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Vertical vs. horizontal transmission of the microbiome in a key disease vector, *Ixodes pacificus*

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Abstract

Vector-borne pathogens are increasingly found to interact with the vector's microbiome, influencing disease transmission dynamics. However, the processes that regulate the formation and development of the microbiome are largely unexplored for most tick species, an emerging group of disease vectors. It is not known how much of the tick microbiome is acquired through vertical transmission vs. horizontally from the environment or interactions with bloodmeal sources. Using 16S *rRNA* sequencing, we examined the microbiome of *Ixodes pacificus*, the vector of Lyme disease in the western USA, across life stages and infection status. We also characterized microbiome diversity in field and laboratory-collected nymphal ticks to determine how the surrounding environment affects microbiome diversity. We found a decrease in both species richness and evenness as the tick matures from larva to adult. When the dominant Rickettsial endosymbiont was computationally removed from the tick microbial community, we found that infected nymphs had lower species evenness than uninfected ticks, suggesting that lower microbiome diversity is associated with pathogen transmission in wild-type ticks. Furthermore, laboratory-reared nymph microbiome diversity was found to be compositionally distinct and significantly depauperate relative to field-collected nymphs. These results highlight unique patterns in the microbial community of *I. pacificus* that is distinct from other tick species. We provide strong evidence that ticks acquire a significant portion of their microbiome through exposure to their environment despite a loss of overall diversity through life stages. We provide evidence that loss of microbial diversity is at least in part due to elimination of microbial diversity with bloodmeal feeding but other factors may also play a role.

KEYWORDS
16S rRNA, *Borrelia burgdorferi*, endosymbiont, *Ixodes pacificus*, life stages, microbiome

1 | INTRODUCTION

Microbial communities, ranging from bacteria to fungi, have been shown to influence animal health and pathogen transmission either directly in the host itself or indirectly through other physiological mechanisms (Gall et al., 2016; Ginsberg, 2008; Hooper & Gordon, 2001). These interactions are particularly intriguing in arthropod species that host complex microbial communities and are also important vectors of plant and animal pathogens. To better predict pathogen transmission dynamics, it is necessary to understand how the microbiome develops, changes and interacts with vector-borne pathogens. However, there is still a lack of mechanistic understanding of how tick microbiomes are formed or developed. This is a critical gap because it is increasingly clear that the composition and overall diversity of vector microbiomes may interact in crucial ways with the acquisition and transmission of important human and animal

pathogens (Cirimotich, Dong, Garver, Sim, & Dimopoulos, 2010; Gonzalez-Ceron, Santillan, Rodriguez, Mendez, & Hernandez-Avila, 2003). In a well-known example, the presence of a nonpathogenic *Rickettsia* species in the tick vector for Rocky Mountain spotted fever (*Dermacentor andersoni*) inhibits the establishment of the pathogen *Rickettsia rickettsii*, thus limiting the distribution of the disease (Burgdorfer, Hayes, & Mavros, 1981). Now, there is increasing evidence that the diversity and composition of the microbial community in general may also affect pathogen transmission dynamics in vector disease systems (Dong, Manfredini, & Dimopoulos, 2009; Narasimhan et al., 2014).

Despite the importance of ticks as vectors of human pathogens (Parola & Raoult, 2001), very little is known about how the tick microbial community is shaped and how the composition and diversity of the microbiome influences pathogen transmission, especially under natural, field conditions. The most commonly reported tick-borne disease in the northern hemisphere is Lyme disease, vectored by *Ixodes* species ticks (Centers for Disease Control and Prevention 2008). Lyme disease is caused by the bacterium *Borrelia burgdorferi*, which is horizontally transmitted between ticks and their bloodmeal hosts. Because *B. burgdorferi* is not transovarially transmitted, ticks can only acquire the pathogen horizontally by feeding on an infected bloodmeal host. The pathogen is carried with the incoming bloodmeal into the tick midgut and then colonizes the midgut lumen, where it resides until the tick's next bloodmeal (Narasimhan et al., 2014). In addition to *B. burgdorferi*, ticks also harbour a diverse plethora of other bacteria, the microbiome, that can form symbiotic, mutualistic, commensal or pathogenic relationships with the tick (Feldhaar, 2011; Noda, Munderloh, & Kurtti, 1997). Some of these are vertically transmitted from one generation to the next, whereas other components may be acquired from their environment. Further, feeding on vertebrate blood has also been shown to affect the composition and diversity of the tick microbiome (Swei & Kwan, 2017). This study seeks to develop a better understanding of the role that vertical vs. horizontal transmission has on the microbiome.

Because ticks are obligate blood-feeding organisms, they rely on close associations with endosymbiotic bacteria to provide essential nutrients that are absent in blood such as biotin and other B vitamins (Feldhaar, 2011; Hunter et al., 2015; Nikoh et al., 2014). As a result of this important function, obligate tick endosymbionts are vertically transmitted from the adult female to her progeny (Niebylski, Peacock, Fischer, Porcella, & Schwan, 1997) and are concentrated in the tick reproductive organs and haemolymph (Beninati et al., 2004; Noda et al., 1997) whereas most of the other bacteria making up the tick microbiome are localized in the midgut or salivary glands (Macaluso, Sonenshine, Ceraul, & Azad, 2001; Socolovschi, Mediannikov, Raoult, & Parola, 2009). *Rickettsia* endosymbionts in *Ixodes* species are obligate and dominate the microbiome, comprising 40%–90% of the microbiome by relative abundance (Benson, Gawronski, Eveleigh, & Benson, 2004; Cheng, Lane, Moore, & Zhong, 2013a). Studies on *Ixodes* spp. show that the absolute abundance of *Rickettsia* increases as ticks mature (Socolovschi et al., 2009) and is higher in female ticks relative to males (Carpi et al., 2011; Narasimhan et al., 2014; Van Treuren et al., 2015; Zolnik, Prill, Falco, Daniels, & Kolokotronis, 2016). However, *Ixodes pacificus*, the primary vector of Lyme disease in the western United States (Burgdorfer, Lane, Barbour, Gresbink, & Anderson, 1985; Clover & Lane, 1995), has a distinct life history compared to other *Ixodes* species and warrants further study. For instance, in contrast to other tick species, our microbiome analysis shows that *I. pacificus* microbial diversity decreases, rather than increases, as the tick matures from larvae to adult, suggesting that the processes that regulate the establishment and development of the *I. pacificus* microbiome are different (Swei & Kwan, 2017).

