HIGH THROUGHPUT TOOLS FOR TICKBORNE DISEASE SURVEILLANCE AND INVESTIGATION OF TICK, PATHOGEN, AND COMMENSAL MICROBIOME ASSOCIATION AT SINGLE-TICK RESOLUTION

A Dissertation Presented

By

Gaurav Chauhan

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This work was undertaken in the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

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September 25th, 2023
DEDICATION

This thesis is dedicated to the unwavering support and encouragement of my family and friends, whose belief in my abilities and endless encouragement have been my pillars of strength throughout this academic journey. I also dedicate this work to my mentors, whose guidance has shaped my intellect and ignited my passion for knowledge. Finally, to all those who have been affected by tick-borne diseases, directly or indirectly. To the tireless researchers, healthcare professionals, and volunteers working to combat tick-borne diseases, your unwavering dedication serves as an inspiration. May this work contribute in some small way to the collective efforts aimed at understanding, diagnosing, and ultimately preventing tick-borne diseases, bringing hope for a healthier future.
ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to all those who have supported and guided me on this challenging academic journey. First and foremost, I would like to express my profound gratitude to my thesis advisor, Dr. Elinor Karlsson, for graciously welcoming me into her lab despite my limited experience in bioinformatics. Thank you for being patient and believing in me. Your invaluable mentorship and insightful feedback have been instrumental in shaping this research. You have been a very strong support, in my professional life and outside of work. Your kindness and support have been instrumental in my successful and fulfilling PhD journey. I owe you a debt of gratitude. Thank you.

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ABSTRACT

The prevalence of tickborne diseases worldwide is increasing virtually unchecked due to lack of effective control strategies. The transmission dynamics of tickborne pathogens are influenced by the tick microbiome, tick co-infection with other pathogens. Understanding this complex system could lead to new strategies for pathogen control, but will require large-scale, high-resolution data. Here we present a strategy that combines citizen science with new molecular strategies to provide the single-tick resolution data urgently needed to inform management of tickborne pathogens.

Our citizen science-based initiative, Project Acari, harnessed the power of volunteers across the US to collect more than 3,000 ticks. To assay collected ticks, we developed a high-throughput screening method using Molecular Inversion Probes (MIPs) that identify tick species, associated pathogens, and the species on which the tick most recently fed. Applying MIPs to 853 individual ticks successfully identified the species of 715 ticks, of which 85 were infected with pathogens of 12 different species. We also detected host DNA in 60 ticks.

We also generated the first comprehensive data on both prokaryotic and eukaryotic microbiome of individual ticks using full-length 16S and 18S sequencing. Our findings corroborate reports of the influence of tick species, sex, and geography on the tick prokaryotic microbiome. We also identify novel associations between the carriage of *B. burgdorferi* and specific microbial taxa.
Our work underscores the power of citizen science, paired with high-throughput processing, to elucidate the ecology of tickborne disease and to guide pathogen-control initiatives.
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<td>Oxford University Press for the Infectious Diseases Society of America</td>
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CHAPTER I: INTRODUCTION
I.1 Tickborne Diseases

In recent decades, tickborne diseases have emerged as a significant global threat to the health of both humans and animals (Couper & Swei, 2018; Saldaña et al., 2017). Ticks are a crucial group of blood-feeding arthropod vectors, known for their capacity to carry and transmit a wide range of pathogens, surpassing other arthropod vectors in this regard (Clay & Fuqua, 2010). Ticks rank second only to mosquitoes as vectors of pathogens affecting humans, and they serve as the primary carriers of pathogens impacting livestock, companion animals, and wildlife (de la Fuente et al., 2008).

Tickborne diseases account for a significant proportion of vector-borne illnesses in temperate North America, Europe, and Asia. In the USA, tickborne diseases account for over 95% of vector-borne disease cases (Rosenberg et al., 2018), with Lyme disease being the most prevalent. From 2016 to 2019, on an average 36,000 Lyme disease cases per year were reported to CDC, and approximately 16,000 cases per year were reported for other tickborne diseases (CDC, 2022). Studies (Kugeler et al., 2021; Mead, 2015) have revealed that cases of Lyme disease have been significantly underreported, and the actual annual incidence is estimated to be 8 to 12 times higher. This would position Lyme disease as the second or third most common reportable infectious disease in the US (Hinckley et al., 2014; Kugeler et al., 2021).
The economic impact of tick-borne diseases is significant and increases every year. In the USA, the economic burden of diagnosed Lyme Disease has been estimated to be nearly $1 billion annually, and would be much higher when including suspected, undiagnosed, or non-acute cases (Hook et al., 2022). Other studies proposed estimates up to 3 billion dollars per year (Adrion et al., 2015). The burden of tick-borne diseases extends beyond the USA, with European countries also facing expenditures of tens of millions of euros in several European countries, attributable to Lyme disease (Mac et al., 2019). Veterinary tick-borne diseases also have a staggering impact, particularly in developing countries (Estrada-Peña & Salman, 2013), where tick-borne diseases affect approximately 80% of the world's cattle population (Rochlin & Toledo, 2020), resulting in substantial economic losses and mortality (de Castro et al., 1997; Kivaria, 2006). Theileriosis, a disease caused by many of several different parasites of genus *Theileria*, a major cause of economic loss and livestock mortality in regions like Tanzania (Kivaria, 2006), was sporadic in the USA until 2017. However, this could change rapidly. Both *Theileria orientalis* (Oakes et al., 2019) and its vector, the *Haemaphysalis longicornis* tick (Rainey et al., 2018), (Hammer et al., 2015), have recently been detected in the USA. The tick and the pathogen has spread rapidly, *Haemaphysalis longicornis* tick has been reported in 19 states (CDC, 2023), and *T. orientalis* has been detected in seven states (Onzere et al., 2023), raising concerns about the potential impact of Theileriosis in the US.
Recent reports suggest that the impacts are tickborne disease are continuing to grow as there has been a consistent rise in the reporting of tickborne diseases cases, as well as the emergence of new viral tickborne diseases (Higuita et al., 2021). The increase in tickborne diseases cases has been substantial, with a remarkable 260% rise in reported cases to the CDC from 2004 to 2017 (CDC, 2022). This trend is particularly alarming as tickborne diseases are also spreading into new regions. For instance, the *Amblyomma americanum* (lone star tick) has been expanding its geographical range northward, leading to identification of novel lone star tick-associated pathogens (Benham et al., 2021; Higuita et al., 2021). Similarly, the *Amblyomma maculatum* (Gulf Coast tick) has also extended far beyond its historical range (Benham et al., 2021). The geographical expansion of these ticks and the associated pathogens poses a significant public health concern, highlighting the need for urgent attention and proactive measures for surveillance to mitigate their impact.

I.1.1 Medically Important Ticks In USA

Ticks are divided into two main taxonomic families: Argasidae (soft ticks) and Ixodidae (hard ticks). They have distinct ecology and public health impacts (Parola & Raoult, 2001), as summarized in Table 1.1. Soft ticks have shorter feeding times and host-seeking strategies, which lead to a lower transmission of human pathogens compared to hard ticks. Hard ticks encounter hosts passively and feed for extended periods, creating prolonged contact with their hosts that
facilitates the transmission of pathogens (Parola & Raoult, 2001). Therefore, hard ticks are the primary concern for human health.

Table 1.1 Distinct Ecologies and Public Health Impacts of Hard and Soft Tick Families

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hard Ticks (Ixodidae)</th>
<th>Soft Ticks (Argasidae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding Time</td>
<td>Extended feeding periods (3-12 days)</td>
<td>Short feeding times (minutes to an hour)</td>
</tr>
<tr>
<td>Feeding Behavior</td>
<td>Engorgement over a single meal</td>
<td>Multiple blood meals per stage</td>
</tr>
<tr>
<td>Developmental Stages</td>
<td>Three active stages (larva, nymph, adult)</td>
<td>Several developmental stages per molting</td>
</tr>
<tr>
<td>Habitat</td>
<td>Found in various natural habitats, suburban and urban areas</td>
<td>Typically inhabit in the habitat of their hosts like wildlife nests, burrows, and caves</td>
</tr>
<tr>
<td>Pathogen Transmission</td>
<td>Usually require over 24 h for efficient transmission</td>
<td>Fewer human pathogens transmitted, due to short feeding times</td>
</tr>
<tr>
<td>Host-Seeking Behavior</td>
<td>Questing behavior, climb on vegetation to find hosts</td>
<td>Active hunters, seek out hosts in their shelters</td>
</tr>
<tr>
<td>Disease Prevalence</td>
<td>Transmit various pathogens to humans and animals</td>
<td>Transmit fewer pathogens to humans</td>
</tr>
<tr>
<td>Public Health Impact</td>
<td>Important vectors for human and animal diseases</td>
<td>Less significant impact on human public health</td>
</tr>
</tbody>
</table>

Medically important hard ticks in the USA include the following:

1. *Ixodes scapularis* (Blacklegged Tick): Also known as the deer tick. It is found primarily in the northeastern, mid-Atlantic, and north-central regions
of the country. It is responsible for transmission of *Borrelia burgdorferi* and *B. mayonii* (which cause Lyme disease), *Anaplasma phagocytophilum* (anaplasmosis), *B. miyamotoi* (a form of relapsing fever), *Ehrlichia muris eauclairensis* (ehrlichiosis), *Babesia microti* (babesiosis), and Powassan virus (Powassan virus disease).

2. *Dermacentor variabilis* (American Dog Tick): It is widely distributed east of the Rocky Mountains. This tick species can transmit *Francisella tularensis* (tularemia) and *Rickettsia rickettsii* (Rocky Mountain spotted fever).

3. *Amblyomma americanum* (Lone Star Tick): These ticks are prevalent in the southeastern and eastern parts of the USA. They can transmit *Ehrlichia chaffeensis* and *E. ewingii* (which cause human ehrlichiosis), *Francisella tularensis* (tularemia), Heartland virus (Heartland virus disease), Bourbon virus (Bourbon virus disease), and Southern tick-associated rash illness (STARI) and can trigger the newly recognized condition known as alpha-gal syndrome, which causes an allergy to red meat.

4. *Rhipicephalus sanguineus* (Brown Dog Tick): It is found worldwide and transmits *Rickettsia rickettsii* (Rocky Mountain spotted fever). This tick species is unique as it can complete its entire lifecycle indoors. It is primarily associated with dogs but can bite humans as well. It can transmit
diseases like Rocky Mountain spotted fever and can cause infestations in homes.

5. *Amblyomma maculatum* (Gulf Coast Tick): Distributed primarily in the southeastern United States, with focal populations in the northeastern, midwestern, and southwestern United States. This tick can transmit *Rickettsia parkeri* (*R. parkeri* rickettsiosis), a milder form of spotted fever.

6. *Dermacentor andersoni* (Rocky Mountain Wood Tick): Found in Rocky Mountain states. It transmits *Rickettsia rickettsii* (Rocky Mountain spotted fever), Colorado tick fever virus (Colorado tick fever), and *Francisella tularensis* (tularemia).

7. *Ixodes pacificus* (Western Blacklegged Tick): It is found in the Pacific Coast states and can transmit *Anaplasma phagocytophilum* (anaplasmosis), *B. burgdorferi* (Lyme disease), and very likely *B. miyamotoi* (*Borrelia miyamotoi* disease, a form of relapsing fever).

Apart from their role in pathogen transmission discussed above and shown in Table 1.2, ticks can give rise to following conditions:

1. Tick Paralysis: When species, including *D. andersoni* and *D. variabilis* in North America (Diaz, 2010; Felz et al., 2000), are attached to the host for prolonged periods (around 5-7 days), it can lead to paralysis of the host due to the presence of neurotoxic substances produced by the engorged
ticks, especially female ticks (Diaz, 2010; Felz et al., 2000; Hall-Mendelin et al., 2011; Morshed et al., 2017).

2. Alpha-gal syndrome: Tick bites can also trigger an allergic reaction known as alpha-gal syndrome, which causes a long-term allergy to red meat. Alpha-gal syndrome is linked to an immune response involving IgE antibodies targeting a specific epitope present in the mammalian oligosaccharide, galactose-alpha-1,3-galactose (alpha-gal) (Steinke et al., 2015). This condition is considered emerging and has been observed globally (Commins & Platts-Mills, 2013; Kwak et al., 2018). In the USA, it is frequently associated with the *A. americanum*, lone star tick.

**Table 1.2 Medically important hard ticks in USA**

<table>
<thead>
<tr>
<th>Tick Species</th>
<th>Geographical Distribution</th>
<th>Pathogens Carried</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. Scapularis</em></td>
<td>Primarily in northeastern, mid-Atlantic, and north-central regions of the USA</td>
<td><em>Borrelia burgdorferi, B. mayonii, Anaplasma phagocytophilum, B. miyamotoi, Ehrlichia muris eauclairensis, Babesia microti, Powassan virus</em></td>
</tr>
<tr>
<td><em>D. Variabilis</em></td>
<td>Widely distributed east of the Rocky Mountains</td>
<td><em>Francisella tularensis, Rickettsia rickettsii</em></td>
</tr>
<tr>
<td><em>A. americanum</em></td>
<td>Prevalent in southeastern and eastern parts of the USA</td>
<td><em>Ehrlichia chaffeensis, E. ewingii, Francisella tularensis, Heartland virus, Bourbon virus, Southern tick-associated rash illness (STARI), alpha-gal syndrome</em></td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Found worldwide</td>
<td><em>Rickettsia rickettsii</em></td>
</tr>
<tr>
<td>Tick Species</td>
<td>Geographic Distribution</td>
<td>Pathogen(s)</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>A. maculatum</em> (Gulf Coast Tick)</td>
<td>Primarily in the southeastern United States, with focal populations in the northeastern, midwestern, and southwestern USA</td>
<td><em>Rickettsia parkeri</em></td>
</tr>
<tr>
<td><em>D. andersoni</em> (Rocky Mountain Wood Tick)</td>
<td>Found in Rocky Mountain states</td>
<td><em>Rickettsia rickettsii</em>, Colorado tick fever virus, <em>Francisella tularensis</em></td>
</tr>
<tr>
<td><em>I. pacificus</em> Western Blacklegged Tick</td>
<td>Found in the Pacific Coast states</td>
<td><em>Anaplasma phagocytophilum</em>, <em>Borrelia burgdorferi</em>, very likely <em>B. miyamotoi</em></td>
</tr>
</tbody>
</table>

I.1.2 Co-infections In Ticks

Co-infection of ticks and humans is a major problem for disease surveillance and clinical care, respectively. Most co-infection studies are focused on co-infection with *B. burgdorferi* in *I. scapularis* ticks and because existing methods typically focus only on the common pathogens, it is possible that additional co-infections remain to be discovered. Ticks can carry multiple bacteria, viruses, and protozoans simultaneously, leading to the transmission of several pathogens in a single bite (Borşan et al., 2021; Boyer et al., 2022; Brooks et al., 2022; Voordouw, 2015; Wormser et al., 2019). Ticks acquire pathogens while feeding from reservoir hosts such as mice, rats, and squirrels. Ticks can then transmit these pathogens when they bite other hosts, including humans, pets, and cattle.

The risk of co-infection is heightened due to the ecological characteristics of the three most common tick-borne pathogens (*Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*). All three pathogens are transmitted by
the same vector, *Ixodes scapularis*, and are carried by many of the same reservoir hosts. Consequently, ticks in Northeast and Midwest regions have a high likelihood of being co-infected with two or more pathogens, increasing the risk of humans acquiring multiple tick-borne diseases (Ostfeld, 2011). Co-infection of multiple tick-borne pathogens can affect the intensity and duration of symptoms in humans (Abrams, 2008; Krause et al., 1996; Martínez-Balzano et al., 2015), making diagnosis and treatment more challenging.

As expected from the capacity of ticks to carry more than one pathogen, human patients with tickborne disease frequently have co-infections. A survey of over 3,000 patients with chronic Lyme disease found that over 50% had coinfections, with 30% reporting two or more coinfections. The most common co-infections included *Babesia, Bartonella, Ehrlichia, Mycoplasma, Rickettsia, Anaplasma,* and *Francisella*, ranging from 1% to 32% (L. Johnson et al., 2014; J. Sperling et al., 2012). In small mammals, ticks (Schulze et al., 2013) and infected humans (Belongia, 2002; Krause et al., 2002), co-infection with *Babesia microti* and *Borrelia burgdorferi* occurred more frequently than expected by chance. However, co-infection with *A. phagocytophilum* and *B. microti* was less common than expected (Hersh et al., 2014).
I.1.3 Outstanding Challenges In Surveillance And Management Of Tickborne Disease

Ticks and tickborne pathogen surveillance presents a formidable challenge due to the vast array of tick species and the multitude of pathogens they carry. Morphological identification of ticks can be an arduous and time-consuming process. Furthermore, understanding the ecological dynamics of ticks and pathogens necessitates knowledge of the hosts upon which these ticks have fed. Additionally, gaining insights into the prevalence of drug-resistant genomic pathogen variants in the wild is crucial for clinical management. Similarly, the current approach for diagnosing TBDs in the clinic involves testing for each pathogen separately, these methods are often not accurate. Moreover, these tests are frequently administered based on a preliminary assessment of the likely causative pathogen, guided by the patient's symptoms. This approach can lead to misdiagnosis or even missed diagnosis, particularly in cases involving complex TBDs with co-infections (Sparagano et al., 2022). There is a pressing need for the rapid and sensitive detection of pathogens, assessment of drug resistance, and identification of co-infections to ensure timely and effective treatment of infectious diseases, as well as for studying spread and distribution of outbreaks (Chen et al., 2015; Ke et al., 2013). In clinical samples detection of co-infections is very crucial as it complicates the symptoms, diseases progression and treatment.
Furthermore, current methods don't look for host DNA in tick samples. Identifying host DNA in ticks which don't have such information available is valuable for understanding tick feeding patterns, assessing disease transmission risk, and planning public health interventions. It helps determine host preferences, feeding stages, and potential disease reservoirs, aiding ecological studies and control strategies.

Given the substantial impact of tickborne diseases, there is an urgent need for high-throughput methods that can rapidly and economically detect all ticks and pathogens, identify the host on which a tick has fed, and determine the presence of drug-resistant variants. Moreover, effective control measures are essential to mitigate the burden of tickborne diseases. Interventions targeting ticks, which could potentially alter pathogen colonization or transmission dynamics, hold promise for bringing about enduring changes in the trajectory of these diseases, thereby influencing their long-term prevalence and geographical distribution.

In the following discussion, we will explore the available methods for tick and pathogen surveillance and strategies for controlling tickborne diseases, while also considering their respective limitations. Subsequently, I will discuss how our innovative approaches of Project Acari surmount these challenges, furnishing us with tools for comprehensive surveillance and the investigation of intricate associations among ticks, pathogens, and tick microbiomes.
I.2 Ticks and Tickborne Pathogen Surveillance

Tick surveillance is of significant public health importance due to its role in monitoring and controlling tick-borne diseases. Tick surveillance involves the systematic collection and testing of ticks to identify the presence of pathogens they may carry. This practice provides valuable information to public health authorities and researchers in several ways:

1. Disease Detection and Monitoring: Tick surveillance helps detect the presence and prevalence of tick-borne pathogens in specific geographic areas. By identifying infected ticks, public health officials can anticipate and respond to disease outbreaks more effectively. This information aids in monitoring the spread of diseases and understanding their epidemiology.

2. Risk Assessment: By tracking the distribution of ticks and the pathogens they carry, risk assessments can be made to determine the likelihood of disease transmission to humans and animals. This allows for targeted prevention and control measures in high-risk regions.

3. Early Warning System: Tick surveillance serves as an early warning system for emerging tick-borne diseases. If new pathogens or novel tick species are detected in certain areas, public health authorities can take proactive measures to prevent their further spread.
4. Targeted Prevention Strategies: Surveillance data helps tailor prevention strategies to specific areas and populations at higher risk. These measures may include public education campaigns, use of repellents, habitat management, and targeted application of acaricides (tick-killing substances).

5. Evaluating Control Measures: Surveillance provides feedback on the effectiveness of control measures. It helps determine if interventions, such as insecticide treatment, are reducing tick populations or pathogen transmission.

6. Research and Data Collection: Tick surveillance data contributes to ongoing research on tick-borne diseases, their ecology, and transmission patterns. It also aids in understanding the behavior of ticks and their interaction with pathogens and hosts.

7. Antimicrobial Resistance Monitoring: Surveillance allows for the monitoring of antimicrobial resistance in tick-borne pathogens. This information is crucial for guiding appropriate treatment protocols and preventing the development of drug-resistant strains.

8. Public Awareness: Sharing tick surveillance findings with the public raises awareness about the risks associated with ticks and tick-borne diseases. It encourages individuals to take preventive measures when engaging in outdoor activities.
Overall, tick surveillance is a critical component of public health strategies aimed at reducing the burden of tick-borne diseases. It enables proactive measures to protect human and animal health and contributes to the development of effective control and prevention strategies.

I.2.1 Methods Of Tickborne Disease Surveillance

Tickborne diseases can be tracked either by monitoring the reported clinical cases or by identifying collecting ticks followed by detecting pathogens carried by the ticks.

1. Tracking reported cases:

   Healthcare providers, laboratories, and public health authorities play a key role in reporting confirmed and suspected cases of tick-borne diseases (Little et al., 2019). When a patient presents with symptoms consistent with a tick-borne disease, healthcare providers conduct diagnostic tests and report positive cases to the relevant public health agency. Tracking reported cases has limitations such as underreporting (Schiffman et al., 2018), diagnostic challenges (Aucott et al., 2009; Kobayashi et al., 2022), and incomplete data.

