

RESEARCH ARTICLE

Cross-kingdom analysis of nymphal-stage *Ixodes scapularis* microbial communities in relation to *Borrelia burgdorferi* infection and load

William J. Landesman^{1,*†}, Kenneth Mulder², L. Page Fredericks³ and Brian F. Allan³

¹Department of Biology, University of Bridgeport, Charles Dana Hall, 169 University Ave., Bridgeport, CT 06604, USA, ²Department of Mathematics, The Long Trail School, 1045 Kirby Hollow Road, Dorset, VT 05251, USA and ³Department of Entomology, University of Illinois at Urbana-Champaign, 320 Morrill Hall, 505 S. Goodwin Ave., Urbana, IL 61801, USA

*Corresponding author: Department of Biology, University of Bridgeport, Charles Dana Hall, 169 University Ave., Bridgeport, CT 06604, USA.
E-mail: wlandesm@bridgeport.edu

One sentence summary: This study describes the microbial communities of *I. scapularis* nymphs, in relation to *B. burgdorferi* presence/absence and identifies specific taxa associated with infection that should be further explored for their role in vector colonization by human pathogens.

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[†]William J. Landesman, <http://orcid.org/0000-0002-2956-4251>

ABSTRACT

The tick microbiota may influence the colonization of *Ixodes scapularis* by *Borrelia burgdorferi*, the Lyme disease bacterium. Using conserved and pathogen-specific primers we performed a cross-kingdom analysis of bacterial, fungal, protistan and archaeal communities of *I. scapularis* nymphs (N = 105) collected from southern Vermont, USA. The bacterial community was dominated by a *Rickettsia* and several environmental taxa commonly reported in *I. scapularis*, as well as the human pathogens *B. burgdorferi* and *Anaplasma phagocytophilum*, agent of human granulocytic anaplasmosis. With the fungal primer set we detected primarily plant- and litter-associated taxa and >18% of sequences were *Malassezia*, a fungal genus associated with mammalian skin. Two 18S rRNA gene primer sets, intended to target protistan communities, returned mostly *Ixodes* DNA as well as the wildlife pathogen *Babesia odocoilei* (7% of samples), a Gregarines species (14%) and a Spirurida nematode (18%). Data from pathogen-specific and conserved primers were consistent in terms of prevalence and identification. We measured *B. burgdorferi* presence/absence and load and found that bacterial beta diversity varied based on *B. burgdorferi* presence/absence. Load was weakly associated with bacterial community composition. We identified taxa associated with *B. burgdorferi* infection that should be evaluated for their role in vector colonization by pathogens.

Keywords: *Borrelia burgdorferi*; *Ixodes scapularis*; Lyme disease; tick microbiota; tick microbiome

INTRODUCTION

The microbiota of arthropod disease vectors are diverse and can be epidemiologically important because they may contain taxa that influence transmission of human pathogens (e.g. Dillon and Dillon 2004; Dong, Manfredini and Dimopoulos 2009; Weiss and

Aksoy 2011; Engel and Moran 2013; Bourtzis et al. 2014; Hughes et al. 2014; Weiss et al. 2019). Tick microbiota, while still poorly understood in comparison to insect vectors of infectious disease, also may influence pathogen transmission (Narasimhan and Fikrig 2015; Bonnet et al. 2017; de la Fuente et al. 2017).

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Specific microbial taxa have been identified that are associated with both increased and decreased pathogen colonization in ticks (Clay et al. 2008; Narasimhan et al. 2014; Gall et al. 2016; Abraham et al. 2017), or that may indirectly affect pathogen transmission by altering tick fitness (Noda, Munderloh and Kurtti 1997; Zhong, Jasinskas and Barbour 2007; Hunter et al. 2015; Smith et al. 2015).

The role of the *Ixodes scapularis* microbiota in pathogen transmission is of particular interest because it is the vector of Lyme borreliosis, a tick-borne disease affecting an estimated 300 000 people per year in the United States (U.S.; CDC 2013; Mead et al. 2013), and several other important pathogens. Research into the *I. scapularis* microbiota could ultimately lead to management strategies to control either tick densities or infection prevalence with pathogens such as *Borrelia burgdorferi* (Narasimhan and Fikrig 2015), the disease-causing agent of Lyme borreliosis in North America. Experimental antibiotic treatments have demonstrated that the *I. scapularis* microbiota indirectly facilitates *B. burgdorferi* colonization by eliciting a tick immune response (Narasimhan et al. 2014). Additionally, some bacterial taxa occasionally present in *I. scapularis* are believed to reduce *B. burgdorferi* establishment through competitive interactions (Ross et al. 2018). *Ixodes scapularis* also contain intracellular bacteria that can potentially improve fitness by provisioning limiting nutrients (Hunter et al. 2015) or inducing production of an antifreeze glycoprotein (Neelakanta et al. 2010; Busby et al. 2012).

Despite this evidence, there are relatively few reports of microbiota-*B. burgdorferi* correlations from natural *I. scapularis* populations, and more information is needed on specific microbial taxa that may facilitate or inhibit pathogen transmission. In addition to *B. burgdorferi* infection prevalence the spirochete infectious dose, or load, is a potential risk factor in the transmission of *B. burgdorferi* (Piesman 1993; Ma et al. 1998). Furthermore, *B. burgdorferi* load may influence the expression pattern of Outer Surface Protein C, essential to *B. burgdorferi* colonization of hosts (Pal et al. 2004), due to density dependent interactions (De Silva et al. 1999; Yang et al. 2000). However, very little is known about the factors driving variation in *B. burgdorferi* loading (i.e. the number *B. burgdorferi* per tick) or whether load influences, or is influenced by, the *I. scapularis* microbiota. An additional challenge to tick microbiota research is that there are several sources of microbial community variation that could mask detection of such a relationship: *I. scapularis* microbiota vary by life stage, sex, geographic location, degree of engorgement and blood meal host identity (Moreno et al. 2006; Rynkiewicz et al. 2015; Van Treuren et al. 2015; Zolnik et al. 2016; Landesman et al. 2019), yet it is often difficult to control for all of these factors in a single study. Additionally, many studies into tick microbial communities focus on bacterial community, while data are generally lacking about communities of fungi, protista and archaea that may inhabit *I. scapularis* as well. Microbial community analyses are especially needed for nymphal stage *I. scapularis*, the life stage responsible for the majority of Lyme disease transmission to humans in the eastern United States (Barbour and Fish 1993).

To address these knowledge gaps, the objectives of this study were to (i) perform a cross-kingdom analysis of *I. scapularis* nymphs to characterize bacterial, archaeal, protistan and fungal inhabitants, (ii) determine if the *I. scapularis* microbial community is correlated with the *B. burgdorferi* presence/absence and/or spirochete load and (iii) identify specific microbial taxa that are correlated with *B. burgdorferi* presence/absence.

METHODS

Site description, tick collection and processing

This research was conducted in Rutland County, Vermont, a region with one of the highest *per capita* rates of Lyme borreliosis reporting in the world (www.healthvermont.gov). Tick specimens for this study were collected from eight deciduous and mixed deciduous-coniferous forest sites from May 18 to July 13 2015, during peak nymphal activity (Fig. 1). Ticks were collected by dragging a white denim cloth (1 m²) across the forest floor on four separate 100 m transects per site. Ticks were removed from the denim cloth every 20 m, placed in 100% ethanol and stored in a -20°C freezer.

Ticks were identified to genus by inspecting mouth parts and patterning of the anal groove. Over 99% of *Ixodes* species collected with the drag cloth method in the northeastern USA, including Vermont, are *I. scapularis* (David N. Allen, personal communication, May 5 2019; Piedmonte et al. 2018; Edwards et al. 2019). Individual nymphs were rinsed with 100% ethanol, air dried and added to 2 mL sample tubes, along with one sterile 2 mm steel bead and homogenized using a Tissue Lyser LT (Qiagen, Alameda, CA) at 55 Hz for 3 minutes. Genomic DNA was extracted using Qiagen's QIAamp DNA Micro Kit following the manufacturer's protocol. Whole nymph DNA was eluted in 60 µL of Qiagen's AE elution buffer solution. To test for laboratory contamination, we performed the same homogenization and DNA extraction procedure using a sterile bead but no tick ('bead blanks'; n = 3). The DNA concentration of isolates was quantified on a Qbit® 2.0 fluorometer (Life Technologies, Carlsbad, CA) using the DS DNA (broad range) assay kit.

