



Ixodes scapularis does not harbor a stable midgut microbiome

Benjamin D. Ross¹ · Beth Hayes² · Matthew C. Radey¹ · Xia Lee³ · Tanya Josek⁴ · Jenna Bjork⁵ · David Neitzel⁵ · Susan Paskewitz³ · Seemay Chou² · Joseph D. Mougous^{1,6}

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Abstract

Hard ticks of the order Ixodidae serve as vectors for numerous human pathogens, including the causative agent of Lyme Disease *Borrelia burgdorferi*. Tick-associated microbes can influence pathogen colonization, offering the potential to inhibit disease transmission through engineering of the tick microbiota. Here, we investigate whether *B. burgdorferi* encounters abundant bacteria within the midgut of wild adult *Ixodes scapularis*, its primary vector. Through the use of controlled sequencing methods and confocal microscopy, we find that the majority of field-collected adult *I. scapularis* harbor limited internal microbial communities that are dominated by endosymbionts. A minority of *I. scapularis* ticks harbor abundant midgut bacteria and lack *B. burgdorferi*. We find that the lack of a stable resident midgut microbiota is not restricted to *I. scapularis* since extension of our studies to *I. pacificus*, *Amblyomma maculatum*, and *Dermacentor* spp showed similar patterns. Finally, bioinformatic examination of the *B. burgdorferi* genome revealed the absence of genes encoding known interbacterial interaction pathways, a feature unique to the *Borrelia* genus within the phylum Spirochaetes. Our results suggest that reduced selective pressure from limited microbial populations within ticks may have facilitated the evolutionary loss of genes encoding interbacterial competition pathways from *Borrelia*.

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- ✉ Benjamin D. Ross
bdross@uw.edu
- ✉ Seemay Chou
seemay.chou@ucsf.edu
- ✉ Joseph D. Mougous
mougous@u.washington.edu

¹ Department of Microbiology, School of Medicine, University of Washington, Seattle, WA 98195, USA

² Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA

³ Department of Entomology, University of Wisconsin, Madison, WI 53706, USA

⁴ Department of Entomology, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA

⁵ Vectorborne Diseases Unit, Minnesota Department of Health, St. Paul, MN 55164, USA

⁶ Howard Hughes Medical Institute, School of Medicine, University of Washington, Seattle, WA 98195, USA

Introduction

Vector-borne pathogens infect over one billion people annually and have expanded at an alarming rate in recent years [1–4]. Lyme disease, which is caused by the tick-borne bacterial pathogen *Borrelia burgdorferi*, is the fifth-most reported infectious disease in the United States, corresponding to over 90% of vector-borne infections in North America [5, 6]. *B. burgdorferi* transits an enzootic cycle between small mammal reservoir hosts, larger mammals, and birds, vectored by ticks of the genus *Ixodes* [6]. Transmission of *B. burgdorferi* to humans via tick bite results in a constellation of inflammatory symptoms requiring an antibiotic treatment regimen for resolution within 2–3 weeks in most cases [7, 8]. Although a vaccine targeting *B. burgdorferi* had been approved by the FDA, it was subsequently withdrawn and currently there is an urgent need for new strategies to control tick-borne disease transmission [9].

Colonization resistance against pathogens mediated by commensal microorganisms is one such proposed strategy [10, 11]. Studies in mosquitos and tsetse flies, among others, have motivated the characterization of the endogenous microbiota associated with vectors in hopes of identifying means by which pathogen transmission can be interrupted

through direct or indirect interactions [12–14]. One mechanism that may govern interactions between pathogens and the microbiota involves direct competition. Many bacteria possess elaborate mechanisms that can mediate interbacterial competition in polymicrobial environments, including specialized pathways such as the type VI secretion system, which delivers toxic effector proteins to target cells [15, 16]. It is thought that the presence and repertoire of interbacterial systems in a given bacterial genome reflects to a certain degree the selective pressures that organism faces in its natural niche [17]. Indeed, these antagonistic systems contribute to bacterial fitness in complex bacterial communities such as the mammalian gut [18–20].

The genus *Borrelia* comprises a group of pathogenic bacteria that rely upon hematophagous arthropods for infectious transmission to humans, including ticks of the order Ixodida [6]. In hard ticks (Ixodidae), recent surveys of the internal microbiota have to-date identified bacteria that could potentially restrict pathogen transmission [21–35]. However, significant variation in the diversity and identity of tick-associated bacteria was observed in these studies—rendering the potential for bacterial interference within ticks uncertain. In this study, we aimed to determine whether *I. scapularis* possesses a diverse midgut microbiota that might be encountered by *B. burgdorferi*. Most previous studies of the microbiome of hard ticks have utilized high-throughput sequencing technologies. We sought to augment sequencing with direct measurements of bacterial load and visualization of bacteria within adult and nymphal *I. scapularis* ticks by confocal microscopy. With these complementary approaches, we provide evidence that hard ticks lack a stable midgut microbiota.

Methods

Tick collection

Ticks were collected from the following sites: oak woodland habitat in Klickitat River Canyon, Washington (*Ixodes pacificus* and *Dermacentor andersoni*); oak-hickory forest in Wolf Creek State Park, Illinois (*Ixodes scapularis* and *Dermacentor variabilis*); oak-dominated forest in Gordie Mikkelson Wildlife Management Area and Carlos Avery Wildlife Management Area, Minnesota (abbreviated GM and CA, *Ixodes scapularis* and *Dermacentor variabilis*); red pine forest in Kettle Moraine State Forest Southern Unit (KM), mixed hardwood forest in Big Eau Pleine County Park (BEP), and oak-hickory forest in Sandberg Woods Conservancy (SC), Wisconsin (*Ixodes scapularis*); oak forest near McPherson Preserve, Oklahoma (*Amblyomma maculatum*). Ticks were collected by the flagging and dragging methods and shipped in 50 mL Falcon tubes with

damp paper towels on wet ice to retain moisture. Species, developmental stage, and sex of individual ticks were determined by visual inspection.