2. Tick Collection methods for Surveillance:

   Several methods are used to determine the number and types of ticks in a given area.
a. Active Collection methods:

Active collection methods in tick surveillance involve targeted approaches to gather ticks from specific high-risk areas or hosts. These methods allow for a focused and detailed analysis of tick populations and the prevalence of pathogens.

i) Tick Drags, Tick Flags, and Tick Walks: This technique involves passing a piece of cloth over or around areas where ticks are known to quest, or a person walking in a designated area while wearing white clothes. It simulates the actual exposure that a person might experience in that area. This is the most reliable method for quantifying the density of host-seeking ticks (Dantas-Torres et al., 2013; Falco & Fish, 1992; Newman et al., 2019). Several studies have used dragging and flagging (Carroll & Schmidtman, 1992; Daniels & Fish, 1990; Falco & Fish, 1992) or Walk sampling (Carey et al., 1980; Schulze et al., 1986) to collect host-seeking ticks in many studies. This method has limitations including limited sampling range and bias towards host-seeking ticks, potentially missing questing ticks in leaf litter or high vegetation.

ii) Carbon Dioxide Trapping: This method exploits the ability of ticks to sense and move toward sources of carbon dioxide (CO2) (Childs & Paddock, 2003; Petry et al., 2010). Dry ice traps are set up by placing dry ice in a vented, insulated container on the ground. The sensitivity to CO2
varies among tick species. For example, it may take up to a day for adult *Ixodes scapularis* ticks to move towards dry ice traps, while many other tick species are attracted more efficiently. So, this method might miss certain species that are less responsive to CO2 or fail to accurately represent tick populations due to variations in CO2 attraction across different tick stages and locations.

iii) Host Trapping and Examination: Host trapping is particularly effective in assessing local tick populations, especially when host nests can be sampled, and their contents extracted using Berlese-Tullgren funnels. For example, in the case of burrowing mammals like field mice, far more ticks can be found in nests than on the host animals themselves (Easton & Goulding, 1974; Foley et al., 2011). However, these methods might not capture all tick species, can underestimate tick abundance due to variations in host behavior and seasonal activity, and could potentially alter tick behavior patterns, leading to biased results.

Active collection methods in tick surveillance certainly have their advantages in providing targeted and detailed data on tick populations and pathogen prevalence. However, they also come with some disadvantages. These methods are labor-intensive, costly, and resource-intensive especially when studying large areas or collecting ticks over extended
periods. They are often restricted to specific locations or hosts, can disturb the natural behavior of ticks, and introduce bias in tick collection.

b. Passive Collection methods:

Passive tick collection methods are techniques that do not involve actively seeking out ticks in specific locations or hosts. Instead, they rely on the natural behavior of ticks to come into contact with hosts (Koffi et al., 2012). These methods are less labor-intensive and allow for a broader and more continuous assessment of tick populations in an area. Some common passive tick collection methods include:

i) Tick Submission by Public or from veterinarians: Encouraging the public to submit ticks they find on themselves or their pets can also be a passive method of tick collection, providing valuable data on tick distribution and potential pathogen transmission. These methods might not cover the entire tick population, as only selected ticks are submitted.

ii) Passive Traps: Passive traps are stationary devices that attract ticks using different baits or attractants. Once ticks come into contact with the trap, they can be collected and analyzed.

iii) Citizen Science: It involves involving the public, including non-professional scientists and volunteers, in the collection and analysis of data related to tick populations and tick-borne pathogens (X. Lee et al.,
2019; Nieto et al., 2018). Here are some ways citizen science can be utilized in tickborne diseases research:

Tick Collection: Citizen science programs often encourage the public to participate in tick collection efforts. Individuals can collect ticks from themselves, their pets, or the environment and submit them to researchers for analysis. This data provides valuable information on tick distribution and helps researchers identify areas of high tick activity.

Tick Identification: Identifying tick species is essential in understanding which ticks are present in a given area and their potential to transmit diseases. Citizen scientists can be trained to identify ticks accurately, contributing to large-scale tick surveillance efforts.

Tick Surveillance and Reporting: Citizen scientists can report tick encounters through various platforms and apps designed for tick surveillance. This data helps researchers track tick activity and map the spread of tick-borne diseases.

Citizen science in tick-borne disease research enhances data collection, increases the spatial coverage of surveillance efforts, and fosters community engagement in public health initiatives. By involving the public in tickborne diseases research, scientists can gain a more comprehensive understanding of tick ecology, disease transmission, and develop better strategies for prevention and control. However, because people and pets
often travel long distances, data derived from them should only be used for tick surveillance when travel histories are assessed (R. J. Eisen & Paddock, 2021).

3. **Ticks and Pathogen Detection Methods for Surveillance:** Detection of pathogens in ticks is a crucial aspect of tick-borne disease surveillance and research. Several methods are employed for tick-borne disease surveillance with each having some advantages and limitations summarized in Table 1.3. Morphological identification, while precise, is time-consuming and requires expertise. Culture and isolation offer live pathogen study but are resource intensive. Serological tests indicate host exposure but can yield false positives. PCR is highly sensitive but has limited multiplexing. Loop-Mediated Isothermal Amplification (LAMP) is faster but faces multiplexing challenges. Targeted sequencing provides accurate detection with prior target knowledge. Metagenomic sequencing identifies diverse microorganisms but can be costly. Multiplex assays are efficient but complex to design.

**Table 1.3 Tick and tickborne pathogen surveillance methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological Identification of Ticks</td>
<td>Ticks can be identified based on their physical characteristics, such as size, color, shape, and the presence or absence of specific structures like mouthparts, scutum (shield-like)</td>
<td>Can provide precise identification when done by experts.</td>
<td>Time consuming and error-prone. Requires expertise and experience.</td>
</tr>
<tr>
<td>Method</td>
<td>Description</td>
<td>Advantages</td>
<td>Limitations</td>
</tr>
<tr>
<td>---------------------------------</td>
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<td>----------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Culture and Isolation</td>
<td>Historically used for identifying tick-borne agents through microscopy and isolation in culture (Burgdorfer et al., 1982; Cowdry, 1925).</td>
<td>May allow for the isolation and study of live pathogens.</td>
<td>Time consuming and requires specialized facilities. Not all pathogens can be grown in culture.</td>
</tr>
<tr>
<td>Serological Tests</td>
<td>Serological tests detect the presence of antibodies produced by hosts in response to tick-borne pathogens. ELISA and IFA are commonly used. IFA can detect pathogens in tick or host tissues (Mans et al., 2015; Raoult &amp; Roux, 1997).</td>
<td>Indirectly indicates host exposure to specific pathogens. Useful for identifying intracellular pathogens.</td>
<td>Some tests may cross-react with antibodies from other pathogens, leading to false positives.</td>
</tr>
<tr>
<td>Polymerase Chain Reaction (PCR)</td>
<td>A sensitive molecular technique that amplifies and detects pathogen DNA or RNA. Uses specific primers to target genetic markers unique to the pathogen (B. J. Johnson et al., 1992; Steiner et al., 1999).</td>
<td>Highly specific and sensitive. Can detect various tick-borne pathogens.</td>
<td>Limitations in multiplexing multiple targets in a single reaction</td>
</tr>
<tr>
<td>Loop Mediated Isothermal Amplification (LAMP)</td>
<td>Amplifies DNA under isothermal conditions. Faster and simpler than PCR. Can be used for on-site detection (Houmansadr et al., 2020; Noden et al., 2018).</td>
<td>Faster and simpler than PCR. Suitable for on-site detection.</td>
<td>Limitations in multiplexing multiple targets in a single reaction</td>
</tr>
<tr>
<td>Targeted Sequencing</td>
<td>Involves selective amplification and sequencing of specific regions of tick or pathogen genomes for accurate detection (Osikowicz et al., 2023; Sanchez-Vicente et al., 2022).</td>
<td>Highly accurate and sensitive detection.</td>
<td>Requires prior knowledge of target regions.</td>
</tr>
</tbody>
</table>
Metagenomic Sequencing

Sequence genetic material present in tick samples without target-specific amplification. Can identify various microorganisms, including pathogens and novel ones.

Can identify a wide range of microorganisms

Can be expensive for surveillance purposes.

Multiplex Assays

Used to simultaneously detect multiple pathogens in a single tick sample, offering efficiency and cost-effectiveness (Michelet et al., 2014; Nigrovic et al., 2023).

Efficient and cost-effective for detecting multiple pathogens.

Designing and optimizing multiplex assays can be complex.

As illustrated in Table 1.3, many methods are developed and used for detection of pathogens for tickborne diseases surveillance. Despite significant progress, a comprehensive, cost-effective, and rapid detection method that can identify all major tick species and their associated pathogens remains elusive. As a result, tickborne diseases surveillance remains a challenging task for researchers and public health officials. The diversity of tick species and the wide range of pathogens they carry create a complex and constantly evolving landscape that requires continuous monitoring and surveillance efforts. While various surveillance techniques have been developed, each with its advantages and limitations, none can fully meet the demands of detecting all tick species and pathogens in an efficient and economical manner. Timely and accurate surveillance enables the identification of high-risk areas, potential outbreaks, and emerging threats, allowing for the implementation of targeted control and prevention measures.
I.3 Controlling Transmission Of Tickborne Diseases

Tickborne diseases can be challenging to control due to the complex ecology of ticks and the diversity of pathogens they carry. However, various strategies can be employed to reduce the risk of tickborne diseases transmission and minimize human exposure to infected ticks. Efforts towards preventing tick-borne infections in humans, like vaccines and repellents, only benefit the individual using them and do not impact disease reservoirs. For effective long-term control, interventions must target the source, either the tick vector or reservoir hosts. By interfering with the life cycle of *B. burgdorferi* or any other tickborne pathogen, these approaches have the potential to permanently alter the disease's incidence and distribution. Below are some promising strategies to disrupt the trajectory of tickborne pathogen infections (Bernard et al., 2020).

I.3.1 Vectors Targeted Strategies

a) Acaricides: Tick-borne pathogens like *B. burgdorferi* rely on ticks as a bridge to transmit from reservoir hosts to humans. Targeting the vector (ticks) rather than specific pathogens offers the advantage of reducing all diseases transmitted by the vector. Reduction of tick populations through acaricides has been recommended, but environmental spraying showed limited impact on Lyme disease transmission when used only on homeowners' properties (Hinckley et al., 2016). Targeted applications, like treating deer with acaricides using a "four-poster device," can decrease
tick populations (Stafford et al., 2009) and potentially reduce human Lyme disease cases (Garnett et al., 2011). However, acaricide resistance is a concern (Eiden et al., 2015; D. B. Thomas et al., 2020), and acaricides can have negative effects on non-target organisms and the environment (van Wieren et al., 2016). Bait boxes and acaricide-impregnated nesting materials have been used to target ticks on mice, but their effectiveness in preventing human disease varies (White & Gaff, 2018). Considering the potential resistance and environmental impact, the benefits of acaricide use need to be carefully assessed.

b) Biopesticides and Biocontrol: Various acaricide strategies have been explored, including natural agents that offer a less toxic approach to tick control. However, some of these natural acaricides and repellents, such as essential oils, garlic, and nootkatone, may have limited effectiveness due to short duration of action or high production costs (Faraone et al., 2019; Jordan et al., 2012; Machtinger & Li, 2017; Nchu et al., 2016). Alternative methods involve interfering with tick mating using sex pheromones or employing traps to capture pests. Successful trials have been conducted on *Rhipicephalus sanguineus* ticks using a gold nanoparticle assembly of a pheromone complex as bait or vapor patches dispersing pheromones (Anish et al., 2017; Gowrishankar et al., 2019). However, no similar products have been developed for *Ixodes* ticks yet.
Another approach involves microbial controls, where natural and engineered fungi, bacteria, and viruses are studied for their tick-killing properties. For instance, the fungus *Metarhizium brunneum*, has shown promise in killing ticks and has less impact on non-target species compared to chemical acaricides (Bharadwaj & Stafford, 2010; Fischhoff et al., 2017). Entomopathogenic nematodes are being considered for *Rhipicephalus microplus* (de Mendonça et al., 2019), and the parasitoid wasp *Ixodiphagus hookeri* shows potential for controlling larval and nymphal stages of *Ixodes* ticks (Krawczyk et al., 2020). Bacterium *Bacillus thuringiensis* has been shown to be toxic to *Ixodes* and *Dermacentor* ticks (Szczepańska et al., 2018).

c) Anti-Tick Vaccines: *Ixodes* ticks are known to carry multiple pathogens, and targeting the vector through vaccination could potentially prevent multiple diseases simultaneously. Research using tick antigens to interfere with tick feeding has shown promise in laboratory settings. For example, a commercial vaccine based on the Bm86 protein from *Boophilus* ticks has been successful in protecting cows against tick feeding (Fragoso et al., 1998). In the case of *Ixodes* ticks, various proteins like subolesin, salivary proteins, tick salivary lectin pathway inhibitor, and tick histamine release factor have been identified as potential vaccine candidates (Bensaci et al., 2012; Narasimhan et al., 2020; Schuijt et al., 2011). However, their effectiveness in preventing disease transmission has been moderate at
best. The co-evolution of ticks and their natural hosts has subjected some of these proteins to high evolutionary pressure, making their development as vaccine targets more challenging than for non-natural hosts like humans. To improve the efficacy of anti-tick vaccination, a combination of several moderately effective antigens with different functions may be explored (Rego et al., 2019).

I.3.2 Reservoir Targeted Approaches

a) Antibiotics: The use of reservoir-targeted antibiotics has proven to be highly effective in reducing the carriage of tick-borne pathogens. For example, deploying doxycycline hyclate baits targeting mice led to a significant reduction in the percentage of B. burgdorferi infected small mammals and nymphal ticks in treated areas. The infection rates were reduced by 89.6% and 94.3% respectively after two years of treatment (Dolan et al., 2011). The strategy also showed a reduction of Anaplasma phagocytophilum carriage by 74% in mice and 92% in nymphal ticks (Dolan et al., 2011).

Despite these promising results, the use of a doxycycline-based strategy raises concerns about the development of antibiotic resistance. Doxycycline is not only used to treat tick-borne infections like Anaplasmosis and Rocky Mountain spotted fever, but it is also effective against other bacterial infections, including methicillin-resistant
Staphylococcus aureus (MRSA). There are worries that widespread distribution of doxycycline could lead to the development of resistance in non-target bacteria. To address this concern, substituting more narrow-spectrum antibiotics that are not critical for treating human diseases may offer a potential solution (Bernard et al., 2020).

b) Reservoir-Targeted Vaccination: Vaccines play a crucial role in preventing diseases in humans. Since the previously available human vaccine for Lyme disease is no longer available efforts are being made to develop newer versions of the Lyme disease vaccine. Currently, efforts to develop a multivalent vaccine targeting B. burgdorferi outer surface protein A (OspA), effective against a wide range of pathogenic Lyme borreliosis Borrelia species are being made (Comstedt et al., 2017; Nayak et al., 2020). A multivalent outer surface protein C (OspC) construct, approved for vaccination of dogs, is the only other protein that is under consideration for human vaccine development (Izac et al., 2020). Since both OspA and OspC are highly variable proteins, developing a vaccine effective against all B. burgdorferi strains is challenging.

The use of the human OspA vaccine in wild reservoirs, particularly Peromyscus mice, has shown a decrease in infection among nymphal ticks when administered subcutaneously (Tsao et al., 2004). Attempts have been made to deliver the OspA vaccine orally, using recombinant
protein or viral vectors. However, the recombinant protein has low immunogenicity, and the use of live viral vectors raises environmental concerns (Bernard et al., 2020).

I.3.3 Future Strategies

a) Genetic Engineering for Reservoir and Vector Incompetence: The advent of CRISPR/Cas technologies allows for targeted engineering of mice. Gene-drive technology, which passes along engineered traits dominantly to offspring, could be used to create B. burgdorferi resistant mice expressing an anti-OspA antibody (Buchthal et al., 2019). This could change the reservoir competence of an entire mouse population. However, concerns about controlling unforeseen events caused by self-replicating engineered mutations present challenges in implementing this approach.

Similar genetic engineering strategies could be applied to ticks, such as engineering ticks to be of a single sex or eliminating specific proteins required for vector competence. These approaches have shown promise in controlling other disease vectors, like mosquitoes for dengue virus (Buchman et al., 2020). However, due to the complex 2-year life cycle of Ixodes ticks, implementing gene-drive approaches for tick control may pose difficulties.
b) Targeting Nutritional Vulnerabilities of *B. burgdorferi*: *B. burgdorferi* possesses a minimal genome and heavily relies on its environment for essential nutrients (Fraser et al., 1997). Unlike many bacteria, it cannot synthesize fatty acids and cholesterol and instead depends entirely on host lipids found in the blood meal. The bacterium also manipulates the tick’s metabolism, suggesting its need to take over tick pathways (Cabezas-Cruz et al., 2019; Hoxmeier et al., 2017). These unique vulnerabilities may offer potential targets for therapeutic intervention. For instance, in a study by Chakraborti et al., it was demonstrated that the use of inhibitory nucleoside analogs to target the purine salvage pathway of *B. burgdorferi* led to the killing of the organism. (Chakraborti et al., 2020). This approach may hold promise for developing new treatment strategies against *B. burgdorferi*.

c) Tick Immunity and the Microbiome: The role of the tick and vertebrate host microbiome in the transmission of organisms is a developing area of research. Studies in mosquitoes have associated features of the human skin microbiome with susceptibility to insect bites (Verhulst et al., 2011). Repellents based on modulating the skin microbiome are being developed for certain disease vectors (Ramírez et al., 2020). It is unknown whether similar effects could repel *Ixodes* ticks by altering the mouse microbiome.
The tick immune system also influences the tick microbiome, and certain tick proteins like Protein of *I. scapularis* with a Reeler domain (PIXR), have been shown to alter the gut microbiome, which in turn affects colonization by pathogenic spirochetes. *Anaplasma phagocytophilum*, another tick-borne pathogen, perturbs the tick gut microbiome to facilitate colonization by inducing a tick protein called *Ixodes scapularis* antifreeze glycoprotein (Abraham et al., 2017). A study showed that *B. burgdorferi* requires high microbiome diversity and thus a well-developed peritrophic membrane in the tick gut to efficiently colonize the tick. Altering the tick microbiome could therefore be a strategy to stop colonization and transmission of *B. burgdorferi* (Narasimhan et al., 2014a).

I.4 Tick Microbiome And Its Association With Tickborne Pathogens

The tick microbiome and its associations with specific tick-borne pathogens have received significant attention in recent years. As discussed in section I.1, ticks are notorious vectors of various pathogens, including bacteria, viruses, and parasites, responsible for transmitting numerous diseases worldwide. However, like other organisms, they also harbor a diverse community of other microorganisms, such as bacteria, viruses, and eukaryotes, collectively referred to as the tick microbiome. As previously discussed in section I.3.3, the tick
microbiome can influence pathogen colonization and transmission, making it a promising avenue for future disease control. Therefore, a thorough investigation of tick microorganisms is crucial.

The advent of next-generation sequencing technologies has revolutionized the study of tick microbiomes. It has become increasingly evident that the microbiome can play crucial roles in tick biology, influencing aspects such as development, reproduction, immunity, and pathogen transmission. Here I provide an overview of the tick microbiome, its composition, dynamics, and known interactions with tickborne pathogens.

I.4.1 Origin And Acquisition Of Tick Microorganisms

Microbial diversity within ticks is greatly influenced by environmental factors, tick species (Lalzar et al., 2012; Van Treuren et al., 2015), tick sex (Duncan et al., 2022; Thapa et al., 2019; Van Treuren et al., 2015), geographical regions (Duncan et al., 2022; Gall et al., 2017; Trout Fryxell & DeBruyn, 2016; Van Treuren et al., 2015), tick life stages (Clay et al., 2008; Moreno et al., 2006; Williams-Newkirk et al., 2014), and feeding status (Heise et al., 2014; Menchaca et al., 2013; Zhang et al., 2014). The microorganisms found in ticks exhibit both taxonomic and ecological diversity, with a wide range of lifestyle strategies for infecting and persisting within tick populations (S. I. Bonnet et al., 2017). They employ various lifestyle strategies, ranging from infectious transmission through tick bites to vertical transmission and maternal transmission enabling persistence.
within tick populations. Certain tick microorganisms rely on maternal transmission, from a tick to its offspring via egg, to ensure persistence across generations of ticks. Moreover, environmental microorganisms can also colonize ticks, either from vertebrate skin surfaces during blood feeding or from the soil and vegetation once ticks drop off their hosts. Tickborne pathogens are primarily acquired \textit{de novo} by ticks via blood feed on animal hosts, primarily rodents, which are reservoirs of the tickborne pathogens. Whereas some tickborne pathogens, like \textit{Babesia} species, \textit{Rickettsia} species, and \textit{Anaplasma} species, can be transmitted vertically across generations, allowing the tick to serve as both a vector and a host (Ravindran et al., 2023). This diverse array of microbial interactions contributes to the complexity of tick microbiomes.

In ticks, as in many arthropod taxa, maternally inherited symbionts employ specific adaptive strategies to persist within their host populations. These symbionts can be classified into two main categories: obligate mutualistic symbionts and facultative symbionts. The first category comprises obligate mutualistic symbionts that are essential for normal host development and provide crucial functions, such as nutritional upgrades by supplying biosynthetic pathways that are absent in the host. For example, in the lone star tick, \textit{Coxiella}-like endosymbiont is a potential source of vitamin not readily available in sufficient quantities from their blood-based diet (Smith et al., 2015). The second category consists of facultative symbionts, which are not necessary for host survival. Some of these symbionts act as defensive agents, providing protection
against natural enemies or heat. Others function as reproductive parasites, manipulating host reproduction by inducing parthenogenesis, feminizing genetic males, causing male-killing, or inducing cytoplasmic incompatibility (S. I. Bonnet et al., 2017).