Borrelia burgdorferi testing

A total of 443 nymphs were tested for *Borrelia burgdorferi* infection and load—that is, both the presence/absence of *B. burgdorferi* and the number of spirochetes per tick—with quantitative real-time PCR (qPCR). We used a primer/probe system developed by Barbour et al. (2009). Forward and reverse primers (5'-GCTGTAAACGATGCACACTTGGT-3' and 5'-GGCGGCACACTTAACACGTTAG-3', respectively) targeted a 69bp region of the *B. burgdorferi* and *B. miyamotoi* 16S rRNA gene. To distinguish between these two species, we utilized a TaqMan™ probe (5'-TTCGGTACTAACTTTTAGTTAA-3'), including the FAM dye and MGBNFQ quencher, that was specific to *B. burgdorferi*. The PCR reaction was performed with a 1X QuantiNova mastermix (Qiagen) and primer and probe concentrations of 0.4 and 0.2 µM, respectively. Each sample and standard was run with 5 µL of template and a total reaction volume of 25 µL. The PCR cycle included a two-minute activation step at 95°C followed by 40 cycles of 95°C for 5 s (denaturation), and a combined annealing/extension step of 51°C for 5 s. PCR amplification was performed on a Qiagen Rotor Gene Q PCR thermal cycler. The qPCR run and qPCR reaction setup were performed in separate, temperature-regulated rooms. Each of the PCR runs included a blank comprised of sterile, RNase free water.

Each PCR run included amplicon standards of known concentration from which standard curves were generated. To prepare the standards, the 69bp target amplicon was synthesized and gel purified by Integrated DNA Technologies, Inc. (Coralville, Iowa). The amplicon was inserted into a StrataClone PCR cloning vector using the StrataClone Blunt PCR Cloning Kit by the

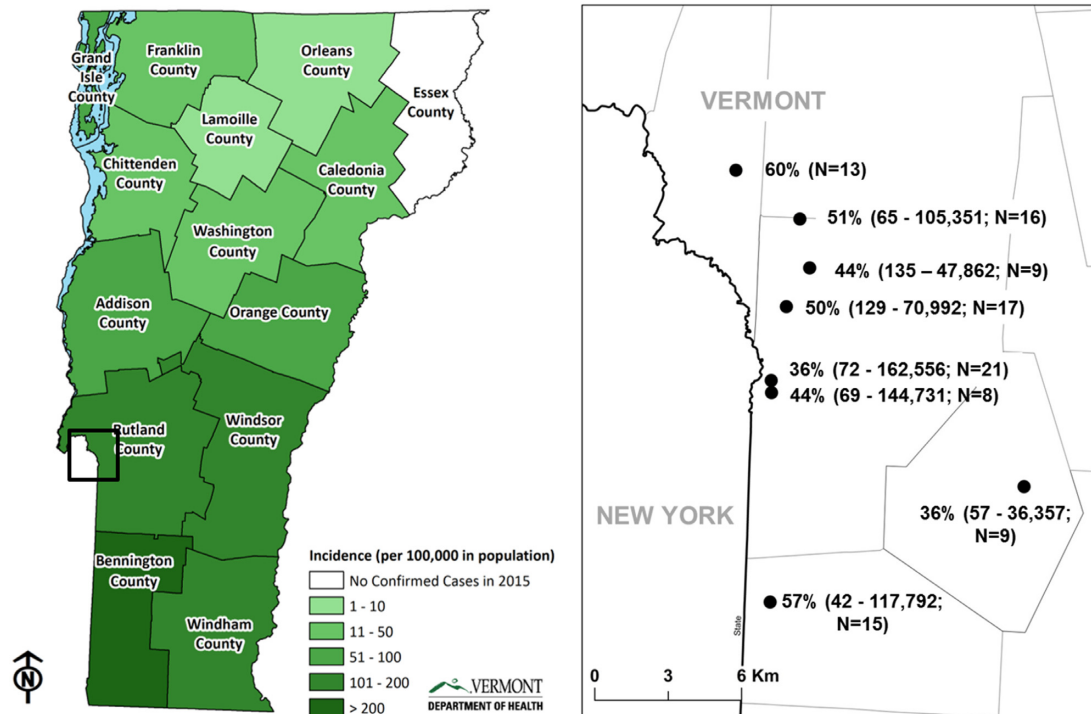


Figure 1. County-level map of Vermont, USA, showing per capita human cases of Lyme disease in 2015, by county. Area of detail shows sample locations, *B. burgdorferi* infection prevalence and spirochete load range (min.—max.). Load was not calculated for one site.

Neuroscience Center of Biomedical Research Excellence of the University of Vermont (Burlington, VT). Plasmids were purified with a Qiagen plasmid maxi prep kit and sequenced by the Advanced Genome Technologies Core Facilities at the University of Vermont to confirm that they contained the correct amplicon. These plasmids were then linearized using BamH1, a high fidelity restriction enzyme (New England Biolabs, Ipswich, MA). Plasmid concentration was determined fluorometrically and 1:10 serial dilutions were prepared with molecular grade TE buffer. Standard concentrations were 41 040; 4104; 410; 41 and 10 plasmids/ μ L.

Spirochete load was calculated using the Rotor Gene Q Series software. Prior to the calculation, for each PCR run we applied a slope correction and removed data from the first five cycles, due to stochastic early amplification in some runs. To reduce the chance of false negatives, any amplification curve with an increase in fluorescence that was less than 5% of the maximum change found in any replicate was removed. Individual amplification curves were visually inspected for linear amplification, and removed if found. Ticks were considered *B. burgdorferi* positive if two out of the three technical replicates amplified and had a Ct value < 37. This threshold was justified by our observation that amplification of water blanks (one per qPCR run) and bead blanks ($n = 3$) was rare, and almost always at a Ct > 37 when it occurred. For *B. burgdorferi*-positive samples we ran three technical replicates to calculate the number of spirochetes per tick, based on the geometric mean of the replicates. Load data from any given run were included in the final data set if the standard curves had a PCR efficiency of 0.90–1.10 and r^2 value > 0.99; load was only calculated if the technical replicates had similar Ct values at the 0.5 Ct threshold. Otherwise, these samples were considered *B. burgdorferi*-positive, but with an undetermined spirochete load.

Microbial community analyses

We used parallel PCR with universal primer sets and barcoding, combined with ultra-high throughput sequencing, to characterize the microbiota of *B. burgdorferi*-infected and uninfected nymphal-stage *I. scapularis*. Among the 443 nymphs tested for *B. burgdorferi* presence/absence and load, we selected 105 for microbial community analysis, including 20 *B. burgdorferi*-negative and 85 *B. burgdorferi*-positive nymphs with a range of spirochete loads (e.g. tens to >100 000). Universal primer sets targeted prokaryotes (515F/806R, v4 region of 16S rRNA gene; Bergmann et al. 2011; Apprill et al. 2015), archaea (Arch349F/806R, 16S rRNA gene; Takai and Horikoshi 2000), fungi (ITS1F/ITS2R, ITS gene; Schoch et al. 2012) and protista (Euk-1391F/EukBr-7R and Euk566F/1200R, targeting the V9 and V4-V5 regions of the 18S rRNA gene, respectively; Amaral-Zettler et al. 2009; Hadziavdic et al. 2014; Table 1).