The most common endosymbiont in *I. pacificus* is the rickettsial phylotype G021, a *Rickettsia* with high homology to the *Ixodes scapularis Rickettsia* endosymbiont (Phan, Lu, Bender, Smoak, & Zhong, 2011). *Rickettsia* G021 is highly abundant in *I. pacificus* and has an average burden of 7.3 Rickettsia cells per tick cell (Cheng, Vigil, Schanes, Brown, & Zhong, 2013b). The high relative abundance of endosymbionts like *Rickettsia* can make it difficult to detect patterns and interactions between other components of the tick microbiome, especially rare taxa. High relative abundance of *Rickettsia* in the East Coast Lyme disease vector, *I. scapularis*, was found to mask detection of DNA sequences of rarer members of the tick bacterial community, including *Coxiella* and *Mycobacterium* (Moreno, Moy, Daniels, Godfrey, & Cabello, 2006). These relatively rare taxa in the microbiome have been shown to disproportionately affect microbial community dynamics (Shade et al., 2014) but may be largely overlooked in vector microbiomes. However, experimental removal of *Rickettsia* to better characterize the rest of the microbiome using antibiotic treatment has not been successful (Kurlovs, Li, Cheng, & Zhong, 2014). Ciprofloxacin-treated adult female *I. pacificus* produced juvenile ticks that still retain *Rickettsia* (Kurlovs et al., 2014). Additionally, when a tick is treated with antibiotics to kill off the main endosymbiont, tick survival is often compromised due to the essential nature of the symbiosis for the tick (Zhong, Jasinskas, & Barbour, 2007). With the limitations of experimental removal of endosymbionts, we employed next-generation deep sequencing and computational removal of *Rickettsia* to perform analysis on the other components of the microbiome. Specifically, we used amplicon-based next-generation sequencing to characterize the microbiome of all life stages and sexes of *I. pacificus*, the western black-legged tick. Then, to better describe the tick microbiome that is in direct contact with horizontally transmitted *B. burgdorferi*, we computationally removed *Rickettsia* reads from sequencing data.

There is emerging evidence that tick microbiome diversity can affect the efficiency of pathogen transmission. A study on *I. scapularis* found that higher tick microbiome diversity facilitates the ability of *B. burgdorferi* to colonize the tick midgut (Narasimhan et al., 2014). Thus, the microbiome, and the factors that structure and shape microbiome diversity have the potential to dramatically influence pathogen transmission dynamics of *B. burgdorferi* and perhaps other tick-borne pathogens (Gall et al., 2016). However, the *I. scapularis* study used ticks that were reared in an artificial laboratory setting, which may not accurately reflect disease dynamics between

pathogens and naturally occurring tick microbiomes. The microbiome composition of laboratory-reared vs. field-collected ticks was examined to evaluate the applicability of laboratory-based studies to predicting microbial interactions of zoonotic pathogens under natural, field conditions. This approach also allowed us to investigate and compare the role of environmental context, laboratory vs. field, on the development of the microbiome through horizontal transmission.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Ixodes pacificus ticks of all life stages were collected from the field using standard drag sampling techniques (Swei, Briggs, Lane, & Ostfeld, 2012). Sample collection took place in Marin County, CA, north of San Francisco from two sites, China Camp State Park (380′9.50″N; 12228′2.53″W) and Marin Municipal Water District Sky Oaks headquarters (3758′5.39″N; 12236′15.20″W). All ticks were collected from oak woodland habitat. Juvenile stages (larvae and nymphs) were collected during peak activity between March and June between 2013 and 2015. Adult ticks were collected December 2014 through February 2015. For ticks collected prior to 2015, the ticks were preserved in 70% ethanol and stored in a −20°C freezer until sample processing and DNA extraction. Ticks collected in 2015 were flash-frozen in liquid nitrogen and stored in a −80°C freezer until sample processing and DNA extraction. Laboratory-reared ticks were collected as engorged larvae from *Peromyscus maniculatus* or *Sceloporus occidentalis*. *S. occidentalis*-fed larvae were collected from field-caught lizards and brought into the laboratory to moult. Due to low tick burdens on wild *P. maniculatus*, larvae were experimentally fed on the deer mice in the lab. The laboratory-reared larvae hatched from field-collected adult ticks that were fed on New Zealand rabbits in the laboratory (Swei et al., 2012). All engorged larvae were reared in the laboratory in 95% relative humidity environmental chambers until they moulted into nymphs (generally 2–3 months). During this time, the engorged ticks did not feed. Molted, laboratory-reared nymphs were immediately flash-frozen after they moulted to preserve their microbiome and stored in 70% ethanol (Swei & Kwan, 2017).

2.2 | Sample processing and DNA extraction

Ticks were thoroughly surfaced sterilized with successive 1 ml washes of 3% hydrogen peroxide (1 min vortex), 70% ethanol twice (30 s each) and de-ionized H_2O (2 min) to remove environmental contamination. The entire tick was individually pulverized with a sterile plastic pestle and digested in lysis buffer overnight. Total nucleic acid was extracted using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's instructions (QIAGEN, Valencia, CA, USA). The 5S-23S rrf-rrl rRNA intergenic spacer of *Borrelia burgdorferi* was targeted with a nested PCR to determine nymphal infection status (Lane, Mun, Eisen, & Eisen, 2005). Hereafter, *B. burgdorferi*-negative nymphs are denoted as Bb− and *B. burgdorferi*-positive nymphs are denoted as Bb+.

2.3 | Microbiome sample preparation and sequencing

Separate amplicon libraries were prepared for each individual tick sample following the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (Klindworth et al., 2013). Amplicon libraries that target the V3–V4 hypervariable regions of the 16S rRNA gene were generated (Illumina, San Diego, CA, USA). Custom primers were used in an amplicon PCR reaction that included 12.5 μl of 2× KAPA HiFi HotStart Ready Mix, 1 μM amplicon forward primer, 1 μM amplicon reverse primer and 5 ng/μl microbial genomic DNA in 10 mM Tris pH 8.5. All ticks were amplified in triplicates and subsequently pooled to minimize PCR bias. Samples were amplified using the following thermocycler conditions: 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and then 72°C for 5 min. The amplicon PCR product was purified using Solid Phase Reversible Immobilization (SPRI) beads to remove primer dimer <60 base pairs (bp) and any nontarget product >700 bp. The resulting PCR product was ca. 550 bp. The individual tick sample was then barcoded using custom dual-indexing primers with Illumina sequencing adaptors from the Nextera XT Index Kit from Illumina. The index PCR reaction included 12.5 μl of 2× KAPA HiFi HotStart Ready Mix, 1 μM Nextera XT Index Primer 1, 1 μM Nextera XT Index Primer 2, 5 μl PCR grade water and 0.25 μM of amplicon PCR product. This PCR was run in duplicate using the following thermocycler conditions: 95°C for 3 min; 12 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min; hold for 4°C. PCR product was purified and visualized as described above. DNA concentrations were quantified using qPCR (KAPA Library Quantification Kit, KAPA Biosystems), and samples were diluted and pooled at equimolar concentrations. Sequencing was performed on an Illumina MiSeq using a MISEQ REAGENT KIT version 2 (Illumina, Inc., San Diego, CA, USA). Each tick was uniquely barcoded to generate microbiome sequence data specific to each tick.

