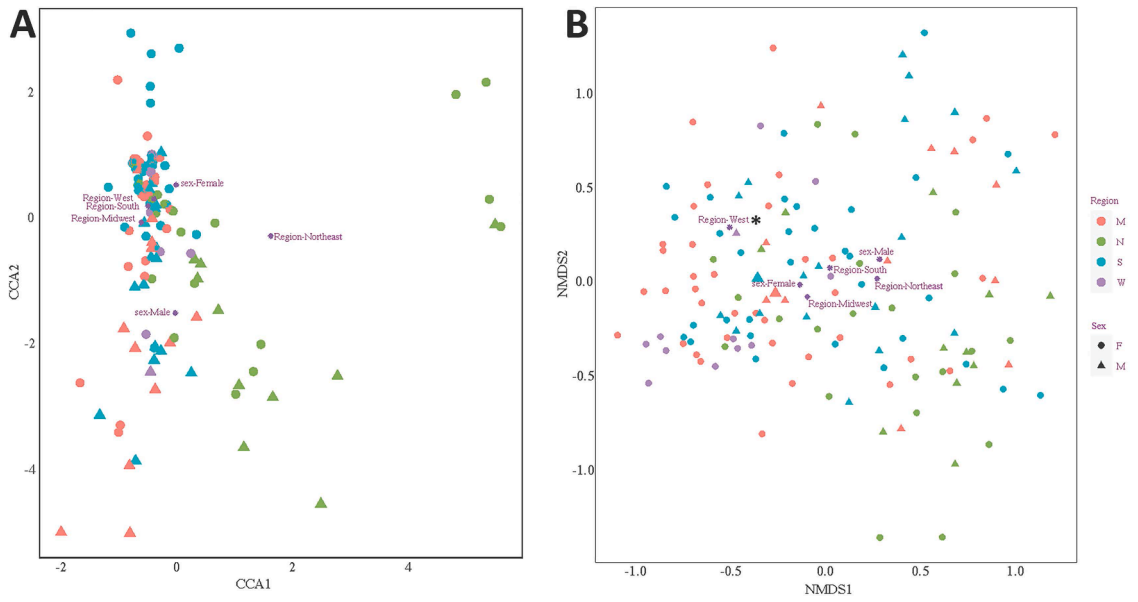

Fig. 5. Plots of multivariate analysis of bacterial beta diversity in *Dermacentor variabilis* through (A) canonical correspondence analysis (CCA) of the top 50 most abundant genera and (B) non-metric multidimensional scaling (NMDS) of all samples based on Bray-Curtis indices. Only significant variables were plotted and labeled as determined by ANOVA for CCA (p-value = 0.001 for region; p-value = 0.05 for sex) and for NMDS (p-value = 0.001 for region; p-value < 0.001 for sex). For plot B, ANOVA determined the West was significantly different from the other three regions and is denoted by an asterisk (*). Region: M = Midwest, N = Northeast, S = South, W = West; Sex: F = female, M = male.

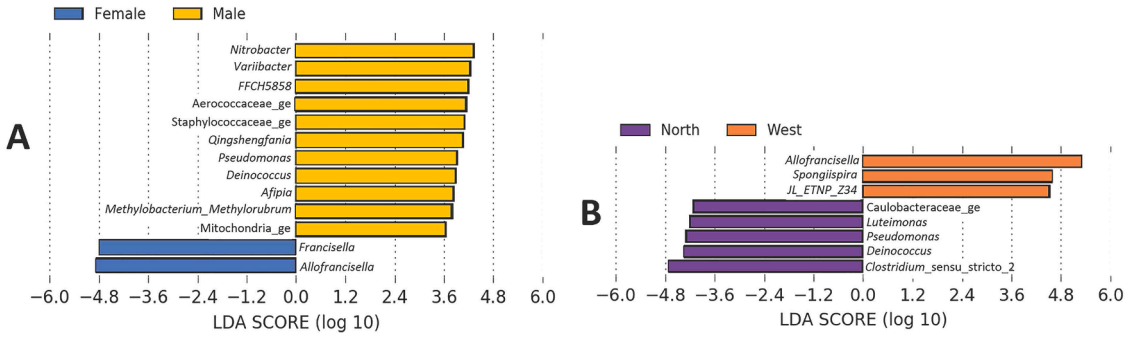

Fig. 6. Plot of LEfSe (Linear discriminant analysis (LDA) Effect Size) results of *Dermacentor variabilis* by tick sex (A) and tick region of origin (B). Only statistically significant (LDA score > 2) genera were included in the plots.

having greater alpha diversity scores than females (Zhang et al., 2019; Sperling et al., 2020; Elias et al., 2021). These findings may be partly explained through the variation of feeding behavior of adult ticks and increased number of male ticks examined in this study compared to previous *D. variabilis* microbiome investigations. In contrast to female ticks, males imbibe less blood during feeding due to their engorgement restrictions and commonly feed several times on the same, or different, host which may provide increased opportunities to acquire a variety of microorganisms (Scoles et al., 2005; Sonenshine, 2005; Nagamori et al., 2019). Male *D. variabilis* sampled here also had significantly different microbial community structure patterns (Fig. 5) than females and 11 genera were identified as the cause of such differences (Fig. 6).

