# ABSTRACT

Thapa, Santosh. Influence of Environmental Temperature on The Bacterial Microbiomes of *Ixodes scapularis* Ticks. Doctor of Philosophy (Biomedical Sciences), May 2018

The blacklegged tick, *Ixodes scapularis*, is the primary vector of Lyme disease in North America. Lyme disease, endemic to approximately 65 countries worldwide, is number one vector-borne illness in the United States. In the eastern United States, Lyme diseases is transmitted by the bite of *I. scapularis* ticks infected with the spirochete bacterium *Borrelia burgdorferi.* If left untreated, Lyme infection can result in a variety of complications related to the joints, heart and nervous systems. Though the disease is highly endemic to the northeastern and upper midwestern regions of the United States, these areas represent only a portion of the vector's and host's total range. The influence of environmental factors (i.e. temperature, humidity) on the seasonal and regional incidence of Lyme disease are well-known, but their impacts on the microbial communities within the tick remain unknown. A better understanding of the influence of environmental variables on the tick microbial community maybe crucial given the recent reports that vector competence and transmission of certain tick-borne diseases can be influenced by the endogenous tick microbial communities. Using high throughput next generation sequencing, the present study investigated the effects of temperature (a major environmental variable) on the tick microbial community by comparing the sequenced amplicons of the bacterial 16S V4 rRNA gene of colony-reared *I. scapularis* ticks exposed to environments with different incubation temperatures (4ºC, 20ºC, 30ºC and 37ºC) at a constant humidity of >80% in a controlled lab setting for 10 days to that of the baseline, unexposed controls. Results showed that the bacterial community composition of colony-reared *I. scapularis* ticks is distinct

between male and female adults. The microbiome of male ticks is more diverse than the females, which are entirely dominated by *Rickettsia.* The bacterial microbiome of *I. scapularis* changed upon incubation at 30ºC for a week and 37ºC for more than five days. Additionally, the male ticks incubated at 37°C revealed a significantly different bacterial diversity compared to the baseline microbiomes, and the change was dependent on the length of exposure. *In-silico* removal of *Rickettsia* from the female data revealed a shift in the underlying bacterial population, with a significantly different bacterial diversity at 37°C compared to that of 4°C and 20°C treatments. These findings led us to further investigate if the bacterial communities in natural populations of *I. scapularis* from diverse geographic and climatic regions also differed. Our results demonstrated that the bacterial community structure in field-collected male ticks from Texas was different compared to the males from Massachusetts. Despite the dominance of *Rickettsia* in female ticks from both states, the females from Massachusetts contained *Borrelia,* and *Anaplasma*, but not from Texas. Interestingly, the bacterial composition in female *I. scapularis* collected from dogs in Texas was more complex in comparison to the field-collected female ticks from both locations. Taken together, these results provide experimental evidence that environmental temperature can impact the bacterial microbiome composition of *I. scapularis* ticks in a controlled laboratory setting and may have implications to the regional differences observed in the bacterial community structures among the natural populations of the tick. Future studies on the mechanisms of how environmental temperature and other abiotic factors influence the tick microbiome will improve our understanding of the impacts of climate change on the ticks' ability to carry and transmit pathogens, with possible ramifications on strategies to control tick-borne and other zoonotic pathogens.

INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE BACTERIAL MICROBIOMES OF *Ixodes scapularis* TICKS

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# INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE BACTERIAL MICROBIOMES OF *Ixodes scapularis* TICKS

DISSERTATION

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For the Degree of

DOCTOR OF PHILOSOHY

By

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 16S V4 | hypervariable region 4 of the 16S subunit of rRNA |
| BL | Baseline |
| BSA | bovine serum albumin |
| DNA | deoxyribonucleic acid |
| µl | microliter |
| µM | micromolar |
| mM | millimolar |
| NOAA | National Oceanic and Atmospheric Administration |
| OSU | Oklahoma State University |
| PCR | polymerase chain reaction |
| PERMANOVA | permutational multivariate analysis of variance |
| pM | picomolar |
| RH | relative humidity |
| rRNA | ribosomal ribonucleic acid |
| TAE | tris (hydroxymethyl) aminomethane, acetic acid, ethylenediamine tetra-acetic acid |
| UV | ultraviolet radiation |

# CHAPTER I

# INTRODUCTION

## Biology and morphology of ticks

Ticks are obligate, hematophagous (blood-sucking) ectoparasites of vertebrates, including mammals, birds, reptiles, and amphibians (1, 2). Taxonomically, ticks are classified in the phylum Arthropoda, class Arachnida, subclass Acari (which also contains mites), superorder Parasitiformes, order Ixodida, and the superfamily Ixodoidea (1, 3) Ticks are further subdivided into three families: Ixodidae ("hard" ticks), Argasidae ("soft" ticks), and Nuttallielidae. Hard ticks are contained within 12 genera, including members of *Ixodes* such as the species under investigation here, are much more prevalent than the soft ticks (6 genera) (4).

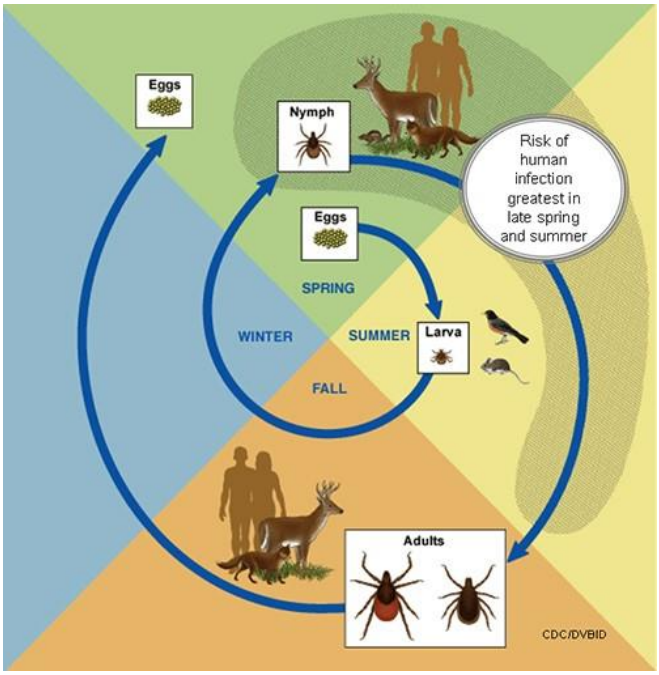
The life cycle of ticks consists of four developmental stages: egg, larva, nymph and adult, the later three being active parasitic phases (5). Each active stage feeds only one time before transitioning to the next stage. Digestion of blood in ticks is intracellular, and is a very slow process, thus a single blood meal remains as a food reserve over months or years, except in ovipositing females (1). Hard ticks have a fairly uniform life cycle within the family, while soft ticks exhibit immense diversity in their development. All ixodid ticks have one nymphal stage, by contrast Argasid, or soft ticks, vary widely in number of nymphal phases, or instars, depending on the species (always more than 1) (6). The majority of ixodid ticks have a 3-host-

life cycle (some are 2- or 1- host ticks), while Argasid ticks typically have a multi-host life cycle. As this research is focused on one of the hard ticks, *Ixodes scapularis* (commonly referred as the 'blacklegged tick'), background on soft ticks is limited here.

In a 3-host-life cycle of ixodid tick, a larva (6-legged) hatches from an egg and quests for a host. Questing is a behavior exhibited by ticks where they crawl up nearby vegetation (such as blades of grass, woody stems, etc.) and wait with their forelegs outstretched for a suitable host to pass within reach. Blacklegged larval ticks commonly feed on white-footed mice, *Peromyscus leucopus*, or other small mammals (7). Upon finding a host, the tick attaches and begins to feed on blood by inserting their mouth parts into the host skin. During the attachment process ixodid ticks secrete a cement-like substance from their salivary glands, which helps the attached tick to bind tightly to the host skin. This is particularly important for ixodid ticks because they feed slowly and have an extended feeding period ranging from a few days to several weeks. For example, blacklegged ticks generally feed for 3-5 days (1). For successful blood feeding, ticks often secret proteins and lipids to minimize pain, itching, and inflammation to the host while maintaining blood flow (8, 9). Once engorged, often resulting in expansion of the tick to three times its original size, the larva drops off from the host, and undergoes ecdysis, thus molting into a nymph (8-legged). The unfed new nymph quests for a host in a similar manner, attaches, feeds, engorges, detaches, and molts to an adult tick. The blacklegged nymphal ticks again feed on small mammals (commonly white-footed mice) and birds. After molting the new adult tick again quests for a host and attaches (blacklegged adults seek larger mammalian hosts, often white-tailed deer). While attached to a host, the adult ixodid ticks mate. Upon completion of mating, females feed rapidly (engorged blacklegged females are often three times their pre-fed size),

drop off the host, seek sheltered sites, lay eggs in protected habitats on the ground (usually under leaf litter), and die shortly thereafter. All ixodid ticks have a single gonotrophic cycle (i.e. the engorged, mated females lay thousands of eggs and then die). Males generally stay on the host in attempts to mate with another female. Male hard ticks usually mate with one or more females and then die, though some may live for months (5). The longevity of life cycles of ixodid ticks in nature generally varies from 2-6 years, which also depends on the geography and climatic conditions (5, 10).

FIG 1. Two-year life cycle of *I. scapularis* (a three-host ixodid tick)




In the spring and summer of year one, as shown in Fig. 1 (11), eggs hatch into larvae which feed once and molt into nymphs. Nymphs undergo dormancy for the fall and winter (i.e. overwintering). In the spring and summer of the tick's second year, primarily from May through early July in northern latitudes, the nymphal ticks become active and take a blood meal from a host. In the fall of the second year, nymphs molt into adult ticks. The adult female ticks feed and mate on mammalian hosts, primarily white-tailed deer, during the fall or early spring. The female lays her eggs (3,000 to 6,000), then dies. Male ticks also attach to the host, but do not feed or become engorged (11, 12).

The size of the *I. scapularis* tick varies depending on the life stage, sex, and feeding state. Unfed females measure about 2.7 mm in length, whereas the males are smaller. A larva is less than 1 mm long, whereas a nymph measures between 1-2 mm (about pin-head sized) in length (12, 13).

Anatomically, tick structure is composed of two parts fused together: the capitulum and body. The mouth parts (capitulum) are composed of the mobile palps; between which are two chelicerae, where lies the hypostome. The palps are 2-4 segmented structures that position the capitulum for feeding. The hypostome, as shown in Fig. 2 (14), is the part of the tick that is actively inserted into the wound of the host. The feeding channel that the hypostome generates is used for the intake of host fluids and injection of the tick's saliva into the host. The hypostome contains barbed *teeth* that help latch the tick to the organism. Ixodid ticks have a hard shield, the scutum, on the dorsal body surface. The body is where the legs are attached; larval ticks are 6-legged while nymphal and adult ticks have eight legs. Also associated to the body is the midgut of the tick, an internal structure constructed of numerous diverticula which are used to digest the blood meal. Ticks breathe air through tiny tubes called spiracles. In general, a tick breathes air between one to fifteen times every hour (15).

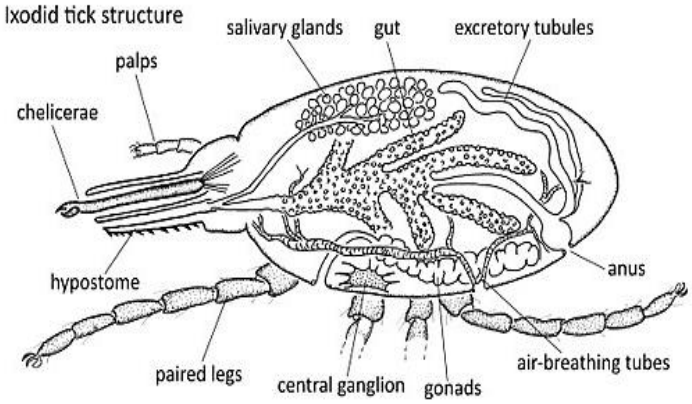



FIG 2. Anatomy of a hard tick

**Ticks and disease**

Ticks are widely distributed throughout most regions of the world and affect animal and human health worldwide. Though ranked second only to mosquitos in total number of cases, ticks are responsible for transmitting a greater variety of pathogens to humans and animals than any other disease vectors (1, 15). One hypothesis for ticks' success as vectors of diverse pathogens is that their midgut is less hostile in comparison of other hematophagous vectors (16). The main reasons for this are the nearly absolute absence of intraluminal proteolytic enzymes (except the hemolytic enzymes that lyse blood cells to release hemoglobin), as the digestive process in ticks is almost entirely intracellular. Consequently, digestion of a blood meal occurs over an extended period of time, thus enabling any ingested pathogens to survive and multiply, and ultimately allowing time for the pathogens to transmit during tick bites (5).

Ticks carry a wide diversity of human infectious agents, including bacterial pathogens which causes Lyme disease, Rocky Mountain spotted fever, tularemia, human granulocytic anaplasmosis, and human monocytic ehrlichiosis; protozoan pathogens including babesiosis, theileriosis; and viral infections such as Colorado tick fever, tick-borne encephalitis, and Powassan encephalitis. Additionally, tick bites can cause irritation at the bite site or elicit allergic reactions, in severe cases including toxicoses and paralysis (1, 15, 17).

Ticks are also responsible for transmitting many diseases to livestock, companion animals, and wild life, including the causative agent of bovine anaplasmosis, babesiosis, Q fever, and dermatophilosis. Ticks also cause toxicosis, paralysis, and allergies to livestock and other animals. Even when ticks do not transmit pathogens, they are of huge concern as pests as severe

tick infestations can lead to livestock skin injuries which may lead to infection of open wounds. Animals subjected to heavy tick burdens can also lead to abortion, weight loss, and decreased milk production (1).

Globally, ticks and tick-borne disease are the cause of significant economic losses (17). In a retrospective study, Adrion et al. (18) (2015) reported that Lyme disease is associated with significant health care costs in the US, as evidenced by a $2,968 higher total health care costs and 87% more outpatient visits over a year, as compared to the healthy controls. In livestock, it has been reported that economic losses due to bovine anaplasmosis, caused by the bacterium *Anaplasma marginale*, which is vectored by the Rocky Mountain wood tick *Dermacentor andersoni*, are over 300 million USD per year in the United States alone (19). Without investments in major tick control, livestock production is almost impossible in some parts of the world; for example, it has been estimated in Tanzania that 384 million USD are lost annually due to tick-borne diseases of livestock (20).

### *I. scapularis*: Natural distribution and Lyme disease

*I. scapularis* is a three-host ixodid tick. These ticks are most commonly found in the leaf litter associated with natural wooded areas frequented by wildlife and in the forest edge, which not only provides shelter for the ticks from harsh environmental conditions but also support a robust wildlife population that serve as a reservoir for the ticks and their associated pathogens. Studies have also suggested that tick abundance is based on the seasonal activity of the ticks in specific regions which varies widely by geographic region and climatic cues, and tick populations can vary dramatically within a given area due to local vegetation and wildlife host abundance. The

greatest risk of being bitten by the ticks exists in the late spring and summer. However, adults may be out searching for a host any time winter temperatures are above freezing (21). A study at the University of Arkansas suggested that nymphal ticks commonly bite humans in northeastern states while adult ticks commonly bite larger animals including humans in southern US states (22).

TABLE 1. Seasonal activity of *I. scapularis* ticks in northeastern and southern US states

| | **Peak Larval Activity** | **Peak Nymphal Activity** | **Peak Adult Activity** |
|---|---|---|---|
| Northeastern | Late summer and early fall (Year 1) | Summer (Year 2) | Fall through winter (Year 2) |
| Southern | Spring and summer (Year 1) | Spring and summer (Year 2) | Fall through winter (Year 2) |

*(Source: /www.capcvet.org/capc-recommendations/ixodes-scapularis-and-ixodes-pacificus/)*

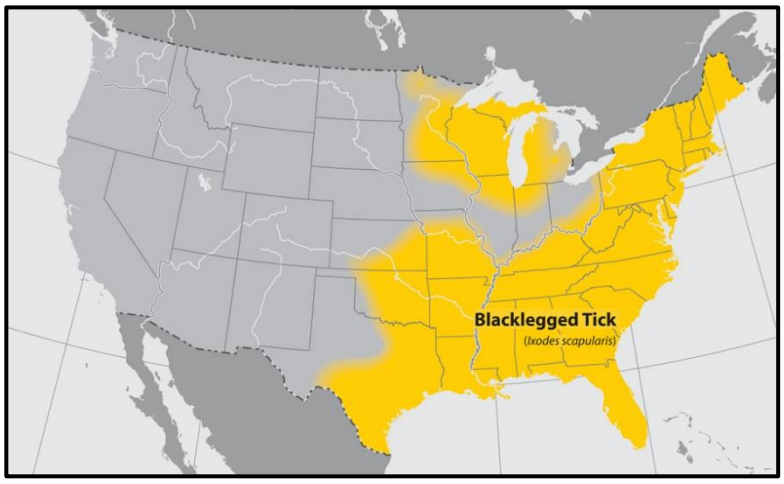



FIG 3. Geographical distribution of natural populations of *I. scapularis* ticks in the US

The CDC map (see Fig. 3 (23)) shows that *I. scapularis* is present in much of the northeastern, mid-Atlantic, southeastern, north central, and south-central states. Over the past two decades, the geographical range of *I. scapularis* in the eastern US has expanded northward (24), which is speculated to be due to changing temperatures along with changes in mammalian host populations (25). Eisen et al. (2016) reported that the number of counties with established population of *I. scapularis* (defined as "6 or more ticks or two or more tick life stages identified in a calendar year") has more than doubled since 1998. While the far south and south-central states remained relatively stable in terms of established populations of the tick, the northeastern and north-central states experienced rapid expansion of the tick's geographical range. As of 2015, *I. scapularis* has been documented from 1,420 (45.7%) of the 3,110 continental US counties, totaling 37 states (24).

Nearly 95% of the ticks from Massachusetts (2088 of the 2203 originated from MA) submitted to the University of Massachusetts during the period from 2006–2012 for tick-borne pathogen testing were *I. scapularis* (26). A similar study of ticks submitted to the University of North Texas Health Science Center for PCR testing during 2008-2014 showed that *I. scapularis* constituted only 5.6% of the ticks removed from human in Texas (27). Another study reported that about 90% of the ticks collected from 250 hunter-killed white-tailed deer (during Oct 2007 to Jan 2008) in Arkansas were *I. scapularis* (28).

Ogden et al. (2004) (29) suggested that temperature and photoperiod are the only regulatory parameters for the activity and development of ticks, as evidenced by the observation of no effect of water stress on *I. scapularis* under the conditions in the northern US. Estrada-Pena et al.

(2012) (30) indicated that, besides the availability of suitable hosts, the major requirements for the development and survival of *I. scapularis* ticks include an air temperature range between -10 to +35 °C, a constant relative air humidity of not less than 80%, and near saturation in the soil.

The blacklegged tick is the primary vector of the Lyme disease spirochete *Borrelia burgdorferi* in the eastern United States and parts of Canada, while a closely related species, *I. pacificus* (the western blacklegged tick), is the major vector of Lyme disease along the Pacific Coast (24, 31, 32). Because incidence of Lyme disease transmission by this tick is significantly less than that caused by *I. scapularis*, the background and discussion on *I. pacificus* is limited here. In addition to Lyme disease, *I. scapularis* is the vector for other pathogens causing anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*), relapsing fever (*Borrelia miyamotoi*), and Powassan encephalitis (Powssan virus) (24, 33, 34).

Lyme disease is transmitted to humans through the bite of infected ticks. Nymphs and adult females are both epidemiologically important for human infection (12). Lyme borreliosis or Lyme disease is a multisystem, tick-borne illness usually characterized by acute flu-like symptoms combined with the distinctive skin rash *erythema migrans* at the site of the tick bite. If untreated, Lyme infection can disseminate to other tissues and organs, causing severe joint pain, heart problems, and various neurological problems in about 10% to 20% of the affected people that may last for years (23) (35). Many of those cases also include co-infections with other tick-borne pathogens (12, 36, 37).

First recognized in the 1980s (31), Lyme disease is the most commonly reported vector-borne illness in the US, and the case counts have increased significantly from over 15,000 cases in 1996 to more than 30,000 cases in 2008 and subsequent years (23, 38, 39). In 2015, it was the sixth most common nationally notifiable disease with 38,069 reported cases (23), yet the true burden of the disease is estimated to be 10-times greater (40, 41). The disease has geographical foci, with the majority of cases concentrated in the northeast and upper midwest regions of the US. In fact, the 14 states of the northeastern and upper midwestern US (Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, Pennsylvania, Rhode Island, Vermont, Virginia and Wisconsin) accounted for 95% of all confirmed Lyme cases in 2015 (Fig. 4) (23).

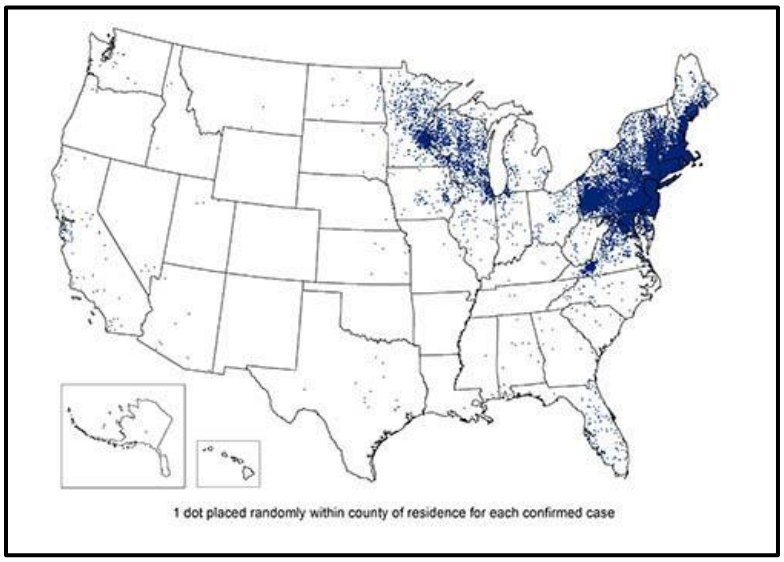



FIG 4. Reported cases of Lyme disease in 2015 in the United States

Lyme disease cases typically surge in June, July, or August but are far less likely from December through March, which corresponds to the seasonal tick activity in the aforementioned regions

(23). In recent decades, Lyme disease cases have increased both in numbers and in geographical distribution across the eastern US, which coincided with the expansion in the range of *I. scapularis* within the region (24).