Library quantification was carried out using qPCR to determine the DNA concentration for each sample and followed the KAPA library quantification kit protocol. The qPCR reaction was a total volume of 10 μl (6 μl KAPA SYBR®FAST qPCR Master Mix, 4 μl diluted library DNA or DNA Standard) with each sample carried out in triplicates. The following thermocycler conditions were used: 95°C for 5 min; 35 cycles of 95°C for 30 s and 60°C for 45 s, with a final dissociation step included. The results of absolute quantification were used to calculate the library concentrations in order to prepare the appropriate dilution for loading onto the MiSeq flow-cell. Each sample was diluted with 10 mM Tris buffer to a final concentration of 2 nM per sample.

All individual tick samples with unique indices were loaded on a MiSeq Sequencer. Sequence libraries were denatured with NaOH, diluted with hybridization buffer, heat denatured and then sequenced on a MiSeq Sequencer using a V2 reagent cartridge (250

base pair, paired-end). PhiX was included at 25% of the total run to serve as an internal control for potentially low-diversity libraries. The final concentration of the loaded library was 8 pM.

2.4 | Statistical analyses of *Ixodes pacificus* life stage microbiome

Illumina FASTQ files were demultiplexed and quality filtered (q20) with Quantitative Insights In Microbial Ecology (QIIME 1.9.1) (Caporaso et al., 2010). Paired-end reads were aligned using PYNAST and assigned to operational taxonomic units (OTU) using a 97% pairwise identity threshold. The GreenGenes taxonomic database was used with an open reference OTU picking strategy for taxonomic assignment (http://greengenes.lbl.gov).

For tick life stage analysis, the sequence data were rarefied to a sequencing depth of 12,141 sequence counts [larva (N = 7), nymph (N = 7), adult male (N = 7), adult female (N = 12)]. A separate analysis of adult males and females was conducted at a sequencing depth of 12,751 sequence reads [adult male (N = 6), adult female (N = 12)]. These results were plotted on a rarefaction curve and an ANOVA-Tukey test was performed to distinguish the differences between tick life stages and between adult male and female tick microbiomes. To characterize microbial alpha diversity, species richness and evenness were calculated manually using the observed OTU table generated in QIIME. We were keen to separate out species richness (the absolute abundance of bacterial genera) and evenness (variation in microbial community between the genera) because standard diversity metrics mask the contributions of richness and evenness to community patterns (Hurlbert, 1971). Beta diversity was examined using weighted and unweighted UniFrac analysis to compare the different groups and plotted in a principal coordinate analysis (PCoA). Unweighted UniFrac looks at only species occurrence in each sample, whereas weighted UniFrac takes into account species composition, or the richness and evenness of the bacterial community. The PCoA graph allows us to visualize the relationship between different tick samples, and whether or not their microbiome is similar or different from one another.

2.5 | Rickettsia diversity in the *Ixodes pacificus* microbiome

Sequence variants at subgenus level were identified from raw Illumina paired-end reads using DADA2 (Callahan et al., 2016a) and PHYLOSEQ (McMurdie & Holmes, 2013) in R (R Core Team 2016; RStudio Team 2015) according to the bioconductor workflow for microbiome analysis (Callahan, Sankaran, Fukuyama, McMurdie, & Holmes, 2016b). After trimming primer sequences, forward and reverse reads were quality filtered, de-replicated, merged, and chimeras were removed resulting in de novo sequence variants. Taxonomy was assigned using a native implementation of the RDP classifier (Wang, Garrity, Tiedje, & Cole, 2007) against a Greengenes release formatted for DADA2 output (DeSantis et al., 2006). Sequences were further classified with BLAST alignments (Altschul, Gish, Miller, Myers, & Lipman, 1990). Individual sequence variants in taxonomic groups of interest were subset and mapped (Dragulescu, 2014) to samples by creating PHYLOSEQ experimental objects (McMurdie & Holmes, 2013). Specifically, the diversity of *Rickettsia* and *Borrelia* populations in our data set was explored the data by visualizing fractional abundance (Wickham, 2009). The workflow presented above is included in our Supporting Information.

2.6 | Statistical analyses of *B. burgdorferi* infection status

Microbiome sequences of ticks that were either infected or uninfected with *B. burgdorferi* were rarefied to a sequencing depth of 6,000 sequence counts [Bb− (N = 9); Bb+ (N = 9)]. These results were plotted on a rarefaction curve, and two-sample Student's t tests were performed in R. *Rickettsia* OTUs were computationally removed using QIIME, and the remaining bacterial OTUs were analysed for composition and evenness as described above in the *I. pacificus* life stage analyses.

2.7 | Statistical analysis comparing field-collected to laboratory-reared nymphs

To evaluate how microbiomes vary between laboratory-reared and field-collected ticks, we compared 15 *I. pacificus* nymphs that were reared in the laboratory from the larval stage to 27 field-collected questing nymphs. Samples were sequenced as described above, and sequences were rarefied to a sequencing depth of 1,621 sequence counts. The statistical analysis followed the procedures described above for *B. burgdorferi* infection status.

3 | RESULTS

3.1 | *Ixodes pacificus* life stage microbiome

Analysis of *Ixodes pacificus* microbiome diversity revealed that species richness is different between life stages and decreases from larvae to adult (ANOVA F = 4.912, df = 3, 29, p < .01, Table 1). There was significantly higher species richness in larvae compared to adult males (p < .01), as well as between larvae and adult females (p = .04) (Figure 1a). Nymphs had intermediate levels of mean species richness but were not significantly different from adult males (p = .10) or adult females (p = .43) or larvae (p = .68). Species evenness (J') was different between all life stages, with evenness decreasing as the tick matures through its life stages (ANOVA F = 22.12, df = 3, 29, p < .001, Table 1). Larval microbiome was significantly more evenly distributed than the adult female microbiome (p < .001) as well as adult male microbiome (p < .001). Species evenness was significantly different between nymphs and adult females (p < .001), but did not differ between nymphs and adult males (p = .06). Between adult female and male ticks, there was no significant difference in species richness (t = 1.58, df = 14.96, p = .13) or species evenness (t = −1.56, df = 6.60, p = .92).