DNA isolation

Live ticks were washed three times with sterile water then dried before immobilization on a glass slide using double-sided tape. 21-gauge needles were used to remove the cuticle, and sterile forceps used to dissect and remove the viscera into 500 μ L sterile deionized water for subsequent DNA purification. Fresh needles were exchanged, and forceps sterilized by washing three times in sterile water and 70% ethanol between each dissection. The first external wash sample was saved, and pooled across individuals of the same species, sex, and geographical collection location. Samples were frozen at -80°C prior to DNA isolation. Frozen tissue samples were thawed and subsequently homogenized via glass bead-beating on a MiniBeadBeater (Biospec Products) for two cycles of one minute duration each. Subsequently, phenol-chloroform-isoamyl alcohol extraction of total nucleic acids was performed with a RNase treatment to remove RNA. Following extraction, DNA pellets were resuspended in 25 μ L sterile deionized water.

Quantitative PCR

The abundance of bacteria in single tick samples was determined by quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Biorad) on a BioRad CFX96 Real Time System C1000. Primers used targeted the 16S rRNA gene (331f and 797r [36]) or the *B. burgdorferi* *flaB* gene [37]. Standard curves were generated from serial dilutions of purified genomic DNA prepared from monocultures of *Escherichia coli* DH5a or *B. burgdorferi* B31-A3. Samples were run in technical triplicates with the mean of each triplicate used for later analysis.

16S rRNA gene sequencing and analysis

Genomic DNA from tick samples, external washes, and water controls was submitted for sequencing and individually barcoded for high-throughput sequencing of V3-V4 16S rRNA amplicons on an Illumina MiSeq, in three separate runs (performed by MrDNA). Sequencing reads were subsequently de-multiplexed and merged into a single fastq file. The UPARSE pipeline was used to process the samples using default settings [38, 39]. Following taxonomy prediction and OTU assignment, the OTU table were filtered to only retain OTUs that appeared at greater than 1% relative abundance in at least one sample. Alpha-diversity and beta-diversity metrics were calculated in QIIME (MacQIIME v1.9.0) [40]. For determination of *B.*

burgdorferi infection status, tick samples in which the sum of the relative abundance of the family Spirochaetaceae exceeded 1% were considered to be infected, while those less than 1% were considered to be uninfected. The sum of the relative abundance of the genera *Bacillus* and *Pseudomonas* was calculated for comparison of *B. burgdorferi* infection status across samples. For LEfSe analysis, the OTU table was converted to relative abundances and, along with associated metadata, was uploaded to the Huttenhower Lab Galaxy web application. Statistical tests and generation of plots were performed in R version 3.3.2 [41].

Histology

Preparing tick sections

Ticks were washed three times in sterile water and immobilized with double-sided tape attached to glass slides. Cuticles were removed with sterile 21-gauge needles. Following removal of the cuticle, dissected ticks were fixed in formalin for at least 5 days at room temperature. Fixed ticks were then embedded in paraffin and four 4-micron sections were prepared from each tick. Slides were de-paraffinized in a series of washes in xylenes, followed by rehydration in washes of decreasing percent ethanol solutions.

Antibody-based microscopy

Deparaffinized slides were first subjected to a 40-min incubation in sodium citrate buffer at 95°C in a vegetable steamer (Black and Decker). Slides were then washed in $2 \times \text{SSC} + 0.3\%$ Tween 20, blocked in PBS + 5% BSA, and incubated with BacTrace fluorescein isothiocyanate goat anti-*Borrelia* whole cell antibodies (Kirkegard and Perry Laboratories) for 1 h at room temperature or overnight at 4 °C. Slides were then washed, dried, and mounted in ProLong Diamond plus DAPI (Thermo Fisher Scientific). Coverslips were sealed with nail polish.

In situ hybridization analysis

Deparaffinized slides were washed in $2 \times \text{SSC} + 0.3\%$ Tween20, then were lightly digested with Proteinase K to allow probe access into tissues, washed and dried. Probes used were EU338 [42] or specific to *Rickettsia*. For *Rickettsia*-specific probes, the nucleotide sequence of the Rick_1442 16 S rRNA probe [43] was aligned to the publicly available REIS genome [44] and was confirmed to be 100% identical. ISH-probes (5' biotin-labeled) were diluted 1:100 to 50 μM in ethylene carbonate hybridization buffer (15% ethylene carbonate, 20% dextran sulfate, 600 mM NaCl, 10 mM sodium citrate [45]). Slides were covered with Parafilm, placed in humidified chambers, and allowed to

incubate for 1 h at 42 °C. Slides were subsequently washed in $2 \times \text{SSC}$ at 37 °C then allowed to air dry. Alexa Fluor 488-coupled tyramide signal amplification (Thermo Fisher Scientific kit T20912) was performed to increase probe signal above background auto-fluorescence from tick midgut digestive products [46, 47]. Slides were mounted in Diamond Prolong mounting medium plus DAPI (Thermo Fisher Scientific) and coverslips sealed with nail polish prior to imaging.

Imaging

Confocal microscopy was performed at the University of Washington Keck Microscopy Center, on a Leica SP8X confocal microscope using Leica Application Suite X. Two-micron z-stacks were imaged at $\times 63$ magnification, using $\times 6$ averaging. Images were extracted from raw.lif files, maximum projected, channels merged, cropped, and pseudocolored using Fiji [48].

