



Endosymbiont interference and microbial diversity of the Pacific coast tick, *Dermacentor occidentalis*, in San Diego County, California

Nikos Gurfield¹, Saran Grewal¹, Lynn S. Cua¹, Pedro J. Torres² and Scott T. Kelley²

¹ Department of Environmental Health-Vector Control Program, County of San Diego, San Diego, CA, United States of America

² Department of Biology, San Diego State University, San Diego, CA, United States of America

ABSTRACT

The Pacific coast tick, *Dermacentor occidentalis* Marx, is found throughout California and can harbor agents that cause human diseases such as anaplasmosis, ehrlichiosis, tularemia, Rocky Mountain spotted fever and rickettsiosis 364D. Previous studies have demonstrated that nonpathogenic endosymbiotic bacteria can interfere with *Rickettsia* co-infections in other tick species. We hypothesized that within *D. occidentalis* ticks, interference may exist between different nonpathogenic endosymbiotic or nonendosymbiotic bacteria and Spotted Fever group Rickettsia (SFGR). Using PCR amplification and sequencing of the *rompA* gene and intergenic region we identified a cohort of SFGR-infected and non-infected *D. occidentalis* ticks collected from San Diego County. We then amplified a partial segment of the 16S rRNA gene and used next-generation sequencing to elucidate the microbiomes and levels of co-infection in the ticks. The SFGR *R. philipii* str. 364D and *R. rhipicephali* were detected in 2.3% and 8.2% of the ticks, respectively, via *rompA* sequencing. Interestingly, next generation sequencing revealed an inverse relationship between the number of *Francisella*-like endosymbiont (FLE) 16S rRNA sequences and *Rickettsia* 16S rRNA sequences within individual ticks that is consistent with partial interference between FLE and SFGR infecting ticks. After excluding the *Rickettsia* and FLE endosymbionts from the analysis, there was a small but significant difference in microbial community diversity and a pattern of geographic isolation by distance between collection locales. In addition, male ticks had a greater diversity of bacteria than female ticks and ticks that weren't infected with SFGR had similar microbiomes to canine skin microbiomes. Although experimental studies are required for confirmation, our findings are consistent with the hypothesis that FLEs and, to a lesser extent, other bacteria, interfere with the ability of *D. occidentalis* to be infected with certain SFGR. The results also raise interesting possibilities about the effects of putative vertebrate hosts on the tick microbiome.

Submitted 2 July 2016

Accepted 19 March 2017

Published 13 April 2017

Corresponding author
Nikos Gurfield,
nikos.gurfield@sdcounty.ca.gov

Academic editor
Irene Newton

Additional Information and
Declarations can be found on
page 17

DOI 10.7717/peerj.3202

© Copyright
2017 Gurfield et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

Subjects Entomology, Microbiology

Keywords Ticks, Endosymbiont, Interference, Microbiome

INTRODUCTION

The Pacific Coast tick, *Dermacentor occidentalis* Marx (henceforth *D. occidentalis*) is the most widely distributed tick in California and is found in chaparral and shrubland areas from northern Baja California to California and Oregon (Furman & Loomis, 1984). *D. occidentalis* is a three-host, hard-shell tick that feeds on a variety of vertebrates, such as rodents, rabbits, cattle, deer, horses and humans. Surveys of this tick have shown its ability to vector human pathogens such as *Francisella tularensis* (tularemia), *Coxiella burnetii* (Q fever), *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Ehrlichia chaffeensis* (human monocytic ehrlichiosis), *Rickettsia rickettsii* (Rocky Mountain spotted fever, RMSF) and *Rickettsia philipii* 364D (hereafter *R. philipii*) as well as the non-pathogenic spotted fever group *Rickettsia*, *R. rhipicephali* (Parker, Brooks & Marsh, 1929; Cox, 1940; Lane et al., 1981; Holden et al., 2003; Wikswa et al., 2008; Shapiro et al., 2010). *Rickettsia philipii*, was originally described as an unclassified *Rickettsia* found by Bell in *D. occidentalis* from California (Philip et al., 1978). It is closely related to *Rickettsia rickettsii* but can be serologically and genetically distinguished (Philip, Lane & Casper, 1981; Karpathy, Dasch & Ereemeeva, 2007). Although discovered in 1966, and long suspected of being able to cause disease, it was only recently confirmed to be associated with eschars and lymphadenopathy in people at the site of a tick bite (Lane et al., 1981; Shapiro et al., 2010; Johnston et al., 2013).

Francisella-like endosymbiotic bacteria (FLEs) have also been detected in *Dermacentor occidentalis* as well as other tick species (Burgdorfer, Brinton & Hughes, 1973; Noda, Munderloh & Kurtti, 1997; Scoles, 2004; Kugeler et al., 2005). FLEs share 16S rRNA gene homology with *Francisella* spp., are vertically transmitted, have been observed within tick ovaries and Malpighian tubules, and vary by tick species (Rounds et al., 2012). Although Burgdorfer et al. demonstrated pathogenicity of a *Francisella* endosymbiont derived from *Dermacentor andersoni* Stiles ticks (previously categorized as *Wolbachia persica*, Forsman, Sandström & Sjöstedt, 1994) to guinea pigs and hamsters via injection, most FLEs are not transmitted by tick bites and are considered non-pathogenic (Burgdorfer, Brinton & Hughes, 1973; Niebylski et al., 1997).

Interestingly, the inability of different endosymbiotic *Rickettsia* species to co-infect the same organ in the same tick, called “interference,” has been demonstrated, although the exact mechanisms are unknown. Early studies seeking to understand the epidemiology of RMSF in the Bitterroot Valley in Montana demonstrated that the non-pathogenic tick endosymbiont *Rickettsia peacockii* (found on the east side of the valley and originally called the East side agent) colonized the ovaries of *D. andersoni* ticks and excluded pathogenic *Rickettsia rickettsii* (more prevalent on the west side of the valley) from infecting the ovaries and being transmitted to eggs (Burgdorfer, Hayes & Mavros, 1981). Similarly, studies of *Dermacentor variabilis* (Say) infected with *R. montanensis* or *R. rhipicephali* demonstrated resistance to transovarial transmission of the reciprocal *Rickettsia* in challenge experiments (Macaluso et al., 2002). Negative influences between co-infecting species of *Rickettsia* and other symbionts has been suggested to occur in other vectors such as fleas (Azad & Beard, 1998; Jones et al., 2012). Interference has been postulated to have significant effects in

altering the distribution of *Rickettsia* pathogens in the environment and, consequently, the presence of human disease (Burgdorfer, Hayes & Mavros, 1981).

The use of next generation sequencing has allowed deeper exploration into endosymbionts and complex bacterial communities that colonize different tick species (Nakao *et al.*, 2013), their organs (Budachetri *et al.*, 2014; Qiu *et al.*, 2014), different life stages (Carpi *et al.*, 2011) and different states of nutrition (Menchaca *et al.*, 2013; Zhang *et al.*, 2014). Attention to the microbiome of ticks has been driven, in part, by the fact that ticks can transmit the broadest range of diseases of any arthropod and the recognition that tick co-infections can have dramatic consequences on both the tick host and human patient (Clay & Fuqua, 2010). Microbiome studies using next generation sequencing techniques have demonstrated that each species of tick harbors its own unique bacterial community often dominated by Proteobacteria and one or two endosymbionts (Clay & Fuqua, 2010; Ponnusamy *et al.*, 2014; Hawlena *et al.*, 2012; Van Treuren *et al.*, 2015; Narasimhan & Fikrig, 2015). Given these findings, we hypothesized that next generation sequence analysis of *Dermacentor occidentalis* ticks microbiomes would reveal patterns of interference or exclusion among pathogenic or non-pathogenic bacteria. We also hypothesized that differences among tick microbiomes would be associated with different geographic locations, and that possible reservoirs of tick pathogens could be found by analyzing ticks for the host origin of prior blood meals or by comparing the tick microbiomes to the skin microbiomes of potential host species. To address these hypotheses, we used culture-independent PCR amplification of the 16S rRNA gene and next-generation sequencing (NGS) to determine whether the microbiomes of SFGR-infected ticks differed from non-SFGR-infected ticks, and if this microbial diversity was consistent with a hypothesis of interference. Our results revealed patterns consistent with partial exclusion between SFGR and FLEs and an association of non-endosymbiotic bacteria with geographic locale. Furthermore, the historical blood meal hosts of the ticks were implicated by the composition of bacterial communities within the ticks and were correlated with SFGR infection. While the precise mechanism of the bacterial interactions (i.e., direct or indirect) need elucidation, our results suggest that carriage of certain pathogenic SFGR in ticks could be modulated by other non-rickettsial endosymbionts, providing a potential non-chemical alternative to SFGR control.

MATERIALS & METHODS

Sample collection

Adult ticks were collected from February to May 2014 from 4 different areas of San Diego County: Escondido Creek, Los Peñasquitos Canyon, Lopez Canyon and Mission Trails Regional Park by dragging a 1 m² piece of canvas over grass and chaparral and then capturing the ticks with forceps and placing them in individual sterile microfuge tubes. The ticks were transported live back to the Vector Disease and Diagnostic Laboratory at the San Diego County Operations Center where, by visual examination, their species and sex were determined and cataloged before freezing them at −80 °C.

DNA extraction, PCR amplification and next generation sequencing

Ticks were processed individually throughout all procedures. The ticks were thawed and washed sequentially in 3% hydrogen peroxide, 100% isopropanol, and sterile distilled water for 1 min in each solution. The final distilled water wash was aspirated from the ticks and then the ticks were sectioned sagittally at midline with a sterile scalpel. Half of the tick was saved at -80°C ; the other half was used for DNA extraction. Briefly, 180 μL of ATL buffer (Qiagen, Valencia, CA, USA) and 20 μL of proteinase K were added to each tick and the ticks lysed overnight at 37°C in an Eppendorf Thermomixer (Hauppauge, NY) with agitation at 1,400 rpm for 15 s every 15 min, before centrifuging the lysate for 3 min at $18,400\times g$. The supernatant was transferred into a sterile microfuge tube and DNA extracted using a Qiagen DNeasy Blood and Tissue kit in a Qiacube using the DNeasy Blood and Tissue protocol for Tissue and Rodent Tails (Qiagen, Valencia, CA, USA). Negative extraction controls consisted of sterile water processed via the same washing, chopping and extraction procedure used on the ticks.

