Assessment and diversity of tick bacterial microbiomes

by

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Abstract

Ticks vector diverse pathogenic bacteria that are important to identify in public health. Recent advances in high throughput sequencing have allowed a comprehensive catalog of these bacteria to be compiled for individual ticks. In this thesis I identify the bacterial assemblages associated with wild-collected *Ixodes scapularis* in three regions of Canada, provide the first microbiome survey for *Ixodes angustus* and expand our knowledge of the microbiome of *Dermacentor albipictus* in Alberta. I also evaluate the strengths and limitations of 16S amplicon sequencing for determining the identity and proportions of bacteria associated with ticks.

Bacterial diversity surveys can give substantially different results depending on the 16S rRNA variable region or regions that are examined, which means that a potential causative agent may be missed even when an illness is known to be associated with a tick bite. I determined that if a single 16S variable region is used, the V4 region generally detects the highest estimated bacterial diversity for ticks. However, more than one marker region or protocol is needed to provide a comprehensive assessment of a tick bacterial assemblage, since any one set of primers or methodology does not detect all bacterial taxa equally. This illustrates the potential for continued discovery of previously unknown microbiome taxa in medically important ticks.

A number of studies have shown very high numbers of bacterial taxa associated with ticks, but recent research has suggested that this high diversity is an artifact of surface contamination. These interpretations are further confounded by microbiome variation that is known to be due to biological factors such as tick species, life stage, blood meal and geographic location. To more rigorously address the extent of bacterial diversity in ticks, I used a one-host tick species, *Dermacentor albipictus*, and constrained sampling to multiple ticks at a single site and one host species, in order to limit the ecological factors normally associated with

microbiome diversity. I still found substantial bacterial diversity, with a complex interaction between richness and evenness in comparisons among tick life stages, as demonstrated by using the Hill number series. Male ticks had a significantly greater number of bacterial genera than females or nymphs, while males had greater evenness than females and similar evenness to nymphs. I concluded that the high diversity of bacteria associated with ticks is probably biologically real and not simply due to technological artifacts. However, the high taxonomic variability of the minor components of the tick microbiome suggests that they should be examined further for functional significance.

Lyme disease is the most common tick-borne disease in Canada and is one of more than a dozen tick-borne illnesses that can occur as single infections or as co-infections in *Ixodes* ticks. The Lyme disease-causing bacterium, *Borrelia burgdorferi,* is the focus of surveillance programs across Canada, including in Alberta where it has been reported in 10-19% of *Ixodes* ticks during the last 7 years. However, the current focus on a single pathogen, *B. burgdorferi*, neglects other pathogens that may be transmitted by these ticks. I performed 16S rRNA bacterial surveys on female *I. scapularis* from Alberta that were previously qPCR-tested in a Lyme disease surveillance program. Both 16S and qPCR methods were concordant for the presence of *Borrelia,* with the 16S studies providing an additional profile of associated bacteria. Ticks that were qPCR-positive for *Borrelia* had significantly greater bacterial diversity than *Borrelia*-negative ticks and no pathogens other than *B. burgdorferi* were identified in these samples. This work supports and extends Lyme surveillance in Alberta, and highlights the potential to investigate the microbial community context and sources of *Borrelia* in Alberta.

Preface

This thesis is my own original work and contains results of collaborative research that have been published or submitted for publication in peer-reviewed journals.

A version of Chapter 2 of this thesis has been published as Sperling JL, Silva-Brandão KL, Brandão MM, Lloyd VK, Dang S, Davis CS, Sperling FAH, Magor KE. 2017. Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks. Ticks and Tick Borne Diseases 8(4): 453-461. doi: 10.1016/j.ttbdis.2017.02.002. I conducted both lab and computer data analyses for this study and wrote the first draft of the paper. Dr. Karina Silva-Brandão and Dr. Marcelo Brandão performed a pilot study as postdoctoral fellows at the University of Alberta and helped to troubleshoot the initial methods. Sophie Dang and Dr. Corey Davis of the Molecular Biology Service Unit at the University of Alberta contributed to lab work at several stages. Dr. Vett Lloyd of Mount Allison University provided specimens with attached metadata and discussion of results. Dr. Felix Sperling and Dr. Katharine Magor provided advice for study design, funding and editing of the manuscript. All authors contributed to writing or editing the manuscript and approved it before publication.

A version of Chapter 3 has been published as Sperling J, MacDonald Z, Normandeau J, Merrill E, Sperling F, Magor K. 2020. Within-population diversity of bacterial microbiomes in winter ticks (Dermacentor albipictus). Ticks and Tick Borne Diseases 11(6): 101535. doi: 10.1016/j.ttbdis.2020.101535. I was responsible for all molecular data collection and analysis as well as drafting the manuscript. Zachary MacDonald provided statistical advice. Jacalyn Normandeau provided tick samples and data curation of metadata associated with these samples. Dr. Evelyn Merrill, Dr. Felix Sperling and Dr. Katharine Magor provided guidance and funding. All authors contributed to writing or editing the manuscript and approved it before publication.

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Dedication

To the people who suffer from tick-borne diseases,

may this research contribute toward better diagnosis and treatment.

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List of Abbreviations

16S rRNA: 16S ribosomal ribonucleic acid

ASV: assigned sequence variant

B- : *Borrelia* negative

B+ : *Borrelia* positive

BLAST: Basic Local Alignment Search Tool

bp: base pair

Cos2: quality of representation of the variables

ESV: exact sequence variant

GLM: Generalized Linear Model

H0, H1, H2, H3, H4: Hill number 0, 1, 2, 3 or 4

MBSU: University of Alberta Molecular Biology Service Unit

MOC: mock community

NMDS: Non-metric multidimensional scaling

NTC: no template PCR control

OTUs: Operational Taxonomic Units

PCA: principal component analysis

PCR: polymerase chain reaction

qPCR : quantitative polymerase chain reaction

SEC: sham extraction control

SMRT: Single Molecule Real Time Sequencing

TROSPA: Tick Receptor for Outer Surface Protein A

V region: 16S rRNA variable region

CHAPTER 1

Introduction

1.1 Background

Ticks harbour diverse microorganisms, many of which cause diseases in the vertebrate hosts that these ticks feed on. The disease-causing organisms include eukaryotes, prokaryotes and viruses that can debilitate humans, companion animals and livestock (Bouchard et al., 2019). Among the most significant diseases vectored by ticks in Canada, 11 of 15 are due to bacteria, one is protozoan and three are viral (Bouchard et al., 2019). Between 2006 and 2010, Lyme disease, the most common tick-borne disease in the USA, cost $3 billion per year in direct human health costs (Adrion et al., 2015) and even more in indirect costs such as reduced productivity and loss of quality of life (Mac et al., 2019). One pest, the cattle tick *Rhipicephalus microplus,* causes production losses of $19 billion per year globally (Hurtado and Giraldo-Ríos, 2018). The microorganisms that help to generate these losses cause multiple cattle diseases, including babesiosis and anaplasmosis, as well as contributing bacterial endosymbionts that allow ticks to live on blood alone. Such nutritional endosymbionts provide vitamins (e.g. vitamin B9, folate) that are not produced by the ticks themselves and are in short supply in the blood diet of ticks (Duron et al., 2018; Nováková and Šmajs, 2018).

In addition to the assemblage of microbes (microbiome) that is internal to ticks, these arachnids also interact with an external microbiome. Ticks spend the majority of their life off-host, where interactions with soil bacteria are critical for survival of the tick (Burtis et al., 2019). Female ticks rub an antibacterial wax onto the surface of freshly laid eggs (Arrieta et al., 2006), and eggs can have a distinct microbiome rich in bacteria with known antifungal and antibacterial compounds, suggesting a protective role for some bacteria (Machado-Ferreira et al., 2015). While no specific soil-associated bacteria are known, as yet, to be pathogens of ticks, the role of soil microbes in the ecology of ticks is an active area of research that seeks to find biological control measures for driving mortality in off-host ticks (Burtis et al., 2019).

The microbes associated with ticks are usually described as highly diverse (Greay et al., 2018; Lado et al., 2018; Obregón et al., 2019). While some bacteria act as endosymbionts or pathogens, others simply reflect the ecological conditions of the tick. The external cuticular microbiome of ticks has high diversity, including microbes from the host's skin as well as off-

host soil bacteria (Binetruy et al., 2019; Ross et al., 2018). In contrast, recent work has established that the gut and ovary of ticks have low microbial diversity (Couper et al., 2019; Ross et al., 2018; Tokarz et al., 2019). Therefore it is critical to consider the scale and location of a tick microbiome before describing its diversity as high or low (Pollet et al., 2020).

Most tick-associated bacteria have highly variable occurrences and no known function, suggesting that they are transient or stochastic members of the microbiome with no role that defines their presence in the community. Several factors and conditions determine the assembly of communities generally, including habitat extent, existing diversity, productivity levels, disturbance and predation (Zhou and Ning, 2017). These factors all apply to the microbiome of ticks but, in the absence of obvious niche partitioning, their assembly may be better described by stochastic processes rather than by deterministic factors (Zhou and Ning, 2017).

Bacteria are the major focus of research on tick microbiomes (Narasimhan and Fikrig, 2015), with recent work revealing substantial bacterial diversity and complexity (Bonnet et al., 2017; Pollet et al., 2020). Consequently, in this thesis I focus on the bacterial component of tick microbiomes. My aims are to evaluate the methods widely used to assess bacterial microbiomes and, in some systems, to more rigorously evaluate the extent of tick microbiome diversity.

1.2 Tick biology and opportunities for bacterial associations

Ticks have complex life cycles that allow diverse associations with bacteria. Ticks are highly effective disease vectors that are small and difficult to detect as they start feeding, first injecting anaesthetic and immunomodulatory molecules with their saliva to avoid detection by their hosts and then using their salivary glands to excrete excess fluid back into their host (Šimo et al., 2017). Numerous tick-borne pathogens are adapted for 'saliva assisted transmission', exploiting the immunomodulation of tick saliva to increase their own transmission and eventual establishment (Nuttall, 2019). This process is facilitated by a long attachment period of several days, during which ticks expand to 100 times their unfed weight (Kaufman, 2007).

Ticks also bide their time as they persist for years in adverse environments, waiting to attach to a new host (Sonenshine and Roe, 2014). The long period between pathogen acquisition in one tick life stage, followed by a moult and transmission by the next life stage, makes ticks highly unusual among arthropod vectors (Dobson, 2014). The evolutionary history of ticks, with fossils dating back 100 million years, has also provided extended opportunities for ticks and

bacteria to adapt to each other, even at the level of proteins (reviewed in de la Fuente et al., 2017). For example, TROSPA (Tick receptor for Outer Surface Protein A) is an intrinsically disordered protein with no known physiological function for the tick (Urbanowicz et al., 2016) but with a critical function for the agent of Lyme disease, allowing *Borrelia* to survive a lengthy interstadial period by attaching to TROSPA in the midgut of the tick (Kenedy et al., 2012).

Protein evolution in ticks is enhanced by gene duplication, as the tick genome is highly duplicated compared to other mites (Mans et al., 2017). This process has allowed secreted salivary gland proteins to diversify in ticks, with many of these proteins involved in a "Red Queen" molecular arms race escalation in which tick responses to host defences accelerate protein evolution (Mans et al., 2017). Such a highly duplicated genome might be expected to favour specialized host associations, particularly under strong selection due to infectious diseases (Fumagalli et al., 2011). However the three-host lifestyle and high host plasticity of ticks also allow ticks to be generalist parasites (McCoy et al., 2013). Their highly duplicated genome may thus facilitate the generalist characteristics of ticks in addition to allowing host specialization. By providing substrate for gene optimization, a duplicated genome can allow gene redundancy and rapid exploitation of novel hosts (Nene, 2009; Sunter et al., 2008). This duality has resulted in ticks being described as global generalists and local specialists (McCoy et al., 2013), with both processes relating to the highly duplicated genome of ticks.

The generalist side of the lifestyle of ticks also relates to their adaptability in fluctuating environments. Such changes involve the ability to remain active despite temperature differences ranging from near 0 ˚C (off-host) to 37 ˚C (on-host), and times between meals measured in years (Sonenshine and Roe, 2014). Ecological variation also occurs by movement of ticks to new habitats via 'superspreader' birds that are ground foraging, larger bodied passerines capable of long distance migration (Brinkerhoff et al., 2019; Scott, 2016). This environmental heterogeneity exposes ticks to diverse bacteria that, in turn, can give access to bacterial genes that confer diverse functions. Evidence for the contributions of bacteria to ticks includes the finding that antibiotic treatment reduces the weight and reproductive success of ticks (Zhong et al., 2007). Such microbiome associations can expand temperature tolerance, increase resistance to desiccation and detoxify heavy metals in other arthropods (Lemoine et al., 2020), and similar roles can be expected in ticks.

1.3 Tick microbiome diversity in relation to typical number of hosts

The extreme environmental differences that many ticks encounter during their life cycle may reflect their survival in varied ecosystems and exposure to divergent host immune systems. But it is not clear if ticks with more complex life cycles are usually associated with more diverse and complex bacterial assemblages (Chicana et al., 2019). Reduced microbial diversity is often associated with negative health outcomes for the host of a microbiome. For example, low microbial diversity is commonly found in human gut 'dysbiosis', which manifests in numerous negative health conditions such as inflammatory bowel disease and *C. difficile* infection (Kriss et al., 2018). However greater diversity is not always better, and nor is it always associated with higher productivity, functioning or stability (Reese and Dunn, 2018). For example, honeybee gut microbial diversity is normally very low (Engel and Moran, 2013; Kešnerová et al., 2020). For ticks, Chicana et al. (2019) found that microbiome richness was very similar among six species that differed in the number of hosts in their life cycle, and diversity measures that included abundance gave mixed results with no relationship to number of hosts. Chicana et al. (2019) nonetheless suggested that broader host range may still be associated with greater microbiome diversity in ticks, setting up an intuitively attractive hypothesis in need of testing.

If ticks with more varied vertebrate hosts have similarly diverse bacterial microbiomes, such a relationship could be due to bacteria expanding the functional range of individual ticks. However, an association is not necessarily functional, since increased bacterial diversity could merely be a result of exposure to more hosts, without metabolic interactions. Regardless, it is essential to first determine whether there even is a discernable general relationship between the number of hosts of a tick species and its microbiome diversity.

To compare bacterial diversity estimates among tick species, I searched all studies in the Web of Science Core Collection that were published during 2000-2020, and kept those containing the terms 'microbio*' AND 'tick*' AND "divers*'. Major methodological differences among studies, such as varied sequencing depth or use of extraction controls, limited the extent to which detailed comparisons were possible among these studies. However, at minimum, each study usually reported the number of genus-level bacterial taxa that were detected, and the typical number of hosts is generally known for the life cycle of each tick species (Estrada-Peña et al., 2017; Horak et al., 2018; Keirans and Durden, 1998; Lindquist et

al., 2016; Sonenshine and Roe, 2014). In Table 1.1, I have compiled reports from 52 publications on genus-level bacterial richness (number of genera or equivalent) detected in ticks. A total of 60 tick species were represented in 104 tick/publication combinations.

To assess the relationship between the number of hosts and microbiome diversity of ticks, each of the tick/publication combinations was categorized by the typical number of different hosts known for each tick species. There were 97 taxonomically or methodologically separate studies on 3-host ticks, 3 on 2-host ticks and 4 on 1-host ticks (Table 1.1). This uneven distribution reflects the uneven number of tick species known in each of these categories (Sonenshein and Roe, 2014). After further subdividing these studies by life stages, the most frequently sampled 3-host ticks were females, followed by unsexed adults, then males. Females constituted all three of the studies on 2-host ticks, but only two on 1-host ticks. Despite the small number of studies on 2-host and 1-host ticks, there was substantial overlap in the number of bacterial genera (taxonomic richness) among the three host groups of ticks (Table 1.1).

Variation in molecular methodologies and life stage sampling both appeared to contribute to greater differences among studies than within studies. In comparisons among studies on the same tick species, it was evident that the number of bacterial genera found among different life stages was usually more consistent within a study than between different studies on the same tick species (Table 1.1, e.g. *Amblyomma americanum*). For the two life stage categories with the largest number of studies, males had higher bacterial richness than females in close to half of the studies (15 of 29) in which both sexes were sampled separately within the same study. Fewer within-study comparisons were done for nymphs against adults (female, male, or mixed), and these showed higher bacterial richness associated with nymphs in 11 of 15 comparisons. However, when all studies were averaged, including those that sampled only one life stage, mean numbers of bacterial genera were lower in nymphs than adult categories (Table 1.1). In general, when the evenness of relative numbers of bacterial taxa was considered (data not shown), fed females were particularly dominated by single endosymbionts that were likely to have a nutritional function (e.g. Brinkerhoff et al., 2020; Gurfield et al., 2017; Ponnusamy et al., 2014).

From these comparisons, it is clear that there is a strong need for greater methodological consistency before bacterial taxonomic richness can be effectively compared among studies. The few studies that have compared bacterial richness in different tick tissue types (Table 1.2) show no clear patterns when all studies are compiled. The number of bacterial taxa detected appears to

depend more on sampling (e.g. whole tick Table 1.1 or tissue specific Table 1.2), the polymerase chain reaction (PCR) primers used in a study (*cf* Menchaca et al., 2013 and Ponuswamy et al., 2014), external cleaning of the tick (Binetruy et al., 2019), feeding status (Brinkerhoff et al. 2020) and even the sequencing platform (reviewed in Greay et al., 2018). Most studies have been based on whole tick extracts and include external as well as internal bacteria, with the most commonly used marker region being 16S rRNA V4. Studies published before 2014 most often used 454 sequencing, after which Illumina sequencing became dominant. Other methodological variables that frequently differed between studies included sequencing depth, subtraction of sequencing reads attributed to extraction controls (e.g. Sui et al. 2017 versus other studies in Table 1.2), and whether the reports refer to phylotypes or OTUs (Operational Taxonomic Units). Such OTUs sometimes refer to a single bacterial phylotype at the species level and consequently are expected to be more numerous than genus level phylotypes (Schloss and Westcott, 2011).

Biological variation is also likely to play a role in differences between studies, which can be reflected in sampling designs. For example, studies with broader environmental and genetic sampling of more than one population should have a greater number of bacterial phylotypes than samples chosen from a single population. However, few direct comparisons have been done and no such pattern is seen in the studies in Table 1.1. Feeding status is another biological variable that may be important, but of the three studies that included some comparison between fed and unfed ticks, two showed higher bacterial richness in unfed ticks (Table 1.1) while one had more in the fed tick (Table 1.2). Therefore, while averages across all studies for 1-host and 2-host tick species may indeed be associated with fewer bacterial genera than three host ticks (Table 1.1), there is no statistical support for the hypothesis of Chicana et al. (2019). The paucity of studies describing the bacterial richness of 1-host and 2-host ticks, as well as the large methodological variation among studies, highlights the need for more rigorous testing of this hypothesis.

1.4 Biodiversity and disease risk

Loss of biodiversity and increased risk of vector-borne diseases has been intensifying with anthropogenic change (Schmeller et al., 2020). Lyme disease has now become known as ‘the first epidemic of climate change’ (Pfeiffer, 2018) based on multiple lines of evidence showing direct links between climate change and Lyme disease (Couper et al., 2020). Further climate change will probably increase the impact of Lyme disease and it is imperative to better

understand the impact of biodiversity loss on disease risk. The dilution effect hypothesis proposes that loss of general biotic biodiversity is directly linked to increased risk of disease (Ostfeld and Keesing, 2000). Various mechanisms contribute to reduced disease incidence, whether by enhanced regulation of susceptible host populations or interruption of disease transmission, while disease risk increases when these mechanisms are short-circuited (Civitello et al., 2015). Research on Lyme disease provided the original impetus for this hypothesis and it was further developed based on ticks as disease vectors (Keesing et al., 2010), and has since been extended to more than 60 host-parasite systems (Civitello et al., 2015). Such systems require a generalist vector, like ticks, and variation in the abundance and behavior of differentially susceptible hosts in order for the dilution effect to operate.