Identification of interbacterial effector-immunity gene homologs in bacterial genomes

Multi-alignments for 220 putative and validated effector and immunity proteins were acquired from the supplemental materials of Zhang et al. [17]. Amino acid seed sequences of interbacterial effector and immunity proteins were queried via tBLASTn against a custom database of 65 complete and draft genomes from the phylum Spirochaetes, 17 complete and draft genomes of tick-associated endosymbionts and pathogens from the phylum Proteobacteria (see Table S1 for accession information), and 2 genomes from the phylum Deferribacteres. All genomes were acquired via download from RefSeq. All hits with $e\text{-values} \leq 10^{-3}$ across the full length of the seed sequence were considered to be homologs of interbacterial effector or immunity proteins. The distribution of effector and immunity genes per genome within each genus was calculated by normalizing the total number of BLAST hit by the number of genomes searched. *Turneriella* genomes were included within the closely-related *Leptospira* genus for this analysis. The number of complete genomes analyzed per clade is as follows: *Anaplasma* [4], *Borrelia* [18], *Brachyspira* [7], *Coxiella* [2], *Deferribacteres* [2], *Erlichia* [8], *Leptospira* [17], *Rickettsia* [3], *Spiriochaeta* [11], *Treponema* [10], *Turneriella* [2].

Results

Borrelia lacks interbacterial effector-immunity genes

Members of the phylum Spirochaetes inhabit diverse environments, from the dense and competitive oral cavity

and gastrointestinal tract of mammals to the midguts of arthropods like termites and ticks. How members of the phylum Spirochaetes engage in interactions with other bacteria is not understood. We therefore sought to characterize the distribution of interbacterial effector and immunity genes in the genomes of Spirochaetes. We first compiled a database of Spirochaetes genomes, encompassing a total of 65 genomes representing all major genera. These genomes were queried for the presence of homologs of 220 interbacterial effector and immunity genes [17], including those with characterized domains found associated with contact-dependent inhibition [49], type VI secretion system [16], and ESX/T7SS antagonistic pathways [50, 51]. This analysis revealed the presence of genes encoding interbacterial effector and immunity domains throughout the Spirochaetes, particularly in species known to inhabit polymicrobial environments such as the mammalian gut microbiome (*Treponema succinifaciens* and *Brachyspira* spp) and the oral microbiome (*Treponema denticola*) (Fig. 1a). In contrast, we failed to detect effector gene homologs and identified only a limited group of immunity gene homologs encoded by any species within the genus *Borrelia*, including representatives from the *sensu stricto* and *sensu lato* genospecies [52]. Since the phylum Spirochaetes is considered to be monophyletic, with extant genera descending from a single common ancestor [53], parsimony supports the conclusion that *Borrelia* lost interbacterial effector genes early in the evolution of the genus. Parallel investigation of the genomes of tick-associated

endosymbionts and tick-transmitted intracellular pathogens including *Rickettsia*, *Coxiella*, *Anaplasma*, and *Ehrlichia* revealed that these genomes also largely lack interbacterial effector–immunity genes (Fig. 1b).

Assessment of bacterial abundance and diversity in *I. scapularis*

The evolutionary loss of effector–immunity genes from the genus *Borrelia* led us to hypothesize that interbacterial interactions might be limited within *I. scapularis*. We therefore sought to quantify the microbial communities associated with wild *I. scapularis*. We first isolated DNA from the dissected viscera (a combination of internal tissues that included midgut, reproductive tissues, and salivary glands) of 61 adult ticks collected from 5 distinct geographic sites in the Midwestern US. We then performed quantitative polymerase chain reaction (qPCR) targeting conserved regions of the 16S rRNA gene. Our analysis demonstrated that the mean internal bacterial load of unfed adult *I. scapularis* ticks varies over several orders of magnitude (Fig. 2a). Male ticks analyzed harbored less bacterial load than did females (4.5×10^5 vs 5.3×10^7 , *t*-test *p*-value < 0.001 , $N = 61$). qPCR with primers specific to the *B. burgdorferi* *flaB* gene revealed infection frequencies similar to that previously reported from the Midwestern geographic regions (Fig. S1A) [54]. To investigate the differences in bacterial community underlying the sex-specific differences that we observed, we performed 16S rRNA gene