TABLE 1 Species richness denoted by average observed OTUs, and evenness (*J'*) values across life stage, *Borrelia burgdorferi* infection status, and laboratory vs. field analyses

	Species richness (*SE*)	Evenness (*SE*)
Larvae (*n* = 7)	380.57 (±26.7)	0.57 (±0.03)
Nymph (*n* = 7)	310.86 (±159.7)	0.41 (±0.19)
Adult female (*n* = 12)	225.33 (±142.8)	0.14 (±0.08)
Adult male (*n* = 7)	162.29 (±47.4)	0.23 (±0.13)
Bb− nymphs (*n* = 9)	250.33 (±107.4)	0.48 (±0.21)
Bb+ nymphs (*n* = 9)	214.00 (±89.1)	0.39 (±0.17)
Field-collected nymphs (*n* = 27)	142.57 (±55.6)	0.49 (±0.20)
Laboratory-reared nymphs (*n* = 15)	65.40 (±24.6)	0.29 (±0.14)

Beta diversity of tick microbiomes was analysed using both weighted and unweighted UniFrac analysis to analyse species composition. Weighted UniFrac analysis found significant compositional differences between larvae and all other life stages: nymphs, adult females and adult males ($t = -10.6$, $df = 29$, $p < .001$; Figure 1c). Unweighted UniFrac analysis also revealed significant differences between life stages with regards to species occurrence ($t = -6.3$, $df = 29$, $p < .001$; Figure 1d). There was a significant difference in species composition between male and female adult ticks (weighted UniFrac $t = -2.5$, $df = 16$, $p = .01$) but not species occurrence (unweighted UniFrac $t = 1.21$, $df = 16$, $p = .23$; Figure 1).

Rickettsia was the dominant genus in all *I. pacificus* life stages and comprised 35% to 82% of total abundance in the tick microbiome. The mean relative abundance of *Rickettsia* was lower in larvae and higher in adult ticks, with nymphs having an intermediate abundance (Figure 1b). In adult males and females, *Methylobacterium* is the second most abundant genus comprising 5.3% and 6.5% of the microbiome, respectively. However, this genus only makes up 0.12% of the total reads in larvae and 1.1% in nymphs. The third most common genus in adult females is *Sphingomonas*, comprising up to 2.2% of the total sequence reads; for adult males, it is an unknown genus in the family *Methylocystaceae*, making up 2.3% of the microbiome. *Sphingomonas* is found in both larvae and nymphs, at 1.8% and 2.6% respectively. Larvae had the most genera present at greater than 1%, with 13 genera identified while nymphs had 11 genera, adult males had five genera, and adult females had four genera (Table 1, Figure 1b).

3.2 | *Borrelia burgdorferi* infection status and microbiome diversity

Neither species composition nor occurrence differed between *Borrelia burgdorferi*-negative and *B. burgdorferi*-positive nymphs [weighted UniFrac, $df = 16$, $t = -0.35$, $p = .74$; unweighted UniFrac $t = -0.67$, $df = 16$, $p = .48$] (Figure 2b,c). Based on infection status, there was no significant difference in species richness ($t = 0.78$, $df = 16$, $p = .45$) or species evenness ($t = 1.0$, $df = 16$, $p = .33$). However, after computational removal of *Rickettsia*, species evenness was significantly lower in Bb+ nymphs ($t = 3.27$, $df = 16$, $p < .01$), but richness was not different ($t = 0.27$, $df = 16$, $p = .79$; Table 2). Microbiome composition was also significantly different (weighted UniFrac $t = -2.36$, $df = 16$, $p = .02$; Figure 3b), although species occurrence was not different (unweighted UniFrac $t = -0.39$, $df = 16$, $p = .7$; Figure 3c).

Rickettsia dominated both Bb− and Bb+ nymphs, although the relative proportion was not significantly different (Bb−: 66.1%, Bb+: 54.8%). For the Bb+ nymphs, *Borrelia* makes up 5.6% of the sequence reads and were confirmed to be *Borrelia burgdorferi* by sequence alignment. *Psuedomonas* and *Sphingomonas* made up 3.3% and 2.5%, respectively, of the Bb− nymph microbiome. Both of these genera were seen at higher proportions in the Bb+ nymphs, 4.2% and 5.0%, respectively (Figures 2a and 3a).

3.3 | Rickettsia endosymbiont and infection prevalence

To examine the diversity of *Rickettsia* reads in our sequences, we developed a computational pipeline to extract all Rickettsia sequences and BLAST them to the GenBank database. We found that across all samples and life stages, all *Rickettsia* sequences displayed 100% pairwise identity to the *I. pacificus* endosymbiont G021 at the V3-V5 16S *rRNA* locus (Cheng et al., 2013b).

3.4 | Laboratory-reared vs. field-collected

Laboratory-reared nymphal ticks had significantly lower species richness ($t = 6.28$, $df = 40.03$, $p < .001$) and evenness ($t = 3.57$, $df = 41$, $p < .001$) than field-collected nymphs (Figure 4a,b). UniFrac analysis showed that laboratory-reared nymphs resembled one another more closely, whereas field-collected nymphs exhibited greater variability (Figure 4). Species composition was significantly different between laboratory and field ticks (weighted UniFrac $t = -2.9$, $df = 41$, $p = .003$; Figure 4c), as well as species occurrence (unweighted UniFrac $t = -11.4$, $df = 41$, $p = .001$; Figure 4d). *Rickettsia* was still the most dominant taxa, representing 62.6% of the microbiome in laboratory-reared nymphs and 60.2% in field-collected nymphs (Figure 4b). Field-collected ticks had significantly higher richness with a total of 143 OTUs compared to 67 OTUs in laboratory-reared ticks (Table 1).