Geographically, western US ticks had the lowest alpha diversity measures while northeastern US ticks held the highest alpha diversity measures. Additionally, the microbial structure of western *D. variabilis* was significantly distinct in comparison to ticks from the other three regions, which is in agreement with a recent study demonstrating western *D. variabilis* hosts a unique microbiome in comparison to *D. variabilis* collected elsewhere (Lado et al., 2020). Across various tick species, other microbiome studies that similarly sampled across a broad geographic area also found the region of tick origin played a significant role in shaping the microbiome (Van Treuren et al., 2015; Jia et al., 2020; Lado et al., 2020). These regional differences may be partially explained by the differing habitat and host availability, and thus a diversity in microorganism exposure (Bonnet et al., 2017; Kwan et al., 2017; Varela-Stokes et al., 2017; Narasimhan et al., 2021; Krasnov et al., 2022). Because different tick species are known to have distinct microbial communities (Kaufman et al., 2018; Chicana et al., 2019), another possible explanation for the significantly different microbiome in western *D. variabilis* may be due to the geographic isolation and adaptation of this population. Until recently, there was minimal connection of the *D. variabilis* population in the eastern and central US with the population along the Pacific Coast (Duncan et al., 2021). Therefore, we hypothesize these two populations may have divergently evolved according to varying environments which has led to distinct bacterial community structure differences between the two groups. Because other molecular work has also suggested this divergence, a name change of the Pacific Coast population of *D. variabilis* to *D. similis* n. sp. was recently proposed as mentioned earlier (Lado et al., 2021). However, until cross-breeding experiments can confirm the species differentiation, the appropriate designation of the Pacific Coast population remains unclear.

Even though the ticks used in the current study were collected from pets—and presumed to have taken a blood meal—tick host (dog or cat)

did not significantly affect the microbiome diversity or structure of this tick population. Active antimicrobial use Many more ticks from dogs ($n = 136$) than cats ($n = 9$) were included in the study and this may have contributed to this conclusion. However, our finding is in agreement with multiple prior studies comparing bacterial communities of ticks and their hosts where geography (i.e., environmental microbes) appeared to explain the tick microbiome specifics better than host characteristics (Rynkiewicz et al., 2015; Estrada-Peña et al., 2018; Lado et al., 2020). In fact, other analyses of various arthropod microbiomes found that many of the taxa identified have an environmental soil origin (Williams-Newkirk et al., 2014; Degli Esposti and Martinez Romero, 2017). Further, some prior studies demonstrating the impact of tick host on the tick microbial structure focused on blood meal effects on immature tick stages when the tick is known to have greater microbial diversity and richness in comparison to adults, and therefore the community is potentially not as stable (Swei and Kwan, 2017; Varela-Stokes et al., 2017; Chandra and Šlapeta, 2020; Narashimhan et al., 2021). Additionally, active antimicrobial use in vertebrate hosts could affect the bacterial community within a feeding tick (Mateos-Hernández et al., 2020); however, this information is unknown for the infested pets in which the sample population was collected from and cannot be factored into the findings. As the tick microbiome continues to be elucidated across various circumstances, these differences may then be more thoroughly explained.

Of the 391 families identified in the sample population, Francisellaceae was the most abundant and prevalent family in the adult *D. variabilis*. Together, two genera from this family, *Allofrancisella* and *Francisella*, constituted 54% of the overall sequences in the top 50 genera identified. Regardless of locale, other *Dermacentor* microbiome studies also found members of Francisellaceae as a common, or most common, taxon (Gall et al., 2016; Chicana et al., 2019; Travanty et al., 2019; Lado et al., 2020; Sperling et al., 2020). *Francisella* and *Francisella*-like organisms have long been associated with *Dermacentor* ticks as endosymbionts, and *Dermacentor* microbiome studies have further strengthened this association by classifying *Francisella* as part of the hypothesized core microbiome of this tick genus (Scoles, 2004; Ahantarig et al., 2013; Varela-Stokes et al., 2017; Kaufman et al., 2018; Chicana et al., 2019; Travanty et al., 2019). *Francisella*-like endosymbionts are found in large quantities in the ovaries and Malpighian tubules and aid in the synthesis of several critical vitamins and co-factors that ticks rarely obtain in high enough quantity from mammalian blood but require for nutrient processing (Rio et al., 2016; Duron et al. 2018; Gerhart et al., 2018; Bonnet and Pollet, 2020). Consequently, obligate endosymbionts are found more abundantly in ticks after feeding, and in adult ticks, particularly female ticks who take a larger blood meal (Duron et al., 2017; Travanty et al., 2019). In fact, *Francisella* was found in the current study to be more consistently abundant in females than in males (Fig. 6). Similarly, female *Dermacentor albipictus* and *D. variabilis* sampled in Alberta and western US, respectively, had higher proportions of *Francisella* than male or immature ticks sampled in the same areas (Chicana et al., 2019; Sperling et al., 2020). *Allofrancisella* is closely related to *Francisella,* and was somewhat recently proposed as a separate genus (Qu et al., 2016), although this position is still in debate (Kumar et al., 2020). Currently, reports on *Allofrancisella* are limited to its isolation from water systems; however, its physiological and biochemical characteristics appear to be similar to *Francisella* (Ottem et al., 2007; Qu et al., 2016; Öhrman et al., 2020). In general, tick-associated endosymbionts are often vertically obtained and appear to predominate the tick microbiome. In comparison to some other arthropods (e.g., fleas), ticks appear to have a less diverse microbial community which may be a result of this endosymbiont dominance (Lively et al., 2005; Hawlena et al., 2013). Since many ticks similarly host a strong endosymbiont community, the functional roles, if any, are hypothesized to also be conserved within a particular tick species (Bonnet and Pollet, 2020; Estrada-Peña et al., 2020).