In contrast to the dilution effect, the amplification effect hypothesis proposes that greater biodiversity increases disease risk, by providing a host community size above a threshold for disease transmission to occur (Roberts and Heesterbeek, 2018). The amplification hypothesis proposes that, below a diversity threshold, a disease cannot persist, but above this threshold, a more diverse community will support more diseases, such as by providing a safe haven for novel parasites (Zargar et al., 2015). Additional complexity arises when different tick life stages have different interactions that lead to dilution or amplification, or hosts vary in their ability to groom or tolerate ticks, independent of diseases (Ogden and Tsao, 2009).

These interactions can also apply to the components of a tick microbiome. The presence of one disease may increase the likelihood of a second pathogen when the first pathogen impairs early defence against the second pathogen (Holden et al., 2005). Competitive interactions as well as facilitation have been demonstrated for *Dermacentor* ticks (Gall et al., 2016; Gurfield et al., 2017). Competitive interactions that reduce the abundance of two strains have been demonstrated by mixed strain infections of a single bacterial species, *Borrelia afzelii* (Genné et al., 2019). Host responses can also influence the composition of a microbiome, with tick gut dysbiosis reducing numbers of some members of the bacterial community and increasing others through interactions with the peritrophic membrane (Abraham et al., 2017; Heisig et al., 2014; Narasimhan et al., 2014) and secreted gut proteins (Narasimhan et al., 2017).

The interpretation of dilution or amplification effects is further complicated by interactions within bacterial microbiomes, since the rare members of a community may be extremely difficult to quantify reliably when there is variation in the total numbers of discrete

niches for the microbiome, rather than variation in relative abundance overall (Reese and Dunn, 2018). Microbiome studies that are designed to test for a possible change in diversity should therefore also consider methodological factors such as detection probability, which may be more important for microorganisms than for macroorganisms.

1.5 Detection of tick-borne disease organisms

The suitability of techniques for detection of tick-borne pathogens depends on the nature of the specimen being tested and the likely quantity of the pathogen present in a sample. The presence of tick-borne pathogens is rarely tested directly in humans, since pathogen numbers may be very small, and yet can have large negative effects on a human host (Committee on Lyme Disease and Other Tick-Borne Diseases, 2011). Destructive biopsy sampling for PCR is not feasible for routine use when detection probability is low (Babady et al., 2008; Mayo Clinic Laboratories, 2020). Direct observation of a pathogen by staining may require culturing to achieve detectable levels of the pathogens (Liveris et al., 2012; Marques, 2015). Not all tick-borne pathogens have the same requirements for culture (Brouqui et al., 2004) and not all pathogens are reliably found in the blood. In particular, Lyme *Borrelia* (*B. burgdorferi*), rapidly leave the blood, in contrast to the relapsing fever spirochete, *Borrelia persica* (Liang et al., 2020), and recent research shows that *Borrelia* is highly concentrated in the platelet fraction of blood (Sanderson et al., 2020). Such variation in tissue specificity accounts for a recent finding of high success using PCR detection in blood for 4 tick coinfections and only 44% success for Lyme *Borrelia* (Buchan et al., 2019). The polymicrobial nature of the pathogens carried by ticks, and the low positivity rates for PCR of Lyme *Borrelia*, has supported continued use of serological detection for humans, in preference to direct detection methods (Babady et al., 2008; Garg et al., 2018; Tilly et al., 2008).

Detection of bacteria in ticks has a number of advantages, including that it is not constrained by the same ethical concerns as for humans. However, detection of a pathogen in a tick does not necessarily indicate that the pathogen would be transmitted to a vertebrate host, since direct detection in the gut of a tick is different from detection in its salivary gland, from which it is more likely to be effectively transmitted (Sonenshine and Roe, 2014). Historically, both *Rickettsia* (Wolbach, 1919) and *Borrelia* were detected in ticks using microscopy and, for Lyme *Borrelia*, by culture of flamed ticks (Barbour and Benach, 2019). An immunofluorescence

assay (IFA) for Lyme disease was in use in passive tick surveillance in Canada by 1982, but detection was restricted to a single species, *B. burgdorferi*. IFA of tick gut contents, culture in BSK-H medium followed by IFA staining of isolates or detection by monoclonal antibodies to *B. burgdorferi* was used from 1990-1998 (Artsob et al., 1992; Ogden et al., 2006).

PCR has become an essential component of detection of tick-borne pathogens, including endpoint PCR and qPCR for specific pathogens (Modarelli et al., 2019) and PCR-amplified 16S rRNA for microbiome scans (Egan et al., 2019). Endpoint PCR of two genes (flagellin and outer surface protein A [OspA]) was used in Canada from January 1998 to January 1999 (Ogden et al., 2006). In January 1999 to October 2003, nested PCR for flagellin genes, followed by OspA, was used for some samples, and starting in 2003 qPCR for flagellin was used to detect *B. burgdorferi* (Ogden et al., 2006). By 2005, multiplex qPCR assays for both *B. burgdorferi* and *Anaplasma phagocytophilium* were used by the Canadian National Microbiology Laboratory for tick surveillance studies (Ogden et al., 2008). In Canada, there is currently no national surveillance for diseases carried by ticks other than *B. burgdorferi* and *A. phagocytophilium* (Bouchard et al., 2019). Such methodological changes have meant that retrospective comparisons of tick infection rates are challenging. However, even when taking into account variation in detection methods, some biological trends are evident, such as the northward expansion of *Borrelia*-positive ticks in North America (Fitzgerald, 2012; Gasmi et al., 2018; Soucy et al., 2018).

Although methods of surveillance for one or a few tick-borne pathogens are well developed, much less attention has been paid to determining whether all potential pathogens have been discovered, or whether interactions between otherwise-minor pathogens can amplify the symptoms of a disease (Diuk-Wasser et al., 2016). Fortunately, it is now possible to more comprehensively survey a microbiome using PCR amplicons that represent a snapshot of a whole community, thereby reducing the risk of concluding that a disease is not tick-borne when a particular single focal pathogen is not found (e.g. Egan et al., 2020). Although 16S rRNA is ubiquitous and commonly used, there are other alternatives such as DNA-directed RNA polymerase subunit beta: rpoB (Case et al., 2007), 60-kDa chaperonin protein subunit: cpn60 (Links et al., 2012) and 50S ribosomal protein: rplP (Lan et al., 2016). The Human Microbiome Project, a major undertaking of the US National Institutes of Health, primarily uses 16S rRNA for its surveys (Turnbaugh et al., 2007). Analysis tools developed for the data created by this project allow a robust understanding of 16S rRNA as a marker gene (Ward et al., 2012).

Comparison of 16S to these other marker genes demonstrates that 16S rRNA is most useful for distantly related species whereas different genes have superior taxonomic resolution for closely related species (Lan et al., 2016).

The overview of a tick microbiome that is provided by high-throughput sequencing of amplicons produced with conserved primers has its own set of shortcomings. For example, 16S rRNA scans of ticks are constrained by variation in primer affinities of different bacteria, as well as sequencing depth and the completeness of the database against which sequences are compared (Greay et al., 2018). Furthermore, in amplicon sequencing it is important to include extraction controls and negative PCR controls, since polymerases amplify DNA from extraction reagents and the lab environment as well as the tick sample (Lejal et al., 2020). Most of the contaminant DNA detected in tick samples arises at the extraction stage (Lejal et al., 2020).

Amplicon sequencing of 16S rRNA is usually preferred to shotgun sequencing because it is more economical for large numbers of samples (Jovel et al., 2016). But shotgun sequencing may have advantages for tick pathogen surveillance by allowing characterization of viruses or organisms that are not readily detected using universal bacterial primers (Jovel et al., 2016). In fact, shotgun sequencing is sometimes viewed as a more effective method for comprehensively sampling microbial communities (Brumfield et al., 2020). However, most tick-borne viruses are RNA viruses (Shi et al., 2018) which necessitates extraction of both DNA and RNA from ticks if detection of viruses as well as bacteria is the goal of the study. Nonetheless, studies that directly compare amplicon sequencing and shotgun sequencing show largely congruent results (e.g. Rausch et al., 2019). Furthermore, amplicon sequencing may be a superior method for detecting bacteria in a sample that is composed of a large proportion of host DNA relative to bacterial DNA (Hillmann et al., 2018).

1.6 Considerations in tick microbiome experimental design

1.6.1 Sampling and DNA extraction

Characterization of a microbiome is a multi-step process that requires careful consideration of many factors at key decision points. At the start of this process, sample collection depends on tick prevalence as well as available resources. Dragging or flagging for ticks is a labour intensive method of collecting and is most successful in areas with high prevalence of ticks (Newman et al., 2019). Less labour-intensive methods use traps, such as dry

ice traps for ticks and host animal traps, but require monitoring and knowledge of the behaviour of the ticks being sampled. For example, tick species vary in their attraction to carbon dioxide and speed of movement towards a source of carbon dioxide (Lindquist et al., 2016). An alternative to active trapping of ticks is passive sampling through tick submissions (Mader et al., 2020; Pak et al., 2019). Passive sampling is based on samples from veterinarians, medical doctors and in some cases, the general public (Lewis et al., 2018; Ogden et al., 2006). Passive sampling has the advantage of surveying a broader geographic area at much lower cost than active sampling (Mader et al., 2020) but may be skewed by factors like human population density or local awareness of ticks.

Prior to DNA extraction, several variables must be recorded, including tick species, life stage and feeding status of the tick. Dragging/flagging generally samples unfed ticks (Newman et al., 2019) and sampling success depends on variation in questing behaviour within species (Arsnoe et al., 2015; Tomkins et al., 2014) as well as among species (Lindquist et al., 2016). Passive sampling of ticks provides a mix of fed and unfed ticks. Ticks removed from humans are about one third engorged and most are not engorged (Nelder et al., 2014). In contrast, ticks passively sampled by veterinarians tend to be well fed, since veterinarians are infrequently asked to remove ticks when they are otherwise easily removed by the owner of the animal (J. Sperling, *personal observation*).

Sample storage before DNA extraction can also affect the results of microbiome studies. Flash freezing of samples is ideal if samples cannot be processed immediately (Hickl et al., 2019; Nebbak et al., 2017). Otherwise, ticks can be stored in the refrigerator, in a -20 ºC freezer, in ethanol or a combination of these methods. Each storage method introduces unique sample biases (Jenkins et al., 2018).

Nucleic acid extraction influences detection of a microbiome, and heavily sclerotized ticks are challenging to extract (Ammazzalorso et al., 2015; Jones et al., 2020). Whole ticks can be bissected or their organs dissected out, but both methods are labour intensive and require sterilizing of equipment between samples. A mechanical tissue homogenizer using beads (steel, glass, or zirconium) is consistent, reproducible and reduces labour and potential for cross contamination (Jones et al., 2020). Mechanical disruption by bead beating also releases nucleic acids from thick walled bacteria such as *Bacillus subtilis* spores and *Mycobacterium bovis* (Vandeventer et al., 2011). However, mechanical disruption can shear DNA (Jones et al., 2020)

which can be a concern for long read technology such as PacBio (SMRT) or Nanopore (Amarasinghe et al., 2020). Freezing in liquid nitrogen and manual homogenization of samples is an option for ticks when bead beating is not feasible (Chauhan et al., 2020).

The resulting template DNA is a composite of host and bacterial DNA. It can be quantified by a great variety of methods, including ultraviolet spectroscopy of dsDNA, ssDNA, RNA or free nucleotides (e.g. Nanodrop ™), fluorescence spectroscopy of intact dsDNA or RNA (e.g. Qubit™), gel electrophoresis (e.g. Bioanalyzer ™, Tapestation™), or qPCR using one or more broad range 'universal 'bacterial probes (Horz et al., 2005).

1.6.2 PCR Amplification

PCR amplification, particularly of 16S rRNA, has been the primary technique used to detect microbial biodiversity, but remains a source of methodological biases at throughout its workflow. The choice of PCR primers contributes the first point of methodological bias in many studies (Hugerth and Andersson, 2017). Primers determine the sensitivity of detection of taxa of interest, and microbiome surveys have targeted multiple different 16S rRNA variable regions with differing results; for example, bacterial diversity based on variable region 2-3 of 16S rRNA may be considerably different from bacterial diversity measured by variable region 3-4 (Bukin et al., 2019). Multiplexing of primers for simultaneous detection of multiple variable regions produces further variation (e.g. Ion Torrent, 2014), which can influence bacterial diversity measures in downstream analyses. In fact, no single variable region appears to adequately discriminate taxa below the genus level, and the use of short read technology for single variable regions likely underestimates bacterial diversity measures (Johnson et al., 2019). One potential solution to the use of short read technologies for microbiome surveys is be to take advantage of the recently increased accuracy of several long read technologies (Amarasinghe et al., 2020) that allow association of all variable regions into a single copy of 16S rRNA. Association of variable regions in a single copy of 16S rRNA also indicates that intragenomic 16S rRNA polymorphisms are prevalent within single bacterial cells and can be clustered into meaningful taxonomic units (Johnson et al., 2019). However, microbiome surveys using short reads of 3-500 bp remain popular despite their drawbacks, and continue to use a variety of different 16S rRNA variable regions. This leads to the question – which region is best? I address this question in Chapter 2.

The parameters used in PCR thermal cycling constitute another series of methodological

biases in the use of 16S rRNA variation to characterize microbial assemblages. In traditional or 'end point PCR', the threshold number of PCR cycles used for detecting low amounts of bacteria mixed with relatively higher amounts of host DNA is generally about 25-35 cycles (Lorenz, 2012). The low end of the range is determined by the need to produce enough product for detection and the upper end of the range is due to degradation of the polymerase, with an increasing number of PCR errors resulting in nonspecific products at the higher end of the range. A further factor specific to short read sequencing of 16S rRNA amplicons is that increasing the number of PCR cycles in high biomass samples (defined as 100,000 16S rRNA copies per µl: Bender et al., 2018) is associated with increased production of chimera, increased sequencing error rate and difficulty in assessing taxon abundance (Sze and Schloss, 2019). In low biomass samples (less than 10 16S rRNA copies per µl, Bender et al., 2018), increased sequencing error may result from the greater number of PCR cycles; however this is a predictable error that can be filtered out during data analysis (Witzke et al., 2020). Both increased sequencing error and chimera production are of less concern in low biomass samples where increased detection is more important.

PCR workflows can also incorporate errors through contamination, and therefore should routinely include extraction controls and negative PCR controls, particularly in low biomass samples (Lejal et al., 2020). Most contaminating bacterial sequences are introduced at the DNA extraction step while fewer contaminating sequences are introduced at the amplification step, and so multiple negative controls (both extraction and amplification) are necessary to gauge the contribution of contaminants (Lejal et al., 2020). Identifying these sources of extraneous bacteria supports more confident interpretation of the composition and quantity of a microbiome (Hornung et al., 2019; Lejal et al., 2020; Sperling et al., 2020).

1.6.3 Sequencing

Methods of library preparation for sequencing can introduce further variation into the detection and identification of bacteria. DNA characteristics such as G/C content and length (in base pairs) are important considerations (Head et al., 2014). Some degree of PCR bias is unavoidable in library preparation (Sato et al., 2019), clonal amplification prior to sequencing using emPCR for PGM (Yang and Sun, 2017), and bridge amplification for Illumina (Ross et al., 2013). Shotgun sequencing has become another major approach to profiling microbiomes, but

can also be influenced by PCR bias during library preparation (Head et al., 2014).

Unlike shotgun sequencing, amplicon sequencing can effectively focus on a microbiome, rather than wasting resources by sequencing tick and host blood meal DNA, which is the greatest proportion of a tick extraction (more than 87% of the DNA: Ravi et al., 2019). However, for rare members of the microbiome, amplicon sequencing is a more efficient use of sequencing reads and can more correctly represent the taxonomic richness and abundance (Tessler et al., 2017).

Several technologies are widely used in high throughput sequencing and vary in their error rates and intrinsic characteristics. For example, Ion PGM sequencing is biased for AT-rich sequences, has a higher error rate after homopolymer runs and has a greater number of indels when compared to Illumina MiSeq data (Allali et al., 2017; Marine et al., 2020; Quail et al., 2012). Ion PGM data is comprised of single end reads and has variable read lengths, whereas Illumina data can be either single end or paired end and is consistent in length. Overall, direct comparisons of microbiome studies using Illumina, 454 and Ion PGM sequencing platforms show that samples cluster by treatment rather than by sequencing platform (Allali et al., 2017). In microbiome studies, Illumina sequencing is now dominant, with an estimated 80% of the global market (Government of the United Kingdom, 2019). PacBio™ sequencing continues to develop, and current databases for long read sequencing of 16S rRNA (including V regions 1-9) are relatively poor compared to those for shorter reads (Whon et al., 2018). Nanopore technology is also an emerging long read technology that is being applied to microbiome studies (Moss et al., 2020) but comparisons with existing 16S rRNA approaches are still limited (Nygaard et al., 2020).

The bioinformatic pipelines that are commonly used for 16S rRNA amplicon sequencing are generally compatible with Illumina data whereas Ion PGM data analysis is less frequently supported. Lack of support for Ion PGM data is due in part to the dominance of Illumina sequencing and in part due to the use of proprietary primers in the Ion PGM microbiome kit (Ion Torrent, 2015). Although Ion PGM sequencing was less expensive than sequencing with comparable Illumina instruments when it first launched in 2011 (Loman et al., 2012) this is no longer the case (Marine et al., 2020). Lack of support for diverse bioinformatic analyses now further limits the use of Ion PGM technology for microbiome studies.

1.6.4 Bioinformatic Analysis

Sequence analysis begins with a quality control step to identify and exclude poor quality sequences. Base calling accuracy is based on Phred scores for 'per base 'sequence quality. Length at a minimum Phred score threshold is a measure of 'per sequence 'read quality. Reads shorter than 50 base pairs or substantially longer than expected amplicons are removed (Prodan et al., 2020). Reads are then clustered and taxonomy assigned (Greay et al., 2018).

Amplicon sequencing aims to identify clusters of closely related sequences and then compare these to a database of known taxonomy. Several bioinformatic pipelines for microbiome data analysis are well established, including QIIME (Caporaso et al., 2010), MOTHUR (Schloss et al., 2009) and DADA2 (Callahan et al., 2016). The three major pipelines vary in their approach to determining the taxonomic identity of sequences. QIIME and MOTHUR have options for phylotyping (binning sequences at a taxonomic level such as genus or family), OTUs and Assigned Sequence Variants (ASVs). DADA2 is based on ASVs rather than phylotypes or OTUs. The original QIIME pipeline (Caporaso et al., 2010) provided reproducible and accessible microbiome analyses based on OTUs but gave inflated diversity measurements (e.g. Edgar, 2017; Prodan et al., 2020). These older QIIME versions are no longer supported and have now been replaced by QIIME2, which uses ASVs (Bolyen et al., 2019). DADA2 currently only processes Illumina amplicon data and is now part of the QIIME2 pipeline (Callahan et al., 2016).

Grouping of sequences is intended to reduce inclusion of sequencing errors that artificially inflate diversity measures. By grouping sequences into phylotypes (taxonomic groupings), multiple subgroupings are included by definition. Each genus may include multiple species that are not distinguishable using current short read technology (Schloss and Westcott, 2011). Within genera, clear clusters of sequences (OTUs) may be discerned but there is no further need to define whether these groupings are at the species level or subgenera. ASVs (single sequences, sometimes referred to as Exact Sequence Variants or ESVs (Glassman and Martiny, 2018) have been popularized by defining an ASV as "an identical group of sequences with biological relevance" (Callahan et al., 2017; Glassman and Martiny, 2018). While OTUs at the level of 97% may group multiple similar sequences into a single OTU, a higher threshold (e.g. 99 % or 100% as is the case of ASVs) risks classifying sequencing error as a novel OTU and producing inflated taxonomic diversity. However single gene taxonomy often does not adequately address species (Dupuis et al., 2012; Will and Rubinoff, 2004), especially bacterial

species (Bapteste and Boucher, 2009) and risks losing biological meaning (Sperling, 2003).

Clustering of sequences to reduce sequencing error can be done with or without a reference comparison. Reference free (*de novo*) clustering of sequences is computationally expensive while closed reference clustering is entirely dependent on a similar sequence being found in the database. With closed reference clustering, novel taxa are lost to classification. 'Open reference 'is a form of hybrid clustering that first clusters sequences by comparing to a database (closed reference), followed by *de novo* clustering of sequences that did not cluster against the reference sequences (Rideout et al., 2014).