4 | DISCUSSION

Hard ticks have long life cycles that span multiple years; thus, their microbiome has the potential to change significantly over the course of their lives. The tick microbiome is initially established by vertically transmitted microbes but later, the microbiome can be altered by the environment and through interactions with bloodmeal hosts. *Ixodes pacificus* has a natural history that is unique compared to

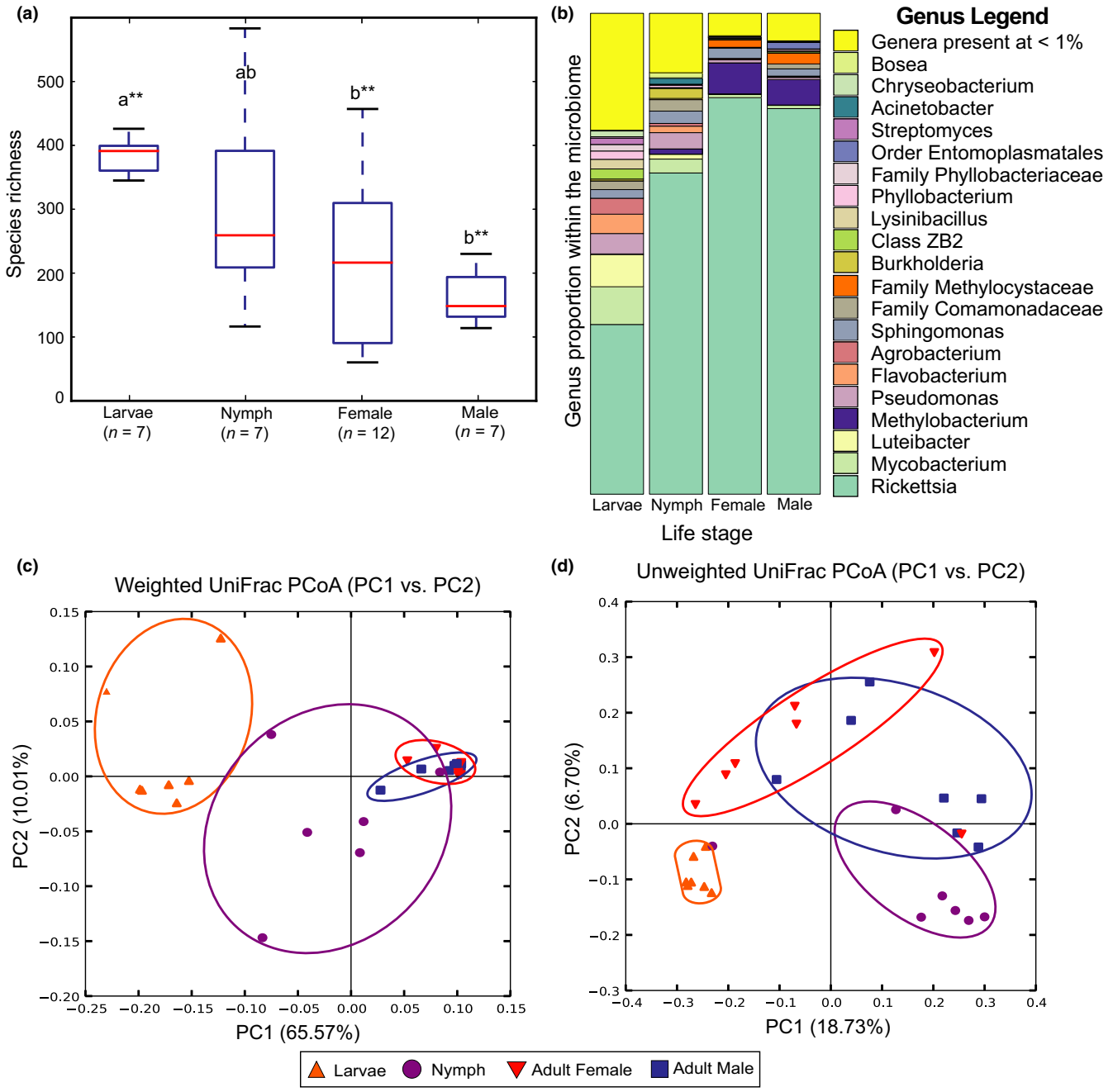
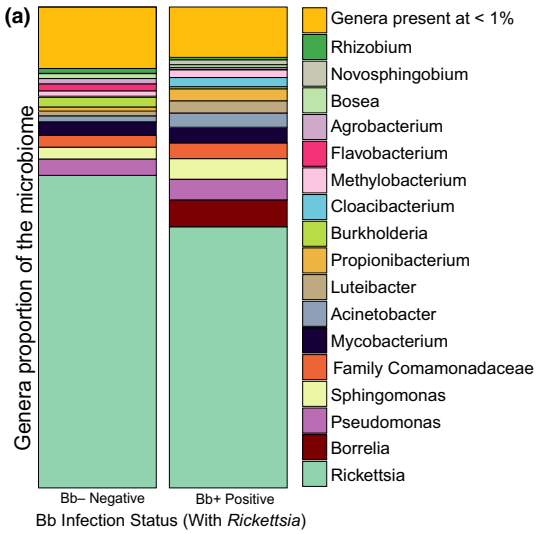
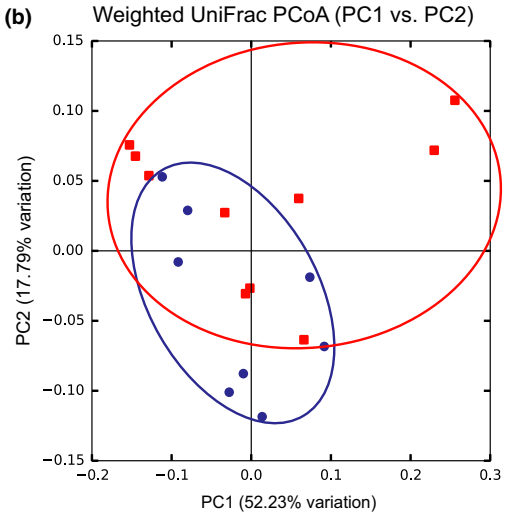


FIGURE 1 Life stage analyses of *Ixodes pacificus* microbiomes. (a) Species richness boxplot showing the average species richness at each life stage: larvae, nymph, adult female and adult male. a** and b** denotes significant differences between the groups, whereas ab means there is not a significant difference between the groups. (b) Taxonomy graph showing the proportions of genera present in each life stage: larvae, nymph, adult female and adult male. (c) Weighted UniFrac principle coordinate analysis (PCoA) graph showing PC1, which accounts for 65.57% variation and PC2 (10.05% variation). (d) Unweighted UniFrac PCoA graph showing PC1 (18.73% variation) and PC2 (6.70% variation). Orange triangles represent larvae, purple circles represent nymphs, red inverted triangles represent adult females, and blue squares represent adult males

other hard ticks, and this is reflected in distinct microbiome patterns reported here. In particular, studies on the microbiome diversity of other *Ixodes* spp. such as *I. ricinus* and *Ixodes scapularis*, two important pathogen vectors of *Borrelia burgdorferi*, display a pattern of increasing microbial species richness as the tick matures from the larval to adult stage, a sharp contrast to our findings (Carpi et al., 2011; Clay et al., 2008; Zolnik et al., 2016). However, our previous work found that *I. pacificus* displays an unusual loss of microbiome species richness through the tick's ontogenic development (Swei & Kwan, 2017). In the present study, we conducted further detailed analysis of adult ticks to examine how male and female adult ticks differ in their microbiome composition and evenness. In contrast to