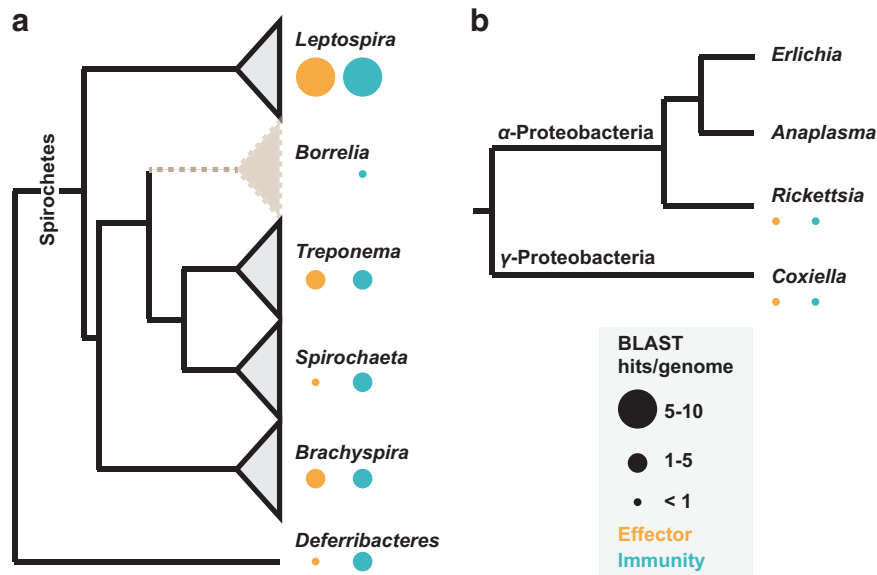
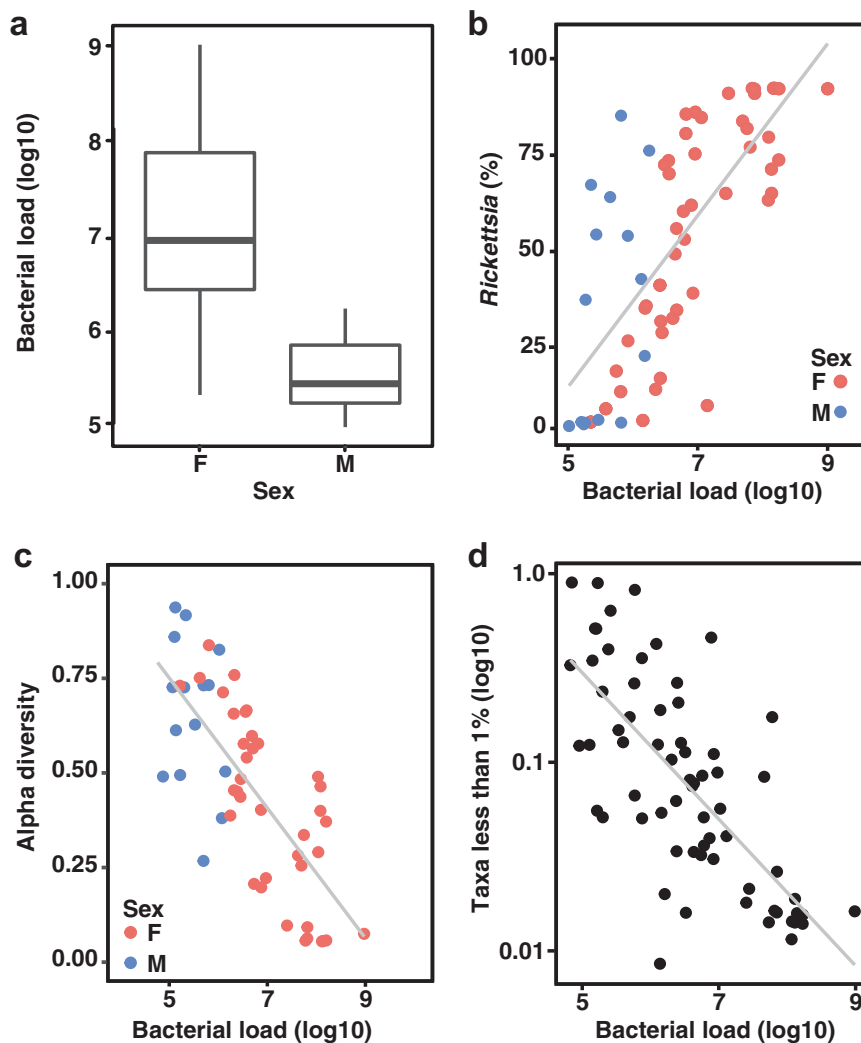


Fig. 1 Distribution of interbacterial effector–immunity gene homologs across the phylum Spirochaetes. **a** The distribution of interbacterial effector and immunity gene homologs in genomes from across a phylogenetic tree of genera within the phylum Spirochaetes. Deferribacteres is shown as the outgroup. BLAST hits are normalized to the

number of genomes analyzed for each genus. The genus *Borrelia* shows a significant deviation from the expected frequency relative to other genera ($p < 0.05$). **b** Interbacterial effector and immunity gene homologs in genomes of bacterial endosymbionts and pathogens associated with Ixodidae ticks from the phylum Proteobacteria.

Fig. 2 Inflation of bacterial diversity in *Ixodes scapularis*. **a** The bacterial load of male and female *I. scapularis* adult ticks, determined by qPCR amplifying the 16S rRNA gene ($N = 61$). Male ticks harbor significantly less bacteria than do females, (t-test $p < 0.001$). **b** Correlation between the relative abundance of *Rickettsia* and the total bacterial load across all adult *I. scapularis* ticks in unfiltered samples (Spearman's $\rho = 0.75$, $p < 0.001$). **c** Alpha diversity measured by the Simpson's value (\log_2) negatively correlates with bacterial load (\log_{10}) across all adult *I. scapularis* samples without filtering of low abundance taxa (Spearman's $\rho = -0.74$, $p < 0.001$). Male (blue) and female (pink) samples appear stratified according to both load and diversity. **d** Relative abundance of the sum of all taxa whose average relative abundance was $< 1\%$ correlates negatively with bacterial load in adult *I. scapularis* samples (\log_{10}), Spearman's $\rho = -0.73$, $p < 0.001$



sequencing on the same samples used for qPCR analysis. In addition, we sequenced the external washes from a subset of ticks, and a water-only control for each group. In total 178 OTUs were detected in the water-only controls, including taxa commonly implicated in reagent contamination and some previously reported to be associated with ticks, such as *Sphingomonas* and *Comamonas* [26, 27, 35]. These OTUs were subsequently eliminated from all other samples in downstream analyses. To describe the taxa that comprise *I. scapularis*-associated bacterial communities following removal of water-borne contamination, we calculated the taxonomic relative abundance averaged across all internal and external samples. This revealed that 258 (95%) of 270 OTUs fell below 1% relative abundance when averaged. In contrast, other taxa exceeded 1% average relative abundance in both internal and external samples, including *Bacillus*, *Pseudomonas*, and *Enterobacteriaceae* (Fig. S2). Importantly, the taxon that was most abundant in internal samples but entirely absent in external samples was the genus *Rickettsia* (average relative abundance of 52%),

which includes the dominant *Rickettsia* endosymbiont of *I. scapularis* [44]. The relative abundance of *Rickettsia* exhibited a positive correlation with total bacterial load (Fig. 2b), indicating that *Rickettsia* is the primary driver of bacterial abundance in most *I. scapularis* ticks. The family Spirochaetaceae also was often abundant in viscera samples but absent in washes (Fig. S2).