In contrast to phylotypes and OTUs, assigned sequence variants (ASVs) are sequences that are defined as 'true sequences' based on probability calculations using an error model for the sequencing run. In short, the most common sequence is expected to have the greatest number of errors and sequences are filtered according to a defined threshold of confidence. Using this single gene taxonomy, ASVs are comparable among studies, and are considered to have higher resolution than OTUs. An ASV is defined as a single exact sequence statistically supported as being biologically present in a sample (Callahan et al., 2017). The argument that ASVs are more appropriate than OTUs relies on the idea that natural variation within a single population is not significant and that a single gene is sufficient for a taxonomic assignment.

Sequence variation occurs not only within populations but also within individuals. Bacteria often have genomes with multiple copies of 16S rRNA that differ from each other, and only a minority of bacterial taxa have a single copy of 16S rRNA (Pei et al., 2010; Větrovský and Baldrian, 2013). Sequence diversity in a species increases with the number of copies of 16S rRNA, and the application of OTUs can both overestimate and underestimate bacterial diversity (Větrovský and Baldrian, 2013).

In order to assign sequences to a taxonomic group, alignment algorithms are used to identify patterns of similarity. Global alignment (using Needleman-Wunsch algorithm e.g. Gao et al., 2017)), local alignment (BLAST family algorithms e.g. Ion Reporter, 2014)) and k-mer matching are options. Wang taxonomy compares k-mers of the query sequence with k-mers of the reference template and calculates probabilities that a sequence will contain specific k-mers. The sequence is assigned to the reference template with the highest probability. The taxonomic assignment algorithms then runs bootstrap replicates to assign a confidence interval (Wang et al., 2007). CLARK-S (Ounit et al., 2015) and Kraken2 (Wood et al., 2019) also use k-mers.

CLARK-S weights k-mers that occur exclusively in a reference sequence and allows mismatches in pre-determined positions. Kraken2 maps pairs of k-mer/pre-computed latest common ancestor kmers of the Ribosomal Database Project taxonomy to a probabilistic hash table.

1.6.5 Biodiversity measures

Once sequences have been classified, data for a microbiome is summarized by calculating diversity, a low dimensional representation of the entire community. Alpha diversity is a summary of a single community and can use richness (number of bacterial taxa present), Shannon Index (an information statistic that includes weighting for abundance of a given taxon or other indices such as Hill numbers. Hill numbers represent 'effective number of species 'and start with Hill Number 0 (= richness). Hill number 0 weights all taxa equally, with rare taxa counted the same as abundant taxa, while contributions of rare taxa diminish with increasing Hill numbers (Haegeman et al., 2013; Jost, 2010; Morris et al., 2014). Hill numbers are intuitively useful for describing microbial diversity measured by DNA sequences since a doubling of OTUs results in a doubling of the diversity measure. In addition, Hill numbers cope with zero inflated data which is a common feature of these datasets (Alberdi and Gilbert, 2019). Zero inflated data does not fit standard statistical distributions due to an excess number of absent taxa, or zeros. Beta diversity describes how different communities relate to each other and generally use distance matrices. These diversity measures distill patterns of microbiome diversity, allowing comparisons among systems that support generalizations at different levels of complexity and better understanding of the drivers of this diversity.

1.7 Profiling Canadian tick bacterial communities

My thesis is focused on the detection and composition of communities of bacteria that are associated with ticks at several locations in Canada. Ticks vector many diseases, some of which probably remain unrecognized. Understanding the identity and relationships of the bacteria associated with ticks is fundamental to developing molecular diagnostics of disease-causing and non-pathogenic variants of bacteria, as well as to understanding many aspects of basic tick biology. Characterization of the full microbiome of ticks using 16S rRNA should provide a comprehensive view of both the known and the previously unknown components of a microbial assemblage. But first it has become clear that greater standardization is needed to ensure

comparability between studies, and it has become increasingly important to resolve whether minor members of a tick microbiome are real or artifactual.

In Chapter 2, I compare the diagnostic utility of different regions of 16S rRNA for describing the complexity of the microbiome of 2 species of *Ixodes* ticks from across Canada. These two tick species are known vectors of *Borrelia burgdorferi*, one of several causative agents of Lyme disease (Sperling and Sperling, 2009). The main vector of Lyme disease in North America, *Ixodes scapularis,* is extending its range and is an increasingly important disease vector in Ontario where it is associated with woodlands with high canopy cover (Slatculescu et al., 2020). *Ixodes angustus* is also able to vector Lyme disease (Damrow et al., 1989) and is associated with moist, cool habitats (Eisen et al., 2006). *I. angustus* is not known to be extending its range (Lindquist et al., 2016). Bacterial diversity in each of the tick species shows dominance of a different species-specific endosymbiont (*Rickettsia* for *I. scapularis* and *Franciscella* for *I. angustus*). Sampling from multiple regions of 16S rRNA gave the most comprehensive diversity estimates. The single 16S rRNA variable region giving the highest diversity estimates for ticks is 16S rRNAvariable region 4 (Sperling et al., 2017).

In Chapter 3, I limit ecological variation among samples to assess whether the diversity of tick microbiomes is high when ecological variables are restricted. I chose a one-host tick, *Dermacentor albipictus,* from a small geographical area of Alberta, the Ya Ha Tinda Ranch. Ticks were removed from radio-collared elk with known pedigrees. Even with restricted ecological variables, microbiome diversity remains high, leading me to question the contribution of methodological limitations of 16S rRNA surveys to diversity estimates. I conclude that the tick microbiome is indeed diverse, with a single dominant endosymbiont and many variable bacterial genera. I propose that examining the functional significance of these variable tick associated bacteria, rather than focusing on taxonomic designations, is an important next step in understanding the microbiome of ticks (Sperling et al., 2020).

In Chapter 4, I compare the consistency of qPCR detection of *Borrelia burgdorferi* and 16S rRNA amplification. I explore the contributions of extraction controls by using a sham extraction, establishing the limits of detection by using a mock community of known composition, and demonstrating that *Borrelia* positive ticks have a more diverse microbiome than those that are *Borrelia* negative. The diverse members of the tick microbiome may have significant effects on the biology of the tick and should be considered if we are to understand

tick biology as well as public health risks.

In the final chapter, I make a general conclusion on the utility of 16S rRNA scans for understanding detection of bacteria associated with ticks, and I consider next steps in tick microbiome analysis.

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Table 1.1: Genus-level bacterial richness of whole ticks, arranged by typical number of hosts per tick, tick species and life stage.

Tick species	Hosts	Egg	Larva	Nymph	Female	Male	Adult	Misc.	Reference
Amblyomma americanum	3			10			7†		Menchaca et al., 2013
Amblyomma americanum	3			185	117	173			Ponnuswamy et al., 2014
*Amblyomma americanum**†	3						184		Trout Fryxell et al., 2016
*Amblyomma americanum**†	3			117	86	99			Brinkerhoff et al., 2020
Amblyomma cajennense - EtOH	3				203				Binetruy et al., 2019
Amblyomma cajennense - bleach	3				15				Binetruy et al., 2019
Amblyomma gemma	3						170		Lee et al., 2019
Amblyomma oblongoguttatum	3							152	Bennett et al., 2019
Amblyomma tapirellum	3							171	Bennett et al., 2019
Amblyomma testudinarium	3			201					Nakao et al., 2013
Amblyomma testudinarium	3						518		Lim et al., 2020
Ambylomma triguttaum	3							140	Egan et al., 2020
Amblyomma variegatum	3				158	223			Nakao et al., 2013
Amblyomma variegatum	3				18	12			Obregon et al., 2019
*Bothriocroton auruginans**	3							15	Egan et al., 2020
*Bothriocroton concolor**	3							40	Egan et al., 2020
Dermacentor astrosignatus †	3						93		Lim et al., 2020
Dermacentor compactus †	3						446		Lim et al., 2020
Dermacentor marginatus	3						343		Tekin et al., 2017
Dermacentor marginatus	3						114		Portillo et al., 2019
Dermacentor marginatus	3				250	240	250		Zhang et al., 2019a
*Dermacentor occidentalis**	3		22	13			14		Chicana et al., 2019
*Dermacentor reticulatus**	3				58	74			Kurilshikov et al., 2015
Dermacentor reticulatus	3				260	350	340		Zhang et al 2019a
Dermacentor steini	3						340		Lim et al., 2020

Table 1.1 continued

Dermacentor variabilis	3						2	Hawlena et al., 2013
*Dermacentor variabilis**	3		14	10				Rynkiewicz et al., 2015
*Dermacentor variabilis**	3						766	Clow et al., 2018
*Dermacentor variabilis**	3						12	Chicana et al., 2019
*Dermacentor variabilis**	3				46	59		Travanty et al., 2019
*Dermacentor variabilis**†	3				5	3		Lado et al., 2020
Haemaphysalis bancrofti *	3						40	Egan et al., 2020
Haemaphysalis bispinosa	3			287	284			Khoo et al., 2016
Haemaphysalis bremneri	3						20	Egan et al., 2020
Haemaphysalis flava	3				273			He and Cheng2018
Haemaphysalis formosensis	3						138	Nakao et al., 2013
*Haemaphysalis humerosa**	3						55	Egan et al., 2020
*Haemaphysalis hystricis**	3				216			Khoo et al., 2016
Haemaphysalis hystricis	3						538	Lim et al., 2020
*Haemaphysalis juxtakochi**	3						81	Bennett et al., 2019
Haemophysalis lagostrophi	3				42			Egan et al. 2020
*Haemophysalis lemuris**	3			69	63	104		Lado et al., 2018
*Haemaphysalis leporispalustris**	3		13					Chicana et al., 2019
Haemaphysalis longicornis	3						156	Nakao et al., 2013
Haemaphysalis longicornis	3				253	208		Zhang et al., 2019a
Haemaphysalis longicornis	3	101	85	79	68	66		Zhang et al., 2019b
Haemaphysalis longicornis	3						40	Egan et al., 2020
*Haemaphysalis punctata**	3						100	Portillo et al 2019
*Haemaphysalis tibetensis**	3				739	885		Yu et al., 2017
*Haemaphysalis wellingtoni**	3		278	289	253			Khoo et al., 2016
Hyalomma aegyptium	3						3	Keskin et al., 2017 (B27 primer)
Hyalomma aegyptium	3						6	Keskin et al., 2017 (B939 primer)

Table 1.1 continued

Hyalomma dromedarii	3						94	Alreshidi et al., 2020
Hyalomma excavatum	3					33		Keskin et al., 2017 (B27 primer)
Hyalomma excavatum	3					42		Keskin et al., 2017 (B939 primer)
Ixodes affinis	3					105		VanTreuren et al., 2015
Ixodes antechini	3						40	Egan et al., 2020
*Ixodes australiensis**	3						45	Egan et al., 2020
*Ixodes fecialis**	3						50	Egan et al., 2020
*Ixodes holocyclus**	3						40	Egan et al., 2020
Ixodes myrmecobii	3						60	Egan et al., 2020
*Ixodes ornithorhynchi**	3						15	Egan et al., 2020
Ixodes ovatus	3			215	143			Nakao et al., 2013
Ixodes ovatus	3			120	105			Obregon et al., 2019
*Ixodes pacificus**	3	381	311	225	162			Kwan et al., 2017
*Ixodes pacificus**	3	380	250			150		Swei et al., 2017
*Ixodes pacificus**	3	26	17			11		Chicana et al., 2019
Ixodes pacificus	3	8						Couper et al., 2019
*Ixodes pavlovskyi**	3			129	136			Kurilshikov et al., 2015
Ixodes persulcatus	3			185	162			Nakao et al., 2013
Ixodes persulcatus, unfed	3			373				Zhang et al., 2014
Ixodes persulcatus, fed	3			289				Zhang et al., 2014
*Ixodes persulcatus**	3			95	76			Kurilshikov et al., 2015
Ixodes persulcatus	3			1153				Sui et al., 2017
Ixodes persulcatus	3			30	28			Obregon et al., 2019
*Ixodes persulcatus**	3			265	843			Li et al., 2020
*Ixodes ricinus**	3		67			71		Carpi et al., 2011
Ixodes ricinus	3			133				Nakao et al., 2013
*Ixodes ricinus**	3			14	7			Estrada-Pena et al., 2018

Table 1.1 continued

*Ixodes ricinus**	3						163		Portillo et al., 2019
Ixodes scapularis	3						2		Hawlena et al., 2013
*Ixodes scapularis**	3		10	6					Rynkiewicz et al., 2015
*Ixodes scapularis**	3						89		VanTreuren et al., 2015
*Ixodes scapularis**	3						773		Clow et al., 2018
Ixodes scapularis	3			358			133		Zolnick et al., 2018
Ixodes scapularis	3				2	21			Thapa et al., 2019a
Ixodes scapularis	3				30	90			Thapa et al., 2019b
Ixodes scapularis	3				28	100			Thapa et al., 2019b
Ixodes scapularis, unfed *†	3	47	48	117	131				Brinkerhoff et al., 2020
Ixodes scapularis, fed*†	3				40				Brinkerhoff et al., 2020
Ixodes scapularis *	3				50	100			Chauhan et al., 2020
Ixodes tasmani *	3							40	Egan et al., 2020
Ixodes trichosuri	3							80	Egan et al., 2020
*Ixodes ventalloi**	3				107				Diaz-Sanchez et al., 2019
Rhipicephalus annulatus	3						482		Tekin et al., 2017
*Rhipicephalus sanguineus**	3			199	232	228			ReneMartellet et al., 2017
*Rhipicephalus sanguineus**	3						102		Portillo et al., 2019
3-host ticks, no. of studies	95	2	11	18	41	27	31	21	
3-host ticks, mean ± SE		74 ± 27	115 ± 46	144 ± 28	177 ± 32	174 ± 41	206 ± 40	72 ± 11	
Hyaloma marginatum	2				30				Keskin et al., 2017 (B27 primer)
Hyaloma marginatum	2				48				Keskin et al., 2017 (B939 primer)
Amblyomma tuberculatum	2				74				Budachetri et al., 2016
2-host ticks, no. of studies	3	0	0	0	3	0	0	0	
2-host ticks, mean ± SE					51 ±13				

Table 1.1 continued

*Dermacentor albipictus**	1		20	14			10		Chicana et al., 2019
Dermacentor albipictus	1			122	114	173	146		Sperling et al., 2020
Ixodes angustus	1		16	13					Chicana et al., 2019
Rhipicephalus microplus	1	54			61	53			Andreotti et al., 2011
1-host ticks, no. of studies	4	1	2	3	2	2	2	0	
1-host ticks, mean ± SE		54	18 ± 7.4	50 ± 33	88 ± 39	113 ± 58	78 ± 51		
	Hosts	Egg	Larva	Nymph	Female	Male	Adult	Misc.	

* tick species was surveyed in more than one population in this study
† mean when multiple values were reported

Table 1.2: Internal genus-level bacterial richness of adult ticks, subdivided by tissue type.

Tick species	**Hosts**	**Salivary gland**	**Saliva**	**Midgut**	**Ovary**	**Viscera**	**Reference**
Rhipicephalus microplus	1	60		61			Segura et al., 2020
Amblyomma maculatum	3	23	16	54			Budachetri et al., 2014
Dermacentor silvatum, partly fed	3		293	99			Duan et al., 2020
Dermacentor silvatum, fully fed	3		445	77			Duan et al., 2020
Ixodes ricinus	3			74	9		Guizzo et al., 2020
Ixodes scapularis	3					6	Ross et al., 2018
no. of studies	6	2	3	5	1	1	
mean ± SE		42 ± 17	251 ± 112	73 ± 15	9	6	

CHAPTER 2

Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks

A version of this chapter has been published as Sperling JL, Silva-Brandão KL, Brandão MM, Lloyd VK, Dang S, Davis CS, Sperling FAH, Magor KE. 2017. Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks. Ticks and Tick Borne Diseases 8(4):453-461. doi: 10.1016/j.ttbdis.2017.02.002.

2.1 Introduction

Ticks are well documented as important vectors of numerous bacterial and viral diseases, and can also debilitate hosts through secondary infections (Sonenshine and Roe, 2014). They may even vector bacteria without causing obvious disease, resulting in asymptomatic infection (e.g. Bouza-Mora et al., 2017). Entire assemblages of microbes associated with ticks can be now surveyed (Narasimhan and Fikrig, 2015), allowing documentation of the influence of bacterial community composition on tick pathogen colonization and vectoring capacity (Gall et al., 2016; Narasimhan et al., 2014). This raises the potential for refined public health risk prediction or even mitigation through microbial management (Crotti et al., 2012). However these applications depend on accurate and consistent identification of microbiomes (Ahantarig et al., 2013; Duron and Hurst, 2013; Gofton et al., 2015; Zhou et al., 2015).

Methods for characterizing tick microbiome assemblages are changing rapidly, with a variety of technologies being used to assay the DNA sequence variation of tick bacterial communities. Initially, Temperature Gradient Gel Electrophoresis/Denaturing Gradient Gel Electorphoresis (Moreno et al., 2006; Schabereiter-Gurtner et al., 2003; Van Overbeek et al., 2008) and Sanger sequencing (Clay et al., 2008; Hartelt et al., 2004; Heise et al., 2010) were applied, while broad-range PCR using electrospray ionization mass spectrometric detection has been used more recently (Eshoo et al., 2015). High throughput sequencing has dominated since Carpi et al. (2011), with 454™ pyrosequencing being the most commonly used platform. Alternative platforms have now replaced 454, each with different error rates for assessing microbial communities (Amore et al., 2016).

PCR amplification of the 16S rRNA gene is now the most commonly used method for

determining bacterial community composition, with the alternating conserved and variable sequence of this gene allowing construction of universal primers that bracket short informative regions. However, there has been no consensus on which of the nine variable regions of 16S rRNA should be targeted in bacterial studies (Barb et al. 2016; Chakravorty et al. 2007; Guo et al. 2013; Kumar et al. 2011; Youssef et al. 2009) and it remains costly to assay the full 1500+ base pairs of 16S rRNA in high throughput sequencing. The use of different 16S rRNA regions has meant that results are often not directly comparable, diminishing the value of inferences that can be drawn. Biases in detection of bacteria for a given region are known to be caused by choice of primers and amplification protocols (Brooks et al., 2015) and estimates of species richness have been shown to vary among 16S rRNA regions (Youssef et al., 2009). Moreover, single "universal" primer pairs may not amplify all bacteria equally, and this problem is exacerbated when preferential amplification of common endosymbionts masks the presence of other important bacteria (Ahantarig et al., 2013; Gofton et al., 2015). Very few studies have examined more than one 16S rRNA variable region or protocol and none has compared diversity estimates for all regions in naturally occurring bacterial assemblages associated with ticks.

My study addresses this gap by comparing the information provided by amplicons from all nine bacterial 16S rRNA variable regions, with the aim of identifying the most appropriate region or regions, as well as primer sets, for comparative studies of tick bacterial community profiles. I used three different library preparations on one sequencing platform: Ion Torrent PGM ™. I assessed the precision of identity match to taxonomic databases, relative abundance of bacterial families, diversity of bacteria detected, and consistency of sample clustering to compare multiple samples of two species of ticks, *Ixodes scapularis*, and *Ixodes angustus*.

2.2 Materials and methods

2.2.1 Samples

We selected a total of 12 tick specimens, including 9 *Ixodes scapularis* (blacklegged tick) and 3 *Ixodes angustus* (Table 2.1). Morphological tick species determinations for Atlantic *I. scapularis* ticks used the key of Keirans and Litwak (1989) and sequence data for the CO1 and 28S genes. All other ticks were identified using the key of Cooley and Kohls (1945). Ticks were removed from hosts before full engorgement or collected from vegetation (Table 2.1). DNA extractions were performed using one of the following commercial kits: Qiagen DNeasy® blood

and tissue (Catalog number 69506), PureLink® Genomic Mini kit (K1820-01) or Aquagenomic™ solution (catalog number 2030T). DNA sample quality was checked using a Nanodrop™, and quantified using a Qubit 2.0™. All specimens were surface cleaned and washed in 70% ethanol or 70% ethanol and sodium hypochlorite before extraction. The microbiome of five of the *I. scapularis* ticks (from Atlantic Canada) was assayed using three independent library preparations. Four *I. scapularis* (from Ontario) were assayed using two of the three libraries, while the *I. angustus* specimens were assayed with only one library.