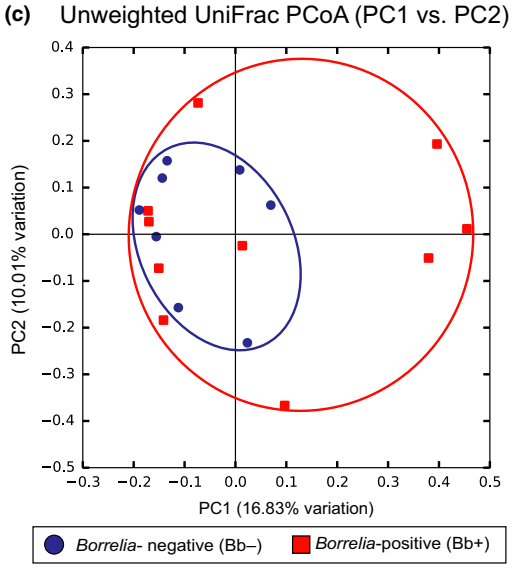


FIGURE 2 *Borrelia burgdorferi* infection status analysis. (a) Taxonomy graph comparing the proportions of genera present between *B. burgdorferi*-negative (Bb−) and *B. burgdorferi*-positive (Bb+) nymphs. (b) Weighted UniFrac PCoA graph showing PC1 (52.23% variation) and PC2 (17.79% variation). (c) Unweighted UniFrac PCoA graph showing PC1 (16.83% variation) and PC2 (10.01% variation). Blue circles denote Bb− nymphs, and red squares denote Bb+ nymphs

TABLE 2 Species Richness denoted by average observed OTUs, and Evenness (J′) values in *Borrelia burgdorferi* nymphal analysis with removal of *Rickettsia*

	Species richness	Evenness
Bb− nymphs (n = 9)	256.33 (±97.3)	0.74 (±0.04)
Bb+ nymphs (n = 9)	245.89 (±62.1)	0.66 (±0.06)

other tick microbiome studies where *I. scapularis* females had a lower microbial diversity than their male counterparts but higher relative *Rickettsia* abundance (Van Treuren et al., 2015; Zolnik et al., 2016), we found that male and female *I. pacificus* have highly similar microbial communities and the relative abundance of endosymbionts did not differ significantly as has been reported in other species. These results suggest that there are processes in the life history and host-microbial interactions of *I. pacificus* and their primary endosymbiont that are distinct from other tick species (Ponnusamy et al., 2014; Van Treuren et al., 2015; Zolnik et al., 2016). As obligate haematophagous organisms, highly specialized symbioses between ticks and bacteria have been formed numerous times to provide ticks with critical nutrients not found in blood. The function of these endosymbionts is considered essential for tick growth and survival; perhaps this dependency is especially strong between *I. pacificus* and their Rickettsial endosymbiont.

Previous work found that the decreasing microbial diversity of more mature ticks was driven by feeding on western fence lizards, *Sceloporus occidentalis*, the preferred juvenile blood meal host for *I. pacificus* (Swei & Kwan, 2017). Field and laboratory studies have shown that ticks infected with *B. burgdorferi*, that then feed on *S. occidentalis*, are cleared of their infection (Kuo, Lane, & Giclas, 2000; Lane & Quistad, 1998). Thus, the loss of microbial species richness in *S. occidentalis*-fed ticks provides strong evidence that lizard blood reduces other bacterial species in addition to *B. burgdorferi*, most likely affecting microbes that it comes in contact with in the tick midgut. The similarity between adult male and female microbiomes may reflect the unique life history *I. pacificus* and its strong preference for feeding on lizards, differentiating it from other *Ixodes* species.

Laboratory experiments that examined the impact of microbiome diversity on the transmission dynamics of *B. burgdorferi* found that higher microbiome diversity resulted in higher colonization success of *B. burgdorferi* in *I. scapularis* (Narasimhan et al., 2014). In contrast, in our examination of field-collected *I. pacificus* nymphs, we did not find that that species richness and composition were significantly different between *B. burgdorferi*-infected and *B. burgdorferi*-uninfected *I. pacificus* nymphs. However, as the dominant taxon in the *I. pacificus* microbiome, the presence of the *Rickettsia* endosymbiont can obscure patterns in the remainder of the