We used primer sets for species-level detection of known pathogens, including *B. burgdorferi* (FBbslF.FBbslR2 5'-CGAGTTCGCGGGAGAGTA and 5'-TCCTAGGCATTMACCATAGMCT-3'; Table 1), *Borrelia miyamotoi*/*Borrelia lonestari* (FBLBM16.23F3.FBLBM16.23R3; 5'-GGTCAAGGGTTCGARTCCCT-3' and 5'-GTTCAACTCCTCTGATCCCA-3'; Fredericks et al., in prep), various strains of *Anaplasma phagocytophilum* (APdsb298F.APdsb670R: 5'-AGGGTTGATAAAATGCACGGC-3' and 5'-TAAGTCGCTGGGTCTCTGGA-3'; APdsb365F.APdsb715R: 5'-AGGTCCTAAGCATCACTCT-3' and 5'-TCTGCCTGTTGAGTCTGGTG-3'; Keesing et al. 2018) and species within the *Babesia* and *Theileria* genera (BT18SF.BT18SR; 5'-GACACAGGGAGGTAGTACAAG-3' and 5'-CTAAGAATTTACCTCTGACAGT-3'; Georges et al. 2001). Finally, we tested a 16S rRNA mitochondrial primer pair (16Splus1.16Sminus1; 5'-CTGCTCAATGATTTTTTAAATTGCTGT-3' and 5'-GTCTGAAGTCAAGT-3'; Nadolny et al. 2011) for potential species-level identification of *Ixodes* species.

Table 1. Summary of primer sets used and the number of reads obtained. ITS = Internal transcribed spacer region of rDNA; IGS = Intergenic spacer region of rRNA; mito = mitochondria; *Only the forward read was analyzed.

Primer Pair (Forward/Reverse)	Gene Region	Target Group	Expected size (nt)	# paired reads	Reference
515F/806R	16S	Prokaryotes	292	23 407 998	1,2
Arch349F/806R	16S	Archaea	457	200 676	3
566F/1200R	18S	Protists	>660*	12 220 122	4
Euk-1391F/EukBr-7R	18S	Protists	95–175	6852 490	5
ITS1F/ITS2R	ITS	Fungi	>580	34 221 582	6
APdsb298F_APdsb670R	dsb	<i>Anaplasma</i>	392	1827 983	7
APdsb365F_APdsb715R	dsb	<i>Anaplasma</i>	370	1985 614	7
BT18SF_BT18SR	18S	<i>Babesia</i>	399–449*	863 988	8
FBbslF_FBbslR2	5S-23S IGS	<i>B. burgdorferi</i>	250	391 485	9
FBLBM16.23F3.FBLBM16.23R3	16S-23S IGS	<i>B. miyamotoi/lonestari</i>	295–350	260 668	9
16Splus1.16Sminus1	16S (mito)	<i>Ixodes</i>	450–454	51 862	10

References: ¹Apprill et al. (2015), ²Bergmann et al. (2011), ³Takai and Horikoshi (2000), ⁴Hadziavdic et al. (2014), ⁵Amaral-Zettler et al. (2009), ⁶Schoch et al. (2012), ⁷Keesing et al. (2018), ⁸Georges et al. (2001); ⁹Fredericks et al., in prep and ¹⁰Nadolny et al. (2011).

Prior to sequencing, PCR amplification was carried out using the Fluidigm Access Array system, which automates the generation of amplicon libraries within an enclosed chamber, reducing preparation time and minimizing potential sources of contamination (Yue et al. 2012; Brown et al. 2016). Briefly, DNA samples were diluted to 2 ng/μL and a mastermix for amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent. Mastermix (4 μL) and target DNA (1 μL) were added to 48 wells of a PCR plate. In a separate plate, 20X primer solutions were prepared by adding 2 μL of each forward and reverse primer (50 μM each), 5 μL of 20X Access Array Loading Reagent and water to a final volume of 100 μL. Mastermix and primer (4 μL each) were added to their respective inlets of a primed Fluidigm LP48.48 IFC, which was placed in a Fluidigm Juno for loading, PCR amplification and harvesting (see Table S1, Supporting Information for PCR conditions). Harvested product was transferred to a new 96 well plate and diluted 1:100 in water, and 1 μL of diluted product was used for a second round of amplification with Illumina linkers and barcodes. Products were quantified on a Qubit fluorometer (Life Technologies, Carlsbad, CA) and stored at –20°C. All samples were run on a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and expected sizes. Samples were then pooled in equal concentrations and these pooled products were size selected on a 2% agarose E-gel and extracted from the isolated gel slice with a Qiagen gel extraction kit. Cleaned size selected products were run on an Agilent Bioanalyzer to confirm appropriate profile and determination of average size. Products were then added to an Illumina HiSeq 2500 for amplicon sequencing of 2 × 250 nt paired end reads. PCR amplification and sequencing were performed at the Roy J. Carver Center for Biotechnology at the University of Illinois (Urbana, IL).

Bioinformatics

We performed a sub-OTU analysis with DADA2 denoising, which generated 100% amplicon sequence variants (ASVs; Callahan et al. 2016). For each primer set, we truncated the sequence at a position where the overall quality generally remained above Q20. All reads for which quality values led to an expectation that more than two errors would occur over that length (max-ee) were removed. Paired reads were joined for all but the Euk566F/1200R and BT18SF_BT18SR primer pairs, for which the analysis was restricted to the forward read, due to non-overlapping amplicons. For the 16S rRNA gene and 18S

rRNA gene data sets, taxonomic classification was performed using 99% OTU sequences from the SILVA database (release 128; Quast et al. 2013). Primer sequences were used to extract relevant subsets of the database, allowing up to 30% mismatch across both primers, and a special naive Bayesian classifier was trained for each primer set. ITS sequences were classified against the UNITE database (v. 01/12/2017; Kõljalg et al. 2013). Initial processing, quality filtering and taxonomy steps were performed at the Center for Bioinformatics and Computational Biology of the University of Delaware (Newark, DE) using Qiime2–2018.2, and additional filtering steps were performed at Green Mountain College (Poultney, VT) with Qiime2–2018-11 (Caporaso et al. 2010; Bolyen et al. 2018).

For pathogen-specific primers, paired or single end (for non-overlapping amplicons) reads were matched to sequences in a custom database of tick-borne pathogens, using vsearch38 (Rognes et al. 2016). Sequences were filtered when percent identity fell below 90% and alignment lengths below 50%. A maximum of 50 hits were retained for each sample-amplicon pair. A pathogen was determined to be present in a sample if there was a greater than 98% match to the database and a minimum of 10 reads present. Representative sequences from the *Ixodes*-specific primer set were manually searched against the NCBI database.

Statistics

Statistical analyses were performed using PRIMER 7 (v. 7.0.13) and R (v 3.4.1). Microbial community data analyses focused on alpha and beta diversity indices derived from the prokaryote (515F/806R) and fungal (ITS1F/ITS2R) data sets. To test the hypothesis that microbial alpha diversity was significantly different between *B. burgdorferi*-infected and -uninfected ticks, we modeled (i) the total number of bacterial or fungal ASVs per sample and (ii) Shannon's diversity index as linear functions of site, sample day and *B. burgdorferi* presence/absence. Sampling day was recorded categorically as one of three time periods of nymphal *I. scapularis* activity: 'early' (May 18 to June 4), 'peak' (June 5–22) and 'late' (June 23 to July 13). Models were fit using maximum likelihood optimization and competing models were compared and weighted using Akaike's Information Criterion (Burnham and Anderson 2002). For beta diversity, we calculated separate log transformed Bray–Curtis similarity matrices from fungal and bacterial ASV tables, and weighted and unweighted UniFrac distance matrices from the bacterial

ASV table. Prior to calculating these matrices, we removed ASVs that had fewer than 10 sequences in the data set and rarefied the tables to 10 700 and 11 400 sequences per sample for bacteria and fungi, respectively. We performed three-way Analysis of Similarity (ANOSIM; Clarke 1993) separately on bacterial and fungal ASV tables with site, sample day and *B. burgdorferi* presence/absence as predictors. For the bacterial ASV table, we removed ASVs classified as Archaea, Mitochondria or Chloroplast. Prior to calculating diversity indices, we also removed all *Borrelia* from the bacterial ASV table, because we (i) had an independent measure of *B. burgdorferi* infection and load as our dependent variables and (ii) wanted to explore any correlation between *B. burgdorferi* presence/absence and the composition of the rest of the microbiota. We used the BEST analysis in Primer to look for correlations between Bray–Curtis similarities and the differences in *B. burgdorferi* load among samples. BEST performs a permutation test for Spearman's rank correlation between sample distances in the Bray–Curtis matrix and pairwise differences in log-transformed *B. burgdorferi* loading.