2.2.2 Library construction

All 12 tick specimens were assayed using the Ion 16S Metagenomics kit (LifeTech A26216) which uses 6 variable regions: V2, V3, V4, V67, V8 and V9 (Figure 2.1a). Amplification occurred in two multiplex pools (one for V2, V4, and V8, and one for V3, V67 and V9), with 25 PCR cycles, and used Ion Xpress Barcoded adapters. Amplicon sizes of the 6 fragments averaged 254 bp, with a range of 215 to 295 bp, based on their sizes in *E. coli* (Ion Torrent, 2014). Emulsion PCR used OneTouch™2 400 bp chemistry (catalog number 4479878). Prior to emPCR, libraries were quantified using QuantStudio™ 6 Flex Real-Time PCR system, normalized and pooled. Sequencing was performed on an Ion Torrent PGM, using a 318 chip, 400 bp read lengths and Torrent Suite 4.2.1. Ion Reporter Metagenomics 16S software version 5.2 was used to trim and process reads. A second library was also constructed for the nine *I. scapularis* specimens. It targeted only 16S rRNA V4, with library preparation and sequencing being performed by the MR DNA™ commercial service (www.mrdnalab.com, Shallowater, TX, USA). One fragment of about 291 bp was amplified using primers 515F/806 (Caporaso et al., 2011) for 30 cycles, and sequenced using an Ion Torrent PGM. Raw sequencing fasta, qual and mapping files were returned and first reassembled into fastq files using make.fastq command in Mothur v.1.36.1 (Schloss et al., 2009) and then converted into BAM files using the FastqToSam 1.126.0 utility of Picard in Galaxy Toolshed at usegalaxy.org (Afgan et al., 2016). The V4 primers were trimmed as forward GTGYCAGCMGCCGCGGTAA and reverse ATTAGAWACCCBNGTAGTCC using Ion Reporter.

DNA from five Atlantic *I. scapularis* was used in the third library preparation, which involved amplification of 16S rRNA V12 and 16S rRNA V56 in two separate reactions using custom primers tailed with “Glenn” (forward) and trP1 (reverse) sequences to serve as primer

binding locations in a second PCR that incorporated Ion Torrent specific emulsion and sequencing sites and a custom barcode. Both pairs of primers were based, in part, on Nossa et al. (2010): Glenn_8F_v1v2 CAGTCGGGCGTCATCAGAGTTTGATCCTGGCTYAG and reverse trP1_357R_v1v2 CCTCTCTATGGGCAGTCGGTGATCTGCTGCCTYCCGTA, and forward Glenn_784F_v5v6 CAGTCGGGCGTCATCAAGGATTAGATACCCT and reverse trP1_1114R_v5v6 CCTCTCTATGGGCAGTCGGTGATGGGTTRCGCTCGTTRC. Locus amplification reactions were performed in 15 µl and contained 1X high fidelity buffer, 2 mM dNTPs, 2mM MgSO4, 0.2 µM of each primer 1 unit of Platinum HiFi Taq Polymerase (Life Technologies) and 10 ng of template DNA. The PCR profile included an initial denaturation at 94 C° for two minutes followed by 35 cycles of 94 C° for 30 seconds, 58 C° for 30 seconds, 68 °C for 60 seconds and final elongation at 68 C° for 7 minutes.

PCR components and cycling conditions for the adapter/barcode reaction were the same as above but used 1 µl of a 1/100 dilution of the first PCR product as template and were performed in 25 µl. Libraries were pooled and gel purified (Qiagen 28704). The final library was quantified using a Qubit 2 fluorometer. Emulsion PCR used an Ion One Touch 2 with 400 bp chemistry. Sequencing was performed on an Ion Torrent PGM using a 316 version 2 chip, 400 bp read chemistry and Torrent Suite 3.4.2. Expected amplicon sizes, based on *E. coli*, averaged 340 bp (Figure 2.1a). As with the other two library preparations, Ion Reporter software 5.2 was used to trim and process reads.

To establish baseline levels of DNA found in the environment and in extraction reagents, the Ion16S kit samples also included a sham tick extraction control. Prior to sequencing, each library preparation was adjusted for equimolar quantity, to obtain similar numbers of reads for each sample (Sanschagrin and Yergeau, 2014).

2.2.3 Taxonomic assignments

Bacterial taxonomic assignments were performed using Ion Reporter Metagenomics 16S software v5.2 (Life Technologies). Base calls entering into the analysis pipeline had a minimum Phred score of Q20 and included only reads with no mismatches in the barcode, up to 3 mismatches in the primer sequence, and a minimum length of 150 bp. Primer detection was set at single end (either forward or reverse) with minimum 90% alignment coverage between hit and query sequences once primer sequences were removed. After primers were removed, sequences

were trimmed to the nearest 20 bp and assigned to a hash table with all unique reads and their copy number.

Taxonomic assignment for unique sequences with a minimum of two copies was based on multistage BLAST® searches using megablast with a maximum evalue of 0.01. The curated Greengenes v13.5 (McDonald et al., 2012) database was used for identifications, as implemented in Ion Reporter. Reads were grouped into 3 bins: 90-97% match (approximating the family level), >97-99% identity (genus level) or ≥ 99% (species level). Taxonomic assignment prior to taxonomic binning is referred to as an 'assignment-first' approach by Siegwald et al. (2017).

2.2.4 Diversity calculations

Diversity calculations for each combination of single specimen and 16S rRNA amplicon used 'vegan' (Galaxy version 0.0.3/vegan 2.3-0;(Oksanen et al., 2015)). Diversity measures were computed separately for all specimen/amplicon combinations as well as for summed data for each library type. Two diversity measures were calculated: 'richness', which is the number of bacterial families estimated in the sample for rarefied data, and the Shannon diversity index, a combined measure of the number and evenness of bacterial families (Oksanen, 2016).

The first two sets of diversity estimates used data rarefied at 3203 reads, which was the smallest read count across all tick individuals (V4 in T120). Region V9 was removed because only two samples had more than 100 reads (JI142 and JI383), with one tick (JI164) not producing any reads. However region V9 reads were included in the analysis for summed regions. Only summed data for the two pairs of custom primers was used, due to low numbers of reads for V56 for some samples.

We also recalculated the two diversity measures for each sample after subtracting the numbers of reads found in the control sample. The numbers of reads for each bacterial family found in the control sample were subtracted from the reads for that same family when found in the tick sample. Bacterial families in the tick sample that had fewer reads than the control sample were set to zero. Only T120's Ion V4 numbers dropped below the previous threshold for rarefaction (from 3203 reads to 3042). For T120 Ion V4, the diversity measures when control reads were removed from the tick sample are reported for rarefaction at 3042 reads.

2.2.5 Clustering

Patterns of variation among ticks and V regions were visualized with principal components analysis (PCA), using the 'princomp' function set to cor=TRUE in R statistical package v3.1.3 (R Core Team, 2015). Analysis was based on 86 combinations of ticks and V regions, including data from 12 ticks and 6 Ion 16S partitions (V2, V3, V4, V67, V8 and summed), 9 ticks for MR DNA V4, and 5 ticks for custom reads summed for V12 and V56. The input data matrix was the percent of total reads for a tick for each of the 12 most frequent bacterial families plus a 13th category of 'Other' including all remaining reads.

2.3 Results

2.3.1 Comparison of 16S rRNA variable regions for assignment of bacterial identity

To evaluate previous use of high throughput sequencing strategies for examining the microbiome of ticks, we surveyed the published literature (Table S2.1). An initial phase of 454 pyrosequencing is now being replaced by other platforms such as Illumina MiSeq and Ion PGM. Different regions of 16S ribosomal RNA (Figure 2.1b) continue to be used to assess diversity and no comparison or standardization of these results has been performed.

We determined which combinations of the 9 variable regions of 16S rRNA (Figure 2.1) detect the greatest bacterial diversity, by generating three libraries using different sets of amplicons (Figure 2.1a) and sequencing them in overlapping sets of 12 ticks. The three multiplexed library preparations, the Ion 16S kit, MR DNA, and a custom library, were compared to give quantitative assessments of bacterial diversity across all nine variable regions recognized in the16S gene. The three different library preparations varied in their proportions of useable reads; the percentage of unfiltered reads that passed quality filters and were at least 150 bp in length was 77% for the Ion 16S kit, 89% for MR DNA, and 39% for the custom library. For assignment of bacterial identities we queried all filtered sequence reads with a minimum of 2 copies against the Greengenes database, where between 47 and 80% of the reads could be mapped (Suppl. Table 2.2). To compare the identity output from each amplicon and library used, we determined the relative proportion of reads that mapped to the six most common families of bacteria at increasing identity thresholds (Figure 2.2). Only a few bacterial families accounted for most of the reads, with 50% overall identified as Rickettsiaceae. Five additional families brought the total to 95%: Pseudomonadaceae, Francisellacae, Enterobacteriaceae,

Spirochaetaceae and Moraxellaceae. The reads from these six families varied greatly in their extent of sequence match to the reference database when they were partitioned by V region amplicon and three matching levels, with ≥99% allowing identification approximating the species level, ≥ 97 to < 99% at genus, and ≥ 90 to < 97% only at family level identifications. Reads from several common bacterial families were not detected in some amplicons; this was best illustrated by V9, which did not detect Rickettsiaceae, the most common bacterial family overall (Figure 2.2). There was also no consistent relationship between fragment length and either number of reads produced or percent match to reference databases. For example, the 12 tick samples coamplified using the set for V2, V4 and V8 in the Ion 16S kit produced the most reads for fragment V8, which was estimated to be the longest. V8 also had fewer identifications at the family level alone than did V2 or V4. Considering this variability in identification level of reads, which depends on V region and bacterial family, we chose to focus our subsequent analyses at the conservative level of family in order to allow more consistent comparisons.

2.3.2 Relative abundance of bacterial families assayed by 16S variable regions

To compare detection rates of different bacterial families between amplicons, we assessed the relative abundance of families identified from reads separately for each tick, 16S rRNA amplicon, or summed combinations of all amplicons for each preparation (Figure 2.3). To maximize diversity we sequenced 12 ticks across different geographical regions representing two *Ixodes* species, including five *I. scapularis* from Atlantic Canada, four *I. scapularis* from Ontario, and two *I. angustus* from Alberta and one from BC. The relative abundance of bacterial families identified in each tick specimen showed substantial differences depending on library assayed, as well as the different V regions assayed from the same library preparations. The bacterial assemblages of five ticks from Atlantic provinces (NB or NS) allow the broadest comparisons, as these were assayed with all three library preparations and across all nine variable regions of the 16S rRNA gene. These five ticks were consistently dominated by Rickettsiaceae, with one specimen (T098) also showing substantial numbers of Spirochaetaceae. This pattern held across all three preparations and almost all amplicons, with the exception of V9. Excluding amplicon V9, the proportion of Spirochaetaceae reads for T098 varied from 78% for V56 to 2% for V8. Few other bacterial families were detected for T098 using the custom primers. The V67 amplicon from the Ion 16S kit amplified reads gave 13% Moraxellaceae and

V56 detected the least.

Comparisons among all nine *I. scapularis* ticks, including the four from Ontario, allow further evaluation of the MR DNA and Ion 16S rRNA preparations (Figure 2.3). The four *I. scapularis* from Ontario had a very different assemblage of bacteria than those from Atlantic Canada. Ontario ticks were mostly dominated by Pseudomonadaceae, although there were still large proportions of Rickettsiaceae in two of the four Ontario ticks, particularly JI315. Other well-represented bacterial families were Enterobacteriaceae (max. 28%, in JI142), Moraxellaceae (max. 14%, in JI142), and Burkholderaceae (max. 11%, in JI315). Except for V9, the proportions of bacterial families were fairly consistent across most amplicons, especially between the summed reads for the Ion 16S kit ("All") and the MR DNA output. The V9 amplicon again gave an anomalous view of bacterial diversity that was dominated by Moraxellaceae and Enterobacteriaceae, but for this amplicon the bacterial proportions were similar between the Ontario and Atlantic ticks. Interestingly, the two duplicate assays of V4 variation provided by the Ion 16S amplicon and the MR DNA service appeared less similar in their bacterial family proportions than the comparison between the summed reads for all amplicons in the 16S kit and MR DNA (e.g. Spirochaetaceae and Moraxellaceae in T098 and Burkholderaceae in JI315), reflecting the possible use of different primers in the V4 amplicons of these two library preparations. The bacterial families identified from reads for the three *I. angustus* ticks from western Canada were only assayed using the six amplicons from the Ion 16S kit (Figure 2.3). As with the Atlantic versus Ontario *I. scapularis*, these ticks had very different assemblages at the bacterial family level. The two Alberta specimens were almost completely dominated by Francisellaceae, while the British Columbia specimen of *I. angustus* had substantial proportions of Francisellaceae but even more Enterobacteriaceae. The V9 amplicon amplified only Enterobacteriaceae, and nothing at all for one of the Alberta *I. angustus*.

To assess the amount and identity of contaminating DNA present in laboratory reagents, we prepared an extraction control sample with a sham tick extraction. Reads were produced for all six amplicons of the Ion 16S kit for the extraction control sample. The numbers of reads for each amplicon were only 8-22% of the averages for a tick sample, except for V9, which was at 37%. The most frequently identified bacterial family in the control sample was Neisseriaceae, which was the 59th most common family in the tick samples overall. The top 12 bacterial families in the control sample comprised 79% of the total, including four families that were in the top 12

from all ticks (Enterobacteriaceae -2nd in control sample, Burkholderaceae – 4th, Pseudomonadaceae – 7th, and Moraxellaceae – 8th).

2.3.3 Comparisons of diversity measures based on each variable region

To quantitatively compare the diversity of bacterial families, we calculated diversity estimates for each tick and variable region amplicon using rarefied data to estimate the number of families (Figure 2.4a) and the Shannon diversity index (Figure 2.4b). The two bacterial diversity measures gave largely consistent patterns and similar relative ranking of the bacterial diversity present among the 12 ticks. Two ticks (T098 and JI315) consistently showed both high numbers of families and Shannon indices compared to other Atlantic or Ontario *I. scapularis*, while *I. angustus* generally had the lowest numbers of families but not Shannon indices. Comparisons among amplicons, representing separate population estimates in the bacterial composition of each tick, showed no uniform patterns for the 16S kit and MR DNA data, although the number of bacterial families per tick was usually high for the V4 Ion amplicon and the summed 16S Ion kit data (Figure 2.4). The summed custom amplicons gave the lowest estimates of bacterial families and Shannon indices. Numbers of families for each tick differed very little or inconsistently among the remaining amplicons.

To determine whether diversity estimates change after removal of possible contaminating reads, we recalculated the two diversity measures after subtracting the number of control sample reads per amplicon from the reads obtained for each amplicon. With this correction, the estimated number of bacterial families declined by approximately half (Figure. S2.1a), largely due to the loss of rare bacterial families. The Ion V4 amplicon gave the highest number of families for almost all specimens. The average decline in estimated number of bacterial families ranged from 62%, for region V2, to 45% for region V3. The Shannon index (Figure S2.1b) declined to an average of 68% of the estimates calculated with the control reads, with the decline ranging from 71%, for region V2, to 65% for region V8. The overall ranking of diversity among ticks remained similar for both the number of families and Shannon index, regardless of whether control reads were included.

Assessment of variation among bacterial family proportions for each amplicon from each tick was provided by a principal component analysis for the frequencies of the most common bacterial families assigned from sequence reads (Figure 2.5). Four bacterial families accounted for most variation assessed in the principal component analysis, with the first two axes

describing 39% of the total variation in bacterial family frequencies. The frequencies of Rickettsiaceae were inversely related to those of Pseudomonadaceae or Enterobacteriaceae in *I. scapularis*. The most common bacterial family was Francisellaceae in *I. angustus* ticks and Pseudomonadaceae in some specimens of *I. scapularis* from Ontario. The principal component analysis also visually displays the consistency of different microbiome assays across variable regions. Assays were generally grouped by tick specimen and geographic source of the specimen, regardless of amplicon used. The most notable exceptions concerned Ion 16S rRNA V4 and V67 from two *I. scapularis* ticks from Ontario (JI315 and JI317) and one from Atlantic (T098). For example, the V4 assay for JI317 grouped more closely to JI314 and JI142 than to all other assays of JI317 (Figure 2.5). Principal components analysis thus largely confirmed the qualitative impressions of bacterial frequencies evident in Figure 2.3, with the detected bacterial assemblages varying by geographical region and species of tick, as well as the 16S rRNA amplicon used.

2.4 Discussion

Our study compared the consistency and effectiveness of replicate bacterial surveys of tick specimens across amplicons for all nine 16S rRNA variable regions. We evaluated three separate library preparations for assaying individual tick DNA samples: the Ion 16S kit based on multiple 16S rRNA variable regions, the MR DNA commercial service based on V4, and custom primers for V12 and V56. The Ion 16S kit generally allowed the greatest number of taxonomic identifications from across the domain Bacteria and five of the six amplicons provided consistent and robust insight into the composition of tick microbiomes. The high sensitivity of this kit to contaminants suggests that extraction controls should be included in sequencing runs in order to interpret both the presence of bacterial taxa and measures of taxonomic richness (number of families) and evenness. However, if cost and convenience are major factors in deciding among preparations, then the MR DNA service may be a cost effective way to survey ticks if local expertise in library preparation and sequencing is unavailable.

We found that our microbiome surveys were limited by a number of methodological factors, such as the specificity of bacterial identifications and inconsistencies in proportional abundances among different assays. However, variation among the bacterial assemblages that were detected depended more on intrinsic biological factors, such as tick species and geographic

origins, as well as the actual bacterial associates of the ticks. Notably, a tick positive for Spirochaetes was detected by all three sequencing strategies. Nonetheless, it remains essential to determine the extent to which variation in bacterial assemblages can be attributed to purely methodological factors rather than the biological variation that is normally the focus of a study. For example, by a similar geographic pattern found in *I. scapularis* ticks that were associated with *Rickettsia* or Enterobacteriaceae at different locations in the eastern U.S.A (Van Treuren et al., 2015). The Van Treuren study used two amplicons (Table S2.1: V1-V3 and V4), finding similar results for both amplicons from different specimens. Our study provided multiple estimates of microbiome composition from across the 16S rRNA gene for single specimens, allowing more controlled comparisons of relative proportions. Amplicons from one Atlantic specimen (T098) consistently contained Spirochaetaceae (*Borrelia*) and in spite of substantial variation in the frequency of reads from this family, this specimen always showed higher amounts of Spirochaetaceae than did other ticks from the same location. The exception was the V9 amplicon of the Ion 16S kit, which gave a very different bacterial composition from all other amplicons. Poor detection of bacterial diversity by V9 was also found by Barb et al. (2016) and Yang et al. (2016).

Variation due to library preparation conditions (including primers) is demonstrated by two comparisons of amplicons with substantial amounts of overlap. The first comparison is between the V2 amplicon of the Ion 16S kit and the custom V12 amplicon of the custom preparation. These two amplicons differed in length and PCR primers, but it is most likely that different preparation conditions account for the substantially lower bacterial diversity assayed with the V12 amplicon since this low diversity was also found with the V56 amplicon. The second comparison is between the V4 amplicons from the Ion 16S kit and the MR DNA service, which are very similar if not identical in size and location (although the primers cannot be compared since those from the Ion 16S kit are proprietary). These two V4 amplicons gave similar diversity measures (Figure 2.4), but the proportions of reads from each bacterial family were substantially different in some cases (e.g. T098 had 60% vs 6% Spirochaetaceae reads for the two V4s).

Uneven matching to reference databases may explain some of the variation in levels of taxonomic assignments for different bacterial families. However, it is difficult to distinguish this factor from biological variation in divergence rates across 16S rRNA variable regions.

Consequently, we maximized consistency of comparisons by focussing on the family level. Nonetheless, most variable regions and common bacterial families gave a substantial proportion of identifications above the 97% matching level. Limitations on microbiome characterizations due to the comprehensiveness of reference databases have been noted in a variety of other studies (Santamaria et al., 2012; Zhou et al., 2015). Such constraints can be expected to be particularly evident in studies of poorly known ecological components, like ticks, which can contain new bacterial taxa that currently cannot be identified beyond higher taxonomic levels (e.g. Van Treuren et al., 2015).