Various external factors such as temperature, humidity, precipitation, and seasons of the year have been suggested to influence the incidence and onset of Lyme disease (30, 42-45).

Given that all of the necessary components for disease transmission, including the hosts and vectors, are present throughout the northern and southern portions of the eastern United States, Lyme disease would be predicted to be present evenly across the geographical range of *I. scapularis*. However, Lyme disease areas in the eastern US do not mimic the geographical distribution of *I. scapularis*, a pattern that is also observed in other tick species. For example, tularemia, caused by the bacterium *Francisella tularensis* and transmitted by the lone star tick (*Amblyomma americanum*), is primarily reported in Arkansas, Kansas, Missouri, and Oklahoma (12), though the vector has a much broader distribution throughout the southeastern US. Studies have suggested that differences in attributes observed between *I. scapularis* from northern and southern states, including density of host-seeking nymphs, rate of infection with *B. burgdorferi*, feeding preference (larvae in northeast feed mainly on white-footed mice, whereas they frequently feed on lizards in the southeast), host-seeking behavior, and variable contact rates between human and nymphal ticks, provide possible explanations of regional differences in incidence of Lyme disease in northern and southern parts of the eastern US (24, 46-50). However, it is also possible that the regional differences observed in Lyme disease incidence between northern and southern portions of the eastern US could have to do with the microbial

community of the ticks themselves and their direct or indirect effects on the disease causative agent. Analogous to the human microbiome, the resident microbial communities of the ticks can have diverse relationships among the members and with the vector (25) that can influence the density and composition of certain tick-borne pathogens, the vector competence and transmission of pathogens to human (51, 52). It is also possible that ecological factors (such as temperature) surrounding the immediate environment of the tick could have a role in shaping the microbial community of the ticks across the geographic range, thus ultimately affecting vector competence and the risk and transmission of the disease.

**Tick Microbiomes**

Over the past decade, tick microbiome research has grown tremendously. As the efforts have been focused towards understanding the composition and diversity of tick-associated microbes, many exciting advancements have already been made. In the late 1990s, PCR assays were used to investigate the few intracellular, endosymbiotic bacteria (*Coxiella*-like endosymbiont and *Francisella*-like endosymbiont) of the ticks (52). With the advent of high-throughput DNA sequencing, a deeper understanding of the microbial diversity in ticks led to the exploration of several complexities of the tick microbial community. For example, one study demonstrated that manipulation of the community of known endosymbiotic bacteria, *Coxiella* spp. in *A. americanum* ticks, can lead to reduced reproductive fitness of the tick (53). Gall et al. (2016) reported that the bacterial microbiome of the Rocky Mountain wood tick, *Dermacentor andersoni*, influenced pathogen susceptibility: an increase in *Rickettsia bellii* proportion and quantity in these ticks was found to be negatively correlated to infection by *Anaplasma marginale,* the most widespread pathogen of livestock (54). Research in this area may ultimately

lead to strategies for bio-control of tick-borne diseases by decreasing the pathogen susceptibility of ticks.

**Techniques for microbiome studies**

Traditional strategies of culturing in laboratory using different nutrient media has been limited to the cultivable type of microorganisms. Recent advancement in molecular methods, such as DNA sequencing, has enabled us to gain insights into the vast spectrum of variability among all types of microbes in the natural world, particularly unculturable microorganisms that are otherwise impossible or difficult to analyze. High-throughput comparative genomics enabled by the advent of next generation sequencing (NGS) platforms such as Roche 454, Illumina (55-58) have facilitated rapid advancement in our understanding of the composition and function of microbial populations in diverse environments, from human (59-63) to insects (64-68) to soil (69-71) to microbial mats of hot springs, and hypersaline ocean (72, 73). The way in which microbiome researches are often carried out is through metagenomic studies. Handelsman (2004) has defined metagenomics as the "genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms" (74). Metagenomics analysis is essentially a sequence-based approach that allows to assess the genetic material of microbes from the environment without the need for isolation and lab cultivation (75, 76). There are two main approaches for analyzing the microbiome: amplicon-based and shotgun metagenomics. The amplicon-based metagenomics approach involves PCR-targeted sequencing of a genetic locus, like one or more hypervariable regions of the 16S ribosomal RNA (rRNA) gene for bacterial microbiomes. On the other hand, shotgun metagenomic analyses are accomplished by unrestricted sequencing of the genome of all microbes present in a given sample. As this study is

based on the 16S rRNA gene amplicons metagenomics, information on shotgun approach is limited here.

**16S rRNA metagenomic analysis**

The 16S rRNA is a component of the 30S small subunit of prokaryotic ribosome and has essential roles both in the structure and function of the ribosome. Due to the slow rates of evolution, the 16S rRNA gene as shown in Fig. 5 (77), that codes for 16S rRNA, is highly conserved among bacteria and archaea, thus making it a prime target for studying these populations (78-80). Because of the presence of both highly conserved regions (allowing for primer design) and variable regions (providing a 'molecular fingerprint' to identify taxa), the 16S rRNA gene is the leading marker gene used to profile diversity of microbial communities (81). Of the nine hypervariable regions (V1 -V9) in 16S rRNA gene interspersed with conserved regions, the V4 (~254 bp) targeted sequencing is the most widely for microbiome studies, including the Earth Microbiome Project (69). Several factors may influence the outcome of 16S microbiome analysis, including, but not limited to, DNA extraction methodologies (82), physiological differences of bacteria (e.g. Gram positive or Gram negative) in a given sample (83), primer binding affinity (84, 85), PCR amplification bias (86) and errors (e.g. chimera formation (87)), 16S rRNA gene copy number variation (88), and environmental contamination (89). However, careful consideration in design, and execution of the experiments can overcome many of these limitations.

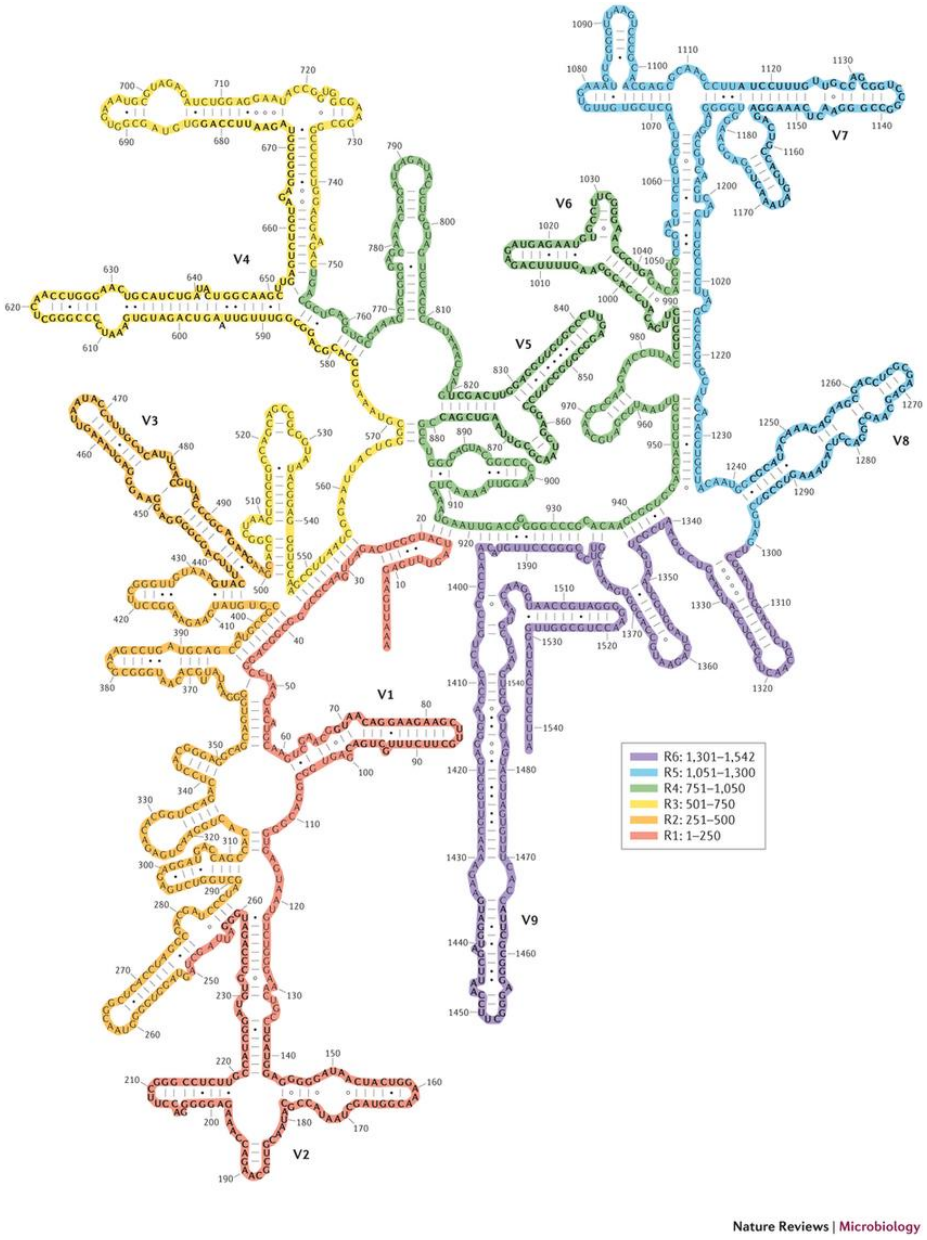



FIG 5. 16S rRNA gene: conserved and hypervariable regions (V1-V9) (reproduced with permission from Dr. Ramon Rossello-Mora).

Sequence data generated by NGS methods requires sophisticated computational techniques for their analysis (90), which often poses a significant challenge for the microbiome researchers. However, development of the user-friendly open-source bioinformatics software programs combined with the availability of comprehensive reference databases (such as the Greengenes

database (91), Ribosomal Database Project (RDP) (92), and SILVA (93) for 16s rRNA genes) has tremendously helped researchers to analyze their NGS datasets. The two most widely adopted open-source software programs in microbiome studies are mothur (94) and QIIME (95). Although there are differences in their software programming, both of these pipelines provide methods for performing taxonomical classification of bacterial sequences, assessment of bacterial diversity within (alpha-diversity) and between samples (beta-diversity), among other features, and both software essentially produce similar results (84). Prior to taxonomic classification, the amplicon sequences of a marker gene such as the V4 region of the bacterial 16S rRNA gene, are clustered into operational taxonomic units (OTUs) (OTU is an arbitrary definition of a taxonomic unit based on sequence divergence) based on their similarity, either *de novo* (96) or to the previously annotated sequences in the reference database (97). In either case, an arbitrary similarity threshold has been used to differentiate clusters in the community. In 1994, a 97% identity was proposed as the canonical clustering threshold as proxy for species (98), which was revised to 99% in 2006 (99). However, it has been reported that this threshold is often insufficient to discriminate closely related species. More recently, Edgar (2018) recommended the identity cutoff of ~99% for full-length 16S rRNA sequences and ~100% for the V4 hypervariable region, for recognition of a bacterial species (100).

Alpha-diversity provides information on presence/absence of taxa, their richness, evenness and other ecological features. A variety of approaches have been adopted (from macro-ecology to microbial ecology) to estimate alpha-diversity within samples, such as rarefaction, observed OTUs, chao1, abundance-based coverage estimators (ace), Shannon, Simpson's index. Rarefaction compares observed richness among habitats, environments, sites, or treatments, which have been sampled unequally. Rarefied curves, resulting from averaging randomizations

of the observed accumulation curves, thus provide a way for comparison of the richness observed in different samples (101, 102). These curves can also help to access the sampling intensity; for example, if a rarefaction curve becomes parallel to the x-axis, one can reasonably be confident that the sampling effort is good enough to trust the observed level of richness. Rarefaction is even suggested as a better measure of diversity than richness (103). The observed OTUs are the unadjusted number of OTUs observed in a sample. The Chao1(104) and ACE (105) richness estimators utilizes correction factor on observed taxa. Chao1 is a robust estimator of data with a high number of low abundant taxa in the community under analysis, while both the chao1 and ACE underestimate true richness at low sample sizes (102). The Shannon diversity index refers to a sum of the proportions of each OTU (or species) relative to the total number of OTUs (or species) in the community and thus takes into account of both the abundance and evenness of taxa (106). On the other hand, the Simpson's index is a measure of dominance as it is weighted toward the most abundant taxa. Furthermore, the inverse Simpson's index ( a reciprocal of Simpson's index) is more commonly used relative to the Simpson's index, but reported to have variance problems (107).

Beta-diversity is commonly calculated using either the phylogeny-based (evolutionary differences between communities) and/or taxon-based approaches. An example of phylogenetic method is UniFrac (unique fraction) distance: unweighted (presence/absence of OTUs) and weighted (abundance of OTUs) (108-110), whereas the Bray-Curtis dissimilarity (111) is a non-phylogenetic one. After calculating the distances between samples, ordination techniques such as principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) are used to visualize their positions in a low-dimensional space (112, 113).

With these background information, the next section will discuss an overview of the dissertation, specific aims and significance. The subsequent chapters will deal with details on the mentioned specific aims and methodology.

## Overview of the dissertation

Recently there has been growing interest in the microbiome of hard ticks due to the potential role that the endogenous microbial community may play in pathogen acquisition and transmission in certain ticks. A major focus on tick microbiome research has been directed towards *I. scapularis* primarily due to the burden of the cases of Lyme disease, the agent of which the tick transmits. Though *I. scapularis* is broadly distributed throughout the northeastern, mid-Atlantic, southeastern, north central and south-central US states, the majority of the Lyme disease cases are concentrated in the northeast and upper midwest regions of the United States (12). The reasons for the rarity of Lyme disease in the southern portions of the eastern US are poorly understood. Analysis of the microbiome is important to the field because the risk of human infection is correlated with the prevalence of natural infection within the tick vectors. Many studies have suggested that environmental factors such as temperature, humidity, precipitation, and seasons of the year play a vital role in the regional onset and transmission of Lyme disease (30) (42). However, a knowledge gap exists in understanding the effect of environmental factors on the tick microbiome composition. We hypothesized that environmental temperature affects the bacterial community composition within the ectoparasite *I. scapularis*, because ticks are ectothermic and more importantly, about 98% of their 2-year life cycle in *I. scapularis* occurs off the host (114).

Additionally, a previous US-based study demonstrated that the microbiota of *Ixodes* ticks vary with sex, species and geographical origin (115). But, those differences are not consistent with those reported from other geographic areas. For example, a recent study on *I. scapularis* ticks from eastern and southern Ontario, Canada found no significant differences in their microbiomes with regard to location, sex, or life stage (116). Though these studies used similar techniques, there are discrepancies in the experimental design with respect to selection of hypervariable regions of the bacterial 16S ribosomal RNA gene (Van Treuren et al (115) targeted V1-V3, while the Clow et al (116) study used V4 region), and inclusion of the ticks collected from distinct climatic areas. These contradicting reports highlight the potential role that, not only the geography, but also the related ecological and environmental factors may have in shaping the microbiome of these ticks.

Specific Aims and Methodology

We focused on the following specific aims and used the described methodology to accomplish these studies:

**Specific Aim 1: Determine the effects of temperature on bacterial microbiomes of *I. scapularis***

The microbial survivorship and diversity in *A. americanum* (three-host-tick belonging to the same hard tick family as that of *I. scapularis*) has been previously shown to be partially dependent on environmental variables (68). Here, colony-reared adult *I. scapularis* ticks were used to investigate the effects of temperature on their microbiome composition. The ticks were exposed to environments with different incubation temperatures (4ºC, 20ºC, 30ºC and 37ºC) at a

constant humidity of 80-95% for 10 days with daily monitoring for their survival. Saturated salt solutions (see Table 2) were used to maintain a constant humidity within each environmental chamber throughout the experiment (117). Genomic DNA was extracted from individual ticks and the bacterial 16S V4 rRNA gene amplicons were sequenced using an Illumina MiSeq platform. After processing of the MiSeq generated raw sequence with a mothur pipeline, bacterial community compositions of the temperature-exposed ticks were analyzed and compared to that of the untreated baseline controls. (Details of the experiments and results are described in Chapter II.)

TABLE 2. Saturated salt solutions used to maintain relative humidity at various temperatures

| **Salt** | **Concentration (g/ml)** | **Temperature** | **Approx. Relative Humidity (estimated)** |
|---|---|---|---|
| Ammonium sulphate [$(NH4)_2SO4$] | 0.79 | 4°C | 82% |
| Potassium chloride [KCl] | 0.40 | 20°C, 30°C | 85% |
| Potassium sulphate [$K_2SO4$] | 0.12 | 37°C | 95% |

**Specific Aim 2: Compare the bacterial microbiomes of *I. scapularis* from two different geographic/climatic regions**

*I. scapularis* ticks were collected from their natural habitat in Kennard, Texas and Cape Cod, Massachusetts (opposing ends of the vector's range) during the years 2016-2017. Standard flagging technique was used for tick collection, which consisted of walking down trails dragging a 1 $m^2$ piece of white cloth attached to a pole gently over and around the vegetation where ticks may be present. All encountered *I. scapularis* ticks were collected with fine-tipped tweezers and placed into sterile collection vials. DNA was extracted from individual ticks and sequencing of

bacterial 16S V4 rRNA gene PCR amplicons was performed using an Illumina MiSeq platform. Bacterial microbiota of these field-collected ticks was assessed for any variations with regard to the collection sites and sex. (Details of the experiments and results are described in Chapter III.)

**Specific Aim 3: Determine if the carriage level of *Borrelia* affects the bacterial microbiome composition in *I. scapularis***

To study if the presence of *Borrelia* affects the structure of bacterial communities in *I. scapularis*, we analyzed the bacterial community compositions of both the *Borrelia* positive and negative field-collected ticks from Massachusetts using 16S V4 rRNA amplicon-based metagenomic sequencing. (For details, see Chapter III.)

Collectively, the data from the temperature exposure study on colony-reared *I. scapularis*, support the conclusion that temperature impacts the tick microbiome community structure. These findings may have implications to the differences that we observed in the bacterial community structures among the natural populations of the ticks from Texas and Massachusetts, two geographically and environmentally distinct regions.

## Significance

Environmental factors such as temperature, humidity, and seasons, have been previously shown to influence the incidence of Lyme disease; however, their impacts on the tick vector microbiota are largely unknown. This study showed that composition of the bacterial microbiomes of colony-reared *I. scapularis* ticks changes upon incubation at 30ºC for a week and 37ºC for more than five days at a relatively constant humidity of >80%. Moreover, there are contradicting

reports that the species, sex and geographic origin substantially affect the microbiome pattern in *Ixodes* ticks collected from the US, but not in the *I. scapularis* ticks collected from Ontario, Canada. Little is known about the microbiomes of wild populations of *I. scapularis* ticks from geographically and environmentally distinct regions. The present study also investigated the composition and diversity of bacterial communities among wild populations of *I. scapularis* collected from natural habitats in Texas and Massachusetts, which encompass two distinct climatic regions from the extreme ends of the tick's US range. We identified differences in the bacterial community composition of the ticks based on gender. Male *I. scapularis* ticks were more diverse than that of the females. Additionally, the male ticks from Massachusetts had a different bacterial community composition compared to the males from Texas. Together our studies are unique in that they provided experimental evidence that environmental temperature can impact the composition of bacterial microbiomes of colony-reared *I. scapularis* ticks in a lab setting and explored the bacterial communities in wild populations of *I. scapularis* from two distinct climatic regions at the opposing ends of the vector's range in eastern US. These findings are significant in understanding the effects of environmental temperature on vector microbiome composition and may have possible ramifications in understanding the role of climate change in the vector's capacity to acquire, propagate, and transmit tick-borne pathogens. These studies also set the groundwork for future research on the functional interactions of the tick microbial communities that are under the influence of various environmental factors and possibly identify future research that could lead to better control of Lyme disease and other tick-borne diseases by exploiting the tick-associated microbial communities.

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CHAPTER II

# EFFECTS OF TEMPERATURE ON BACTERIAL MICROBIOME COMPOSITION IN *Ixodes scapularis* TICKS

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Submitted for Publication

## Abstract

*Ixodes scapularis* is the principal vector of Lyme disease in North America. The disease is endemic to the northeastern and upper midwestern regions of the United States, but these areas represent only a subset of the total range of the vector and hosts. Environmental factors are known to influence regional and seasonal incidence of Lyme disease. More recently reports have also shown that competence and transmission of certain tick-borne illnesses may be influenced by the endogenous tick microbial communities. However, the influence of environmental variables on the tick microbial community and its potential effects on vector competence remain largely unexplored. With a goal to understand the impact of environmental temperature on microbial community composition within the tick, we investigated the bacterial microbiome of colony-reared *I. scapularis* ticks incubated at different temperatures (4ºC, 20ºC, 30ºC and 37ºC) at a constant humidity of 80-95% in a controlled lab setting by comparison of sequenced amplicons of the bacterial 16S V4 rRNA gene to that of the baseline controls. The microbiomes of colony-reared *I. scapularis* were distinct between male and female adults. Male ticks were more diverse than the females, which were entirely dominated by *Rickettsia*. *In silico* removal of *Rickettsia* sequence from female data revealed a more complex bacterial community, which is consistent in complexity with those seen among the male ticks. The bacterial community composition of these ticks changes upon incubation at 30ºC for a week and 37ºC for more than five days. Moreover, the male ticks incubated at 37°C exhibited significantly different bacterial diversity compared to the baseline microbiome, and the change in bacterial diversity was dependent upon duration of exposure. *Rickettsia*-free data revealed a significantly different bacterial diversity in female ticks incubated at 37°C compared to that of 4°C and 20°C

treatments. These results provide experimental evidence that environmental temperature can quickly impact the tick bacterial microbiome in a laboratory setting.

**Keywords:** tick microbiome, 16S rRNA, microbial ecology, *Ixodes scapularis*, blacklegged tick, tick-temperature behavior

## Importance

In North America, the blacklegged tick (*Ixodes scapularis*) is the primary vector of Lyme disease. Although environmental factors (such as temperature, humidity) are known to influence the incidence of Lyme disease, their impact on the tick microbial community is largely unknown. Given reports that non-pathogenic bacteria within ticks can influence vector competence for certain tick-borne diseases, our findings of changes in bacterial community compositions in blacklegged ticks upon incubation at higher temperatures provides valuable information that will guide future studies to understand the impacts of environmental factors and climate change on vector competence and the risk and spread of zoonotic diseases.