RESULTS

We obtained 6.8 to 34.2 million sequence reads from the 515F/806R, ITS1F/ITS2R, Euk566F/1200R and Euk-1391F/EukBr-7R primer pairs, and 200 000 reads from Arch349F/806R (Table 1). For two of the three bead blanks there were few (e.g. <20) to no sequences. However, for one bead blank there were 654 sequences obtained from the Arch349F/806R primer pair, 172 705 sequences from the Euk-1391F/EukBr-7R primer pair, 38 sequences from the Euk566F/1200R primer pair and 4087 sequences from the 515F/806R primer pair. These sequences were classified as unidentified bacteria, unidentified Mammalia (for both 18S rRNA gene primers) and *Lactococcus*, respectively.

We based our detection limit (Ct = 37) for qPCR on the behavior of sample blanks, since we consistently observed amplification below our lowest standard, in which case amplification was rarely observed and almost always above Ct 37 when found. Quantitative PCR runs used for load analysis had efficiencies that ranged from 0.93 to 1.11 and technical replicates with < 0.50 Ct difference. The overall *B. burgdorferi* infection prevalence, determined by qPCR, was 47% (208/443 positive), with a range of 36%–60% by site. The spirochete load among all *B. burgdorferi*-positive nymphs tested ranged from 42 to 162 556 (Fig. 1).

Prokaryotes (515F/806R)

With the 515F/806R primer pair we obtained 2128 bacterial ASVs, of which 1852 were present in individual samples (hereafter referred to as 'rare ASVs'). The most abundant bacterial genus was *Rickettsia*, which represented 31.7% of all bacterial sequences and was present in 90.3% of samples. There were 258 ASVs identified as *Rickettsia*, with a single dominant ASV that was found in all *Rickettsia*-positive samples. The other *Rickettsia* ASVs were rare, and all but four had >97% similarity to the most common *Rickettsia* ASV (the other four were present in single samples with <10 copies). A nucleotide BLAST search (blastn algorithm) of the most common, and several of the next most common ASVs, indicated *Rickettsia buchneri* as the most likely match. Other abundant taxa were *Borrelia* (8.8% of sequences and 54% of samples, according to the HiSeq data), *Burkholderia-Paraburkholderia* (5.3% of sequences and 53% of samples), *Sphingomonas* (5.1% of sequences and 68% of samples), *Mycobacterium* (4.1% of sequences and 68% of samples) and *Anaplasma* (4.2% of sequences and 9.7% of samples; Fig. 2

and Table S2, Supporting Information). Additional taxa that comprised >1% of all sequences, and were found in at least 10% of samples, were *Pseudomonas*, *Comamonadaceae*, *Luteibacter*, *Staphylococcus* and *Methylobacterium*. We found two archaeal ASVs in a single sample that were from the Woesearchaeota DHVEG-6 phylum (data not shown).

All samples determined to be *B. burgdorferi*-negative with the 515F/806R primer set were also determined to be *B. burgdorferi* negative by qPCR analysis. An additional 13 samples were *B. burgdorferi* negative with 515F/806R; these corresponded to qPCR samples that were *B. burgdorferi*-positive but with the lowest loads in the data set (Table S3, Supporting Information).

Fungi (ITS1F/ITS2R)

We obtained 1712 fungal ASVs, of which 1537 were present in individual samples. The most abundant fungal taxon was *Malassezia restricta*, which comprised 12.4% of fungal sequences and was found in 36.6% of samples (Fig. 2 and Table S2, Supporting Information). *Malassezia globosa* and unidentified *Malassezia* were found in 23.8% and 32.7% of samples and comprised 3.2% and 2.5% of sequences, respectively. Other abundant taxa were from the orders Xylariales (3.5% of sequences and 22.8% of samples), *Capnodiales* (3.0% and 17.8%), *Helotiales* (2.5% and 18.8%), the family *Didymellaceae* (2.6% and 20.8%) and the genera *Cladosporium* (2.3% and 22.8%) and *Ramularia* (8% and 16.8%). We found the entomopathogenic fungi *Beauveria bassiana*, *Lecanicillium fusisporum* and unidentified *Lecanicillium*, each in three different tick samples. Unidentified Ascomycota and unidentified fungi comprised 9.1% and 7.4% of sequences, respectively; for each taxon, 93% of these unidentified taxa were rare ASVs. A blastn search of a subset of these rare ASVs indicated that they represented a diversity of taxa, rather than a small number of dominant ASVs.

Eukaryotes (Euk566F-1200R and Euk-1391F/EukBr-7R)

The Euk566F-1200R primer pair returned > 99.5% of sequences classified as *Ixodes* and an additional 0.3% of the sequences were other non-target taxa (e.g. unidentified Eukaryota and unidentified Opisthokonta). There were 36 fungal and 12 protistan taxa which comprised 73% and 17.5% of the sequences, respectively, after removing the *Ixodes* sequences. The most abundant fungi were *Dothideomycetes* (4.3% of sequences in 16.5% of samples, after filtering *Ixodes* sequences), *Leotiomyces* (3.1% of sequences in 12.6% of samples) and *Pleosporales* (1.3% of sequences in 11.7% of samples; Fig. 2 and Table S2, Supporting Information). The most abundant protists were two *Babesia* ASVs, which comprised 8.5% of the non-*Ixodes* sequences and were found in 7/103 (7%) of samples. A blastn search revealed *Babesia odocoilei* as the closest match for both of these ASVs. Other relatively abundant protists were an unidentified *Colpodida* (2.8% of sequences in four samples), *Bromelothrix* (1.2% of sequences in one sample), *Phascolodon vorticella* (0.5% of sequences in three samples) and *Vulcanochloris guanchorum* (0.5% of sequences in one sample; Table S2, Supporting Information).

With the Euk566F-1200R primer pair we also found an unidentified Spirurida (Phylum: Nematoda) in 18% of samples, and an unidentified Tylenchida (Phylum: Nematoda) in one sample. A blastn search of the Spirurida taxa indicated *Dirofilaria repens* as the best match (208/210 nt matched) for all ASVs. Other top matches were *Onchocercidae*, *Loa loa*, *Rumenfilaria andersoni*, *Elaeophora schneideri*, *Filarioidea* and *Cercopithifilaria baina* (206–207 nt matched).