I.4.2 Tick Microbiome And Tick Biology
The effect of tick microbiome on tick biology is significant and has been increasingly recognized in recent studies. Symbiotic interactions between ticks and microorganisms play a crucial role in normal tick biology and ecological specialization to a blood diet. Tick symbionts, including maternally inherited bacteria, are remarkably diverse, with at least 10 distinct genera identified in ticks(Duron et al., 2017). Tick symbionts associated with each of the most common tick species in the USA are summarized in Table 1.4.

One of the most widespread and biologically relevant tick symbionts is Coxiella-LE, which is found in various tick species (Almeida et al., 2012; Duron et al., 2017; Jasinskas et al., 2007; Lalzar et al., 2012; Machado-Ferreira et al., 2011). It is crucial for the survival and reproduction of the Amblyomma americanum, the lone star tick (Zhong et al., 2007a). This symbiont shows pronounced tissue tropism in various tick species, primarily infecting the ovaries, enabling maternal transmission, and the distal part of Malpighian tubules, suggesting potential for impacts on nutrition, osmoregulation, and excretion (S. I. Bonnet et al., 2017). Coxiella-LE is transmitted to over 99% of tick progeny,
ensuring its persistence within tick populations (Lalzar et al., 2014; Machado-Ferreira et al., 2011). The Coxiella-LE genome encodes protein active in synthesis of major B vitamins that are not typically obtained in sufficient quantities from a blood-based diet (Gottlieb et al., 2015; Smith et al., 2015). This nutritional upgrading has allowed ticks to adapt to their hematophagy lifestyle, enabling them to subsist on an unbalanced dietary resource (S. I. Bonnet et al., 2017).

Recent studies have suggested the existence of alternative obligate symbionts in some tick species. These symbionts, such as Francisella-LE and Rickettsia, may also play a vital role in tick survival by encoding genetic capabilities for B vitamin synthesis in A. maculatum (Gerhart et al., 2016) and Ixodes species (Ixodes scapularis and I. pacificus (Hunter et al., 2015) respectively. The presence of beneficial Rickettsia symbionts has been shown to influence tick larval motility in A. americanum, Dermacentor variabilis, and I. scapularis ticks. Additionally, some tick-borne pathogenic bacteria, including Anaplasma phagocytophilum and Ehrlichia chaffeensis, can also synthesize essential vitamins, potentially contributing to tick adaptation (Dunning Hotopp et al., 2006).

Along with obligate symbionts, ticks commonly harbor facultative symbionts belonging to a variety of bacterial genera. The role of facultative symbionts in ticks is not fully understood, but recent findings suggest various effects on tick biology like tick motility, reproduction, and adaptation to blood feeding. However,
their exact roles and effects on tick health and behavior require further research (S. I. Bonnet et al., 2017).

### Table 1.4 Symbionts associated with common hard tick species

<table>
<thead>
<tr>
<th>Tick vector</th>
<th>Predominant endosymbionts</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ixodes scapularis</em></td>
<td><em>Rickettsia buchneri</em></td>
<td>(Al-Khafaji et al., 2020; Oliver et al., 2021)</td>
</tr>
<tr>
<td><em>Ixodes pacificus</em></td>
<td><em>Rickettsia buchneri</em>-like (GO21)</td>
<td>(Bagheri et al., 2017; Kwan et al., 2017)</td>
</tr>
<tr>
<td><em>Dermacentor andersoni</em></td>
<td><em>Rickettsia peacockii, Rickettsia bellii, Francisella spp, Arsenophonus spp</em></td>
<td>(Gall et al., 2016)</td>
</tr>
<tr>
<td><em>Amblyomma americanum</em></td>
<td><em>Coxiella-like, Rickettsia spp</em></td>
<td>(Klyachko et al., 2007a; Maldonado-Ruiz et al., 2021)</td>
</tr>
<tr>
<td><em>Amblyomma maculatum</em></td>
<td><em>Francisella-like endosymbiont and Candidatus Midichloria mitochondrii</em></td>
<td>(Budachetri et al., 2014, 2018)</td>
</tr>
<tr>
<td><em>Haemaphysalis longicornis</em></td>
<td><em>Coxiella-like, Francisella-like</em></td>
<td>(M. Wang et al., 2018; Y. Wang et al., 2018)</td>
</tr>
<tr>
<td><em>Dermacentor variabilis</em></td>
<td><em>Francisella spp., Sphingomonas spp., Delftia spp., Hymenobacter spp.</em></td>
<td>(Travanty et al., 2019)</td>
</tr>
<tr>
<td><em>Rhipicephalus sanguineus</em></td>
<td><em>Coxiella spp., Rickettsia spp., Bacillus spp.</em></td>
<td>(René-Martellet et al., 2017)</td>
</tr>
</tbody>
</table>
I.4.3 Tick-Microbiome-Pathogen Interactions

Tick-microbiome-pathogen interactions have been investigated across various tick species, shedding light on these complex relationships. In *Ixodes scapularis* ticks, unfed larvae hatched and raised under sterile conditions had altered bacterial microbiome and reduced microbiome diversity. This dysbiosis led to altered tick gut peritrophic membrane integrity via decreased expression of STAT, resulting in decreased *B. burgdorferi* colonization, suggesting a higher microbiome diversity is needed for the *B. burgdorferi* to colonize in the tick gut (Narasimhan et al., 2014). However, observations from ticks collected in the wild have been mixed. Some studies have reported that the presence or absence of *B. burgdorferi* in *I. scapularis* ticks correlates with variations in bacterial diversity (Landesman et al., 2019; J. L. H. Sperling et al., 2020). On the other hand, other studies have found no correlation between the microbiome diversity of infected and uninfected ticks. Instead, these studies have revealed notable associations between the persistence of spirochetes and specific microbial taxa (Brinkerhoff et al., 2020; Chauhan et al., 2019). These findings suggest that *B. burgdorferi* requires a specific gut microbial environment for successful colonization, though the intricate mechanisms governing these interactions remain to be fully elucidated (Kurokawa et al., 2020).

The interaction between *B. burgdorferi* and the tick gut microbiome is facilitated by tick gut proteins, as evidenced by studies involving RNA interference. When the gene encoding Protein of *I. scapularis* with a Reeler domain (PIXR), a
secreted gut protein, was silenced using RNA interference or when mice were immunized against PIXR, there was a significant reduction in *B. burgdorferi* colonization in the tick gut. This suggests that the bacterium induces PIXR to enhance its colonization within the tick (Narasimhan et al., 2017). In vitro and in vivo experiments indicated that PIXR has an inhibitory effect on bacterial biofilm formation, leading to the possibility that alterations in biofilm formation could influence the adherence of spirochetes to the gut epithelium (Narasimhan et al., 2017). Dysbiosis of the tick gut microbiome disrupts the formation of the peritrophic matrix (PM), a glycan-rich structure that lines the gut epithelial cells. The diminished expression of a key structural component of the PM, known as peritrophin, mediated by the STAT protein, results in a thinner and permeable PM. This disruption reduces the ability of *B. burgdorferi* to colonize the tick gut, suggesting that the PM plays a critical role in protecting spirochetes from harmful substances within the gut environment (Narasimhan et al., 2014a).

While these studies shed light on the interactions between the tick, its microbiome, and *B. burgdorferi*, there are still other unexplored mechanisms through which the tick microbiome may influence the persistence of *B. burgdorferi* in the gut. Notably, the genome of *B. burgdorferi* lacks several genes necessary for the synthesis of essential nutrients, making it dependent on its tick vector and vertebrate host for these nutrients (Kurokawa et al., 2020). Hence, gut endosymbionts or commensals in ticks may play a vital role in supporting the survival of spirochetes by providing the deficient nutrients. Additionally, *Borrelia*
spirochetes might actively manipulate the microbial composition to create a favorable environment for their colonization. The infection could induce the expression of specific genes coding for antimicrobial peptides, altering the tick microbiome composition in favor of the spirochetes' establishment in the gut. An example of this is the antimicrobial molecule Dae2, found in *I. scapularis* ticks, which selectively kills harmful mammalian skin microbes without affecting *B. burgdorferi* (Hayes et al., 2020). Further research is needed to fully elucidate these complex networks of interactions between the tick microbiome and *B. burgdorferi* in the gut.

In contrast, *Anaplasma phagocytophilum*, another pathogen carried by *Ixodes scapularis*, has distinct requirements for successful colonization. It necessitates a thin and permeable peritrophic matrix (PM) for rapid passage from the tick guts to the salivary glands. When *A. phagocytophilum* infects ticks, it induces the expression of tick antifreeze glycoprotein. This protein binds to Gram-positive bacterial peptidoglycan, resulting in the alteration of bacterial biofilm formation and a reduction in the abundance of such taxa within the tick microbiome. This antimicrobial activity plays a pivotal role in the modulation of the tick gut microbiome and decreases the structural integrity of the PM, thereby facilitating *A. phagocytophilum* colonization (Abraham et al., 2017).
Interactions between tick microbiota and pathogenic bacteria in tick species other than *Ixodes* have been relatively understudied. However, some research has shed light on these relationships as listed in Table 1.5. Disruptions in the tick microbiome caused by antibiotics can influence pathogen susceptibility in *D. andersoni*: A negative correlation between *Rickettsia bellii* and *Anaplasma marginale* burden, as well as a positive correlation between *Francisella*
endosymbionts and Francisella novicida infection levels was observed (Gall et al., 2016). Similarly, a negative relationship between Francisella-like endosymbiont levels and Spotted Fever Group Rickettsia was observed in D. occidentalis, suggesting possible interference between Francisella-like endosymbiont and Spotted Fever Group Rickettsia in this tick species (Gurfield et al., 2017). A study by Budachetri et al. (2018) indicated that decreased Francisella-like endosymbiont levels and increased Candidatus Midichloria mitochondrii levels were associated with Rickettsia parkeri infection in A. maculatum. In a study by Burgdorfer et al., found that an ovary infection of R. peacockii resulted in limited R. rickettsii infection (Burgdorfer et al., n.d.). Subsequently, it was demonstrated that, in D. variabilis, R. montana prevented both the establishment and vertical transmission of the related Rickettsia, R. rhipicephali (Macaluso et al., 2002).

The mechanisms underlying the regulation of pathogen infection by endosymbiont bacteria remain relatively unclear, but it has been hypothesized that endosymbionts may directly or indirectly impact pathogen growth. Direct mechanisms could involve endosymbionts secreting molecules that either enhance or limit pathogen replication, while indirect mechanisms might include competition for essential host resources, restricting pathogen replication, or inhibiting immune factors that promote pathogenic bacteria growth (Gall et al., 2016).
The study of the tick microbiome has opened exciting avenues of research with profound implications for our understanding of tick physiology, vector capacity, and tickborne pathogen transmission. The complex interactions between ticks, their microbial communities, and the pathogens they carry are critical factors in shaping the epidemiology and dynamics of tickborne diseases.

1.5 Goals Of This Thesis

The burden of tickborne diseases is increasing as ticks and their associated pathogens spread. To unravel the complex and dynamic interactions within these ecosystems, we require extensive data. By studying individual ticks' interactions at a single-tick resolution, rather than on a population level, we can gain a deeper understanding of specific tick-pathogen-microbiome interactions. This level of detail is crucial for comprehending the patterns of tick and pathogen spread into new areas, identifying novel tick-pathogen associations, detecting co-infections, and designing effective interventions. Addressing this pressing issue necessitates high-throughput methods in tickborne disease surveillance and the investigation of associations among ticks, pathogens, and commensal microbes at a single-tick resolution.

In this thesis, I discuss my work to address these challenges. I begin by introducing Project Acari, a citizen science project, which allows for the rapid collection of a large number of ticks from various locations across the United States. Next, I discuss how I developed a method using Molecular Inversion
Probes (MIP) for rapid and economical tickborne diseases surveillance. Using this method, I show it is feasible to monitor changes in the distribution and abundance of ticks and assess the presence and prevalence of tickborne pathogens. This data can be used to provide actionable, evidence-based information on infection risk to clinicians, the public, and policy makers. Finally, I introduce a new full length 16S and 18S rDNA sequencing method that provides comprehensive insights into the tick microbiome, including symbiotic bacteria, protozoa, and fungi. This data will guide our understanding of the role of the tick microbiome in tick physiology, vector capacity, and the transmission of tickborne pathogens, potentially paving the way for interventions to control pathogen transmission by modulating the tick microbiome.

Together, nationwide collection of ticks through Project Acari, pathogen assessment via MIPs, and microbiome characterization by 16S and 18S rDNA sequencing data provide previously inaccessible insights on the association among ticks, their pathogens, and their microbiome. These comprehensive insights will be instrumental in formulating approaches to modulate the tick microbiome, aiming to reduce the vectorial capacity of ticks by impeding pathogen acquisition, development, and horizontal and vertical transmission. This data is crucial for guiding efforts to disrupt the transmission of tick-borne pathogens, ultimately leading to a decrease in the occurrence of tick-borne diseases and associated morbidity and mortality. The subsequent chapters of this thesis will delve deeper into the methods, findings, and implications of these
cutting-edge approaches, with the aim of contributing to the advancement of our knowledge and the improvement of public health strategies in combating tickborne diseases.
CHAPTER II: CITIZEN SCIENCE ENABLES EXPLORATION OF VECTOR-PATHOGEN DYNAMICS IN TICKBORNE DISEASE AT SINGLE-TICK RESOLUTION
II. 1 Preface

The findings discussed in this chapter have been published previously:
Chauhan G., McClure J., Hekman J., Marsh P. W., Bailey J. A., Daniels R. F.,
Genereux D. P., & Karlsson E. K. (2020). Combining citizen science and
genomics to investigate tick, pathogen, and commensal microbiome at
single-tick resolution. Front Genet, 10:1322.
https://doi.org/10.3389/fgene.2019.01322

The Project Acari website and SQL database was set up by Jesse McClure and
maintained by Jessica Hekman. 16S microbiome sequencing was performed at
Umass Chan Medical School Sequencing core. I performed all the experiments
and analysis described in this chapter.
II.2 Abstract

The prevalence of tickborne diseases worldwide is increasing and virtually unchecked, due to the lack of effective control strategies. Transmission dynamics of tickborne pathogens are influenced by the tick microbiome, tick co-infection with other pathogens, and environmental features. Understanding this complex system could lead to new strategies for pathogen control, but requires large-scale, high-resolution data. Here, we introduce Project Acari, a citizen science-based project to assay, at single-tick resolution, species, pathogen infection status, microbiome profile, and environmental conditions for tens of thousands of ticks collected from numerous sites across the United States. In the first phase of the project, we collected more than 2400 ticks wild-caught by citizen scientists and developed novel, high-throughput methods to process and sequence them individually. Applying these methods to 192 Ixodes scapularis ticks collected in a region with high incidence of Lyme disease, we found 62% colonized by Borrelia burgdorferi, the Lyme disease pathogen. In contrast to previous reports, we did not find an association between the microbiome diversity of a tick and its probability of carrying B burgdorferi. However, we did find undescribed associations between B. burgdorferi carriage and the presence of specific microbial taxa within individual ticks. Our findings underscore the power of coupling citizen-science with high-throughput processing to reveal pathogen
dynamics. Our approach can be extended for massively parallel screening of individual ticks, offering a powerful tool to elucidate the ecology of tickborne disease and to guide pathogen-control initiatives.

II.3 Introduction

Tickborne diseases are an emerging public health threat in the United States and worldwide (Institute of Medicine (US) Committee on Lyme Disease & Other Tick-Borne Diseases: The State of the Science, 2011; Paules et al., 2018)(Table 2.1). The US Centers for Disease Control and Prevention (CDC) lists seven tick species known to transmit 19 human pathogens in the US alone (Tickborne Diseases of the United States | Ticks | CDC, n.d.). The CDC recorded a total of 59,349 confirmed and probable tickborne diseases cases in 2017, up from 22,527 cases in 2004, and the real burden of tickborne infections is estimated to exceed reported cases by as much as ten-fold (Christina A. Nelson et al., 2015; Schiffman et al., 2018).

In parallel, the geographic distribution of ticks and the pathogens they carry is expanding, and new pathogens continue to emerge (Paddock et al., 2016; Rosenberg et al., 2018). *Ixodes scapularis*, the blacklegged tick that transmits Lyme Disease, is found in ~50% more US counties than in 1996, and Lyme disease is now widespread throughout New England and the Midwest (Paules et al., 2018). Cases of spotted fever rickettsiosis have been reported throughout the contiguous United States. Eight new tick-borne pathogens have been identified
since 2010 in the US (S. Bonnet et al., 2014; Tokarz et al., 2014; Vayssier-Taussat et al., 2013). Indeed, it is estimated that up to half of all tickborne diseases may be caused by pathogens not previously known to be associated with ticks, or not yet identified at all (Feder et al., 2007).

**Table 2.1 CDC reports of tickborne disease caused by bacterial and protozoan pathogens increased markedly from 2004 to 2016** (Rosenberg et al., 2018)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Year 2004</th>
<th>Year 2016</th>
<th>% increase</th>
<th>Pathogens</th>
<th>Tick vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyme disease</td>
<td>19,804</td>
<td>36,429</td>
<td>84%</td>
<td>B. burgdorferi, B. mayonii</td>
<td>I. pacificus, I. scapularis</td>
</tr>
<tr>
<td>Tularemia</td>
<td>134</td>
<td>230</td>
<td>72%</td>
<td>F. tularensis</td>
<td>D. variabilis, D. andersoni, A. americanum</td>
</tr>
<tr>
<td>Spotted fever rickettsiosis</td>
<td>1713</td>
<td>4269</td>
<td>149%</td>
<td>R. rickettsii, R. parkeri, Rickettsia sp 364D, R. parkeri</td>
<td>D. variabilis, D. andersoni, R. sanguineus</td>
</tr>
<tr>
<td>Anaplasmosis / Ehrlichiosis</td>
<td>875</td>
<td>5750</td>
<td>557%</td>
<td>A. phagocytophilum, E. chaffeensis, E. ewingii, E. muris eauclairensis</td>
<td>I. pacificus, I. scapularis, A. americanum</td>
</tr>
<tr>
<td>Babesiosis**</td>
<td>1126</td>
<td>1910</td>
<td>70%</td>
<td>B. microti, B. divergens, B. duncanii, strain MO-1</td>
<td>I. scapularis</td>
</tr>
</tbody>
</table>

** Cases reported in 2011. No data for 2004.

Given the substantial and increasing impact of tickborne diseases on human health, and their expanding geographic ranges, there is an urgent need for effective control strategies. While some approaches have proven effective locally and for the short-term, few have produced clear evidence of enduring benefit. Public education campaigns to raise awareness of tick disease and promote
personal protection have little long-term impact on the incidence of disease (Beaujean et al., 2016). Targeting culling of species that help sustain tick populations, such as deer, reduces risk only in discrete areas, requires a longer time frame, and may face sociopolitical obstacles (Jordan et al., 2007; Telford, 2017). Application of acaricides, pesticides that kill organisms from the Acari (the subclass of arachnids that includes ticks), while somewhat effective in the short term, fails to decrease household risk and can lead to the evolution of resistance (George et al., 2004; Hinckley et al., 2016). Efforts to vaccinate individuals against locally prevalent tickborne pathogens have yet to be deployed at scale, and limited data are available to assess their safety, efficacy, and durability (Beaujean et al., 2016; Richardson M, n.d.). Under most circumstances, then, no single method is likely to be effective in reducing disease risk to humans and sustainable for the long term (Stafford et al., 2017).

Advances in genome editing technology are enabling powerful new control strategies. For example, species that provide reservoirs for pathogens can be genetically modified to be immune, an approach proposed to control tickborne pathogen transmission by white-footed mice on the islands of Nantucket and Martha’s Vineyard (Najjar et al., 2017). An alternative strategy, paratransgenesis, targets the microbiome of the disease vector species to reduce transmission rates. This approach has already been used to control dengue (Moreira et al., 2009; Walker et al., 2011) and has been proposed to control malaria (Hughes et
al., 2011), Chikungunya (Moreira et al., 2009; Mousson et al., 2010), Zika (Dutra et al., 2016), and Chagas disease (Durvasula et al., 1997).

At present, though, application of these approaches for controlling tickborne pathogens is impeded by the limited understanding of pathogen-transmission dynamics. Ticks are inhabited by communities of commensal and symbiotic microorganisms that can influence, or even drive, tickborne pathogen transmission in a complex web of interactions in which the same bacterial species that promotes colonization by one tickborne pathogen may impede colonization by others (S. I. Bonnet et al., 2017; Dong et al., 2009; Narasimhan et al., 2014b; B. Weiss & Aksoy, 2011; B. L. Weiss et al., 2012; Zouache et al., 2012). The tick microbiome varies by species, sex, and even by sampling location (Van Treuren et al., 2015). Studies to date have been limited by their focus on microbes from relatively small numbers of ticks collected in limited geographic areas (S. Lee et al., 2014; Salkeld et al., 2014; Sayler et al., 2017; Tokarz et al., 2014). Furthermore, many studies have pooled ticks before processing, preventing investigation of the individual-level dynamics of tick-pathogen associations fundamental to understanding disease transmission (Tokarz et al., 2014; Vayssier-Taussat et al., 2013).