Proportions of identifications at the levels of 97-99% (genus) and above 99% (species) varied both among groups of bacteria and amplicons (Figure 2.2). Most amplicons yielded a majority of reads with at least 97% match to Greengenes sequences but not above 99%. It is widely accepted that taxonomy based solely on 16S rRNA should not normally extend beyond the level of genus (Srinivasan et al., 2015; Rossi-Tamisier et al., 2015). At the genus level, none of the amplicons was superior to the others across all bacterial families, although V8 usually ranked high (Figure 2.2). However, proportionally very few reads for Spirochaetaceae were produced by V8 (Figure 2.3). Multi-locus sequence typing or more specific primers are normally considered to be necessary for species level distinctions (Emerson et al., 2008; Youssef et al., 2009). Further analysis of this level of taxonomic specificity is beyond the scope of the current study and will provide opportunities for downstream investigations.

Extreme variation in detection probability raises the question of whether the presence of particular bacteria is an artefact or that they are present but difficult to detect. For example, we found *Candidatus Midichloria mitochondrii* at low frequency in four Atlantic ticks, but only from the forward primer of the custom V56 amplicon and not at all for V4 from MR DNA or any of the Ion 16S rRNA amplicons (data not shown). *Midichloria* have been described as the dominant bacterium in *Ixodes ricinus* ticks (Klubal et al., 2016; Gofton et al., 2015) and *Ixodes holocyclus* (Gofton et al., 2015) and it would be instructive to determine whether these bacteria are generally difficult to detect. This case demonstrates the potential biases of single marker identifications for bacteria and serves as a caution against making generalizations based on single amplicons.

We calculated diversity measures as a way of summarizing data and detecting potential biases due to reliance on different 16S rRNA variable regions. We expected to find consistently

higher numbers of bacterial families derived from the summed totals for all amplicons of the Ion 16S kit, since the six amplicons (in reality five amplicons since V9 gave so few reads) should provide multiple opportunities to sample bacterial families that might not be detected by particular primer combinations. We found that the 16S Ion summed data did usually give a high estimate of numbers of bacterial families for each tick, but interestingly the V4 estimate from the 16S kit was highest for five ticks, and the V3 estimate was highest for four ticks (Figure 2.4). Siegwald et al. (2017) demonstrated that sequencing errors had a significant effect on overestimation of microbiome diversity estimates, especially richness (number of families), and Ion PGM data is especially sensitive to this effect. Low diversity values obtained for the custom primer sets reflects the paucity of low-frequency families detected, although the common families (Rickettsiaceae and Spirochaetaceae) largely mirrored the proportions found with Ion 16S amplicons and MR DNA.

Removal of reads in proportion to their detection in an extraction control sample for the 16S Ion kit reduced both the expected number of bacterial families and their Shannon index by a substantial margin (Figure S2.1). However the relative ranking of the diversity measures for each specimen remained essentially the same, as did the dominant bacterial families detected. Such subtraction of control reads is a conservative measure since the small amount of DNA in the control sample will allow more efficient amplification and thus may overestimate the presence of environmental contaminants in the extraction and amplification protocols. Nonetheless, the extraction control sample in our study clearly demonstrated the importance of considering contamination in microbiome surveys, especially the potential for erroneous identification of contaminating DNA as being present in the tick sample rather than in the extraction kit.

Principal component analysis illustrated the consistency of bacterial frequency profiles for each tick, as well as allowing visualization of trends among specimens and geographic regions (Figure 2.5). Different amplicons for each tick usually clustered by specimen, although the Ion V4 and Ion V67 amplicons gave ambiguous clustering for several specimens. These results further highlight the importance of obtaining multiple assays of bacterial assemblages. Interestingly, in these cases the MR DNA V4 assay clustered more closely to the summed Ion assay than the Ion V4 assay did.

The observation that MR DNA data gave estimates of bacterial read proportions and diversity measures that were generally similar to those obtained from the Ion 16S kit raises the

question of whether the single V4 MR DNA assay can replace the multiple assays of the Ion 16S kit. The MR DNA service cost was $50 US per sample, which translated into a cost per read that was about 2.5X more than that of the Ion 16S kit after DNA extraction and quantification. However the Ion 16S kit costs more than twice as much per tick DNA sample, if the same number of reads per amplicon are expected, since this kit gives information on six amplicons rather than one. An additional factor to consider is that use of the MR DNA service did not require the higher level of skill needed to make libraries and operate an Ion Torrent PGM. Technologies and costs continue to change and so these calculations serve only as a rough comparative estimate relevant to our study. A commercial service may be sufficient for fast comparisons of sample microbiomes, assuming that a single amplicon is deemed sufficient for such estimates. However, we consider that the added value of sampling a microbiome assemblage more comprehensively and precisely by reliance on multiple amplicons will more than compensate for the reduced cost of sampling a single amplicon.

Several biologically relevant patterns of bacterial associations for ticks across Canada emerge from our study despite our limited sampling of specimens from four geographic locations and two tick species. First, there is a clear difference between *I. scapularis*, which was dominated by Rickettsiaceae or Pseudomonadaceae in our samples, and *I. angustus*, which was dominated by Francisellaceae. A marked difference of this magnitude was also found by Van Treuren et al., (2015) for *I. scapularis* compared to *Ixodes affinis* in North Carolina. Second, there were marked differences in bacterial composition in samples of the same species but from different geographic regions, again paralleling the findings of Van Treuren for *I. scapularis* in the eastern USA. Finally, our results are consistent with the hypothesis of competitive interactions or niche partitioning between different groups of bacteria in ticks (Van Treuren et al., 2015), in that Rickettsiaceae common in Atlantic ticks were more decisively replaced by Pseudomonadaceae than Enterobacteriaceae in some Ontario samples. All of these patterns remain to be confirmed with more extensive sampling of populations and locations, but already provide a strong indication of the potential value of such microbiome surveys in ticks that are known to be important vectors of disease. In future, symbionts such as *Wolbachia*, *Rickettsia* and *Spiroplasma* (Duron and Hurst, 2013) may potentially be manipulated to control ticks and change the disease dynamics of tick-borne diseases. However, it is clear that the first step must be to accurately identify the bacteria involved, as well as to determine their relative proportions.

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Table 2.1. Samples included, indicating tick species, life stage, host and sample handling. Ticks were removed before full engorgement or from vegetation. Prep refers to A, Ion 16S Metagenomics kit; B, MRDNA service; and C, custom library.

	Tick ID	Species	Life stage	Source	Date collected	location	Tick surface cleaning	DNA extraction	Prep
1	T095	*Ix. scapularis*	adult female	dog	12-Oct-12	Aulac, NB	70% EtOH	AquaGenomic™	A,B,C
2	T098	*Ix. scapularis*	adult female	dog	15-Oct-12	Amherst, NS	70% EtOH	AquaGenomic™	A,B,C
3	T115	*Ix. scapularis*	adult female	dog	17-Oct-12	St. George, NB	70% EtOH	AquaGenomic™	A,B,C
4	T120	*Ix. scapularis*	adult female	dog	19-Oct-12	Mohannes, NB	70% EtOH	AquaGenomic™	A,B,C
5	T121	*Ix. scapularis*	adult female	dog	23-Oct-12	Aulac, NB	70% EtOH	AquaGenomic™	A,B,C
6	JI142	*Ix. scapularis*	nymph	deer	02-Nov-13	Turkey Point, ON	70% EtOH + NaClO	DNeasy®	A,B
7	JI314	*Ix. scapularis*	adult female	dog	15-Apr-14	Long Point, ON	70% EtOH + NaClO	DNeasy®	A,B
8	JI315	*Ix. scapularis*	nymph	vegetation	13-Apr-14	Forestville, ON	70% EtOH + NaClO	DNeasy®	A,B
9	JI317	*Ix. scapularis*	nymph	vegetation	05-Apr-14	Forestville, ON	70% EtOH + NaClO	DNeasy®	A,B
10	JI164	*Ix. angustus*	nymph	dog	12-Dec-13	Edmonton, AB	70% EtOH + NaClO	PureLink®	A
11	JI165	*Ix. angustus*	nymph	dog	30-Nov-13	Edmonton, AB	70% EtOH + NaClO	PureLink®	A
12	JI383	*Ix. angustus*	nymph	vegetation	05-Sep-14	Williams Lake, BC	70% EtOH + NaClO	PureLink®	A
13	control	n/a	n/a	lab areas	18-Nov-14	Edmonton, AB	n/a	PureLink®	A

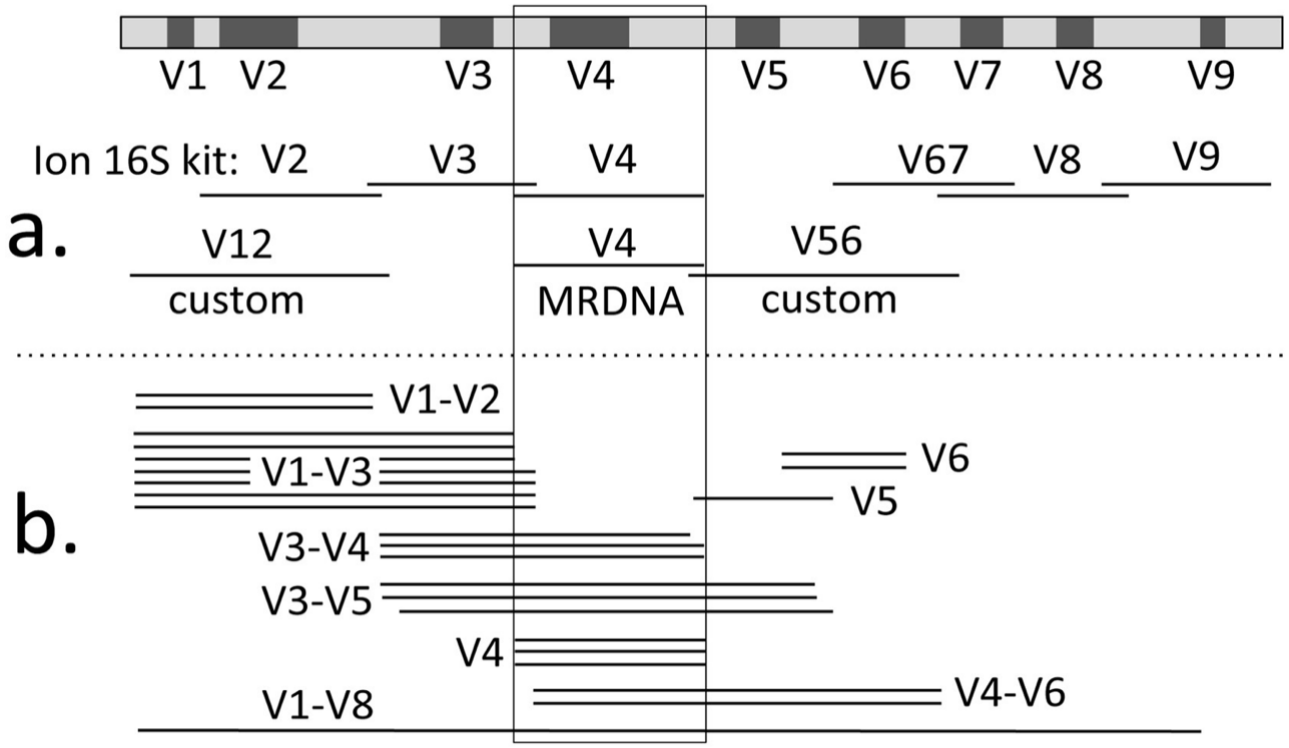

Figure 2.1. 16S rRNA regions sampled in tick microbiome analyses: a) arrangement of 16S rRNA conserved and variable regions, using E. coli numbering, with expected length and locations of amplicons sampled in this study, including six from the Ion 16S kit, one from the MR DNA service, and two custom amplicons; and b) previous tick microbiome studies (details in Table S2.1)

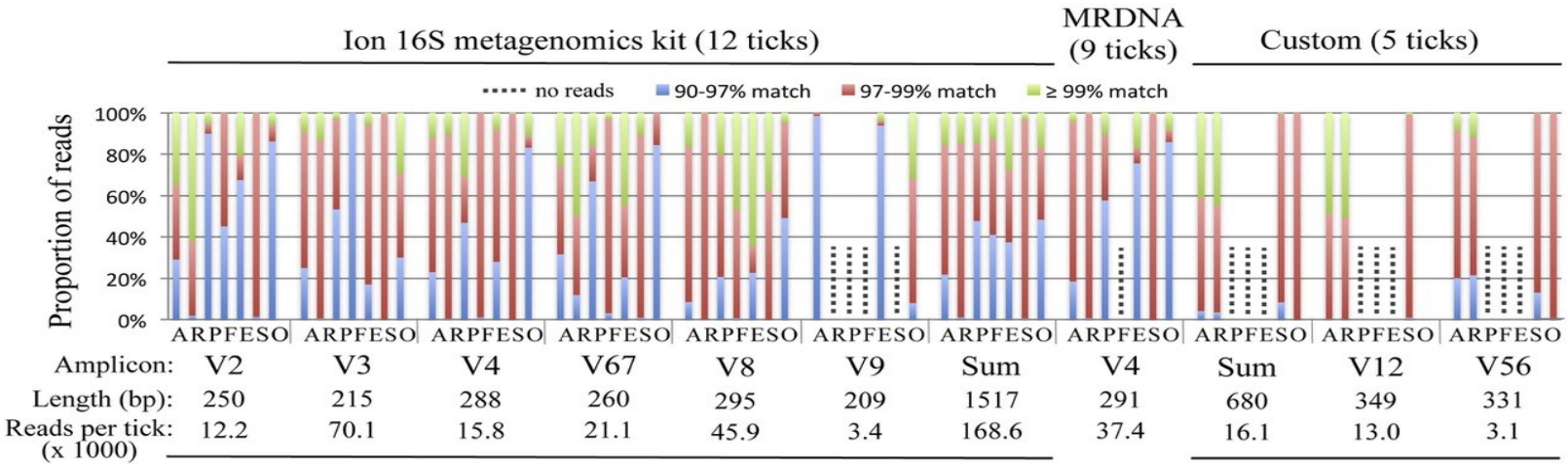

Figure 2.2. Sequence identity of reads for the six most frequently represented bacterial families, shown as percent match to Greengenes databases. A = All reads summed, R= Rickettsiaceae, P=Pseudomonadaceae, F= Francisellaceae, E=Enterobacteriaceae, S= Spirochaetaceae, O=Moraxellaceae.

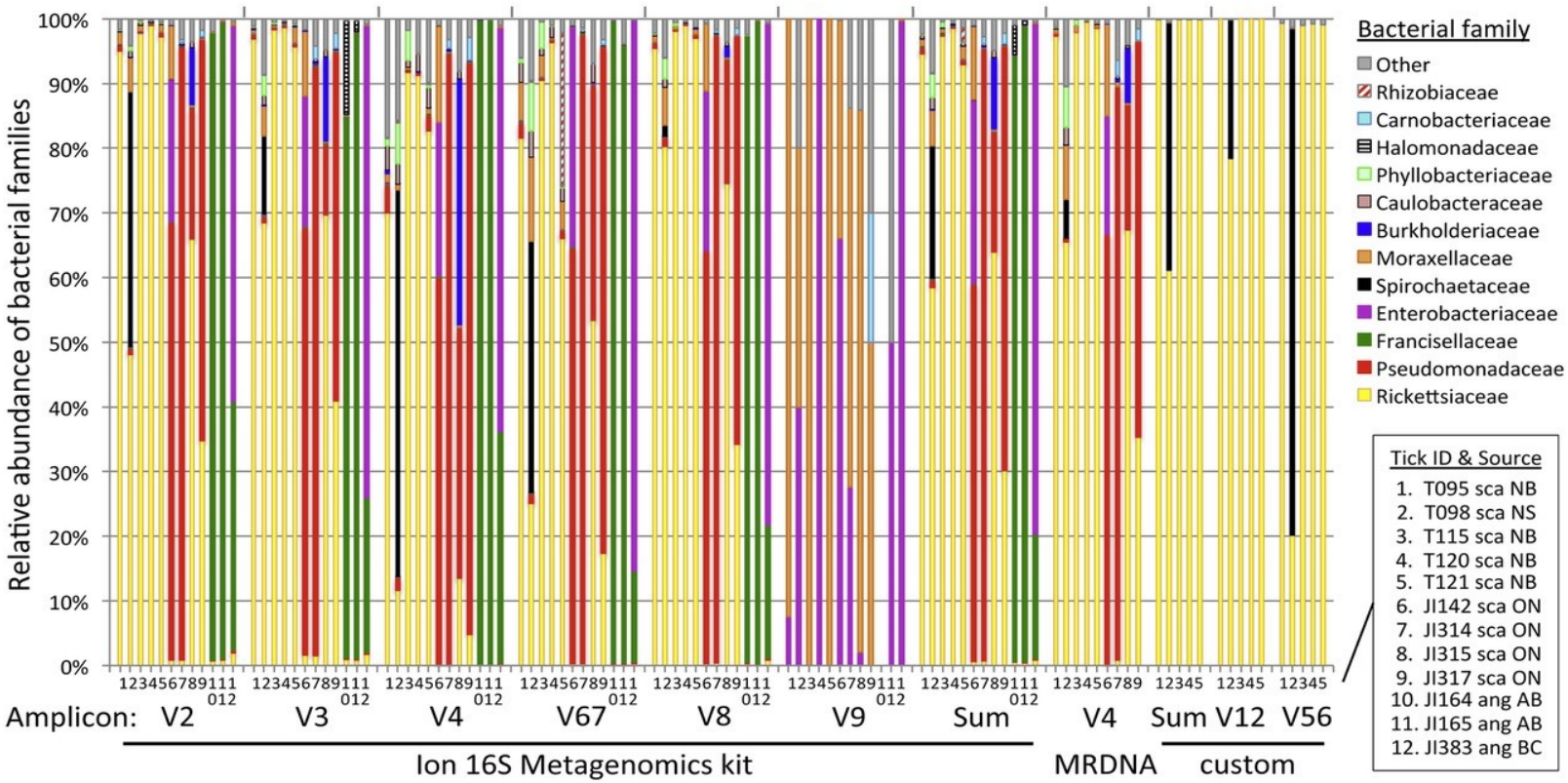

Figure 2.3. Proportions of bacterial reads detected in 12 ticks, arranged by 16S region. The 12 most common bacterial families are distinguished and the remaining low-frequency families are grouped as 'Other'. Only 9 ticks were sampled for MR DNA V4 and 5 ticks for the custom datasets. Tick 10 (JI164) had no V9 region reads.