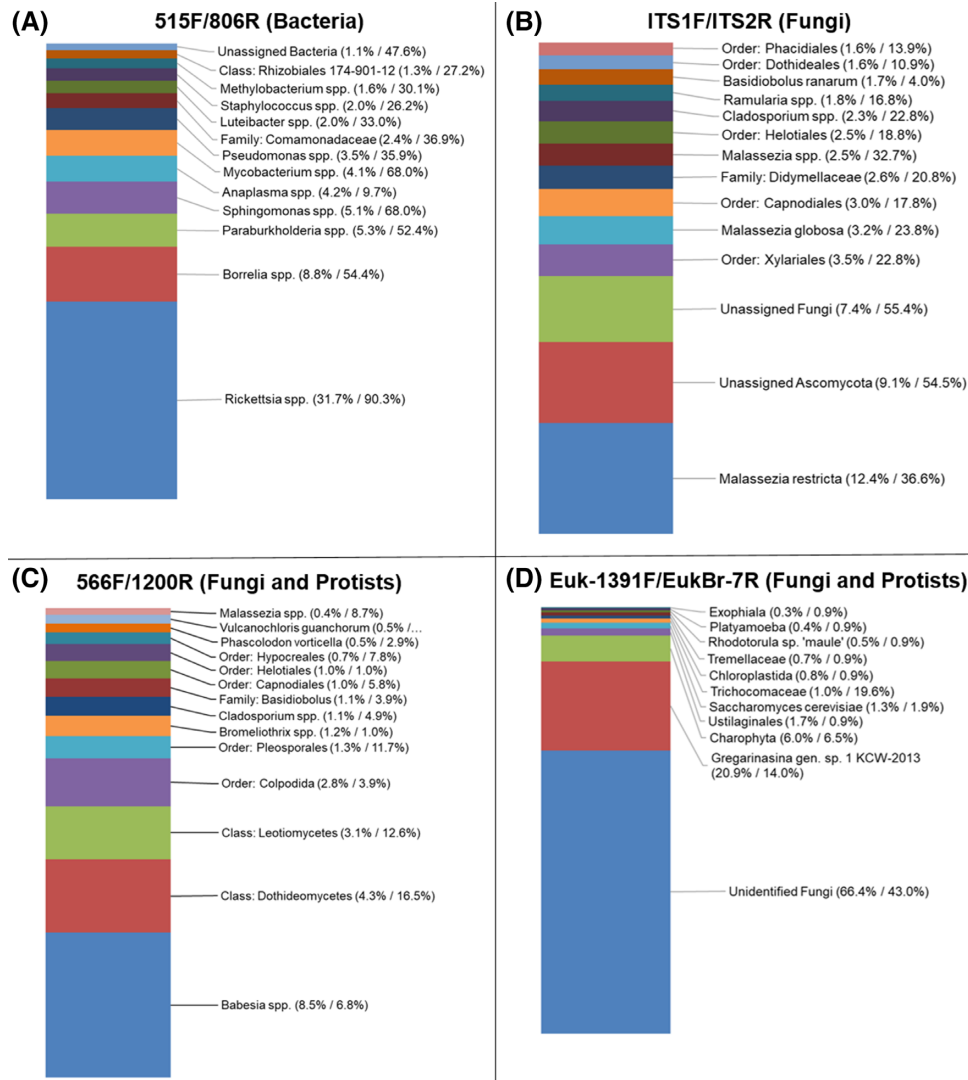


Figure 2. Taxonomy summaries for the 515F/806R, ITS1F/ITS2R, 566F/1200R and Euk-1391F/EukBr-7R primer sets. In parentheses: number of sequences as a percentage of total, and percentage of samples in which the taxon was found.

With the Euk-1391F/EukBr-7R primer-pair we found > 95% of sequences were unidentified Acari and an additional 4.2% of sequences were unidentified Animalia or unidentified Mammalia. The most abundant protists were an unidentified Charophyta, found in seven samples, and *Gregarinasina* gen. sp. 1 KCW-2013, found in 15 samples. There were seven fungal taxa, each found in 1–2 samples and unidentified fungi found in 46 samples (Fig. 2 and Table S2, Supporting Information).

Archaea (Arch349F/806R)

With the Arch349F/806R primer pair we found an unidentified Archaea in the same sample in which we found *Woeisearchaeota* DHVEG-6 with the 515F/806R primer set. Additionally, we found the same dominant bacterial taxa identified with the 515F/806R primer set (e.g. *Rickettsia*, *Borrelia*, *Anaplasma*, *Burkholderia-Paraburkholderia*, etc.; Table S2, Supporting Information). Unidentified bacteria comprised 40% of the sequences; many of these were revealed by a blastn search to be from the 18S rRNA gene region of *Ixodes*.

Pathogen- and tick-specific primer sets

Using pathogen-specific primers we found *Borrelia burgdorferi* in 62/103 samples and no *B. miyamotoi* (Table S2, Supporting Information). We found *A. phagocytophilum* in 11/103 samples with both *Anaplasma*-specific primer sets and *Babesia odocoilei/Babesia* spp. RD1 (equal e scores) in 6/103 samples. For each sample-primer pair these species hits almost always comprised >97% of reads. The 16Splus1.16Sminus1 primer set returned sequences that matched the 18S region of *Ixodes* species, which was not the intended target, and thus no confirmation of identifications could be made.

Potential drivers of alpha diversity

AIC analysis indicated that *B. burgdorferi* presence/absence was very weakly predictive of the number of bacterial ASVs (Δ AIC = 1.28; data not shown) and not a significant predictor of Shannon's Diversity Index. Neither site nor sampling day was predictive of bacterial alpha diversity. AIC analysis indicated that fungal alpha diversity was affected by both site and sampling day but not *B. burgdorferi* presence/absence; models that

Table 2. Summary of fungal alpha diversity modeling using Akaike's Information Criterion and Shannon's Index and Number of ASVs as response variables.

Model	Shannon's Index			
	Null	Day	Site	Site + Day
# parameters	2	4	8	10
Negative Log Likelihood	414.9	408.3	406.3	400.6
AIC	833.7	824.6	828.7	821.2
Δ AIC	12.5	3.4	7.5	0
AIC weight	0	0.18	0.02	1
P(Best Model)	0	0.15	0.02	0.83
No. of ASVs				
Model	Null	Day	Site	Site + Day
# parameters	2	4	8	10
Negative Log Likelihood	158.5	148.8	149.3	140.6
AIC	321	305.6	314.6	301.1
Δ AIC	19.9	4.4	13.5	0
AIC weight	0	0.11	0	1
P(Best Model)	0	0.098	0.001	0.901

Table 3. Three-way ANOSIMs of bacterial and fungal community similarity with site, sample day (three equal time periods from May 18 to July 13, 2015) and *B. burgdorferi* presence/absence as factors. *Borrelia* spp. were filtered from the data set and Bray-Curtis distance matrices were log (x+1) transformed. Significance was determined by comparing the ANOSIM R to the R values obtained from 999999 random permutations. For negative R values, the P-value was calculated as (100-P) * 2 (two-tail test).

Kingdom	Data	Seqs/sample	N	Site		Sample Day		<i>B. burgdorferi</i>	
				R	P	R	P	R	P
Bacteria	Bray-Curtis	10 700	102	0.123	0.002	0.209	0.0006	0.188	0.014
	Weighted UniFrac			0.069	0.051	0.046	0.213	0.246	0.012
	Unweighted UniFrac			0.043	0.144	0.096	0.05	0.142	0.055
Fungi	Bray-Curtis	11 400	101	0.063	0.026	0.05	0.169	-0.145	0.028

combined both site and day as predictors of the number of ASVs as well as Shannon's Diversity Index had the greatest model support (Table 2).

Drivers of bacterial and fungal beta diversity

After quality filtering and rarefaction, 103 samples, of which 83 were *B. burgdorferi*-positive, were retained for bacterial beta diversity analysis. The BEST analysis provided evidence of a weak correlation between the pairwise Bray-Curtis distance between samples and the difference in their *B. burgdorferi* loads ($\rho = 0.052$, $P = 0.066$). Using a three-way ANOSIM, we found a significant correlation between *B. burgdorferi* presence/absence and bacterial community similarity as estimated with the Bray-Curtis similarity matrix ($R = 0.188$, $p = 0.014$) and the weighted UniFrac distance matrix ($R = 0.246$, $P = 0.012$). Similarity estimated by the unweighted UniFrac distance matrix was also correlated with *B. burgdorferi* presence/absence ($R = 0.142$, $P = 0.055$; Table 3). After removing rare ASVs, the effect was significant with the weighted UniFrac Matrix ($P = 0.036$), but weaker with the Bray-Curtis matrix ($P = 0.072$) and not significant with the unweighted UniFrac matrix ($P = 0.405$, Table S4, Supporting Information). Differences in site and sample day were both significant factors of beta diversity, with and without the rare ASVs (Table 3; Table S4, Supporting Information).

We hypothesized that *Spirurida* presence may also influence the *I. scapularis* microbial community, either due to the presence of its own unique microbiota or other interactions with *I.*

scapularis. Using a two-way ANOSIM of the Bray-Curtis matrix, with *B. burgdorferi* and *Spirurida* presence/absence as factors, we found that bacterial assemblages varied significantly between *Spirurida*-positive and *Spirurida*-negative ticks ($R = 0.153$; $p = 0.022$; Table 4). This was also the case with *B. burgdorferi* presence/absence ($R = 0.162$, $P = 0.012$). After removing rare ASVs, significance for *B. burgdorferi* presence/absence was similar, and the results for *Spirurida* presence/absence showed a higher R value and stronger significance ($R = 0.176$, $P = 0.007$; Table S5, Supporting Information). There was a significant effect of *B. burgdorferi* infection with the weighted UniFrac metric ($R = 0.144$, $P = 0.039$). For *Spirurida* presence/absence, we found a negative correlation with the unweighted UniFrac metric ($R = -0.12$; $P = 0.036$), implying greater average similarity between groups than within groups. However, these results with the UniFrac matrices were not significant after removing the rare ASVs (Table S5, Supporting Information).