Developing new approaches to effectively control tickborne diseases will require understanding, in detail, how the tick microbiome modulates the probability of pathogen carriage and transmission. Furthermore, given the rapid increase in tickborne diseases, longitudinal monitoring is critical to identify the spread of
tickborne pathogens and inform management strategies. The need for higher-resolution data is increasing rapidly as human and animal movement, as well as altered interactions of ticks with pathogen reservoirs and human populations, change the epidemiology of tickborne diseases (S. Bonnet et al., 2014).

Here, we describe Project Acari, a program designed to collect microbiome and pathogen data from large numbers of individual ticks collected from both broad geographic areas and over extended time periods. Citizen scientists mail ticks encountered during their normal activities, each labeled with a geographic location, using our inexpensive “Tick Kit”. We then extract and process DNA in 96-well plates, attaching sequence barcodes that uniquely identify the tick, microbiome, and pathogen DNA extracted from each tick. This allows us to analyze the co-occurrence of pathogens and particular microbiome species with single-tick resolution. Applying this approach to a small pilot study of *I. scapularis* ticks collected in Grafton, Massachusetts, revealed a previously unknown association between pathogen infection and particular components of the microbiome, demonstrating the potential of this approach to enable discoveries when scaled up to tens of thousands of ticks collected from a broad geographic area over extended periods of time.
II.4 Materials And Methods

II.4.1 Sample Collection

We used two different strategies for tick collection. First, for our pilot study of *I. scapularis* ticks, we needed a sample set with a high proportion of pathogen-infected *I. scapularis* ticks. We asked a small cohort of volunteers living in an area of Massachusetts with a high reported incidence of Lyme disease to place any ticks they encountered in a resealable zipper storage bag, rather than discarding them. These ticks were delivered directly to our research lab by the participants in November 2017. We confirmed by visual inspection that all collected ticks were adult *I. scapularis*.

Second, to expand the geographic range of the project, we developed a “Tick Kit” that was inexpensive to produce and ship and that conformed to the dimensions and requirements for standard U.S. Postal Service First Class Mail Letters. Each Tick Kit costs $0.60 and includes 1) a Tick Card with individual spots for 14 ticks; 2) stickers to affix and seal the ticks to the Tick Card; 3) a sealable biohazard-labeled polypropylene bag to hold the Tick Card after tick collection; and 4) a pre-paid postage sealable return envelope to send the Tick Card to us (Figure 1A). Each Tick Card is uniquely barcoded for easy tracking.

To maximize the collection of adult ticks, which have the greatest probability of carrying at least one species of pathogens and are easiest to see and, therefore,
collect, we emphasized the collection of ticks in the late fall. Project Acari started enrolling participants in October 2017 and kits were sent out in October and November of both 2017 and 2018. We advertised the project through social media (Facebook, Instagram, and Twitter), and through email to the community of citizen scientists enrolled in the Darwin’s Ark project (DarwinsArk.org). People interested in participating visited our website (ProjectAcari.org) to register and request a Tick Kit. Participation is free.

Upon receipt, each Tick Kit is incubated at -20°C for 24 h to ensure that no ticks are still alive. Each Tick Card is then scanned, its image saved, and stored at -20°C until processing. We store the Tick Card data, including the kit requestor’s name and date of kit receipt, as well information on each tick, including collection location (as zip code) and date of collection, in a custom SQL database. We update the database with tick species, infection status, and microbiome profile as information becomes available.

II.4.2 High-throughput DNA Extraction

For DNA extraction, one tick was placed into each well of a 96-well deep-well plate (Squisher-96, Zymo research Cat No.: H1004), then washed with 70% ethanol, followed by 3 washes with sterile water. Next, the Squisher-96 tool was inserted into plates, which were then placed in a container filled with liquid nitrogen and incubated for 5-10 mins. The frozen ticks were completely crushed and homogenized using the Squisher-96 tool. The homogenized ticks were
incubated in Buffer ATL and proteinase K (Qiagen Blood and Tissue Kit, Cat No.: 69581) for 3 hours at 55°C according to the manufacturer’s instructions. The DNA was then extracted following the manufacturer's protocol. The DNA concentration is measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). To avoid cross-contamination, each Squisher-96 was used only once.

II.4.3 Analysis Of 192 *Ixodes scapularis* Ticks

II.4.3.1 Screening For *Borrelia burgdorferi*.

We screened for *B. burgdorferi* by polymerase chain reaction (PCR), using primers specific to Outer Surface Protein A (ospA2 and ospA4) and Flagellin (fla1 and fla3) (Persing et al., 1990). Each 20 μl PCR reaction mixture contained 25 ng genomic DNA, 200 μM of each dNTP, 0.5 μM each forward and reverse primers, 1 μl HotStarTaq DNA Polymerase (Qiagen Cat No.: 203203), and 1X PCR buffer. Thermal amplification was performed in an Eppendorf Mastercycler Pro S (Eppendorf North America, Inc.) using the following cycling conditions: 15 min of initial denaturation at 94°C followed by 25 cycles consisting of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min and indefinite hold at 4°C. The resulting amplicons were visualized using SYBR Gold on a 2% agarose gel following electrophoresis.
II.4.3.2 16S rDNA Library Preparation.

To sequence the microbiome of each tick, we amplified the V3-V4 region of 16S rDNA and added barcodes and Illumina adapters according to Illumina’s protocol for 16S Metagenomic Sequencing Library Preparation (Illumina, 2013). The resulting libraries were quantified using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Cat No.: P11496) from ThermoFisher Scientific. For the 128 libraries (99 females, 29 males; 88 infected, 40 uninfected) yielding DNA concentrations above 10nM, we normalized the concentrations to 4nM and pooled the samples in equimolar concentrations. We sequenced the amplicons using Illumina Miseq v3 reagent kit using 10% PhiX, following the standard protocol for 16S sequencing. The resulting sequence reads were filtered for read quality, base-called, and demultiplexed using bcl2fastq (v2.20).

II.4.3.3 Quality Control And Filtering Of 16s Sequencing Reads.

We imported and processed the demultiplexed paired-end sequencing reads using the ‘quantitative insights into microbial ecology’ pipeline of Qiime2 v.2018.2.0 (Bolyen et al., 2018). The reads were denoised using the ‘divisive amplicon denoising algorithm’ DADA2 (Callahan et al., 2016) plugin in Qiime2. This step filtered out noise and corrected errors in marginal sequences, removed chimeric sequences and singletons, merged paired-end reads, and finally dereplicated the resulting sequences, resulting in high-resolution amplicon sequence variants (ASVs) for downstream analysis. ASVs assume that biological
sequences are present in the sample and can resolve single-nucleotide differences in sequence variants. Using the denoise-paired command, the DADA2 options passed were trim-left-f: 17, trim-left-r: 21, trunc_len_f: 300, trunc_len_r: 240, with all other options left as default. Additionally, ASVs with minimum sample frequency of 2 and a total minimum frequency of 10 were removed.

II.4.3.4 Assessment Of Overall Bacterial Diversity

We assessed bacterial diversity within (alpha diversity: observed ASVs, Shannon index (Shannon, 1948) and Faith PD index (Faith, 1992)) and between (beta diversity: weighted (C. A. Lozupone et al., 2007) and unweighted UniFrac (C. Lozupone & Knight, 2005)) ticks using the q2-diversity plugin in QIIME2. Principal coordinates plots of beta diversity were visualized using the EMPeror tool (Vázquez-Baeza et al., 2013). PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) analysis was used to measure the effect size and significance on beta diversity for grouping variables (Balakrishnan et al., 2014). As alpha and beta diversity metrics are sensitive to uneven sampling depths, multiple rarefactions were performed prior to computing the diversity indices. The number of ASVs per sample was randomly selected (without replacement) at an even depth of 0 to 20,000 sequences. For alpha and beta diversity analysis the ASVs were rarefied at 8,333 sequences per sample.
II.4.3.5 Taxonomic Annotation And Relative Abundance Of Identified Bacterial Taxa.

We performed taxonomic annotation of ASVs in QIIME 2 using a pre-trained Naïve Bayes classifier and the q2-feature-classifier plugin (Bokulich et al., 2018). Prior to annotation, the classifier was trained on the QIIME-compatible 16S SILVA reference (99% identity) database v.128 (Quast et al., 2013). The reference sequences were trimmed to span the amplified v3-v4 region of the 16S rDNA gene using the extract-reads command of the q2-feature-classifier plugin in QIIME2. The resulting relative abundance tables of annotated ASVs were exported into R to generate stacked barplots to visualize the relative abundance of bacterial taxa across sample types.

II.4.3.6 Identification Of Bacterial Markers Potentially Associated With B. burgdorferi Infection.

To identify possible bacterial taxa associated with B. burgdorferi infection, we used the linear discriminant analysis (LDA) effect size method (LEfSe) v.1.7 (Segata et al., 2011). Relative abundance tables of annotated ASVs were imported into LEfSe. B. burgdorferi infection was entered as the class vector (the main condition under investigation) and the sex of each tick as a subclass (as a biologically meaningful grouping within each class).

To identify associated taxa, LEfSe first uses pairwise non-parametric factorial Kruskal Wallis sum-rank tests to detect ASVs with significant differential
abundance between classes. Then, for biological consistency, it performs pairwise unpaired Wilcoxon rank-sum tests to compare differentially abundant ASVs from the previous step between subclasses (gender). The default p-value of <0.05 was used for both tests and ASVs with consistent significant differential abundance among the two classes and across both subclasses were considered possible taxa associated with Borrelia infection. Lastly, the associated taxa were used to build a latent Dirichlet allocation (LDA) model from which the relative differences among classes were ranked to obtain the effect size. The LDA model was built using default parameters and the resulting logarithmic scores of the analyses are presented. An LDA score of ≥2.0 was set as the threshold to retain any taxa.

II.5 Results

In the first phase of Project Acari, we sought to demonstrate the potential of large-scale, citizen-science as a tool for investigating the dynamics of tickborne diseases. Our initial tick collection, focused on an area of Massachusetts with a high rate of Lyme disease, yielded the 192 I. scapularis ticks used in our pilot genomics project. We then extended Project Acari to the entire United States using mail-in Tick Kit cards provided for free. Finally, we developed high-throughput methods for processing ticks using 96-well plates, enabling rapid scale-up.
II.5.1 Tick Collection By Citizen Scientists

We completed a two-stage trial launch in October 2017 and 2018, receiving requests for 1027 kits. Our kit is designed to be inexpensive to produce and mail and easy for citizen scientists to use and return (Figure 2.1A). Each kit has space for 14 ticks labeled with collection data and zip code, and we specifically request unengorged ticks (Figure 2.1B). As of January 15, 2019, 31% (322) of kits have been returned from 302 zip codes and 32 states, with an average of 7.5 ticks/card. (Figure 2.1C). Of the 2417 total ticks received, 2171 (90%) were unengorged and 246 (9.9%) were engorged. Most (1996 ticks; 82.5%) were collected in the late fall season (August - November), and the remainder either during the winter (December - March; 126 ticks) or spring (April - June; 295 ticks). Visual inspection of a subset of Tick Cards showed that while the majority of ticks appeared to be adult *I. scapularis*, the cards also included other life stages and other species, including *Dermacentor variabilis* (American dog tick), *Amblyomma americanum* (lone star tick), *Ixodes pacificus* (Western blacklegged tick), and *Rhipicephalus sanguineus* (Brown dog tick) (Figure 2.1D). In 29 zipcodes, ticks were collected in both 2017 and 2018, highlighting the potential for longitudinal sampling.
Figure 2.1 Citizen science approach enables large-scale collection of many tick species from a broad geographic area. (A) Our custom-designed “Tick Kit” includes a Tick Card, clear stickers to adhere ticks to the Tick Card, as well as a sealable biohazard bag and prepaid plastic envelope for return shipping. All components fit into standard first-class mail envelopes for fast and inexpensive shipping in both directions. (B) A returned Tick Card with 14 ticks, the maximum capacity of the Tick Card, each sealed on the card with a clear sticker and labeled by the volunteer with collection data and location (zip code). Each Tick Card has a unique barcode and instructions are printed on the card adjacent to the tick collection section. (C) Over 900 people volunteered for Project Acari during two recruitment windows (October and November of both 2017 and 2018); we received 322 Tick Cards with 2417 ticks from 32 states. Most (2027 ticks) came from states in the northeast (inset), the geographic region with the highest rates of TBD in the United States. (D) Visual inspection of a subset of tick cards suggested that, while most of the ticks collected were adult female (left) or male (right) *Ixodes scapularis*, we also received (E) diverse life stages, including adults (left) and nymphs (right) as well as other species, including (F) female and male *Amblyomma americanum* (lone star tick) and (G) female and male *Dermacentor variabilis* (American dog tick).
II.5.2 Higher-Throughput Tick DNA Extraction

We developed a method for efficient extraction of DNA from large numbers of individual ticks. Initially, we tried bead beating, the standard approach for extracting tick DNA. While this method is effective for extraction from small numbers of ticks in screw-cap tubes using homogenization speeds ~4000 rpm, it could not be scaled up to permit extraction within the 96-well format, and so was not amenable for higher-throughput processing. Commercially available homogenizers that accommodate 96-well plates generally have a maximum speed of 1600 rpm. At these speeds, the hard exoskeletons of ticks remain intact after ten minutes of bead beating. We therefore developed an alternative approach to address this challenge. We first froze the ticks by placing the 96-well plate containing the ticks in liquid nitrogen and then manually homogenized the ticks using the 96-well Zymo Research Squisher-96.

This method enabled DNA extraction both from ticks collected locally through our pilot project and from ticks mailed on Tick Cards from various locations throughout the United States, then stored at -20°C for up to one year. Among the 192 adult *I. scapularis* ticks included in our pilot study, the DNA yield per tick ranged from 0.074 to 3ug, averaging 1.033 μg from female ticks and 0.535 μg from the smaller male ticks. Among the 188 ticks received on Tick Cards mailed from across the United States, the yields ranged from 0.079 to 7.6 μg per tick, averaging 0.692 and 0.362 μg from female and male *Ixodes*, respectively. The
average DNA yield from non-Ixodes ticks, which are generally larger, was 2.13 ug. We also successfully extracted RNA from Tick Card ticks, indicating that ticks collected by citizen scientists could also be used to survey for RNA viruses (data not shown).

II.5.3 Microbiome Sequencing Pilot Study In 192 Ixodes scapularis Ticks

We used PCR to screen for B. burgdorferi in all 192 Ixodes scapularis ticks in our pilot study. We identified B. burgdorferi infection in 119 (61.9%) of them, consistent with infection rates reported in this region (Laboratory of Medical Zoology | Tick-Borne Diseases Passive Surveillance, n.d.). We prepared a 16S metagenomic sequencing library for each tick, then sequenced the 128 libraries with concentrations >= 10nM (99 females and 29 males; 88 positive for B. burgorferi, 40 uninfected). For each sample, we obtained an average of 32,629 DADA2 (Callahan et al., 2016)-processed quality sequences (±41,579 SD) and 66 (±36 SD) unique amplicon sequence variants (ASVs).

II.5.3.1 Coinfection

We used the 16S sequencing data to identify ticks coinfected with multiple pathogens in addition to B. burgdorferi, in which infection was defined as at least 0.1% of microbiome reads coming from the tickborne pathogen. Consistent with previous reports, we observed several cases of double and even triple infection
Six ticks were coinfected with *B. burgdorferi* and *B. miyamotoi*, and seven ticks were coinfected with *B. burgdorferi* and *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis. Of these, two ticks carried all three of these pathogens. Because our PCR process targeted the eubacterial 16S region, our data do not indicate whether these ticks were also coinfected with *Babesia*, a eukaryotic pathogen previously reported to be present in coinfections (Hersh et al., 2014; Krause et al., 1996; Swanson et al., 2006).

II.5.3.2 Alpha Diversity

Consistent with previous reports, individual male ticks had greater microbial diversity than that in female ticks (Thapa et al., 2019; Van Treuren et al., 2015). Male ticks had a larger total number of microbial species (Figure 2.2A; $p=1.01e-10$); had higher Faith phylogenetic diversity index scores (Faith, 1992) (Figure 2.2C; $p=7.0e-6$), a measure of total phylogenetic branch length spanned by species present; and had higher Shannon index scores, a measure of species count and abundance (Figure 2.2E; $p=6.45e-10$) (Shannon, 1948).

Alpha diversity did not differ significantly between ticks infected versus uninfected with *B. burgdorferi* based on either absolute number of species (Figure 2.2B; $p=0.301$) or Faith phylogenetic diversity index score (Figure 2.2D; $p=0.56$). *B.burgdorferi*-infected ticks had a slightly higher Shannon index score (Figure
2.2F; p=0.024), but this difference disappeared after removing \textit{B. burgdorferi} reads (p=0.58).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.2.png}
\caption{Alpha diversity of the microbiome differs significantly between females and males, but not between \textit{B. burgdorferi}-infected and uninfected ticks. We measured alpha diversity using three different metrics, including (A) the count of observed ASVs by sex (p=1.01e-10) and (B) by infection status (p=0.30), (C) the Shannon index, a non-phylogenetic alpha diversity metric, by sex (p=6.45e-10) and by (D) infection status (p=0.02), and (E) Faith's Phylogentic Diversity index, a phylogenetic alpha diversity metric by sex (p=6.45e-10) and (F) by infection status (p=0.56). While infection status was significant using the Shannon Index, this difference disappeared after removing \textit{B. burgdorferi} reads from the analysis (p=0.58).}
\end{figure}
The greater alpha diversity in male ticks was evident in the phylum abundance of the microbiomes (Figure 2.3A). *Proteobacteria* comprised the highest relative abundance in all ticks, averaging 89.7% in *B. burgdorferi*-uninfected male ticks, 62.9% in infected male ticks, 97.07% in uninfected female ticks, and 92.9% in infected female ticks. The differences between *B. burgdorferi* infected and uninfected *I. scapularis* ticks of both sexes, however, can be explained by the different abundance of *Spirochaetes*, the phylum that includes *B. burgdorferi*. Among infected ticks, *Spirochaetes* was the second most abundant phylum, averaging 28.1% in males and 4.22% in females; however, this phylum was essentially absent in uninfected ticks (0.062% in males and 0.034% in females). Genera-level analysis confirmed that infected male ticks had a significantly higher abundance of *B. burgdorferi* than that in infected females (19.89% vs 3.75%; Welch’s t-test = 4.0505, p = 0.00048), and that most of the *Spirochaetes* in the infected ticks were *B. burgdorferi* (Figure 2.3B). After removing *B. burgdorferi* from the analysis, infected and uninfected ticks had similar microbiome profiles on the genera-level, with a much higher proportion of *Rickettsia* in female ticks, consistent with other published results (Thapa et al., 2019; Van Treuren et al., 2015). However, *Rickettsia* did not explain all of the difference between males and female ticks. Even after removing *B. burgdorferi* and *Rickettsia* species, the diversity in the male ticks remained significantly higher than that in female ticks (Figure 2.3C).
Figure 2.3 Abundance of microbial taxa in male and female ticks with and without *B. burgdorferi* infection reflects the greater diversity of the male microbiome. (A) At the phylum level, *B. burgdorferi* is evident in the abundance of *Spirochaetes* in the infected ticks, and males have higher frequency non-Proteobacteria phyla, reflecting the greater diversity of their microbiome. Here, "Others" (grey) represents all reads unclassifiable using the 16S SILVA reference database with a 99% identity cutoff. (B) The greater microbiome diversity in males, and higher abundance *B. burgdorferi* in infected males (19.89% vs 3.75%; t-test = 4.0505, p = 0.00048), is also evident at the level of genera. Females have a higher frequency of *Rickettsia*, potentially due to the high abundance of that genus in the ovaries (Noda et al., 1997). (C) The greater diversity of male microbiome on the genera level persists even when both *B. burgdorferi* and *Rickettsia* are removed from the analysis.
II.5.3.3 Beta Diversity

We also observed differences in the microbiome profiles of *B. burgdorferi*-positive and -negative ticks, with a number of specific taxa over and underrepresented in the infected ticks. Beta diversity differed significantly between infected and uninfected ticks when measured by relative abundance (weighted Unifrac distance (C. A. Lozupone et al., 2007), pseudo-F test=8.28366, p = 0.001; Figure 2.4A). However, there was no difference when beta diversity was measured by occurrence (unweighted UniFrac distance (C. Lozupone & Knight, 2005), pseudo-F test=1.197, p = 0.161) or when measured as relative abundance after *B. burgdorferi* was removed from the analysis (pseudo-F test=1.53959, p = 0.206; Figure 2.4B).
**Figure 2.4** Overall beta diversity of the microbiome does not differ by *B. burgdorferi* infection status; however, specific taxa are significantly over and underrepresented in infected ticks. (A) Beta diversity, measured by weighted Unifrac distance, differed significantly between infected (blue) and uninfected (red) ticks (pseudo-F test=8.28366, P = 0.001), but (B) this difference disappears once *B. burgdorferi* is removed from the analysis (pseudo-F test=1.53959, P = 0.206). (C) LEfSe discriminant analysis shows that reads from *B. burgdorferi* and *Cutibacterium* are significantly more abundant (orange) in *B. burgdorferi*-positive ticks, while reads from three taxa are significantly less abundant (purple), including *Rickettsia*. (D) The underabundance of *Rickettsia* persists in LEfSe analysis of female ticks only, and the number of significantly more (orange; 5 taxa) and less (purple; 6 taxa) abundant taxa increases.
Our LEfSe discriminant analysis (Segata et al., 2011) revealed previously unreported associations between *B. burgdorferi* infection and the presence of specific microbial taxa. The abundance of reads from two taxa (*B. burgdorferi* itself and the genus *Cutibacterium*) was significantly higher in *B. burgdorferi*-positive ticks, while reads from three taxa (genus *Rickettsia*, family *Diplorickettsiaceae* and family *Beijerinckiaceae*) were significantly less abundant (Figure 2.4C). When LEfSe analysis was restricted to female ticks, the number of taxa significantly more or less abundant in *B. burgdorferi*-infected ticks increased markedly (Figure 2.4D). We observed *Rickettsiella* to be more abundant in *B. burgdorferi*-negative female ticks, but this pattern was largely driven by a single uninfected tick with an exceptionally high abundance of *Rickettsiella* (79.4%, compared to 0 to 3.6% in other ticks). *Rickettsiella* is an intracellular bacterium known to be pathogenic in some arthropods, but its pathogenicity in ticks is unknown. Finally, we searched for bacterial species known to be pathogenic in humans and detected four pathogens not previously reported to be associated with ticks (Table 2.2).
Table 2.2 Microbiome analysis detected bacterial species pathogenic to humans and not previously reported in ticks

<table>
<thead>
<tr>
<th>Pathogen</th>
<th># of ticks infected</th>
<th># coinfected with B. burgdorferi</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chryseobacterium indologenes</em></td>
<td>3 (2.3%)</td>
<td>2</td>
<td>Opportunistic; can infect immunocompetent humans; exhibits resistance to multiple antibiotics (Cascio et al., 2005; Chou et al., 2011; Hsueh et al., 1996; Lin et al., 2010; McKew, 2014)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>22 (17.2%)</td>
<td>14</td>
<td>Associated with trauma or immunocompromised state; lethal in rare cases (Carpenter et al., 2008; B. S. Thomas et al., 2013)</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>15 (11.7%)</td>
<td>10</td>
<td>Infects immunocompromised individuals (Bagdure et al., 2012; Vernazza et al., 1991)</td>
</tr>
<tr>
<td><em>Streptococcus suis</em></td>
<td>2 (1.6%)</td>
<td>2</td>
<td>Can cause systemic disease in humans (Huong et al, n.d.)</td>
</tr>
</tbody>
</table>

II.6 Discussion

Efforts to control the transmission and spread of tickborne diseases have typically been limited by the lack of data on the presence, abundance, and dynamics of pathogens in individual ticks. We sought to develop a robust platform, Project Acari, to provide large-scale, high-resolution data on tickborne pathogen. We focused initially on four essential components: (1) enrollment of large numbers of volunteers through social media; (2) economical collection of
ticks that can provide high quality DNA, regardless of distance from research site; (3) laboratory methods for tick processing that are readily scalable for thousands of samples; and (4) a pilot genomic dataset to validates essential findings of earlier studies and offers new insights.