Co-occurrence analysis via correlation coefficients was performed to examine the ecological interactions between genera through their coexistence within the tick microbial community. In total, 5 co-occurrences were found significantly strong (correlation coefficient $>$ 0.5 and p-value $<$ 0.05). These associations were between *Spongiispira* and *JL-ETNP-Z34* of the Family Thioglobaceae, *Spongiispira* and *Allofrancisella*, unclassified genera in Family Staphylococcaceae and unclassified genera in Family Aerococcaceae, unclassified genera in Family Rickettsiaceae and *Rickettsia*, and *Moellerella* and *Arsenophonus*. On the other hand, no significantly strong negative correlations were identified. In spite of limited previous reports on microbial co-occurrence patterns in the tick microbiome, our observed pattern of more positive than negative associations was similar to prior studies performed in other tick species (Williams-Newkirk et al., 2014; Couper et al., 2019). Although not fully understood, these co-occurrence relationships may be attributed to concurrent vertical or horizontal transmission over many generations (Degnan et al., 2009). If relationships hold true throughout a tick population over time, these symbiotic bacteria may present possible avenues of novel tick control such as genetic engineering of vectors or as vaccine targets (de la Fuente et al., 2017; Couper et al., 2019; Bonnet and Pollet, 2020; Mateos-Hernández et al., 2020; Wu-Chuang et al., 2021; Maitre et al., 2022). For instance, negative co-occurrence (i.e. co-infection found less often than expected) could imply infection with one microorganism prevents or discourages infection with the other (i.e. competitive exclusion), suggesting the potential use of microbial manipulation of tick endosymbionts in tick control efforts (Taylor et al., 2012; Williams et al., 2014; Bonnet and Pollet, 2020). Experimentally, nonpathogenic *Rickettsia peacockii*, an endosymbiont of *D. andersoni*, blocks establishment of the pathogenic *R. rickettsii* within the tick, and this is assumed to also occur in nature as suggested by the lack of Rocky Mountain spotted fever cases in a region where *R. peacockii* infected *D. andersoni* predominate (Burgdorfer et al., 1981). This concept is also supported by more recent work demonstrating the conserved nature of bacterial endosymbionts in individual tick species which potentially mask or limit the transmission of taxa between tick and host (Lively et al., 2005; Hawlena et al., 2013; Rynkiewicz et al., 2015; Bonnet and Pollet, 2020). Though promising, further work is likely still needed to determine the full function of these tick microorganisms before they can be used safely and effectively in tick or TBD control.

5. Conclusion

Understanding how the tick microbiome may affect pathogen acquisition is critical, given the fact that vector-borne diseases continue to increase in prevalence and diversity across North America. This study examined the microbial community structure in a large number ($n = 145$) of ticks collected from 32 states and all four US regions (West, Midwest, South, and Northeast). Our analysis demonstrated the role of geographic origin and tick sex in shaping the microbial community. These observations have the potential to serve as a basis for additional research on the mechanisms through which tick microbiome impacts the maintenance and transmission of medically significant microbes. Additionally, the continuing research on genera co-occurrence patterns could identify possible microbiome-manipulating strategies for novel tick-borne disease control.

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CRediT authorship contribution statement

Kathryn T Duncan: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Project administration. **Mostafa S Elshahed:** Writing – original draft, Writing – review & editing, Supervision. **Kellee D Sundstrom:** Investigation, Project administration.

Susan E Little: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Noha H Youssef:** Methodology, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Project administration, Supervision.

Declaration of Competing Interest

KD, ME, KS, SL, and NY have no competing interests to declare in relation to the subject matter contained within this manuscript.

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Supplementary materials

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