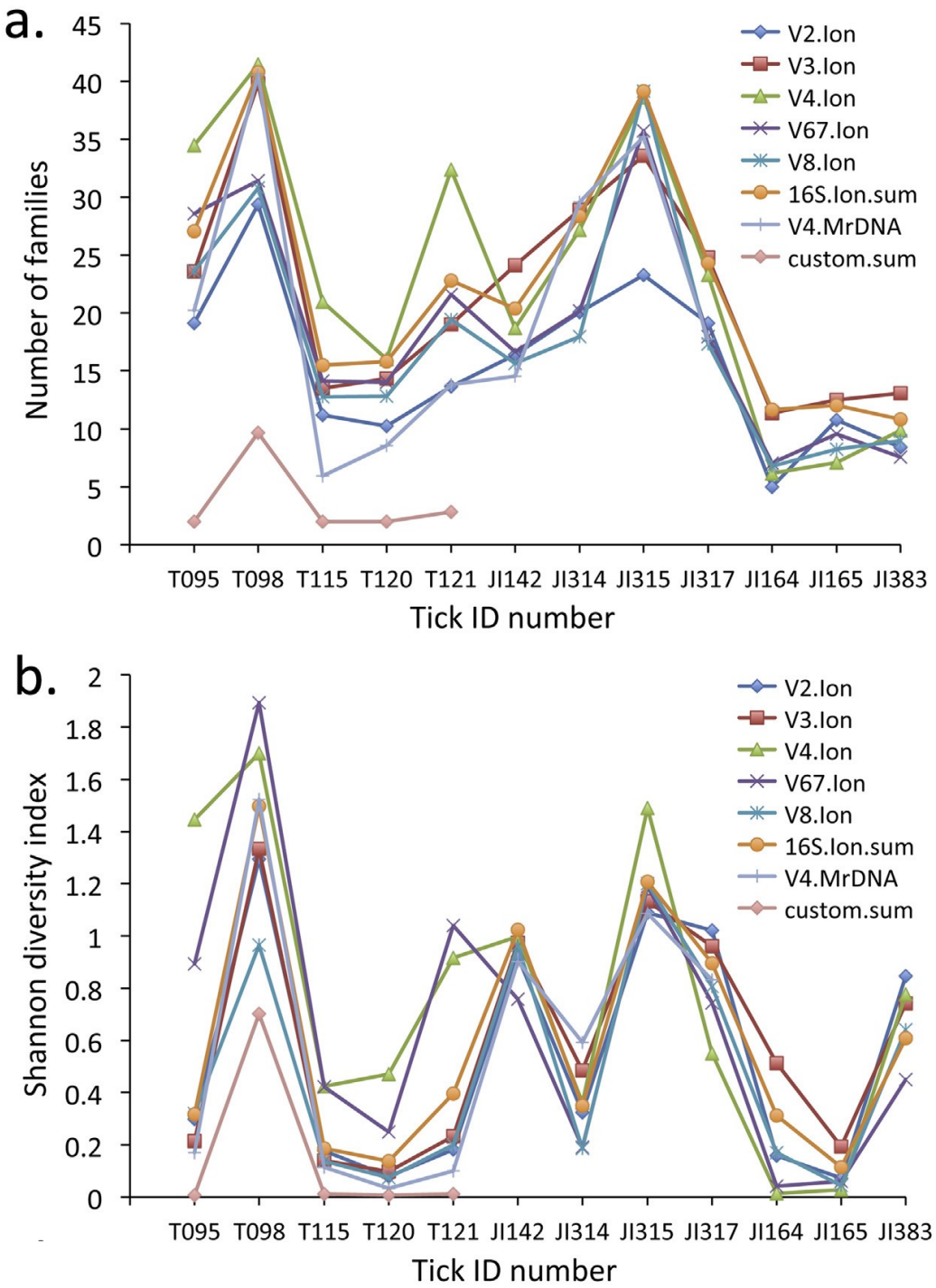

Figure 2.4. Diversity indices for 12 ticks and 8 assays of 16S rRNA variable regions: a) expected number of families ('richness' as implemented in Galaxy version 0.0.3/vegan 2.3-0;(Oksanen et al.,2015)), and b) Shannon diversity index (Galaxy version 0.0.3/vegan 2.3-0, Oksanen 2015). Data in all cases is rarefied to 3203 reads. Ion V9 samples are excluded and custom V12 and V56 samples are summed due to low read numbers

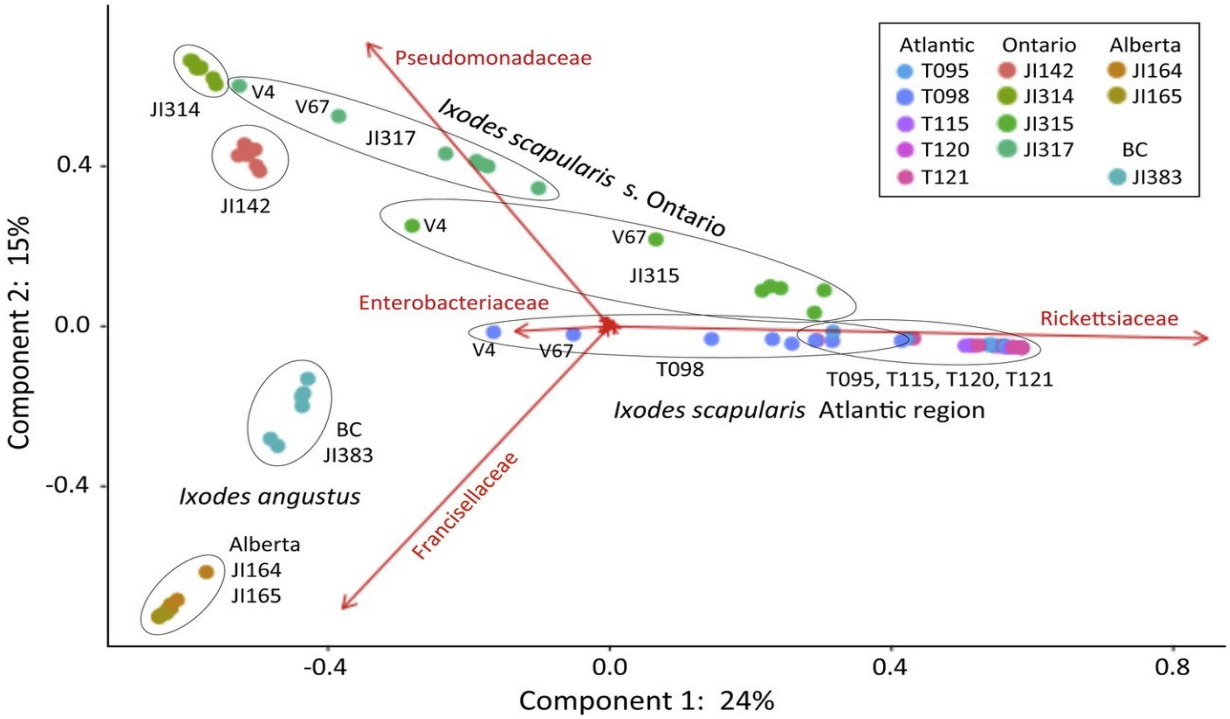

Figure 2.5. Principal component analysis of proportions of the 12 most common bacterial families in 107 tick and variable region combinations. Ellipses show clusters from individual or grouped ticks, distinguished by dot color. Arrows show eigenvector contributions of the four most frequent bacterial familie

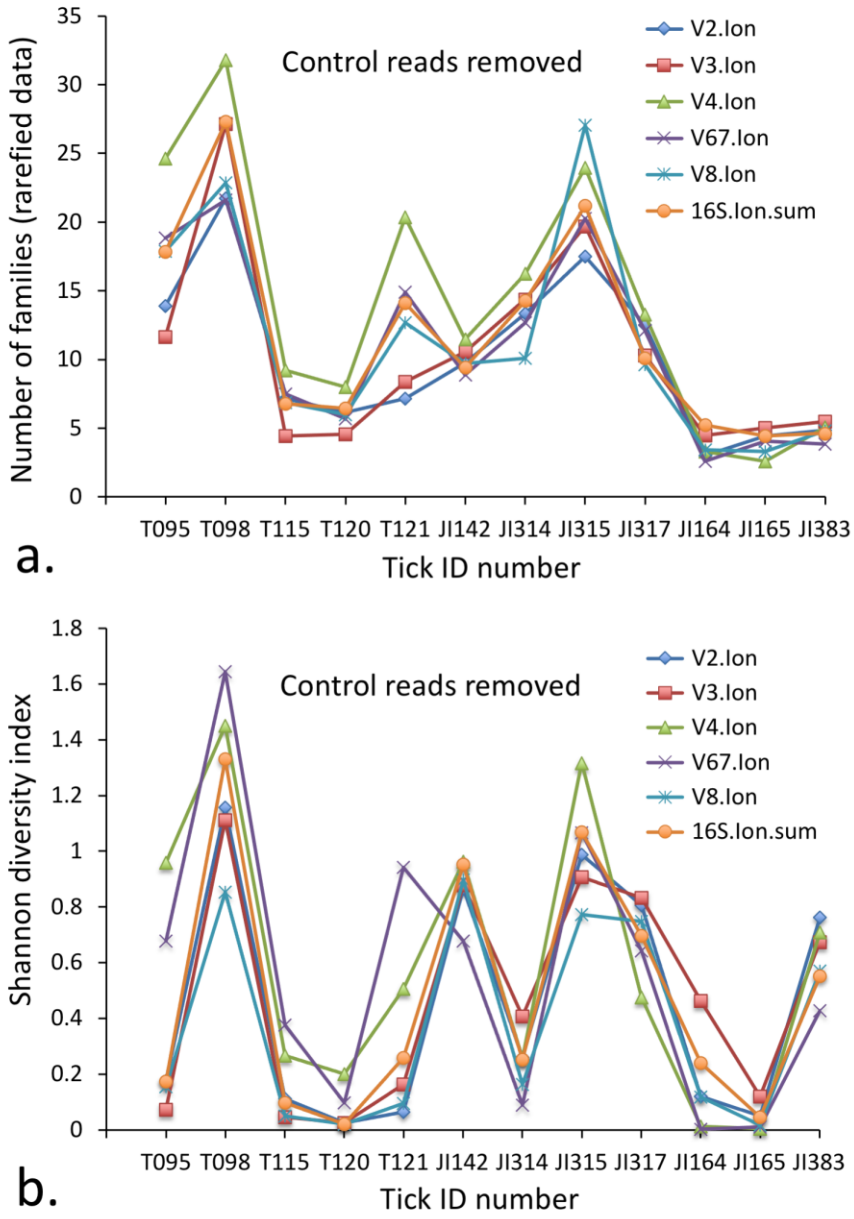

Figure S2.1. Diversity indices for the same 12 ticks as in Figure 2.4 with the 6 assays of the Ion 16S kit recalculated with control read numbers removed from data: a) expected number of families and b) Shannon diversity index. The number of reads for Ion 16S kit V4 of sample T120 fell below the previous threshold for rarefaction (3203 reads) and indices are therefore reported for rarefaction at 3042 reads

Table S2.1. Survey of previous tick microbiome studies.

16S region	Amplicon*	Tick species	Reference	Sequencing platform
V1-V2	27-338	*Ixodes scapularis* (pooled)	Narasimhan et al. 2014	454
V1-V2	27-338	*Ixodes holocyclus* & *I. ricinus*	Gofton et al., 2015	Ion PGM 400 bp
V1-V3	23-519	*Amblyomma maculatum*	Budachetri et al., 2014	454
V1-V3	27-518	*I. ovatus, I. persulcatus, Haem. flava*	Qiu et al., 2014	454
V1-V3	27-519	*Rhipicephalus microplus*	Andreotti et al., 2011	454
V1-V3	27-534	*Dermacentor variabilis, Ix. scapularis*	Hawlena et al., 2013	454
V1-V3	27-534	*Amblyomma americanum*	Ponnusamy et al., 2014	454
V1-V3	27-534	*D. variabilis, I. scapularis*	Rynkiewicz et al., 2015	454
V1-V3	27-534	*I. scapularis, I. affinis*	Van Treuren et al., 2015	454
V3-V4	341-785	*Amblyomma americanum*	Trout Fryxell & DeBruyn, 2016	Illumina MiSeq
V3-V4	343-806	*Ambl. loculosum, Carios capensis*	Wilkinson et al., 2014	454
V3-V4	341-805	*I. scapularis*	Zolnick et al., 2014	Illumina MiSeq
V3-V5	338-907	*Amblyomma cajennense*	Machado-Ferreira et al., 2015	MegaBACE 1000
V3-V5	357-926	*Amblyomma americanum*	Williams-Newkirk et al., 2014	454
V3-V5	not reported	*I. persulcatus, I. pavlovskyi, D. reticulatus* (pooled)	Kurilshikov et al., 2015	Illumina MiSeq
V4	515-806	*Ixodes persulcatus*	Zhang et al., 2014	Illumina MiSeq
V4	517-806	*Dermacentor andersoni*	Clayton et al., 2015	454
V4	515-806	*Ixodes scapularis, I. affinis*	Van Treuren et al., 2015	Illumina MiSeq
V4-V6	530-1100	*Amblyomma tuberculatum*	Budachetri et al., 2016	454
V4-V6	530-1100	*R. turanicus, R. sanguineus*	Lalzar et al., 2012	454
V5	786-939	*Amblyomma americanum*	Menchaca et al., 2013	Ion PGM 100bp
V6	872-1052	*Ixodes ricinus*	Carpi et al., 2011	454; Illum. Gaiix
V6	872-1052	*Haemaphysalis wellingtoni, H. hystricis, H. bispinosa*	Khoo et al., 2016	Ion PGM 200 bp
V1-V8	27-1435	*Dermacentor andersoni*	Gall et al., 2016	PacBio Circ Con

Table S2.2. Number of reads at three stages of data processing. See Methods for filtering parameters. Mapped reads are only counted if at least two identical reads were found.

Tick ID	16S kit unfiltered	MrDNA unfiltered	Custom unfiltered	16S kit filtered	MrDNA filtered	Custom filtered	16S kit mapped	MrDNA mapped	Custom mapped
T095	259662	51964	44817	212270	46260	17675	179007	37501	8286
T098	281783	50009	41191	227221	43697	19150	178975	32433	8710
T115	257251	52590	42860	204379	47851	15678	174928	39891	7255
T120	238605	45873	48750	194065	42092	17930	168628	35131	8261
T121	290338	48568	40105	237290	44867	16244	200267	37155	7733
JI142	297641	55074	210919	47164	149109	33440			
JI314	301754	61255	239217	53561	191074	41628			
JI315	260387	44299	206420	39524	162851	29315			
JI317	259566	72906	204282	64921	161587	51190			
JI164	243934	177767	139287						
JI165	245398	185229	151375						
JI383	283865	206231	166668						
control	55299	41945	27374						
total	2476787	327975	88855	1903365	292129	34174	1518220	227859	15994
% retained				76.80%	89.10%	38.50%	79.80%	78.00%	46.80%
Ave./tick	274715	54663	44428	215366	48688	17087	172253	37977	7997

CHAPTER 3

Within-population diversity of bacterial microbiomes in winter ticks (*Dermacentor albipictus)*

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3.1 Introduction

Ticks can vector wildlife disease agents ranging from bacteria to viruses and piroplasms (Greay et al., 2018). The bacterial diversity associated with ticks is of particular interest, as it affects wildlife via individual pathogens, as well as through interactions among different microbes (Bonnet et al., 2017; Portillo et al., 2019). These microbial assemblages are often considered to have high diversity that enhances the adaptability of ticks (Lado et al., 2018; Obregón et al., 2019; Stewart and Bloom, 2020). However, there has been little consensus on the composition or determinants of microbial diversity (richness or variability) of tick bacterial assemblages (Couper et al., 2019). Bacteria are the best-studied component of tick microbiomes due to the importance of bacterial pathogens, presence of bacterial endosymbionts and the advent of high throughput sequencing of 16S rRNA (Bonnet et al., 2017; de la Fuente et al., 2017; Greay et al., 2018). Sequencing of 16S rRNA amplicons has allowed bacteria to be binned into phylotypes, effectively assigning sequences to taxonomic units that approximate the genus level (Schloss and Westcott, 2011).

The view that tick microbiomes have high taxonomic and functional diversity has recently been questioned (Ross et al., 2018; Couper et al., 2019; Tokarz et al., 2019). For example, Ross et al. (2018) reported extremely low bacterial diversity in the dissected gut of ticks, and instead proposed that the high taxonomic diversity of microbiomes typically reported for whole ticks is an artifact of sequencing technologies detecting contaminants either on tick exoskeletons or in laboratory reagents. To compensate for such artificially inflated diversity

estimates, Ross et al. (2018) recommended pooling tick population samples to reduce phylotype sampling biases in PCRs on small quantities of starting DNA. However, pooling ticks obscures detection of the variation in microbiomes of different individual ticks in a population, which may be of great ecological interest. It is therefore important to further evaluate the basis for this recommendation.

Our study assesses the diversity and variability of the bacterial microbiome of a one-host tick, *Dermacentor albipictus* (the winter tick), in a system chosen to reduce variability in the ecological conditions experienced by individual ticks. All life stages of *D. albipictus* feed on a single host individual, in this case the North American elk, *Cervus elaphus*. As a one-host tick, most of the life cycle of *D. albipictus* occurs on its host (~7 mo of the year; Samuel, 2004). In contrast, many other species of ixodid ticks, particularly those known as zoonotic vectors of disease agents (e.g., *Ixodes scapularis,* which transmits the agents of Lyme disease), have a three-host life cycle with different vertebrate host species for each tick life stage. Hosts for *I. scapularis* range from small rodents to birds (Lindquist et al., 2016), enabling these three-host ticks to have long-distance dispersal across substantially different habitats, potentially leading to higher microbiome diversity. The microbiome of *I. scapularis* is also affected by host blood meal (Landesman et al., 2019). In contrast, we expect microbiome diversity in the *D. albipictus* system to consist of fewer bacterial phylotypes.

To evaluate the microbiome of *D. albipictus*, we collected individuals from a single landscape in southwestern Alberta, Canada, and surveyed their bacterial diversity using 16S rRNA amplicon sequence variation. From these data, we estimated both richness and relative abundance of bacterial taxa for each tick. Quantifying microbiome diversity with an individual tick-based approach allowed us to relate variation in diversity measures to ecological and life history factors that differed among ticks. As in other tick species, we detected hundreds of bacterial phylotypes, but with lower diversity in females than other tick life stages.

3.2 Materials and methods

3.2.1 Sample collection

Ticks were collected at Ya Ha Tinda Ranch in southwestern Alberta as part of a long-term elk monitoring project led by the Universities of Alberta and Montana. Female elk were sampled in late February or early March of 2013 and 2018, with age range of 3-15 yr, while male

elk, aged 2-3 yr, were sampled in January 2018 (Table 3.1). Animal handling procedures were in accordance with the Canadian Council on Animal Care Guidelines and approved by the University of Alberta Biosciences Animal Care and Use Committee (Protocol #AUP00000624). Ticks were stored in 95% ethanol upon removal from elk and kept at -20 °C for long-term storage. Ticks were organized into three functional classes (adult female, adult male, nymph) and identified to species, life stage, and sex using keys in Lindquist et al., (2016).The feeding status of ticks (engorgement) was scored as either flat or partially fed. No ticks were scored as fully fed.

3.2.2 DNA extraction

A total of 44 ticks were selected for microbiome analyses. Each tick was surface cleaned in 10% sodium hypochlorite by holding the tick in freshly disinfected tweezers and vigorously moving the tick back and forth through the bleach solution at least 8 times. Each tick was then rinsed twice in distilled water, air dried and diced with a razor blade before extraction using the QIAmp DNA Minikit (catalog #51306, Qiagen,Germantown, MD, USA). Zirconia/silica beads (1 mm diameter) were added to samples prior to overnight incubation with Proteinase K. Samples were subjected to bead beating before and again after incubation, then cooled on ice. One sham extraction was included as a control for identifying the 'kitome' (Hornung et al., 2019) and background contaminants found in the lab and sequencing facility (Eisenhofer et al., 2019; Weyrich et al., 2019).

3.2.3 PCR and sequencing

The 16S rRNA variable region V4 was amplified with PCR primers 515/806 (Caporaso et al., 2011) in a single-step PCR of 30 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen). Library preparation and sequencing were performed by MR DNA^{TM} (www.mrdnalab.com, Shallowater, TX, USA) using Ion PGM. Input DNA was a mix of tick DNA and microbial DNA, with quantification by Nanodrop^{TM}. Ion PGM data was generated as single-end sequence.

3.2.4 Sequence filtering

Raw fastq sequences (= reads) with Ion Torrent software quality scores above Q25 were trimmed to 277 bp using FastX Trimmer (Hannon 2010 fastx-toolkit/0.0.14). Mothur 1.43.0

(Schloss et al., 2009) was used in subsequent sequence analyses including demultiplexing. The forward primer (GTGCCAGCMGCCGCGGTAA) was used to identify sequence during trimming. Reads were removed from further analyses if they had homopolymers > 6 bp, lengths < 200 bp, > 2 primer differences, or >1 barcode difference. Reads with up to 2 differences were merged into the numerically dominant sequence before being aligned. Silva.nr_v132 database was used to align sequences using default k-mer searching with flip=T. Alignments outside the expected V4 region were removed using screen.seqs optimize=start, criteria=97. Chimeras were removed using Vsearch v2.13.3 (Rognes et al., 2016), with the most abundant sequence as reference and scored with the default Needleman-Wunsch algorithm, dereplicate=T.