Results from the first phase of Project Acari underscore the power of citizen science as a tool for sampling large numbers of ticks from geographically diverse locations. Using our custom-designed Tick Kit, we collected nearly 2500 ticks from across the United States, including individuals of diverse species at various life stages (Figure 1). Through our pilot project, we processed 192 *I. scapularis* ticks collected by volunteers in and around Grafton, Massachusetts. This area is reported to have rates of *B. burgdorferi* infection in the *I. scapularis* tick population exceeding 50%, suggesting we would have sufficiently high infection rates in our pilot sample set to compare the microbiomes of infected and uninfected ticks (*Laboratory of Medical Zoology | Tick-Borne Diseases Passive Surveillance*, n.d.). We validated previously reported correlations between tick sex and microbiome profiles, discovered significant associations between specific microbiome components and *B. burgdorferi* infection, and detected known human pathogens that had not previously been reported to be carried by ticks.

As part of this pilot project, we developed methods for higher throughput processing of ticks. One major challenge was simply penetrating the small, hard tick exoskeleton without damaging the DNA. Previous studies used
microdissection, bead beating, or both, to homogenize ticks (Budachetri et al., 2014; Moreno et al., 2006; Narasimhan et al., 2014b). While these approaches are effective, they are also labor intensive, imposing severe constraints on the total number of individual ticks that can be processed.

Our new method is simple. We first place each tick in each well of a 96-well deep well plate, then flash-freeze the ticks by placing the plate in a dish of liquid nitrogen. We then use the Zymo Research Squisher-96 to shatter and homogenize the ticks. This allows 96 samples to be processed in parallel, providing the rapid throughput needed for large-scale tick genomics. We confirmed that this method could be used to extract high-quality DNA both from the 192 ticks in the pilot set and from 188 ticks mailed in by volunteers using our custom-designed Tick Card. We note, however, that this approach precludes inference of associations between microbes and specific tick organs and organ systems, information that would be attainable using microdissection (Narasimhan et al., 2014b).

For our pilot project, we determined *B. burgdorferi* infection status and 16S microbiome sequence data for 128 wild-caught *I. scapularis* ticks. We validated previously published findings that, for both laboratory and wild caught ticks, overall microbial diversity in higher is males than in females, and the abundance of *Rickettsia* much higher in the female (Thapa et al., 2019; Van Treuren et al., 2015; Zolnik et al., 2016). Previous work found *Rickettsia* to be abundant in the ovaries of female ticks, where it may play an important developmental role (Noda
et al., 1997). In contrast to previous findings in laboratory-raised ticks, however, we found that in wild caught ticks the difference in the microbiomes of males and females persists even after *Rickettsia* was removed (Figure 3C) (Thapa et al., 2019).

Studying wild-caught ticks provides real world context for findings made in laboratory-reared ticks. For example, previous work showed that *Borrelia* did not colonize ticks raised in sterile environments and with very low microbiome diversity (Narasimhan et al., 2014b). In our field collected samples, however, we observe no correlation of microbiome diversity and *Borrelia* infection status, suggesting all wild-caught ticks have sufficient diversity to allow *Borrelia* colonization. Thus, while lab-reared ticks can provide insight into the dynamics of tickborne pathogens not accessible using field-collected samples, tickborne pathogen interventions developed based on findings from laboratory ticks may not be applicable to real-world populations.

With microbiome data from just 128 ticks, we discovered components correlated with infection status that had not been previously described, highlighting the urgent need for larger scale tick research. Our findings also illustrate why, even as projects scale up, data at single-tick resolution remains essential. Our finding that *Rickettsiella* was significantly more abundant in uninfected female ticks was driven almost entirely by a single tick with exceptionally high abundance of *Rickettsiella* (79.4%, compared to 0-3.6% in other ticks), a phenomenon only detectable because we were able to distinguish the DNA from each tick. The
capacity of our approach to process a large number of ticks and to sample from many different geographic locations also revealed some human pathogens not previously associated with ticks; further work will be needed to determine the frequencies of these associations.

Data at single-tick resolution also enabled us to detect 13 instances of ticks infected with multiple pathogens. Co-infection is well documented in tickborne pathogens, with between 10-40% of ticks reported to carry multiple pathogens (Diuk-Wasser et al., 2016; Hersh et al., 2014; Swanson et al., 2006), and transmission of multiple pathogens can increase both the duration and intensity of tickborne diseases symptoms, and complicate diagnosis (Sanchez et al., 2016). Understanding the prevalence of tickborne pathogen co-infection in the tick population for a given region could immediately impact diagnostic approaches and treatment decisions.

In the next phase of Project Acari, we will screen ticks for a broader set of pathogens and use genomics to collect detailed information on the sex, species, and genomic variants of each tick. In the work reported here, we assessed tick species and sex through visual inspection. This approach is laborious and potentially error-prone, and would ideally be replaced by genomic approaches. However, in contrast to the intense focus on using genomics for controlling diseases in arthropod vectors (Hill et al., 2005), tick genomics is much less developed. Only a single species, *I. scapularis*, has a reference genome, and sequences are not yet anchored to chromosomes (Gulia-Nuss et al., 2016).
Without high quality genome assemblies for tick vector species, we cannot easily identify genomic markers that indicate tick species and sex, or capture known pesticide-resistance loci. Thus, there is an urgent need for “genomic infrastructure” to enable the development of new technologies for tickborne pathogen surveillance and interventions.

tickborne pathogens are increasing in prevalence worldwide, likely exacerbated by ongoing climate change (USGCRP, 2016). Here, we have demonstrated the potential of citizen science to support large-scale, longitudinal tickborne pathogen genomic analysis across broad geographic areas. Through Project Acari, we collected ticks from across the United States and also resampled the same locations in multiple years. While the initial phase described here focused on a single tick species and pathogen, our genomic approaches are readily extendable to other tick and pathogen species. We envision Project Acari as an open data resource that engages directly with communities at risk from tickborne pathogen to provide real-time information on the spread of pathogens, provide a rich source of data for investigating the factors that drive this spread, and inform the development of new control strategies.
Chapter III: Development of new molecular tools for comprehensive identification of ticks, pathogens, microbiome and their associations
III. 1 Preface

In MIPs study, library sequencing was performed by Rebecca DeFeo from Jeffrey Bailey's Lab. Following sequencing, the initial quality control, demultiplexing, Unique molecular identifier correction and clustering was performed by Ozkan Aydemir from Jeffrey Bailey's Lab.

In Microbiome study, we used lab services from Loop Genomics. They did partial library preparation of pooled 16S and 18S amplicons, sequencing of the library, quality control, demultiplexing and reads stitching to provide full length 16S 18S amplicons post sequencing. They further contributed by creating the ASV tables and assigning taxonomy to ASVs.

I performed all the experiments and analysis described in this chapter.
III.2 Abstract

Globally, ticks transmit more pathogens than any other arthropod lineage. Known tickborne pathogens include around 60 bacteria, 30 eukaryotic parasites and 100 viruses, one-third of which cause zoonoses.

Existing methods for tick and pathogen detection are generally time-consuming, error-prone and do not detect all co-infections and novel tick-pathogen associations. Moreover, economical methods for detecting pathogens in individual ticks are lacking. The absence of surveillance methods restricts the information available to clinicians regarding the pathogens and coinfections prevalent in a given region, making diagnosis and treatment difficult. Thus, new epidemiologic investigative tools are needed to simultaneously identify tick species and tick-borne pathogens. Furthermore, in samples from poorly characterized sources without tick engorgement status, it's challenging to determine if ticks were questing for a host or already feeding. Detecting host DNA in non-engorged ticks is crucial for assessing their feeding status and identifying their host. To enable high-throughput screening at single-tick resolution, we developed a targeted sequencing-based method using molecular inversion probes (MIPs) to screen for all known pathogens and hosts in individual ticks.

We designed 215 host (tick and mammalian host) and 320 pathogen MIPs to identify about 25 Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes
and *Rhipicephalus* tick species, 25 mammalian host species to assess tick feeding patterns and engorgement status, about 100 tick-borne pathogens (prokaryotic: *Borrelia, Borrelia*, *Ehrlichia, Anaplasma, Rickettsia, Francisella, Bartonella* and *Coxiella*, eukaryotic: *Babesia* and *Theileria*, and Viruses: Powassan, Heartland and Bourbon virus), and 5 drug resistant genomic variants of *Babesia microti* known to be associated with clinical relapse in humans.

We evaluated the sensitivity of MIPS using positive control samples representing target levels ranging from 1,000 and 10,000 genomes per reaction mixture and found that they have sensitivity as low as 1000 copies per reaction or 200 copies/μl) and were highly selective. We then applied our MIPs assay to 853 visibly unengorged individual adult ticks collected by citizen scientists, through Project Acari, from across the USA and successfully identified the species of 715 ticks of which 85 individual ticks were infected with pathogens. The set of pathogens detected included *Borrelia burgdorferi, Borrelia miyamotoi, Anaplasma phagocytophilum, Ehrlichia chaffeensis, Francisella tularensis*, five different pathogenic species of *Rickettsia*, and parasite species *Babesia microti* and *Babesia duncani*. We detected human DNA in 12 tick samples and dog DNA in 48 tick samples, despite our request for participants to send only unfed ticks and our selection of visibly unengorged ticks for analysis. This suggests that these ticks might have started feeding before they were collected and sent to us.
Next, we performed full length 16S and 18S sequencing to generate the first comprehensive data on microbiome of individual ticks, including symbiotic bacteria, protozoa, and fungi. We found that geographic regions had a consistent effect on prokaryotic microbiome diversity, with ticks from the Northeast having a significantly greater diversity. We did not see such an effect on eukaryotic diversity. We also observed that male ticks in general had a higher prokaryotic diversity than female ticks. Finally using LEfSe analysis on *Borrelia*-infected vs uninfected blacklegged ticks, we detected previously undescribed associations between *B. burgdorferi* carriage and the presence of specific microbial taxa.

Our successful application of MIPs and microbiome assay to identify pathogens in ticks collected via Project Acari highlights the power of integrating citizen science and high-throughput processing to discern pathogen dynamics. This methodology is readily extensible to enable large-scale screening of individual ticks, providing a valuable tool for understanding the ecology of tick-borne diseases and informing efforts to control pathogens.

**III.3 Introduction**

Tickborne diseases are the leading cause of vector-borne diseases in the US, accounting for 95% of the reported cases in 2013, making ticks the most medically important group of arthropods in the United States. Lyme disease accounted for almost 75 percent of all reported cases of indigenously acquired
vector-borne disease. With 8 new tickborne pathogens identified since 2010 (Paddock et al., 2016), there are now 20 known pathogens in the US carried by ticks. With clear evidence of the emergence of new pathogens (Tokarz et al., 2014; Vayssier-Taussat et al., 2013), it is estimated that up to half of all tickborne diseases may be caused by unknown pathogens (Feder et al., 2007). Tickborne diseases are reported all across the US, but most of the cases are reported in the Northeast and Midwest. The numbers of reported cases of tickborne diseases in the United States have followed consistent upward trends (R. Eisen et al., 2017). In parallel, increases in ticks’ distribution and abundance have been reported (R. Eisen et al., 2017). This spread is attributed to increased human and animal movement, as well as altered interactions between reservoirs, pathogens, and human population. This leads to changing epidemiology and emergence of new tickborne diseases (S. Bonnet et al., 2014).

III.3.1 Surveillance And Diagnosis

Many studies have sought to document the distribution of ticks and the epidemiology of tickborne diseases in the U.S. Unfortunately, the utility of these studies has been limited by study of only a small number of ticks and by collection from a limited geographic areas (S. Lee et al., 2014; Salkeld et al., 2014; Sayler et al., 2017; Tokarz et al., 2014) during a limited time interval. We overcame these limitations and problems, by using “Project Acari”, our citizen science project discussed in Chapter II, to rapidly collect a large number of ticks
across the U.S over multiple years. Using this approach, we captured the ticks that are normally encountered by individuals in their daily activities. Moreover, the hard exoskeleton of ticks is resistant to the lysis buffers used in DNA extraction protocols, making extraction laborious, and forcing researchers to pool ticks for preprocessing. This pooling has severely limited opportunities to collect the high-resolution data needed to assess the pathogen prevalence and to determine the incidence of coinfections (Narasimhan et al., 2014b; Tokarz et al., 2014). To overcome this challenge, we developed a high throughput method to preprocess and extract DNA from large numbers of individual ticks.

Previous studies have used morphological methods to identify tick species (Thompson et al., 2022). These approaches are time consuming, require expertise, and can be error prone. Furthermore, current surveillance methods screen exclusively for pathogens known to be vectored by a given tick species or found in a particular geographical area. Thus, these methods are unable to detect novel tick-pathogen associations. Although existing molecular approaches can target individual known pathogens, there is not yet a high throughput and yet economical method for detection of all known tick-borne pathogens in a tick sample. Moreover, for samples that come from poorly characterized sources and did not include engorgement status of the tick, it is very difficult to determine if the tick was collected when it was questing for a host or if the tick had already attached to the host and just started feeding. In such samples, ticks that are not visibly engorged, detection of host DNA is the only way that could inform the
engorgement status of the tick and identification of its host. Shotgun metagenomic sequencing can, in principle, address this challenge, but is prohibitively expensive due to the high cost of sequencing DNA isolated from ticks or humans, which have very large genomes.

Furthermore, the current approach for diagnosing tickborne diseases in clinics involves testing for each pathogen separately, which are often not reliable. Also, mostly the tests are done only for the pathogens that are believed to be the cause of the diseases based on the symptoms of the patient, thus leading to improper diagnosis of complex tickborne diseases involving co-infections. Rapid and sensitive detection of pathogens along with any drug resistance and co-infections is of great importance for timely treatment of infectious diseases, as well as for studying spread and distribution of outbreaks (Chen et al., 2015; Ke et al., 2013). In clinical samples detection of co-infections is very crucial as it complicates the symptoms, diseases progression and treatment.

In Lyme disease transmission, Tick Receptor for Outer Surface Protein A (TROSPA) gene plays an important role, as it is essential for colonization of B. burgdorferi in the tick gut (Fikrig et al., 2004; Pal et al., 2000; Yang et al., 2004). OspA interacts specifically with the tick receptor TROSPA, which is located on the luminal surface of gut epithelial cells and is upregulated when spirochaetes are ingested (Pal et al., 2004). The blockade of TROSPA by TROSPA antisera or by the repression of TROSPA expression via RNA interference reduced B.
*B. burgdorferi* adherence to the *I. scapularis* gut in vivo, thereby preventing efficient colonization of the vector and subsequently reducing pathogen transmission to the mammalian host (Pal et al., 2004). Many studies have looked at the natural variants of OSPA, that often are referred to as “serotypes;” these serotypes are of direct relevance to developing OspA based vaccines. OspA serotype 1 is the predominant serotype of *B. burgdorferi* s.s. in North America. However, in Europe there is considerable variation in the Osp A serotypes that cause Lyme disease (Sam R. Telford, 2023). However, no study has looked at natural variants of TROSPA in *I. scapularis* ticks. Identification of a natural variant that is naturally resistant or has a reduced binding to OSPA might be useful in controlling the transmission of *B. burgdorferi*.

Polymerase chain reaction (PCR) is the most popular technique for molecular detection of pathogens, due to its specificity and sensitivity. But its use for detection of pathogens is greatly limited by its inability to multiplex beyond a few targets. Currently, there is no technology available that can efficiently and economically test for tick species, pathogens, drug resistant markers, host DNA and TROSPA variants in large numbers of samples in parallel, and this is urgently needed both for well-powered epidemiological studies and in clinical medicine.

Here I will present a high throughput method based on Molecular Inversion Probes, that allows for massively parallel targeted capture, in large number of
tick samples, allowing for identification of tick species, detection of pathogens and co-infections. Our method also detects the presence of potential drug resistant markers in *B. microti* (*Lemieux et al., 2016*), mammalian host DNA as a marker for tick engorgement status and identification of host, as well as TROSPA variants. Our method is a powerful, broadly applicable new approach for high-throughput genomic surveillance and has a potential to be used as a diagnostic method for numerous targets within any nucleic acid sample of interest, allowing for diagnosis of co-infections and detection of drug resistance.

Studying individual ticks will enable us to investigate the dynamics of tick-pathogen associations, an approach fundamental to understanding disease transmission. A better understanding of the geographic distribution of known pathogens could ultimately improve diagnosis, treatment, and prevention of tickborne diseases. Identification of naturally occurring OSPA resistant TROSPA variants might pave ways to methods to control Lyme disease transmission.

III.3.2 Tick-Pathogen-Microbiota Association And Disease Control

Despite the clear impact of tickborne diseases on public health, there is a lack of methods for controlling the transmission of tickborne diseases. Developing preventive strategies against this disease is critical in reducing its negative impact on people’s health and countries’ economies. A wide variety of prevention and control strategies have been utilized or investigated to reduce tick abundance, the prevalence of tick-borne pathogens, and/or the risk of human
exposure to tick-associated pathogens, but none of these have found to be sufficiently effective to control tickborne diseases. Ongoing endeavors focus on preventing human infection, but these initiatives won't impact the disease reservoirs. Human-targeted interventions like vaccines, repellents, and prophylactic treatments necessitate continuous investment since they solely safeguard individuals rather than curbing infection risk at a larger scale. To ensure lasting control over tick-borne infections, addressing the root source becomes imperative (Bernard et al., 2020). Several investigations have indicated that endosymbionts within ticks play a pivotal role in the host tick's viability and overall fitness (Zhong et al., 2007b). Furthermore, there is a possibility that these endosymbionts could also impact the host tick's vector competence (Klyachko et al., 2007b; Narasimhan et al., 2014b). Interventions directed at either the vector or these reservoir hosts could potentially bring about enduring changes in the disease's trajectory, thereby altering its long-term prevalence and geographical spread.

Paratransgenesis, a promising alternative strategy to control vector borne diseases, that uses genetically modified bacteria to inhibit pathogen carrying capacity of vectors, is currently being tested in many diseases carrying vectors. Paratransgenesis can control Dengue (Moreira et al., 2009; Walker et al., 2011) in the field (Our Research | World Mosquito Program, n.d.), and has shown promise against Malaria (Hughes et al., 2011), Chikungunya (Moreira et al., 2009; Mousson et al., 2010), Zika (Dutra et al., 2016) and Chagas disease
(Durvasula et al., 1997) in lab settings, suggesting that it could be exceptionally powerful in limiting the spread of tickborne diseases.

To be able to use microbes to control transmission of tickborne diseases, it is necessary that the strategies can be applied to natural populations of ticks. For this to be achieved, studies assessing the diversity of tick-associated microbes across diverse ecological niches are required. Thus, to examine both the host-microbe and host-microbe-pathogen tripartite interactions from ticks collected from differing environmental conditions is crucial.