The ticks were screened for spotted fever group rickettsia using a Power SYBR Green *real-time* PCR Mastermix kit (Life Technologies, Carlsbad, CA, USA) and primers for the *romp A* gene (Eremeeva *et al.*, 2003). Reactions were carried out in a total volume of 20 μL composed of 10 μL Power SYBR Green Mastermix, 0.125 μL each of primers RR190.547F (20 μM) and RR190.701R (20 μM), 7.75 μL of nuclease-free water, and 2 μL of template DNA (Eremeeva *et al.*, 2003; Wikswa *et al.*, 2008). *Real-time* PCR cycling conditions were: 3 min at 95°C ; 40 cycles of: 20 s at 95°C , 30 s at 57°C , 30 s at 65°C ; a holding cycle of 5 min at 72°C ; and a continuous cycle of: 15 s at 95°C , 1 min at 55°C , 30 s at 95°C , 10 s at 55°C ; and a final holding temperature of 4°C .

DNA from ticks that screened positive for SFGR were subjected to semi-nested PCR amplification of *rompA* using primers Rr190-70, Rr190-701, and Rr190-602 and the intergenic region (IGR) using primary and nested primers RR0155-*rpm B* (Eremeeva *et al.*, 2006; Shapiro *et al.*, 2010; Wikswa *et al.*, 2008). Briefly, 20 μL of 2X Taq Master Mix (Qiagen, Valencia, CA), 2 μL of forward primer Rr190-70 (20 mM), 2 μL of reverse primer Rr190-701/Rr190-602 (20 mM), 14 μL of nuclease-free H_2O , and 2 μL of DNA was amplified using PCR cycling conditions of 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 57°C for 30 s, and 68°C for 2 min and then 72°C for 5 min before holding the products at 4°C . For the IGR PCR amplification, 20 μL of 2X Taq Master Mix (Qiagen, Valencia, CA, USA), 1 μL of forward primer RR 0155 PF (20 mM), 1 μL of reverse primer 0155 PR (20 mM), 16 μL of nuclease-free H_2O , and 2 μL of DNA was amplified using PCR cycling conditions of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 68°C for 1 min and then 72°C for 7 min before holding the products at 4°C .

Amplification products were visualized in a 1% agarose gel stained with ethidium bromide on a UV illuminator and subsequently purified using the PureLink PCR Purification Kit, following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and purified using the BigDye XTerminator Purification Kit following the manufacturer's protocols on an AB 3500xL Genetic Analyzer (Applied Biosystems, Grand Island, NY, USA). Due to highly conserved 16S rRNA gene sequences between *Francisellaceae*, DNA

extracts of the ticks were also tested specifically for the presence of *Francisella tularensis* using a multi-target *real-time* PCR test employing primers ISFtu 2, *iglC* and *tul4* that are specific for *F. tularensis* as described in [Kugeler et al. \(2005\)](#) and [Versage et al. \(2003\)](#). All reactions were performed in a final volume of 20 μ l and contained LightCycler FastStart DNA Master HybProbe mix (Roche, Mannheim, Germany) at a 1 \times final concentration, 500 nM forward and reverse primers, 100 nM probes, and 1.25 U of uracil-DNA glycosylase per reaction. For the *iglC* and *tul4* the final MgCl₂ concentration was 4 mM, and for the ISFtu2 assay, the final concentration was 5 mM. *Real-time* PCR cycling conditions were: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of: 95 °C for 10 s, 60 °C for 30 s; and 45 °C for 5 min.

PCR amplification of the cytochrome b gene was used to query the DNA from the ticks for determining the hosts of their prior blood meals using the primers UNFOR403 and UNREV1025 ([Kent & Norris, 2005](#); [Lah et al., 2015](#)). PCR reactions were conducted using 2X Taq PCR Master Mix (Qiagen, Valencia, CA) with primer concentrations at 0.2 μ M, 8 μ L of template per reaction and a total reaction volume of 40 μ L. PCR cycling conditions were: denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; then final extension at 72 °C for 7 min before holding the PCR products at 4 °C.

For the bacterial community analysis, a segment of the conserved bacterial 16S rRNA gene was amplified from the individual tick DNA extractions using universal primers 515F and 806R that flank the V4 region ([Caporaso et al., 2012](#)). The 806R primers also contained a unique 12-nucleotide Golay “barcode” for each sample that allowed us to pool the PCR products from all the samples into one Illumina MiSeq sequencing run but then to identify sequences derived from each individual tick. PCR reactions were conducted in a total volume of 40 μ L using Taq98[®] Hot Start 2X Master Mix (Lucigen, Middleton, WI, USA) with primer concentrations at 0.2 μ M. PCR cycling conditions were: denaturation at 98 °C for 2 min followed by 35 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; then final extension at 72 °C for 10 min before holding the PCR products at 4 °C. The PCR products were visualized under UV light on 1% agarose gels stained with ethidium bromide before being normalized and sequenced on an Illumina MiSeq instrument by The Scripps Research Institute DNA Array Core Facility using their standard protocols (TSRI, San Diego, CA, USA).

Computational and statistical analyses

The sequence data was analyzed using the QIIME (Quantitative Insights Into Microbial Ecology) version 1.8.0 software program ([Caporaso et al., 2010b](#)). Raw sequence data was demultiplexed into samples by barcode and filtered by mean quality score below 25, homopolymers greater than 6, uncorrected barcodes, barcodes not found in the mapping file, chimeric sequences and mismatched primers. Sequences were grouped into operational taxonomic units (OTUs) at the 97% sequence similarity level using UCLUST ([Edgar, 2010](#)) and a consensus taxonomic classification was assigned to each representative OTU using the UCLUST classifier with a Greengenes 13_8 reference database ([DeSantis et al., 2006](#)) in which at least 90% of the sequences within the OTU matched the consensus taxonomic

classification 16S rRNA gene. Sequences were aligned using PyNAST ([Caporaso et al., 2010a](#)) against the Greengenes 13_8 reference core set and a phylogenetic tree of the OTUs inferred using FastTree ([Price, Dehal & Arkin, 2010](#)). In order to remove spurious OTU's and samples with low numbers of sequences, OTU's that occurred only once in the data and samples with less than 150 OTUs were removed. *Rickettsia*, *Francisella* and other selected taxonomic sequence identifications were crosschecked against the NCBI nucleotide database using BLASTn. Sequence, OTU table and map files can be downloaded from Figshare: [10.6084/m9.figshare.2056275](#), [10.6084/m9.figshare.2068644](#), and [10.6084/m9.figshare.2056272](#), respectively.

The OTU dataset was rarefied to an even sampling depth of 150 and weighted and unweighted UniFrac distance measures between all pairs of microbial communities were calculated and visualized by principal coordinate analyses (PCoA) ([Lozupone & Knight, 2005](#)). Rarefying at 1,500 even sampling depth resulted in similar results. Several analyses were performed to determine possible factors related to microbiome differences observed within the ticks and if interference between bacteria was observed. To determine if microbial profiles were consistent with the hypothesis of interference between bacteria, the Pearson product-moment correlation coefficient (PPMC) was calculated using R to determine if a statistically significant relationship existed between the number of sequences of *Rickettsia* and *Francisella* found in the various locations. Faith's phylogenetic diversity measure (PD) was used to compare the alpha diversity between male and female ticks. Unlike other ecological diversity metrics (Shannon, chao1) that rely on species counts, Faith's PD is calculated based on phylogenetic tree branch lengths which captures an additional aspect of diversity, namely evolutionary diversity. To elucidate which bacteria in the microbiome were associated with the presence of SFGR (as identified by *rompA* and IGR sequences), Random Forest supervised learning was performed in QIIME using 1,000 trees and 10 times cross validation. The ratio of *Rickettsia* to *Francisella* was classified as high (ratio > 2), even (0.5–2) or low (<0.5) in each tick. Correlations between non-*Rickettsia*, non-*Francisella* genera in the tick microbiomes and the *Rickettsia* to *Francisella* ratios (high, even, or low) was determined via a Kruskal–Wallis *H* test. Associations between microbiome phylogenetic distances with physical separation of the sampling locations was investigated by comparing microbiome UniFrac measures to the physical distances between sampling locations using the Isolation by Distance (IBD) web service <http://ibdws.sdsu.edu/~ibdws/distances.html> ([Jensen, Bohonak & Kelley, 2005](#)). IBD tests the linear relationship between geographic distance and genetic distance of a population or, in our case, geographic distance and the microbial community phylogenetic distance. It uses a pairwise Mantel test to assess the relationship between location and UniFrac phylogenetic distances. To determine which of the abundant genera were responsible for differences in UniFrac measures between locations, OTUs that occurred in less than 10% of the samples were removed and the null hypothesis that abundances of OTUs were the same for all locations was tested using a Kruskal–Wallis *H* test in QIIME. A Procrustes least squares orthogonal mapping analysis was performed in QIIME to determine if the *beta* diversity of *Rickettsia* and *Francisella* populations was similar to non-*Rickettsia* non-*Francisella* populations with respect to location ([Gower, 1975](#)). Procrustes analysis is a statistical scaling

method that transforms multidimensional shape data, in this case, *beta* diversity matrices, into maximal superimposition (least squared distances) to determine the concordance between the matrices. Furthermore, Analysis of Similarity (ANOSIM), which compares the ranked Bray–Curtis similarity between and within groups, was used to determine whether microbial population *beta* diversity between locations differed significantly. We also compared inter- and intra-subject microbial community variability using PERMANOVA (Anderson, 2001), a non-parametric multivariate analysis of variance test that employs a permutation procedure to test the null hypothesis that there is no difference between and within subjects. We used Bray–Curtis distance and 999 permutations in R studio (Version 0.99.893, vegan package).