3.2.5 Taxonomic assignments

Reads were clustered at 97% identity using the OptiClust algorithm (Westcott and Schloss, 2017), then assigned to a bacterial "phylotype" using Silva.nr_v132 taxonomy at Mothur level 1, corresponding to genus level. The Wang method (Wang et al., 2007) was applied to classify sequences using Mothur's default bootstrap confidence value of 80 to assign phylotypes to the genus level. Phylotypes that assigned reliably to a bacterial family but did not correspond closely to a single genus within the family were considered unclassified family level reads and counted as one taxonomic unit. Sequence reads for our study were reported without using the 16S rRNA gene copy number correction, as recommended by Louca et al. (2018). Matches to all sequences with >5000 identical copies were found using megaBLAST searches of the nucleotide (nr/nt) database of NCBI (Agarwala et al., 2016).

3.2.6 Extraction control adjustment

Extraction control adjustment: After phylotype assignments were completed, read numbers for each tick sample in the community matrix generated by Mothur 1.43.0 were then adjusted by subtracting the number of reads for each phylotype found in the extraction control sample (Nguyen et al., 2015). Negative numbers were reset to zero. The resulting community matrix was then subsampled to 25000 reads with the rrarefy function in vegan 2.5-6 (Oksanen et al., 2019) in R version 3.5.3. Specimens with < 25000 reads were removed. The final dataset comprised 38 samples: 12 females, 11 males and 15 nymphs. Rarefaction curves are shown in Figure S2.1.

3.2.7 Diversity measures

Phylotype richness was estimated as the number of observed phylotypes present in each microbiome, approximately corresponding to genus level. Unclassified family level reads were assumed to represent a single taxonomic unit. To account for uncertainty in detection of rare bacterial taxa associated with ticks, we report Hill numbers as a generalized measure of bacterial diversity. Successive Hill numbers in the series give less weight to rare taxa, and so are recommended for more conservative estimation of the effect of rare taxa on diversity measures (Kang et al., 2016). Hill numbers were computed using vegan 2.5-6 (Oksanen et al., 2019) in R version 3.5.3. Hill numbers 0 – 2 correspond to transformations of common diversity indices; H0 = richness, H1 = the exponential of Shannon's entropy, and H2 = the inverse Simpson's index. As a further exploration of bacterial diversity we calculated Pielou evenness, as it is less dependent on the number of phylotypes than Hill numbers and represents a pure measure of relative evenness (Ehsani et al., 2018; MacDonald et al., 2017). Pielou evenness was estimated as the natural log of H1 (Shannon's entropy) divided by the natural log of phylotype richness (Pielou, 1966). Estimation of all diversity indices was completed using the microbiome matrix in Table S3.2.

3.2.7 Multivariate visualization

To assess patterns of beta diversity, we used a rank based ordination, non-metric multidimensional scaling (NMDS), that applied metaMDS to a Bray-Curtis distance matrix calculated in vegan 2.5-6 and plotted with ggplot version 3.2.1. Principal components analysis (PCA) was used to visualize the contributions of the 7 major groupings of bacterial phylotypes: 1. Francisellaceae as the sum of both *Francisella* and Francisellaceae_unclassified, 2. *Pseudomonas*, 3. *Ehrlichia*, 4. *Asinibacterium,* 5. *Acinetobacter*, 6. *Streptococcus* and 7. all "Other" bacterial phylotypes present at < 1% in the total dataset. Principal components were computed in R version 3.5.3 using FactoMineR 1.42 (Lê et al., 2008) and factoextra 1.0.5.

3.2.8 Bacterial diversity models

A series of generalized linear models (GLMs) was applied to assess the extent to which bacterial diversity, measured as richness (H0), the exponential of Shannon's entropy (H1), and

evenness, varied with amount of DNA input into PCR, number of sequences prior to subsampling, and engorgement level. GLMs addressing richness were fitted to a beta regression model (log-link function) using the R package betareg version 3.1-3, as a Poisson distribution did not adequately account for the dispersion observed in integer richness values. GLMs addressing H1 and evenness were fitted using a Gamma distribution (log-link function) in R package MASS version 7.3.51.1, as these values are continuous and necessarily positive. For each model, we included predictor variables: tick class (female, male or nymph), feeding status, and amount of input DNA. To test for the significance of possible unevenness in sequencing prior to subsampling for the richness model, we also include the number of reads prior to subsampling (Table S3.1).

3.3 Results

3.3.1 Bacterial sampling and major phylotypes

We sampled the microbiome of a total of 44 ticks, including 12 females, 14 males and 18 nymphs (Table S3.1). Amplification and Ion PGM sequencing of the V4 region of 16S rRNA was performed on all 44 tick samples, giving 43 with greater than 10,000 reads, ranging from 38,826 to 214,812. Among the 43 ticks, a total of 1073 genus-level bacterial phylotypes were detected, each represented by one or more reads. To reduce the influence of possible contaminants and mis-assigned sequences, the number of reads per phylotype found in the extraction control was removed from each tick sample, leaving 954 taxa in the tick samples. To equalize sampling effort, samples with less than 25,000 reads were removed, and all further analyses were based on subsampling to 25,000 reads per tick, leaving 38 ticks (12 female, 11 male and 15 nymphs). In the aggregate of all ticks, *Francisella* comprised 67% of total reads, followed by *Pseudomonas* (6.0%), *Ehrlichia* (5.0%), unclassified Francisellaceae (4.0%), *Asinibacterium* (2%), *Acinetobacter* (1%) and *Streptococcus* (1%) (Figure 3.1; Table S2.2). All other phylotypes each contributed < 1% of the total reads and together comprised 14%.

3.3.2 BLAST search matches

Results for the 40 most common sequences (>5000 copies) are reported in Table S3.3. One phylotype, *Francisella*-like endosymbiont (FLE), was represented by 23 of the 40 sequence variants. One variant (length 250 bp) comprised most of the FLE total, while the other FLE

sequences differed only by substitutions, indels or length variants of one or at most two nucleotides. The best matches for all 23 sequence variants were FLEs of *Dermacentor variabilis* or *D. albipictus.* There was more variation in taxonomic assignments for *Pseudomonas* sequences, whereas the two *Ehrlichia* sequences both matched a single set of sequences identified as uncultured *Ehrlichia* sp. clones or *Ehrlichia minasensis* (Table S3.3).

3.3.3. Extraction Control

The single extraction control sample that we sequenced to estimate bacterial read proportions arising from kit contaminants and other artifacts resulted in 39,279 reads and 92 phylotypes (Table S3.4). Eight phylotypes were found uniquely in the extraction control sample, and 14 other phylotypes were found as singleton or doubleton reads. The most abundant were *Pseudomonas* and *Escherichia-Shigella*, and another 34 phylotypes each had more than 100 reads in the control sample, including *Francisella* with 3038 reads.

3.3.4 Bacterial assemblages and tick life stages

Although Francisellaceae (*Francisella* plus unclassified Francisellaceae) comprised the overwhelming majority of reads (71%) across all ticks, the proportion of Francisellaceae reads varied among individual ticks and between tick classes (Figure 3.1). Francisellaceae reads comprised 0% to > 99.5% of all reads for individual ticks, with an average of 88% in female ticks, 65% in males, and 66% in nymphs. All tick specimens that had less than 50% of reads assigned to Francisellaceae were nymphs or males. Fed ticks, all life stages combined, had significantly greater abundance of Francisellaceae compared to flat ticks (Wilcoxon rank sum $p=0.033$). Compared by life stage, unfed nymphs had noticeably fewer Francisellaceae reads than fed nymphs, while fed nymphs were not significantly different from females or males (Figure S3.2). Six samples had *Pseudomonas* reads above the level found in the control sample. Four other bacterial phylotypes that each made up more than 1% of reads in the aggregate total – *Ehrlichia, Asinibacterium*, *Acinetobacter* and *Streptococcus* - were detected inconsistently and at varying proportions in individual samples. Of the 38 ticks, 15 had at least one *Ehrlichia* read, with the highest proportion comprising 52% of the reads in one sample. A total of 27 ticks carried *Asinibacterium* (up to 41%), 32 had *Acinetobacter* (up to 15%) and 36 had *Streptococcus* (up to 5%). NMDS analysis of bacterial assemblages showed that the most consistent similarity

was among female ticks, while males and nymphs were more variable (Figure 3.2). PCA analysis showed that variation was largely driven by Francisellaceae (Figure S3.3).

3.3.5 Bacterial sampling variability

Fourteen elk each provided a single tick, while two tick classes were collected on 7 elk, and all three tick classes were taken on 3 elk. Comparisons of ticks removed from the same elk individual did not always give the same proportions of bacterial phylotypes (Table S3.5). For example, two nymphs sampled from the same elk were very different from each other (YFN29, YFN34). Also one elk individual was sampled in both 2013 and 2018, providing one male and one female tick each year (Table S3.5). Each year, only one of the two ticks had an unusually high proportion of *Pseudomonas*. In 2013 it was the female tick (37% for OFF10 vs 0% for OFM23) while in 2018 it was the male (38% for OFM20 vs 0% for OFF9).

3.3.6 Bacterial diversity estimates

Mean richness (H0) of the bacteria associated with *D. albipictus* was greater for male ticks than both females and nymphs (Figure 3.3). H1, the first in the Hill series of diversity estimators, includes components of both richness and evenness and gave a median value for females that was lower than for males or nymphs. Subsequent H2-4 estimators continued this pattern; regardless of the weight placed on rare taxa, female bacterial assemblages were less diverse than either males or nymphs (Figure 3.3). Females also had the lowest Pielou evenness estimate, while males and nymphs were similar to each other (female average = 0.11. s.e.= 0.02, male = 0.27, s.e.= 0.04, nymph = 0.32, s.e.= 0.05).

3.3.7 Microbiome composition correlates

To test the influence of ecological and methodological factors, we fitted generalized linear models addressing diversity measures, H0, H1, and Pielou's evenness, with explanatory variables: tick class (female, male or nymph), tick feeding status, amount of input DNA and number of reads prior to subsampling (Table 3.1; Table S3.6). For H0 (richness), only tick class was significant. For tick class, nymphs and female ticks did not differ in phylotype richness, but males had a significantly greater number of phylotypes than females or nymphs. The amount of input DNA and feeding status of the tick were not significantly related to phylotype richness

(Table 3.1). Results for H1 (exponential of Shannon's entropy) showed significant differences for females compared to both nymphs and males (Table S3.6). For evenness, nymphs and male ticks did not differ, but the distribution of phylotypes in females was, on average, significantly less even. Amount of input DNA was not significantly related to phylotype evenness (Table 3.1). However, *Francisella* plus unclassified Francisellaceae reads were significantly more abundant in fed ticks than in flat ticks (Wilcoxon p=0.033).

3.4 Discussion

3.4.1 Microbiome of D. albipictus ticks

The microbiome of *D. albipictus* ticks is dominated by FLE, but this is manifested differently among tick life stages. Within life stages, we found that individual tick samples had considerable variation in bacterial assemblages (Figure 3.1; Table S3.2), and between life stages, females had high abundance of Francisellaceae and lower overall microbiome diversity than nymphs. Males and nymphs had greater diversity of bacterial phylotypes, both in terms of numbers of phylotypes and more even abundance of these phylotypes. However, the fact that the minor microbiome component of this elk/tick system was both stochastic and diverse supports recent profiling of tick microbiomes that emphasizes shared pathways among taxonomically diverse bacteria that can functionally replace each other (Estrada-Peña et al., 2020).

3.4.2 *D. albipictus microbiome composition*

We found FLE in all except one tick specimen, with no females having less than 50% Francisellaceae reads while 3 of 14 male ticks and 6 of 17 nymphs were below 50% (Table S3.2). As FLE is a vertically transmitted endosymbiont, transmitted primarily from female to egg (reviewed in Ahantarig et al., 2013) the high abundance of FLE in females is expected. In contrast, Scoles (2004) detected FLE in only 4 of 11 specimens of *D. albipictus* collected in southern Alberta and Texas; however sex was not reported. Using the primers of Scoles (2004), Leo et al. (2010) also found FLE in only a subset of *D. albipictus* ticks from Alberta, with 20 of 46 *D. albipictus* containing FLE based on either 16S rRNA or 17 kDA lipoprotein gene markers. These differences from our study could be due to biological differences between tick populations, feeding status of the tick and technological constraints, including primer design and PCR conditions. The variable effect of primer choice for detection of tick bacterial

endosymbionts, including *Francisella,* has been documented by Sperling et al. (2017). Further, the 250-bp 16S rRNA phylotype for FLE that we found in our study is also present in numerous other tick species, and its 16S rRNA sequence is indistinguishable from mammalian pathogenic *Francisella* (reviewed by Zellner and Huntley, 2019). Consequently, the short sequences that we used may mask genetic variation in FLEs both within *D. albipictus* as well as between tick species. Nonetheless, most of our 250 bp sequences were 100% identical to the FLE of *D. variabilis* and *D. andersoni*, as reported by Leo et al. (2010). Variation in sequences assigned to FLE did not exceed the published per read error rate of 1.4-1.5 errors per 100 bases for Ion PGM sequencing (Salipante et al., 2014), and so only a single taxonomic FLE unit was distinguished using a 250 bp fragment of 16S rRNA region V4.

After FLE, *Pseudomonas* was the second most abundant bacterial phylotype found in *D. albipictus* from elk at the Ya Ha Tinda Ranch (Table S3.2). *Pseudomonas* has been reported as a kit contaminant (Lejal et al., 2020) as well as a ubiquitous bacterium with antifungal properties (Agaras et al., 2018) and, occasionally, entomopathogenic properties (Dieppois et al., 2015). Although the significance of *Pseudomonas* associations with ticks remains untested, this bacterial genus has commonly been described as a component of the microbiota of ticks (e.g. Clow et al., 2018; Zolnik et al., 2018; Couper et al., 2019; Thapa et al., 2019a). *Pseudomonas* may be found on the surface of the tick or internally (Ross et al., 2018) and is an example of a taxon that could be removed if bioinformatic filtering is based solely on the presence of a related congener in the extraction control (Eisenhofer et al., 2019). Such filtering of *Pseudomonas* reads needs to be considered cautiously in the context of the abundance of reads and potential ecological roles of these bacteria. In our study we followed the recommendations of Nguyen et al. (2015) by subtracting the number of sequences found in the negative control from the number of sequences associated with each biological sample, to allow us to better distinguish biological signal from sequencing noise. Sequences assigned to *Pseudomonas* represent several possible taxonomic units and sequence variants, but 16S rRNA is known to have low discriminatory power for species level assignments unless specific primers are used (Pereira et al., 2018). We were thus unable to confidently distinguish between sequencing error or low discriminatory power of region V4 for the 16S rRNA taxonomic assignments of *Pseudomonas,* but conclude that there is likely to be more than one type of *Pseudomonas* in our samples.

In addition to FLE, and more broadly Francisellaceae, as well as *Pseudomonas*, four other phylotypes each comprised at least 1% of the total bacterial microbiome reads (Table S3.2). The most interesting of these is *Ehrlichia*, which made up 5% of the reads and was present in 15 tick samples. Ten of these ticks had >1% reads for *Ehrlichia*, with one as high as 52%. The short 16S rRNA gene fragment that we sequenced revealed a single taxonomic unit that nonetheless had a range of possible species assignments (Table S3.3). The closest matches included an unnamed *Ehrlichia* species from China, *Ehrlichia minasensis* from Brazil, an unnamed *Ehrlichia* species from Malaysia, and most notably a novel species of *Ehrlichia* from the neighbouring Canadian province of British Columbia (Gajadhar et al., 2010). However, our most common sequence had only 3 nucleotide differences from *Ehrlichia chaffeensis*, a pathogen of mammals that has been detected during post-mortem analysis in an elk (Stoffel et al., 2015). Since *D. albipictus* ticks found on a deceased elk did not test positive for *E. chaffeensis*, Stoffel et al. (2015) speculated that a different tick species was responsible for the infection; however, our results support the possibility that *D. albipictus* could be a potential vector of *Ehrlichia* for elk.

Not much is known about *Asinibacterium*, a member of the family Chitinophagaceae, except that members of this genus are also described in the microbiome of *Ixodes scapularis* ticks in Texas and Massachusetts (Thapa et al., 2019a). Of the remaining bacteria represented by at least 1% of reads across the 38 ticks (*Streptococcus* and *Acinetobacter*) their variable presence suggests that they are not core members of the *D. albipictus* microbiome. However, low abundance bacteria may have important roles as drivers of microbiome composition after disturbances to the community (Benjamino et al., 2018). *Acinetobacter* has been described in other tick microbiome studies (e.g Kwan et al., 2017; Thapa et al., 2019a). By contrast, *Streptococcus* and many bacteria comprising less than 1% of reads within the dataset are likely to be soil contaminants. Nonetheless, the importance of soil bacteria to off-host survival in ticks cannot be underestimated (Burtis et al., 2019).

3.4.3. Extraction Control Adjustment

Published lists of common kit contaminants (Eisenhofer et al., 2019; Glassing et al., 2016; Lejal et al., 2020; Salter et al., 2014) show that the presence of *Pseudomonas*, *Escherichia/Shigella* and *Lactobacillus* is unsurprising. However, the presence of *Francisella* in the extraction control underscores the value of including such controls (Eisenhofer et al., 2019;

Hornung et al., 2019; Lejal et al., 2020). Since *Francisella* has not been reported elsewhere as a contaminant, but is present at high frequency in ticks, its detection in the extraction control sample is most likely to be due to contamination at the extraction stage (Lejal et al., 2020; Minich et al., 2019) (Weyrich et al., 2019). The amount of input DNA for the extraction control was orders of magnitude less than the amount of DNA for any of the tick samples in the initial rounds of PCR, which could result in relative overamplification of aerosol contamination. This may also reflect the dominance of *Francisella* in *D. albipictus*, not only via aerosols. Index or 'tag switching' can also be a significant source of mis-assigned reads (Costello et al., 2018; Sinha et al., 2017) and the pattern is stochastic (Yao et al., 2018). The Ion Torrent sequencing platform that we used has up to 0.167% index switching (Palmer et al., 2018). In our dataset, prior to subsampling, 3,807,726 reads were assigned to a single taxon, *Francisella*. If index switching is responsible for detection of *Francisella* in the control sample, the number of reads potentially mis-assigned to the control sample would be up to 141 reads per tick sample (6359 reads across 45 indexed samples). This is less than the number detected in our control sample but is a noticeable quantity nonetheless, and the possibility of mis-assigned reads should be considered in datasets dominated by a single taxon before corresponding reads are removed for individuals.

Extraction controls can also be essential for interpreting reports of novel bacteria in ticks removed from wildlife (e.g. Egan et al. 2019). In our study, *Ehrlichia* and *Asinibacterium* were found in numerous tick samples but not in the extraction control. However, filtering for possible index switching of up to 0.167% of any taxon in our dataset (Palmer et al., 2018) allows a threshold value to be calculated across the entire dataset. For *Ehrlichia*, this threshold is 2 reads per tick sample (42044 *Ehrlichia* total reads may result in up to 70 mis-assigned reads spread across 45 samples).

3.4.4 Microbiome variability and drivers

Some pairs of ticks from the same individual elk had substantially different numbers of reads for the same phylotypes. This may reflect true biological variation among ticks, but the high inconsistency of phylotype abundance that we found for even the more common bacteria like *Ehrlichia* and *Asinibacterium* means that potential sampling artifacts must also be considered. This inconsistency indicates that bacterial community structure and population

trends cannot be reliably interpreted from one or even a few samples. Although it may be cheaper to pool multiple ticks from the same cohort, this practice assumes that variation among ticks is biologically inconsequential or due to experimental artifacts. We feel that it is too early to discount the possibility of true biological variation in tick bacterial associations from the same host animal, particularly as these can involve potential pathogens like *Ehrlichia* (Dumler et al., 2015).

Except for differences between tick life stages, and the effect of feeding status on evenness of the microbiome, we did not find that any other life history or ecological factors were related to the observed variation in microbiome diversity of *D. albipictus*. The bacterial assemblage of ticks may be inherently stochastic and therefore unpredictable. However, it is clear that there is substantial variation between individual ticks that is significantly related to tick sex and life stage, even among these ticks collected from single host animals. Since this variation would not be evident in pooled samples, following the recommendation for pooling by Ross et al. (2018) would diminish the opportunity to detect functionally significant variation in the microbiome of ticks. For example, although the microbiome of female *D. albipictus* was dominated by FLE, significant functional differences may still exist between the tick sexes in the remainder of the reduced microbiome. Both *Asinibacterium* and *Ehrlichia* were not consistently present in our samples, including in ticks removed from the same animal at the same time. In addition, biologically relevant variation in infection rates of ticks feeding on the same infected host animal is confounded by possible methodological factors, such as overamplification of FLE which may reduce our ability to detect relatively rare bacteria and barcode switching which may result in detection of taxa dominant in the overall dataset but not present in a given sample.