Using a two-way Similarity Percentages (SIMPER) analysis, we found that a total of 466 ASVs were needed to explain 70% of the community dissimilarity based on *B. burgdorferi* presence/absence, and a total of 390 ASVs were needed to explain 70% of the dissimilarity based on *Spirurida* presence/absence. ASVs making the largest contribution to community dissimilarity, and comprising at least 0.5% of sequences and at least 5% of samples, are plotted in Fig. 3. ASVs showing the largest differences based on *B. burgdorferi* presence/absence were a *Pseudomonas* ASV (#3 in Fig. 3A) and a *Staphylococcus* ASV, which were more abundant in *B. burgdorferi*-negative nymphs, and a

Table 4. Two-way ANOSIMs of bacterial and fungal community similarity with *B. burgdorferi* presence/absence and Spirurida infection status as factors. *Borrelia* spp. were filtered from the dataset and Bray–Curtis distance matrices were log (x+1) transformed. Significance was determined by comparing the ANOSIM R to the R values obtained from 999 999 random permutations. For negative R values, the P-value was calculated as (100-P) * 2.

Kingdom	Data	Seqs/sample	N	<i>B. burgdorferi</i>		Spirurida	
				R	P	R	P
Bacteria	Bray–Curtis	10 700	102	0.162	0.012	0.153	0.022
	Weighted Unifrac			0.144	0.039	0.028	0.346
	Unweighted Unifrac			0.042	0.209	–0.12	0.036
Fungi	Bray–Curtis	11 400	101	–0.067	0.072	0.047	0.126

Sphingomonas ASV (#2 in Fig. 3A), which was more abundant in *B. burgdorferi*-positive nymphs. ASVs showing the largest differences based on Spirurida presence/absence were a *Luteibacter* and a *Mycobacterium* ASV (#1 in Fig. 3B), which were more abundant in Spirurida-positive nymphs, and a *Rickettsia* ASV, which was more abundant in Spirurida-negative nymphs (Fig. 3B). These qualitative comparisons may inform the development of more targeted PCR analyses using species presence/absence by *B. burgdorferi* infection status.

After quality filtering and rarefaction, 101 samples were retained for fungal beta diversity analysis. With the three-way ANOSIM we found a negative correlation measure between *B. burgdorferi*-infected and -uninfected nymphs ($R = -0.145$, $P = 0.028$, Table 3). PERMDISP indicated that average deviations from the centroid were 69.2 (\pm SE 0.19) and 67.1 (\pm SE 0.53) for *B. burgdorferi*-infected and -uninfected ticks respectively ($p_{perm} = 0.037$), suggesting the negative correlation was due to greater dispersion in *B. burgdorferi*-positive ticks. When removing the rare ASVs, the negative correlation due to *B. burgdorferi* presence/absence was weaker ($R = -0.115$, $P = 0.146$), as was evidence for greater dispersion ($p_{perm} = 0.113$). Fungal communities were significantly different by site ($R = 0.063$, $P = 0.026$), but not by sampling day ($P = 0.169$), and these results for site and day were similar after removing the rare ASVs (Table S4, Supporting Information).

Due to the large number of non-target taxa (e.g. Acari and Ixodes), statistical analyses were not performed on data obtained from the 18S rRNA gene and Arch349F/806R primer sets.

DISCUSSION

Using parallel PCR with universal primer sets, followed by high-throughput sequencing, we performed a cross-kingdom analysis of the *I. scapularis* microbiota of field-collected ticks. This analysis uncovered several pathogens of human and wildlife significance and insights into the potential source of colonizing microbes. Below we discuss our taxonomic findings from each of the five universal primer sets tested, and identify specific ASVs correlated with *B. burgdorferi* presence/absence that should be further studied for potential positive or negative roles in vector colonization by human pathogens. Our analysis provides insight into how tick microbiota may be affected by the presence of pathogens, information that may ultimately inform management strategies that reduce pathogen exposure risk (Narasimhan and Fikrig 2015).

We found that *B. burgdorferi* presence/absence was correlated with bacterial beta diversity, more strongly due to differences in the relative abundance of taxa, rather than differences in species occurrence (Table 3). Fungal beta diversity also varied by *B. burgdorferi* presence/absence, due to differences in the

dispersion of samples (Anderson et al. 2006), and this pattern with fungi appeared to have been driven by the rare ASVs (Table 3; Table S3, Supporting Information). Importantly, our analysis controlled for a number of sources of microbial community variation, including life stage, degree of engorgement (all were questing ticks) and geographic location (Moreno et al. 2006; Rynkiewicz et al. 2015; Van Treuren et al. 2015; Zolnik et al. 2016), thereby enabling us to focus primarily on microbial taxonomic correlations with *B. burgdorferi* infection. Sex is also a driver of microbial community variation—but one that is difficult to determine in nymphs—and our large sample size was expected to reduce any potential sample bias due to sex.

The 20 *B. burgdorferi*-negative samples, as determined by qPCR, were also negative by Illumina sequencing (both the FBbsIF_FBbsIR2 and 515F/806R primer sets; Table S3, Supporting Information). The 12 samples that were positive by qPCR, but negative by Illumina sequencing, had the 12 lowest *B. burgdorferi* load estimates in the data set, accounting for most of the discrepancies among primer sets. These results suggest that qPCR was the most sensitive test for *B. burgdorferi* presence/absence. The FBbsIF_FBbsIR2 primer set detected seven more *B. burgdorferi*-positive samples than 515F-806R; this somewhat lower sensitivity of 515F/806R may be due to greater competition among targets for this conserved primer set. The number of Illumina reads were lower than qPCR loads because (i) Illumina read counts, unlike qPCR samples, were based upon 1 μ L of sample and not adjusted for DNA extraction elution volume, (ii) there could have been a loss of sample during gel extraction and (iii) samples for Illumina sequencing were diluted to 2 ng/ μ L prior to sequencing, in order to reduce PCR inhibition. Because the dilution was based on whole tick DNA, and not microbial DNA specifically, quantitative comparisons of the primer sets were not performed. Two samples exhibited large discrepancies among primer sets: samples with loads of 31 352 (T1194) and 82 985 (T1168) had no hits with the FBbsIF_FBbsIR2 primer set (for T1194 the 515F-806R primer set detected 142 reads). The cause for these large differences between qPCR and the FBbsIF_FBbsIR2 primer set is unknown.

Although our data concerning microbiome-*B. burgdorferi* interactions are correlative, several other studies also indicate that *I. scapularis* microbiota may influence pathogen colonization of ticks. Ross et al. (2018) found that colonization by *B. burgdorferi* may be impeded by antagonistic interactions with species such as *Pseudomonas*, which possess genes for a Type VI secretion system. Consistent with their findings, we identified a relatively abundant and influential *Pseudomonas* ASV (Fig. 3A) that was present in 20% of *B. burgdorferi*-negative (4/20) but only 3.7% of *B. burgdorferi*-positive nymphs (3/82; $P = 0.026$ based on a test with 20 000 permutations). In addition to a *Pseudomonas* ASV, we found several other genera that should be evaluated for

