

Ixodes scapularis does not harbor a stable midgut microbiome

Benjamin D. Ross^{1,*}, Beth Hayes², Matthew C. Radey¹, Xia Lee³, Tanya Josek⁴, Jenna Bjork⁵, David Neitzel⁵, Susan Paskewitz³, Seemay Chou^{2,*}, Joseph D. Mougous^{1,6,*}

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

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

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

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

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

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

*To whom correspondence should be addressed

1 Abstract

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

18

1 **Introduction**

2 Vector-borne pathogens infect over one billion people annually and have expanded at an
3 alarming rate in recent years (Harvell et al., 2002; Jones et al., 2013; Keesing et al., 2010; Vora,
4 2008). Lyme disease, which is caused by the tick-borne bacterial pathogen *Borrelia burgdorferi*,
5 is the fifth-most reported infectious disease in the United States, corresponding to over 90% of
6 vector-borne infections in North America (Kugeler et al., 2015; Radolf et al., 2012). *B.*
7 *burgdorferi* transits an enzootic cycle between small mammal reservoir hosts and other
8 mammals, vectored by ticks of the genus *Ixodes* (Radolf et al., 2012). Transmission of *B.*
9 *burgdorferi* to humans via tick bite results in a constellation of inflammatory symptoms
10 requiring an antibiotic treatment regimen for resolution within 2-3 weeks in most cases (Berende
11 et al., 2016; Wormser et al., 2006). While a vaccine targeting *B. burgdorferi* had been approved
12 by the FDA, it was subsequently withdrawn and currently there is an urgent need for new
13 strategies to control tick-borne disease transmission (Nigrovic and Thompson, 2007).

14 Colonization resistance against pathogens mediated by commensal microorganisms is
15 one such proposed strategy (Buffie and Pamer, 2013; Finney et al., 2015). Studies in mosquitos
16 and tsetse flies, among others, have motivated the characterization of the endogenous microbiota
17 associated with vectors in hopes of identifying means by which pathogen transmission can be
18 interrupted through direct or indirect interactions (Cirimotich et al., 2011a; Cirimotich et al.,
19 2011b; Wang et al., 2017). One mechanism that may govern interactions between pathogens and
20 the microbiota involves direct competition. Many bacteria possess elaborate mechanisms that can
21 mediate interbacterial competition in polymicrobial environments, including specialized
22 pathways such as the type VI secretion system, which delivers toxic effector proteins to target
23 cells (Hibbing et al., 2010; Russell et al., 2014). It is thought that the presence and repertoire of

1 interbacterial systems in a given bacterial genome reflects to a certain degree the selective
2 pressures that organism faces in its natural niche (Zhang et al., 2012). Indeed, these antagonistic
3 systems contribute to bacterial fitness in complex bacterial communities such as the mammalian
4 gut (Kommineni et al., 2015; Verster et al., 2017; Wexler et al., 2016).

5 The genus *Borrelia* comprises a group of pathogenic bacteria that rely upon
6 hematophagous arthropods for infectious transmission to humans, including ticks of the order
7 *Ixodida* (Radolf et al., 2012). In hard ticks (*Ixodidae*), surveys of the internal microbiota have to-
8 date identified bacteria that could potentially restrict pathogen transmission (Andreotti et al.,
9 2011; Budachetri et al., 2014; Budachetri et al., 2016; Clay et al., 2008; Clayton et al., 2015;
10 Hawlena et al., 2012; Khoo et al., 2016; Nakao et al., 2013; Narasimhan et al., 2014; Rynkiewicz
11 et al., 2015; Swei and Kwan, 2016; Trout Fryxell and DeBruyn, 2016; van Treuren et al., 2015;
12 Williams-Newkirk et al., 2014; Zolnik et al., 2016). However, significant variation in the
13 diversity and identity of tick-associated bacteria was observed in these studies – rendering the
14 potential for bacterial interference within ticks uncertain. In this study, we aimed to determine
15 whether *I. scapularis* possesses a diverse midgut microbiota that might be encountered by *B.*
16 *burgdorferi*. Most previous studies of the microbiome of hard ticks have utilized high-throughput
17 sequencing technologies. We sought to augment sequencing with direct measurements of
18 bacterial load and visualization of bacteria within adult and nymphal *I. scapularis* ticks by
19 confocal microscopy. With these complementary approaches, we provide evidence that hard
20 ticks lack a stable midgut microbiota.

21

22

23

1 **Results**

2 *Borrelia* lacks interbacterial effector–immunity genes

3 Members of the phylum *Spirochaetes* inhabit diverse environments, from the dense and
4 competitive oral cavity and gastrointestinal tract of mammals to the midguts of arthropods like
5 termites and ticks. How members of the phylum *Spirochaetes* engage in interactions with other
6 bacteria is not understood. We therefore sought to characterize the distribution of interbacterial
7 effector and immunity genes in the genomes of *Spirochaetes*. We first compiled a database of
8 *Spirochaetes* genomes, encompassing a total of 63 genomes representing all major
9 genera. These genomes were queried for the presence of homologs of 220 interbacterial effector
10 and immunity genes (Zhang et al., 2012), including those with characterized domains found
11 associated with contact-dependent inhibition (Hayes et al., 2010), type VI secretion system
12 (Russell et al., 2014), and ESX/T7SS antagonistic pathways (Cao et al., 2016; Whitney et al.,
13 2017). This analysis revealed the presence of genes encoding interbacterial effector and
14 immunity domains throughout the *Spirochaetes*, particularly in species known to inhabit
15 polymicrobial environments such as the mammalian gut microbiome (*Treponema succinifaciens*
16 and *Brachyspira* spp) and the oral microbiome (*Treponema denticola*) (Figure 1A). We failed to
17 detect effector gene homologs and identified only a limited group of immunity gene homologs
18 encoded by any species within the genus *Borrelia*, including representatives from the *sensu*
19 *stricto* and *sensu lato* genospecies (Becker et al., 2016). Since the phylum *Spirochaetes* is
20 considered to be monophyletic, with extant genera descending from a single common ancestor
21 (Paster et al., 1991), parsimony supports the conclusion that *Borrelia* lost interbacterial effector
22 genes early in the evolution of the genus. Parallel investigation of the genomes of tick-associated
23 endosymbionts and tick-transmitted intracellular pathogens including *Rickettsia*, *Coxiella*,

1 *Anaplasma*, and *Ehrlichia* revealed that these genomes also largely lack interbacterial effector–
2 immunity genes (Figure 1B).

3

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

5 The evolutionary loss of effector–immunity genes from the genus *Borrelia* led us to hypothesize
6 that interbacterial interactions might be limited within *I. scapularis*. We therefore sought to
7 quantify the microbial communities associated with wild *I. scapularis*. We first isolated DNA
8 from the dissected viscera (a combination of internal tissues that included midgut, reproductive
9 tissues, and salivary glands) of 61 adult ticks collected from 5 distinct geographic sites in the
10 Midwestern US. We then performed quantitative polymerase chain reaction (qPCR) targeting
11 conserved regions of the 16S rRNA gene. Our analysis demonstrated that the mean internal
12 bacterial load of unfed adult *I. scapularis* ticks varies over several orders of magnitude (Figure
13 2A). Male ticks analyzed harbored less bacterial load than did females (4.5×10^5 vs 5.3×10^7 , t-test
14 p-value < 0.001, N = 61). qPCR with primers specific to the *B. burgdorferi* *flaB* gene revealed
15 infection frequencies similar to that previously reported from the Midwestern geographic regions
16 (Figure S1A) (Hamer et al., 2014). To investigate the differences in bacterial community
17 underlying the sex-specific differences that we observed, we performed 16S rRNA gene
18 sequencing on the same samples used for qPCR analysis. In addition, we sequenced the external
19 washes from a subset of ticks, and a water-only control for each group. 178 OTUs were detected
20 in the water-only controls, including taxa commonly implicated in reagent contamination and
21 some previously reported to be associated with ticks, such as *Sphingomonas* and *Comamonas*
22 (Nakao et al., 2013; Narasimhan et al., 2014; van Treuren et al., 2015). These OTUs were
23 subsequently eliminated from all other samples in downstream analyses. To describe the taxa

1 that comprise *I. scapularis*-associated bacterial communities following removal of water-borne
2 contamination, we calculated the taxonomic relative abundance averaged across all internal and
3 external samples. This revealed that 258 (95%) of 270 OTUs fell below 1% relative abundance
4 when averaged. In contrast, other taxa exceeded 1% average relative abundance in both internal
5 and external samples, including *Bacillus*, *Pseudomonas*, and *Enterobacteriaceae* (Figure S2).
6 Importantly, the taxon that was most abundant in internal samples but entirely absent in external
7 samples was the genus *Rickettsia* (average relative abundance of 52%), which includes the
8 dominant *Rickettsia* endosymbiont of *I. scapularis* (Gillespie et al., 2012). The relative
9 abundance of *Rickettsia* exhibited a positive correlation with total bacterial load (Figure 2B),
10 indicating that *Rickettsia* is the primary driver of bacterial abundance in most *I. scapularis* ticks.
11 The family *Spirochaetaceae* also was often abundant in viscera samples but absent in washes
12 (Figure S2).

13 The number of taxa with low relative abundance could indicate that the internal
14 environment of *I. scapularis* is particularly well suited to fostering diverse microbial
15 communities. However, an alternate possibility is that low abundance OTUs represent signal
16 from contamination. Indeed, several studies have examined the effect of low biomass input on
17 diversity metrics in 16S rRNA gene sequencing surveys, with the notable result that alpha
18 diversity (within-sample diversity) correlated negatively with the abundance of input DNA
19 (Jervis-Bardy et al., 2015; Lauder et al., 2016; Salter et al., 2014). We therefore examined the
20 relationship between alpha diversity and bacterial load as quantified by qPCR in our *I. scapularis*
21 samples. When examined across all 61 *I. scapularis* samples, we found a strong negative
22 correlation between total bacterial load and alpha diversity that was independent of geographic
23 origin (Spearman's rho = -0.74, $p < 0.001$) (Figure 2C and S3). These data imply that the low

1 bacterial biomass associated with adult *I. scapularis* ticks can impact the interpretation of 16S
2 rRNA gene sequencing surveys by artificially inflating alpha diversity. We further observed a
3 strong negative correlation between bacterial load and the relative abundance of the group of
4 taxa contributing to 1% or less of the total across all *I. scapularis* samples (Figure 2D). We
5 found only six taxa to be present in all *I. scapularis* viscera samples, represented by eight OTUs,
6 including the genera *Rickettsia*, *Borrelia*, *Pseudomonas*, *Francisella*, and *Escherichia*, and the
7 family *Enterobacteriaceae* (Figure S2). Linear discriminant analysis effect size (LEfSe) revealed
8 the taxa most likely to explain differences between external wash samples and internal viscera
9 samples. External samples are characterized by taxa from the phyla *Proteobacteria* and
10 *Actinobacteria*, while viscera samples are distinguished by the orders *Spirochaetales* and
11 *Rickettsiales* (Figure S4) (Segata et al., 2011).