SourceTracker was used to compare the tick microbial profiles to microbiome datasets of dog, fish, iguana, human, pigeon, rat, and soil. SourceTracker is a tool that uses Bayesian methods to predict the source(s) of microbial communities in a set of samples (sink) (Knights et al., 2011). (The inclusion of human microbiome information, especially skin, also allowed us to test for investigator-introduced contamination since skin bacteria is the most common source of laboratory and indoor contamination.) To test for sources of the tick microbiomes (sink), microbial source tracking was performed on the merged sink and source OTU file. A logistic regression was performed using the general linear model function in R, was used to determine if *Rickettsia* presence or absence (binary outcome) in the tick was associated with a particular host source. SourceTracker was also used as a quality control measure to identify possible sample contamination. SourceTracker version 1.0 was implemented in QIIME (version 1.9.1) with default settings. As source datasets, we used publicly available sequence data in QIITA (<https://qiita.ucsd.edu/>) that included 16S rRNA data from a wide range of samples such as canine skin, mouth, and feces (Study ID 1684), human skin, mouth and stool (Study ID 1684), soil (Study ID 1684, 10363), fish, frog, iguana, pigeon, and rat skin (Study ID 1748) and negative water controls (Study ID 10363) as sources. All source and sink samples were sequenced using Illumina and the same 16S rRNA V4 region primers.

RESULTS

Four hundred seventy-four adult *D. occidentalis* ticks were collected. No immature ticks were caught. Although no ticks were positive for *Francisella tularensis*, 39 ticks (8.2%) were positive for *R. rhipicephali* and 12 (2.3%) were positive for *R. philipii* 364D as identified by sequencing of the *rompA* gene and IGR. No significant difference in infection rate between male and female ticks by *R. rhipicephali* and *R. philipii* was observed (Fisher's exact test; $P = 0.47$). From this group, 114 ticks were selected for Illumina sequencing. Amplification and gel electrophoresis of the V4 segment of the 16S rRNA gene produced visible PCR products of the expected 300 bp size from all ticks, while negative PCR and DNA extraction controls yielded no visible bands and were not sequenced. After quality filtering, 102 ticks remained: 44 positive for SFGR (as identified by *rompA* and IGR sequencing) and 59 negative for SFGR (forty-five male and fifty-seven female) from the four locations (Table 1); the total number of sequences was 6,799,927 with sample depths ranging from 2013 to

Table 1 Tick collection locations, number of ticks infected with Spotted Fever group *Rickettsia*, and number of male and female *D. occidentalis* ticks collected at each location.

| Location | GPS Coordinates | <i>R. rhipicephali</i> | <i>R. philipii</i> | Negative | M/F ^a |
|--------------------|------------------------|------------------------|--------------------|----------|------------------|
| Escondido Creek | 33.060700, -117.179500 | 7 | 1 | 9 | 8/9 |
| Lopez Canyon | 32.906776, -117.202964 | 14 | 9 | 22 | 23/22 |
| Mission Trails | 32.834444, -117.045833 | 4 | 1 | 19 | 7/17 |
| Peñasquitos Canyon | 32.938638, -117.130351 | 7 | 1 | 8 | 7/9 |

Notes.

^aNo statistically significant association between SFGR infection and male versus female, Fisher's exact test; $P > 0.5$. M, male; F, female.

250403 reads (Table S1). Clustering sequences at the 97% level of similarity and discarding OTUs that occurred only once yielded 105,174 different OTUs and 535 different taxa including one unassigned taxon. *Rickettsia* and *Francisella* genera were the most prevalent genera present in the ticks, representing 46.8% and 41.4% of all genera, respectively. The next most frequently occurring genera were *Sphingomonas* (3%), *Methylobacterium* (1%) and *Hymenobacter* (0.4%) (Fig. 1).

One *Rickettsia* sp. (OTU 83718) accounted for 89% of all *Rickettsia* OTUs and matched 99–100% to *R. rhipicephali* (GenBank accession numbers CP013133.1, NR_074473.1, CP003342.1, NR_025921.1, and U11019.1). It also had 99% identity to other *Rickettsia*; however, *E* values were 100× higher to these other *Rickettsia* sequences. The second most abundant OTU (553807) accounted for 0.7% of all *Rickettsia* OTUs and was 99% identical to several different *R. rickettsii* strains including *R. philipii* str. 364D (GenBank: NR_074470.1) as well as other strains of *R. rickettsii* (including GenBank accession numbers CP006010.1, NR_102941.1, and CP003311.1). All other *Rickettsia* OTUs comprised less than 0.09% of total *Rickettsia* OTUs. OTU 840032 comprised 87.4% of all *Francisella* OTUs and matched 100% with *Francisella*-like endosymbiont (FLE) of *D. occidentalis* (GenBank accession numbers AY805304.1, and AY375402.1). The next closest matches were *Francisella* endosymbionts of other tick species *D. albipictus*, *D. andersoni* and *D. variabilis* (GenBank accession numbers GU968868.1, FJ468434.1, and AY805307.1, respectively). The next most abundant *Francisella* OTU (399541) (GenBank acc. KU355875.1, this paper) accounted for 3.1% of all *Francisella* OTUs and matched 97% with gene sequences of endosymbionts previously determined from a spectrum of *Dermacentor* species including *Dermacentor occidentalis* (AY375403.1), *D. albipictus* (GU968868.1), *D. variabilis* (AY805307.1), *D. nitens* (AY375401.1) and *D. andersoni* (AY375398.1). All other *Francisella* OTUs accounted for less than 0.4% of the total *Francisella* OTUs.

Female ticks had significantly less microbial diversity (alpha diversity) than male ticks as measured by Faith's Phylogenetic Diversity which measures diversity based on phylogenetic tree lengths (Faith's PD, two sample *t*-test; $t = 3.63$, $P < 0.01$; Fig. 2). Although there was no significant difference between the mean number of *Rickettsia* and *Francisella* sequences in male versus female ticks (Student's *t* test $P = 0.36$, 0.06, respectively), *Rickettsia* and

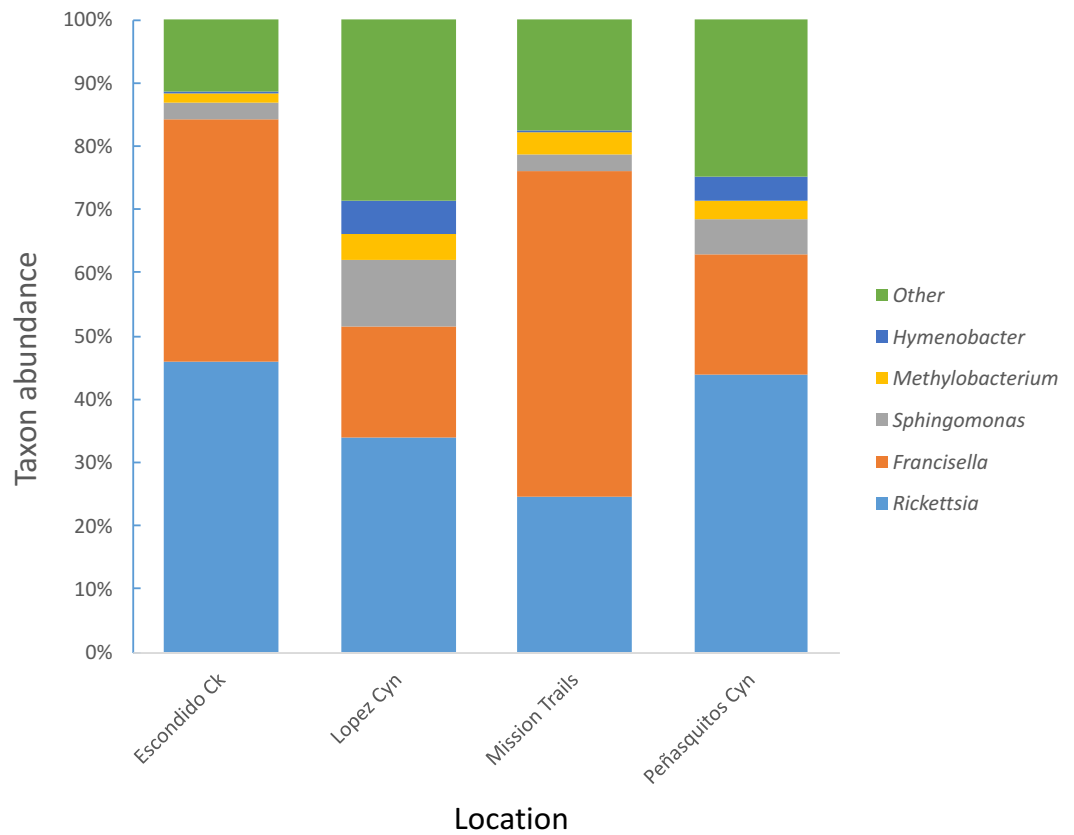


Figure 1 Most abundant bacterial genera detected in *D. occidentalis* from four different locations in San Diego County.

Francisella endosymbionts comprised a greater percentage of the microbiome of female ticks than male ticks 74.9% and 60.1%, respectively (Student's *t* test $P = 0.02$; Fig. 3).