## Introduction

*Ixodes scapularis* (commonly known as the 'blacklegged' or 'deer' tick) is the primary North American vector of the spirochete bacterium *Borrelia burgdorferi*, the etiological agent of Lyme disease and the most commonly reported vector-borne illness in the United States (1-5). The tick is also a vector for a number of other human pathogens, including *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Babesia microti* (babesiosis), and Powassan virus (3, 6, 7). In addition, these ticks carry a variety of commensal and endosymbiotic bacteria of unknown pathogenicity (8, 9).

*I. scapularis* is widely distributed throughout the eastern United States (3), but the majority of the Lyme disease cases reported in the country are from the northeastern and upper midwestern regions that correspond to areas where *I. scapularis* is the dominant tick species (2, 5, 10-12). Despite a steady increase in distribution of *I. scapularis* ticks in the southeastern U.S. over the last two decades (13) and the presence of competent hosts, there are comparatively few cases of Lyme disease from this geographic area (14, 15). The reasons for the low prevalence of Lyme disease in the southeastern U.S. are not completely understood. Studies have suggested that the occurrence of Lyme disease and its transmission is influenced by multiple factors, including vector density, pathogen carriage levels, life stage, differences in seasonal tick behavior and potential for interaction with humans (14, 16-19), and climatic/environmental factors such as temperature, humidity, and precipitation (20-25). Additionally, the host preference is also variable depending on life stage and geographic region. Adult *I. scapularis* commonly feed on large mammals such as white-tailed deer (*Odocoileus virginianus*). Larval and nymphal *I. scapularis* feed primarily on white-footed mice (*Peromyscus leucopus*) in the northeast, but

feed increasingly on lizards in the southeast (14, 26, 27). The latter may be poor or non-competent hosts for *B. burgdorferi* (14, 28).

Recent studies have increasingly shown important roles of the tick microbiome in vector competence and pathogen transmission dynamics for many tick-borne diseases (29) (reviewed in (8, 9, 30)). Other studies have found that endosymbiotic bacteria within the ticks play crucial roles not only in reproductive fitness (30) and provision of nutrients (31), but can also influence pathogen acquisition, virulence, and transmission (32). One prior study showed that the bacterial microbiomes of *Ixodes* ticks varied by geographical origin, species, sex, and life-stages (33), yet the factors that drive the composition and diversity of the tick microbial community have not been thoroughly investigated. Previous work from our lab has demonstrated that the microbial survivorship and diversity of bacteria within *Amblyomma americanum* ticks (the Lone star tick, a three-host-tick belonging to the same hard tick family as that of *I. scapularis)* are partially dependent on environmental variables (34). However, no published studies have systematically assessed the direct effects of environmental factors such as temperature on the composition and diversity of the microbiomes of *I. scapularis* ticks.

Ticks are ectothermic (35, 36) and spends the majority of their life cycle (98% of the 2-year life cycle in *I. scapularis)* off host (37). Bacteria also exhibit difference in optimal growth rates (35). To better understand the effects of environmental temperature on the microbial communities of ticks, we investigated the bacterial microbiomes of colony-reared *I. scapularis* ticks statically incubated at different temperatures (4°C, 20°C, 30°C and 37°C) at a constant relative humidity (RH) of > 80% in a controlled lab setting, by comparison of sequenced amplicons of the hypervariable region 4 of the bacterial 16S rRNA gene. To provide a more uniform microbiome population for the experiment, we used *I. scapularis* adult ticks reared in a single facility. The

resultant data provide information regarding the plasticity of the tick microbiome and the impact of environmental temperature on the underlying bacterial community compositions, which may provide insight into disease risk and aid in establishing effective interventions to combat tick-borne diseases.

## Materials and Methods

### Tick samples, processing and incubation

Approximately 4-month-old live unfed *I. scapularis* adults (n=90) were purchased from a colony maintained at the Tick Rearing Facility at Oklahoma State University (OSU), Stillwater, OK, USA. These adult ticks had not been fed a blood meal, but nymphs were previously fed on New Zealand white rabbits (*Oryctolagus cuniculus*) in accordance with the OSU Institutional Animal Care and Use Committee (IACUC) Animal Use Protocol No. AG-12-14 (Lisa Coburn, personal communication). The adult ticks in the facility were held at ~22.8ºC at a constant relative humidity of ~90% with a photo period of 15 light/9 dark (Lisa Coburn, personal communication).

The live ticks were sent overnight to our laboratory in plastic containers with moist paper towels to maintain a humid environment. Immediately after receiving the ticks, individuals were sorted by sex (45 males, 45 females), based on the Centers for Disease Control and Prevention (CDC) morphological classification criteria (38). Ten individual ticks (5 males and 5 females) were immediately stored at -20°C in separate sandwich bags (16.5 cm x 8.2 cm) until DNA extraction, and these ticks served as a baseline control for the study. Eighty remaining ticks were placed into eight separate autoclaved glass mason jars (n=10 per jar, either all males or all females)

containing strips of sterile meshed fabric to provide housing material for the ticks and to reduce condensation. The tops of the jars were also covered with fabric to allow the conditions in the jar to equilibrate with that of the environmental chambers in which they were placed while containing the ticks.

Appropriate saturated salt solutions were used to maintain a constant relative humidity of 80-95% at each given temperature as follows: ammonium sulfate [$(NH4)_2SO_4$], 4°C; potassium chloride (KCl), 20°C and 30°C; and potassium sulfate ($K_2SO_4$), 37°C (39-41). The mason jars containing ticks, along with vessels of appropriate saturated salt solutions were then placed in four different treatment conditions: two static incubators set at 37°C and 30°C, respectively, a drawer from the lab refrigerator (4°C) and a plastic container placed at room temperature in the lab (20°C). To maintain a constant relative humidity, jars holding ticks and vials of salt solutions were first placed into three separate Styrofoam boxes before putting them at 4°C, 30°C and 37°C. The plastic container set at room temperature was further sealed with Saran wrap to maintain constant humidity. Temperature and humidity in different environmental chambers were monitored throughout the experiment using four Onset HOBO Temp/RH Data Loggers (Onset Computer Corp., Cape Cod, Massachusetts, USA) with a logging interval of every 30 minutes (see Table S1 for experimental data). Dead ticks, if present, were removed from the chamber every day, and placed at -20°C in separate sandwich bags until DNA extraction. By day 10, only 15% of the total ticks were alive at higher temperatures (30°C and 37°C combined) (see Fig. 1), and thus we terminated the experiment by preserving all the remaining ticks at -20°C until processing.

TABLE S1. Average temperature and relative humidity recorded at different environmental chambers (groups) over 10 days using Onset HOBO Data Loggers. (SEM= Standard error of the mean, RH= relative humidity)

| **Groups** | **No. of *I. scapularis* ticks** | **Mean Temp °C (± SEM)** | **Mean RH % (± SEM)** |
|---|---|---|---|
| 4°C | males (10), females (10) | 4.03 (± 0.15) | 80.66 (± 0.14) |
| 20°C | males (10), females (10) | 20.00 (± 0.13) | 85.07 (± 0.17) |
| 30°C | males (10), females (10) | 29.97 (± 0.00) | 86.40 (± 0.02) |
| 37°C | males (10), females (10) | 36.70 (± 0.01) | 93.36 (± 0.03) |

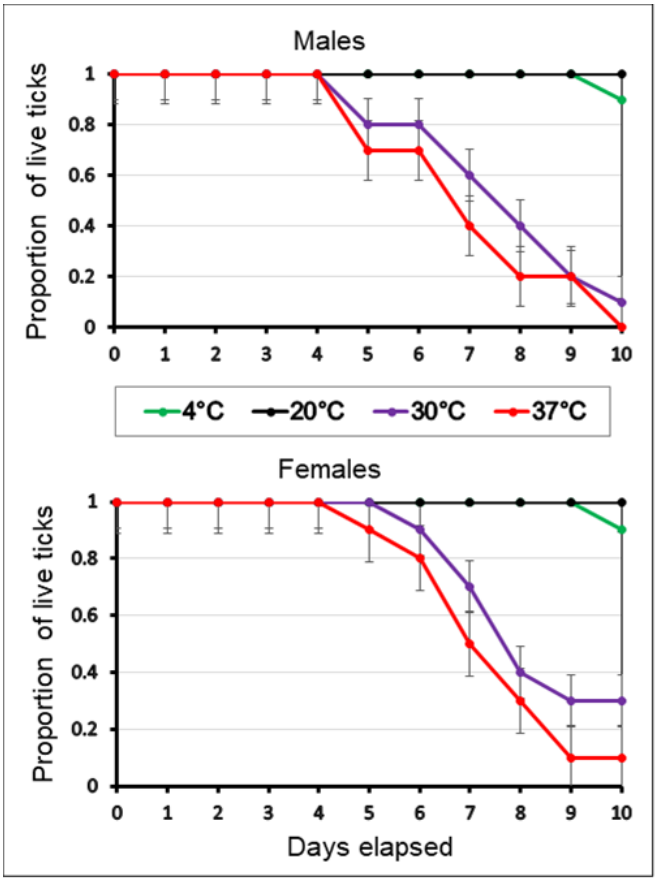


FIG 1. Survival of *I. scapularis* males (upper panel) and females (lower panel) under different incubation temperatures at a constant relative humidity of 80-95%.

**Selection of incubation temperature and humidity**

Temperature selection was determined by comparison of geographical distribution maps of *I. scapularis* (3) and reported cases of Lyme disease in the US (4, 5), the summer and winter temperatures of the areas that possessed the same vectors but with differing rates of Lyme infection (42), published studies (20, 43) and feasibility within the lab.

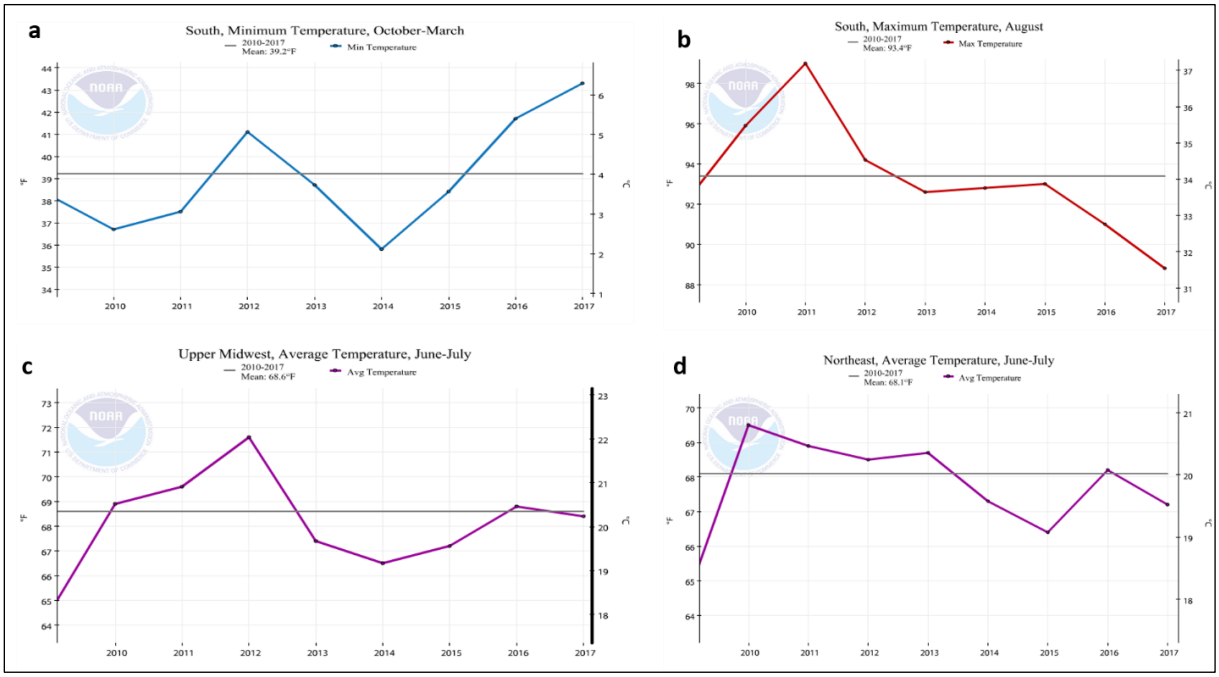


FIG S1. Temperatures recorded in upper midwest, northeast and south US climatic regions during 2010-2017

The 4°C corresponds to the average minimum temperature across the US south climatic region during October to March (peak activity period of adult *I. scapularis* in Texas) (44) for the years 2010-2017 (Fig. S1a), while 37°C is the maximum average temperature of the US south climatic region during August of the same years (Fig. S1b) (42). A temperature of 20°C corresponds to an average daily temperature across the upper midwest (Fig. S1c) and northeast (Fig. S1d) states of

the US during June-July of the years 2010-2017 (42), which in turn corresponds to the peak nymphal activity in the region (5). The humidity of >80% was chosen because high relative humidity is critical for survival and activity of *I. scapularis* ticks, with optimal level ranging from 80% to 95% depending on temperature (45-47).

**DNA extraction from ticks**

Genomic DNA was extracted individually from 90 adult *I. scapularis* ticks using the Mag-Bind® Plant DNA Plus Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's protocol with minor modifications as described below. Prior to extraction, ticks were surface sterilized by dipping in 10% (v/v) sodium hypochlorite solution for 30 seconds, followed by rinsing with molecular biology grade water for 1 min. Sterilization techniques, such as using sodium hypochlorite solution, have previously been demonstrated to significantly eliminate the bacteria and DNA in tick surface (34). Then, each tick was thoroughly air dried on a microscopic slide, cut into at least 8 different sections with a sterile scalpel, and the entire tick was placed in a 2 ml screw-capped FastPrep tube (MP Biomedicals, LLC., Santa Ana, CA) containing 550 µl CSPL® buffer (Omega Bio-Tek, Norcross, GA) and about 8-10 sterile 2.8 mm ceramic beads (Mo Bio Laboratories Inc., Carlsbad, CA). The entire tick was pulverized at a speed of 7 m/s for 60s (3 cycles) using the FastPrep-24™ 5G Instrument (MP Biomedicals, LLC., Santa Ana, CA) and subsequently incubated at 56ºC for 2 hours. Resultant genomic DNA was quantified using a high sensitivity dsDNA assay on Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA) and stored at -20ºC until future use. A 'blank extraction', containing only the extraction reagents and beads, was also prepared for every set of DNA extractions.

**Tick mitochondrial 16S rRNA gene amplification**

As a sample positive control, each DNA extract was PCR amplified with 16S-1 and 16S+2 primers specific for tick mitochondrial 16S rRNA gene (48), using 5-µl of template DNA in a 25-µl reaction mixture containing 5-µl 10X ThermoPol® reaction buffer (New England Biolabs, Inc., Ipswich, MA), 2.5-µl 10X bovine serum albumin, 2-µl of 2.5 mM DNTPs , 0.5-µl forward primer, 0.5-µl reverse primer, 0.25-µl *Taq* DNA polymerase and 11.75-µl molecular biology grade water. Amplification was performed in a BioRad C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) as follows: 10 min of initial denaturation at 94ºC followed by 9 cycles consisting of denaturation at 92ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 1.30 min, then followed by 31 cycles consisting of denaturation at 92ºC for 1 min, annealing at 56ºC for 1 min, and extension at 72ºC for 1.30 min, with a final extension at 72ºC for 10 minutes and indefinite hold at 4°C. The PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide, and subsequently visualized under UV light.

**16S rRNA gene library preparation and sequencing**

All samples were PCR amplified in duplicates using 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers with Illumina sequencing adaptor, that target the hypervariable region four (V4) of the bacterial 16S rRNA gene, as described in the Earth Microbiome Project (EMP) 16S Illumina Amplification Protocol (49) with minor modifications as mentioned below. Briefly, a master mix solution was prepared per 25 µl PCR reaction volume with 2.5 µl 10X Accuprime™ PCR Buffer II (Invitrogen, Carlsbad, CA), 2.5 µl 10X Bovine Serum Albumin (New England Biolabs, Inc., Ipswich, MA), 1 µl 50 mM $MgSO_4$, 0.5 µl 10µM forward primer , 0.5 µl 10µM reverse primer, 0.1 µl of 5U/µl Accuprime™ *Taq*

DNA Polymerase High Fidelity, 10 µl of template DNA and 7.9 µl molecular biology grade water. Amplification was carried out in a BioRad C1000 Touch™ thermal cycler with the following cycling parameters: an initial denaturation at 94ºC for 2 min followed by 30 cycles (35 cycles for all male samples) consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 40 s, and extension at 68ºC for 40 s, with a final extension at 68ºC for 5 minutes and a 4°C indefinite hold. Amplicon quality was visualized under UV light after separation via gel electrophoresis (1.5% agarose).

PCR products were purified using AMPure XP magnetic beads and separate 16S libraries were prepared for each amplicon sample following the Illumina 16S metagenomic sequencing library preparation guide. Each sample were labeled with specific indices through index PCR using Accuprime High-fidelity DNA polymerase. The master mix solution for index PCR (per 50 µl reaction) included 5-µl 10X Accuprime™ PCR Buffer II, 5-µl Nextera XT Index Primer 1, 5-µl Nextera XT Index Primer 2, 0.2-µl Accuprime™ *Taq* DNA Polymerase High Fidelity (5U/µl), 5-µl 16S V4 PCR amplicon product and 29.8-µl molecular grade water. The PCR parameters were: an initial denaturation step at 94ºC for 3 min followed by 8 cycles consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 68ºC for 30 s, with a final extension at 68ºC for 5 min and an indefinite hold at 4ºC. Purified 16S libraries were quantified using a high sensitivity dsDNA assay on Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA) and stored at -20ºC until further processing. The libraries were pooled in equimolar amounts. The final concentration of the library loaded in the MiSeq Reagent Kit v2 (Illumina Inc, San Diego, CA) was 10 pM. The MiSeq run also included a 5% PhiX DNA as an internal control for potentially

low diversity libraries. Paired-end (2×250) high-throughput sequencing (500 cycles) was performed according to the manufacturer's recommendations an Illumina MiSeq® instrument.

**Sequence processing and analysis**

Raw sequence data were processed with the mothur v1.36.1 (50), as previously described (51). Quality filtered contigs were aligned to the SILVA database (52), and screened for chimeras using UCHIME algorithm (53). Sequences with 97% similarity were then grouped into operational taxonomic units (OTUs) (54) and assigned to taxonomic groups by comparison to the Greengenes reference database (version 13.8.99) (55, 56). Relative abundances of bacterial taxa in each sample were calculated and compared among groups (see Fig. S2). Taxa with a relative abundance of ≥ 1% in at least one sample were analyzed individually, while those with <1% relative abundance in all samples were grouped together into 'Others' category. 'Unclassified (phylum level)' were the OTUs that did not match to any of the sequences in the Greengenes database.

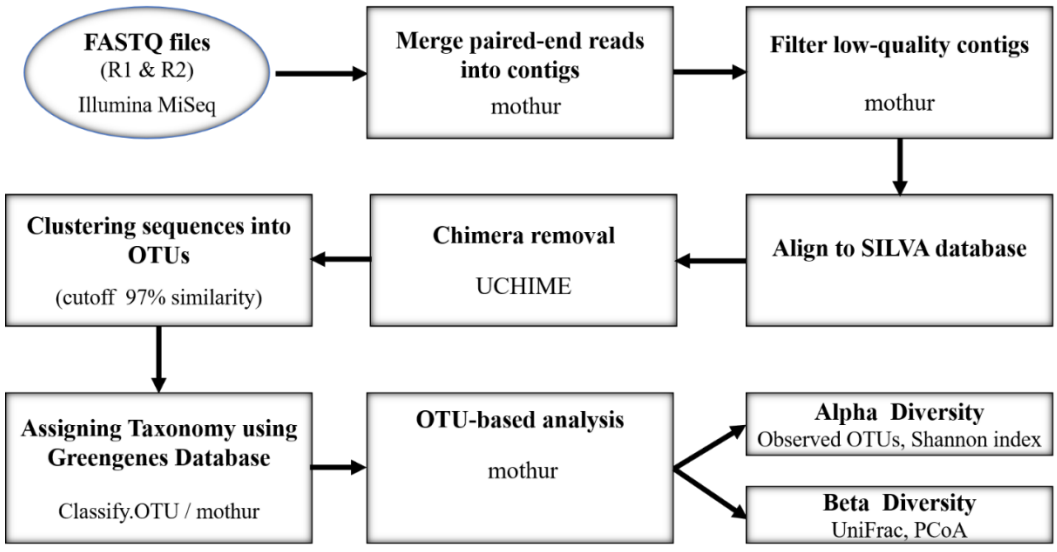


FIG S2. Bioinformatics workflow for processing of 16S rRNA gene sequences using mothur

Bacterial diversity was estimated from the normalized data: each sample was rarefied according according to the size of our smallest library to reduce the bias of uneven sequencing depth Various metrics, including Observed OTUs and Shannon index, were used to estimate alpha-diversity of all the samples, while beta-diversity between samples was assessed using principal coordinates analysis (PCoA) plots of the weighted and unweighted UniFrac distances.

**Statistical analysis**

The Microsoft Excel 2016 for windows (Microsoft Corporation, Redmond, WA, USA), XLSTAT-Ecology (Addinsoft SARL, New York, USA), and R software (version 3.4.1) was used for statistical analyses. As our data did not meet normality assumptions (using Shapiro-Wilk test), non-parametric analysis was performed for this study. Kruskal Wallis group significance testing was employed to analyze the bacterial community composition between various groups. Additionally, comparisons between bacterial community compositions were performed using a permutational multivariate analysis of variance (PERMANOVA) in the vegan package of R (57, 58). Comparisons of bacterial diversity (Shannon index) among more than two groups utilized a Kruskal-Wallis test, while analysis of molecular variance (AMOVA) (51, 59) test was used for analyzing the UniFrac distance matrices of the female ticks after *in-silico* removal of *Rickettsia* sequences. Shannon diversity between two groups was compared using a Mann-Whitney test. Where multiple comparison testing was performed, a Bonferroni correction was applied (60), and a corrected $p < 0.05/n$ (where n= number of comparisons) was considered significant for all pairwise comparisons.