12 While the internal bacteria associated with most wild adult *I. scapularis* are dominated by
13 *Rickettsia* and *B. burgdorferi*, a minority of samples exhibit high relative abundances of three
14 putatively environmental taxa, including the genera *Bacillus* and *Pseudomonas*, and the family
15 *Enterobacteriaceae* (Figure 3A) all of which have been previously described as associated with
16 *I. scapularis* (Moreno et al., 2006; Steinhaus, 1946; van Treuren et al., 2015). Colonization of
17 ticks appeared to be independent for each taxon since, although co-occurrence was detected in a
18 few samples, most ticks harbored only one dominant environmental taxon. While total bacterial
19 load was not correlated with colonization by environmental bacteria, samples with high
20 abundance of these bacteria – deriving from each of the five geographically-isolated collection
21 sites – were less frequently infected by *B. burgdorferi* than expected (Figure 3B, C). This
22 observation suggests that colonization by *Bacillus*, *Pseudomonas*, and *Enterobacteriaceae* may
23 limit *B. burgdorferi* infection.

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

2 To orthogonally and directly validate our findings of a limited internal microbiome in ticks, we

3 turned to confocal microscopy in order to visualize bacteria distribution and localization within *I.*

4 *scapularis*. Our sequencing results suggest that *Rickettsia* comprise the dominant internal

5 microbial inhabitants of most wild adult *I. scapularis* ticks. However, we questioned i) if our

6 analyses failed to detect low-abundance yet highly diverse communities of midgut-associated

7 bacteria masked by the high abundance of *Rickettsia* and ii) if the limited midgut colonization by

8 environmental bacteria that we observed could be visualized and therefore validated. Ticks

9 present a number of challenges that hinder standard microscopy techniques. These challenges

10 include the physical barrier of the thick outer cuticle, a high degree of internal auto-fluorescence

11 derived from the cuticle, and remnants of previous blood-meals including heme crystals (Sojka et

12 al., 2013). In order to preserve tissue integrity and organization, we utilized formalin-fixation

13 and paraffin embedding of dissected ticks, followed by thin sectioning and staining for

14 visualization. Use of biotin-labeled ISH probes allowed us to perform tyramide-signal

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

16 With these methods, we characterized the distribution of bacteria within tissues of

17 individual adult *I. scapularis* ticks (N=41). Abundant and DAPI-intense cocci that co-stained

18 with probes targeting universally conserved 16S rRNA sequences (EU338) were observed in the

19 cytoplasm of ovarian cells of all female *I. scapularis* ticks (Figure S5). These are presumed to be

20 vertically transmitted *Rickettsia* (Noda et al., 1997). In striking contrast to ovaries, the midgut of

21 most unfed adult *I. scapularis* lacked bacilli or cocci as visualized by DAPI signal or ISH signal

22 localized to the lumen or luminal epithelium (Figure 5A, 90%, N=41 total ticks). This differed

23 for *B. burgdorferi*-infected *I. scapularis* ticks, in which spirochete cells constituted the only

1 detected midgut bacteria (Figure 4A, S5). However, a minority of *I. scapularis* ticks from a
2 single site (CA) exhibited midgut colonization by bacterial cells with distinct cocci and bacilli
3 cellular morphologies (10% of total *I. scapularis* examined, N=41) (Figure 4B). Since *I.*
4 *scapularis* nymphs have been reported to possess a diverse midgut biofilm-forming microbiome,
5 with very low relative abundance of *Rickettsia* (Abraham et al., 2017; Narasimhan et al., 2014),
6 we considered the possibility that while adult *I. scapularis* ticks may lack a diverse internal
7 microbiome, earlier life stages could be colonized to a greater extent. To test this possibility, we
8 imaged sections prepared from whole-mounted *I. scapularis* nymphs, using similar formalin
9 fixation and staining methodology as before. We found that *I. scapularis* nymphs also exhibit
10 low DAPI and ISH probe staining within midgut tissues (Figure S6). Since nymphs are
11 considerably smaller than adults, they may be more sensitive to artefactual noise associated with
12 low-biomass high-throughput sequencing. In support of this notion, the alpha diversity of the
13 *Ixodes pacificus* microbiome negatively correlates with life stage progression from larvae to
14 adults as body size increases (Swei and Kwan, 2016).

15

16 *16S rRNA gene sequencing of diverse wild adult ticks*

17 We next sought to test whether our findings of a limited internal bacterial load and restricted
18 diversity in *I. scapularis* were generalizable across *Ixodidae*. Vector competence for *Borrelia*
19 spp. varies across genera within the *Ixodidae*. Ixodid ticks also vector pathogenic *Anaplasma* and
20 *Ehrlichia* spp which must transit the midgut during their enzootic cycles (Ismail et al., 2010). We
21 collected six species of hard tick from multiple geographic locations, including species from the
22 genera *Amblyomma*, *Dermacentor*, and *Ixodes*. qPCR-based assessment of internal bacterial load
23 revealed a variability similar to that of *I. scapularis*, with *Amblyomma maculatum* exhibiting the

1 highest load and broadest variation across individual ticks (Figure 5A). As observed for *I.*
2 *scapularis*, the dominant bacterial taxa associated with each tick species were species-specific
3 endosymbionts (Figure 5B). These include *Francisella* and *Rickettsia* in *Amblyomma*
4 *maculatum*, *Francisella* in *Dermacentor* spp, and *Rickettsia* in *Ixodes* spp, as has been previously
5 reported (Budachetri et al., 2014; Rynkiewicz et al., 2015). Although alpha diversity varies
6 broadly across species (Figure 5C), negative correlations with total bacterial load across hard tick
7 samples (an exception being *I. pacificus*) supported a similar pattern as seen for *I. scapularis*
8 (Figure 5D).

9 Beta diversity (between-samples) analysis using the weighted UniFrac metric (which
10 accounts for differences in taxon abundance between samples) revealed significant clustering of
11 samples by tick genus (ADONIS, $p=0.001$) (Figure S7A), as expected for stable microbiomes or
12 those largely composed of endosymbionts. In contrast, external wash samples cluster together
13 regardless of which tick they were associated with. This suggests that external microbiomes
14 share common features (eg. the presence of diverse low-abundance taxa and lack of highly
15 abundant endosymbionts) that differ from internal microbiomes of ticks. This interpretation is
16 supported by beta diversity analyses using the unweighted UniFrac metric (in which each OTU is
17 given equal weight regardless of relative abundance in the sample) which revealed no such
18 internal-external (Figure S7B) or genus-level clustering, suggesting that the low-abundance taxa
19 in samples do not sufficiently drive differences between tick genera.

20 Finally, we sought to extend our microscopy findings across hard tick species. Similar to *I.*
21 *scapularis*, we found that *A. maculatum*, *D. variabilis*, and *I. pacificus* ticks lack abundant
22 midgut-associated bacteria, while females belonging to these species contained abundant DAPI-
23 intense cocci within ovaries (Figure S8). We also observed that midgut sections near ovaries

1 sometimes contained localized micro-colonies, similar to those shown to be *Rickettsia* in *I.*
2 *scapularis*. These may be species-specific endosymbionts, as previously reported (Clay et al.,
3 2008; Klyachko et al., 2007).

4

5 **Discussion**

6 Here we have provided multiple lines of evidence showing that unfed wild hard ticks possess a
7 limited internal microbiome. This is in contrast to reports that possession of a diverse
8 microbiome is a characteristic feature of hard ticks across species and life stages (Abraham et al.,
9 2017; Andreotti et al., 2011; Budachetri et al., 2014; Budachetri et al., 2016; Nakao et al., 2013;
10 Narasimhan et al., 2014; Swei and Kwan, 2016; Trout Fryxell and DeBruyn, 2016; van Treuren
11 et al., 2015; Zolnik et al., 2016). Our report adds to the growing literature suggesting that not all
12 animals are intimately associated with a complex and abundant internal microbiome (Hammer et
13 al., 2017; Jing et al., 2014). We find that measurements of high diversity derived from high-
14 throughput sequencing of tick samples is largely a function of low bacterial biomass. This is a
15 widespread problem inherent in high-throughput sequencing studies that rely upon PCR-
16 amplification during the sample preparation process (Glassing et al., 2016; Kim et al., 2017). We
17 propose that pooling multiple samples to increase input biomass is one method that might lower
18 noise from reagent-based or external contamination (Gall et al., 2016).

19 There is great interest in exploiting natural microbial communities to benefit human
20 health and combat disease. One such avenue of active research involves leveraging the native
21 microbial communities associated with medically-relevant arthropod vectors (like mosquitos and
22 ticks) to prevent pathogen transmission (Cirimotich et al., 2011b; Narasimhan and Fikrig, 2015).

1 We note that *B. burgdorferi* may be particularly susceptible to inhibition by competitor bacteria
2 if encountered due to a paucity of genetic mechanisms for direct interbacterial interactions. This
3 is supported by our findings that the increased abundance of *Bacillus*, *Enterobacteriaceae*, and
4 *Pseudomonas* within the midgut is associated with decreased *B. burgdorferi* infection. Notably,
5 these bacteria were also detected in external wash samples. This intriguing pattern suggests that
6 some tick-associated external bacteria can colonize the *I. scapularis* midgut and, in doing so,
7 might competitively exclude *B. burgdorferi*, either by displacement or by inhibition of infection
8 during a subsequent blood meal. Members of these taxa encode an arsenal of mechanisms with
9 which to compete with other bacterial cells, including type IV, VI, and VII secretion systems,
10 contact-dependent inhibition (CDI) mechanisms, and the ability to produce bacteriocins (Cao et
11 al., 2016; Hayes et al., 2010; Riley and Wertz, 2002; Russell et al., 2014; Souza et al., 2015). It is
12 unknown if environmental bacteria can persist within the midgut during transstadial molts, but it
13 is worth noting that detection of *Bacillus* and other bacteria within ticks long preceded the 16S
14 rRNA gene sequencing era (Steinhaus, 1946). Direct inhibition of *B. burgdorferi* by tick-
15 associated bacteria remains an active area of investigation by our laboratories.