Escondido Canyon had lower average alpha diversity than Lopez and Peñasquitos canyons, $P = 0.05$ (Fig. 4). Beta diversities of unweighted and weighted tick microbiomes had small but statistically significant associations with location as measured by analysis of similarity (ANOSIM) of UniFrac distances and visualized on Principal coordinate analysis (PCoA) (Figs. 5A and 5B). When only *Rickettsia* and *Francisella* were assessed for association with location, ANOSIM results were not statistically significant (ANOSIM, unweighted UniFrac; $R = -0.06$, $P = 0.92$; ANOSIM, weighted UniFrac $R = 0.02$, $P = 0.13$). However, in order to determine if location influenced the non-dominant species separately from the dominant *Rickettsia* and *Francisella* endosymbionts, *Rickettsia* and *Francisella* were removed from the data and the analysis was repeated. After *Rickettsia* and *Francisella* were removed, the remaining microbiome association with location was low but statistically significant (ANOSIM, unweighted UniFrac; $R = 0.20$, $P < 0.01$; ANOSIM, weighted UniFrac; $R = 0.28$, $P < 0.01$). Procrustes analysis also demonstrated that the beta diversity of microbiomes in which *Rickettsia* and *Francisella* were removed had a different association with location than *Rickettsia* and *Francisella* endosymbionts (error, $M^2 = 0.91$, $P < 0.01$). Isolation by distance (IBD) analysis using unweighted UniFrac distances that incorporated

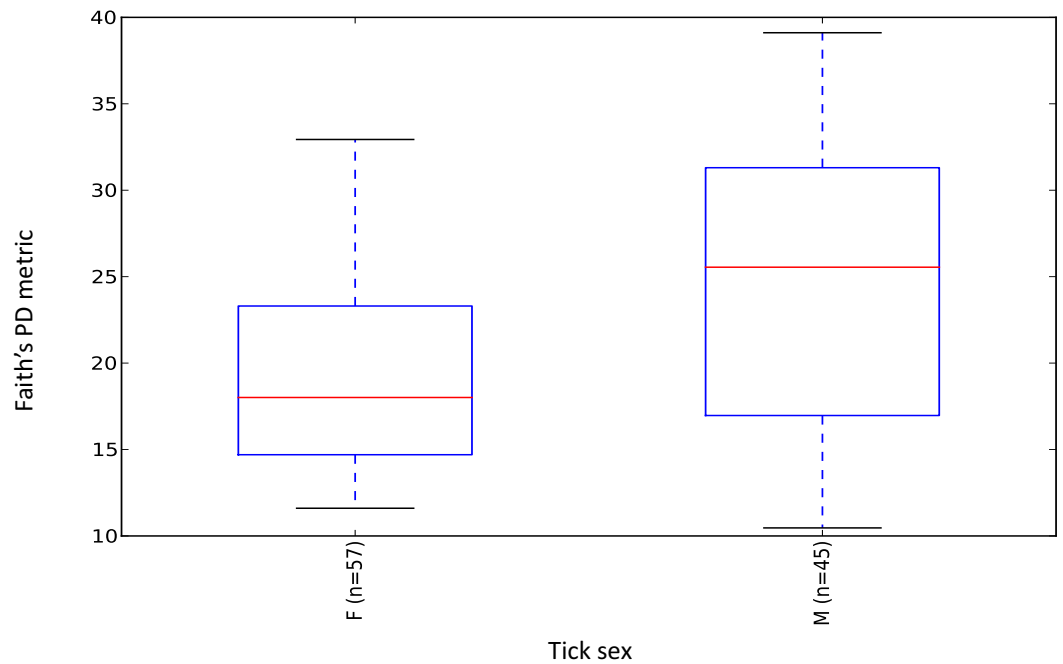


Figure 2 Boxplot of microbiome alpha diversity in *D. occidentalis* ticks measured by Faith's phylogenetic diversity (PD) whole tree as implemented in QIIME of male and female *D. occidentalis*. Faith's PD, two sample *t*-test, male versus female; $t = 3.63$, $P < 0.01$.

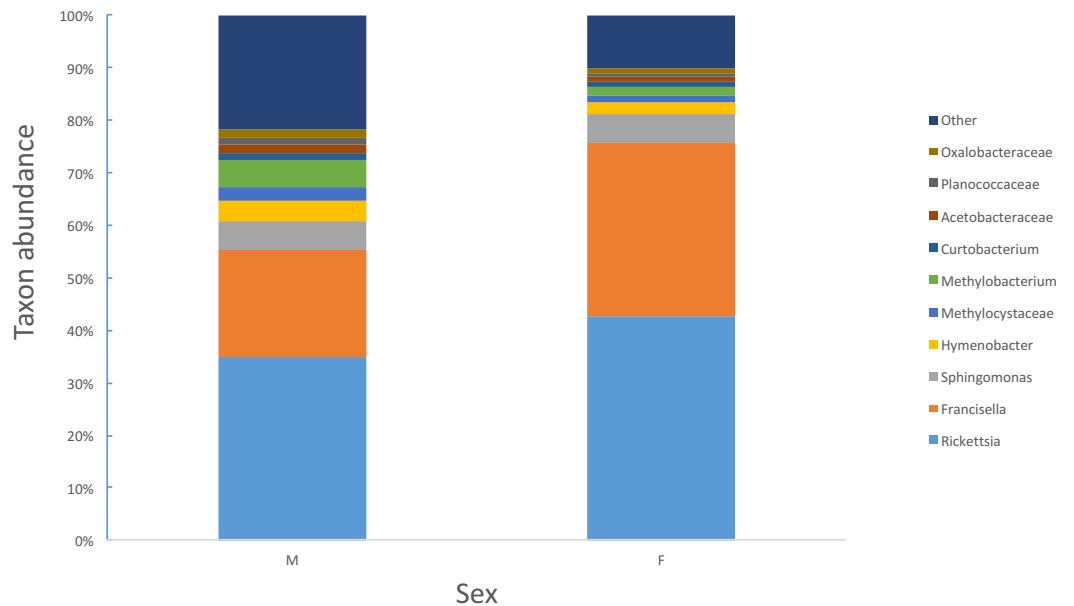


Figure 3 Percent abundance of taxa that comprise at least 1% or greater of the total microbiome in male and female ticks.

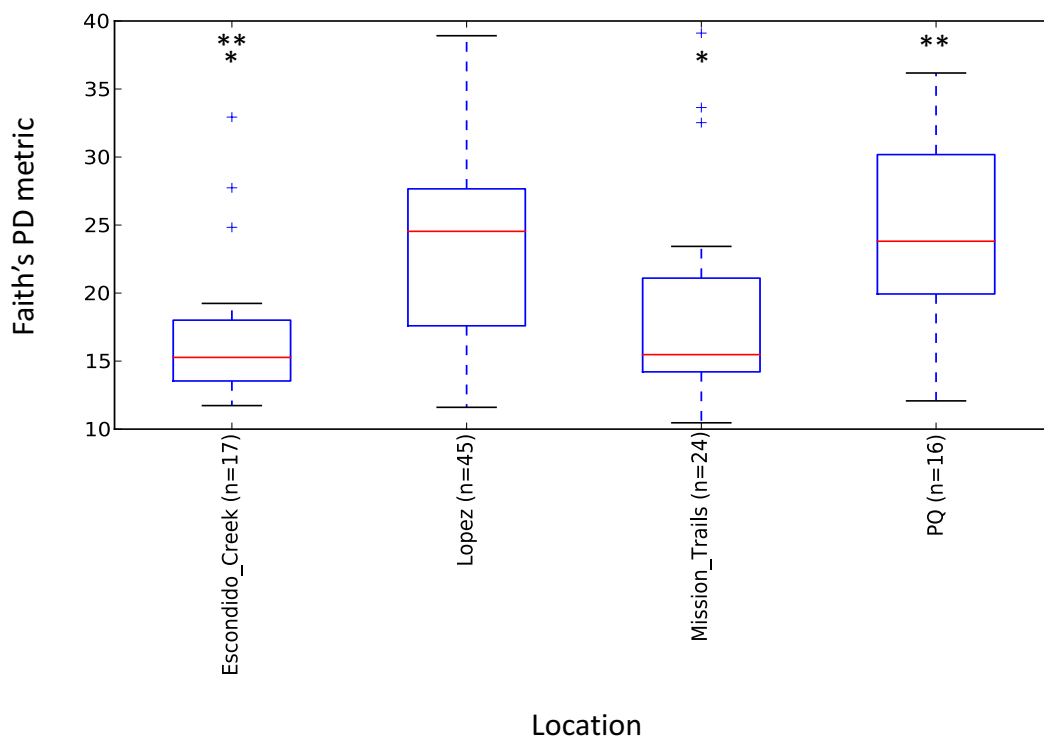


Figure 4 Boxplot of microbiome alpha diversity in *D. occidentalis* ticks measured by Faith's phylogenetic diversity (PD) whole tree as implemented in QIIME of four different hiking areas in San Diego County. Stars indicate statistically significant differences between samples; Faith's PD, two sample *t*-test, Escondido Creek versus Lopez Canyon; $t = -3.28$, $P = 0.02$; Escondido Creek versus PQ, $t = -3.31$; $P = 0.04$; other comparisons were not statistically significant. PQ = Peñasquitos Canyon.

all members of the microbiome revealed little geographic IBD (Mantel test, unweighted UniFrac, $R = 0.09$, $P < 0.01$). However, a pattern of IBD was significant after excluding *Rickettsia* and *Francisella* (Mantel test, unweighted UniFrac, $R = 0.14$, $P < 0.01$). (Unlike weighted UniFrac, unweighted UniFrac distances only incorporate the presence or absence of microbial taxa and do not take into account the abundance of the particular taxa. This allowed us to focus on the taxonomic differences among locations rather than the abundance differences of particular taxa. In a separate analysis, we found significant differences in the relative abundances of three bacterial genera, *Nevskia*, *Curtobacterium* and *Sphingomonas*, between locations (Kruskal–Wallis $H = 25.7, 24.2, 22.9$; Bonferroni corrected $P < 0.01$, respectively). Ticks in Peñasquitos and Lopez Canyons had higher abundances of *Nevskia* than ticks collected in Mission Trails. Peñasquitos and Lopez Canyons ticks had higher relative abundances of *Curtobacterium* and *Sphingomonas* than ticks in Escondido Creek and Mission Trails (Table 2).