The number of taxa with low relative abundance could indicate that the internal environment of *I. scapularis* is particularly well suited to fostering diverse microbial communities. However, an alternate possibility is that low abundance OTUs represent signal from contamination. Indeed, several studies have examined the effect of low biomass input on diversity metrics in 16S rRNA gene sequencing surveys, with the notable result that alpha diversity (within-sample diversity) correlated negatively with the abundance of input DNA [55–57]. We therefore examined the relationship between alpha diversity and bacterial load as quantified by qPCR in our *I. scapularis* samples. When examined across all 61 *I. scapularis*

samples, we found a strong negative correlation between total bacterial load and alpha diversity that was independent of geographic origin (Spearman's $\rho = -0.74$, $p < 0.001$) (Fig. 2c and S3). These data imply that the low bacterial biomass associated with adult *I. scapularis* ticks can impact the interpretation of 16 S rRNA gene sequencing surveys by artificially inflating alpha diversity. We further observed a strong negative correlation between bacterial load and the relative abundance of the group of taxa contributing to 1% or less of the total across all *I. scapularis* samples (Fig. 2d). We found only six taxa to be present in all *I. scapularis* viscera samples, represented by eight OTUs, including the genera *Rickettsia*, *Borrelia*, *Pseudomonas*, *Francisella*, and *Escherichia*, and the family Enterobacteriaceae (Fig. S2). Linear discriminant analysis effect size (LEfSe) revealed the taxa most likely to explain differences between external wash samples and internal viscera samples. External samples are characterized by taxa from the phyla Proteobacteria and Actinobacteria, while viscera samples are distinguished by the orders *Spirochaetales* and *Rickettsiales* (Fig. S4) [58].

While the internal bacteria associated with most wild adult *I. scapularis* are dominated by *Rickettsia* and *B. burgdorferi*, a minority of samples exhibit high relative abundances of three putatively environmental taxa, including the genera *Bacillus* and *Pseudomonas*, and the family Enterobacteriaceae (Fig. 3a) all of which have been previously described as associated with *I. scapularis* [35, 59, 60]. Colonization of ticks appeared to be independent for each taxon since, although co-occurrence was detected in a few samples, most ticks harbored only one dominant environmental taxon. While total bacterial load was not

correlated with colonization by environmental bacteria, samples with high abundance of these bacteria—deriving from each of the five geographically isolated collection sites—were less frequently infected by *B. burgdorferi* than expected (Fig. 3b, c). This observation suggests that colonization by *Bacillus*, *Pseudomonas*, and Enterobacteriaceae may limit *B. burgdorferi* infection.

Immuno-staining and confocal microscopy reveals *I. scapularis* midgut biogeography

To orthogonally and directly validate our findings of a limited internal microbiome in ticks, we turned to confocal microscopy in order to visualize bacteria distribution and localization within *I. scapularis*. Our sequencing results suggest that *Rickettsia* comprise the dominant internal microbial inhabitants of most wild adult *I. scapularis* ticks. However, we questioned (i) if our analyses failed to detect low-abundance yet highly diverse communities of midgut-associated bacteria masked by the high abundance of *Rickettsia* and (ii) if the limited midgut colonization by environmental bacteria that we observed could be visualized and therefore validated. Ticks present a number of challenges that hinder standard microscopy techniques. These challenges include the physical barrier of the thick outer cuticle, a high degree of internal auto-fluorescence derived from the cuticle, and remnants of previous blood-meals including heme crystals [46]. In order to preserve tissue integrity and organization, we utilized formalin-fixation and paraffin embedding of dissected ticks, followed by thin sectioning and staining for visualization. Use of biotin-labeled ISH-probes allowed us to perform tyramide-signal

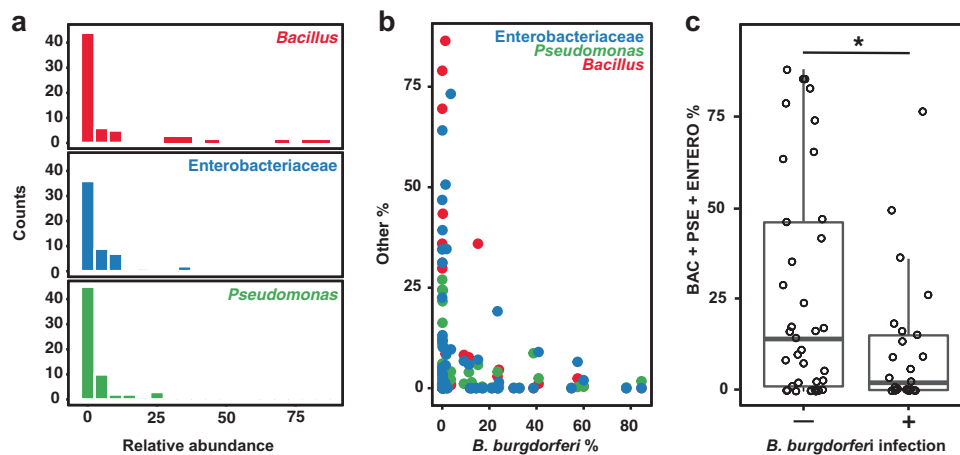


Fig. 3 Transient environmental bacteria limit *B. burgdorferi* infection. **a** Histograms depict the relative abundance of bacteria (*Bacillus*, red; Enterobacteriaceae, blue; *Pseudomonas*, green) detected in *I. scapularis* viscera samples, in bins of 5%. Counts indicate number of samples. **b** Scatter plot of the relationship between the relative abundance of environmental bacteria detected in *I. scapularis* viscera and