16 Despite the detection of environmental bacteria within ticks, our findings suggest that the
17 midgut of hard ticks may largely be an environment that is ill-suited for bacterial growth,
18 perhaps due in part to the exceedingly low levels of the vital nutrient thiamin in *I. scapularis*
19 (Zhang et al., 2016). While *B. burgdorferi* has evolved unique metabolic strategies in order to
20 persist in this thiamin-limited environment, other less-specialized bacteria may not be able to do
21 so. Additional factors that might limit the overall bacterial load of hard ticks are the possession
22 of conserved or unique innate immunity factors that could target bacteria (Chou et al., 2014;
23 Palmer and Jiggins, 2015), heme toxicity (Anzaldi and Skaar, 2010), and the effects of nutrient

1 limitation and desiccation during the extended period between blood meals (Radolf et al., 2012;
2 Sonenshine and Roe, 2014).

3 *Borrelia burgdorferi* may encounter a limited diversity of bacteria within the *I.*
4 *scapularis* midgut, yet co-infections with other pathogens such as *Anaplasma* are common
5 (Hamer et al., 2014). The mechanisms by which *B. burgdorferi* co-exists with other microbial
6 pathogens within the tick remain unexplored. While *Anaplasma*, *Ehrlichia*, and *Rickettsia* spp
7 are intracellular and may not engage in direct physical interactions with *Borrelia* spp, these
8 bacteria may influence colonization through other means (Kocan et al., 2010; Simhadri et al.,
9 2017). *B. burgdorferi* may not frequently encounter diverse bacteria within the midgut of the
10 tick, however, multi-strain infections may be common within single ticks (Durand et al., 2017;
11 Durand et al., 2015; Herrmann et al., 2013; Strandh and Råberg, 2015; Voordouw, 2015).
12 Furthermore, it is as yet unclear the extent to which different *B. burgdorferi* strains engage in
13 interactions or how multi-strain infections might influence transmission to humans and
14 pathogenicity.

15 Our bioinformatic analyses suggests that the genus *Borrelia* lost genes for mediating
16 interbacterial interactions during the course of its evolution. While *Ixodes* are the only vectors
17 for *B. burgdorferi* in North America, ticks of the genus *Ornithodoros* (soft ticks) vector the
18 relapsing fever spirochete *B. hermsii* (Dworkin et al., 2002). We did not detect interbacterial
19 effector–immunity gene homologues encoded by *B. hermsii*. Although soft ticks were not
20 included in our 16S rRNA gene sequencing or microscopy, we speculate that *Ornithodoros*, like
21 *Ixodidae*, harbors a limited internal microbiome. Our results further imply that lice of the order
22 *Phthiraptera* (class Insecta), which vectors *B. duttonii* and *B. recurrentis*, may also possess a
23 limited internal microbiome (Lescot et al., 2008). *Borrelia* spp may have therefore evolved to

1 exploit evolutionarily divergent hematophagous arthropods that share the common lack of a
2 stable midgut microbiota.

3

4 **Methods**

5 **Tick collection**

6 Ticks were collected from the following sites: oak woodland habitat in Klickitat River Canyon,
7 Washington (*Ixodes pacificus* and *Dermacentor andersonii*); oak-hickory forest in Wolf Creek
8 State Park, Illinois (*Ixodes scapularis* and *Dermacentor variabilis*); oak-dominated forest in
9 Gordie Mikkelsen Wildlife Management Area and Carlos Avery Wildlife Management Area,
10 Minnesota (abbreviated GM and CA, *Ixodes scapularis* and *Dermacentor variabilis*); red pine
11 forest in Kettle Moraine State Forest Southern Unit (KM), mixed hardwood forest in Big Eau
12 Pleine County Park (BEP), and oak-hickory forest in Sandberg Woods Conservancy (SC),
13 Wisconsin (*Ixodes scapularis*); oak forest near McPherson Preserve, Oklahoma (*Amblyomma*
14 *maculatum*). Ticks were collected by the flagging and dragging methods and shipped in 50mL
15 Falcon tubes with damp paper towels on wet ice to retain moisture. Species, developmental
16 stage, and sex of individual ticks were determined by visual inspection.

17

18 **DNA isolation**

19 Live ticks were washed three times with sterile water then dried before immobilization on a glass
20 slide using double-sided tape. 21-gauge needles were used to remove the cuticle, and sterile
21 forceps used to dissect and remove the viscera into 500uL sterile deionized water for subsequent
22 DNA purification. Fresh needles were exchanged, and forceps sterilized by washing three times
23 in sterile water and 70% ethanol between each dissection. The first external wash sample was

1 saved, and pooled across individuals of the same species, sex, and geographical collection
2 location. Samples were frozen at -80°C prior to DNA isolation. Frozen tissue samples were
3 thawed and subsequently homogenized via glass bead-beating on a MiniBeadBeater (Biospec
4 Products) for two cycles of one minute duration each. Subsequently, phenol-chloroform-isoamyl
5 alcohol extraction of total nucleic acids was performed with a RNase treatment to remove RNA.
6 Following extraction, DNA pellets were resuspended in 25uL sterile deionized water.

7

8 **Quantitative PCR**

9 The abundance of bacteria in single tick samples was determined by quantitative PCR using
10 SsoAdvanced Universal SYBR Green Supermix (Biorad) on a BioRad CFX96 Real Time
11 System C1000. Primers used targeted the 16S rRNA gene (331f and 797r(Nadkarni et al., 2002))
12 or the *B. burgdorferi* *flaB* gene (Jewett et al., 2007). Standard curves were generated from serial
13 dilutions of purified genomic DNA prepared from monocultures of *Escherichia coli* DH5a or *B.*
14 *burgdorferi* B31A3. Samples were run in technical triplicates with the mean of each triplicate
15 used for later analysis.

16

17 **16S rRNA gene sequencing and analysis**

18 Genomic DNA from tick samples, external washes, and water controls was submitted for
19 sequencing and individually barcoded for high-throughput sequencing of V3-V4 16S rRNA
20 amplicons on an Illumina MiSeq, in three separate runs (performed by MrDNA). Sequencing
21 reads were subsequently demultiplexed and merged into a single fastq file. The UPARSE
22 pipeline was used to process the samples using default settings (Edgar, 2010, 2013). Following
23 taxonomy prediction and OTU assignment, the OTU table were filtered to only retain OTUs that

1 appeared at greater than 1% relative abundance in at least one sample. Alpha-diversity and beta-
2 diversity metrics were calculated in QIIME (MacQIIME v1.9.0) (Caporaso et al., 2010). For
3 determination of *B. burgdorferi* infection status, tick samples in which the sum of the relative
4 abundances of the family *Spirochaetaceae* exceeded 1% were considered to be infected, while
5 those less than 1% were considered to be uninfected. The sum of the relative abundance of the
6 genera *Bacillus* and *Pseudomonas* was calculated for samples for comparison of *B. burgdorferi*
7 infection status. For LEfSe analysis, the OTU table was converted to relative abundances and,
8 along with associated metadata, was uploaded to the Huttenhower Lab Galaxy web application.
9 Statistical tests and generation of plots were performed in R version 3.3.2 (R Development Core
10 Team, 2013).

11

12 **Histology**

13 *Preparing tick sections*

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

20

21 *Antibody-based microscopy*

22 Deparaffinized slides were first subjected to a 40 minute incubation in sodium citrate buffer at
23 95°C in a vegetable steamer (Black and Decker). Slides were then washed in 2X SSC + 0.3%

1 Tween 20, blocked in PBS + 5% BSA, and incubated with BacTrace fluorescein isothiocyanate
2 goat anti-*Borrelia* whole cell antibodies (Kirkegaard and Perry Laboratories) for 1 hour at room
3 temperature or overnight at 4°C. Slides were then washed, dried, and mounted in ProLong
4 Diamond plus DAPI (Thermo Fisher Scientific). Coverslips were sealed with nail polish.

5

6 *In situ hybridization analysis*

7 Deparaffinized slides were washed in 2X SSC + 0.3% Tween20 then were lightly digested with
8 Proteinase K to allow probe access into tissues, washed and dried. Probes used were EU338
9 (Amann et al., 1995) or specific to *Rickettsia*. For Rickettsia-specific probes, the sequence of the
10 Rick_1442 16S rRNA probe (Vannini et al., 2005) was aligned to the publically available REIS
11 genome (Gillespie et al., 2012) and confirmed to be identical. ISH-probes (5' biotin labeled)
12 were diluted 1:100 to 50uM in ethylene carbonate hybridization buffer (15% ethylene carbonate,
13 20% dextran sulfate, 600mM NaCl, 10mM sodium citrate (Matthiesen and Hansen, 2012). Slides
14 were covered with parafilm, placed in humidified chambers, and allowed to incubate for 1 hour
15 at 42°C. Slides were subsequently washed in 2X SSC at 37°C then allowed to air dry. Alexa
16 Fluor 488-coupled tyramide signal amplification (Thermo Fisher Scientific T20912) was
17 performed to increase probe signal above background autofluorescence from tick midgut
18 digestive products (Biegala et al., 2002; Sojka et al., 2013). Slides were mounted in Diamond
19 Prolong mounting medium plus DAPI and coverslips sealed with nail polish.

20

21 **Imaging**

22 Confocal microscopy was performed at the University of Washington Keck Microscopy Center,
23 on a Leica SP8X confocal microscope. 2-micron stacks were imaged at 63x magnification, using

1 6X averaging. Images were extracted from raw .lif files, maximum projected, channels merged,
2 cropped, and pseudocolored using Fiji (Schindelin et al., 2012).

3

4 **Identification of interbacterial effector–immunity gene homologues in genomes**

5 Multi-alignments for 220 putative and validated effector and immunity proteins were acquired
6 from the supplemental materials of Zhang *et al.* (Zhang et al., 2012). Amino acid seed sequences
7 of interbacterial effector and immunity proteins were queried via tBLASTn against a custom
8 database of 63 *Spirochetes* genomes acquired from RefSeq, or a database of 22 genomes for tick-
9 associated endosymbionts and pathogens from the phylum *Proteobacteria*. All hits with e-values
10 less than or equal to 10^{-3} across the full length of the seed sequence were considered to be
11 homologues of interbacterial effector or immunity proteins. The distribution of effector and
12 immunity genes per genome within each genus was calculated by normalizing the total number
13 of BLAST hit by the number of genomes searched.