Rickettsia and *Francisella* were negatively correlated in the ticks (Pearson's product moment correlation; $R = -0.44$, $P < 0.01$; Fig. 6). In order to assess whether the tick microbiomes were predictive of infection with spotted fever group *Rickettsia* (as determined by *real-time* PCR of the *rompA* gene and IGR sequences), a Random Forests supervised learning analysis using 1,000 trees and $10\times$ cross validation was performed on the OTU

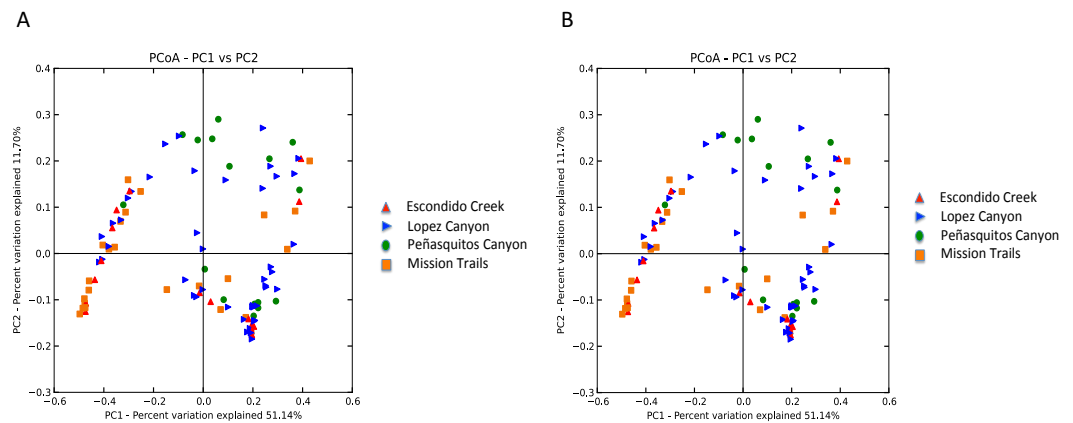


Figure 5 (A) Unweighted beta diversity of *D. occidentalis* microbiomes at four different locations in San Diego County. ANOSIM, unweighted UniFrac; $R = 0.14$, $P < 0.01$. (B) Weighted beta diversity of *D. occidentalis* microbiomes at four different locations in San Diego County. ANOSIM, weighted UniFrac; $R = 0.12$, $P = 0.01$.

Table 2 OTUs and genera associated with different locations.

| OTU | Genus | H^a | P^b | Escondido Creek ^c | Mission Trails ^c | Peñasquitos Cyn ^c | Lopez Cyn ^c |
|--------|-----------------------|-------|--------|------------------------------|-----------------------------|------------------------------|------------------------|
| 73481 | <i>Nevskia</i> | 25.7 | 0.0002 | 1.59 | 0.04 | 2.31 | 1.09 |
| 643513 | <i>Curtobacterium</i> | 24.2 | 0.0004 | 0.18 | 0.25 | 3.06 | 1.91 |
| 489455 | <i>Sphingomonas</i> | 22.9 | 0.0007 | 0.12 | 0.13 | 3.69 | 2.09 |

Notes.

^aKruskal–Wallis H value.

^bBonferroni correction (Bonferroni correction is used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data because the probability of identifying at least one significant result due to chance increases as more hypotheses are tested).

^cAverage number of OTU occurrences per sample.

dataset minus *Rickettsiaceae* and *Rickettsia* OTUs. The ratio of baseline error to the estimated generalization error was 8.8 (i.e., more than 8 times greater than random chance). The most predictive OTU was the FLE OTU 840032 and it accounted for 13% of the model. OTUs 866436 and 639277 each accounted for 3% of the model and the closest database matches to it were the Firmicutes *Geobacillus* and *Aeribacillus* (*Geobacillus*), respectively (*Minana-Galbis et al., 2010*). Non-*Rickettsia*, non-*Francisella* bacteria associated with *Francisella* to *Rickettsia* > 2 (range 2.4–119.0) were *Planococcaceae* and *Geobacillus* (Kruskal–Wallis test; $H = 23.8, 14.2$, Bonferroni $P < 0.001$ and $P = 0.011$, respectively). In addition, PERMANOVA with Bray–Curtis distances was used to test for the impact of tick sex, collection site and endosymbiotic infection status on the composition of microbial communities between samples (*Anderson, 2001*). These environmental differences showed a significant influence on the tick associated microbial communities. SFG ($P = 0.001$), tick sex ($P = 0.021$), location ($P = 0.001$), and *Rickettsia* to *Francisella* ratio ($P = 0.008$) all had significant effects on the bacterial communities.

Amplification of vertebrate cytochrome b gene was attempted to determine the origin of the ticks' host blood meals; however, no cytochrome b was amplified from the ticks.

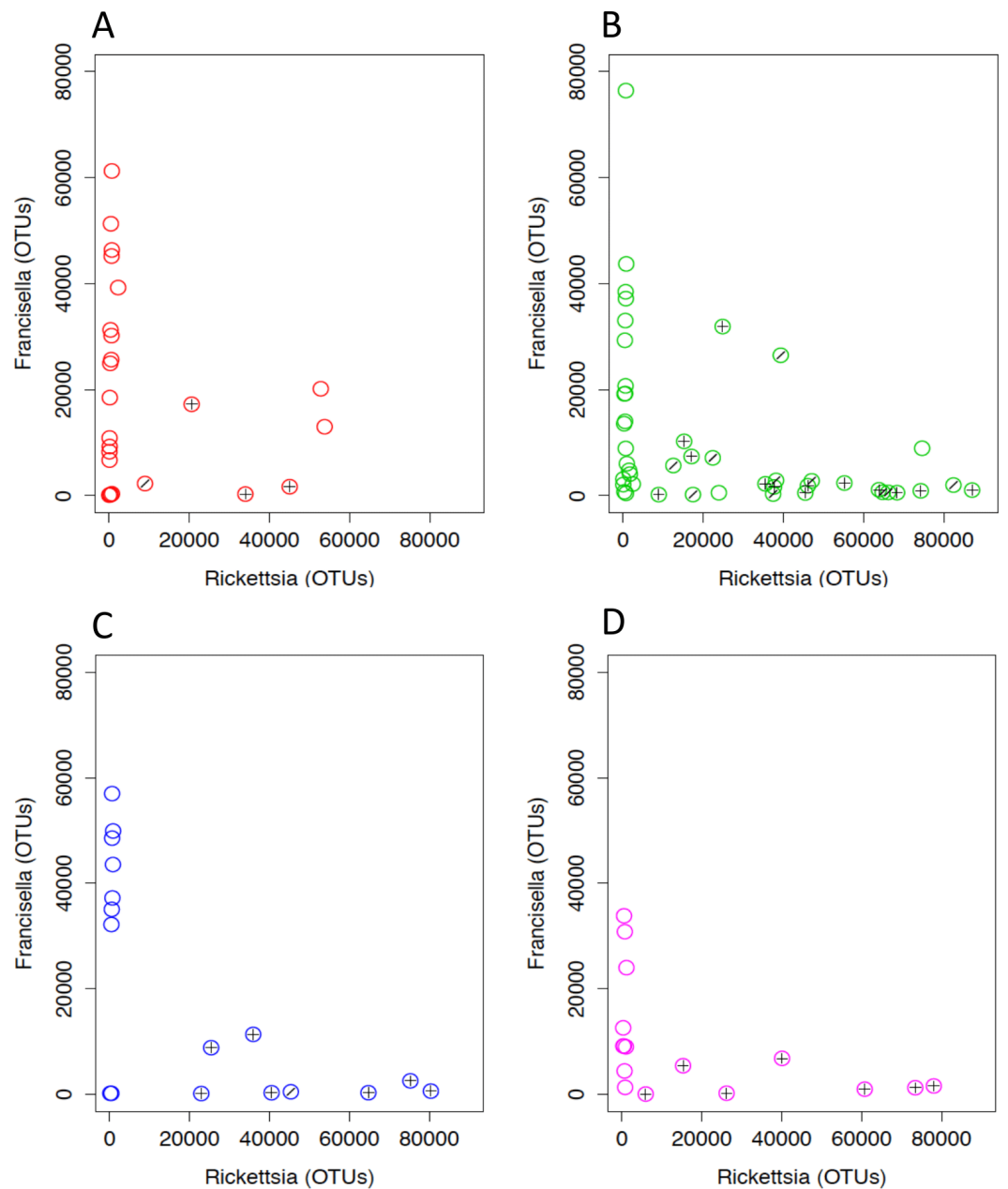


Figure 6 *Rickettsia* and *Francisella* OTU abundance in *D. occidentalis* ticks in San Diego County. Plus signs (+) indicate ticks infected with *R. rhipicephali* and slashes (/) indicate ticks infected with *R. philipii* 364D. Pearson product moment correlation; $R = -0.44$, $P < 0.01$. (A) Mission Trails; (B) Lopez Canyon; (C) Escondido Canyon; (D) Peñasquitos Canyon.

This may have been due to ticks being captured before feeding as they were questing for a blood meal. SourceTracker analysis revealed that 31% of ticks had microbiomes that were between 1.1 and 27.4% similar to dog skin microbiomes (Table S2). Ticks negative for *R. philipii* or *R. rhipicephali* were more likely to have microbiomes similar to dog skin than ticks that were infected with *R. philipii* or *R. rhipicephali* (Generalized Linear Model; $P = 0.023$). *Sphingomonadaceae*, *Oxalobacteraceae*, and *Comamonadaceae* were the most abundant families of bacteria shared between tick and dog skin microbiomes. The tick microbiomes were less than 1% similar to microbiomes of the skins of fish, iguana, pigeon, rat, and human, as well as human oral, plant and soil microbiomes (Table S2).