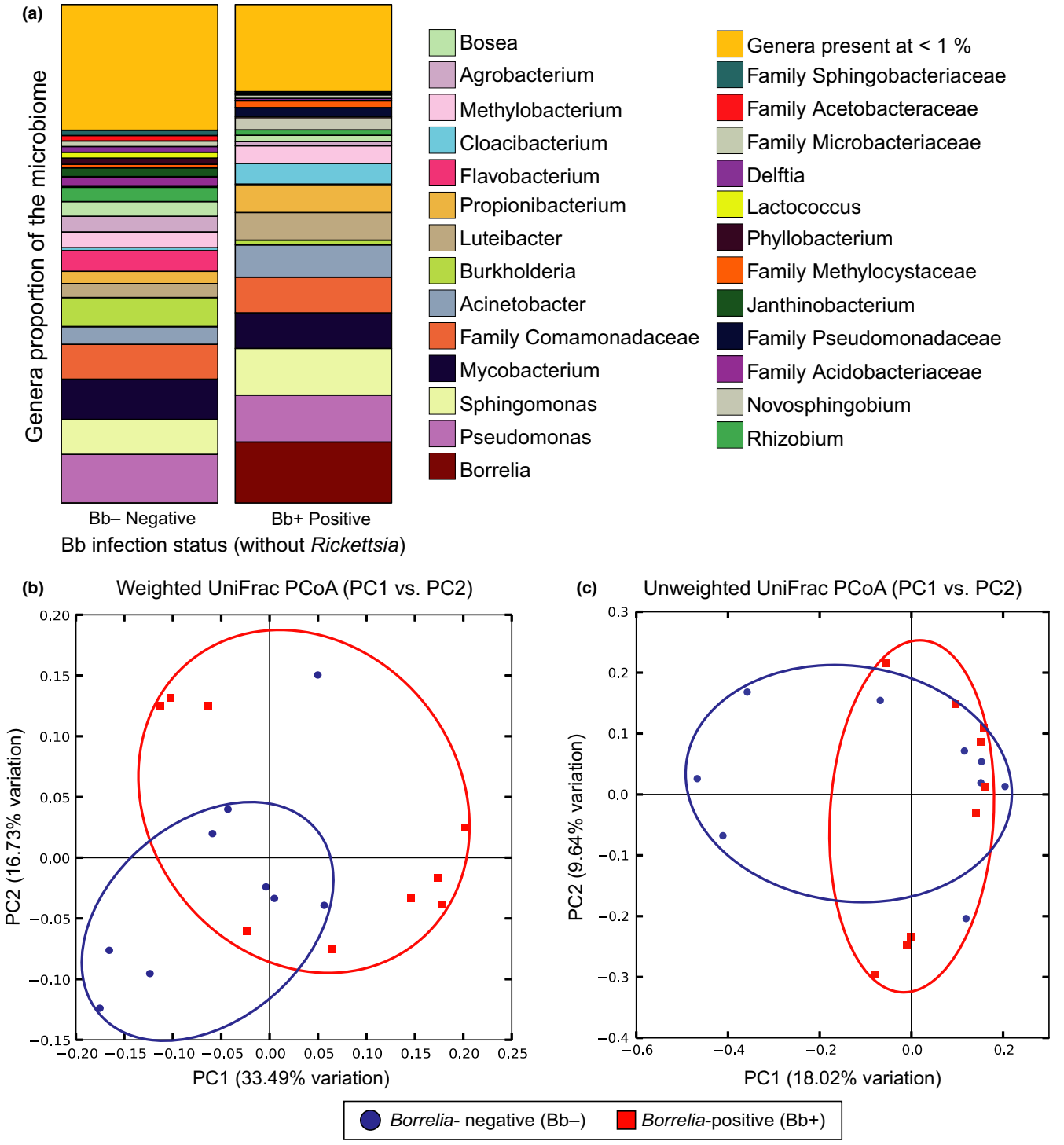


FIGURE 3 *Borrelia burgdorferi* Infection Status Analysis without *Rickettsia*. (a) Taxonomy graph comparing the proportions of genera present between *B. burgdorferi*-negative (Bb−) and *B. burgdorferi*-positive (Bb+) nymphs. (b) Weighted UniFrac PCoA graph showing PC1 (33.49% variation) and PC2 (16.73% variation). (c) Unweighted UniFrac PCoA graph showing PC1 (18.02% variation) and PC2 (9.64% variation). Blue circles denote Bb− nymphs, and red squares denote Bb+ nymphs

tick microbiome. The dominance of endosymbionts in ticks has been shown to partially be facilitated by the endosymbiont's ability to avoid the tick innate immune system while other microbes are reduced (Mattila, Munderloh, & Kurtti, 2007). Endosymbiotic *Rickettsia* species are intracellular microbes that have only been shown to be localized in the reproductive tissues and haemolymph (Beninati et al., 2004; Noda et al., 1997). It is therefore unlikely that they are coming into direct contact with lizard bloodmeals in the midgut. Efforts to experimentally remove *Rickettsia* endosymbionts from *Ixodes* spp. ticks have been unsuccessful (Kurlovs et al., 2014)

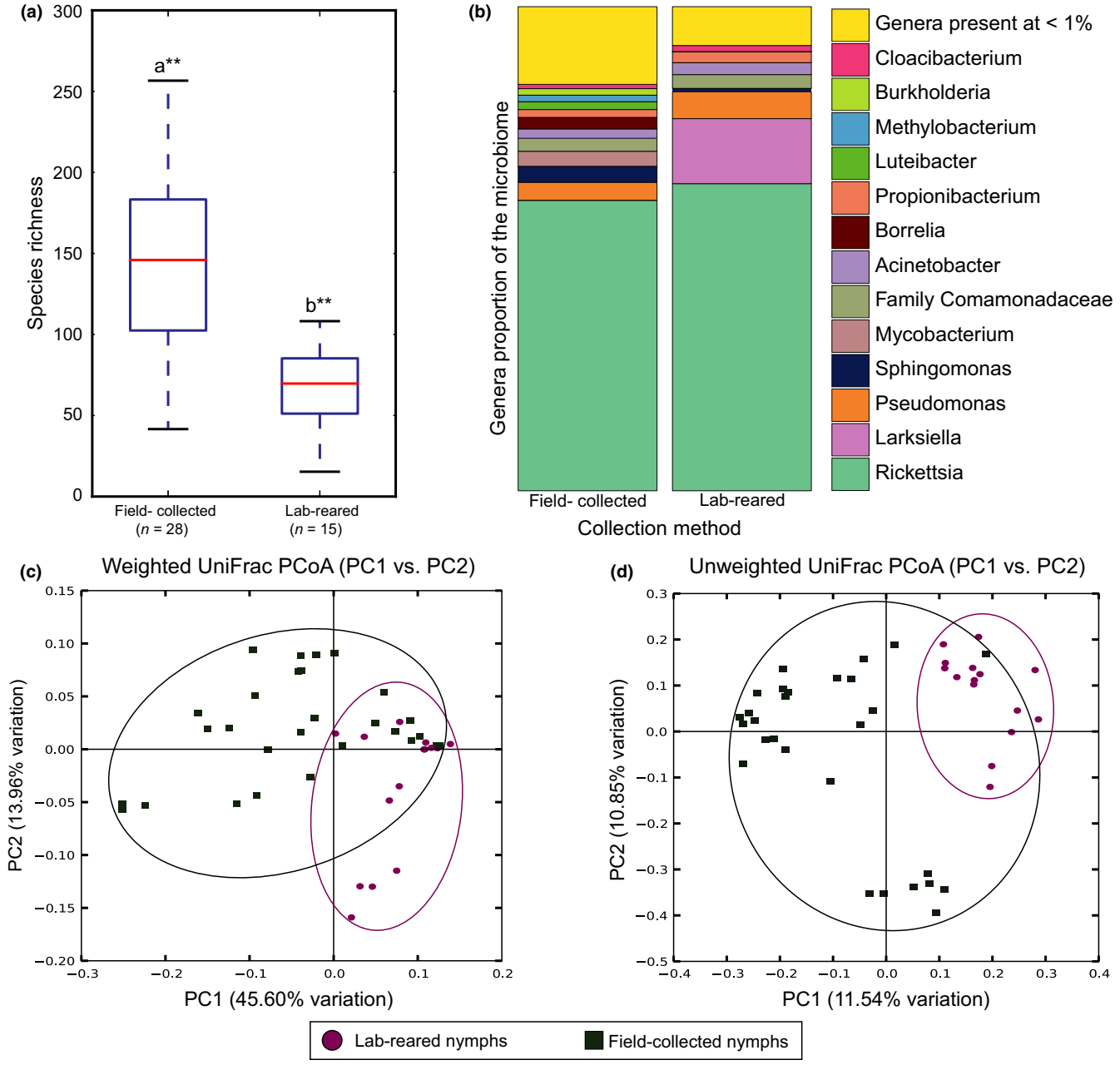


FIGURE 4 Laboratory-reared vs. Field-collected nymph analysis. (a) Species richness boxplot comparing the average species richness between field-collected nymphs and laboratory-reared nymphs. a** and b** denote significant differences between the groups. (b) Taxonomy graph comparing the proportions of genera present between laboratory-reared and field-collected nymphs. (c) Weighted UniFrac PCoA graph showing PC1 (45.60% variation) and PC2 (13.96% variation). (d) Unweighted UniFrac PCoA graph showing PC1 (11.54% variation) and PC2 (10.85% variation). Purple circles denote laboratory-reared nymphs, and green squares denote field-collected nymphs