14

15 **Author contributions**

16 *BDR, SC, and JDM conceived the study. BDR, SC, and JDM designed the study. BDR, SC, BH,*
17 *and MCR conducted experimental work. XL, TJ, DN, and JB collected ticks. BDR, SC, and JDM*
18 *wrote the paper. All authors read and approved the paper.*

19

20 **Acknowledgements**

21 *We thank the Fred Hutch Experimental Histopathology Core facility and Dan Long at Rocky Mountain*
22 *Labs for tick sectioning and slide preparation. We thank Barbara Simon, Jim Ruppa, David Simon, and*
23 *June Reznikoff for their indefatigable efforts in collection of ticks from the Klickitat River “Ant Ranch”,*
24 *and Susan Little for collection of Amblyomma ticks. We thank the UW Keck Imaging Center for providing*

1 *equipment and assistance in confocal microscopy and acknowledge its support from the NIH (S10*
2 *OD016240). We thank Mr. DNA for sequencing support. We are grateful to colleagues for careful review*
3 *of the manuscript and members of the Mougous lab for helpful discussions. This work was supported by*
4 *National Institutes of Health grant R21AI114923 (JDM), and the Burroughs Wellcome Fund (JDM). BDR*
5 *was supported by a Simons Foundation-sponsored Life Sciences Research Foundation postdoctoral*
6 *fellowship.*

7

8 **Data accessibility:**

9 Sequencing data was deposited at the NCBI SRA, BioProject accession # pending.

10

11 **Author information:**

12 The authors declare that no competing financial information exists. Correspondence should be directed to
13 bdross@uw.edu, seemay.chou@ucsf.edu, and mougous@uw.edu.

14

15

16

1 **References**

2 Abraham, N.M., Liu, L., Jutras, B.L., Yadav, A.K., Narasimhan, S., Gopalakrishnan, V., Ansari,
3 J.M., Jefferson, K.K., Cava, F., Jacobs-Wagner, C., *et al.* (2017). Pathogen-mediated
4 manipulation of arthropod microbiota to promote infection. *Proc Natl Acad Sci U S A* *114*,
5 E781-E790.

6 Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and in situ
7 detection of individual microbial cells without cultivation. *Microbiol Rev* *59*, 143-169.

8 Andreotti, R., Perez de Leon, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G., and Scoles, G.A.
9 (2011). Assessment of bacterial diversity in the cattle tick *Rhipicephalus* (*Boophilus*) microplus
10 through tag-encoded pyrosequencing. *BMC Microbiol* *11*, 6.

11 Anzaldi, L.L., and Skaar, E.P. (2010). Overcoming the heme paradox: heme toxicity and
12 tolerance in bacterial pathogens. *Infect Immun* *78*, 4977-4989.

13 Becker, N.S., Margos, G., Blum, H., Krebs, S., Graf, A., Lane, R.S., Castillo-Ramirez, S., Sing,
14 A., and Fingerle, V. (2016). Recurrent evolution of host and vector association in bacteria of the
15 *Borrelia burgdorferi* sensu lato species complex. *BMC Genomics* *17*, 734.

16 Berende, A., ter Hofstede, H.J., Vos, F.J., van Middendorp, H., Vogelaar, M.L., Tromp, M., van
17 den Hoogen, F.H., Donders, A.R., Evers, A.W., and Kullberg, B.J. (2016). Randomized Trial of
18 Longer-Term Therapy for Symptoms Attributed to Lyme Disease. *N Engl J Med* *374*, 1209-
19 1220.

20 Biegala, I.C., Kennaway, G., Alverca, E., Lennon, J.-F., Vaulot, D., and Simon, N. (2002).
21 IDENTIFICATION OF BACTERIA ASSOCIATED WITH DINOFLAGELLATES
22 (DINOPHYCEAE) ALEXANDRIUM spp. USING TYRAMIDE SIGNAL AMPLIFICATION-
23 FLUORESCENT IN SITU HYBRIDIZATION AND CONFOCAL MICROSCOPY1. *J Phycol*
24 *38*, 404-411.

25 Budachetri, K., Browning, R.E., Adamson, S.W., Dowd, E., Chao, C.-c., Ching, W.-m., Karim,
26 S., and Dowd, S.E. (2014). An Insight Into the Microbiome of the *Amblyomma maculatum* (Acari :
27 Ixodidae) An Insight Into the Microbiome of the *Amblyomma maculatum* (Acari :
28 Ixodidae). *Journal of Medical Entomology* *51*, 119-129.

29 Budachetri, K., Williams, J., Mukherjee, N., Sellers, M., Moore, F., and Karim, S. (2016). The
30 microbiome of neotropical ticks parasitizing on passerine migratory birds. *Ticks Tick Borne Dis*
31 *8*, 170-173.

32 Buffie, C.G., and Pamer, E.G. (2013). Microbiota-mediated colonization resistance against
33 intestinal pathogens. *Nat Rev Immunol* *13*, 790-801.

34 Cao, Z., Casabona, M.G., Kneuper, H., Chalmers, J.D., and Palmer, T. (2016). The type VII
35 secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor
36 bacteria. *Nat Microbiol* *2*, 16183.

1 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.,
2 Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010). QIIME allows analysis of high-
3 throughput community sequencing data. *Nat Methods* *7*, 335-336.

4 Chou, S., Daugherty, M.D., Peterson, S.B., Biboy, J., Yang, Y., Jutras, B.L., Fritz-Laylin, L.K.,
5 Ferrin, M.a., Harding, B.N., Jacobs-Wagner, C., *et al.* (2014). Transferred interbacterial
6 antagonism genes augment eukaryotic innate immune function. *Nature*.

7 Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M., and
8 Dimopoulos, G. (2011a). Natural microbe-mediated refractoriness to *Plasmodium* infection in
9 *Anopheles gambiae*. *Science* *332*, 855-858.

10 Cirimotich, C.M., Ramirez, J.L., and Dimopoulos, G. (2011b). Native microbiota shape insect
11 vector competence for human pathogens. *Cell Host Microbe* *10*, 307-310.

12 Clay, K., Klyachko, O., Grindle, N., Civitello, D., Oleske, D., and Fuqua, C. (2008). Microbial
13 communities and interactions in the lone star tick, *Amblyomma americanum*. *Molecular Ecology*
14 *17*, 4371-4381.

15 Clayton, K.A., Gall, C.A., Mason, K.L., Scoles, G.A., and Brayton, K.A. (2015). The
16 characterization and manipulation of the bacterial microbiome of the Rocky Mountain wood tick,
17 *Dermacentor andersoni*. *Parasites & vectors* *8*, 632-632.

18 Durand, J., Herrmann, C., Genne, D., Sarr, A., Gern, L., and Voordouw, M.J. (2017). Multistrain
19 Infections with Lyme Borreliosis Pathogens in the Tick Vector. *Appl Environ Microbiol* *83*.

20 Durand, J., Jacquet, M., Paillard, L., Rais, O., Gern, L., and Voordouw, J. (2015). Cross-
21 Immunity and Community Structure of a Multiple-Strain. *81*, 7740-7752.

22 Dworkin, M.S., Schwan, T.G., and Anderson, D.E., Jr. (2002). Tick-borne relapsing fever in
23 North America. *Med Clin North Am* *86*, 417-433, viii-ix.

24 Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST.
25 *Bioinformatics* *26*, 2460-2461.

26 Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads.
27 *Nat Methods* *10*, 996-998.

28 Finney, C.A., Kamhawi, S., and Wasmuth, J.D. (2015). Does the arthropod microbiota impact
29 the establishment of vector-borne diseases in mammalian hosts? *PLoS Pathog* *11*, e1004646.

30 Gall, C.A., Reif, K.E., Scoles, G.A., Mason, K.L., Mousel, M., Noh, S.M., and Brayton, K.A.
31 (2016). The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen
32 susceptibility. *The ISME Journal*, 1-10.

33 Gillespie, J.J., Joardar, V., Williams, K.P., Driscoll, T., Hostetler, J.B., Nordberg, E., Shukla, M.,
34 Walenz, B., Hill, C.a., Nene, V.M., *et al.* (2012). A rickettsia genome overrun by mobile genetic

1 elements provides insight into the acquisition of genes characteristic of an obligate intracellular
2 lifestyle. *Journal of Bacteriology* 194, 376-394.

3 Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B., and Chiodini, R.J. (2016). Inherent bacterial
4 DNA contamination of extraction and sequencing reagents may affect interpretation of
5 microbiota in low bacterial biomass samples. *Gut Pathog* 8, 24.

6 Hamer, S.A., Hickling, G.J., Walker, E.D., and Tsao, J.I. (2014). Increased diversity of zoonotic
7 pathogens and *Borrelia burgdorferi* strains in established versus incipient *Ixodes scapularis*
8 populations across the Midwestern United States. *Infection, Genetics and Evolution* 27, 531-542.

9 Hammer, T.J., Janzen, D.H., Hallwachs, W., Jaffe, S.P., and Fierer, N. (2017). Caterpillars lack a
10 resident gut microbiome. *Proc Natl Acad Sci U S A* 114, 9641-9646.

11 Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., and Samuel,
12 M.D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* 296,
13 2158-2162.

14 Hawlena, H., Rynkiewicz, E., Toh, E., Alfred, A., Durden, L.a., Hastriter, M.W., Nelson, D.E.,
15 Rong, R., Munro, D., Dong, Q., *et al.* (2012). The arthropod, but not the vertebrate host or its
16 environment, dictates bacterial community composition of fleas and ticks. *The ISME Journal* 7,
17 221-223.

18 Hayes, C.S., Aoki, S.K., and Low, D.A. (2010). Bacterial contact-dependent delivery systems.
19 *Annu Rev Genet* 44, 71-90.

20 Herrmann, C., Gern, L., and Voordouw, M.J. (2013). Species co-occurrence patterns among
21 Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. *Applied and environmental
22 microbiology* 79, 7273-7280.

23 Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B. (2010). Bacterial competition:
24 surviving and thriving in the microbial jungle. *Nature Reviews Microbiology* 8, 15-25.

25 Ismail, N., Bloch, K.C., and McBride, J.W. (2010). Human ehrlichiosis and anaplasmosis. *Clin
26 Lab Med* 30, 261-292.

27 Jervis-Bardy, J., Leong, L.E., Marri, S., Smith, R.J., Choo, J.M., Smith-Vaughan, H.C.,
28 Nosworthy, E., Morris, P.S., O'Leary, S., Rogers, G.B., *et al.* (2015). Deriving accurate
29 microbiota profiles from human samples with low bacterial content through post-sequencing
30 processing of Illumina MiSeq data. *Microbiome* 3, 19.

31 Jewett, M.W., Lawrence, K., Bestor, A.C., Tilly, K., Grimm, D., Shaw, P., VanRaden, M.,
32 Gherardini, F., and Rosa, P.A. (2007). The critical role of the linear plasmid lp36 in the
33 infectious cycle of *Borrelia burgdorferi*. *Mol Microbiol* 64, 1358-1374.