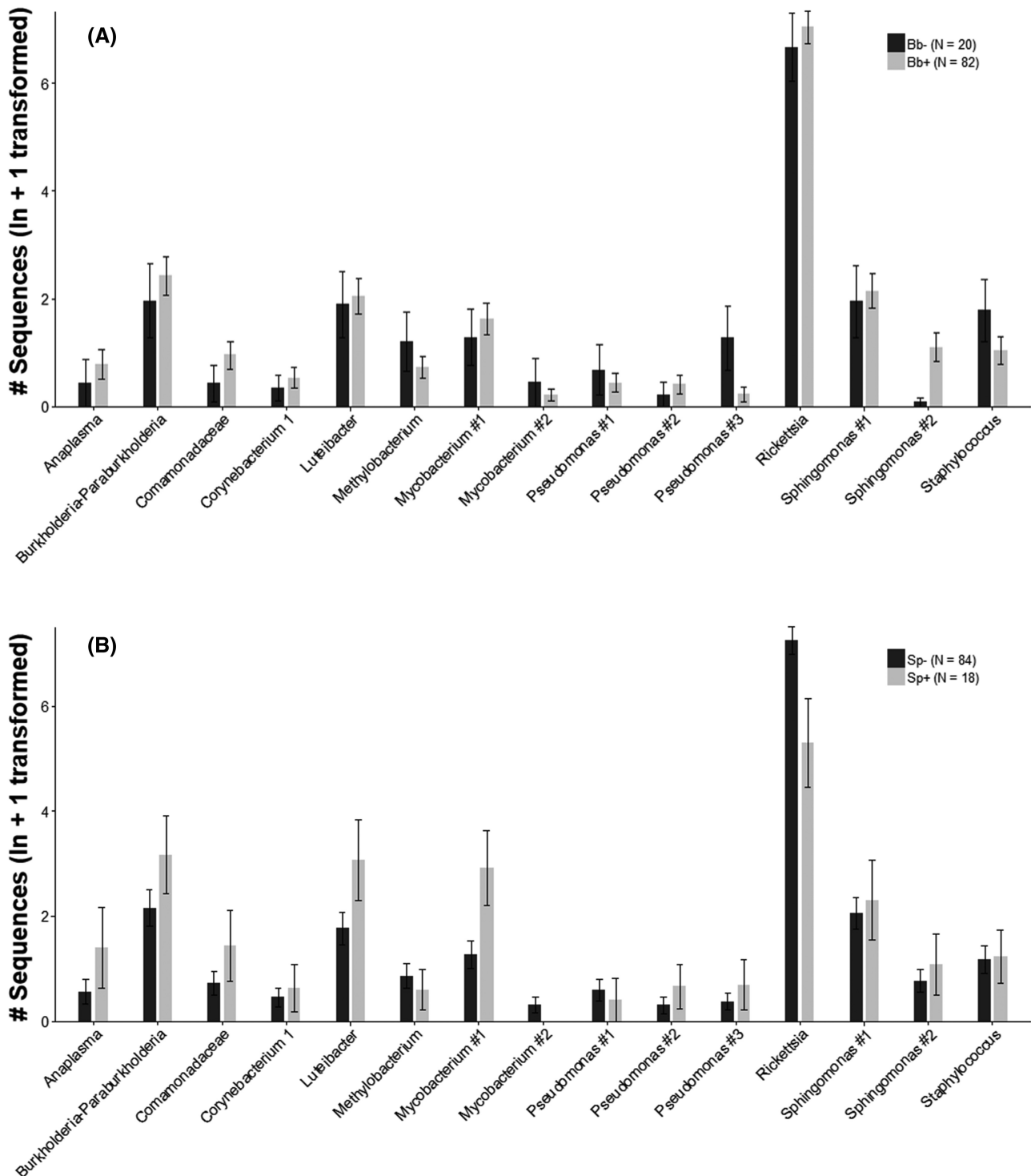


Figure 3. (A), SIMPER Plot of ln+1 transformed ASV sequence counts (\pm SE) by (A) *B. burgdorferi* presence/absence (Bb+/Bb-) and (B), Spirurida presence/absence (Sp+/Sp-) for individual ASVs with an overall frequency >0.5% and contribution to community similarity of at least 0.5%, as determined by two-way SIMPER. The taxonomic identity of the ASVs appear in the X-axis. Y-axis values are low due in part to the large number of samples with zeroes, representing samples not infected by *B. burgdorferi*.

their potential to either inhibit (e.g. *Staphylococcus*) or facilitate (e.g. *Sphingomonas*) *B. burgdorferi* colonization (Fig. 3A). The tick microbiota is also believed to trigger an immune response in *I. scapularis* that facilitates *B. burgdorferi* colonization of the midgut (Narasimhan et al. 2014). Similarly, the *I. scapularis* microbiota can be altered by presence of *A. phagocytophilum*, which appears

to facilitate colonization of this pathogen, potentially at the expense of *B. burgdorferi* colonization (Abraham et al. 2017). The *I. scapularis* microbiota may also indirectly affect pathogen transmission by altering tick fitness, due to the presence of *R. buchneri*, a nutritional endosymbiont that has the genetic machinery for folate synthesis (Hunter et al. 2015).

Both the *I. scapularis* microbiota, as well as *B. burgdorferi* colonization of *I. scapularis*, are correlated with the identity of the blood meal host (LoGiudice et al. 2003; Ostfeld and Keesing 2012; Landesman et al. 2019). Therefore, our observed microbiota-*B. burgdorferi* correlations may also be an indicator of host identity, rather than, or in addition to, species interactions. Furthermore, parasitic nematodes from the Spirurida order also infect a limited range of hosts (e.g. Joseph et al. 2011) and our SIMPER analysis of Spirurida-microbiota correlations uncovered several influential taxa (*Luteibacter*, a *Mycobacterium*, *A. phagocytophilum* and *Rickettsia*; Fig. 3B) that are from orders shown to be correlated with host species identity (Landesman et al. 2019). We therefore suggest that knowledge of the host blood meal, which is difficult to discern from questing ticks (Allan et al. 2010), is a critical missing factor in this and other analyses of tick microbiota.

Spirochete load is an important but under-studied risk factor for transmission of *B. burgdorferi* (e.g. De Silva et al. 1999; Yang et al. 2000), and large variation in *B. burgdorferi* loads in *I. scapularis* were reported here and by Wang et al. (2003). Our sampling design included a large proportion (80%) of infected nymphs, allowing us to study microbial community composition across orders of magnitude of spirochete loads for detection of potential microbiota-*B. burgdorferi* load correlations. BEST analysis suggested that nymphs with a higher spirochete load tend to have more distinct microbiota than nymphs with a lower spirochete load, although the correlation was weak. However, there was a considerable amount of noise in the data set, as evidenced by the large number of rare ASVs, and our analysis is limited by the fact that sequence counts, while useful for comparing relative abundances between groups, are not a reliable estimate of absolute abundances (Krehenwinkel et al. 2017). Thus, our evidence for a weak correlation between microbial community composition and spirochete load, combined with data showing differences in presence/absence by *B. burgdorferi* infection status (e.g. a *Pseudomonas*, a *Staphylococcus* and a *Sphingomonas*), indicate taxa which should be the focus of continued analyses to determine their potential relationships to *B. burgdorferi* infection and transmission. Such an analysis would aid in the search for microbial taxa that may be interacting with *B. burgdorferi* within *I. scapularis*.

Borrelia burgdorferi load values should be interpreted with caution, as these estimates are prone to pipetting error, especially at low load values, as well as error due to variation in qPCR efficiency among separate runs (efficiency ranged from 0.93 to 1.11 among runs used for load estimates). Additionally, the presence of *B. miyamotoi*, which also binds to our primer set, could reduce the reaction efficiency, although we found little evidence that this pathogen was present in samples.

The most commonly encountered bacteria was a *Rickettsia*—most likely *Rickettsia buchneri*—an obligate intracellular bacterium that is transmitted transovarially and trans-stadially (Kurtti et al. 2015) and that is believed to be one of the few stable members of the *I. scapularis* microbiota (Ross et al. 2018). *Anaplasma phagocytophilum*, found in 9.7% of samples, is the agent of human granulocytic anaplasmosis and the second most common tick-borne disease in Vermont (www.healthvermont.gov). In 2015, there were reported 55 cases per 100 000 individuals of anaplasmosis in Vermont, and this increased to 189 cases per 100 000 in 2017 (Natalie A. Kwit, Vermont Department of Health, Personal Communication).