the relative abundance of *B. burgdorferi*. Color scheme as in **a**. **c** Barplots indicate the sum of the relative abundance for each of three abundant taxa between samples in which *B. burgdorferi* relative abundance exceeds 1% (infected) and those samples for which abundance is <1% (uninfected). The difference is statistically significant (Mann Whitney U test, $p < 0.001$)

amplification (TSA), dramatically increasing the signal to noise ratio within tick sections.

With these methods, we characterized the distribution of bacteria within tissues of individual adult *I. scapularis* ticks ($N = 41$). Abundant and DAPI-intense cocci that co-stained with probes targeting universally conserved 16S rRNA sequences (EU338) were observed in the cytoplasm of ovarian cells of all female *I. scapularis* ticks (Fig. S5). These are presumed to be vertically transmitted *Rickettsia* [61]. In striking contrast to ovaries, the midgut of most unfed adult *I. scapularis* lacked bacilli or cocci as visualized by DAPI signal or ISH signal localized to the lumen or luminal epithelium (Fig. 5a, 90%, $N = 41$ total ticks). This differed for *B. burgdorferi*-infected *I. scapularis* ticks, in which spirochete cells constituted the only detected midgut bacteria (Fig. 4a, S5). However, a minority of *I. scapularis* ticks from a single site (CA) exhibited midgut colonization by bacterial cells with distinct cocci and bacilli cellular morphologies (10% of total *I. scapularis* examined, $N = 41$) (Fig. 4b). Since *I. scapularis* nymphs have been reported to possess a diverse midgut biofilm-forming microbiome, with very low relative abundance of *Rickettsia* [26, 62], we considered the possibility that while adult *I. scapularis* ticks may lack a diverse internal microbiome, earlier life stages could be colonized to a greater extent. To test this possibility, we imaged sections prepared from whole-mounted *I. scapularis* nymphs, using similar formalin-fixation and

staining methodology as before. We found that *I. scapularis* nymphs also exhibit low DAPI and ISH probe staining within midgut tissues (Fig. S6). Since nymphs are considerably smaller than adults, they may be more sensitive to artefactual noise associated with low-biomass high-throughput sequencing. In support of this notion, the alpha diversity of the *I. pacificus* microbiome negatively correlates with life stage progression from larvae to adults as body size increases [31].

16S rRNA gene sequencing of diverse wild adult ticks

We next sought to test whether our findings of a limited internal bacterial load and restricted diversity in *I. scapularis* were generalizable across Ixodidae. Vector competence for *Borrelia* spp. varies across genera within the Ixodidae. Ixodid ticks also vector pathogenic *Anaplasma* and *Ehrlichia* spp which must transit the midgut during their enzootic cycles [63]. We collected six species of hard tick from multiple geographic locations, including species from the genera *Amblyomma*, *Dermacentor*, and *Ixodes*. qPCR-based assessment of internal bacterial load revealed a variability similar to that of *I. scapularis*, with *Amblyomma maculatum* exhibiting the highest load and broadest variation across individual ticks (Fig. 5a). As observed for *I. scapularis*, the dominant bacterial taxa associated with each

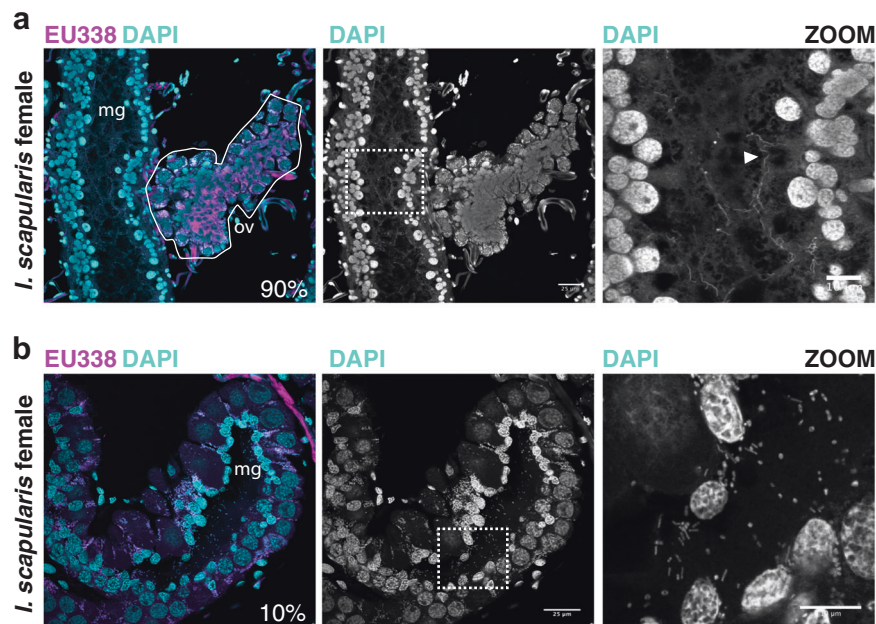


Fig. 4 Midgut-associated bacteria in *I. scapularis*. **a** Cross-section of the ovary and midgut of a *B. burgdorferi*-infected adult female *I. scapularis* tick. Region highlighted by zoom indicated by white box. “mg” indicates midgut lumen. Ovary indicated by white outline labeled with “ov”. Percent indicates the proportion of analyzed ticks with similar internal bacterial biogeography ($N = 41$). Arrowhead in