Currently, most studies analyze ticks from a specific and small geographical location, oftentimes pooling multiple ticks together (S. Lee et al., 2014; Salkeld et al., 2014; Sayler et al., 2017; Tokarz et al., 2014). Moreover, the predominant approach in tick research involves sequencing one or two variable regions of the 16S rDNA gene. However, this method fails to provide insights into the eukaryotic community of the tick microbiome, and the resolution of the bacterial community is limited as only a few sequences can be correctly aligned to species level. The biggest challenge in studying the eukaryotic community is the presence of high abundance of tick 18S rDNA, which gets amplified along with microbial 18S rDNA thus reducing the cost effectiveness of the assay.

Here, I introduce an innovative methodology that offers an extensive coverage of the tick microbiome, enabling the exploration of host-microbe-pathogen tripartite interactions in ticks collected from various geographic and environmental
contexts. Our approach involves amplification of full-length 16S and 18S rDNA sequences, supplemented by peptide nucleic acid (PNA) blocker designed to specifically inhibit tick 18S rDNA amplification. Through this technique, we achieve comprehensive insights into the microbiome of individual ticks spanning diverse species across the United States. This encompassing method effectively captures both prokaryotic and eukaryotic communities, all the while maintaining an economical and feasible approach. Deciphering interactions between ticks and their microbiomes can potentially unlock new strategies to control tick-borne diseases via modulation of tick microbiome.

III.4 Methods And Materials

III.4.1 Sample Collection And High-Throughput Nucleic Acid Extraction

We collected ticks from across the US through Project Acari, as discussed in Chapter II.4.1. Briefly, we sent out a Tick Kit to each participant who requested a Kit through our website ProjectAcari.org. Participants then sent back the Tick Card with a tick attached to the barcoded spots in Tick Card. We recorded information on each tick, including collection location (as zip code) and date of collection, in a custom SQL database. The ticks were stored at -20 degree Celsius until processed.

For nucleic acid extraction, we homogenized individual ticks using liquid nitrogen and 96-well Zymo Research Squisher-96 (Cat No.: H1004) as described in
Chapter II.4.2. We used All Prep DNA/RNA Kit in 96 well format (Cat. No.: 80311) for simultaneous extraction of both DNA and RNA from each tick sample following the manufacturer's protocol. In this study we only used DNA for our assays. The DNA was quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Cat. No.: P7589) following the manufacturer's protocol.

III.4.2 Molecular Inversion Probes

III.4.2.1 Molecular Inversion Probes Design And Target Selection

Genomic targets, of length 100 to 300 bp, unique to target species were identified and used to design MIPs. Target Species, targeted genes and MIPs sets are listed in Table 3.1. For each pathogen (pathogen species and antibacterial resistance variants) and host (tick species, TROSPA gene and mammalian host species) target, MIPs were specifically designed for this study. MIPs were designed using MIPTools as described in Aydemir et.al. 2018 (Aydemir et al., 2018). We designed a total of 215 host (tick and mammalian) and 320 pathogen probes (File 3.1).
### Table 3.1 Target species and MIP target gene

#### A. Pathogen targets

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaplasma centrale</em>, <em>Candidatus Neoehrlichia mikurensis</em>, Ehrlichia ewingii, Ehrlichia platys</td>
<td>groEL</td>
</tr>
<tr>
<td><em>Anaplasma marginale</em></td>
<td>msp1b</td>
</tr>
<tr>
<td><em>Anaplasma ovis</em></td>
<td>msp4</td>
</tr>
<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>groEL, msp4, msp2</td>
</tr>
<tr>
<td><em>Babesia bigemina</em></td>
<td>cytb</td>
</tr>
<tr>
<td><em>Babesia bovis</em>, <em>Babesia major</em></td>
<td>CCTeta</td>
</tr>
<tr>
<td><em>Babesia microti</em></td>
<td>CCTeta, cytb</td>
</tr>
<tr>
<td><em>Babesia caballi</em></td>
<td>RAP1</td>
</tr>
<tr>
<td><em>Babesia canis</em>, <em>Babesia gibsoni</em>, <em>Babesia vogeli</em></td>
<td>cox1</td>
</tr>
<tr>
<td><em>Babesia divergens</em></td>
<td>hsp70</td>
</tr>
<tr>
<td><em>Babesia duncani</em>, <em>Theileria parva</em>, <em>Theileria velifera</em></td>
<td>CoxIII</td>
</tr>
<tr>
<td><em>Babesia equi</em></td>
<td>ema1</td>
</tr>
<tr>
<td><em>Bartonella henselae</em></td>
<td>pap31</td>
</tr>
<tr>
<td><em>Bartonella quintana</em></td>
<td>bqtR</td>
</tr>
<tr>
<td><em>Borrelia afzelii</em>, <em>Borrelia andersonii</em>, <em>Borrelia burgdorferi</em>, <em>Borrelia garinii</em>, <em>Borrelia japonica</em>, <em>Borrelia mayonii</em>, <em>Borrelia bavariensis</em>, <em>Borrelia bissetii</em>, <em>Borrelia chilensis</em>, <em>Borrelia valaisiana</em></td>
<td>Fla, rpoB</td>
</tr>
<tr>
<td><em>Borrelia americana</em>, <em>Borrelia californiensis</em>, <em>Borrelia caucasica</em>, <em>Borrelia lanae</em>, <em>Borrelia merionesi</em>, <em>Borrelia microti</em>, <em>Borrelia sinica</em>, <em>Borrelia spielmanii</em>, <em>Borrelia tanukii</em>, <em>Borrelia turdi</em>, <em>Borrelia yangtzensis</em>, <em>Borrelia carolinensis</em>, <em>Borrelia finlandensis</em>, <em>Borrelia lusitaniae</em></td>
<td>Fla</td>
</tr>
<tr>
<td>Organism</td>
<td>Genes/Proteins</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
</tr>
<tr>
<td><em>Borrelia anserina</em>, <em>Borrelia parkeri</em>, <em>Borrelia theileri</em></td>
<td>Fla, rpoB, qlpQ</td>
</tr>
<tr>
<td><em>Borrelia coriaceae</em>, <em>Borrelia hermsii</em>, <em>Borrelia johnsonii</em>, <em>Borrelia lonestari</em>, <em>Borrelia miyamotoi</em>, <em>Borrelia persica</em>, <em>Borrelia turcica</em>, <em>Borrelia turicatae</em></td>
<td>Fla, qlpQ</td>
</tr>
<tr>
<td><em>Borrelia crocidurae</em>, <em>Borrelia duttonii</em>, <em>Borrelia recurrentis</em></td>
<td>Fla, rpoB, qlpQ, recN, recC</td>
</tr>
<tr>
<td><em>Borrelia hispanica</em></td>
<td>Fla, qlpQ, recC</td>
</tr>
<tr>
<td><em>Borrelia lusitaniae</em></td>
<td>rpoB</td>
</tr>
<tr>
<td><em>Borrelia venezuelensis</em></td>
<td>qlpQ</td>
</tr>
<tr>
<td>Bourbon virus</td>
<td>PB1, NP</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>idc, IS1111</td>
</tr>
<tr>
<td>Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia muris, Ehrlichia ruminantium</td>
<td>groEL, dsb</td>
</tr>
<tr>
<td>Ehrlichia Muris Eauclairensis</td>
<td>Dsb, GroEL</td>
</tr>
<tr>
<td><em>Francisella tularensis</em> subsp. holarctica, <em>Francisella tularensis</em> subsp. novicida, <em>Francisella tularensis</em> subsp. tularensis</td>
<td>tul4</td>
</tr>
<tr>
<td>Heartland virus</td>
<td>segmentS (long and small)</td>
</tr>
<tr>
<td>Powassan virus</td>
<td>genome</td>
</tr>
<tr>
<td>Rickettsia aeschlimannii, Rickettsia africana, Rickettsia amblyommatis, Rickettsia asiatica, Rickettsia conorii, Rickettsia heilongjiangensis, Rickettsia helvetica, Rickettsia japonica, Rickettsia massiliæ, Rickettsia monacensis, Rickettsia montanensis, Rickettsia parkeri, Rickettsia philipii, Rickettsia raoultii, Rickettsia rhipecephali, Rickettsia slovaca</td>
<td>23S-5S-ITS</td>
</tr>
<tr>
<td>Rickettsia rickettsii</td>
<td>genome, 23S-5S-ITS</td>
</tr>
<tr>
<td>Theileria annulata, Theileria lestoquardi, Theileria sp. Yokoyama</td>
<td>ms1</td>
</tr>
<tr>
<td>Theileria orientalis</td>
<td>MPSP</td>
</tr>
</tbody>
</table>
B. Mamalian host targets

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bos indicus</em>, <em>Bos taurus</em>, <em>Capra hircus</em>, <em>Odocoileus virginianus</em>, <em>Ovis aries</em>, <em>Peromyscus leucopus</em></td>
<td>12S</td>
</tr>
<tr>
<td><em>Camelus bactrianus</em>, <em>Camelus dromedarius</em>, <em>Camelus ferus</em></td>
<td>16S</td>
</tr>
</tbody>
</table>

C. *Babesia microti* Relapse targets

<table>
<thead>
<tr>
<th>Species</th>
<th>Target gene</th>
<th>Target Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Babesia microti</em></td>
<td>Cytochrome b</td>
<td>LN871600.2:4881-4883, LN871600.2:4956-4958, LN871600.2:4998-5000, LN871600.2:5016-5018, LN871600.2:5427-5429</td>
</tr>
</tbody>
</table>

D. Tick species targets

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
</tr>
</thead>
</table>

E. TROSPA gene targets

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ixodes scapularis</em>, <em>Ixodes ricinus</em></td>
<td>TROSPA gene</td>
</tr>
</tbody>
</table>

We created two target files: one for hosts and one for pathogens. The host file consists of unique target sequences for all host targets (tick species, TROSPA gene and mammalian host species), while the pathogen file includes unique
target sequences for all pathogen targets (pathogen species and antibacterial resistance variants) for which MIPs were to be designed. We then created a pseudo reference file which contains all reference genomes available for each target pathogen species we were interested in. Furthermore, since reference genomes are not available for all tick species, we created a “meta” reference genome. This consisted of, for the tick species of interest, all available reference genomes as well as all genomic sequences available for the species for which the reference genome was not available.

To capture all known variations in the target regions, we obtained all closely related sequences to target sequences through BLASTn search for each of the sequences in the target files using default parameters. Some of these sequences belong to the target species, these were added to the pseudo reference file of each target pathogen. While some sequences belong to closely related species that might be present in tick samples, but we were not interested in and would want to avoid (such as tick microbiome species), these were added to the “meta” reference genome.

Many of the sequences from the BLASTn search contain ambiguous IUPAC nucleotides. This can be a problem in the alignments, primer design, etc. We created an expanded pseudo reference genome by creating all alternative sequences for each ambiguous base. We limited this to the sequences that have a maximum of 4 ambiguous bases to avoid an exponential increase in the number of sequences generated. For example, a sequence that has 1 N will
generate 4 sequences that have A, T, G or C in place of N. A sequence with 2 Ns would generate 16.

We designed MIPs to bind to all the target sequences in the pseudo reference genome (covering all the known variations), and to avoid all of the sequences in the “meta” reference genome. Similarly, the MIPs designed for the host targets (tick species, TROSPA gene and mammalian host species) are specific and also capture all natural variations in the target sequences. We also specified a high "single_mip_threshold" to force all designs to output only a single MIP for all designs except for the TROSPA gene where we want to cover the entire gene and thus multiple MIPs were designed to capture the full-length sequence of the gene. To capture all known variation in the probe binding site, we use multiple probes similar to degenerate primers used in regular PCR.

III.4.2.2 Molecular Inversion Probe Capture, Amplification, And Sequencing

Our approach to molecular inversion probe library sequencing follows a multi-step protocol adapted from previously published protocol (Aydemir et al., 2018), tailored for the identification of both tick hosts and pathogens. This protocol involves several key stages. Firstly, a panel is constructed by combining desired MIPs, encompassing 215 host-related (including tick species, TROSPA gene, and mammalian host species) and 320 pathogen-related (incorporating pathogen species and antibacterial resistance variants) targets. The panel is then subjected to 5’ end phosphorylation. Depending on requirements, panels can be utilized individually or amalgamated for a larger panel. In our assays, we merged
both panels in a 1:1 ratio. Each capture reaction is executed as a singular reaction per MIP panel pool for each sample. This involves combining sample DNA, MIP panel pool, polymerase, and ligase. Under isothermal conditions, MIPs bind to their respective targets, followed by polymerase extension and the formation of single-stranded circles by ligase. Post-capture, exonuclease treatment is employed to eliminate any residual linear DNA (unbound probes, original template DNA). All captured products subsequently undergo amplification through a forward and reverse primer, which includes Illumina sequencing adapters and 8-nucleotide-long sample barcodes. Following barcoding, samples are combined into a single tube to generate a sequence-ready library, which is further refined before sequencing using solid-phase reversible immobilization (SPRI) beads and agarose gel purification (Figure 3.1).
Figure 3.1 MIP workflow and experimental multiplexing. A Molecular Inversion Probe comprises two arms that match its target, connected by a 36-base constant backbone sequence shared among all MIPs. Fourteen random bases between the target-specific arms and the backbone create unique molecular identifiers (UMIs). MIPs are designed...
and synthesized for diverse targets (1). To massively parallelize the capture process all MIPs are pooled into a single MIP panel (2). A 96-well workflow minimizes errors and streamlines sample processing. During the capture reaction, MIPs hybridize with their specific targets, followed by DNA polymerase filling the gap and ligase closing it (4). Exonucleases are then employed to remove non-circularized probes and template DNA, leaving only the circularized target DNA (6). PCR reaction is set up to add sample-specific barcodes to each sample using primers with the universal sequences that bind to the probe backbone (7, 9). PCR product is pooled and after cleanup, libraries are sequenced using Illumina's sequencing platform with dual indexing (11).

### III.4.2.3 Molecular Inversion Probe Data Processing

The sequencing data was processed using MIPTools as detailed in Aydemir et al. 2018 ([Aydemir et al. 2018](#)). In a concise overview, the initial step involved demultiplexing sequences based on their dual sample barcode using bcl2fastq software from Illumina. Subsequently, the paired-end reads were merged together using FLASH (Magoč & Salzberg, 2011) and subjected to filtering based on expected length as well as per-base quality scores. A sequence would be discarded if the fraction of quality scores >30 was <70% (Q30 < 70%). Following this, quality-filtered merged sequences were further demultiplexed by specific targets using the extension and ligation arm sequences. This procedure resulted in the generation of individual files for each target within every sample.

For each sample, target sequences were then refined using their distinctive molecular identifiers (UMIs). This process entailed clustering sequences based on their UMIs, thereby generating a consensus sequence for each unique UMI.

The incorporation of UMIs facilitated the elimination of a significant portion of PCR errors, particularly those arising in later cycles, encompassing polymerase stutter and subsequent sequencing errors. Following UMI correction, the
sequences were additionally subjected to clustering utilizing the qcluster algorithm from SeekDeep (Hathaway et al., 2018).

III.4.2.4 Molecular Inversion Probe Target Identification

The quality filtered sequences were then subjected to BLASTn search to identify the targets that were present in each sample. The species of the hits with the lowest e-value (>95% query coverage and >95% identity) were assigned to the sequences. In rare cases when the sequences didn’t get any hits based on above criteria, these sequences were neglected.

For detection of the Babesia variants associated with antimicrobial resistance, the sequences were aligned to the reference genome and checked for SNPs at the locus associated with antimicrobial resistance published by Lemieux et al. 2016 (Lemieux et al., 2016).

III.4.3 Tick Microbiome Study

III.4.3.1 16S And 18S Primers And PNA Blocking Primer

We used 16S/18S HT Service from Loop Genomics using custom primers (Table 3.2). To reduce tick 18S amplification, we used PNA blocker.

We searched for the tick-specific sequences where the blocker nucleic acids can bind, which results in the inhibition of tick DNA amplification while allowing the amplification of other eukaryotic DNA by PCR. We downloaded sequences for 18S rDNA of Ixodidae (taxid: 6939) from NCI GenBank. The blocker site was
selected based on the criteria shown in Table 3.3, as well as low self-complementarity, no mismatches among tick sequences, closest site to the reverse primer site and no cross reactivity beyond phylum Arthropoda. The PNA blocker, TickR32_PNA “CAAGTTTGGTCATCTTTCCA” start site was 32 bp away from the reverse primer start site. PNA (TickR32_PNA) was synthesized by PNA BIO INC.

We tested PNA blocker on six tick species (*Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor andersoni*, *Dermacentor variabilis*, *Rhipicephalus sanguineus*, *Ixodes scapularis*).

We amplified the 18S rDNA gene using the primers listed above with and without PNA blocker. PNA blocker was used in a ratio of 1:4 (primers to PNA blocker). We used 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems Cat No.: KK2602) and followed the manufacturer’s protocol for amplification. The reaction conditions were set at 95 °C for 3 min, followed by 30 cycles of 98 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and 72°C for 10 min. The amplified product was visualized using SYBR Gold on a 2% agarose gel following electrophoresis.

**Table 3.2. Primers used in Microbiome Analysis**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td>AGAGTTTGATCMTGGCTYAG</td>
<td>TACCTTGTTACGACTT</td>
</tr>
<tr>
<td>18S rDNA</td>
<td>YCYGGTTGATCCTGCC</td>
<td>GMWACCTTGTTACGACTT</td>
</tr>
<tr>
<td></td>
<td>CTTTCYGCAGGTTACCTAC</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. PNA blocker selection criteria and properties

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Requirement</th>
<th>PNA Blocker: TickR32_PNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm at 4μM</td>
<td>70-80°C</td>
<td>74.5°C</td>
</tr>
<tr>
<td>GC content</td>
<td>&lt;50%</td>
<td>40%</td>
</tr>
<tr>
<td>Purine content (AG)</td>
<td>&lt;50%</td>
<td>35%</td>
</tr>
<tr>
<td>Max stretch of purine</td>
<td>&lt;6</td>
<td>3</td>
</tr>
</tbody>
</table>

III.4.3.2 16s And 18s Rdna Library Preparation, Sequencing, And Taxonomy Assignment

Loop Genomics provided us with 96 well Multiplex Oligo Plate with barcoded custom 16S/18S primers. We processed 853 samples and 11 blanks in 9 plates. For each sample, 10 μl of Q5 Master Mix was added to a unique well in the reaction plate. To each well, 25ng tick DNA was added followed by 5 μl of a unique index mix taken from the Multiplex Oligo Plate. The 16S/18S genes were amplified using PCR following the manufacturer’s protocol. Following amplification, each well was quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Cat. No.: P7589) following the manufacturer’s protocol and diluted to 0.2ng/μl. For each 96 well plate, 5 μl of each sample was pooled together to a final volume of 480μl. Each of the 9 pools were purified following SPRIgnore Cleanup protocol from Loop Genomics. The purified pools were shipped to Loop Genomics for library preparation, sequencing. Loop Genomics performed quality
control on sequencing data and taxonomy assignment. We received representative ASVs within each sample along with their counts and taxonomy.

III.4.3.3 Assessment Of Overall Bacterial Diversity

We assessed bacterial diversity as described in Section II.4.3.4. For alpha and beta diversity analysis, the ASVs were rarefied at 300 sequences per sample for prokaryotic communities and 50 sequences per sample for eukaryotic communities.

III.4.3.4 Identification Of Bacterial Markers Potentially Associated With B. burgdorferi Infection

Bacterial markers were identified as described in Section II.4.3.5

III.5 Results

In this chapter, I will describe the development of two important methods in our research: Molecular Inversion Probes (MIPs), and full length 16S and 18S rDNA sequencing, and assess their capacity to serve as rapid and economical tools for high-throughput surveillance of tickborne diseases and provide a comprehensive understanding of the tick microbiome, encompassing both prokaryotic and eukaryotic communities respectively.
III.5.1 Tick Selected And Analyzed In This Study

As of October 2021, we received 410 mail-in kits back from participants, containing a total of 3,102 ticks. Among these ticks, 2,782 (90%) were unengorged, 293 (9.4%) were engorged, and 27 (0.87%) were not in either of these categories and were classified as "unknown". In this study we applied MIPs and microbiome analysis methods to 853 ticks (529 ticks from the *Ixodes* species and 324 ticks from other genera). The majority of the ticks received and subsequently selected for our study were from the Northeast region of the USA, where tick abundance is known to be highest. However, in order to ensure a comprehensive representation of tick populations and tick-borne diseases, we took great care in selecting ticks from diverse species, geographical locations, and various collection times. We initially chose ticks from states outside the Northeast and Midwest regions. Next, we selected ticks from areas where we had received tick samples over multiple years (2017 to 2019), with the majority coming from the Northeast and Midwest regions. Finally, we included ticks from both the Northeast and Midwest regions, encompassing both the *Ixodes* genus and other genera.

This selection process allowed for a comprehensive investigation into the diversity and dynamics of tickborne diseases across various tick species and locations, as well as a detailed exploration of the microbiome interactions within these ticks. By employing MIPs and full length 16S and 18S rDNA sequencing,
we aimed to gain valuable insights into tick epidemiology and the complex relationships between ticks, their pathogens, and the microbiome that coexist within their ecosystem.

III.5.2 Molecular Inversion Probes And Tickborne Diseases

Epidemiology

III.5.2.1 Mips Design And Validation

We designed a total of 535 MIPs, which have the capability to detect approximately 25 tick species, 100 pathogens (comprising around 75 prokaryotic, 20 eukaryotic pathogens, and 3 viruses), 25 mammalian hosts, and 5 variants associated with *Babesia microti* infection relapse in humans. Additionally, we included MIPs to sequence the full-length TROSPA gene of *Ixodes* species in 7 smaller segments. This allows us to identify variants in the TROSPA gene that might be associated with high or low *B. burgdorferi* infection rates in *Ixodes* ticks.