## Results

### 16S V4 sequencing results

A total of 89 *I. scapularis* tick samples (except for the 4°C male group where one sample failed to amplify) were sequenced for bacterial 16S V4 rRNA gene using an Illumina MiSeq. A total of 9,431,153 standard-quality sequences were obtained. On average, we obtained 105,968 quality-sequences (± 41,579 standard deviation) per sample. The blank extraction control resulted in about 0.0005% of all quality-filtered sequences and was excluded from further analysis. Rarefaction curves at a depth from1,000 to 30,000 sequences suggested sufficient sequencing coverage as shown by the representative curves of observed OTUs reaching a plateau (Fig. S3) (except 4°C male tick1, and 37°C male- tick1, tick2, tick3; but all of these 4 samples had coverage rates of greater than 98% as shown in Table S2).

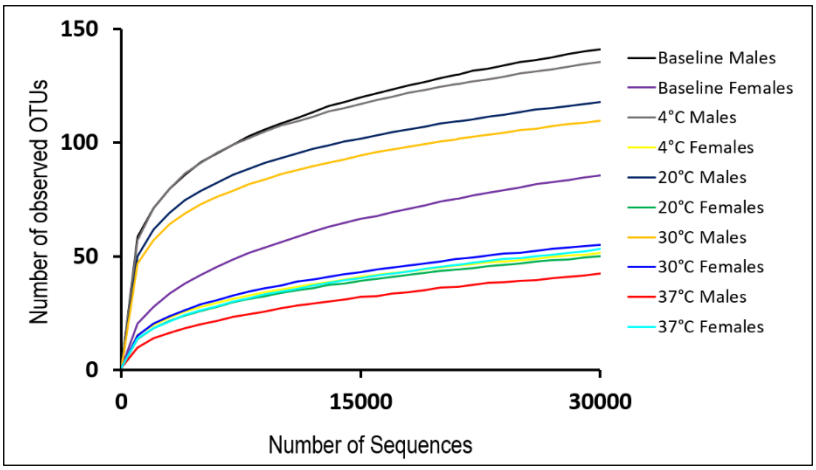


FIG S3. Rarefaction curves of observed OTUs versus increasing number of sequence**.** The curves represent the average of 30 iterations sampled every 1,000 sequences starting from 1,000 to 30,000 sequences.

TABLE S2**.** Coverage depth and other measures of alpha diversity per gender at baseline and various temperatures. (ACE: abundance-based coverage estimator; BL: baseline ticks; 4C, 20C, 30C and 37C denotes the ticks incubated at 4ºC, 20ºC, 30ºC and 37ºC temperatures respectively; m: male; f: female)

| **Sample** | **Coverage (%)** | **ACE value** | **Inverse Simpson index** |
|---|---|---|---|
| *I. scapularis* males | | | |
| BLm tick1 | 99.05 | 161.74 | 11.83 |
| BLm tick2 | 99.34 | 110.76 | 14.96 |
| BLm tick3 | 99.37 | 108.41 | 11.18 |
| BLm tick4 | 99.28 | 108.94 | 7.25 |
| BLm tick5 | 98.96 | 179.29 | 4.53 |
| 4Cm tick1 | 98.88 | 167.19 | 12.10 |
| 4Cm tick2 | 99.12 | 136.10 | 8.54 |
| 4Cm tick3 | 99.16 | 126.22 | 12.13 |
| 4Cm tick4 | 98.85 | 160.35 | 13.00 |
| 4Cm tick5 | 99.13 | 130.61 | 7.36 |
| 4Cm tick6 | 99.28 | 134.78 | 2.52 |
| 4Cm tick7 | 99.31 | 112.04 | 6.22 |
| 4Cm tick8 | 99.15 | 123.94 | 6.10 |
| 4Cm tick9 | 99.39 | 104.11 | 3.45 |
| 20Cm tick1 | 99.16 | 124.06 | 5.96 |
| 20Cm tick2 | 99.37 | 112.54 | 3.14 |
| 20Cm tick3 | 99.12 | 137.49 | 5.41 |
| 20Cm tick4 | 99.37 | 100.84 | 6.83 |
| 20Cm tick5 | 99.44 | 87.40 | 4.36 |
| 20Cm tick6 | 99.23 | 111.54 | 5.13 |
| 20Cm tick7 | 99.21 | 117.62 | 4.63 |
| 20Cm tick8 | 99.29 | 114.04 | 4.60 |
| 20Cm tick9 | 99.18 | 125.35 | 5.52 |
| 20Cm tick10 | 99.28 | 108.42 | 6.90 |
| 30Cm tick1 | 99.20 | 120.70 | 4.93 |
| 30Cm tick2 | 99.24 | 116.03 | 4.80 |
| 30Cm tick3 | 99.33 | 102.66 | 6.17 |
| 30Cm tick4 | 99.37 | 97.58 | 4.15 |
| 30Cm tick5 | 99.31 | 107.27 | 5.27 |
| 30Cm tick6 | 99.32 | 111.55 | 8.78 |
| 30Cm tick7 | 99.19 | 144.48 | 4.37 |
| 30Cm tick8 | 99.32 | 101.00 | 2.38 |
| 30Cm tick9 | 99.29 | 113.65 | 3.37 |
| 30Cm tick10 | 99.79 | 94.79 | 1.03 |

| Sample | Coverage (%) | ACE value | Inverse Simpson index |
|---|---|---|---|
| 37Cm tick1 | 99.29 | 99.48 | 4.37 |
| 37Cm tick2 | 98.86 | 199.13 | 4.63 |
| 37Cm tick3 | 99.14 | 127.75 | 4.34 |
| 37Cm tick4 | 99.63 | 89.99 | 1.08 |
| 37Cm tick5 | 99.65 | 101.63 | 1.08 |
| 37Cm tick6 | 99.90 | 49.05 | 1.01 |
| 37Cm tick7 | 99.84 | 68.79 | 1.57 |
| 37Cm tick8 | 99.76 | 108.37 | 1.59 |
| 37Cm tick9 | 99.85 | 57.66 | 1.02 |
| 37Cm tick10 | 99.86 | 45.18 | 1.02 |
| | | | |
| *I. scapularis* females | | | |
| BLf tick1 | 99.73 | 71.12 | 1.03 |
| BLf tick2 | 99.76 | 64.77 | 1.04 |
| BLf tick3 | 98.78 | 262.57 | 1.09 |
| BLf tick4 | 99.80 | 61.03 | 1.02 |
| BLf tick5 | 99.82 | 69.76 | 1.02 |
| 4Cf tick1 | 99.76 | 44.29 | 1.04 |
| 4Cf tick2 | 99.75 | 46.08 | 1.05 |
| 4Cf tick3 | 99.78 | 55.19 | 1.03 |
| 4Cf tick4 | 99.80 | 70.48 | 1.02 |
| 4Cf tick5 | 99.79 | 86.38 | 1.02 |
| 4Cf tick6 | 99.72 | 88.45 | 1.05 |
| 4Cf tick7 | 99.76 | 76.32 | 1.02 |
| 4Cf tick8 | 99.77 | 70.66 | 1.03 |
| 4Cf tick9 | 99.71 | 64.70 | 1.04 |
| 4Cf tick10 | 99.67 | 68.38 | 1.21 |
| 20Cf tick1 | 99.80 | 81.61 | 1.02 |
| 20Cf tick2 | 1.00 | 74.64 | 1.02 |
| 20Cf tick3 | 1.00 | 57.44 | 1.08 |
| 20Cf tick4 | 1.00 | 69.46 | 1.03 |
| 20Cf tick5 | 0.99 | 104.54 | 1.36 |
| 20Cf tick6 | 1.00 | 73.63 | 1.02 |
| 20Cf tick7 | 1.00 | 57.97 | 1.01 |
| 20Cf tick8 | 1.00 | 62.46 | 1.07 |
| 20Cf tick9 | 1.00 | 68.12 | 1.01 |
| 20Cf tick10 | 1.00 | 88.11 | 1.02 |
| 30Cf tick1 | 99.70 | 78.20 | 1.04 |
| 30Cf tick2 | 99.92 | 11.46 | 1.01 |
| 30Cf tick3 | 99.87 | 55.49 | 1.02 |
| 30Cf tick4 | 99.69 | 75.31 | 1.08 |
| 30Cf tick5 | 99.64 | 95.79 | 1.08 |

| Sample | Coverage (%) | ACE value | Inverse Simpson index |
|---|---|---|---|
| 30Cf tick6 | 99.65 | 88.23 | 1.07 |
| 30Cf tick7 | 99.75 | 83.68 | 1.09 |
| 30Cf tick8 | 99.69 | 84.36 | 1.14 |
| 30Cf tick9 | 99.69 | 72.21 | 1.23 |
| 30Cf tick10 | 99.74 | 64.03 | 1.36 |
| 37Cf tick1 | 99.83 | 52.05 | 1.02 |
| 37Cf tick2 | 99.80 | 92.15 | 1.04 |
| 37Cf tick3 | 99.68 | 85.20 | 1.06 |
| 37Cf tick4 | 99.73 | 94.22 | 1.04 |
| 37Cf tick5 | 99.68 | 72.95 | 1.05 |
| 37Cf tick6 | 99.64 | 69.71 | 1.28 |
| 37Cf tick7 | 99.79 | 77.33 | 1.39 |
| 37Cf tick8 | 99.71 | 75.06 | 1.72 |
| 37Cf tick9 | 99.78 | 129.25 | 1.62 |
| 37Cf tick10 | 99.88 | 22.16 | 1.25 |

**Baseline bacterial microbiome of *I. scapularis* ticks**

The microbiome results showed that the bacterial community composition differed by gender. Male ticks had a more complex bacterial microbiome than females. At the phylum level, *Proteobacteria* comprised the highest relative abundance across all baseline male ticks (range=54.7-83.4%, mean=67.4%), followed by *Actinobacteria* (4.3-41.4%,16.6%), *Bacteroidetes* (1.6-13.2%, 9.3%) and *Firmicutes* (1.7-17.8%, 6.7%). By comparison, baseline females had the OTUs assigned almost entirely to a single phylum *Proteobacteria* (range=95.9-99.6%, mean=98.6%) (Fig. S4).

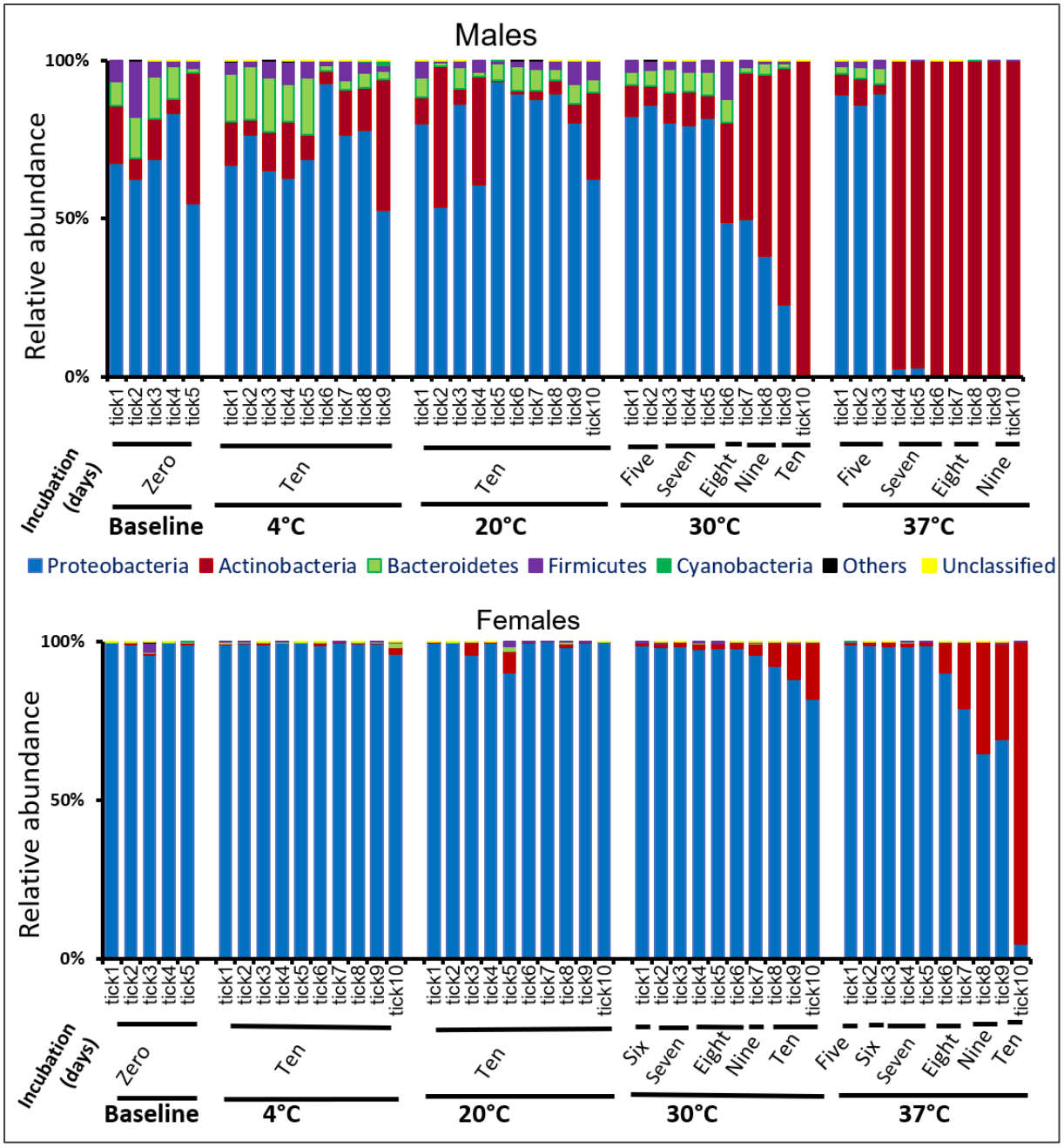


FIG S4. Phylum level bacterial microbiome of individual *I. scapularis* male (upper panel) and () female (lower panel) ticks. Microbiome of both the baseline and temperature treated ticks are shown in the stacked bar charts where each bar represents an individual tick.

Consistent with the pattern of phylum-level results, 21 genera (plus one order-level taxon, and two family-level taxa) were found with a relative abundance of ≥ 1% in at least one of the baseline male samples, while the females had only 2 genera with 1% or greater abundance (Fig. 2). The top ten genera (mean relative abundances) in baseline male tick samples was comprised of *Pseudomonas* (21.2%), *Brevibacterium* (11.2%), *Bradyrhizobium* (9.5%), *Sediminibacterium* (8.6%), *Phenylobacterium* (8.2%), *Ralstonia* (6.6%), *Sphingomonas* (4.8%), *Acinetobacter* (4.6%), *Rhodoplanes* (4.1%) and *Staphylococcus* (2.3%). A small proportion of the male microbiome constituted genera such as *Corynebacterium*, *Brachybacterium*, *Rickettsia*, and others. In contrast, the microbiome of baseline female ticks was dominated entirely by the genus *Rickettsia* (range=94.6-98.5%, mean=97.2%), which had a significantly (Kruskal-Wallis test $p$ =0.009) very low abundance (0.1-3.7%, 0.9%) among the males.

Because the microbiomes of female ticks were completely dominated by amplicons likely derived from the rickettsial endosymbiont known to occur in this tick species, and because this endosymbiont resides primarily in the ovaries (61), we performed *in silico* removal of *Rickettsia* sequence from the female data to assess the underlying inherent gut bacterial microbiome of the female ticks. Removal of *Rickettsia* from baseline female data sets resulted in an average of 4,155 reads per sample (range=1,809-6,600). Interestingly, *Rickettsia*-free baseline female data revealed a total of 34 taxa (including 25 genera) with 1% or greater abundance in at least one of the samples. The bacterial community composition in *Rickettsia*-free baseline females (Fig. 2 right panel) were consistent with those observed among full profiles (i.e. *Rickettsia* included) of baseline male ticks (Fig. 2 left panel).

For comparison, we also deleted *Rickettsia* sequences from baseline male samples (see Appendix); however, removal of *Rickettsia* from the male data sets did not affect the overall community composition in male ticks.

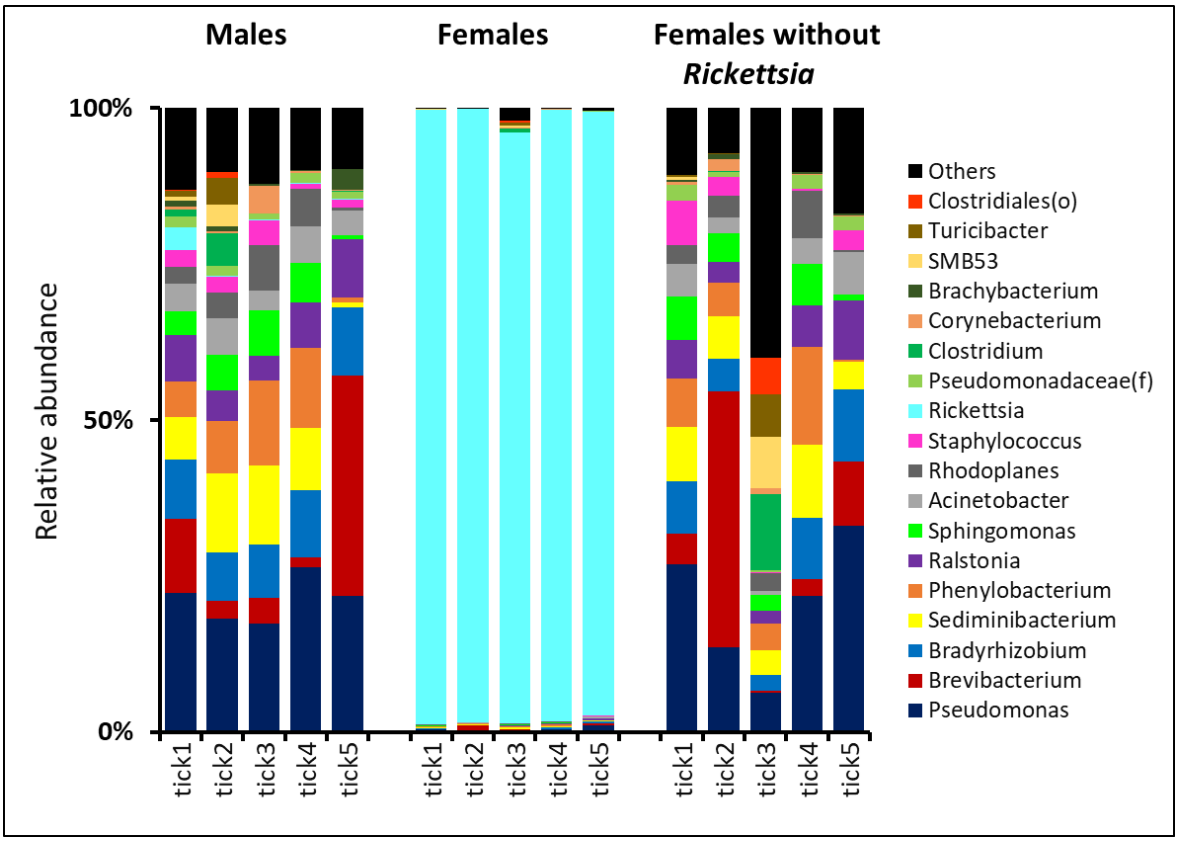


FIG 2. Bacterial microbiome in baseline populations of the colony-reared *I. scapularis* adult ticks. Top fifteen taxa with ≥ 1% relative abundance are shown. Each bar represents an individual whole tick sample, where different color indicates the relative proportions of various bacteria characterized to the genus level (wherever possible) based on 97% OTU similarity threshold of bacterial 16S V4 rRNA gene using Greengenes reference database. Baseline ticks were not exposed to any temperatures. Left panel: male ticks, middle panel: female ticks and right panel: female ticks after removal of *Rickettsia*.

**Bacterial microbiome of *I. scapularis* ticks incubated at different temperatures**

Live *I. scapularis* adult ticks were incubated at different temperatures (4ºC, 20ºC, 30ºC and 37ºC) at a constant humidity of 80-95% for ten days, with daily monitoring of their survival (see Fig. 1 for survival curves), and their bacterial microbiome was characterized.

The phylum *Proteobacteria* had the highest abundance in all male ticks incubated at 4ºC for 10 days (mean=71.1%), 20ºC for 10 days (78.3%), 30ºC for a week (81.9%), and 37ºC for 5 days (88.2%) (Fig. S4), which was preceded by the phyla *Actinobacteria, Bacteroidetes,* and *Firmicutes.* On the other hand, *Actinobacteria* was highly abundant among male ticks held at 30ºC for 8-10 days (mean=61.9%), and 37ºC for 7-9 days (98.9%). In contrast, *Proteobacteria* was the dominant phylum across all temperatures in the female samples, except a few individuals incubated at 30ºC for 10 days, and 37ºC for 9-10 days (where ~ 50% of the total bacterial phyla was represented by *Actinobacteria*) (Fig. S4).

In the male ticks, *Pseudomonas, Phenylobacterium, Sediminibacterium, Bradyrhizobium, Brevibacterium, Ralstonia, Rhodoplanes, Sphingomonas, Acinetobacter,* and *Staphylococcus* constituted the top-ten genera at 4°C, accounting for more than three-fourths of the bacterial community (Fig. 3a). In addition, many of these bacteria were also the major genera in the males held at 20°C, where forty percent of the tick population had high abundance of *Rickettsia* (mean=32.6%). Furthermore, *Pseudomonas* had the highest relative abundance in males held at 30°C for 5-7 days (mean=37.9%), whereas *Brevibacterium* (48.8%) and *Streptomyces* (72.3%) were the dominant genera in the day 9 and day 10 samples. Even at 37°C, *Pseudomonas* represented the most highly abundant genus (45.2%) in day 5 males and was found in all other

male samples held at 37°C, but with a low relative abundance (0.5%). *Brevibacterium* (97.9%) and *Streptomyces* (99.3%) were the two genera that entirely dominated the day 7 and 9 male samples, respectively (Fig. 3a).

In female ticks with all sequences, *Rickettsia* was the only dominant genus (64.5-99.5%) across all temperature treatments regardless of the duration of incubation, except one sample incubated at 37°C for 10 days which contained *Brevibacterium* as the highest proportion (89.2%) (Fig. 3b). Female ticks incubated at 30°C for 10 days had an average relative abundance of *Brevibacterium* of 11.4% while those kept at 37°C for 8 and 9 days had 13.6% and 29.5% *Brevibacterium*, respectively (Fig. 3b). Analyses of the underlying bacterial microbiome composition remaining after *in silico* removal of *Rickettsia* sequences from the female data set revealed a diverse bacterial community in the female ticks with the dominance of *Brevibacterium* in both 30°C and 37°C treatment groups (Fig. 3c). These results were consistent with those seen among male samples.