zoom indicates *B. burgdorferi* cells. **b** Staining of an *I. scapularis* female tick collected from CA site with midgut-associated bacteria. White dashed box indicates the region highlighted by zoom. Percent indicates the proportion of analyzed ticks with similar internal bacterial content. Scale bars indicate 25 microns for wide view and 10 microns for zoomed panels

tick species were species-specific endosymbionts (Fig. 5b). These include *Francisella* and *Rickettsia* in *Amblyomma maculatum*, *Francisella* in *Dermacentor* spp, and *Rickettsia* in *Ixodes* spp, as has been previously reported [25, 28]. Although alpha diversity varies broadly across species (Fig. 5c), negative correlations with total bacterial load across hard tick samples (an exception being *I. pacificus*) supported a similar pattern as seen for *I. scapularis* (Fig. 5d).

Beta diversity (between-samples) analysis using the weighted UniFrac metric (which accounts for differences in taxon abundance between samples) revealed significant clustering of samples by tick genus (ADONIS, $p = 0.001$) (Fig. S7A), as expected for stable microbiomes or those largely composed of endosymbionts. In contrast, external wash samples cluster together regardless of which tick they were associated with. This suggests that external microbiomes share common features (e.g., the presence of diverse low-abundance taxa and lack of highly abundant

endosymbionts) that differ from internal microbiomes of ticks. This interpretation is supported by beta diversity analyses using the unweighted UniFrac metric (in which each OTU is given equal weight regardless of relative abundance in the sample) which revealed no such internal-external (Fig. S7B) or genus-level clustering, suggesting that the low-abundance taxa in samples do not sufficiently drive differences between tick genera.

Finally, we sought to extend our microscopy findings across hard tick species. Similar to *I. scapularis*, we found that *A. maculatum*, *D. variabilis*, and *I. pacificus* ticks lack abundant midgut-associated bacteria, while females belonging to these species contained abundant DAPI-intense cocci within ovaries (Fig. S8). We also observed that midgut sections near ovaries sometimes contained localized micro-colonies, similar to those shown to be *Rickettsia* in *I. scapularis*. These may be species-specific endosymbionts, as previously reported [23, 64].

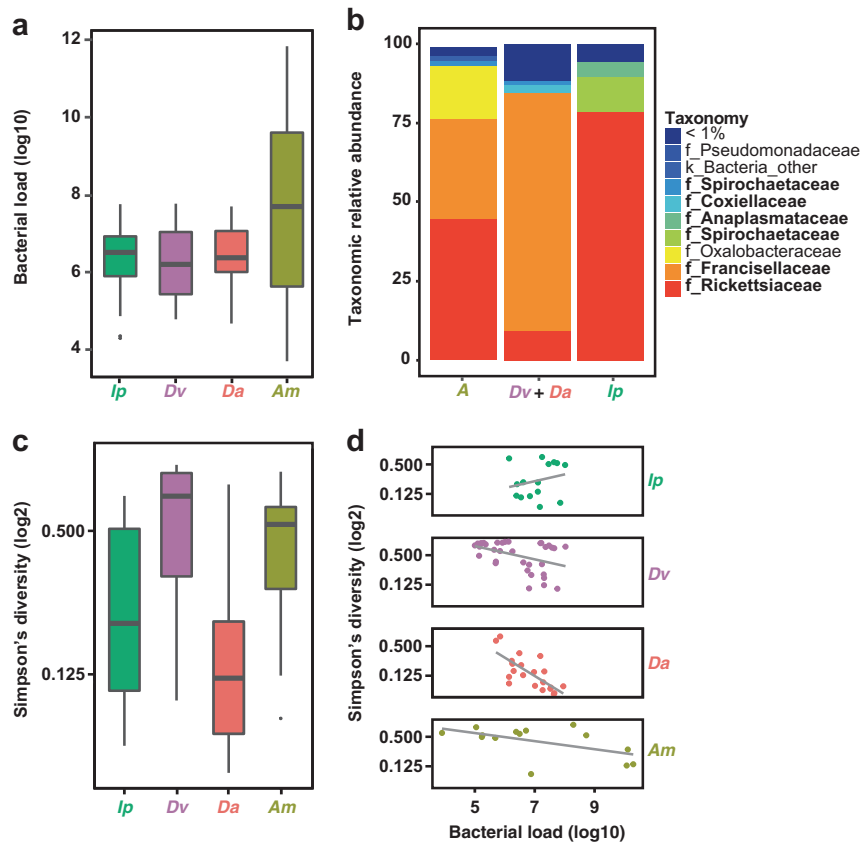


Fig. 5 Impact of limited bacterial load on bacterial diversity estimates in hard ticks. **a** Box and whisker plots describing the internal bacterial load (log₁₀) across all hard ticks sampled ($N = 139$), as determined by qPCR. Species names abbreviated to *Ip* (*Ixodes pacificus*), *Dv* (*Dermacentor variabilis*), *Da* (*Dermacentor andersoni*), and *Am* (*Amblyomma maculatum*). **b** Relative taxonomic abundance for bacteria detected by 16 S rRNA gene sequencing averaged across all samples for *A. maculatum*, *D. variabilis* and *D. andersoni*, and *I.*

pacificus. Taxa previously known to be internally associated with these species are bolded in legend. **c** Box and whisker plots describing Simpson's Diversity (log₂) of all hard tick internal samples without filtering, with same color scheme as in **a**. **d** Bacterial load plotted against Simpson's Diversity (log₂). Spearman's rank correlation: *Ip* $\rho = 0.07$, $p > 0.1$; *Dv* $\rho = -0.88$, $p < 0.001$; *Da* $\rho = -0.73$, $p < 0.001$; *Am* $\rho = -0.62$, $p < 0.05$