34 Jing, X., Wong, A.C., Chaston, J.M., Colvin, J., McKenzie, C.L., and Douglas, A.E. (2014). The
35 bacterial communities in plant phloem-sap-feeding insects. *Mol Ecol* 23, 1433-1444.

1 Jones, B.A., Grace, D., Kock, R., Alonso, S., Rushton, J., Said, M.Y., McKeever, D., Mutua, F.,
2 Young, J., McDermott, J., *et al.* (2013). Zoonosis emergence linked to agricultural intensification
3 and environmental change. *Proc Natl Acad Sci U S A* *110*, 8399-8404.

4 Keesing, F., Belden, L.K., Daszak, P., Dobson, A., Harvell, C.D., Holt, R.D., Hudson, P., Jolles,
5 A., Jones, K.E., Mitchell, C.E., *et al.* (2010). Impacts of biodiversity on the emergence and
6 transmission of infectious diseases. *Nature* *468*, 647-652.

7 Khoo, J.J., Chen, F., Kho, K.L., Ahmad Shanizza, A.I., Lim, F.S., Tan, K.K., Chang, L.Y., and
8 AbuBakar, S. (2016). Bacterial community in *Haemaphysalis* ticks of domesticated animals from
9 the Orang Asli communities in Malaysia. *Ticks Tick Borne Dis* *7*, 929-937.

10 Kim, D., Hofstaedter, C.E., Zhao, C., Mattei, L., Tanes, C., Clarke, E., Lauder, A., Sherrill-Mix,
11 S., Chehoud, C., Kelsen, J., *et al.* (2017). Optimizing methods and dodging pitfalls in
12 microbiome research. *Microbiome* *5*, 52.

13 Klyachko, O., Stein, B.D., Grindle, N., Clay, K., and Fuqua, C. (2007). Localization and
14 visualization of a *Coxiella*-type symbiont within the lone star tick, *Amblyomma americanum*.
15 *Applied and Environmental Microbiology* *73*, 6584-6594.

16 Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., and Ewing, S.A. (2010). The natural
17 history of *Anaplasma marginale*. *Vet Parasitol* *167*, 95-107.

18 Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y.,
19 Bousounis, P., Kristich, C.J., and Salzman, N.H. (2015). Bacteriocin production augments niche
20 competition by enterococci in the mammalian gastrointestinal tract. *Nature* *526*, 719-722.

21 Kugeler, K.J., Farley, G.M., Forrester, J.D., and Mead, P.S. (2015). Geographic Distribution and
22 Expansion of Human Lyme Disease, United States. *Emerg Infect Dis* *21*, 1455-1457.

23 Lauder, A.P., Roche, A.M., Sherrill-Mix, S., Bailey, A., Laughlin, A.L., Bittinger, K., Leite, R.,
24 Elovitz, M.A., Parry, S., Bushman, F.D., *et al.* (2016). Comparison of placenta samples with
25 contamination controls does not provide evidence for a distinct placenta microbiota. *Microbiome*
26 *4*, 29-29.

27 Lescot, M., Audic, S., Robert, C., Nguyen, T.T., Blanc, G., Cutler, S.J., Wincker, P., Couloux,
28 A., Claverie, J.M., Raoult, D., *et al.* (2008). The genome of *Borrelia recurrentis*, the agent of
29 deadly louse-borne relapsing fever, is a degraded subset of tick-borne *Borrelia duttonii*. *PLoS*
30 *Genet* *4*, e1000185.

31 Matthiesen, S.H., and Hansen, C.M. (2012). Fast and non-toxic in situ hybridization without
32 blocking of repetitive sequences. *PLoS One* *7*, e40675.

33 Moreno, C.X., Moy, F., Daniels, T.J., Godfrey, H.P., and Cabello, F.C. (2006). Molecular
34 analysis of microbial communities identified in different developmental stages of *Ixodes*
35 *scapularis* ticks from Westchester and Dutchess Counties, New York. *Environmental*
36 *Microbiology* *8*, 761-772.

1 Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. (2002). Determination of bacterial
2 load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* *148*,
3 257-266.

4 Nakao, R., Abe, T., Nijhof, a.M., Yamamoto, S., Jongejan, F., Ikemura, T., and Sugimoto, C.
5 (2013). A novel approach, based on BLSOMs (Batch Learning Self-Organizing Maps), to the
6 microbiome analysis of ticks. *ISME Journal* *7*, 1003-1015.

7 Narasimhan, S., and Fikrig, E. (2015). Tick microbiome: the force within. *Trends in Parasitology*
8 *31*, 315-323.

9 Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-Epstein, R.,
10 Deponte, K., Fish, D., and Fikrig, E. (2014). Gut microbiota of the tick vector *Ixodes scapularis*
11 modulate colonization of the Lyme disease spirochete. *Cell host & microbe* *15*, 58-71.

12 Nigrovic, L.E., and Thompson, K.M. (2007). The Lyme vaccine: a cautionary tale. *Epidemiol
13 Infect* *135*, 1-8.

14 Noda, H., Munderloh, U.G., and Kurtti, T.J. (1997). Endosymbionts of ticks and their
15 relationship to Wolbachia spp. and tick-borne pathogens of humans and animals. *Appl Environ
16 Microbiol* *63*, 3926-3932.

17 Palmer, W.J., and Jiggins, F.M. (2015). Comparative Genomics Reveals the Origins and
18 Diversity of Arthropod Immune Systems. *Mol Biol Evol* *32*, 2111-2129.

19 Paster, B.J., Dewhirst, F.E., Weisburg, W.G., Tordoff, L.A., Fraser, G.J., Hespell, R.B., Stanton,
20 T.B., Zablen, L., Mandelco, L., and Woese, C.R. (1991). Phylogenetic analysis of the
21 spirochetes. *J Bacteriol* *173*, 6101-6109.

22 R Development Core Team (2013). R: A Language and Environment for Statistical Computing.

23 Radolf, J.D., Caimano, M.J., Stevenson, B., and Hu, L.T. (2012). Of ticks, mice and men:
24 understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nature reviews Microbiology*
25 *10*, 87-99.

26 Riley, M.A., and Wertz, J.E. (2002). Bacteriocins: evolution, ecology, and application. *Annu
27 Rev Microbiol* *56*, 117-137.

28 Russell, A.B., Peterson, S.B., and Mougous, J. (2014). Type VI secretion system effectors:
29 poisons with a purpose. *Nat Rev Microbiol* *12*, 137-148.

30 Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C., and Clay, K. (2015). Concordance of
31 bacterial communities of two tick species and blood of their shared rodent host. *Molecular
32 Ecology* *24*, 2566-2579.

33 Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P.,
34 Parkhill, J., Loman, N.J., and Walker, A.W. (2014). Reagent and laboratory contamination can
35 critically impact sequence-based microbiome analyses. *BMC Biology* *12*, 87-87.

1 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch,
2 S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for
3 biological-image analysis. *Nat Methods* *9*, 676-682.

4 Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower,
5 C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol* *12*, R60.

6 Simhadri, R.K., Fast, E.M., Guo, R., Schultz, M.J., Vaisman, N., Ortiz, L., Bybee, J., Slatko,
7 B.E., and Frydman, H.M. (2017). The Gut Commensal Microbiome of *Drosophila melanogaster*
8 Is Modified by the Endosymbiont *Wolbachia*. *mSphere* *2*.

9 Sojka, D., Franta, Z., Horn, M., Caffrey, C.R., Mareš, M., and Kopáček, P. (2013). New insights
10 into the machinery of blood digestion by ticks. *Trends Parasitol* *29*, 276-285.

11 Sonenshine, D.E., and Roe, R.M. (2014). *Biology of ticks*, 2nd edn (New York: Oxford
12 University Press).

13 Souza, D.P., Oka, G.U., Alvarez-Martinez, C.E., Bisson-Filho, A.W., Dunger, G., Hobeika, L.,
14 Cavalcante, N.S., Alegria, M.C., Barbosa, L.R., Salinas, R.K., *et al.* (2015). Bacterial killing via
15 a type IV secretion system. *Nat Commun* *6*, 6453.

16 Steinhaus, E.A. (1946). *Insect microbiology; an account of the microbes associated with insects*
17 and ticks, with special reference to the biologic relationships involved (Ithaca, N.Y.: Comstock
18 publishing company, inc.).

19 Strandh, M., and Råberg, L. (2015). Within-host competition between *Borrelia afzelii* *ospC*
20 strains in wild hosts as revealed by massively parallel amplicon sequencing. *Philosophical*
21 *transactions of the Royal Society of London Series B, Biological sciences* *370*, 20140293--
22 20140293-.

23 Swei, A., and Kwan, J.Y. (2016). Tick microbiome and pathogen acquisition altered by host
24 blood meal. *The ISME Journal*, 1-4.

25 Trout Fryxell, R.T., and DeBruyn, J.M. (2016). The Microbiome of *Ehrlichia*-Infected and
26 Uninfected Lone Star Ticks (*Amblyomma americanum*). *PLoS One* *11*, e0146651.

27 van Treuren, W., Ponnusamy, L., Brinkerhoff, R.J., Gonzalez, A., Parobek, C.M., Juliano, J.J.,
28 Andreadis, T.G., Falco, R.C., Ziegler, L.B., Hathaway, N., *et al.* (2015). Variation in the
29 microbiota of *Ixodes* ticks with regard to geography, species, and sex. *Applied and*
30 *Environmental Microbiology* *81*, 6200-6209.

31 Vannini, C., Petroni, G., Verni, F., and Rosati, G. (2005). A bacterium belonging to the
32 Rickettsiaceae family inhabits the cytoplasm of the marine ciliate *Diophrys appendiculata*
33 (Ciliophora, Hypotrichia). *Microbial Ecology* *49*, 434-442.

34 Verster, A.J., Ross, B.D., Radey, M.C., Bao, Y., Goodman, A.L., Mougous, J.D., and
35 Borenstein, E. (2017). The Landscape of Type VI Secretion across Human Gut Microbiomes
36 Reveals Its Role in Community Composition. *Cell Host Microbe* *22*, 411-419 e414.

1 Voordouw, M.J. (2015). Co-feeding transmission in Lyme disease pathogens. *Parasitology* *142*,
2 290-302.

3 Vora, N. (2008). Impact of anthropogenic environmental alterations on vector-borne diseases.
4 *Medscape J Med* *10*, 238.