DISCUSSION

D. occidentalis is one of the most common tick species found in San Diego and is a vector of human pathogens including *Francisella tularensis* and *Rickettsia philipii*. This is the first study of the microbiome of *D. occidentalis* ticks using NGS technologies to examine pathogen interference within the tick microbiome. Although *Francisella tularensis* has been detected previously in ticks in San Diego (Kugeler et al., 2005), none of the ticks harbored this bacterium or genera of other recognized zoonotic tick-borne pathogens such as *Borrelia*, *Anaplasma*, *Ehrlichia*, *Babesia* or *Bartonella*; however, a low percentage of the ticks were infected with spotted fever group *Rickettsia*: 2.5% with *R. philipii* and 8.2% with *R. rhipicephali*. This is a slightly lower prevalence of *R. philipii* than surveys of ticks performed in Orange, Riverside, Los Angeles, Santa Barbara and Ventura counties north of San Diego, that reported an overall 7.5% prevalence of *R. philipii* (Wikswow et al., 2008) but is within the range of *R. philipii* prevalence reported from northern California of 0.4–5.1% (Lane et al., 1981; Philip, Lane & Casper, 1981). Similar to other tick species, the microbiome of *D. occidentalis* was dominated by Proteobacteria, primarily *Rickettsia* or *Francisella*, with lesser amounts of *Sphingomonas*, *Methylobacterium* and *Hymenobacter* (*Bacteroidetes*). These last three genera are all decomposer microbes found in the soil and except for *Hymenobacter*, have been detected in other tick microbiome studies. Even though the ticks were washed multiple times before DNA extraction, the possibility that some of these represent surface bacteria cannot be completely excluded. Although not performed in this study, removal of any OTUs detected by sequencing a sterile water negative control would also improve the sequence quality of future analyses. However, it is worth noting that members of the genus *Sphingomonas* were also found in two different studies of *Ixodes* ticks (Van Treuren et al., 2015), including one that studied larvae, suggesting that this genus may be arthropod-associated.

Although 58 of the ticks were negative for SFGR by real-time PCR of the *rompA* gene and IGR, all of the ticks contained OTUs whose partial 16S rRNA gene segments aligned with SFGR in GenBank. The cause of this discrepancy may be due to the increased sensitivity of the Illumina sequencing platform compared to real-time PCR of *rompA* and IGR sequences and/or the presence of other *Rickettsia* spp. with highly conserved 16S rRNA genes but that lack *rompA* and IGR sequences complementary to the PCR primers used. Analysis of other genes would be required to resolve them at the species level (Eremeeva,

Yu & Raoult, 1994; Regnery, Spruil & Plikaytis, 1991). Additional data support that more than two different *Rickettsia* species were present within the tick population tested. *R. rhipicephali* was detected by real-time PCR of the *rompA* gene and/or IGR in ticks that had OTU 837189 counts greater than 5900/tick, except for two ticks, T14-0667 and T14-0769 that had high OTU 837189 counts of 73,527 and 53,714, respectively, but were negative for *R. rhipicephali*. Similarly, *R. philipii* was detected in ticks with OTU 553807 counts ranging from 11 to 2,158, except for one sample, T14-0667, that had 884 counts of OTU 553807 yet was negative for *R. philipii* by real-time PCR of *rompA* gene and IGR. These findings are consistent with the presence of species of *Rickettsia* different from *R. rhipicephali* and *R. philipii* that could not be discriminated by the partial 16S rRNA gene or *rompA* and IGR sequences. The two most abundant *Francisella* OTUs, 840032 and 399541, accounted for over 90% of all *Francisella* OTUs and were 100% identical to *Francisella*-like endosymbionts (FLE) of *D. occidentalis* (GenBank accession numbers [AY805304](#) and [AY375402](#) for OTU 840032, and [KU355875](#) for OTU 399541). Taken as a whole, these results are consistent with tick co-infection with a mixture of *Rickettsias* and FLEs.

The number of unique OTUs detected in *D. variabilis* was 6.4 times higher than found in a study of *Ixodes* ticks, although, sequence depth was approximately two times greater in our study and, as noted, the vast majority of the OTUs occurred at very low frequencies ([Van Treuren et al., 2015](#)). OTUs that occurred only once in the data were removed. However, a presence threshold (i.e., requiring OTUs to be present in more than one tick) was not applied so that rare species that contributed to microbiome differences between locations would not be filtered out. This resulted in a large number of rare OTUs analyzed in our data, although, the interesting inverse relationship of the dominant endosymbiont species was not affected.

The low frequency OTUs found in the study could have partially been the result of contamination. Although negative water controls did not yield visible products when subjected to 16S rRNA PCR, these controls were not subjected to NGS sequencing. SourceTracker analysis did not find evidence of skin contaminants commonly found in reagents or samples ([Hewitt et al., 2013](#)). SourceTracker was originally designed to analyze laboratory and built environment contamination and skin bacteria are the most common contaminants in these environments ([Kelley & Gilbert, 2013](#)), suggesting that investigator introduced contamination was not a major contributor to the low frequency OTU numbers. Nonetheless, in future studies would be much improved by the incorporating negative water extraction controls carried through to NGS. Future studies would also be significantly improved by following a more rigorous contamination protocol procedure such as that outlined by [Kozich et al. \(2013\)](#): https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP_v4.md.

Similar to *Ixodes scapularis* and *Amblyomma americanum* ticks, female *D. occidentalis* ticks harbored a less diverse array of bacteria than males ([Fig. 2](#)) ([Ponnusamy et al., 2014](#); [Van Treuren et al., 2015](#)). Endosymbionts belonging to *Rickettsia*, *Coxiella*, *Francisella* and *Arsenophous* genera have been found in different tick species and are thought to interfere with and partially exclude other bacteria and pathogenic forms of closely related organisms from transovarial transmission leading to lower alpha diversity in female

ticks than males (Burgdorfer & Brinton, 1975; Macaluso et al., 2002; Niebylski, Peacock & Schwan, 1999; Noda, Munderloh & Kurtti, 1997; Reinhardt, Aeschlimann & Hecker, 1972; Telford III, 2009). In *D. occidentalis*, a higher percentage of *Rickettsia* and *Francisella* in the microbiomes of female ticks than male ticks may have similarly decreased species richness in female ticks compared to males (Fig. 2).

The beta diversity of the endosymbionts and non-endosymbionts differed with respect to location. Although non-endosymbionts demonstrated a small association with location, geographic association was not observed by the *Rickettsia* and *Francisella* endosymbionts. In addition, Procrustes analysis results demonstrated that *Rickettsia* and FLE beta diversities had different relationships to geographical locations than the other microbiome components, illustrating that different factors shape *Rickettsia* and FLE components of the *D. occidentalis* microbiome than other non-endosymbiont microbiome members. One factor that appeared to contribute to the geographical differences in the non-endosymbiont microbiome was isolation by distance. Geographical differences in bacterial community composition in the same hematophagous insect species has been seen in fleas and ticks, however, the causes are not completely known (Jones, Knight & Martin, 2010; Van Treuren et al., 2015). Differential geographic localization of *Nevskia*, *Curtobacterium* and *Sphingomonas*, genera that are associated with environmental sources such as the air-water interface (*Nevskia*) and soils (*Curtobacterium* and *Sphingomonas*), may be the result of differences in soil microbial ecology at each location (Van Treuren et al., 2015). Alternatively, non-endosymbiont microbiome differences could be the result of stochastic or different populations of ticks at each location. In contrast, the dependency of *Rickettsia* and *Francisella* endosymbionts on their *D. occidentalis* host may have restricted the degree of variation that population separation could impart upon these endosymbionts (Budachetri et al., 2014).

One of the primary hypotheses of this study was to determine if negative associations between bacteria, suggestive of interference, occurred within ticks especially with respect to pathogens. Indeed, a strong inverse relationship was observed between *Rickettsia* and FLE infection (Fig. 6) and a Random Forests supervised learning model successfully predicted the absence of SFGR within the ticks. Not surprisingly, FLE OTU 840032 contributed most to the model. FLE and different uncategorized *Rickettsia* co-infection in ticks has been previously observed but not enumerated (Niebylski et al., 1997; Scoles, 2004) and partial interference between co-infection by different *Rickettsia* species has been demonstrated (Burgdorfer, Hayes & Mavros, 1981; Macaluso et al., 2002). Although the quantitative 16S rRNA gene sequence data of FLE and *Rickettsia* co-infection in this study do not directly measure interference between the organisms, they are consistent with interference between FLE and *Rickettsias* and require further experimental studies for confirmation.

The mechanisms by which *Rickettsia* and *Francisella* interfere with each other in co-infections are not known and there can be many kinds of interaction, direct or indirect, between microbes and the tick host which can be influenced by the infection behavior of the different bacterial species. Although the localization of *R. rhipicephali* and *R. philipii* within ticks has not been determined, FLEs have been found in female tick reproductive tissues and hemolymph (Goethert & Telford, 2005; Scoles, 2004). In addition, non-*Francisella*

bacteria were also associated with low *Rickettsia* to *Francisella* ratios. *Planococcaceae* and *Geobacillus* were associated with greater abundance of *Francisella* relative to *Rickettsia* within the ticks. Although blood meals of the ticks could not be detected by amplification of vertebrate cytochrome b gene from the ticks, 31% of the tick microbiomes had microbiome components similar to canine skin which may suggest the source of a prior blood meal if they incorporated some of the skin flora into their own microbiome as has been shown with host blood microbiomes following feeding (Zhang *et al.*, 2014). Use of SourceTracker for comparison of tick and skin microbiomes is a novel approach and, interestingly, generalized linear models showed that ticks with canine skin microbiome components were less likely to be infected with *Rickettsia* which is consistent with *R. rhipicephali* and *R. philipii* being endosymbionts without a canine host.