To confirm the capacity of MIPs to identify their intended targets, we utilized DNA from 9 ticks of known species and from 4 known pathogens as positive controls (Table 3.4). For tick species, we used DNA extracted from 8 ticks received from the tick rearing facility of Oklahoma State University. For *I. scapularis*, we used DNA isolated from an individual collected from the wild and identified morphologically by sight. For pathogens, we ordered DNA of *Babesia duncani*, *Babesia microti* and *Francisella tularensis* from BEI Resource. For *Borrelia*
burgdorferi, we utilized DNA from an I. scapularis tick previously identified as infected in our earlier PCR screening (Chapter 2).

For the control assay, to evaluate the sensitivity of the MIPs, we used positive control samples representing target levels ranging from 200 to 2,000 target genomes/μl in 5μl, equivalent to 1,000 and 10,000 genomes per reaction mixture. This involved testing individual ticks, pathogens, and individual ticks spiked with a pool of 3 pathogen DNAs: Babesia duncani, Babesia microti, and Francisella tularensis.

In the control assay for tick species identification, the MIPs detected tick species in approximately 95% (68 out of 72) of positive control samples and in all samples with tick DNA copy numbers greater than 1000 per reaction mixture. For pathogen detection and identification, the MIPs detected pathogens in about 88% of positive control samples and in 92% of samples with tick DNA copy numbers greater than 1000 per reaction mixture (Figure 3.2 and Table 3.4).

We unexpectedly detected Candidatus Rickettsia andeanae and Rickettsia amblyommatis in Amblyomma maculatum and Amblyomma americanum, respectively, even though the samples were not spiked with these pathogen DNA. This suggests that the ticks received from the rearing facility might have been infected with these bacteria. Additionally, our MIPs successfully sequenced the TROSPA gene segments from I. scapularis and I. ricinus, as well as the loci responsible for Babesia microti infection relapse in humans. While we did not test
other tick species and pathogens due to lack of access to positive controls, we assume that MIPs designed for these targets would exhibit similar efficiency in detecting the tick species and pathogens, given that they were designed in the same manner.

Table 3.4. Ticks and Pathogens used as positive controls for MIPs assay validation

<table>
<thead>
<tr>
<th>Control</th>
<th>Species</th>
<th>Source</th>
<th>Form</th>
<th>Detection rate (above 1000 copies/rxn) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tick</td>
<td><em>Amblyomma americanum</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Amblyomma maculatum</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Dermacentor andersoni</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Dermacentor variabilis</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Haemaphysalis longicornis</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Ixodes pacificus</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Ixodes ricinus</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td><em>Rhipicephalus sanguineus</em></td>
<td>Tick Rearing Facility, Oklahoma State University</td>
<td>Live tick</td>
<td>100%</td>
</tr>
<tr>
<td>Tick</td>
<td>Pathogen</td>
<td>Source</td>
<td>Type</td>
<td>Detection Rate</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>Ixodes scapularis</td>
<td>Babesia duncani</td>
<td>BEI Resources</td>
<td>DNA</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Babesia microti</td>
<td>BEI Resources</td>
<td>DNA</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Francisella tularensis</td>
<td>BEI Resources</td>
<td>DNA</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>Borrelia burgdorferi</td>
<td>Infected Tick</td>
<td>Live tick</td>
<td>83%</td>
</tr>
</tbody>
</table>

* For each target, calculated from Figure 3.2 as (Only samples with copy number >1000copy/rxn are counted here):
Detection rate = (#Green boxes / (#Green boxes + #Red boxes)) * 100
Figure 3.2. MIPs validation: MIPs successfully and selectively detected ticks (A) and pathogens (B) in the tick metagenomic DNA. Tick metagenomic DNA was spiked with pathogen DNA and tested at 200 - 2000 genomic copies/μl. Targets that were expected/known to be present in the sample are shown in red (not detected by MIPs assay) or green (successfully detected by MIPs assay). Targets that were not expected/unknown to be present in a sample are shown in yellow (detected by MIPs) or gray (not detected by MIPs).

III.5.2.2 Implementation Of Mips For Ticks Species And Pathogen Detection.

I analyzed 853 individual ticks collected by citizen scientists from across the USA. MIPs successfully identified the species of 715 tick samples (Figure 3.3, File 3.1). Identified ticks belonged to species Amblyomma americanum (Lone star tick) (n = 26), Dermacentor andersoni (Rocky mountain wood tick) (n = 6), Dermacentor variabilis (American dog tick) (n = 240), Haemaphysalis longicornis (Asian longhormed tick) (n = 1), Ixodes pacificus (Western blacklegged tick) (n = 51), Ixodes scapularis (Blacklegged tick) (n = 376), Rhipicephalus sanguineus (Brown dog tick) (n = 15). From here on I will discuss only about the ticks for which the species was identified using MIPs.

A total of 376 Ixodes scapularis (blacklegged ticks) were analyzed, with 269 ticks collected from the Northeast region of the USA and 107 ticks collected from other regions of the country. Among the Ixodes scapularis from the Northeast, 48 ticks (17.84%) tested positive for Borrelia burgdorferi, the causative agent of Lyme disease. In the rest of the USA, 5 ticks (4.67%) tested positive for B. burgdorferi. Additionally, 10 ticks (3.72%) from the Northeast and 1 tick (0.93%) from the rest of the USA tested positive for Anaplasma phagocytophilum, a pathogen causing human granulocytic anaplasmosis. For Babesia microti, a protozoan parasite
responsible for babesiosis, 3 ticks (1.12%) from the Northeast and none from the rest of the USA tested positive. Whereas, 1 tick (0.37%) from the Northeast and 2 ticks (1.9%) from other regions of the USA were positive for *Borrelia miyamotoi*, a recently identified tick-borne pathogen. Furthermore, 1 tick (0.37%) from the Northeast and 1 tick (0.93%) from the rest of the USA tested positive for *Francisella tularensis*, the causative agent of tularemia. On the other hand, in no *I. scapularis* tick tested was infected with *Rickettsia montanensis* and *Rickettsia tillamookensis* in the Northeast region, whereas 4 ticks (3.7%) and 2 ticks (1.9%) from the rest of the USA region were tested positive respectively.

Among the 51 *Ixodes pacificus* (Western blacklegged ticks) analyzed, 1 tick (1.96%) tested positive for *F. tularensis*, 1 tick (1.96%) for *Ehrlichia chaffeensis*, and 1 tick (1.96%) for *Rickettsia tillamookensis*. The *I. pacificus* ticks analyzed were from Oregon (3 ticks) and California (48 ticks).

For the 240 *Dermacentor variabilis* (American dog ticks) analyzed, 5 ticks (2.08%) tested positive for *F. tularensis*, 1 tick (0.42%) for *Babesia duncani*, and 4 ticks (1.67%) for *Rickettsia montanensis*.

Among the 26 *Amblyomma americanum* (Lone star ticks) analyzed, 1 tick (3.85%) tested positive for *Ehrlichia chaffeensis* and 1 tick (3.85%) for *Rickettsia amblyommatis*.

Furthermore, we analyzed 6 *Dermacentor andersoni* ticks (Rocky mountain wood ticks), out of which 2 ticks (33.33%) tested positive for either *Rickettsia parkeri* or
Rickettsia peacockii, or both. The obtained sequences from the amplified target region were not unique enough to distinguish which of these Rickettsia species was present in the ticks.

No pathogenic agents were detected in the 15 Rhipicephalus sanguineus (Brown dog ticks) and the 1 Hamaphysalis longicornis (Asian longhorned tick) analyzed in this study.

Overall, 18.88% of I. scapularis, 5.88% of I. pacificus, 3.33% of D. variabilis, 3.84% of A. americanum and 33.33% of D. andersoni were infected with pathogens.

Figure 3.3: Citizen science, in combination with MIPs, facilitates tick identification and surveillance at the zip code level resolution across the USA. We successfully identified tick species for 715 out of 853 tested ticks, encompassing 7 tick species. Each colored dot on the map represents the tick species identified at a specific zip code. In cases where multiple species were detected at the same zip code, the dots are overlaid. Most ticks were received and assayed from states in the northeast (inset), the geographic region with the highest rates of tickborne diseases in the United States. Map is further zoomed in to the state of Massachusetts to show the zip code level resolution.
III.5.2.3 Mips Successfully Detected Multiplicity Of Tick Infection

Co-infections among ticks are prevalent and hold significant implications for various fields, including ecology, public health, and epidemiology. Detecting these co-infections accurately is of paramount importance for understanding their impacts on both ticks and the potential transmission of pathogens to humans and animals. A robust surveillance method capable of identifying all possible combinations of co-infections is essential. Such a method would enable researchers and healthcare professionals to comprehensively assess the complexities of tick-borne diseases, predict potential synergistic effects between pathogens.

Using MIPs, we were able to identify 10 ticks that were co-infected with more than one pathogen. Among these co-infected ticks, 7 were of the species *Ixodes scapularis*, accounting for 10% of the total infected *I. scapularis* ticks (Figure 3.4). Additionally, 2 co-infected ticks were identified as *Dermacentor variabilis*, representing 25% of the total infected *D. variabilis* ticks, and 1 co-infected tick was *Amblyomma americanum*.

The single *A. americanum* tick that was found to be infected carried two pathogens: *Ehrlichia chaffeensis* and *Rickettsia amblyommatis*. This tick was collected from New Hill, NC 27562. For *D. variabilis*, one tick collected from Mineral Bluff, GA 30559, carried *F. tularensis* and *Rickettsia montanensis*, while
another tick collected from West Buxton, ME 04093, carried *F. tularensis* and *Babesia duncani*.

As anticipated, *I. scapularis* ticks showed the highest number of co-infections. Among the 7 co-infected *I. scapularis* ticks, 3 carried both *B. burgdorferi* and *A. phagocytophilum* (two from Spencer, NY 14883, and one from Westborough, MA 01581), 2 carried *B. burgdorferi* and *B. microti* (collected from Sterling, MA 01564, and Suncook, NH 03275), and 1 tick carried *B. burgdorferi* and *B. miyamotoi* (collected from Windsor, VT 05089). The only co-infected *I. scapularis* tick that did not have *B. burgdorferi* as one of the pathogens and was not collected from the Northeastern states was found in Chesterton, IN 46304. This tick carried *Rickettsia montanensis* and *Rickettsia tillamookensis*. The co-infection data obtained through MIPs provides valuable insights into the prevalence and distribution of multiple pathogens within tick populations, which is crucial for understanding the complex dynamics of tickborne diseases.
Figure 3.4. MIPs detected multiplicity of tick infection. (A) Infection status of tick species. Ticks analyzed ranged from being uninfected to carrying 2 pathogens. For example, nearly 19% of I. scapularis ticks were infected, 10% of these carried 2 pathogens (B), (C) and (D).
III.5.2.4 Mips Detected Novel Tick-Pathogen Associations

In our assay, we employed MIPs to systematically investigate the presence of each pathogen in each tick sample, regardless of tick species or their geographical origin. This approach enabled us to uncover previously unreported and novel tick-pathogen associations (Table 3.5). This discovery highlights the significance of utilizing unbiased and comprehensive methods to expand our knowledge of tick-pathogen interactions. Among the 4 associations we identified, 2 were found in *Ixodes scapularis* ticks, and 1 each in *Ixodes pacificus* and *Dermacentor variabilis* ticks.

Specifically, our assay revealed that two *Ixodes scapularis* ticks collected from New Market, AL 35761, and Chesterton, IN 46304, were infected with *Rickettsia tillamookensis*. This finding is particularly intriguing as *Rickettsia tillamookensis* had been previously associated with *Ixodes pacificus* ticks in California (Gauthier et al., 2021; Paddock et al., 2022). Notably, we also detected *Rickettsia tillamookensis* in one of the *Ixodes pacificus* ticks collected from Santa Cruz, California 95060 during our study. This novel association finding suggests that this pathogen may not be restricted to the western USA and *Ixodes pacificus* ticks.

Another novel association we uncovered involved *Ixodes scapularis* and *Francisella tularensis*. MIPs detected *F. tularensis* in two *Ixodes scapularis* ticks collected from Rusk, TX 75785, and Belfast, ME 04915. This finding aligns with
previous research by Reif et al. (2018), which demonstrated that cell lines DAE100, derived from *Dermacentor andersoni* ticks, and ISE6, derived from *Ixodes scapularis*, were permissive to *F. tularensis* ssp. novicida infection. However, DAE100 cells exhibited significantly higher bacterial levels and mortality compared to ISE6 cells. (Reif et al., 2018).

Similarly, we identified an *Ixodes pacificus* tick from Santa Cruz, California 95060, that was infected with *F. tularensis*. Lastly, our assay detected *B. duncani* in a *Dermacentor variabilis* tick collected from West Buxton, ME 04093. *B. duncani* has recently been shown to be vectored by *Dermacentor albipictus* ticks (Swei et al., 2019).

**Table 3.5. Novel Tick-pathogen associations detected by MIPs assay**

<table>
<thead>
<tr>
<th>Host</th>
<th>Pathogen</th>
<th>Relevant Info</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. scapularis</em></td>
<td><em>F. tularensis</em></td>
<td>ISE6 cell line permissive to <em>F. tularensis</em> infection</td>
<td>Reif K. et. al. Sci Rep. 2018 Aug 23;8(1):12685</td>
</tr>
<tr>
<td><em>I. pacificus</em></td>
<td><em>F. tularensis</em></td>
<td><em>I. scapularis</em> cell lines permissive to <em>F. tularensis</em> infection</td>
<td>Reif K. et. al. Sci Rep. 2018 Aug 23;8(1):12685</td>
</tr>
<tr>
<td><em>D. variabilis</em></td>
<td><em>B. duncani</em></td>
<td><em>B. duncani</em> vectored by <em>Dermacentor albipictus</em> ticks</td>
<td>Swei A et. al. Int J Parasitol. 2019 Feb; 49(2):95–103</td>
</tr>
</tbody>
</table>
These findings contribute valuable insights into the diversity of tick-pathogen associations and highlight the significance of using MIPs as a powerful tool for the identification of novel tick-pathogen associations. Such knowledge can play a crucial role in enhancing our understanding of tick-borne diseases and improving strategies for their surveillance and management.

III.5.2.5 Mips Successfully Detected Tick Mammalian Host

Although we tried to select unengorged ticks in our study, our MIPs assay detected 60 ticks that carried host DNA from humans (12 ticks), dogs (48 ticks) or cattle (1 tick), suggesting that these ticks had already started feeding when they were collected. More precisely, among the ticks carrying host DNA, we observed 30 *I. scapularis* ticks, 3 *I. pacificus* ticks, 22 *D. variabilis* ticks, 1 *A. americanum* tick, and 4 *R. sanguineus* ticks, as delineated in Table 3.6. Surprisingly, 1 tick carried DNA from both a human and a dog.

**Table 3.6. Host DNA detected in ticks**

<table>
<thead>
<tr>
<th>Host</th>
<th><em>I. scapularis</em></th>
<th><em>I. pacificus</em></th>
<th><em>D. variabilis</em></th>
<th><em>A. americanum</em></th>
<th><em>R. sanguineus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs</td>
<td>21</td>
<td>3</td>
<td>20</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Human</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cattle</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
III.5.2.5 Mips Can Screen Pathogen Variants That Cause Clinical Relapse In Humans

In a study conducted by Lemieux J. et al. in 2016, they identified 5 variants of *Babesia microti* that were associated with clinical relapse in human patients (Lemieux et al., 2016). These variants were specifically located in the Atovaquone-binding regions of the cytochrome b (cytb) gene. In our MIPs assay, we detected *B. microti* in 3 *Ixodes scapularis* ticks, and interestingly, each of these *B. microti* samples had a wild-type (WT) variant at the specific cytb loci known to be linked to clinical relapse.

This finding highlights the valuable role of MIPs in the detection of drug-resistant variants of pathogens in wild populations. By specifically targeting genetic regions associated with drug resistance, MIPs allow for the rapid and accurate identification of such variants in various tick species and geographical locations. The ability to detect drug-resistant strains is crucial for public health, as it helps to monitor the spread and prevalence of these variants in natural tick populations. This knowledge can inform healthcare providers and policymakers in implementing effective treatment strategies and developing new therapeutics to combat drug-resistant tick-borne infections.

The use of MIPs holds tremendous potential as a significant advancement in tick-borne disease surveillance. Its high-throughput capabilities allow for the simultaneous screening of multiple pathogens across numerous tick samples,
enabling the detection of novel tick-pathogen associations and the identification of drug-resistant variants in wild populations. This powerful tool not only provides valuable data for monitoring the prevalence and distribution of tick-borne pathogens but also enhances our understanding of the intricate interactions between ticks, pathogens, and their environments.

By utilizing MIPs, researchers and public health authorities can gain comprehensive insights into the epidemiology of tick-borne diseases, helping to identify emerging threats and assess the risk of disease transmission. This knowledge is essential for implementing effective prevention and control measures, ultimately reducing the burden of tick-borne illnesses on human and animal populations.

III.5.3 Tick Microbiome And Its Association With Tickborne Pathogens

In tick research, a considerable amount of attention has been directed towards investigating the prokaryotic symbionts within these arthropods. However, the understanding of eukaryotic symbionts in ticks has remained relatively limited, primarily due to the challenges associated with selectively amplifying microbial 18S rDNA in the presence of tick 18S rDNA. The investigation of prokaryotic microbiome is mostly done by sequencing only 1 or 2 variable regions (mostly V3-V4 region) of 16S rDNA. Our study sought to bridge this knowledge gap by adopting a comprehensive approach. In our assay, we meticulously examined both the prokaryotic and eukaryotic communities residing within ticks by
sequencing full length 16S and 18S rDNA. This concerted effort aimed to illuminate the entirety of the tick microbiome, shedding light on the often-overlooked eukaryotic aspects. By delving into both these domains, we aimed to provide a more holistic understanding of the intricate interactions that shape the tick microbiome.

III.5.3.1 Tickr32_pna Successfully Inhibited The Amplification Of Tick 18s Rdna

I sequenced full length 16S rDNA, to capture prokaryotic communities, and 18S rDNA, to capture eukaryotic communities, for our microbiome analysis. To avoid the amplification of tick 18S rDNA by the universal primers, we used Peptide nucleic acid (PNA), TickR32_PNA, specific to tick 18S rDNA to block its amplification. We tested the PNA on 6 tick species, and successfully blocked the amplification of the ticks 18S rDNA. The 18S amplicon bands in the lanes without PNA blocker, run a little faster (implying that it is smaller than it really is) due to the high amount of amplicon DNA present in the lane. We confirmed this by loading 1/10 dilution of I. scapularis sample from lane 13 in lane 17. This diluted sample in lane 17 ran almost at the same size as actual 18S rDNA in other lanes. We also confirmed, using I. scapularis tick, that the PNA does not have any effect on amplification of 16S rDNA by 16S primers alone as well as when used in combination with 18S primers (Figure 3.5).
Figure 3.5 TickR32_PNA inhibits the amplification of Tick 18S rDNA. TickR32_PNA didn’t have any effect on 16S amplification when tested in *I. scapularis* ticks. Lane 17 was loaded with 1:10 dilution of Lane 13, to confirm that the 18S bands are running fast only because of high amplicon concentration in samples loaded to the wells.

For our study, we used 715 ticks from the MIPs analysis for which we were able to identify tick species. Since sequencing depth was not what we had expected from our assay, we had to omit samples from our analysis due to low sequencing reads. After quality control and removal of tick 18S sequences, we used samples with at least 300 reads for prokaryotic communities and 50 reads for eukaryotic communities for further analysis. The rarefaction curve indicated that these cutoffs provided sufficient sequencing depth to adequately capture the diversity of ASVs in selected samples. For microbiome analyses we used data obtained from MIPs analysis of ticks. To determine the infection status of the tick, we combined the data from MIPs analysis and our microbiome data. Samples with more than 1% abundance of *B. burgdorferi* or *A. phagocytophilum* were considered positive for respective pathogens. Ticks used in microbiome analysis are listed in File 3.3.
III.5.3.1 Microbiome Diversity Varies By Species And Gender

In our study, consistent with findings in other studies, we observed variations in the tick microbiome attributed to different tick species and genders (Ponnusamy et al., 2014; Van Treuren et al., 2015; Zolnik et al., 2016). Assessing prokaryotic alpha diversity, we determined that *D. variabilis* displayed the highest microbiome diversity, characterized by substantial species richness, evenness, and phylogenetic diversity as indicated by observed features, the Shannon index, and Faith’s Phylogenetic Diversity (Figure: 3.6). Conversely, *D. andersoni*, *I. pacificus*, *I. scapularis*, and *R. sanguineus* exhibited significantly lower diversity in terms of observed features. Moreover, both *I. pacificus* and *I. scapularis* demonstrated significantly reduced Shannon index and Faith’s PD values in comparison to *D. variabilis*.

Likewise, within individual tick species, male ticks showcased higher microbial diversity than their female counterparts in *D. variabilis* (Shannon Index), *I. scapularis* (Shannon Index and Faith’s PD), and *I. pacificus* (Shannon Index and Faith’s PD). A similar pattern emerged in *D. andersoni* ticks, although the difference lacked statistical significance due to insufficient sample size. An exception to this trend was observed in *R. sanguineus*, where diversity levels between genders were nearly equal. In *A. americanum*, the diversity in female ticks surpassed that in male ticks; however, this discrepancy did not reach statistical significance.
Figure 3.6 Alpha diversity of the microbiome varies between species and gender. We measured alpha diversity using three different metrics including observed features, Shannon Index and Faith PD. Alpha diversity varies in both prokaryotic (A and B) and eukaryotic (C and D) communities. p-values are listed in File 3.4.
Contrary to prokaryotic diversity findings, eukaryotic alpha diversity did not exhibit significant differences among tick species or gender. In *I. scapularis* ticks, we observed a subtle elevation in eukaryotic alpha diversity in female ticks compared to males, yet this variation did not achieve statistical significance.