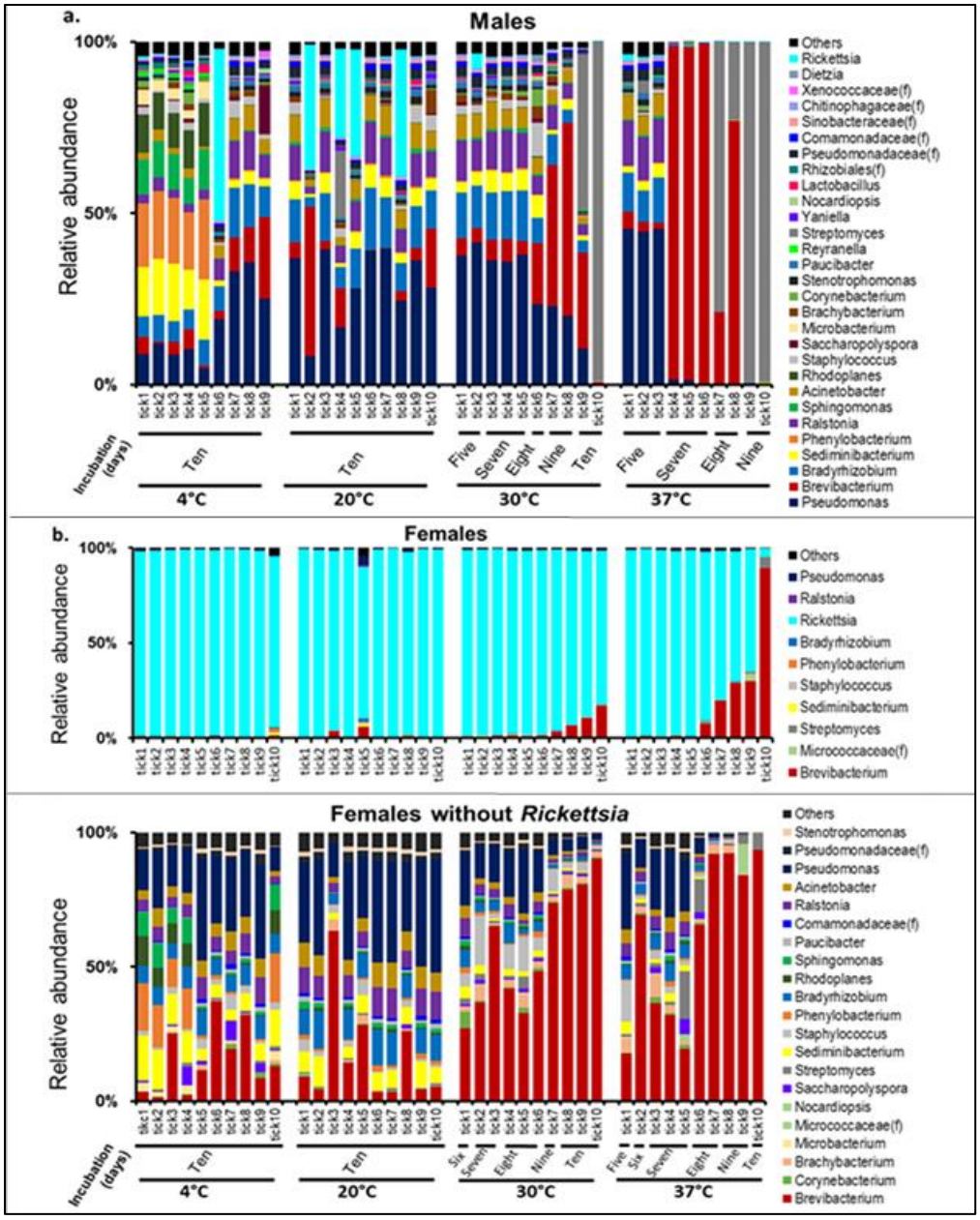


FIG 3. Bacterial microbiome of colony-reared *I. scapularis* adult (**a**) male, and (**b**) female ticks incubated at 4ºC, 20ºC, 30ºC and 37ºC, for up to 10 days at a constant humidity of 80-95%. Genus-level (wherever possible) taxonomic composition of bacteria in ticks showing their relative abundances. (**c**) Bacterial abundance in female ticks after *in-silico* removal of *Rickettsia*. Each stacked bar represents an individual tick whose DNA was extracted individually from the whole tick, and the numbers below each bar denote the tick ID number in that sample population.

**Comparison of the bacterial microbiome in *I. scapularis* ticks incubated at different temperatures with that of baseline ticks**

There was no significant change in the bacterial composition of *I. scapularis* ticks incubated at 4°C and 20°C (at constant humidity of >80%) for ten days, compared to that of the baseline samples. However, the microbiome composition of the baseline *I. scapularis* ticks was different in comparison to those incubated at 30°C for more than a week or at 37°C for more than 5 days. The differences were more prominent in male ticks compared to the full female profiles (i.e. *Rickettsia* included). The phylum *Proteobacteria*, which represented the highest relative abundances across all baseline samples, showed a significantly decreased (Kruskal-Wallis test $p$ =0.004) mean abundance (from 67.4% to 1%) in male ticks incubated for 7-9 days at 37ºC. Conversely, males held at 37ºC for 7-9 days showed a significant increase ($p$ =0.004) in relative abundance of the phylum *Actinobacteria* (from a mean relative abundance of 16.6% to 98.9%) (Fig. S4).

In male ticks held at 37°C, the relative abundance of *Pseudomonas* was significantly decreased (Kruskal-Wallis test $p$ =0.017) from day 5 (mean=42.5%) to day 9 (average for 7-9 days=0.5%). Significant difference ($p$ =0.004) was also observed when abundances of *Pseudomonas* in baseline males (21.2%) were compared to the males held at 37°C for more than 5 days (0.5%). Relative percent of *Brevibacterium* dramatically increased (from the baseline average of 11.2% to 97.9%, Kruskal-Wallis test $p$ =0.025) in male ticks incubated at 37ºC for 7 days, but show a decreased abundance in day 8 samples (mean=48.9%), and almost 0.3% abundance by day 9 (Fig. 2 and Fig. 3a). Additionally, *Streptomyces*, with a very low abundance at baseline (0.5%) was highly represented in day 8 samples (mean=50.5%) and dominated the bacterial community

in ticks held at 37°C for 9 days. The genus *Streptomyces* was significantly differently abundant between the unexposed (baseline) male ticks and 37°C treated males for 8-9 days ($p$ =0.014), but not between the baseline males and those held at 37°C for 5 days ($p$ =0.297).

The mean abundance of *Rickettsia,* the only dominant genus across all females, slightly decreased from 97.2% (baseline) to 92.1% in 8-10 days at 30°C. Additionally, there was a relatively low percentage of *Rickettsia* (75%) in females incubated at 37°C for 8-9 days, which corresponds to an increase in abundance of *Brevibacterium* (from a baseline value of 11.2% to 21.6%). Based on PERMANOVA analysis, the bacterial community of males incubated at 37°C showed a significant difference compared to baseline samples (Adonis statistic, F= 6.0044, $p$ = 0.001).

In *Rickettsia*-free female data, the dominance of *Brevibacterium* was prominent in both 30°C (mean=57.6%) and 37°C (60.3%) treatment groups, compared to the baseline females (11.9%). Multiple pairwise comparisons (Bonferroni corrected significance level= 0.005) demonstrated that the abundance of *Brevibacterium* in *Rickettsia*-deleted baseline female samples was significantly different than those incubated at 37°C for more than 7 days ($p$ =0.0001).

**Effect of temperature on the bacterial diversity of ticks**

For diversity analyses, the data were normalized by rarefying to a sequencing depth of 2,399 reads. Our analyses showed that male ticks exhibited the higher bacterial richness (number of observed OTUs) across all conditions compared to the full profiles of the females (i.e. *Rickettsia* included) (Fig. 4a). Similar results were obtained with other measures of alpha diversity (see Table S3).

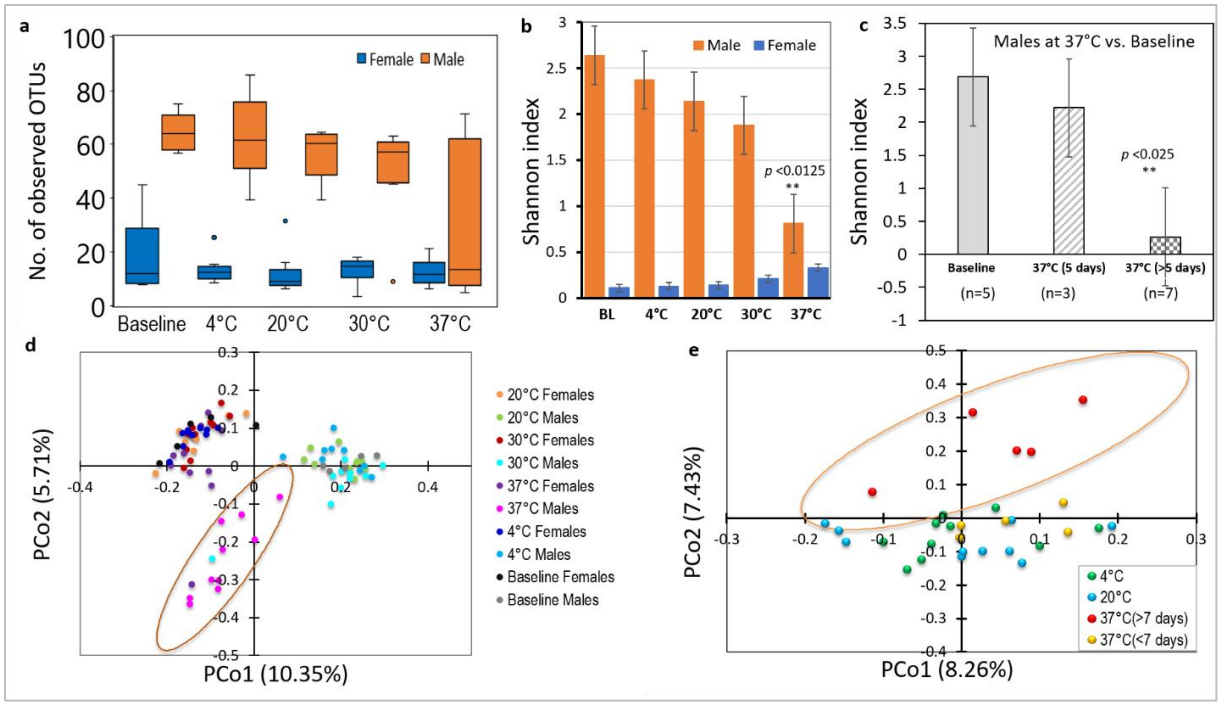


FIG 4. Effect of temperatures on diversity of *I. scapularis* microbiomes as measured by (**a**) observed number of OTUs, (**b**) and (**c**) Shannon index, and PCoA plots of unweighted UniFrac distances of all the samples (**d**) and females without *Rickettsia* sequences (**e**). The samples inside the ellipses are clustered separately from the remaining groups (95% confidence intervals). Each dot in the PCoA plot represents the bacterial microbiome of an individual tick.

However, the diversity (Shannon index) was generally decreased in males across all temperature groups, compared to the baseline (Fig. 4b). Furthermore, males incubated at 37°C exhibited significantly different (Mann Whitney test $p < 0.0125$) Shannon index values compared to the baseline. Interestingly, the Shannon diversity in males decreased with increased length of incubation at 37°C, evidenced by a significantly different (Mann Whitney test $p < 0.025$) Shannon index in male ticks incubated for more than 5 days at 37°C as compared to the baseline (Fig. 4c).

The PCoA of unweighted UniFrac distances of bacterial communities showed that the first two axes (PCo1 and PCo2) explained 10.35% and 5.70% of the variation in the data, respectively, with the 37°C male samples clustering separately from others indicating that male ticks incubated at 37C° had a distinct bacterial composition compared to other ticks (Fig. 4d). Furthermore, the PCoA of the unweighted UniFrac distances of the female bacterial communities after *in-silico* removal of *Rickettsia* revealed a significantly different bacterial diversity at 37°C compared to that of the 20°C treatment group (AMOVA test $p < 0.005$) which clustered separately in the plot (Fig. 4e). Similarly, the weighted UniFrac distances of the female ticks incubated at 37°C had a significantly different bacterial diversity compared with that of the 4°C (AMOVA test $p = 0.00010$) and 20°C ($p=0.0001$) treatment groups.

## Discussion

Ticks are important vectors of many human and animal pathogens. Additionally, they carry a higher proportion of endosymbionts and commensals, which may provide nutrient supplements to the tick and also affect vector competence (reviewed in (8, 9, 29)). Studies have reported that the tick microbial community is variable depending on several factors such as source of host blood meal (62), feeding status (34, 63, 64), tick species, life stage, gender, and geographical origin (33, 65, 66). However, very few studies have focused on the tick microbiome differences based on environmental conditions such as seasons of collection (67) or the immediate environment (34).

In this study, we investigated the effect of environmental temperature on the bacterial microbiome of *I. scapularis* ticks, the primary Lyme disease vector in North America. To the best of our knowledge, a systematic study to evaluate temperature effects on tick microbiomes has not yet been reported. Thus, the findings of the present study help enhance our understanding of the impacts of changes in environmental temperature in the shaping of microbial community structure in ectothermic tick vectors. This, in turn, is vital in predicting the potential effect of warming climate on pathogen acquisition and vector competence in ticks, thereby affecting the transmission dynamics of the diseases they carry.

Despite the same age (approx. 4 months), rearing environment, and blood meals (in early life stages), the adult male and female *I. scapularis* ticks exhibited a distinct bacterial community structure. Male ticks displayed more heterogeneous bacterial communities than females, which contained almost exclusively *Rickettsia* (Fig. 2). The high relative abundance of *Rickettsia* in the female ticks appears to be a sex difference (61). Other than *Rickettsia*, *Pseudomonas* was the only genus found with a relative abundance of ≥1% in females, even though 37 genus-level taxa (plus more higher level taxonomic groups) were present. In contrast, baseline male ticks had a very low abundance of *Rickettsia* (mean =0.9%). Besides *Rickettsia*, 21 genera (plus one order-level taxon, and two family-level taxa), were found with ≥ 1% relative abundance in at least one of the baseline male samples. Relatives of many of these bacteria identified in male ticks (*Pseudomonas*, *Brevibacterium*, *Bradyrhizobium*, *Sediminibacterium*, *Phenylobacterium Ralstonia*, *Sphingomonas*, *Acinetobacter*, *Rhodoplanes*) are associated with soil, water and plants, suggesting that these bacteria may be acquired from their environments and maintained through molting.

Although microbial community diversity is different between lab-reared and wild-caught ticks (68), Zolnik et al. (30) also found a high relative abundance of *Rickettsia* in field-collected whole adult female blacklegged ticks (mean=97.9%), but comparatively low abundance in the males. Furthermore, *Rickettsia* was abundantly found in a lab-reared larval population of *I. scapularis* (30, 69). Another study on *I. scapularis* also reported a low abundance of *Rickettsia* in males, possibly due to loss of the *Rickettsia* endosymbionts while transitioning from through life stages in males (70). These reports were corroborated by the presence of *Rickettsia* in ovaries of female ticks, and its absence in the testes of adult males (70). Thus, our findings of *Rickettsia* in both male and female blacklegged ticks, with high levels in the females, is in concordance with previous studies (30, 33).

*Rickettsia*, which contains a number of tick-borne pathogenic species, including *Rickettsia rickettsii, R. japonica, R. akari* (71), and *R. parkeri* (72), also contains many non-pathogens. The rickettsial endosymbiont of *I. scapularis, R. buchneri* (61), has been reported to provide a source of vitamins to the tick (73). Additionally, the *Rickettsia* endosymbiont of *I. pacificus*, closely related to *I. scapularis,* has been shown to contribute to the synthesis of folic acid (73). Because the microbiomes of female ticks were entirely dominated by the *Rickettsia*, the likely endosymbiont known to occur in *I. scapularis*, and because this endosymbiont resides primarily in the ovaries, we removed *Rickettsia* sequences from the female data sets to further explore the underlying gut microbiome of the female ticks. Removal of *Rickettsia* from female data set revealed a total of 34 taxa (including 25 genera) with 1% or greater abundance in at least one of the samples, consistent in complexity with those seen among the males, suggesting that

*Rickettsia* is potentially masking the underlying diversity in female ticks. Our findings of a more complex bacterial community composition in female *I. scapularis* after *Rickettsia* are removed, indicates that this approach may be useful to understand the underlying gut microbiome in the whole female tick samples. In our experimental study, all ticks survived 10 days at 20ºC. One tick each was found dead on the 10$^{th}$ day for both male and female groups incubated at 4°C. By contrast, ticks of both sexes began dying at day 5 in both the 30°C and 37°C, with slightly better survivorship among females. This is likely due to heat stress, with increased female body size possibly providing some level of protection. A recent study by Ginsberg et al. (24) also showed a decreased survival of immature larval *I. scapularis* at 32.2 °C compared to 22.2°C with a relatively similar humidity of ~85%.

We found that colony-reared *I. scapularis* adult ticks incubated for ten days at 4°C and 20°C (with >80% humidity) showed no significant change in bacterial community composition when compared to the untreated baseline microbiomes, suggesting that these temperatures may have very little to no effect in the adult tick microbiomes. We also found a fairly standard bacterial community in the first days of incubation at 30ºC and 37ºC, but the community complexity of the ticks drops dramatically upon prolong incubation, particularly after more than a week at 30ºC and longer than 5 days at 37ºC. Our revelation of decreased relative abundance of *Pseudomonas* in male ticks held at 30°C for more than a week and at 37°C for more than 5 days, with a concomitant increase in relative proportion of *Brevibacterium*, suggesting potential impacts of warmer temperature in eliminating certain groups of bacteria while favoring others. This is also the case with the genus *Streptomyces*, which showed a significant increase in mean relative abundance in male ticks incubated across high temperatures (from the 0.5% at baseline to 72.3%

at 30°C for 10 days, to 99.3% at 37ºC for 9 days). Our findings of significantly high abundance of *Brevibacterium* in *Rickettsia*-free female data treated at 37°C for more than 7 days, compared to the *Rickettsia*-deleted baseline female ticks, further supports the idea that warmer temperature incubation may have preference to certain groups of bacteria over others. Taken together, these data provide evidence that the bacterial microbiome of *I. scapularis* changes upon extended incubation at 30°C and 37°C.

Additionally, the microbiome change in *I. scapularis* ticks could not be directly attributed to the death of the ticks, because, firstly even at the same temperature treatment (for example, 37°C male groups), the microbiome of the ticks that died early (less than 6 days) had a different bacterial composition than those that died later (by day 8 or 9); secondly the microbiome of the ticks that died early (for example, less than 7 days at 30ºC) had a similar microbiome to that of the baseline ticks (which were live ticks that were preserved at -20°C immediately upon receipt in our lab).

We also found a general decrease in the bacterial richness (Fig. 4a) and diversity (Fig. 4b) of the male ticks as the temperature increased from 4°C to 37°C. When compared to the baseline, male ticks exhibited a significantly lower diversity (Shannon index, $p < 0.0125$) at 37°C, which was dependent upon the duration of exposure. More specifically, males incubated at 37°C for more than 5 days showed a significant decrease ($p < 0.025$) in the bacterial diversity compared to that of the baseline population, most likely due to the higher abundances of either *Brevibacterium* or *Streptomyces* (see Fig. 3a). Because the nature of the experimental design provided information on relative abundances rather than absolute numbers, it is not known if the shift in population

resulted from an increase in absolute numbers of *Brevibacterium* and/or *Streptomyces*, decreases in absolute numbers of other bacteria, or some combination of the two. *Streptomyces* spp. are notable for their natural production of a number of antibiotics. It is interesting to speculate if this may also have played a role in the microbial population shifts among these ticks.

The low number of observed OTUs in baseline females (with all sequences considered), as well as across all temperature treatments (see Fig. 4a), is concomitant with the dominance of *Rickettsia* throughout all groups. Our results are based on the analysis of the total microbiome of the whole tick, so one could predict different results if various organs of the ticks were assessed separately. However, a previous study on field-collected *I. scapularis* found the highest observed OTUs in whole females compared to the salivary glands and midguts (30), suggesting that our approach was relevant to assess the natural microbiome of the female ticks.

However, *in-silico* removal of the sequences of *Rickettsia* from the female data revealed a shift in the remaining bacterial population and dramatic increase in *Brevibacterium* levels at 30°C and 37°C (see Fig. 3c), with a significantly different microbiome at 37°C compared to the 4°C and 20°C treatments (Fig. 4e), suggesting that the rickettsial super-dominance is masking the effects of temperature in the underlying diversity of the bacterial population in female *I. scapularis* ticks. The bacterial community revealed after removal of the rickettsial sequences indicates that the gut microbiome (because ovaries are the main reservoir of *Rickettsia* in the female ticks) of the female tick is highly diverse.

## Conclusion

The bacterial microbiome of the colony-reared *I. scapularis* was distinct between male and female ticks, with more diverse bacterial community in males compared to the females. The bacteria *Rickettsia* almost exclusively dominated the female ticks, whereas several environmental related genera were found in the males. *In silico* removal of *Rickettsia* sequences revealed the underlying hidden bacterial community in the female ticks, which is consistent in complexity with that of the males. We found that the bacterial microbiome of *I. scapularis* males changes upon incubation in controlled lab settings of 30°C for more than a week and 37°C for more than 5 days and a constant humidity of >80%. More specifically, the major findings of our study include: *Pseudomonas*, the predominant genus found across all male ticks, showed a low relative abundance (0.5%) when incubated at 37°C for more than 5 days. This was significantly low in comparison to the baseline abundance (21.2%), and when compared to those ticks incubated for only for 5 days at 37°C (45.2%). In addition, the genera *Brevibacterium* (97.9%) and *Streptomyces* (99.3%) dominated the male ticks held at 37°C for 7 and 9 days respectively. Exposure of male *I. scapularis* ticks at 37°C for more than 5 days significantly decreased the bacterial richness and diversity. On the other hand, *Rickettsia*, the most abundant genus for all female *I. scapularis* samples, showed a decreased abundance after 8 days of incubation at 37ºC. The non-*Rickettsia* taxa with increased abundance in females treated at 37ºC for >8 days were represented by the genera *Brevibacterium* and *Streptomyces*, similar to that seen in males. *In-silico* deletion of *Rickettsia* bacteria revealed a significantly different bacterial diversity in the female ticks incubated at 37°C compared to that of the 4°C and 20°C treatments.