Many of the abundant bacterial taxa found (e.g. *Sphingomonas*, *Mycobacterium*, *Pseudomonas*, Comamonadaceae, *Luteibacter*, *Staphylococcus*, *Methylobacterium* and Rhizobiales; Fig. 2) have been previously reported in *I. scapularis* (e.g. Benson

et al. 2004; Moreno et al. 2006; Narasimhan et al. 2014; Rynkiewicz et al. 2015; Van Treuren et al. 2015) and in other *Ixodes* species (Heise, Elshahed and Little 2010; Carpi et al. 2011; Kwan et al. 2017; Diaz-Sanchez et al. 2019), suggesting that they may be members of the *I. scapularis* internal microbiota. Many of these abundant genera contain species that inhabit plants and soil, suggesting that some may have colonized the tick surface (e.g. Ross et al. 2018; Zolnik et al. 2018) where they are less likely to interact with ticks or their internal microbiota. However, *Luteibacter*, *Sphingomonas*, *Pseudomonas* and *Staphylococcus* have been found in salivary glands of *Ixodes ovatus* and *Ixodes persulcatus* (Qiu et al. 2014). Furthermore, *Pseudomonas* was found on both the surface and within the viscera of *I. scapularis* (Ross et al. 2018). Surface washing with bleach may be more effective than ethanol at removing surface-dwelling organisms, thereby allowing for an analysis of primarily internal microbes (Binetruy et al. 2019). However, for this study we used an ethanol wash because we were also interested in surface-associated taxa, such as entomopathogenic fungi (see below), that may play a role in reducing tick fitness (e.g. Kirkland, Westwood and Keyhani 2004). Furthermore, it has been suggested that surface microbes may potentially enter internal structures via surface openings (Hynes 2014; Ross et al. 2018), although this mechanism remains untested.

Environmental microbes may also be acquired from the forest (Zolnik et al. 2018) and other tick environments. Many of the dominant fungi in our data set, including the Xylariales, Capnodiales (including *Cladosporium* and *Ramularia*), Pleosporales and Helotiales (Fig. 2) are plant-associated fungi (Webster and Weber 2007; Hyde et al. 2013), providing additional evidence that the tick microbiota contains many environmental taxa. The most commonly encountered fungi were from the genus *Malassezia*, which contains species that frequently colonize mammalian skin (Galuppi and Tampieri 2008; Boekhout et al. 2010). The absence of *Malassezia* from sample blanks suggests that they may have a non-human origin, and raises the possibility that *I. scapularis* acquire *Malassezia*, as well as other environmental microbes, due to physical contact with their vertebrate host during blood feeding (Hynes 2014). We found *Beauveria bassiana*, an entomopathogenic fungus shown to reduce fitness of several non-*Ixodes* tick species (Samish and Rehacek 1999; Kaaya and Hassan 2000; Gindin et al. 2002), in three samples. *Lecanicillium fusisporum*, an entomopathogenic fungus of aphids (Vu, Hong and Kim 2007), and unidentified *Lecanicillium* were each found in three other samples. Although we found no evidence that any of these taxa affected tick densities (data not shown), the ability to screen large numbers of ticks for entomopathogenic fungi may aid in the collection of natural specimens to be evaluated as biological control agents.

Only five protistan taxa were found with the Euk566F/1200R primer set, despite obtaining over 12 million reads (Table S2). This may be due to competition for primer binding by *Ixodes* DNA, which comprised the vast majority of sequence reads, and/or low abundance of protists inhabiting *I. scapularis*. The protists detected in this study were likely a mixture of environmental species as well as true residents of the *I. scapularis* microbiota. The Euk-1391F/EukBr-7R primer set was also dominated by *Ixodes* taxa, and returned even fewer protistan species, with less taxonomic resolution than the Euk566F/1200R primer set.

With the Euk-1391F/EukBr-7R primer set we detected a Gregarine species (*Gregarinasina* gen. sp. 1 KCW-2013), which are frequently found associated with invertebrates, including arthropods (Rueckert, Betts and Tsaousis 2019). The most abundant protist was a *Babesia* spp., with *Babesia odocoilei* as the most

likely species (Fig. 2 and Table S2, Supporting Information). This classification is supported by a blastn search of amplicons from the Euk566F/1200R primer set, for which the two *Babesia* ASVs had greater than 99% identity to *B. odocoilei* in 7/103 samples. We also found evidence for *B. odocoilei* with the *Babesia*-specific primer set in 6/103 samples (Table S2, Supporting Information). *B. odocoilei* has established populations in *I. scapularis* in New England (Armstrong et al. 1998; Steiner et al. 2014) and nearby regions of Canada (e.g. Milnes et al. 2019). *Babesia odocoilei* is transmitted between *I. scapularis* and the white-tailed deer (*Odocoileus virginianus*; Emerson and Wright 1968; Emerson and Wright 1970; Waldrup et al. 1990) and is the agent of cervid babesiosis, affecting captive animals and, potentially, wild populations of Elk and Caribou (Mathieu et al. 2018; Milnes et al. 2019). Interestingly, 5 of 7 *Babesia odocoilei* positive samples were also *B. burgdorferi* positive, suggesting that an unexpectedly high proportion of larva fed upon, and were infected with *B. burgdorferi*, by white-tailed deer. Alternatively, this finding may indicate that there are additional unknown hosts for *B. odocoilei* or that *B. odocoilei* is transmitted transovarially. To the best of our knowledge the latter mechanism has not been evaluated for this species. *Babesia microti*, the causative agent of human Babesiosis, is endemic in the northeastern United States but was not detected in our samples. In 2015 there were approximately two cases (3.4 per 100,000 people) of human Babesiosis in Rutland County, Vermont (Natalie A. Kwit, Vermont Department of Health, Personal Communication), suggesting that *B. microti* prevalence in nymphs was low during the study period.

With the Euk566F/1200R primer set (Table S2, Supporting Information) we also found a Spirurida nematode. *Dirofilaria repens* (Table S2, Supporting Information), one of the causative agents of heartworm and human dirofilariasis (Joseph et al. 2011; Kartashev et al. 2011), was the closest match in NCBI. However, using longer sequencing reads from samples collected in New York and Connecticut, USA Tokarz et al. (2019) found Spirurida more closely matched to *Acanthocheilonema viteae*. A comparison of our shorter (210 nt) amplicons revealed 99%–100% similarity to the corresponding sequence in the Tokarz et al. sequences (Rafal Tokarz, personal communication, May 3, 2019). It remains to be determined if nematode DNA found in *I. scapularis* is from living organisms and whether *I. scapularis* is a competent vector for transmission.

The archaea primer set returned bacteria, but no archaea, and the 515F/806R primer pair returned one archaeon (Table S2, Supporting Information), suggesting that archaea are largely absent from these ticks. However, Nakao et al. (2013) identified archaea in their tick samples, which may indicate that our primer sets lack the sensitivity to detect additional archaeal species.

CONCLUSIONS

Advances in PCR and DNA sequencing technologies now allow for the cost-effective analysis of large numbers of tick specimens, enabling new insights into the composition and function of tick microbiota. Using the Fluidigm Access Array system, combined with Illumina's HiSeq platform, we performed a cross-kingdom analysis of the microbiota of *I. scapularis* nymphs in southern Vermont, a region with one of the highest rates of Lyme disease reported in the USA. In addition to commonly studied human pathogens (*B. burgdorferi* and *A. phagocytophilum*), we identified several taxa of potential veterinary and wildlife significance in *I. scapularis*, including *B. odocoilei* and a Spirurida

nematode. Entomopathogenic fungi were found in nine samples. With the fungal primer set, we also found an abundance of *Malassezia*, suggesting that animal skin and fur may be an important source of environmental microbes within the whole tick microbial community. Finally, we found differences in the relative abundances of bacteria inhabiting *I. scapularis* based on *B. burgdorferi* presence/absence. Most notably a *Pseudomonas* ASV, a potential competitor with *B. burgdorferi* in the *I. scapularis* midgut, was more common in *B. burgdorferi*-negative nymphs. However, despite testing >80 *B. burgdorferi*-positive ticks across a large range of spirochete loads, we found only a weak association between microbial community composition and spirochete load. Nevertheless, these findings offer considerable promise for the discovery of microbial taxa that may influence the transmission of tick-borne pathogens and future research should seek to identify mechanisms by which microbial interactions may be harnessed to disrupt disease transmission pathways.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://www.femsec.org) online.

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Conflict of interest. None declared.

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