5 Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., Agre, P., and
6 Jacobs-Lorena, M. (2017). Driving mosquito refractoriness to *Plasmodium falciparum* with
7 engineered symbiotic bacteria. *Science* *357*, 1399.

8 Wexler, A.G., Bao, Y., Whitney, J.C., Bobay, L.-M., Xavier, J.B., Schofield, W.B., Barry, N.A.,
9 Russell, A.B., Tran, B.Q., Goo, Y.A., *et al.* (2016). Human symbionts inject and neutralize
10 antibacterial toxins to persist in the gut. *Proc Natl Acad Sci U S A* *113*, 3639-3644.

11 Whitney, J.C., Peterson, S.B., Kim, J., Pazos, M., Verster, A.J., Radey, M.C., Kulasekara, H.D.,
12 Ching, M.Q., Bullen, N.P., Bryant, D., *et al.* (2017). A broadly distributed toxin family mediates
13 contact-dependent antagonism between gram-positive bacteria. *Elife* *6*.

14 Williams-Newkirk, A.J., Rowe, L.A., Mixson-Hayden, T.R., and Dasch, G.A. (2014).
15 Characterization of the bacterial communities of life stages of free living lone star ticks
16 (*Amblyomma americanum*). *PLoS ONE* *9*.

17 Wormser, G.P., Dattwyler, R.J., Shapiro, E.D., Halperin, J.J., Steere, A.C., Klempner, M.S.,
18 Krause, P.J., Bakken, J.S., Strle, F., Stanek, G., *et al.* (2006). The Clinical Assessment,
19 Treatment, and Prevention of Lyme Disease, Human Granulocytic Anaplasmosis, and
20 Babesiosis: Clinical Practice Guidelines by the Infectious Diseases Society of America. *Clinical
Infectious Diseases* *43*, 1089-1134.

22 Zhang, D., de Souza, R.F., Anantharaman, V., Iyer, L.M., and Aravind, L. (2012). Polymorphic
23 toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of
24 action, immunity and ecology using comparative genomics. *Biol Direct* *7*, 18-18.

25 Zhang, K., Bian, J., Deng, Y., Smith, A., Nunez, R.E., Li, M.B., Pal, U., Yu, A.M., Qiu, W.,
26 Ealick, S.E., *et al.* (2016). Lyme disease spirochaete *Borrelia burgdorferi* does not require
27 thiamin. *Nat Microbiol* *2*, 16213.

28 Zolnik, C.P., Prill, R.J., Falco, R.C., Daniels, T.J., and Kolokotronis, S.-O. (2016). Microbiome
29 changes through ontogeny of a tick pathogen vector. *Molecular Ecology*.

30

31

1 **Figure Legends**

2 **Figure 1. Distribution of interbacterial effector–immunity gene homologues across the**
3 **phylum *Spirochaetes*.** **(A)** The distribution of interbacterial effector and immunity gene
4 homologs in genomes from across a phylogenetic tree of genera within the phylum *Spirochaetes*.
5 *Deferribacteres* is shown as the outgroup. BLAST hits are normalized to the number of genomes
6 analyzed for each genus. *Borrelia* shows a significant deviation from the expected frequency
7 relative to other genera ($p < 0.05$). **(B)** Interbacterial effector and immunity gene homologs in
8 genomes of endosymbionts and pathogens associated with *Ixodidae* from the phylum
9 *Proteobacteria*.

10

11 **Figure 2. Inflation of bacterial diversity in *Ixodes scapularis*.** **(A)** The bacterial load of male
12 and female *I. scapularis* adult ticks, determined by qPCR amplifying the 16S rRNA gene
13 (N=61). Male ticks harbor significantly less bacteria than do females, (t-test $p < 0.001$). **(B)**
14 Correlation between the relative abundance of *Rickettsia* and the total bacterial load across all
15 adult *I. scapularis* ticks in unfiltered samples (Spearman's rho = 0.75, $p < 0.001$). **(C)** Alpha
16 diversity measured by the Simpson's value (log2) negatively correlates with bacterial load
17 (log10) across all adult *I. scapularis* samples without filtering of low abundance taxa
18 (Spearman's rho= -0.74, $p < 0.001$). Male (blue) and female (pink) samples appear stratified
19 according to both load and diversity. **(D)** Relative abundance of the sum of all taxa whose
20 average relative abundance was less than 1% correlates negatively with bacterial load in adult *I.*
21 *scapularis* samples (log10), Spearman's rho = -0.73, $p < 0.001$.

22

1 **Figure 3. Transient environmental bacteria limit *B. burgdorferi* infection.** **A)** Histograms
2 depict the relative abundance of bacteria (*Bacillus*, red; *Enterobacteriaceae*, blue; *Pseudomonas*,
3 green) detected in *I. scapularis* viscera samples, in bins of 5%. Counts indicate number of
4 samples. **B)** Scatter plot of the relationship between the relative abundance of environmental
5 bacteria detected in *I. scapularis* viscera and the relative abundance of *B. burgdorferi*. Color
6 scheme as in (A). **C)** Barplots indicate the sum of the relative abundance for each of three
7 abundant taxa between samples in which *B. burgdorferi* relative abundance exceeds 1%
8 (infected) and those samples for which abundance is less than 1% (uninfected). The difference is
9 statistically significant (Mann Whitney U test, $p < 0.001$).
10

11 **Figure 4. Midgut-associated bacteria in *I. scapularis*.** **(A)** Cross-section of the ovary and
12 midgut of a *B. burgdorferi*-infected adult female *I. scapularis* tick. Region highlighted by zoom
13 indicated by white box. “mg” indicates midgut lumen. Ovary indicated by white outline labeled
14 with “ov”. Percent indicates the proportion of analyzed ticks with similar internal bacterial
15 biogeography (N=41). Arrowhead in zoom indicates *B. burgdorferi* cells. **(B)** Staining of an *I.*
16 *scapularis* female tick collected from CA site with midgut-associated bacteria. White dashed box
17 indicates the region highlighted by zoom. Percent indicates the percent of total ticks analyzed
18 with similar internal bacterial content. Scale bars indicate 25 microns for wide view and 10
19 microns for zoomed panels.
20

21 **Figure 5. Impact of limited bacterial load on bacterial diversity estimates in hard ticks.**
22 **(A)** Box and whisker plots describing the internal bacterial load (log10) across all hard ticks
23 sampled (N=139), as determined by qPCR. Species names abbreviated to *Ip* (*Ixodes pacificus*),

1 *Dv* (*Dermacentor variabilis*), *Da* (*Dermacentor andersonii*), and *Am* (*Amblyomma maculatum*).
2 **(B)** Relative taxonomic abundance for bacteria detected by 16S rRNA gene sequencing averaged
3 across all samples for *A. maculatum*, *D. variabilis* and *D. andersonii*, and *I. pacificus*. Taxa
4 previously known to be internally associated with these species are bolded in legend. **(C)** Box
5 and whisker plots describing Simpson's Diversity (log2) of all hard tick internal samples without
6 filtering, with same color scheme as in A. **(D)** Bacterial load plotted against Simpson's Diversity
7 (log2). Spearman's rank correlation: Ip rho = 0.07, p > 0.1; Dv rho = -0.88, p < 0.001; Da rho = -
8 0.73, p < 0.001; Am rho = -0.62, p < 0.05.

9

10 **Figure S1. Characterization of *Borrelia burgdorferi* infection in wild *I. scapularis*. (A)**
11 *Borrelia* infection frequency as determined by qPCR for *B. burgdorferi* *flab* gene, plotted by
12 collection site. **(B)** The relative abundance of *B. burgdorferi* as measured by qPCR with *flaB*
13 primers calculated as a percent of total 16S rRNA gene counts exhibits a strong positive
14 correlation between the relative abundance of *Borrelia* by 16S rRNA gene sequencing (Pearson
15 p < 0.0001, r = 0.96). **(C)** Alpha diversity as quantified by the Simpson's diversity metric,
16 plotted for *B. burgdorferi* infected and uninfected ticks. No significant difference is observed (t-
17 test, p > 0.07).

18

19 **Figure S2. Taxonomic characterization of the *I. scapularis* microbiota.** Relative taxonomic
20 abundance for bacteria detected by 16S rRNA gene sequencing averaged across all adult *I.*
21 *scapularis* internal viscera samples and external washes. Taxa previously known to be associated
22 with *I. scapularis* are bolded in legend.

23

1 **Figure S3. Bacterial load and diversity in adult female *I. scapularis* ticks across sample**

2 **collection sites.** Shannon diversity (y-axis) and bacterial load as measured by qPCR (x-axis) of

3 adult female *I. scapularis* ticks. Samples cluster by sex, but not by geographic collection site.

4 Big Eau Pleine (BEP), Carlos Avery (CA), Gordie Mikkelson (GM), Kettle Moraine (KM),

5 Sandberg Conservancy (SC).

6

7 **Figure S4. LEfSe plot of *I. scapularis* samples.** Taxa enriched in either the viscera (yellow) or

8 the wash samples (blue) as measured by the LDA score are indicated.

9

10 **Figure S5. Detection of internal bacteria within *I. scapularis* by confocal microscopy. A)**

11 DAPI (blue) and EU338-TSA-ISH (magenta) staining of the central section of the ovary of a

12 female adult *I. scapularis* tick. The ovary is outlined in white and region of inset zoom is

13 indicated by a dashed white rectangle. **(B)** Anti-*B. burgdorferi* (magenta) and DAPI (blue)

14 staining of a midgut cross-section and ovary of an infected *I. scapularis* adult female tick. The

15 midgut lumen is indicated by “mg” and ovary is outlined in white. Scale bars are 25 microns.

16

17 **Figure S6. Confocal microscopy of *I. scapularis* nymphs.** DAPI (blue) and EU338-TSA-ISH

18 (magenta) staining of an *I. scapularis* nymph. Midgut indicated by arrowhead. Scale bar is 10

19 microns.

20

21 **Figure S7. Beta diversity analysis of adult hard tick samples. A)** Beta diversity analysis using

22 weighted UniFrac (which accounts for taxon relative abundance within samples) reveals

23 clustering by external wash samples and by tick species. **B)** Beta diversity analysis using the

1 unweighted UniFrac metric (in which the relative abundance of taxa within samples is not taken
2 into account) shows a lack of species-specific clustering.

3

4 **Figure S8. Confocal microscopy of non-*I. scapularis* hard ticks. (A)** *Amblyomma maculatum*
5 adult female, **(B)** *Dermacentor andersonii* adult female, **(C)** and *Ixodes pacificus* adult female.
6 All ticks stained with DAPI (blue) and EU338-TSA-ISH (magenta), with the midgut lumen
7 indicated with “mg” and ovary with “ov”. Arrowheads indicate bacterial micro-colonies within
8 midgut. Ovaries are outlined in white in A and C. Scale bars are 25 microns.