In summary, the environmental temperature can impact the tick bacterial microbiome of *I. scapularis* in a laboratory setting. Future studies on how environmental variables influence vector microbiome composition, and the possible ramifications of the ticks' ability to carry and transmit pathogens, are needed to better understand the impact of climate change on risk and spread of tick-borne and other zoonotic diseases.

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## Conflict of interests

The authors declare that they have no competing interests.

## Authors' contributions

ST and MSA conceived and designed the study. MSA supervised the study. ST prepared samples for sequencing, performed PCR and sequencing experiments YZ processed the data using mothur. ST, YZ, and MSA analyzed data. ST and YZ performed statistical analyses. ST drafted the initial manuscript, and all authors provided feedback and insights into the manuscript. All authors read, edited, and approved the final version of the manuscript.

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CHAPTER III

# BACTERIAL MICROBIOMES OF *Ixodes scapularis* COLLECTED FROM TEXAS AND MASSACHUSETTS, UNITED STATES

## Abstract

**Background:** The blacklegged tick (*Ixodes scapularis*) is the primary vector of Lyme disease in the North America, which also carries numerous human pathogens, including the causative agent of anaplasmosis and babesiosis. Though *I. scapularis* is found across the eastern United States, Lyme disease is endemic to the northeast and upper midwest, whereas the disease is very rare or absent in the southeastern portion of the region. Many factors associated with the tick, hosts and environment have been suggested to influence the onset of Lyme disease, yet the factors driving the regional preference of Lyme disease is not fully understood. Recent studies on various hard ticks have demonstrated that vector competence and transmission of certain tick-borne pathogens can be influenced by the resident microbial community of the ticks. Additionally, there are conflicting reports on microbiome variation of *Ixodes* ticks based on species, gender, and geographic origin. More recently, we have demonstrated that the composition of the endogenous tick microbial community in *I. scapularis* can be influenced by the environmental temperature. The present study assesses the microbiomes in adult *I. scapularis* collected from two geographically and climatically distinct regions (Texas and Massachusetts) of the eastern United States, representing extreme ends of the vector's range.

**Methods:** In an effort to better understand the microbiome of ticks from diverse geographic and environmental regions, we analyzed the bacterial community of 115 adult *I. scapularis* collected from the natural vegetation in Kennard, Texas and North Truro, Massachusetts on Cape Cod over two years (2016-2017), by sequencing the 16S V4 rRNA gene using an Illumina MiSeq sequencer. In addition, 7 female *I. scapularis* collected from dogs in North Texas were included in the study.

**Results**: Field-collected male *I. scapularis* ticks generally had a diverse bacterial microbiome in comparison to the females. The genus *Rickettsia* dominated the microbiomes of field-collected *I. scapularis* females from both regions and the bacterial composition of the male ticks from Massachusetts were different than that of the males from Texas. Fifty percent of the males from Texas were dominated by *Rickettsia*, with the major non-rickettsial bacteria in Texas males represented by *Mycobacterium, Acinetobacter, Sphingomonas, Methylobacterium, Corynebacterium* and many others of environmental origin. By comparison, the five most numerically dominant genera in the male ticks from Massachusetts were *Pseudomonas*, *Borrelia, Rickettsia, Anaplasma,* and *Bradyrhizobium*. *In-silico* removal of *Rickettsia* from Massachusetts female data sets revealed the hidden bacterial composition, where presence of *Borrelia* was prominent compared to the full female profiles (i.e. *Rickettsia* included). Interestingly, *Wolbachia,* a known endosymbiont of insects, was also detected in more than 25% of the ticks from Massachusetts. Overall, *Borrelia* and *Anaplasma,* which contain several pathogenic species, were detected in about 63%, and 15% of all *I. scapularis* ticks (males and females combined) respectively collected from Massachusetts. Furthermore, a total of about 11.3% ticks from Massachusetts were co-infected with both *Borrelia* and *Anaplasma*. None of the *I. scapularis* ticks from Texas were found to be positive for *Borrelia*, *Anaplasma* or *Wolbachia.* Interestingly, female ticks from dogs in Texas had a more complex bacterial community than the wild-caught females.

**Conclusions:** Our results indicate that the microbiome of *I. scapularis* ticks varies by gender. Additionally, field-collected male *I. scapularis* from Massachusetts had a different microbiome composition than that of the males from Texas. We also found that the microbiome of the female

ticks varied based on sample source, suggesting potential roles of the habitat and its immediate environment in shaping the tick microbiome.

**Keywords**: Tick microbiome, *Ixodes scapularis*, 16S rRNA, bacterial diversity, Blacklegged tick, Ecology

## Introduction

Ticks transmit a greater variety of human and animal pathogens than any other disease vector and have significant public health impacts worldwide (1-3). The blacklegged tick, *Ixodes scapularis*, is the major vector of the Lyme disease agent, *Borrelia burgdorferi*, in North America (4). This tick also carries a number of other pathogens that cause human disease, including anaplasmosis and babesiosis (5-7).

With more than 30,000 reported cases and an estimated 10-fold greater burden than the reported case counts, Lyme disease is the most common vector-borne illness in the U.S. (1, 8-10). Despite a broad geographic distribution of *I. scapularis* across the eastern United States, Lyme disease cases are concentrated in the northeastern and upper midwestern states, whereas the disease is very rare or absent in the southern portion of the vector's range (5, 10). The prevalence of *B. burgdorferi* among *I. scapularis* in the northeastern U.S. has been reported to be as high as 30-50% (11-13), while it is rarely (<1%) detected in the ticks from the southern United States (14-16). Over the past two decades, the incidence of Lyme disease has increased in numbers and

geographical area across the eastern U.S., which coincides with a significant northward range expansion of *I. scapularis* in the northeastern and midwestern regions (5, 17).

Many factors, including the density of host-seeking *B. burgdorferi*-harboring ticks, availability of the *B. burgdorferi* competent hosts, tick-behavior, seasonal activity of the ticks, and environmental variables have been suggested to influence the infection rate of Lyme disease (5, 18-24), although the reasons behind the regional incidence of Lyme disease are not fully understood. In recent years, studies on various ixodid ticks have demonstrated that the microbial community of the ticks can influence not only the reproductive fitness and physiological processes of the tick, but may also influence acquisition, establishment and transmission of certain tick-borne pathogens (25-31). In addition, a U.S. based study has demonstrated that the microbiome of *Ixodes* ticks vary with sex, species and geography (32), although the differences are not consistent with these ticks in other geographic areas. For example, a recent study on *I. scapularis* ticks from eastern and southern Ontario, Canada found no significant differences in their microbiomes with regard to geographic origin, sex and life stages (33). These contradicting reports highlight the potential role that the geography and related ecological and environmental factors may have in shaping the microbiome of ixodid ticks. More recently, we have demonstrated that the composition of the endogenous tick microbial community in colony-reared *I. scapularis* can be influenced by the environmental temperature (Thapa et al. 2018, unpublished data). Here, we assess the bacterial microbiomes of adult *I. scapularis* ticks collected from their natural habitat in Texas and Massachusetts, opposing ends of the vector's range in the eastern U.S., each with distinct climates.

## Materials and methods

### Tick sampling and processing

During 2016 and 2017, a total of 115 adult *I. scapularis* were collected from around Trinity county of the Davy Crockett National Forest in Kennard, Texas and in North Truro area of Cape Cod, Massachusetts. Due to the difference in activity levels of ticks in different local environments, sample collection in Massachusetts was done during the summer while ticks from Texas were collected during autumn. *I. scapularis* is endemic to Cape Cod, located in the northeast U.S. (11, 34), and Trinity county in Texas, part of the southeastern U.S., has established populations of the ticks as well (5). Adult ticks were chosen to provide a fair comparison of the tick microbiomes from two regions with different geography and climate and to provide ample DNA per sample without the need for pooling of multiple, smaller life stages. Standard flagging technique was used for tick sampling, which consisted of walking down trails dragging a 1 $m^2$ piece of white cloth attached to a pole gently over and around the vegetation where ticks may be present. All encountered ticks were collected with fine-tipped tweezers and placed into sterile collection vials containing cotton fabric for housing. Ticks were categorized by location (TX or MA) and sex (male or female). All ticks were then preserved at -20°C until DNA extraction. In addition, seven *I. scapularis* females collected from dogs in North Texas were included in the study. Details of the collection sites and dates are provided in Table 1.

TABLE 1. *Ixodes scapularis* ticks collected from different sites

| Collection sites | Source | Collection Year | # of ticks by gender | Total # of ticks |
|---|---|---|---|---|
| Davy Crockett National Forest, Kennard, Texas | Vegetation | December 2017<br>November 2017<br>December 2016 | males (14), females (11)<br>males (4), females (11)<br>males (7), females (6) | 25<br>15<br>13 |
| Hurst, Texas | Dogs | December 2017 | Females (7) | 7 |
| Cape Cod, Massachusetts | Vegetation | May 2017 | Males (32), females (30) | 62 |

**DNA extraction**

All tick samples were treated in sequence with 10% sodium hypochlorite and molecular biology grade water to reduce surface contamination. Each whole tick was then cut into sections with a sterile scalpel on a glass microscope slide. The all resultant sections of a tick were placed in a 2 ml screw-capped FastPrep tube (MP Biomedicals, LLC., Santa Ana, CA) containing 550 µl CSPL® buffer (Omega Bio-Tek, Norcross, GA) and 8-10 sterile 2.8 mm ceramic beads (Mo Bio Laboratories Inc., Carlsbad, CA). Following pulverization (3 cycles of 7 m/s for 60s) in a FastPrep-24™ 5G Instrument (MP Biomedicals, LLC., Santa Ana, CA), each sample was incubated at 56ºC for 2 hours. Total DNA was then extracted from 122 individual ticks using a Mag-Bind® Plant DNA Plus Kit (Omega Bio-tek, Norcross, GA) as per the manufacturer's instructions. A blank extraction control with reagents and beads was also prepared for each lot of DNA extractions. The extracted genomic DNA was quantified with a Nanodrop spectrophotometer (Invitrogen, Carlsbad, CA) and stored at -20ºC until further processing.

**Tick mitochondrial 16S rRNA gene amplification**

Each DNA extract was first assessed by PCR to amplify the 16S region of tick mitochondrial DNA using 16S-1 and 16S+2 primers, as a sample positive control (35). A 25-µl reaction mixture consisted of 5-µl 10X ThermoPol® buffer (New England Biolabs, Inc., Ipswich, MA), 2.5-µl 10X bovine serum albumin, 2-µl of 2.5 mM DNTPs, 0.5-µl forward primer, 0.5-µl reverse primer, 0.25-µl *Taq* DNA polymerase, 5-µl template DNA and 11.75-µl molecular biology grade water. Amplification was performed in a BioRad C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with 10 min of initial denaturation at 94ºC followed by 9 cycles consisting of denaturation at 92ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 1.30 min, followed by 31 cycles consisting of denaturation at 92ºC for 1 min, annealing at 56ºC for 1 min, and extension at 72ºC for 1.30 min, with a final extension at 72ºC for 10 minutes and indefinite hold at 4°C. Each PCR products were evaluated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, and subsequent visualization under UV. Successful amplification of the tick mitochondrial 16S fragment was deemed proof of successful extraction and an absence of excessive quantities of PCR inhibitors, and therefore suitable for further analyses.

**Bacterial 16S rRNA gene amplification**

DNA was amplified in duplicates by PCR using 515F/806R primers that target the hypervariable region four (V4) of the bacterial 16S rRNA gene. The primer set (forward: 5'-GTGCCAGCMGCCGCGGTAA-3' and reverse 5'-GGACTACHVGGGTWTCTAAT-3') had overhanging Illumina sequencing adaptors. Earth Microbiome Project (EMP) 16S Illumina

Amplification Protocol was followed (36) with minor modifications as described below. In brief, a master mix solution was prepared per 25 µl PCR reaction volume with 2.5 µl 10X Accuprime™ PCR Buffer II (Invitrogen, Carlsbad, CA), 2.5 µl 10X Bovine Serum Albumin (New England Biolabs, Inc., Ipswich, MA), 1 µl 50 mM $MgSO_4$, 0.5 µl 10µM forward primer, 0.5 µl 10µM reverse primer, 0.1 µl of 5U/µl Accuprime™ *Taq* DNA Polymerase High Fidelity, 10 µl of template DNA and 7.9 µl molecular biology grade water. PCR was carried out in a BioRad C1000 Touch™ thermal cycler with the following cycling parameters: an initial denaturation at 94ºC for 2 min followed by 30 cycles (35 cycles for all male samples, with few exceptions: 40 cycles) consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 40 s, and extension at 68ºC for 40 s, with a final extension at 68ºC for 5 minutes and a 4°C indefinite hold. Amplicon quality was evaluated by visualizing under UV light after separation in 1.5% agarose gel electrophoresis. No template negative controls were used during the PCR runs.

**16S rRNA gene library preparation and sequencing**

PCR amplicons in duplicate sets were combined for each sample. Purification of the PCR products were completed using AMPure XP magnetic beads and 16S libraries for a total of 122 samples were prepared following the Illumina 16S metagenomic sequencing library preparation protocol with the following minor modifications: the reaction mixture for index PCR (per 50 µl reaction) consisted of 5-µl 10X Accuprime™ PCR Buffer II, 5-µl Nextera XT Index Primer 1, 5-µl Nextera XT Index Primer 2, 0.2-µl Accuprime™ *Taq* DNA Polymerase High Fidelity (5U/µl), 5-µl PCR product and 29.8-µl molecular biology grade water. Amplification was performed in a BioRad C1000 Touch™ thermocycler with the following program: 94ºC for 3 min followed by 8 cycles of 94ºC for 30 s, 55ºC for 30 s, 68ºC for 30 s, and 68ºC for 5 min, followed by holding at

4ºC. The final purified PCR products were quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA) and libraries were pooled in equimolar amounts. The pooled library was sequenced with an Illumina MiSeq instrument for 500 cycles per manufacturer's recommendations. Two negative controls (a blank extraction control and a no-template PCR negative control) were sequenced alongside of the samples to monitor the background noise. The final concentration of the library pool loaded in the MiSeq Reagent Kit v2 (Illumina Inc, San Diego, CA) was 10 pM. A 5% PhiX DNA was also included in the run as an internal control.

**Data analysis**

Data was analyzed in the Windows version of Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA), and XLSTAT-Ecology (Addinsoft SARL, NY), as well as in mothur software (37). Raw sequences generated out of the Illumina MiSeq Instrument were processed using mothur v1.36.1. Quality filtered contigs were aligned to the SILVA database (38) and chimeras were removed using uchime (39) algorithm. Sequences with 97% identitty threshold were binned into operational taxonomic units (OTUs) (40) and taxonomic groups was assigned by comparison to the Greengenes reference database v13.8.99 (41, 42).

The negative controls (one blank extraction control and another no-template PCR negative control) sequenced with the samples resulted in about 0.054% of the total reads. Therefore, the total number of reads in negative controls were subtracted from each of the samples as a further quality control. Additionally, for genus level data analysis, a total of 0.085% of the reads from each sample were considered as zero to minimize putative background contaminations. Relative abundances of bacterial taxa were then compared between groups based on location (Texas vs

Massachusetts), sex (male vs female), and source (vegetation vs dogs). Statistical analyses of the differently abundant taxa among groups were performed using the Kruskal Wallis test. Taxa with a relative abundance of ≥ 1% in at least one sample were analyzed individually, while those with <1% relative abundances were grouped together into an 'Others' category. Prior to diversity analyses, subsampling at a depth of the lowest library size was performed to normalize the number of sequences in each sample (43). Alpha diversity within samples was calculated using Observed OTUs, ACE estimator, Chao1 index, and Shannon index (44). Comparison between groups was performed using the Wilcoxon signed rank test, with adjustment of *p*-value for multiple comparisons using Bonferroni correction (45). The level of significance used in these analyses was 0.05. Beta-diversity beween samples was quantified with weighted and unweighted UniFrac distance matrices and the bacterial community structure was visualized using principal coordinates analysis (PCoA) plots.

## Results

All the 122 *I. scapularis* samples sequenced had libraries with adequate depth for further analysis, as evident from the mean Good's coverage of 99.9% (range=99.9-100%). Additionally, rarefaction curves of the number of observed OTUs plotted at a depth from 1,000 to 30,000 sequences reaching plateau suggested sufficient sample coverage. Rarefaction curves of the number of observed OTUs as a function of sampling effort are illustrated in Fig 1.

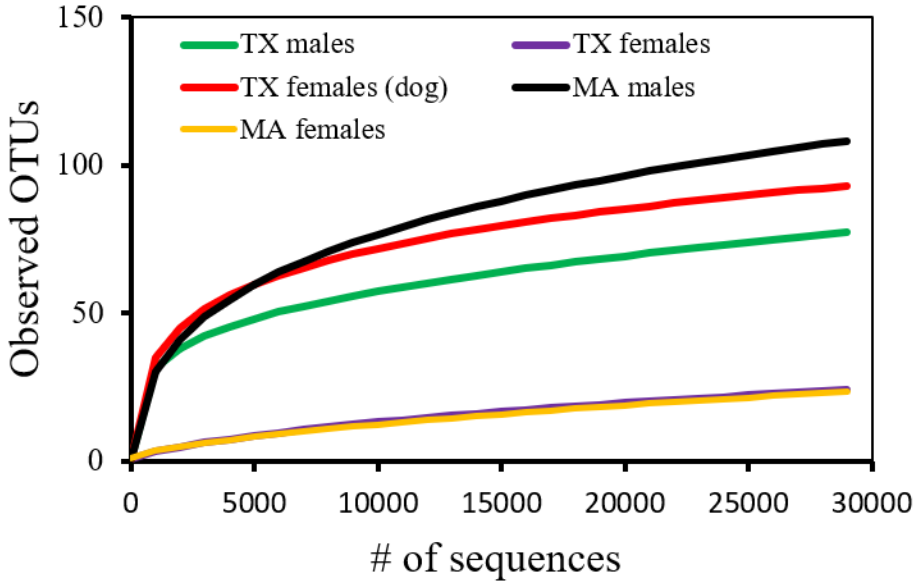


FIG 1. Rarefaction curves of the number of OTUs observed in male and female *I. scapularis*

**Microbiome structure of *I. scapularis***

The phylum *Proteobacteria* dominated the *I. scapularis* microbiome in both locations under study; entire OTUs were assigned to this phylum in all field-collected females (n=28) from Texas and 50% each of the Texas males and Massachusetts females. More than 65% of the male samples from Massachusetts had a very high abundance of *Proteobacteria* (mean= 92.6%). Likewise, *Proteobacteria* dominated the female ticks (n=7) collected from dogs in Texas (range=86.0 -100%). Other common phyla in the microbiomes of the Texas ticks (both field-collected males and the females from dogs) included various proportions of *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*. In addition to these non-proteobacterial phyla found in the Texas ticks, Massachusetts ticks were represented by the *Spirochaetes*. Among the female ticks from Massachusetts, 36.7% had *Spirochaetes* with relative abundance of greater than 1% (range=1.1-11.0%, mean=3.3%), whereas 62.5% of the Massachusetts males included >1% abundance of *Spirochaetes* (3.0-85.7%, 37.5%) (see Fig. 2).

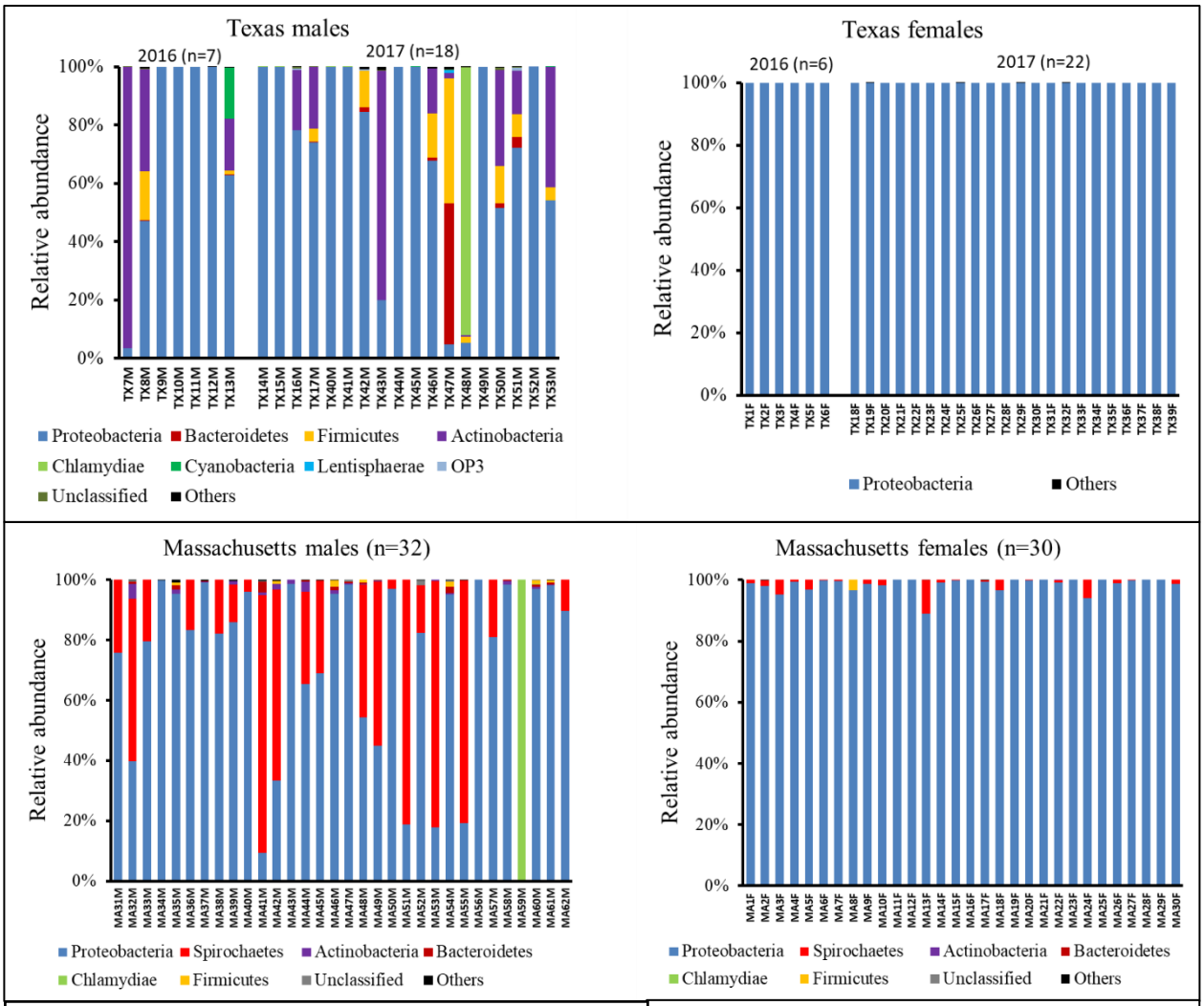


FIG 2. Relative abundance of bacterial phyla in *I. scapularis* ticks from Texas and Massachusetts. Unless otherwise indicated, all ticks analyzed were field-collected from vegetations. Each column represents an individual tick.