**Figure 3.7 Beta diversity of the prokaryotic microbiome differs by Species.** Bray Curtis plot of the prokaryotic communities shows beta diversity of ticks varied by species. The *I. scapularis* ticks found in the West coast (green rings) had a microbiome more closely related to the *I. pacificus* ticks (purple rings).

Prokaryotic beta diversity, quantified through the Bray-Curtis matrix, revealed a significant influence of tick species on the microbiome structure (Figure 3.7). Interestingly, we observed a distinct clustering pattern wherein *I. scapularis* ticks
collected from the West displayed a close clustering with *I. pacificus* ticks. This intriguing clustering pattern suggests that they may have acquired similar microbial communities from the shared environment or the host they had fed on.

III.5.3.2 Microbiome Diversity Varies By Geography

Upon investigating prokaryotic alpha diversity within *I. scapularis* ticks, a consistent trend emerged, although not all contrasts were statistically significant (Figure 3.8). Ticks collected from the Northeast region of the USA exhibited notably higher microbiome diversity in contrast to those from other regions such as the Midwest, South, and West. This disparity was evident across various diversity metrics, including observed features, the Shannon index, and Faith's Phylogenetic Diversity. Particularly in the case of Faith's Phylogenetic Diversity, the distinction was accentuated, implying that the microbiome of Northeastern ticks encompasses a broader phylogenetic range.
Figure 3.8 Microbial Diversity in *I. scapularis* ticks varies by geography. We measured alpha diversity using three different metrics including observed features, Shannon Index and Faith PD. Alpha diversity varies in both prokaryotic (A and B) and eukaryotic (C and D) communities. In prokaryotic communities, the general trend observed is that the diversity is highest in ticks collected from Northeast. For eukaryotic communities, although the ticks from south have much lower diversity, the comparisons didn’t reach significance due to small sample size except when measured as observed features. p-values are listed in File 3.5.
Similar observations were made when comparing alpha diversity between male and female ticks from each geographic region, although not all contrasts were statistically significant. Conversely, in the realm of eukaryotic alpha diversity within *I. scapularis* ticks, those from the South exhibited lower diversity in comparison to their Midwest and Northeast counterparts.

Upon analyzing prokaryotic alpha diversity within *D. variabilis* ticks, a slightly different pattern emerged. Ticks collected from the Western region of the USA displayed significantly lower microbiome diversity compared to those from the other three regions.

We did not observe any significant difference in microbiome diversity based on tick infection status or the host species whose DNA was detected through our MIPs assay.

**III.5.3.3 Identification Of Bacterial Markers Potentially Associated With *B. burgdorferi* Infection**

While our examination of microbial diversity didn't uncover any disparities among ticks infected with *B. burgdorferi*, we proceeded to conduct LEfSe discriminant analysis on both male and female *I. scapularis* ticks. This was aimed at identifying potential bacterial taxa linked to *B. burgdorferi* colonization. In our analysis of female ticks, we discovered that six taxa were more prevalent in uninfected ticks, whereas four taxa were more abundant in ticks infected with *Borrelia*, with one of these taxa being *B. burgdorferi* itself (Figure 3.9 A).
Figure 3.9: LEfSe discriminant analysis identified bacterial markers potentially associated with *B. burgdorferi* infection. LDA analysis in female *I. scapularis* ticks (A) shows that six taxa (green) are enriched in uninfected ticks, while five taxa (orange) are enriched in *B. burgdorferi* infected ticks, including *B. burgdorferi* itself. Similarly in male ticks (B), 15 taxa (orange) are enriched in *B. burgdorferi* infected ticks, including *B. burgdorferi* itself, with none enriched in uninfected ticks.
Applying the same analysis to male ticks (Figure 3.9 B), we identified 14 taxa that were enriched in *Borrelia*-infected ticks, once again with *B. burgdorferi* itself being among these taxa. This analysis sheds light on potential associations between specific bacterial taxa and *B. burgdorferi* infection, suggesting that certain microbes may exhibit preferential relationships with this pathogen in different sexes of *I. scapularis* ticks.

III.5.3.4 Common Symbionts Of Tick Vectors

Many studies have investigated the prokaryotic symbionts of ticks, but not much information is available about the eukaryotic symbionts of the ticks. In our assay we looked at both prokaryotic and eukaryotic communities of the ticks to shed some light on the complete microbiome of ticks (Figure 3.10).

Prokaryotic Microbiome (Figure 3.10 A):

Consistent with previous studies, the common prokaryotic symbionts of *R. sanguineus* (97% in female & 92% in male) and *A. americanum* (82% in females and 25% in males) were from *Coxiella* genus. We also detected *Arsenophonus* (24%) and *Cutibacterium* (10%) in *A. americanum* male ticks. Unlike previous studies (Clay et al., 2008; Maldonado-Ruiz et al., 2021), we did not detect any *Rickettsia* in male *A. americanum* ticks and only 2% in female ticks.
In *D. andersoni* ticks, the most abundant genus was *Francisella* (82% in females and 85% in males). Female ticks also carried 17% reads from the *Nocardiopsis* genus.

In contrast to previous studies that reported *Francisella* to be the most abundant genus in *Dermacentor variabilis* ticks (Duncan et al., 2022), we observed only 26% and 8.4% reads from *Francisella* genus in female and male ticks respectively. In our study, *Pseudomonas* in female ticks (52.5%), and *Arsenophonus* (40%) were most abundant in *D. variabilis* ticks.

As reported in previous studies, in *Ixodes* species *Rickettsia* was the most abundant genus (*I. pacificus*: females 95% and male 60%, *I. scapularis*: female 88% and male 38%). Males from *I.pacificus* and *I. scapularis* species also carried *Sphingomonas* at 11% and 6% reads respectively. *I. scapularis* male ticks carried 20% *Borrelia* and 11% *Cutibacterium* reads.

*Sphingomonas* was found to be most abundant in the only *Haemaphysalis longicornis* tick we analyzed. Other genera observed were *Methylobacterium/Methylorubrum* and *Williamsia* at 19% and 8% respectively.

Eukaryotic Microbiome (Figure 3.10 B):

Analyzing the eukaryotic communities of ticks, we found that *Cladosporium* was the most common genus among the tick species analyzed and is the only genus to be detected in each of the tick species we analyzed.
In *A. americanum* female ticks there were a total of 14 genera of which 7 genera had percent abundance greater than 1%. 18% ASVs belong to uncultured genera. The most common eukaryotic genera were *Piskurozyma* (26%), *Cladosporium* (23%) and *Colletotrichum* (14%).

In *D. variabilis* female ticks, a total of 93 genera were detected, but only 5 genera had an abundance of more than 1%. *Fusarium* (79%) was the most abundant genus, followed by *Alternaria* (6%). Similarly in male *D. variabilis* ticks, a total of 91 genera were detected, but only 5 genera had an abundance of more than 1%. Unlike the female ticks, *Colletotrichum* (48%) was the most abundant genus, followed by *Fusarium* (31%) and *Alternaria* (13%). There were 16 genera in female ticks (each abundance less than 0.03%) that were not detected in male ticks. Similarly, there were 14 genera in male ticks (each abundance less than 0.06%) that were not detected in female ticks. We also analyzed 1 nymph *D. variabilis* tick in our study. In this tick we detected 9 genera and 1.4% ASVs belonged to uncultured genera. The most common genera were *Rhogostoma* (50%), *Leucosporidium* (18.5%), *Cladosporium* (10%). Each of the genera detected in this nymph tick, were also detected in adult ticks, but at a much lower abundance. Out of all the genera detected in this nymph tick, only *Cladosporium* genus, which had an abundance of 10%, had an abundance of more than 1% in adult male (2.2%) and female (2.4%) *D. variabilis* ticks. All other genera were at less than 1% abundance in adult ticks.
Figure 3.10: Commonly observed prokaryotic (A) and eukaryotic (B) communities in tick species.
In the only *H. longicornis* tick we analyzed, we detected 9 genera in total and 15% ASVs belonged to uncultured genera. The most common genera were *Cladosporium* (32%), *Phoma* (29%) and *Fusarium* (10%).

*I. Pacificus* carried eukaryotes from 17 genera, with only 8 genera representing more than 1% abundance. *Cladosporium* (61%) was most abundant in *I. pacificus* male ticks followed by *Piskurozyma* (11%) and *Rhynchogastremataceae* (10%). In *I. scapularis* female ticks, 72 genera were detected of which 17 genera had percent abundance greater than 1% and 6% ASVs were from uncultured genera. *Cladosporium* (28.7%) and *Ceramothyrium* (7%) were the most common genera. In *I. scapularis* male ticks, 17 genera were detected of which 12 genera had percent abundance greater than 1% and 15% ASVs belonged to uncultured genera. *Cladosporium* (28%), *Phoma* (15%) and *Pestalosphaeria* (12%) were the most common genera. There were 14 genera that were detected in both male and female *I. scapularis* ticks.

**III.6 Discussion**

Efforts aimed at curtailing the transmission and proliferation of tick-borne diseases have often encountered a bottleneck due to a paucity of data regarding tick microbiomes, as well as the presence, abundance, and dynamics of pathogens within individual ticks across geographical regions. Addressing this research gap, our study endeavors to establish a robust platform, Project Acari,
which is dedicated to furnishing expansive and high-resolution data pertaining to ticks and tick-borne pathogens. In the previous chapter we showed that using citizen scientists we economically collected and processed a large number of ticks from across the USA (for details see Chapter II). In this chapter, we further our efforts and concentrate on three pivotal components: (1) the formulation of advanced laboratory protocols for the surveillance of ticks and tickborne pathogens; (2) the development of high-throughput laboratory techniques to generate an all-encompassing microbiome profile of ticks; and (3) the introduction of a pilot genomic dataset that not only validates earlier crucial findings but also augments our understanding with new insights.

Harnessing the power of MIPs to detect hundreds of genomic targets in a DNA sample, we developed a high-throughput assay for surveillance of ticks and tick-borne pathogens. Our MIPs assay can detect: (a) 25 tick species, (b) 100 tick-borne pathogens, (c) 25 mammalian hosts, (d) 5 drug resistant variants in *B. microti*, and (e) *Ixodes* species TROSPA gene variants. Employing our MIPs assay, we successfully identified the species of 715 ticks collected from diverse locations across the USA. These ticks span six out of the seven most prevalent tick species found in the USA; the notable exception being *A. maculatum* (gulf coast tick), which was absent among the aforementioned 715 ticks. Each of these ticks is accompanied by collection site information down to the level of zip codes. Among these ticks, 85 ticks were infected with at least 1 pathogen and 10 ticks were co-infected with 2 pathogens. In total we detected 11 species of
pathogens. We observed that the ticks collected from the Northeast region of the USA had a higher prevalence of pathogens compared to the rest of the country.

Our assay's findings reveal that the prevalence of diverse pathogens identified in various tick species across distinct geographical regions tends to align with the lower ends of prevalence previously reported (Nieto et al., 2018; TickReport, n.d.). This can be attributed to a couple of factors. Firstly, the possibility arises that data sourced from citizen scientists may not always be flawless. Evidence has shown instances where ticks collected by citizen scientists did not carry pathogens in regions where these pathogens are confirmed to exist, and human cases have been documented (Nieto et al., 2018). Secondly, our MIPs method could conceivably lack the requisite sensitivity to detect pathogens present in tick DNA at exceedingly low abundance. This is exemplified by our microbiome data, wherein we identified *B. burgdorferi* 16S rDNA sequences in 17 tick samples that tested negative in our MIPs assay.

Given the comprehensive scope of our MIPs assay that facilitates screening for every target across all samples, we have successfully identified 4 novel tick-pathogen associations (Table 3.5). These observations could potentially be attributed to the fact that these tick species feed on the reservoir hosts for these pathogens. However, it's worth noting that these newfound pathogen-tick species associations either exhibit some level of evidence connecting the pathogen with other tick species within the same genus (Gauthier et al., 2021; Swei et al.,
2019), or studies have demonstrated the permissiveness of cell lines derived from the tick species to the respective pathogen (Reif et al., 2018). These circumstances suggest the potential for vector competence. Nevertheless, the verification of pathogen transmission cycles in the respective tick species would necessitate further experimental investigations.

Our developed method exhibits the capability to detect DNA from the host species, even when ticks have initiated feeding. In our study, we identified host DNA in 60 ticks. Notably, a majority of these ticks carried dog DNA, followed by human DNA, with only 1 tick containing cattle DNA. This observation is consistent with the circumstances under which these ticks were collected by citizen scientists, many of whom were part of our Darwin's Dog citizen science initiative, specifically targeting dog owners. These ticks were often gathered during routine activities such as walks with pets in natural settings like woods, parks, and backyards. This information holds potential significance, especially in clinical scenarios, where it could aid in discerning whether an infected tick collected from humans or pets had commenced feeding. This, in turn, enables the assessment of the risk of pathogen exposure. Beyond clinical implications, the host detection capability of our assay extends to offering insights into vector ecology and the circulation of pathogens.

In our pursuit of microbiome analysis, our initial anticipation was to achieve an average sequencing depth of 10,000 reads per sample. However, we were
confronted with an outcome that fell short of this target, amounting to less than half the expected count. As a result, we were compelled to confine our analysis to samples that exhibited a minimum of 300 reads for prokaryotic communities and 50 reads for eukaryotic communities. Despite this constraint, our assessment indicated that this sequencing depth sufficed to effectively capture the diversity of ASVs in the selected samples, as supported by alpha diversity indexes and rarefaction plots. Nonetheless, it’s plausible that a handful of low abundance species might have been overlooked due to this limitation. Notably, our microbiome study data validates and reinforces pivotal findings from earlier investigations. We have corroborated that the microbial diversity within ticks is substantially influenced by various factors, encompassing environmental variables, tick species (Lalzar et al., 2012; Van Treuren et al., 2015), tick sex (Duncan et al., 2022; Thapa et al., 2019; Van Treuren et al., 2015), geographical regions (Duncan et al., 2022; Gall et al., 2017; Trout Fryxell & DeBruyn, 2016; Van Treuren et al., 2015). Furthermore, our study has ventured into uncharted territory by offering comprehensive insights into the eukaryotic communities of diverse tick species found within the United States.

Our research endeavor involved the collection of ticks spanning various regions across the United States. Moreover, we adopted a longitudinal approach by revisiting the same locations between 2017 and 2019. Intriguingly, despite this three-year span, we did not discern any significant variations in terms of pathogen prevalence or the composition of tick microbiomes. Nonetheless, this
extended data collection strategy holds promise for enabling sustained longitudinal surveillance of tick-borne pathogens and facilitating comprehensive genomic analysis across diverse geographic expanses.

Employing the MIPs technology, we embarked on sequencing the complete TROSPA gene by fragmenting it into smaller sections. Our aim was to identify any TROSPA variants that might exhibit a negative correlation with the colonization of \textit{B. burgdorferi} within \textit{I. scapularis} ticks. Among the ticks we analyzed, we did not uncover any such associations. However, continued exploration involving a larger pool of ticks could potentially yield the identification of specific variants. The revelation of a naturally occurring variant that exhibits inherent resistance or reduced binding to OSPA could hold significant implications for controlling the transmission of \textit{B. burgdorferi}.
CHAPTER IV: CONCLUSION
IV.1 Conclusion

Here I showcase Project Acari, as a robust and comprehensive platform that delivers extensive, high-resolution data pertaining to ticks and tick-borne pathogens. This innovative initiative encompasses several key elements that collectively contribute to its efficacy:

Firstly, the engagement of a large number of volunteers through social media has enabled the efficient enrollment of participants, facilitating widespread data collection. Secondly, the economical collection of ticks from diverse locations, regardless of their proximity to research sites, has been achieved, ensuring the availability of high-quality DNA samples for analysis. Thirdly, our scalable laboratory methods for tick processing and DNA extraction have enabled the processing of thousands of samples, streamlining the workflow. Fourthly, our advanced laboratory protocols for tick and pathogen surveillance. Furthermore, our method for an exhaustive microbiome profiling technique for ticks provides unprecedented insights into the complex interactions between ticks and their microbiomes.

Through the amalgamation of these components, Project Acari facilitates an enhanced comprehension of the geographic distribution of ticks and pathogens. Additionally, it offers valuable insights into the prevalence of drug-resistant variants in the wild, a critical aspect for informed diagnosis and treatment of tick-borne diseases. Moreover, our investigation into naturally occurring TROSPA
variants potentially linked to *B. burgdorferi* colonization and the intricate dynamics between ticks and their microbiomes holds the promise of unlocking novel strategies for controlling tick-borne diseases. This could involve the incorporation of genetically modified TROSPA or interventions focusing on the modulation of tick microbiomes.

In conclusion, by providing a comprehensive and adaptable framework, Project Acari lays the foundation for continued research that can significantly contribute to the mitigation of the impact of tick-borne diseases. By combining citizen science, cutting-edge laboratory techniques, and innovative data analysis offers a powerful toolkit to reshape the landscape of tick-borne disease research, diagnosis, and prevention.

Project Acari, as established and elucidated within this thesis, holds the promise of continued and expansive tick and tick-borne pathogen surveillance. The methodologies outlined provide a robust framework for ongoing data collection and analysis, ensuring a comprehensive understanding of these complex ecological systems. In this section, we showcase the application of our methods to a subset of collected ticks, underscoring the potential for broader implications.

The fusion of citizen science participation with our advanced laboratory techniques offers a unique vantage point in unraveling the intricacies of tick ecology and pathogen dynamics. The extensive reach of citizen scientists facilitates data collection on an unprecedented scale, spanning diverse
geographical regions that may be challenging for traditional research efforts to cover comprehensively. By coupling this participatory approach with precise pathogen screening, we not only gain insights into the geographic distribution of ticks and pathogens but also uncover patterns and associations that might otherwise remain concealed.

At a local level, the integration of citizen science data with our surveillance methods yields valuable information about the phenology of human-tick interactions. This granular understanding of when and where people encounter ticks can inform public health campaigns, helping individuals adopt precautionary measures during high-risk periods. Furthermore, the genetic diversity of ticks and pathogens across different regions can offer clues about the evolution and adaptation of these organisms, aiding in the prediction of disease outbreaks and potentially guiding intervention strategies.

Project Acari extends its capabilities beyond surveillance, offering a dataset with vast potential for diverse research avenues. The comprehensive microbiome data, encompassing eukaryotic communities, holds the promise of uncovering intricate host-microbe-pathogen interactions, which could drive innovative intervention strategies, including paratransgenesis. Furthermore, our exploration of naturally occurring variants of TROSPA linked to reduced *B. burgdorferi* colonization might hold the key to novel Lyme diseases control strategies, involving genetically modified TROSPA. Armed with our comprehensive
methodology and dataset, we possess a practical tool to delve into the complex connections among ticks, their microbiomes, and harbored pathogens, thus shaping the landscape of disease control.

In conclusion, Project Acari embodies a collaborative and multidisciplinary approach that leverages the power of citizen science and cutting-edge molecular techniques to tackle the challenges posed by tick-borne diseases. The methods, insights, and data generated through this endeavor stand not only as a testament to the success of our present research but also as a foundation for continued exploration, enabling us to safeguard public health, advance scientific knowledge, and develop effective strategies for disease prevention and management.

IV.2 Future Directions

As I conclude this thesis, several avenues for future research and exploration emerge, building upon the foundation laid by the work presented. These potential directions encompass both the refinement of current methodologies and the expansion into new frontiers of tick-borne disease research.

Longitudinal Studies: While our study covered a span of three years, longer-term longitudinal studies could provide deeper insights into the temporal dynamics of tick populations, pathogen prevalence, and microbiome composition. Such
extended studies could unravel patterns of change and adaptation, critical for understanding the evolving landscape of tick-borne diseases.

Pathogen-Vector Dynamics: Investigating the intricate relationships between pathogens and their vector ticks could shed light on factors influencing pathogen transmission efficiency. Further research into TROSPA variants and their impact on *B. burgdorferi* colonization could potentially yield novel targets for disease control strategies.

Microbiome Interactions: Delving deeper into the interactions between tick microbiomes and pathogens could lead to the development of innovative strategies for disease management. Understanding how the microbiome influences vector competence could open avenues for the manipulation of microbial communities to reduce disease transmission. Bacterial species, identified by our LEfSe analysis, that are negatively correlated with *B. burgdorferi* colonization need further studies to confirm their association.

Geographical Expansion: Expanding the geographical scope of tick collection beyond the United States could provide a global perspective on tick-borne diseases. This would contribute to a more comprehensive understanding of the factors driving disease prevalence and distribution.

Advanced Molecular Techniques: Continued advancements in molecular techniques could enhance the sensitivity and resolution of our surveillance methods, for example coupling MIPs with low cycle PCR for pre-amplification of
targets. Furthermore, MIPs panel can be extended to incorporate probes to detect and identify new pathogens, drug resistant variants as they are discovered. For example, recently, Lemieux et.al. identified linked blocks of accessory genome elements located on plasmids associated with human dissemination in *B. burgdorferi* using whole genome sequencing (Lemieux et al., 2023). Probes designed to detect these genome elements can be incorporated into our MIP panel to surveil *B. burgdorferi* variants, in the wild, associated with human dissemination.
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