Discussion

Here we have provided multiple lines of evidence showing that unfed wild hard ticks possess a limited internal microbiome. Our findings extend upon early studies which found little evidence for culturable bacteria within *I. scapularis* and add to the growing literature suggesting that not all animals are intimately associated with a complex and abundant internal microbiome [65–68]. This is in contrast to recent reports that suggest a diverse microbiome is a characteristic feature of hard ticks across species and life stages [21, 26–29, 31, 33–35, 62]. We find in our samples that measurements of elevated diversity derived from high-throughput sequencing of tick samples are largely a function of low bacterial biomass. This is a widespread problem inherent in high-throughput sequencing studies that rely upon PCR-amplification during the sample preparation process [69, 70]. We propose that pooling multiple samples to increase input biomass is one method that might lower noise from reagent-based or external contamination [71].

There is great interest in exploiting natural microbial communities to benefit human health and combat disease. One such avenue of active research involves leveraging the native microbial communities associated with medically relevant arthropod vectors (like mosquitos and ticks) to prevent pathogen transmission [14, 72]. We note that *B. burgdorferi* may be particularly susceptible to inhibition by competitor bacteria if encountered due to a paucity of genetic mechanisms for direct interbacterial interactions. This is supported by our findings that the increased abundance of *Bacillus*, *Enterobacteriaceae*, and *Pseudomonas* within the midgut is associated with decreased *B. burgdorferi* infection. Notably, these bacteria were also detected in external wash samples. This intriguing pattern suggests that some tick-associated external bacteria can colonize the *I. scapularis* midgut and, in doing so, might competitively exclude *B. burgdorferi*, either by displacement or by inhibition of infection during a subsequent blood meal. Members of these taxa encode an arsenal of mechanisms with which to compete with other bacterial cells, including type IV, VI, and VII secretion systems, contact-dependent inhibition (CDI) mechanisms, and the ability to produce bacteriocins [16, 49, 50, 73, 74]. It is unknown if environmental bacteria can persist within the midgut during transstadial molts, but it is worth noting that detection of *Bacillus* and other bacteria within ticks long preceded the 16 S rRNA gene sequencing era [59]. Direct inhibition of *B. burgdorferi* by tick-associated bacteria remains an active area of investigation by our laboratories.

Despite the detection of environmental bacteria within ticks, our findings suggest that the midgut of hard ticks may largely be an environment that is ill-suited for bacterial growth, perhaps due in part to the exceedingly low levels of

the vital nutrient thiamin in *I. scapularis* [75]. While *B. burgdorferi* has evolved unique metabolic strategies in order to persist in this thiamin-limited environment, other less-specialized bacteria may not be able to do so. Additional factors that might limit the overall bacterial load of hard ticks are the possession of conserved or unique innate immunity factors that could target bacteria [76, 77], heme toxicity [78], and the effects of nutrient limitation and desiccation during the extended period between blood meals [6, 79].

Borrelia burgdorferi may encounter a limited diversity of bacteria within the *I. scapularis* midgut, yet co-infections with other pathogens such as *Anaplasma* are common [54]. The mechanisms by which *B. burgdorferi* co-exists with other microbial pathogens within the tick remain unexplored. While *Anaplasma*, *Ehrlichia*, and *Rickettsia* spp are intracellular and may not engage in direct physical interactions with *Borrelia* spp, these bacteria may influence colonization through other means [80, 81]. *B. burgdorferi* may not frequently encounter diverse bacteria within the midgut of the tick, however, multi-strain infections may be common within single ticks [82–86]. Furthermore, it is as yet unclear the extent to which different *B. burgdorferi* strains engage in interactions or how multi-strain infections might influence transmission to humans and pathogenicity.

Our bioinformatic analyses suggests that the genus *Borrelia* lost genes for mediating interbacterial interactions during the course of its evolution. While *Ixodes* species are the only vectors for *B. burgdorferi* in North America, ticks of the genus *Ornithodoros* (soft ticks) vector the relapsing fever spirochete *B. hermsii* [87]. We did not detect interbacterial effector–immunity gene homologs encoded by *B. hermsii*. Although soft ticks were not included in our 16 S rRNA gene sequencing or microscopy, we speculate that *B. hermsii* encounters a limited internal microbiome within *Ornithodoros* ticks. Our results further imply that lice of the order Phthiraptera (class Insecta), which vectors *B. recurrentis*, may also possess a similarly limited internal microbiome [88]. *Borrelia* spp may have therefore evolved to exploit evolutionarily divergent hematophagous arthropods that share the common lack of a stable internal microbiota.

Data accessibility

Sequencing data were deposited at the NCBI Sequence Read Archive, BioProject accession PRJNA414923.

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Author contributions B.D.R., S.C. and J.D.M. conceived the study. B.D.R., S.C. and J.D.M. designed the study. B.D.R., S.C., B.H. and M.C.R. conducted experimental work. X.L., T.J., D.N. and J.B. collected ticks. B.D.R., S.C. and J.D.M. wrote the paper. All authors read and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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