At the genus level, OTUs of all field-collected *I. scapularis* females (n=28) from Texas belonged almost entirely to *Rickettsia* spp. Although 66 taxa, including 48 genera, had an abundance of 1% or greater in at least one of the 25 male samples from Texas, about half of them were also dominated by *Rickettsia*. The remaining Texas male samples (n=13) had a more complex microbiome comprised of *Mycobacterium* (mean=23.3%, range=0.1-94.7%), and *Acinetobacter* (22.3%, 0.8-45.9%), followed by *Sphingomonas* (mean=4.6%), *Methylobacterium* (3.9%), *Corynebacterium* (2,4%), *Staphylococcus* (2.3%), *Escherichia* (2.0%), *Rickettsia* (1.8%), *Sphingobium* (1.6%), *Rhizobium* (1.4%), *Pseudomonas* (1.3%), and others (Fig. 3). There was no variation in the microbiomes of ticks from Texas based on the year of collection (Kruskal-Wallis test $p > 0.05$ for both male and female ticks). Female ticks collected from dogs in Texas revealed a more complex bacterial communitycompared to the wild-caught females, comprising of *Rickettsia* (mean=53.2%), *Pseudomonas* (19.6%), *Bradyrhizobium* (6.5%), *Sediminibacterium* (5.3%), *Ralstonia* (4.3%), *Acinetobacter* (1.9%), and others (Fig. 3).

In Massachusetts *I. scapularis* ticks, *Rickettsia* also dominated the female microbiome (mean abundance=97.0%, range=68.7-100%), followed by *Borrelia* (overall mean=1.3%), *Wolbachia* (1.1%), *Anaplasma* (0.1%), and others (Fig. 3). While *Rickettsia* was present in all female ticks from Massachusetts, *Borrelia* and *Anaplasma* were detected in 60% and 10% of the female samples, respectively. More specifically, the mean abundance (range) of *Borrelia* and *Anaplasma* among the positive female samples was 2.2% (0.2-10.7%) and 1.4%, (1.9-79.3%) respectively. Interestingly, *Wolbachia* was found in more than 25% of the females from the region.

Microbiomes of the male ticks captured in Massachusetts were represented by multiple genera, including *Pseudomonas* (overall mean abundance=23.0%), *Borrelia* (22.9%), *Rickettsia* (20.1%), *Anaplasma* (9.3%), *Bradyrhizobium* (4.9%) *Wolbachia* (3.8%), *Ralstonia* (3.5%), *Acinetobacter* (1.1%), and others. Altogether, 24 taxa (including 17 genera) had 1% or greater abundance in at least one of the 32 male samples from Massachusetts. *Borrelia* (mean abundance among positive samples=34.9%, range=0.1%-83.6%) was found in 65.6% of the Massachusetts males, while *Anaplasma* (49.7%, 1.9-79.5%) was detected in about 19% of the males. Moreover, *Wolbachia* was also found in about 28% of the male ticks from Massachusetts.

Differences were identified in the distribution of bacterial genera between the male ticks from Massachusetts and Texas, as evidenced by a significant difference in mean relative abundance of some of the genera that are present in male ticks across both states, such as *Pseudomonas* (Kruskal-Wallis test $p = 0.0001$), *Acinetobacter* ($p = 0.006$) and *Mycobacterium* ($p = 0.004$). Additionally, *Anaplasma*, *Borrelia* and *Wolbachia* bacteria were found in both male and female ticks from MA, but not from TX.

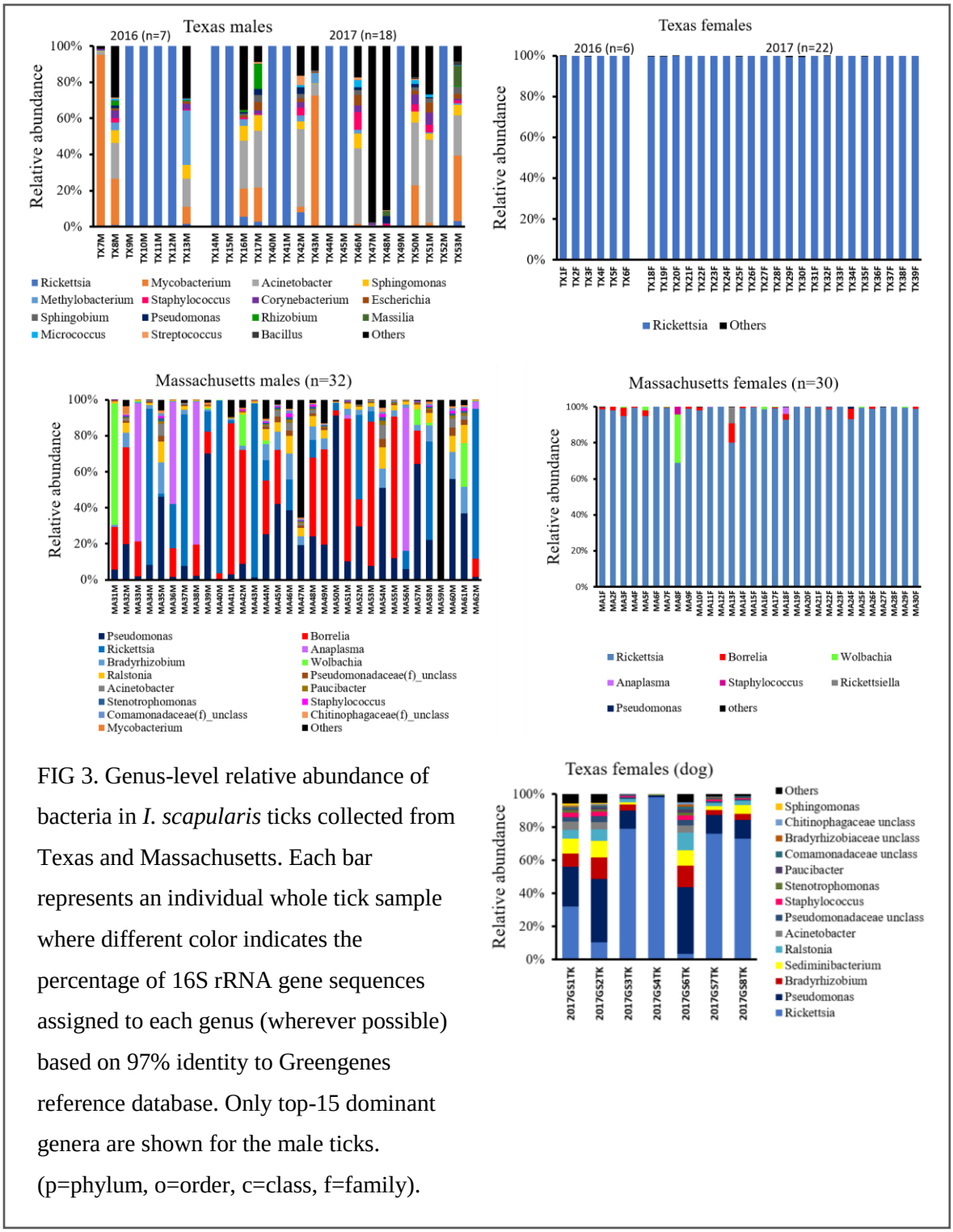


FIG 3. Genus-level relative abundance of bacteria in *I. scapularis* ticks collected from Texas and Massachusetts. Each bar represents an individual whole tick sample where different color indicates the percentage of 16S rRNA gene sequences assigned to each genus (wherever possible) based on 97% identity to Greengenes reference database. Only top-15 dominant genera are shown for the male ticks. (p=phylum, o=order, c=class, f=family).

Because the microbiomes of female ticks were entirely dominated by amplicons likely derived from the rickettsial endosymbiont known to occur in this species, and because this endosymbiont resides primarily in the ovaries (46), we removed *Rickettsia* sequences from the female data sets to further explore the inherent (predominately) gut microbiome of the female ticks. After *in-silico* removal of *Rickettsia* sequences from the female dataset, none of the field-collected female samples from Texas had sufficient sequences remaining to pass the inclusion criteria (as described in the data analysis portion of the materials and methods section) needed to proceed for further analysis. Five female samples from Massachusetts also did not meet the inclusion criteria for further analysis after removing *Rickettsia*. Of the remaining 25 female samples, all collected from Massachusetts, only 13 had more than 1000 reads (mean=7439, range=1,149-29,487) after deleting *Rickettsia*. Fig. 4 shows the relative abundance of bacteria in this subset (n=13) of Massachusetts females ticks before and after removal of *Rickettsia* sequence from their datasets. *In-silico* removal of *Rickettsia* from the Massachusetts female datasets revealed the previously hidden bacterial composition, where presence of *Borrelia* was prominent compared to the full female profiles (i.e. *Rickettsia* included). In contrast to a very low distribution of *Borrelia* in their full profiles (range=0.5-10.7%, mean=3.3%), relative abundance of *Borrelia* in *Rickettsia*-deleted 11 female samples (two of the 13 were negative for these bacteria) ranged from 44.7% to 100% (mean=78.7%) (Fig. 4). The high relative abundance of *Borrelia* in the *Rickettsia*-deleted female group was significantly different (Kruskal-Wallis test $p < 0.0001$) compared to those with *Rickettsia*. Likewise, removal of *Rickettsia* from two *Anaplasma*-positive females resulted in a significant increase in relative proportions of *Anaplasma*, from 3.7% (with *Rickettsia*) to 52.2% (without *Rickettsia*) in sample MA18F and 0.2% to 16.6% in MA22F.

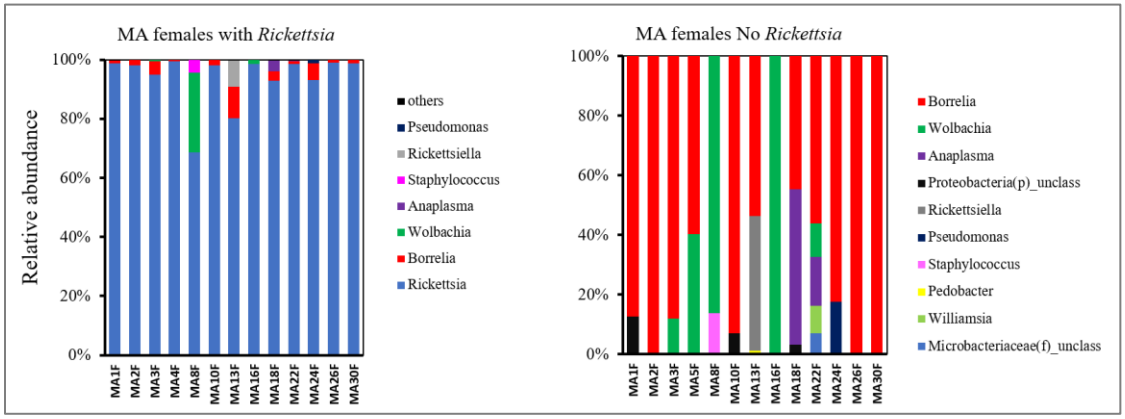


FIG 4. Composition of bacterial taxa in field-collected female *I. scapularis* ticks from Massachusetts with *Rickettsia* (left panel) and after *in-silico* removal of *Rickettsia* (right panel). Taxa are classified to the genus level wherever possible and each bar represents an individual tick identified on the x-axis (F=female). p=phylum, f=family.

**Alpha diversity**

Regardless of the geographical origin, field-collected male ticks exhibited significantly higher bacterial richness (number of OTUs observed) than that of the females (Wilcoxon signed-rank test $p<0.0001$). However, female ticks collected from dogs in Texas had a higher number of observed OTUs in comparison to the wild-caught females from either Texas or Massachusetts (Fig. 5, upper panel). Similar results were found with ACE (abundance-based coverage estimator) value and Chao1 index (data shown in Appendix).

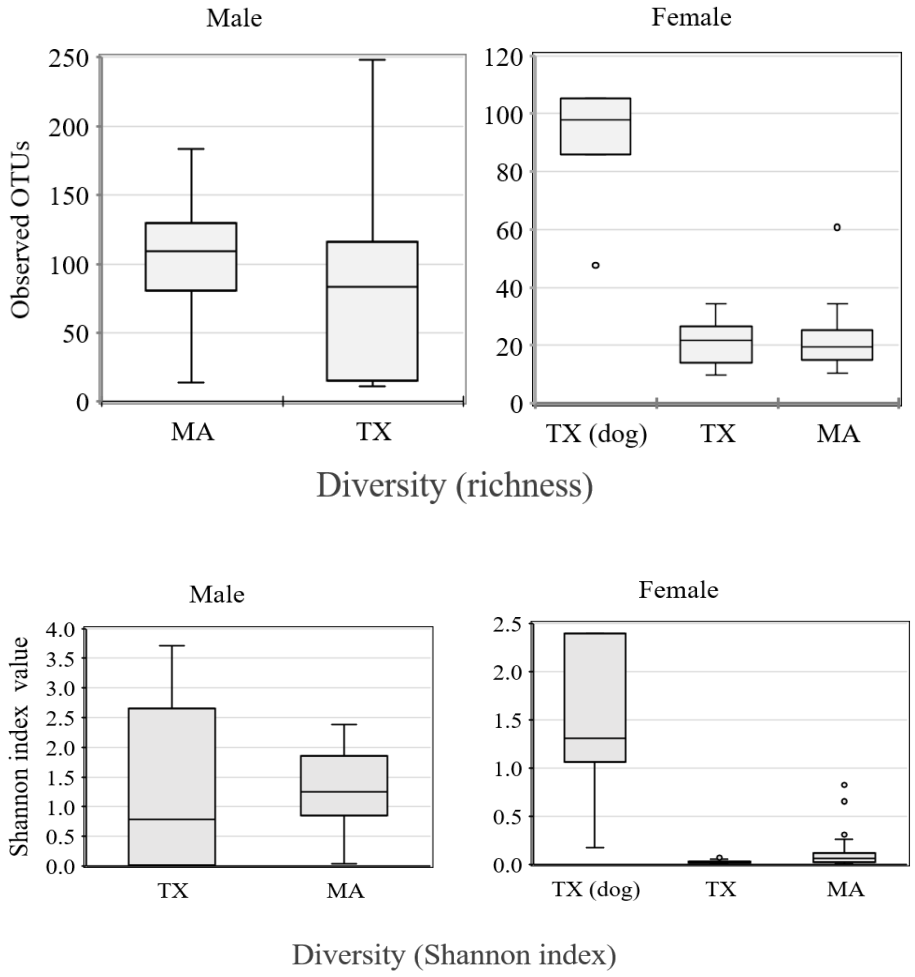


FIG 5. Alpha diversity measures for male and female ticks.

The Shannon diversity index of male ticks was significantly higher than the females (Wilcoxon signed-rank test $p<0.0001$) from both Texas and Massachusetts (Fig. 5), and field-collected female ticks from both locations had a lower bacterial diversity in comparison to the female ticks collected from dogs in Texas. Further multiple comparison analyses revealed no significant differences in the bacterial diversity of female ticks originated from dogs with that of the male ticks collected from wild-vegetation in both states.

**Beta diversity**

Weighted and unweighted UniFrac distance measures were calculated for the diversity between samples. Although a clear separation of the ticks by the state of collection and sex was not observed in all cases, the majority of the male *I. scapularis* ticks captured from Massachusetts clustered separately from others, as did half the males from Texas in an unweighted PCoA plot of axis-1 vs axis-2. The PCoA of unweighted UniFrac distances of bacterial communities showed that the first two axes (PCo1 and PCo2) explained 10.5% and 3.7% of the variation in the data, respectively (Fig. 6). The plot also demonstrated that the male samples from TX clustered separately when compared to the males from Massachusetts, except one outlier from MA within the cluster of TX males. Interestingly, female ticks collected from dogs in Texas clustered close to the field-captured males from Massachusetts.

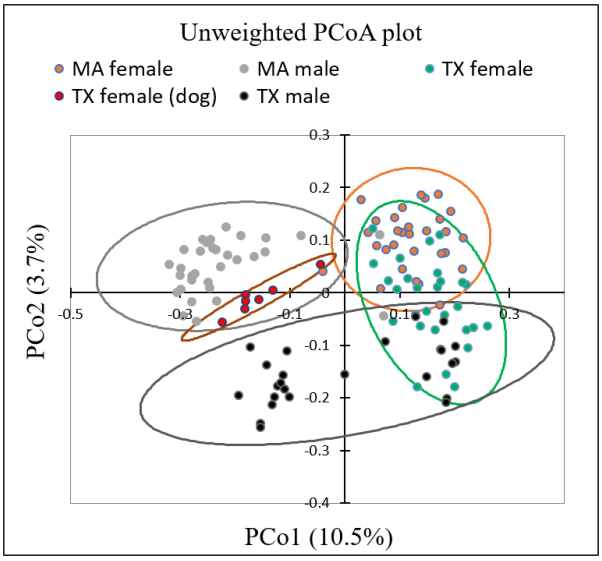


FIG 6. PCoA plot using unweighted UniFrac distance of the bacterial composition in male, and female ticks. In a few cases, samples inside the ellipses are clustered separately from the remaining groups (95% confidence intervals). Each dot represents the bacterial microbiome of an individual tick.

**Co-infection of *Borrelia* and *Anaplasma* in Massachusetts ticks**

The overall *Anaplasma-Borrelia* co-infection rate was found to be about 11.3% in the field-collected *I. scapularis* from Massachusetts (see Table 2).

TABLE 2. Distribution of *Borrelia* and *Anaplasma* in field-collected *I. scapularis* ticks from Massachusetts

| Gender | No. of *I. scapularis* | *Borrelia* +ve | *Anaplasma* +ve | *Borrelia-Anaplasma* +ve |
|---|---|---|---|---|
| Male | 32 | 21 (65.6%) | 6 (18.8%) | 5 (15.6%) |
| Female | 30 | 18 (60.0%) | 3 (10.0%) | 2 (6.7%) |
| **Total** | **62** | **39 (62.9%)** | **9 (14.5%)** | **7 (11.3%)** |

**Microbiome of *Borrelia*-positive and *Borrelia*-negative *I. scapularis* ticks**

As shown in Table 2, two of the 18 *Borrelia*-positive Massachusetts *I. scapularis* females were found to be co-infected with *Anaplasma* (with relative abundances of MA18F=3.7%, MA22F=0.2%). On the other hand, among the 12 *Borrelia*-negative female samples from MA, only one had *Anaplasma* (MA11F= 0.1% abundance). *Wolbachia* was detected in both the *Borrelia*-positive and -negative MA female ticks.

Of the 21 *Borrelia*-positive MA males, five were co-infected with *Anaplasma* (abundance range=1.9-76.6%). Sixty percent (3 out of 5) had an average abundance of 70.9% *Anaplasma* bacteria. In contrast, only one *Borrelia*-negative MA male tick contained *Anaplasma* (with 79.5% abundance), which also harbored *Rickettsia* (10%), *Pseudomonas* (6%), *Bradyrhizobium*

(1.9%), *Ralstonia* (1.6%), *Paucibacter* (0.2%), and others. Like the female samples, *Wolbachia* was also detected in both *Borrelia*-positive and -negative male ticks captured from Massachusetts.

Although the distribution of individual bacterial taxa varied among male and female ticks from Massachusetts, there was no significant difference in the bacterial microbiome composition between *Borrelia*-positive and *Borrelia*-negative groups within each sex (see Fig.7). However, the bacterial community structure of *Borrelia*-positive males from Massachusetts was different from that of the Texas males (none of the ticks from Texas were positive for *Borrelia*, *Anaplasma*, or *Wolbachia* bacteria). Likewise, *Borrelia*-negative males from MA had a different community structure of bacteria when compared to the Texas males. In terms of female ticks, *Rickettsia* was almost exclusively dominant in both regions, but MA females contained sequences of *Borrelia* and *Anaplasma,* among others, while these bacteria were absent from the ticks collected in Texas.

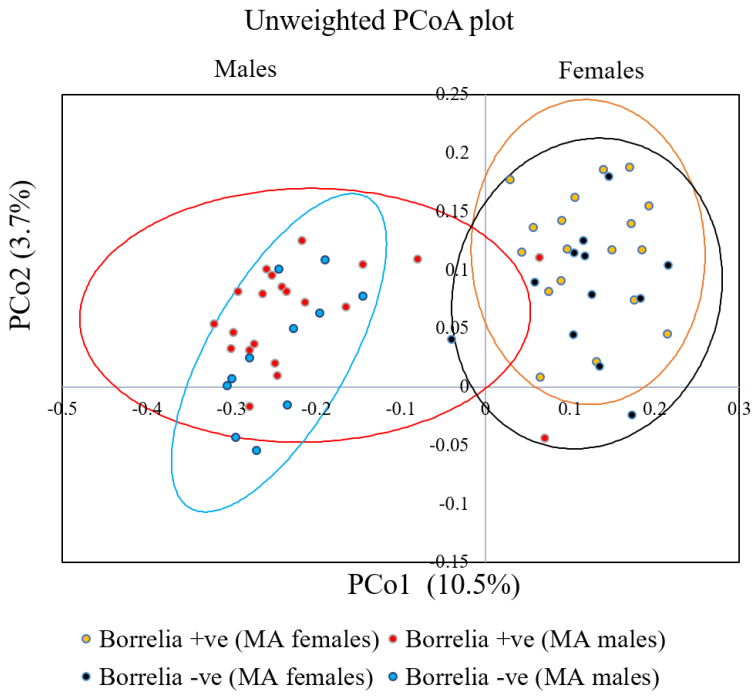


FIG 7. Unweighted PCoA plot of *Borrelia*-positive and *Borrelia*-negative *I. scapularis* males and females collected from Massachusetts. The ellipses are drawn at 95% confidence intervals, where each dot represents the bacterial microbiome of an individual tick.