and resulted in nonviable ticks. The *I. pacificus* microbiome is heavily dominated by *Rickettsia*. We found that 100% of all ticks were uniformly infected with the same *Rickettsia* phylotype G021 (Hunter et al., 2015). However, the non-*Rickettsia* component of the tick microbiome may be more important to overall diversity patterns associated with pathogen transmission dynamics. Rare taxa in the microbiome may play a disproportionally important role in community structure and response to disturbance (Mikkelson, Bokman, & Sharp, 2016; Shade et al., 2014). To examine the components of the tick microbiome that may be obscured by the dominant endosymbiont, we computationally removed *Rickettsia* sequence reads from nymphal *I. pacificus* ticks. We found that *B. burgdorferi*-infected nymphs have similar microbiome species richness but significantly lower microbiome evenness compared to *B. burgdorferi*-negative nymphs. From these results, we suggest that ecological factors that decrease tick microbiome species evenness may facilitate higher transmission success of *B. burgdorferi*. *B. burgdorferi* is not known to affect the composition of the tick microbiome but

because this study was descriptive, we cannot rule out the possibility that *B. burgdorferi* induced the patterns we present. Mechanistic experimental studies are required to definitively determine the potential interactions between *B. burgdorferi* and *I. pacificus* microbiome diversity.

Our results differ significantly from other microbiome studies on hard ticks, including a laboratory study that associated greater microbiome diversity with higher *B. burgdorferi* colonization success in *I. scapularis* (Narasimhan et al., 2014). Our study used field-collected ticks and found that *B. burgdorferi*-infected *I. pacificus* displayed significantly lower microbiome diversity, primarily driven by lower species evenness. These results were not detected until the dominant *Rickettsia* endosymbiont was computationally removed. These findings may reflect the introduction of microbes from pathogen reservoirs such as dusky footed woodrats (*Neotoma fuscipes*) or western grey squirrels (*Sciurus griseus*) (Eisen, Eisen, & Lane, 2004). Although uninfected ticks may have fed as larvae on both uninfected pathogen reservoirs or nonreservoirs, the majority of *I. pacificus* larval bloodmeals are taken from western fence lizards (Casher, Lane, Barrett, & Eisen, 2002; Lane & Loye, 1989; Swei et al., 2012; Tälleklint-Eisen & Eisen, 1999); thus, the lowering of species richness may fundamentally affect microbial dynamics and drive higher evenness on average in the tick microbiome. Our study did not determine whether the detected differences in evenness in *B. burgdorferi*-positive and *burgdorferi*-negative *I. pacificus* ticks are causal or correlational but follow-up studies could help clarify the precise nature of such interactions.

The discrepancy between our study and other laboratory-based studies may reflect significant differences in microbial diversity between laboratory-reared and field-collected ticks. There is strong evidence that ticks acquire microbial diversity from the environment, which structures their microbiomes' species richness and composition (Carpi et al., 2011; Van Treuren et al., 2015; Zolnik et al., 2016). We sought to directly compare microbiome composition and diversity of nymphal ticks that were either collected from the field or reared in the laboratory from engorged larval ticks through the moulting process to the nymphal stage. The laboratory-reared ticks only spent a life stage or two, at the most, in the laboratory compared to field-collected ticks; thus, our findings represent differences in the tick environment and not drastically different rearing histories. We found that field-collected ticks have significantly higher species richness and evenness, with the average species richness of field-collected ticks more than twice as high as laboratory-reared ticks. Microbiome species evenness was also nearly double that of laboratory ticks. Furthermore, when looking at beta diversity, laboratory-reared nymphs clustered more closely, indicating that their microbial compositions were more similar to one another's (Figure 4c). In contrast, field-collected nymphs were more highly divergent between tick samples. Our results reflect the patterns and dynamics of *I. pacificus* microbiomes and associated zoonotic pathogens from field-collected ticks and are therefore more relevant for realized entomological disease risk. While laboratory studies are useful for measuring certain interactions, we argue that results from such studies may not accurately reflect processes in the field and caution against relying solely on laboratory studies for understanding microbiome mediated interactions with vector-borne pathogens.

Here, we present data on patterns of microbiome diversity in *I. pacificus*, the primary vector of Lyme disease in the western United States. Like other blood-feeding hard ticks, *I. pacificus* microbiome is dominated by endosymbiotic bacteria (*Rickettsia* G021), but we present data that demonstrate an unusually high representation of *Rickettsia* in both female and male ticks suggesting that this relationship is especially critical in *I. pacificus*. We also show that a great deal of the *I. pacificus* microbiome is acquired horizontally from their environment with our comparison of field-collected and laboratory-reared ticks. These strong differences in species richness and composition may affect the transmission of important human pathogens such as *B. burgdorferi*. Our study on field-collected ticks in a natural oak woodland habitat found that *B. burgdorferi* infection is associated with lower microbiome diversity, particularly species evenness. Thus, the processes that regulate the vertical and horizontal transmission of microbes into the tick vectors are critically important for pathogen transmission and vector competency.

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DATA ACCESSIBILITY

Microbiome DNA sequences filed under BioProject ID PRJNA352452. Biosample Accession nos: SAMN05981172–SAMN05981184, SAMN07556896–SAMN07556914, SAMN07569721–SAMN07569748, SAMN07569671–SAMN0756985. Pipeline for *Rickettsia* Identification is available in the Supplemental Information.

AUTHOR CONTRIBUTIONS

J.Y.K. designed the project, performed the field collections and laboratory research, performed research protocol troubleshooting, analysed the data, and wrote the manuscript; R.G. wrote and ran the script for identifying the *Rickettsia* species; B.C. helped with field collection and laboratory research; C.M. helped with the field collection and laboratory research; A.S. designed the project, analysed data, contributed new reagents and analytical tools, and co-wrote the manuscript.

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SUPPORTING INFORMATION

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