Figure 1

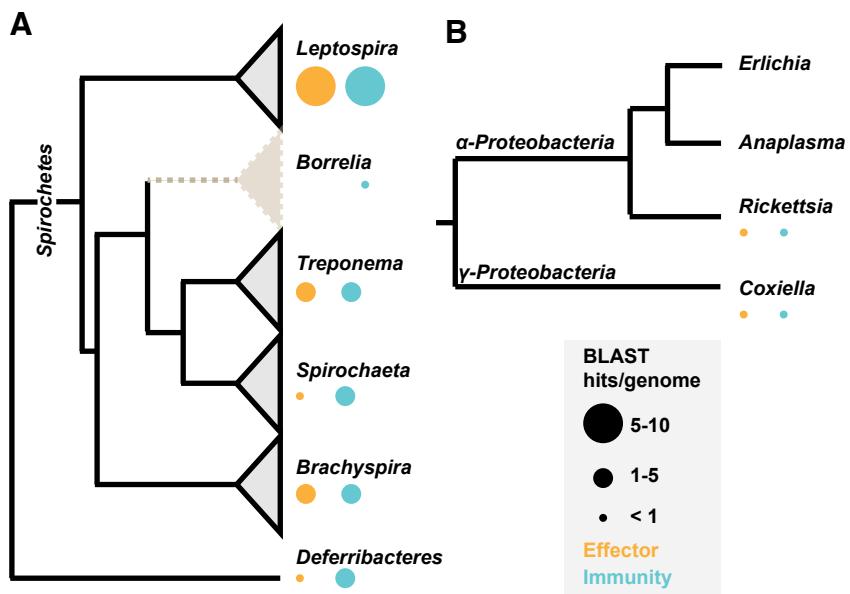


Figure 2

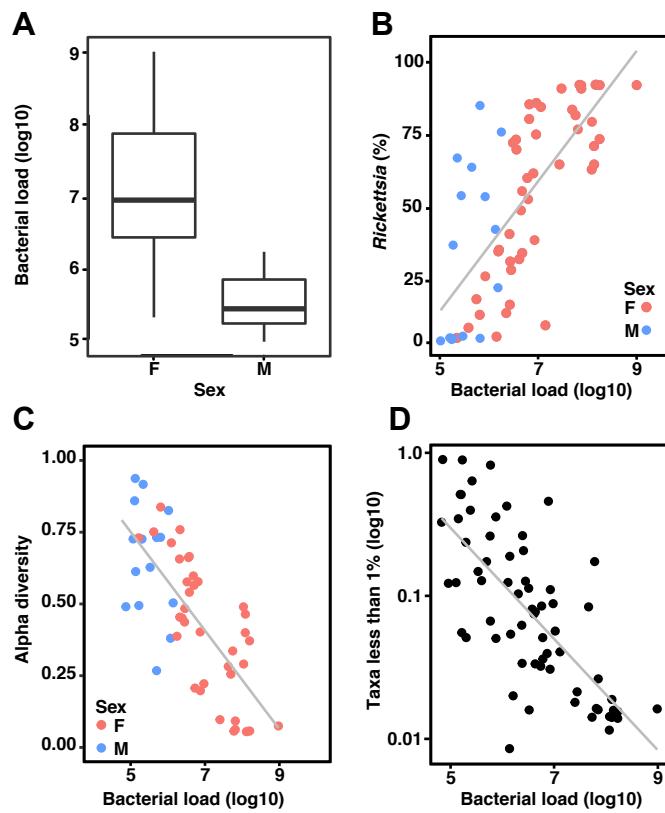


Figure 3

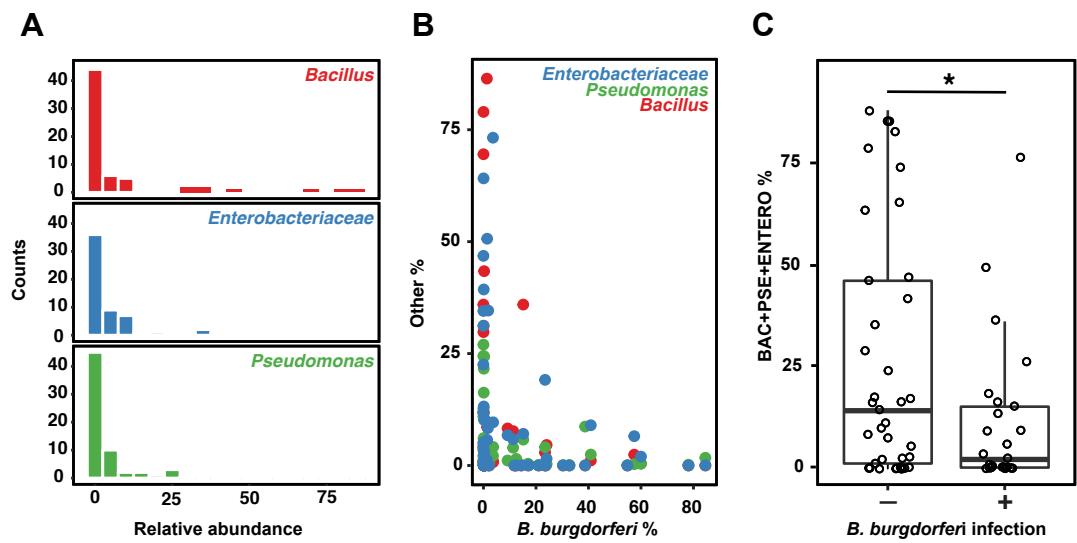


Figure 4

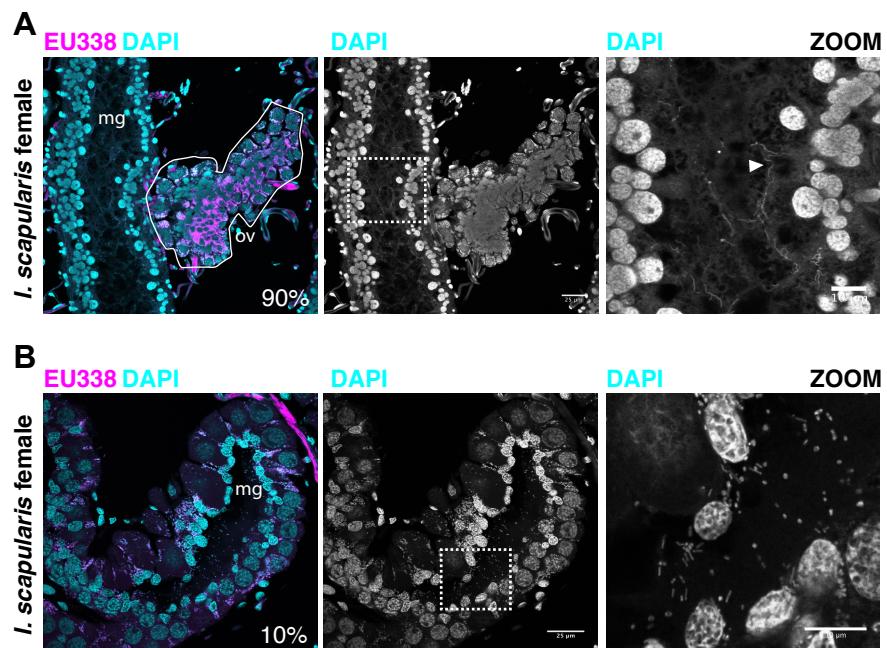


Figure 5

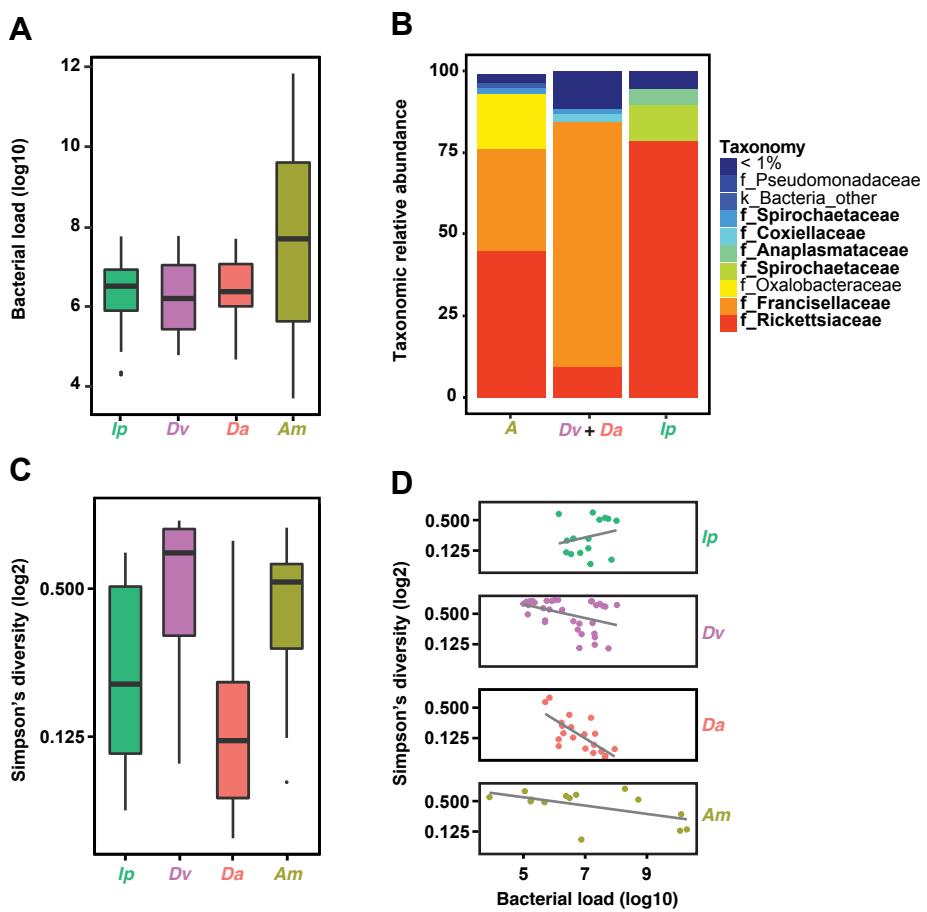


Figure S1

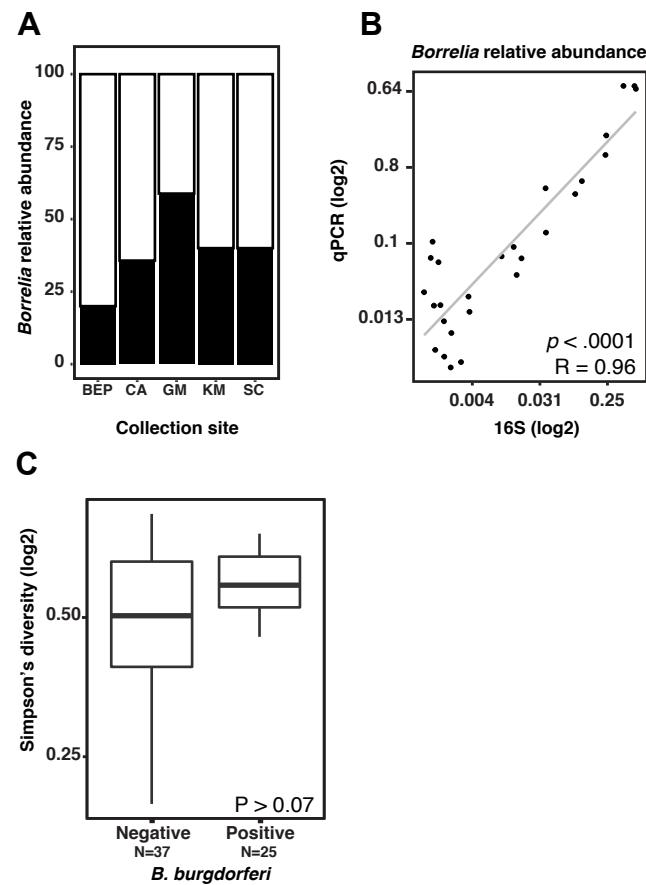


Figure S2