## Discussion

Tick microbiome research is of particular importance in the field of vector biology, considering the roles many ticks play as carriers of numerous pathogens of human and animal health significance (3, 25, 27, 47, 48). The microbial community of *I. scapularis* ticks has increasingly been studied in recent years (27, 33, 49-51), given the importance of the tick as the primary vector of Lyme disease pathogen (*B. burgdorferi*) (4) in North America (10), and as a vector for many pathogens, including *Anaplasma phagocytophilum* (52), *Babesia microti* (52, 53), *B.*

*miyamotoi* (54-56) and Powassan virus (5). Here we conducted an exploratory study of the bacterial microbiomes in field-collected adult *I. scapularis* ticks from Texas and Massachusetts, two regions in the eastern US with different geography and climate, and the female ticks collected from dogs in Texas.

We found dominance of the phylum *Proteobacteria* in *I. scapularis* ticks from both Texas and Massachusetts and in both genders. A previous study of wild-caught ticks from several U.S. states also found >80% of the reads to be assigned to *Proteobacteria*. Other phyla found in this study, including *Spirochaetes*, were also previously reported in these ticks (32). Consistent with the phylum-level results, our finer taxonomic characterization of the data showed a high abundance of *Rickettsia,* a member of the *Proteobacteria*. Our finding of exclusive abundance (100%) of *Rickettsia* in all field-collected female ticks from Texas, and a very high dominance (mean=97%) in females from Massachusetts, is consistent with previous findings in *I. scapularis* (32, 49-51). High prevalence of *Rickettsia* in larvae and nymphal *I. scapularis* has also been observed in other studies (13, 25).

*Rickettsia*, which contains many potentially pathogenic species, including *Rickettsia rickettsii, R. japonica, R. akari* (57) and *R. parkeri* (58), also contains a number of non-pathogenic species. The only known rickettsial endosymbiont of *I. scapularis*, *R. buchneri* (46), has been shown to provide a source of vitamins to the tick (59). High prevalence of *Rickettsia* endosymbionts in female ticks has been established to be associated with the ovaries. However, our findings of absolute dominance by this bacterium in about half of the wild-caught male ticks from Texas and variable in abundance (as high as 96%) in more than half of the males from Massachusetts

contradicts the previous findings (49, 60), suggesting that *Rickettsia* bacteria may be residing in other body parts of the male ticks, such as the gonads. Additionally, our findings of a high relative abundance of *Borrelia* (range=44.7%-100%, mean=78.7%) in a subset (n=11) of Massachusetts female ticks after *in-silico* removal of *Rickettsia* compared to their full profiles (0.5-10.7%, 3.3%), suggest that the abundance of *Rickettsia* bacteria are potentially masking the inherent gut microbiome of the female ticks.

In our study, about half of the male ticks from Texas and the majority of the males (27 of 32) from Massachusetts had a complex microbiome consisting of many genera, including *Pseudomonas, Mycobacterium, Acinetobacter, Sphingomonas, Methylobacterium*, and others. Several relatives of these bacteria have been found to be associated with the soil, plants, and water, suggesting that these bacteria may be acquired from the environment and maintained through the developmental stages. Differences were identified in the distribution of bacterial genera between the male ticks from Massachusetts and Texas, as evidenced by a significant variation in overall mean relative abundance of some of the genera that are present in the male ticks from both states, such as *Pseudomonas* (MA= 23% vs TX=1.3%), *Acinetobacter* (MA= 1.1% vs TX =22.3%), *Mycobacterium* (MA=0.4% vs TX=23.3%). More specifically, *Borrelia* and *Anaplasma* sequences were found in both male and female ticks from MA but not from TX. These findings provide evidence that the bacterial microbiomes of male ticks from MA were different from that of the TX male ticks, suggesting a geographical and/or ecological variation of the microbiota in these ticks. Although the distribution of individual bacterial taxa varied among male and female ticks from Massachusetts, there was no significant difference in the bacterial microbiome composition between *Borrelia*-positive and *Borrelia*-negative groups within each

sex. The mean abundance of *Borrelia*, which could include pathogenic *B. burgdorferi* and *B. miyamotoi*¸ was higher in males (34.9%) in comparison to the females (2.2%). In a previous study, an average of 7.2% abundance of *Borrelia* was detected in female salivary glands of field-collected *I. scapularis* ticks (49). Several previous studies from the northeastern and midwestern U.S. have routinely detected these bacteria in *I. scapularis* ticks (32, 49).

Overall, the genera *Borrelia* and *Anaplasma* were found in about 63% and 15% of the *I. scapularis* ticks collected from Massachusetts, but none from Texas. One reason for the high number of *Borrelia* sequences in the present study may be due to methodological differences. In comparison to the traditional PCR-based approaches used previously, the 16S rRNA gene sequencing used here cannot discriminate between species. It is highly likely that the samples that yielded *Borrelia* 16S rRNA gene sequences could be of *B. burgdorferi* (the causative agent of Lyme disease) or *B. miyamotoi* (relapsing fever group bacterium). In fact, *B. miyamotoi* has also been identified in this area, albeit at substantially lower numbers, with 2.3% of ticks tested from Cape Cod in 2016 found positive for this bacterium (61). Furthermore, it has been previously demonstrated that not all *I. scapularis* samples yielding *Borrelia* reads from 16S rRNA Illumina sequencing produce amplicons in PCR testing of the *B. burgdorferi* specific *ospC* gene (32). Similar results of discordance between traditional PCR assays and Illumina MiSeq sequencing was also observed in another study on the *A. americanum* tick (62). However, our findings of about 63% *Borrelia* in *I. scapularis* ticks collected from Cape Cod, MA is in line with the findings of Xu et al. (2016) (11), who also reported that 62.5% of *I. scapularis* ticks tested from Nantucket county in Massachusetts were *Borrelia* positive, and unpublished work conducted in our laboratory using nested PCR methods (data not shown).

In this study, the *Anaplasma-Borrelia* co-infection rate was 11.3% in the ticks from Massachusetts. In a previous study (11) on human-biting *I. scapularis* from Massachusetts, 1.8% of the ticks were coinfected by *B. burgdorferi* and *A. phagocytophilum*. The higher rate of co-infection in this study could be attributed to the overall higher prevalence of *Borrelia* found. The presence of *A. phagocytophilum* in *I. scapularis* may be advantageous to the tick, based on the findings of Neelakanta et al. (63) that *A. phagocytophilum* in *I. scapularis* enhanced the tick's survival in the cold environment.

We also found *Wolbachia* present in more than 25% of the ticks from Massachusetts. *Wolbachia,* a known endosymbiont predominately of insects (64, 65), has been previously reported in ticks (66, 67). Although *Wolbachia* has been known to induce resistance to dengue virus in *Aedes aegypti* mosquitoes (65) and other insects (64), Plantard et al. (2012) showed that *Wolbachia* in the *I. ricinus* tick, a major European vector of the Lyme disease agent, is due to the presence of the endoparasitoid wasp *Ixodiphagus hookeri,* and not a true endosymbiont of the tick (68). Thus, the prevalence of *Wolbachia* reported here may not be a true symbiont of the *I. scapularis* ticks, but rather the findings suggest that those ticks may have been parasitized by unidentified insect such as wasps.

It is important to note that the female ticks collected from dogs in Texas had a more complex microbiome, (consisting of *Rickettsia, Pseudomonas, Bradyrhizobium, Sediminibacterium, Ralstonia, Acinetobacter* and others) in comparison to the wild-caught females from both the states, suggesting that the tick microbiome may differ depending on the habitat or the result of a

recent blood meal. Interestingly, microbiomes of the female ticks that originated from dogs in Texas closely matches that of the male ticks from Massachusetts in terms of diversity but not in community membership, further supporting the idea that the bacterial microbiomes of female *I. scapularis* ticks varies with their sample source.

Bacterial richness was generally significantly higher in male ticks compared to female ticks, regardless of the geographical origin. This is consistent with our results of *Rickettsia* dominating all female ticks studied. Furthermore, a significantly higher Shannon diversity in male ticks suggests that the community of male ticks were more diverse as well as even, compared to the females. These differences in part may be explained by the observation of different relative abundance of bacterial taxa in male and female ticks. For example, *Proteobacteria* almost dominated the entire female population of the wild-caught ticks. By comparison, half of the males from Texas and the majority of the males in Massachusetts contained other phyla including *Bacteroidetes, Spirochaetes, Actinobacteria*, *Firmicutes* and others.

## Conclusions

Analyses of the microbiomes of field-collected adult *I. scapularis* ticks from Texas and Massachusetts demonstrated that the bacterial microbiota of male ticks was generally different and more diverse in comparison to females. The phylum *Proteobacteria* dominated the *I. scapularis* microbiome of both sexes in both Texas and Massachusetts. The bacteria *Rickettsia* almost exclusively dominated the field-collected female ticks from both Texas and Massachusetts, as well as half of the male ticks from Texas. *In-silico* removal of *Rickettsia* from Massachusetts female data sets revealed a hidden bacterial composition, where the presence of

*Borrelia* was frequently prominent compared to the full female profiles (i.e. *Rickettsia* included). Several environmental related genera, including *Mycobacterium, Acinetobacter, Sphingomonas* were found as the next dominant bacteria in male ticks from Texas. In contrast, the microbiome of the male ticks collected from Massachusetts were dominated by both environmental and pathogenic genera. In particular, highly abundant bacterial genera found in the male ticks from MA were *Pseudomonas, Borrelia, Rickettsia, Anaplasma*, and, *Bradyrhizobium*. *Wolbachia* was found in more than 25% of the Massachusetts ticks. *Borrelia* and *Anaplasma*, both of which contains pathogenic species, were detected in about 63% and 15% of the *I. scapularis* ticks collected from Massachusetts, but neither were found in those from Texas. Of the total ticks from Massachusetts, about 11.3% were coinfected with both *Borrelia* and *Anaplasma*, which is higher than previously reported by others.

Overall, the microbiomes of *I. scapularis* collected from natural vegetation in Texas and Massachusetts varied with sex. Additionally, male ticks from Massachusetts had a different microbiome composition than the males from Texas. Furthermore, the microbiome of the female *I. scapualris* ticks from dogs in Texas was found to be more complex than those of the wild-caught females from both Texas and Massachusetts.

Future studies on the functional and mechanistic aspects of the tick microbiome, including the possible roles of the ecological factors in shaping the tick microbiome will help us understand the microbiome biology of the resident microbial community of ticks, and the potential roles of habitat ecology in the ability of ticks to acquire, maintain and transmit pathogens. This will ultimately help development of strategies to control the risk and transmission of tick-borne diseases.

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# CHAPTER IV

# CONCLUSIONS AND FUTURE DIRECTIONS

The major findings of these studies include:

- The bacterial microbiome of colony-reared *I. scapularis* ticks is distinct between male and female adults. The microbiome of male blacklegged ticks is more diverse than the females, which are dominated almost exclusively by *Rickettsia.*
- Because the microbiomes of female ticks were entirely dominated by amplicons likely derived from the rickettsial endosymbiont known to occur in this species, and because this endosymbiont resides primarily in the ovaries (1), we removed *Rickettsia* sequences from the female data sets to further explore the inherent gut microbiome of the female ticks. *In silico* removal of *Rickettsia* revealed a complex bacterial community in female ticks, which is consistent in complexity with those seen among the male ticks.
- All *I. scapularis* ticks survived 10 days at 20ºC at a constant relative humidity of >80%, whereas one tick each was found dead on the 10$^{th}$ day for both male and female groups incubated at 4°C. By contrast, ticks of both sexes began dying at day 5 in both 30°C and 37°C treatment groups, with slightly better survivorship among females. All male ticks had died by the 10$^{th}$ day at 37ºC, with only one survivor on the 10$^{th}$ day at 30ºC. The most likely cause of death of these ticks is due to heat stress.

- The bacterial microbiome of *I. scapularis* changes upon incubation at 30°C for more than a week and 37°C for more than 5 days, at a constant humidity of >80%. *Pseudomonas*, the predominant genus found across all male ticks, showed a low relative abundance (0.5%) when incubated at 37°C for more than 5 days. This was significantly lower in comparison to the baseline abundance (21.2%) and also compared to those ticks incubated for only 5 days at 37°C (45.2%). The genera *Brevibacterium* (97.9%) and *Streptomyces* (99.3%) dominated the male ticks held at 37°C for 7 and 9 days respectively. Exposure of male *I. scapularis* ticks at 37°C for more than 5 days significantly decreased the bacterial richness and diversity.
- *Rickettsia*, the most abundant genus for all female *I. scapularis* samples, showed a decreased abundance after 8 days of incubation at 37ºC. The non-*Rickettsia* taxa with increased abundance in females treated at 37ºC for >8 days were represented by the genera *Brevibacterium* and *Streptomyces*, similar to that seen in males. *In-silico* deletion of *Rickettsia* from the female data revealed a shift in the remaining bacterial population and dramatic increase in *Brevibacterium* levels at 37°C, with a significantly different bacterial diversity at 37°C compared to that of 4°C and 20°C treatments.
- The microbiome change in *I. scapularis* ticks could not be directly attributed to the death of the ticks, because, firstly even at the same temperature treatment (for example, 37°C male groups), the microbiome of the ticks that died early (less than 6 days) had a different bacterial composition than those that died later (by day 8 or 9); secondly the microbiome of the ticks that died early (for example, less than 7 days at 30ºC) had a similar microbiome to that of the baseline unexposed ticks.

- Bacterial diversity in field-collected male *I. scapularis* ticks was more diverse in comparison to the females.
- The bacterial microbiome in field-collected female *I. scapularis* ticks from both Texas and Massachusetts was almost exclusively dominated by a single genus, *Rickettsia*. This was also the case in half of the wild-caught male ticks from Texas.
- Several environmental related genera, including *Mycobacterium, Acinetobacter,* and *Sphingomonas* were the dominant non-rickettsial bacteria in the male ticks from Texas. In contrast, *I. scapularis* males captured in Massachusetts were mainly represented by *Pseudomonas, Borrelia, Rickettsia, Bradyrhizobium, Ralstonia, Acinetobacter* and *Anaplasma*.
- *In-silico* removal of *Rickettsia* from Massachusetts female data sets revealed the hidden bacterial composition, where presence of *Borrelia* was prominent compared to the full female profiles (i.e. *Rickettsia* included).
- *Borrelia* and *Anaplasma* were detected in about 63% and 15%, respectively of the *I. scapularis* ticks collected from North Truro, Massachusetts, but none from Texas. Overall, 11.3% of the *I. scapularis* ticks from Massachusetts were co-infected with both *Borrelia* and *Anaplasma*.
- Additionally, bacterial composition and diversity in female *I. scapularis* collected from dogs in Texas was more complex (consisting of many bacterial genera, including *Rickettsia, Pseudomonas, Bradyrhizobium, Sediminibacterium, Ralstonia,* and *Acinetobacter*) in comparison to the field-collected females from both Texas and Massachusetts.

Taken together, these results provide experimental evidence that environmental temperature can impact the bacterial microbiome composition of *I. scapularis* ticks in a controlled laboratory setting and may have implications for the regional differences observed in the bacterial community structures among the natural populations of the tick.

**Future directions**

Future microbiome studies on *I. scapularis* covering other geographical areas, with inclusion of all life stages of *I. scapularis* (larvae, nymphs and adults), will provide more insights into the microbiome of the tick. Additional research on interactions among the bacterial community of the tick will help provide information about the functional consequences of differences in microbial composition of the tick microbiome. Functional metagenomic (2, 3) analysis using techniques such as transcriptomics (4, 5), and metabolomics (6, 7), will help inform how the environmental temperature (and other abiotic factors) influence the tick microbiome. For example, understanding the expression level of genes (8) in ticks treated at different temperatures and their impacts on microbial interactions in ticks may provide further information about the functional consequences of high temperature exposure in these ectothermic arthropods. These types of studies may improve our understanding of the impacts of climate change on the ticks' ability to carry and transmit pathogens, with possible ramifications on strategies to control tick-borne and other zoonotic pathogens.

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# APPENDIX

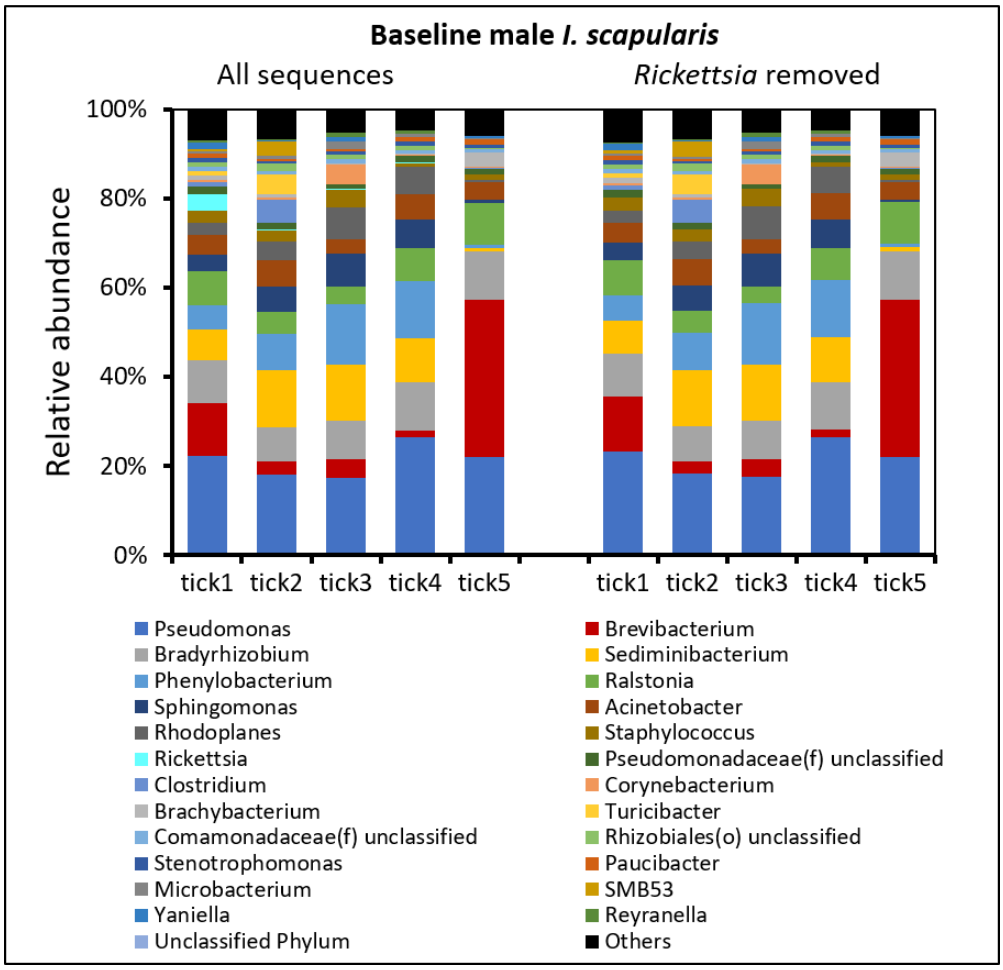


FIG 1. Relative abundance of bacterial taxa in colony-reared baseline *I. scapularis* males with all sequences (left panel) and with *Rickettsia* removed (right panel). Sequences with ≥ 1% relative abundance in at least one of the samples are reported individually, while remaining taxa are grouped together in an 'others' category.

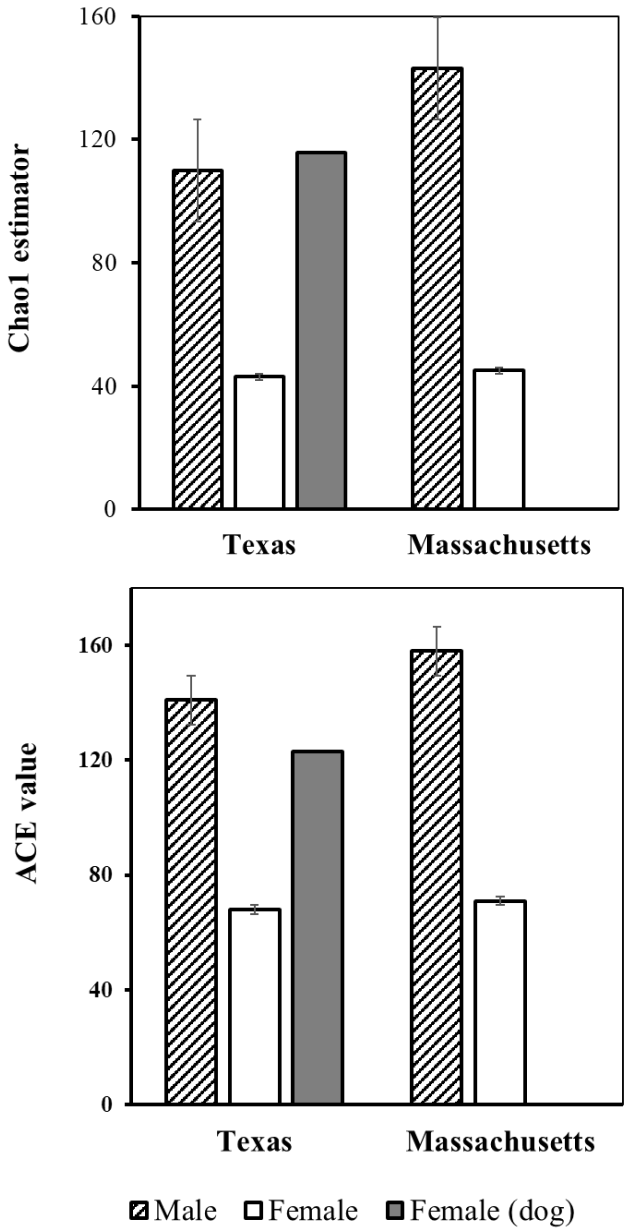


FIG 2. Bacterial richness (ACE and Chao1 estimator) in *I. scapularis* ticks. All ticks were collected from natural vegetation in Texas and Massachusetts, except female (dog) group where ticks were collected from dogs in North Texas.