CONCLUSIONS

The results of this study suggest that FLE and *Rickettsia* endosymbionts partially exclude each other in co-infections of the same *D. occidentalis* tick. Although interference between *Rickettsia* co-infections has been known for many years, this is the first study that suggests possible exclusion between different endosymbiont genera in ticks. Whether FLEs can be shown to inhibit *Rickettsia* co-infection in the laboratory, the mechanisms for their interaction and whether they could be propagated through a tick population as a means to render ticks unable to vector pathogenic *Rickettsia* are intriguing prospects that warrant further investigation. In addition to more mechanistic investigations, future research should also apply approaches such as shotgun metagenomics that can be used to assemble long sequence reads and even complete genomes from whole microbiome samples for more accurate species and strain identification.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Nikos Gurfield conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Saran Grewal and Lynn S. Cua performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- Pedro J. Torres performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

- Scott T. Kelley conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

GenBank: [KU355875.1](#).

Data Availability

The following information was supplied regarding data availability:

Gurfield, Nikos; Kelley, Scott (2015): Dermacentor occidentalis microbiome 16S rRNA gene sequences from San Diego County. figshare.

<https://doi.org/10.6084/m9.figshare.2056275.v2>;

Gurfield, Nikos; Kelley, Scott (2016): Dermacentor occidentalis 16S rRNA OTU table. figshare.

<https://doi.org/10.6084/m9.figshare.2068644.v1>;

Gurfield, Nikos (2015): Mapping file for Dermacentor occidentalis 16S rRNA microbiome dataset from San Diego County. figshare.

<https://doi.org/10.6084/m9.figshare.2056272.v2>.

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.3202#supplemental-information>.

REFERENCES

- Anderson MJ. 2001.** A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**:32–46 DOI [10.1111/j.1442-9993.2001.01070.pp.x](https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x).
- Azad AF, Beard CB. 1998.** Rickettsial pathogens and their arthropod vectors. *Emerging Infectious Diseases* **4**(2):179–186 DOI [10.3201/eid0402.980205](https://doi.org/10.3201/eid0402.980205).
- Budachetri K, Browning RE, Adamson SW, Dowd E, Chao CC, Ching WM, Karim S, Dowd SE. 2014.** An insight into the microbiome of the *Amblyomma maculatum* (Acari: Ixodidae). *Journal of Medical Entomology* **51**(1):119–129 DOI [10.1603/ME12223](https://doi.org/10.1603/ME12223).
- Burgdorfer W, Brinton B, Hughes LE. 1973.** Isolation and characterization of symbiotes from the Rocky Mountain wood tick *Dermacentor andersoni*. *Journal of Invertebrate Pathology* **22**:424–434 DOI [10.1016/0022-2011\(73\)90173-0](https://doi.org/10.1016/0022-2011(73)90173-0).
- Burgdorfer W, Brinton LP. 1975.** Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. *Annals of the New York Academy of Sciences* **266**(1):61–72 DOI [10.1111/j.1749-6632.1975.tb35088.x](https://doi.org/10.1111/j.1749-6632.1975.tb35088.x).
- Burgdorfer W, Hayes SF, Mavros AJ. 1981.** Nonpathogenic Rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsii*. In: Burgdorfer W, Anacker RL, eds. *Rickettsiae and Rickettsial Diseases*. 1st edition. 58. New York: Academic Press, 585–594.

- Caporaso JG, Bittinger K, Bushman FD, Desantis TZ, Andersen GL, Knight R. 2010a. PyNAST: a flexible tool for aligning sequences from a template alignment. *Bioinformatics* 26(2):266–267 DOI 10.1093/bioinformatics/btp636.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010b. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7(5):335–336 DOI 10.1038/nmeth.f.303.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* 6(8):1621–1624 DOI 10.1038/ismej.2012.8.
- Carpi G, Cagnacci F, Wittekindt NE, Zhao F, Qi J, Tomsho LP, Drautz DI, Rizzoli A, Schuster SC. 2011. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. *PLOS ONE* 6(10):e25604 DOI 10.1371/journal.pone.0025604.
- Clay K, Fuqua C. 2010. The tick microbiome: diversity, distribution and influence of the internal microbial community for a blood-feeding disease vector. In: *Draft background paper written to stimulate discussion for the institute of medicine committee on lyme disease and other tick-borne diseases: the state of the science workshop entitled: Critical needs and gaps in understanding prevention, amelioration, and resolution of lyme and other tick-borne diseases: the short-term and long-term outcomes*. Washington, D.C.: Institute of Medicine.
- Cox HR. 1940. *Rickettsia diaporica* and American Q fever. *American Journal of Tropical Medicine and Hygiene* 20:463–469.
- DeSantis TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL. 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research* 34(Web Server):W394–W399 DOI 10.1093/nar/gkl244.
- Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):2460–2461 DOI 10.1093/bioinformatics/btq461.
- Eremeeva M, Yu X, Raoult D. 1994. Differentiation among Spotted Fever Group *Rickettsia* species by analysis of restriction fragment length polymorphism of PCR-amplified DNA. *Journal of Clinical Microbiology* 32(3):803–810.
- Eremeeva ME, Bosserman E, Zambrano M, Demma L, Dasch GA. 2006. Molecular typing of novel *Rickettsia rickettsii* isolates from Arizona. *Annals of the New York Academy of Sciences* 1078(1):573–577 DOI 10.1196/annals.1374.114.
- Eremeeva ME, Dasch GA, David J, Silverman DJ. 2003. Evaluation of a PCR assay for quantitation of *Rickettsia rickettsii* and closely related Spotted Fever Group *Rickettsia*. *Journal of Clinical Microbiology* 41(12):5466–5472 DOI 10.1128/JCM.41.12.5466.

- Forsman M, Sandström G, Sjöstedt A. 1994.** Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *International Journal of Systematic Bacteriology* **44**(1):38–46 DOI [10.1099/00207713-44-1-38](https://doi.org/10.1099/00207713-44-1-38).
- Furman DP, Loomis EC. 1984.** The ticks of California (Acari: Ixodida). In: *Bulletin of the California Insect Survey*, vol. 25. Berkeley: University of California Press.
- Goethert HK, Telford SR. 2005.** A new *Francisella* (Beggiatiales: Francisellaceae) inquiline within *Dermacentor variabilis* Say (Acari: Ixodidae). *Journal of Medical Entomology* **42**(3):502–505 DOI [10.1603/0022-2585\(2005\)042\[0502:anfbfi\]2.0.co;2](https://doi.org/10.1603/0022-2585(2005)042[0502:anfbfi]2.0.co;2).
- Gower JC. 1975.** Generalized procrustes analysis. *Psychometrika* **40**(1):33–51 DOI [10.1007/BF02291478](https://doi.org/10.1007/BF02291478).
- Hawlana H, Rynkiewicz E, Toh E, Alfred A, Durden LA, Hastriter MW, Nelson DE, Rong R, Munro D, Dong Q, Fugua C, Clay K. 2012.** The arthropod, but not the vertebrate host or its environment, dictates bacterial community composition of fleas and ticks. *The ISME Journal* **7**(1):221–223 DOI [10.1038/ismej.2012.71](https://doi.org/10.1038/ismej.2012.71).
- Hewitt KM, Mannino FL, Gonzalez A, Chase JH, Caporaso JG, Knight R, Kelley ST. 2013.** Bacterial diversity in two neonatal intensive care units (NICUs). *PLOS ONE* **8**(1):e54703 DOI [10.1371/journal.pone.0054703](https://doi.org/10.1371/journal.pone.0054703).
- Holden K, Boothby JT, Anand S, Massung RF. 2003.** Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in ticks (Acari: Ixodidae) from a coastal region of California. *Journal of Medical Entomology* **40**:534–539 DOI [10.1603/0022-2585-40.4.534](https://doi.org/10.1603/0022-2585-40.4.534).
- Jensen JL, Bohonak AJ, Kelley ST. 2005.** Isolation by distance (web service). *BMC Genetics* **6**(1):13 DOI [10.1186/1471-2156-6-13](https://doi.org/10.1186/1471-2156-6-13).
- Johnston SH, Glaser CA, Padgett K, Wadford DA, Espinosa A, Espinosa N, Eremeeva ME, Tait K, Hobson B, Shtivelman S, Hsieh C, Messenger SL. 2013.** Rickettsia spp. 364D causing a cluster of Eschar-associated illness, California. *The Pediatric Infectious Disease Journal* **32**(9):1036–1039 DOI [10.1097/INF.0b013e318296b24b](https://doi.org/10.1097/INF.0b013e318296b24b).
- Jones RT, Bernhardt SA, Martin AP, Gage KL. 2012.** Interactions among symbionts of *Oropsylla* spp. (Siphonoptera: Ceratophyllidae). *Journal of Medical Entomology* **49**(3):492–496 DOI [10.1603/ME11244](https://doi.org/10.1603/ME11244).
- Jones RT, Knight R, Martin AP. 2010.** Bacterial communities of disease vectors sampled across time, space and species. *The ISME Journal* **4**:223–231 DOI [10.1038/ismej.2009.111](https://doi.org/10.1038/ismej.2009.111).
- Karpathy SE, Dasch GA, Eremeeva ME. 2007.** Molecular typing of isolates of *Rickettsia rickettsii* by use of DNA sequencing of variable intergenic regions. *Journal of Clinical Microbiology* **45**(8):2545–2553 DOI [10.1128/JCM.00367-07](https://doi.org/10.1128/JCM.00367-07).
- Kelley ST, Gilbert JA. 2013.** Studying the microbiology of the indoor environment. *Genome Biology* **14**:202 DOI [10.1186/gb-2013-14-2-202](https://doi.org/10.1186/gb-2013-14-2-202).
- Kent RJ, Norris DE. 2005.** Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome b. *American Journal of Tropical Medicine and Hygiene* **73**(2):336–342 DOI [10.1055/s-0029-1237430](https://doi.org/10.1055/s-0029-1237430).

- Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, Bushman FD, Knight R, Kelley ST. 2011.** Bayesian community-wide culture-independent microbial source tracking. *Nature Methods* **8**(9):761–763 DOI [10.1038/nmeth.1650](https://doi.org/10.1038/nmeth.1650).
- Kozich J, Schloss P, Baxter N, Jenior M, Koumpouras C. 2013.** MiSeq wet lab SOP, 16S rRNA sequencing with the illumina MiSeq: library generation, QC, & sequencing. Available at https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP_v4.md.
- Kugeler KJ, Gurfield N, Creek JG, Mahoney KS, Versage JL, Petersen JM. 2005.** Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. *Applied and Environmental Microbiology* **71**(11):7594–7597 DOI [10.1128/AEM.71.11.7594-7597.2005](https://doi.org/10.1128/AEM.71.11.7594-7597.2005).
- Lah EFC, Yaakop S, Ahamad M, Nor SM. 2015.** Molecular identification of blood meal sources of ticks (Acari: Ixodidae) using cytochrome b gene as a genetic marker. *ZooKeys* **43**(478):27–43 DOI [10.3897/zookeys.478.8037](https://doi.org/10.3897/zookeys.478.8037).
- Lane RS, Emmons RW, Dondero DV, Nelson BC. 1981.** Ecology of tick-borne agents in California I. Spotted fever group rickettsiae. *American Journal of Tropical Medicine and Hygiene* **30**(1):239–252.
- Lozupone C, Knight R. 2005.** UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**(12):8228–8235 DOI [10.1128/AEM.71.12.8228](https://doi.org/10.1128/AEM.71.12.8228).
- Macaluso KR, Sonenshine DE, Ceraul SM, Azad AF. 2002.** Rickettsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. *Journal of Medical Entomology* **39**(6):809–813 DOI [10.1603/0022-2585-39.6.809](https://doi.org/10.1603/0022-2585-39.6.809).
- Menchaca AC, Visi DK, Strey OF, Teel PD, Kalinowski K, Allen MS, Williamson PC. 2013.** Preliminary assessment of microbiome changes following blood-feeding and survivorship in the amblyomma americanum nymph-to-adult transition using semi-conductor sequencing. *PLOS ONE* **8**(6):e67129 DOI [10.1371/journal.pone.0067129](https://doi.org/10.1371/journal.pone.0067129).
- Minana-Galbis D, Pinzon DL, Loren JG, Manresa A, Oliart-Ros RM. 2010.** Reclassification of *Geobacillus pallidus* (Scholz et al., 1988) Banat et al., 2004 as *Aeribacillus pallidus* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **60**(7):1600–1604 DOI [10.1099/ijs.0.003699-0](https://doi.org/10.1099/ijs.0.003699-0).
- Nakao R, Abe T, Nijhof AM, Yamamoto S, Jongejan F, Ikemura T, Sugimoto C. 2013.** A novel approach, based on BLSOMs (Batch Learning Self-Organizing Maps), to the microbiome analysis of ticks. *The ISME Journal* **7**(5):1003–1015 DOI [10.1038/ismej.2012.171](https://doi.org/10.1038/ismej.2012.171).
- Narasimhan S, Fikrig E. 2015.** Tick microbiome: the force within. *Trends in Parasitology* **31**(7):315–323 DOI [10.1016/j.pt.2015.03.010](https://doi.org/10.1016/j.pt.2015.03.010).
- Niebylski ML, Peacock MG, Schwan TG. 1999.** Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). *Applied and Environmental Microbiology* **65**(2):773–778.
- Niebylski ML, Schrumpp ME, Burgdorfer W, Fischer ER. 1997.** *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western

- Montana. *International Journal of Systematic Bacteriology* **47**(2):446–452
[DOI 10.1099/00207713-47-2-446](https://doi.org/10.1099/00207713-47-2-446).
- Noda H, Munderloh UG, Kurtti TJ. 1997.** Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. *Applied Environmental Microbiology* **63**(10):3926–3932.
- Parker RR, Brooks CS, Marsh H. 1929.** The occurrence of *Bacterium tularensis* in the wood tick (*Dermacentor occidentalis*) in California. *Public Health Reports* **44**:1299–1300.
- Philip RN, Casper EA, Burgdorfer W, Gerloff RK, Hughes LE, Bell EJ. 1978.** Serologic typing of rickettsiae of the spotted fever group by microimmunofluorescence. *Journal of Immunology* **121**(5):1961–1968.
- Philip RN, Lane RS, Casper EA. 1981.** Serotypes of tick-borne spotted fever group rickettsiae from western California. *The American Journal of Tropical Medicine and Hygiene* **30**(3):722–727.
- Ponnusamy L, Gonzalez A, Van Treuren W, Weiss S, Parobek CM, Juliano JJ, Knight R, Roe RM, Apperson CS, Meshnick SR. 2014.** Diversity of *Rickettsiales* in the microbiome of the lone star tick, *Amblyomma americanum*. *Applied and Environmental Microbiology* **80**(1):354–359 [DOI 10.1128/AEM.02987-13](https://doi.org/10.1128/AEM.02987-13).
- Price MN, Dehal PS, Arkin AP. 2010.** FastTree 2—approximately maximum-likelihood trees for large alignments. *PLOS ONE* **5**(3):e9490
[DOI 10.1371/journal.pone.0009490](https://doi.org/10.1371/journal.pone.0009490).
- Qiu Y, Nakao R, Ohnuma A, Kawamori F, Sugimoto C. 2014.** Microbial population analysis of the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. *PLOS ONE* **9**(8):e103961 [DOI 10.1371/journal.pone.0103961](https://doi.org/10.1371/journal.pone.0103961).
- Regnery RL, Spruil CL, Plikaytis BD. 1991.** Genotypic identification of rickettsiae and estimation of intra-specific sequences divergence for portion of two rickettsial gene. *Journal of Bacteriology* **173**(5):1576–1589 [DOI 10.1128/jb.173.5.1576-1589.1991](https://doi.org/10.1128/jb.173.5.1576-1589.1991).
- Reinhardt C, Aeschlimann A, Hecker H. 1972.** Distribution of *Rickettsia*-like microorganisms in various organs of an ornithodoros moubata laboratory strain (Ixodoidea: Argasidae) as revealed by electron microscopy. *Zeitschrift für Parasitenkunde* **39**:201–209 [DOI 10.1007/BF00329456](https://doi.org/10.1007/BF00329456).
- Rounds MA, Crowder CD, Matthews HE, Philipson CA, Scoles GA, Ecker DJ, Schutzer SE, Eshoo MW. 2012.** NIH public access. *Journal of Medical Entomology* **49**(4):843–850 [DOI 10.1016/j.surg.2006.10.010](https://doi.org/10.1016/j.surg.2006.10.010).Use.
- Scoles GA. 2004.** Phylogenetic analysis of the *Francisella* -like endosymbionts of *Dermacentor* Ticks. *Journal of Medical Entomology* **41**(3):277–286
[DOI 10.1603/0022-2585-41.3.277](https://doi.org/10.1603/0022-2585-41.3.277).
- Shapiro MR, Fritz CL, Tait K, Paddock CD, Nicholson WL, Abramowicz KF, Karpathy SE, Dasch GA, Sumner JW, Adem PV, Scott JJ, Padgett KA, Zaki SR, Ereemeeva ME. 2010.** *Rickettsia* 364D: a newly recognized cause of eschar-associated illness in California. *Clinical Infectious Diseases* **50**(4):541–548 [DOI 10.1086/649926](https://doi.org/10.1086/649926).

- Telford III SR. 2009.** Status of the ‘East Side Hypothesis’ (transovarial interference) twenty five years later. *Annals of the New York Academy of Sciences* **1166**:144–150 DOI [10.1111/j.1749-6632.2009.04522.x](https://doi.org/10.1111/j.1749-6632.2009.04522.x).Status.
- Van Treuren W, Ponnusamy L, Brinkerhoff RJ, Gonzalez A, Parobek CM, Juliano JJ, Andreadis TG, Falco RC, Ziegler LB, Hathaway N, Keeler C, Emch M, Bailey JA, Roe RM, Apperson CS, Knight R, Meshnick SR. 2015.** Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. *Applied and Environmental Microbiology* **81(18)**:6200–6209 DOI [10.1128/AEM.01562-15](https://doi.org/10.1128/AEM.01562-15).
- Versage JL, Severin DDM, Chu MC, Petersen JM. 2003.** Development of a multi-target real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *Journal of Clinical Microbiology* **41**:5492–5499 DOI [10.1128/JCM.41.12.5492-5499.2003](https://doi.org/10.1128/JCM.41.12.5492-5499.2003).
- Wikswow ME, Hu R, Dasch GA, Krueger L, Arugay A, Jones K, Hess B, Bennett S, Kramer V, Eremeeva ME. 2008.** Detection and identification of spotted fever group rickettsiae in *Dermacentor* species from southern California. *Journal of Medical Entomology* **45(3)**:509–516 DOI [10.1603/0022-2585\(2008\)45\[509:DAIOSF\]2.0.CO;2](https://doi.org/10.1603/0022-2585(2008)45[509:DAIOSF]2.0.CO;2).
- Zhang XC, Yang ZN, Lu B, Ma XF, Zhang CX, Xu HJ. 2014.** The Composition and transmission of microbiome in hard tick, *Ixodes Persulcatus*, during blood meal. *Ticks and Tick-Borne Diseases* **5(6)**:864–870 DOI [10.1016/j.ttbdis.2014.07.009](https://doi.org/10.1016/j.ttbdis.2014.07.009).