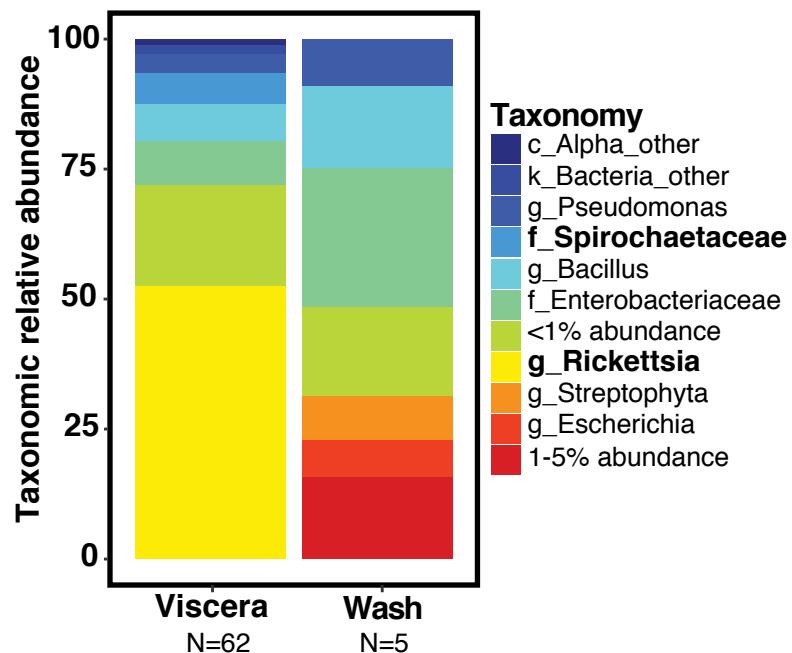


Figure S3

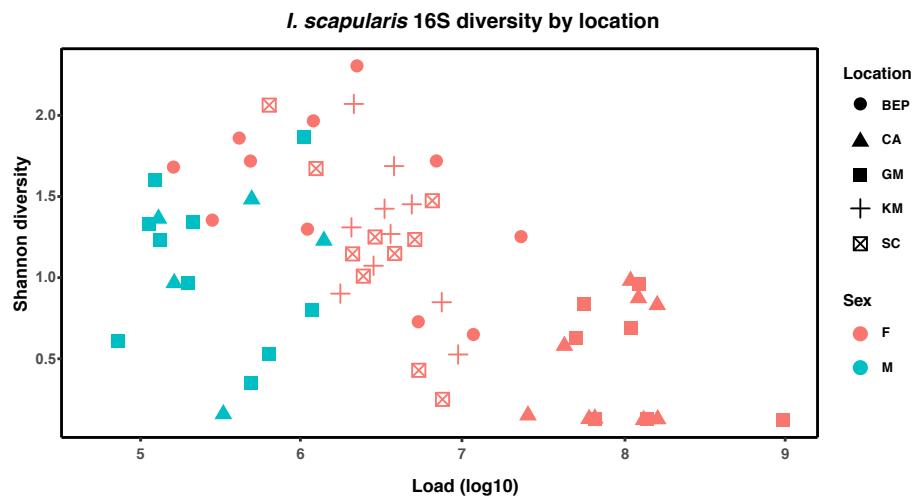


Figure S4

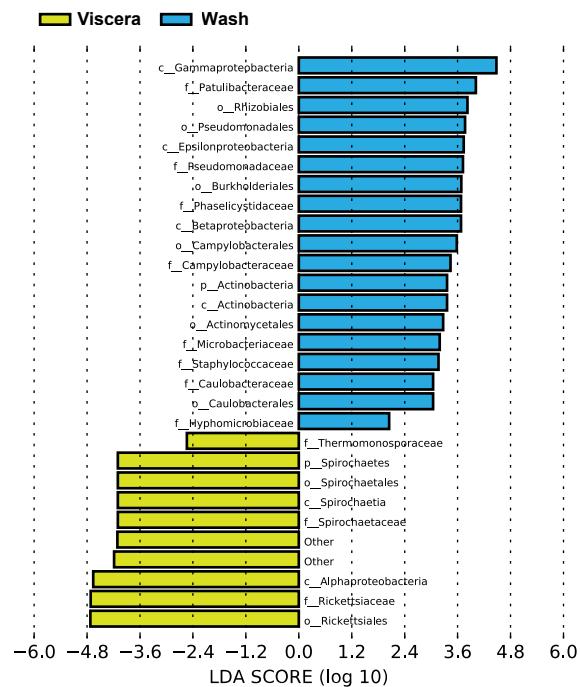


Figure S5

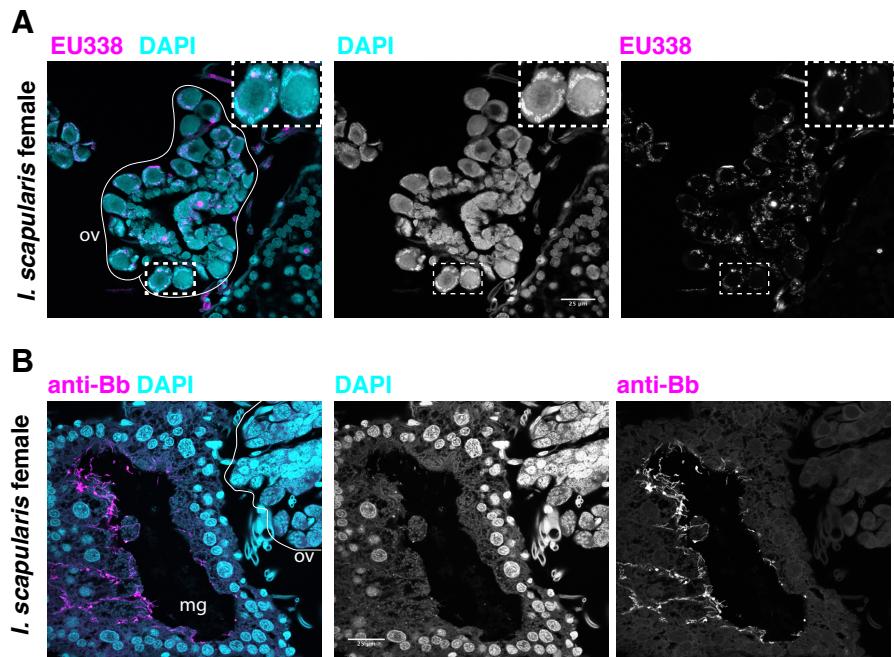


Figure S6

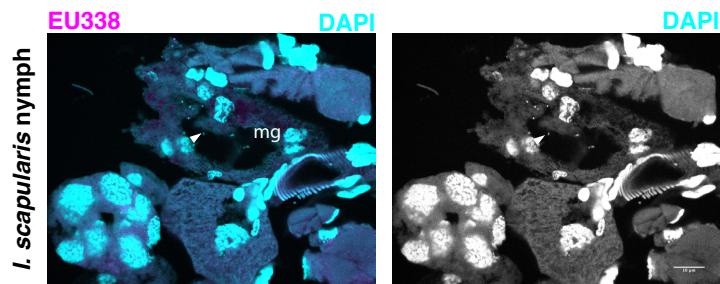


Figure S7

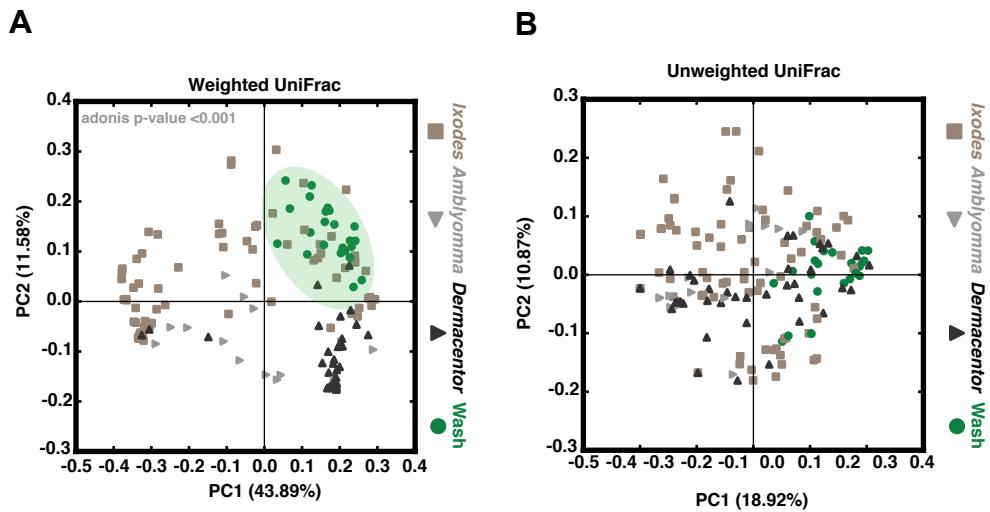


Figure S8