3.4.5 Is the microbiome of D. albipictus highly diverse?

Until 2018, many authors described tick microbiome diversity as high, with most emphasizing phylotype richness. In contrast, five recent studies diverged markedly from this trend (Couper et al., 2019; Ross et al., 2018; Thapa et al., 2019a, 2019b; Tokarz et al., 2019), reporting that “individual ticks harbour low diversity microbiomes” (Couper et al., 2019). This difference highlights alternate interpretations of lower frequency bacterial sequences as significant components of functional bacterial assemblages *versus* technical artifacts or functionally insignificant surface contaminants on ticks. Patterns of diversity also depend on the

scale at which the microbiome is being studied (Pollet et al., 2020). Our study focussed on the microbiome of the whole tick, but was restricted to a single geographic region. The high variability of bacterial phylotype diversity associated with *D. albipictus* in our study raises several methodological questions about how best to distinguish biological signals from methodological error or noise. We found that repeated samples from a single host and extraction controls were particularly informative in highlighting potentially stochastic variation.

Tick surface decontamination with sodium hypochlorite is recommended as being more efficient than ethanol and other commercial products (Binetruy et al., 2019; Hoffmann et al., 2020). This efficiency means that the species richness of bacteria contributed by the external tick microbiome to diversity measures in our study is likely to be less than for studies that use ethanol to clean the outside of the tick. The external microbiome of ticks is composed of bacteria found in and on the cuticle, which in other arthropods is known to protect the animal from external stressors and may be used in host recognition (e.g. Dosmann et al., 2016). However, the biological effect of the external microbiome of ticks remains poorly understood (Bonnet et al., 2017), despite its potential importance to overwintering success and pheromone production of ticks. Furthermore, insect-associated *Streptomyces* have been shown to be especially promising sources of antifungals (Chevrette et al., 2019).

If the microbiome diversity of ticks is a functional reflection of their environmental conditions, then the use of single hosts by *D. albipictus* should result in lower microbiome diversity than other ticks, most of which have a three-host life cycle. In California, *D. albipictus* had lower bacterial diversity when compared to sympatric populations of *Dermacentor variabilis* and *Dermacentor occidentalis* (Chicana et al., 2019). These authors suggest that the broader host range of the latter two species may have contributed to their greater microbiome diversity at the population level. However our diversity measures for *D. albipictus* (average adult female H0= 91, adult male= 139 and nymph= 85) were comparable to the three-host tick *Amblyomma americanum* (H0 86 (f); 99 (m); 117(n): (Brinkerhoff et al., 2020) and greater than *D. variabilis* (H0 46(f); 59 (m): Travanty et al., 2019). A direct comparison of one-host versus three-host ticks, using the same lab methods and bioinformatic filtering thresholds, will be required to more rigorously test the hypothesis that greater host range contributes to higher taxonomic diversity for ticks.

3.4.6 Functional roles of microbiome components

Francisella-like endosymbionts in ticks have reduced genomes but intact pathways for the synthesis of several B vitamins and cofactors that are lacking in mammal blood (Duron et al., 2018; Gerhart et al., 2018). If FLEs are nutritionally essential for some tick species, then these bacteria should be present in all blood-feeding life stages. Increased numbers of FLE are found in *Haemophysalis* ticks after feeding (Liu et al., 2016), and FLEs are also known to dominate the microbiome of female ticks in many *Dermacentor* species (Duron et al., 2017; Travanty et al., 2019). In this context, the lower relative abundance of FLE in nymphs than adult ticks is a consequence of feeding status, with fed nymphs having similar FLE numbers to other life stages when feeding is factored out (Figure S3.2) . However variation in detection of endosymbionts by tick species and life stage has also been noted by Rounds et al., (2012) and reviewed in (Stewart and Bloom, 2020). Such apparent variation in abundance of taxonomically diverse bacteria raises the possibility of functional replacement by different groups that have shared biochemical pathways (Estrada-Peña et al., 2020).

Our finding of greater abundance of FLE in fed ticks is consistent with a nutritional role for FLE. Interestingly, the one specimen that lacked FLE (OFN30) had the greatest abundance of *Ehrlichia.* Since *Ehrlichia* contain genes for biotin synthase (Dunning Hotopp et al., 2006), this suggests that ticks infected with *Ehrlichia* may have less need for FLE as a nutritional symbiont. We did not, however, detect a significant relationship between numbers of *Ehrlichia* and other bacteria, including FLE (Table S3.2 and Figure S3.3). Nonetheless, the high variability and diversity of bacterial taxa in the one-host tick, *D. albipictus* suggests that potential functional redundancy in core nutritional pathways of tick microbiomes, regardless of the number of host species, should receive further attention.

3.5 References

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Table 3.1. Summary statistics, using nymph as reference, for generalized linear models of microbiome phylotype diversity. GLMs for female and male as reference are in Table S3.6.

H0, richness (log-link)	Estimate	Std Err	P-Value
tick class (female)	0.0117	0.2065	0.9550
tick class (male)	0.5271	0.2007	**0.0086**
engorgement (flat)	0.2589	0.1658	0.1185
input DNA	0.0002	0.0038	0.9578
number of sequences	0.0000	0.0000	0.1851
Pielou evenness (log-link)			
tick class (female)	-0.6684	0.1907	**0.0005**
tick class (male)	0.0115	0.1572	0.9418
engorgement (flat)	0.3573	0.1483	**0.0160**
input DNA	0.0028	0.0028	0.3050
H1, expShannon (log-link)			
tick class (female)	-0.8030	0.3094	**0.0140**
tick class (male)	0.2258	0.3227	0.4889
engorgement (flat)	0.4226	0.2636	0.1184
input DNA	0.0046	0.0059	0.4412

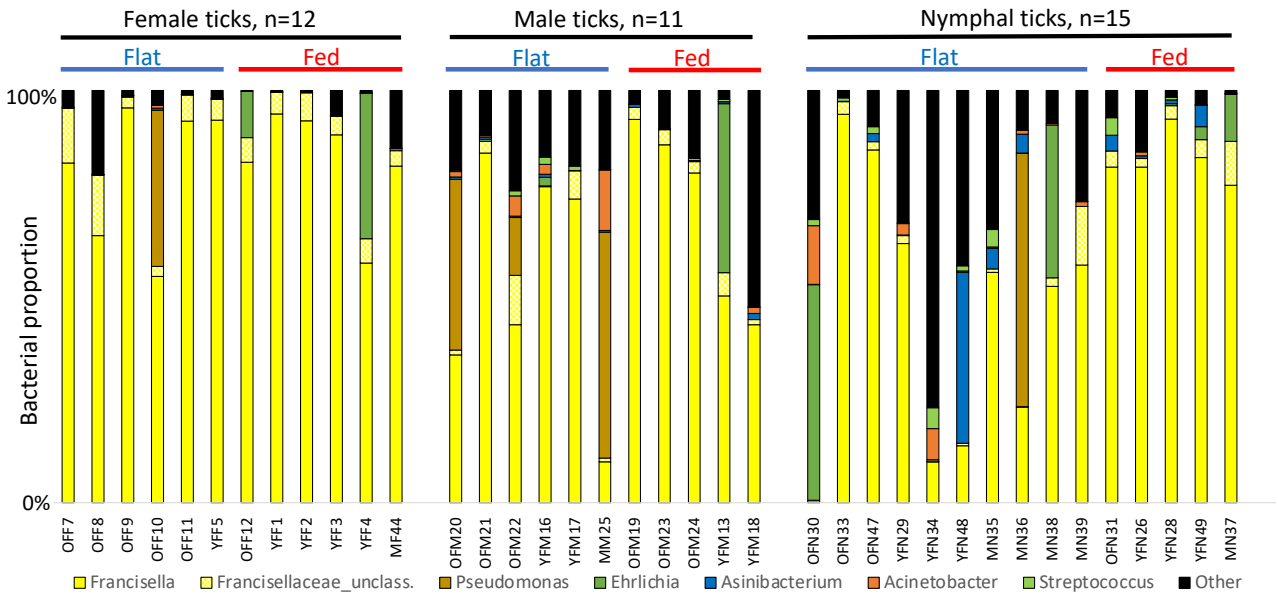

Figure 3.1. Proportions of bacterial phylotypes comprising >1% of the total dataset for all *Dermacentor albipictus*. Tick samples are grouped by life stage/sex, and feeding status

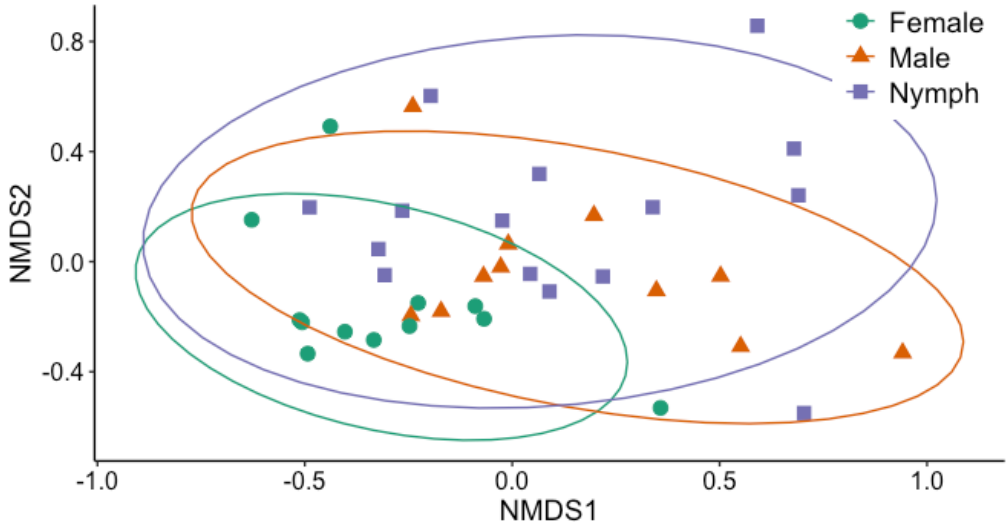

Figure 3.2. Non-metric multidimensional scaling (NMDS) of Bray-Curtis distance. Ellipses assume a multivariate t-distribution at the 0.95 level.

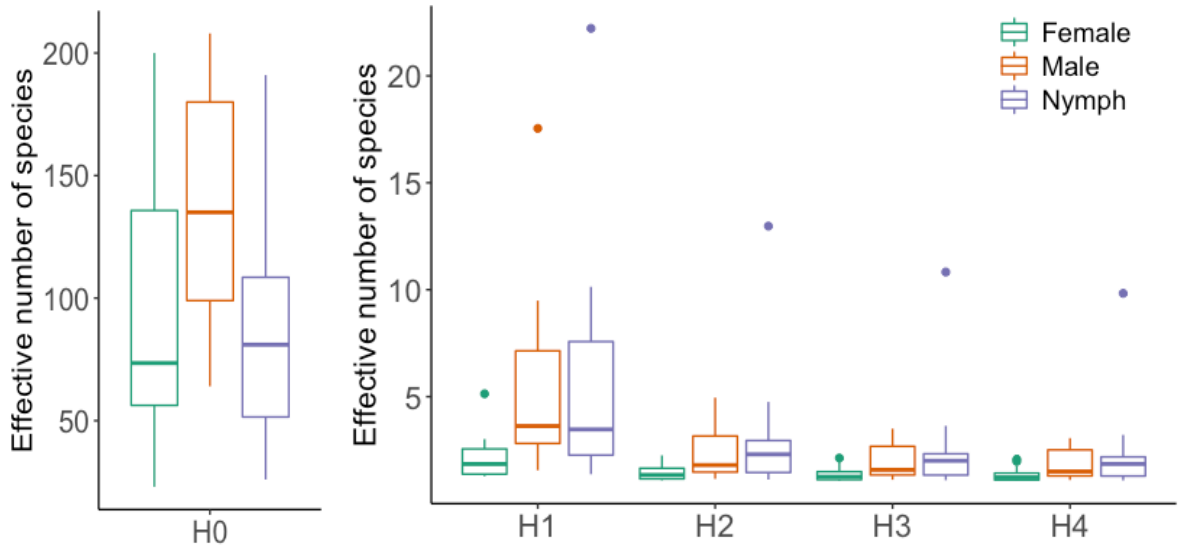

Figure 3.3. Box plots for effective number of bacterial phylotypes at Hill numbers H0-H4, grouped by tick life stage/sex. Increasing Hill numbers have decreasing emphasis on rare phylotypes. Females have the lowest richness and evenness. Males have the highest richness but, when evenness is taken into account, are intermediate to females and nymphs.

Table S3.1. Ticks and associated metadata included in this study.

Tick ID number	Tick stage	Tick sex	Feeding status	Elk ID number	Elk sex	Age (yrs)	Sample year	# reads pre-subsampling
OFF7	adult	female	flat	YL167	female	15	2018	27828
OFF8	adult	female	flat	YL154	female	10	2018	36025
OFF9	adult	female	flat	OR99	female	15	2018	208883
OFF10	adult	female	flat	OR99	female	10	2013	122404
OFF11	adult	female	flat	YL167	female	15	2018	80123
OFF12	adult	female	fed	OR56	female	13	2018	69249
OFM19	adult	male	fed	YL167	female	15	2018	94834
OFM20	adult	male	flat	OR99	female	15	2018	68894
OFM21	adult	male	flat	OR81	female	13	2018	91265
OFM22	adult	male	flat	YL112	female	9	2018	66158
OFM23	adult	male	fed	OR99	female	10	2013	108902
OFM24	adult	male	fed	OR56	female	13	2018	86568
OFN30	nymph	-	flat	OR90	female	9	2013	49610
OFN31	nymph	-	fed	OR81	female	13	2018	61689
OFN32	nymph	-	flat	OR65	female	14	2018	11214
OFN33	nymph	-	flat	YL112	female	9	2018	85750
OFN47	nymph	-	flat	OR91	female	12	2013	43058
YFF1	adult	female	fed	OR94	female	7	2013	83264
YFF2	adult	female	fed	OR89	female	3	2013	77548
YFF3	adult	female	fed	OR83	female	6	2013	226193
YFF4	adult	female	fed	OR97	female	6	2013	68349
YFF5	adult	female	flat	OR41	female	4	2013	95699
YFM13	adult	male	fed	OR89	female	3	2013	104280
YFM14	adult	male	fed	OR94	female	7	2013	8379
YFM15	adult	male	flat	OR83	female	6	2013	10319
YFM16	adult	male	flat	OR97	female	6	2013	39689
YFM17	adult	male	flat	OR98	female	3	2013	67021
YFM18	adult	male	fed	OR41	female	4	2013	64981
YFN26	nymph	-	fed	OR94	female	7	2013	29009
YFN27	nymph	-	flat	OR83	female	6	2013	17013
YFN28	nymph	-	fed	YL161	female	5	2018	106456
YFN29	nymph	-	flat	OR83	female	6	2013	32813
YFN34	nymph	-	flat	OR83	female	6	2013	51612
YFN48	nymph	-	flat	YL170	female	5	2018	50282
YFN49	nymph	-	fed	OR88	female	4	2013	94288

MF44	adult	female	fed	YLM1813	male	3	2018	103193
MM25	adult	male	flat	YLM1806	male	3	2018	45700
MM45	adult	male	fed	YLM1813	male	3	2018	15436
MN35	nymph	-	flat	YLM1815	male	2	2018	53093
MN36	nymph	-	flat	YLM1823	male	2	2018	108652
MN37	nymph	-	fed	YLM1824	male	3	2018	38956
MN38	nymph	-	flat	YLM1816	male	3	2018	118266
MN39	nymph	-	flat	YLM1827	male	3	2018	45515
MN46	nymph	-	flat	YLM1813	male	3	2018	9998

Table S3.2. Numbers of reads for each tick and bacterial phylotype, with phylotypes arranged by total abundance. Top 7 bacterial phylotypes are listed separately with remaining phylotypes grouped as 'Others'. Numbers for each phylotype are from data subsampled to 25000 for each sample.

sample	No. reads	*Fran-cisella*	*Pseudo-monas*	*Ehrli-chia*	*Franc* _uncl	*Asiniba-cterium*	*Acineto-bacter*	*Strepto-coccus*	*Others*
OFF7	25000	20608	0	0	3302	0	49	39	1002
OFF8	25000	16186	0	0	3661	42	4	20	5087
OFF9	25000	23939	0	0	657	76	1	11	316
OFF10	25000	13729	9473	0	592	132	169	3	902
OFF11	25000	23147	0	2	1582	0	2	2	265
OFF12	25000	20649	0	2795	1497	0	0	15	44
OFM19	25000	23231	0	0	735	180	26	12	816
OFM20	25000	8960	10338	0	298	163	314	89	4838
OFM21	25000	21212	0	114	702	156	91	26	2699
OFM22	25000	10795	3504	0	2989	102	1222	306	6082
OFM23	25000	21708	0	0	918	14	16	14	2330
OFM24	25000	20003	0	1	691	0	57	118	4130
OFN30	25000	0	161	13072	0	3	3563	374	7827
OFN31	25000	20360	0	0	974	955	0	1048	1663
OFN33	25000	23561	0	0	756	0	25	207	451
OFN47	25000	21397	0	0	499	494	0	420	2190
YFF1	25000	23583	0	0	1293	6	0	61	57
YFF2	25000	23165	0	0	1690	17	0	33	95
YFF3	25000	22309	0	0	1133	6	10	7	1535
YFF4	25000	14529	0	8808	1485	3	20	32	123
YFF5	25000	23189	0	0	1280	8	24	4	495
YFM13	25000	12541	0	10248	1418	104	19	147	523
YFM16	25000	19160	0	513	55	178	609	439	4046
YFM17	25000	18419	0	0	1699	0	17	287	4578
YFM18	25000	10788	0	0	309	382	367	12	13142
YFN26	25000	20359	0	0	506	174	219	78	3664
YFN28	25000	23266	0	139	797	233	0	151	414
YFN29	25000	15726	0	0	478	38	681	50	8027
YFN34	25000	2471	0	126	2	0	1906	1258	19237
YFN48	25000	3460	0	1	143	10368	91	306	10631
YFN49	25000	20937	0	787	1081	1286	10	28	871
MF44	25000	20420	0	0	930	0	116	8	3526
MM25	25000	2477	13703	0	221	107	3669	16	4807
MN35	25000	13969	0	0	213	1235	91	1065	8427

MN36	25000	5808	15359	16	15	1147	233	0	2422
MN37	25000	19252	0	2876	2654	0	27	14	177
MN38	25000	13115	0	9279	519	0	96	0	1991
MN39	25000	14407	0	0	3547	0	291	38	6717
sum	950000	632835	52538	48777	41321	17609	14035	6738	136147
percent		66.61%	5.53%	5.13%	4.35%	1.85%	1.48%	0.71%	13.73%

Table S3.3. BLAST results for sequences with greater than 5000 copies.

***Francisella*-like Endosymbiont (FLE)**	no. of reads	Best E-value	Percent identity
1QZJ7_00916_05865	1559916	3.00E-126	100.00%
1QZJ7_00941_02256	118896	9.00E-127	100.00%
1QZJ7_02858_07511	117743	5.00E-124	99.60%

FLE sequence matches: GenBank accession nos. and names for set of identical best E-value matches to FLE sequences obtained in this study

MG834270.1	Francisella endosymbiont of Dermacentor variabilis isolate Dv0080_NB 16S ribosomal RNA gene, partial sequence
GU968872.1	Francisella-like endosymbiont of Dermacentor albipictus haplotype 11 16S ribosomal RNA gene, partial sequence
GU968871.1	Francisella-like endosymbiont of Dermacentor albipictus haplotype 10 16S ribosomal RNA gene, partial sequence

***Pseudomonas* 1**	no. reads	Best E-value	Percent identity
1QZJ7_04714_05252	119477	3.00E-126	100.00%
1QZJ7_01134_05149	12706	9.00E-127	100.00%
1QZJ7_03110_05067	10272	1.00E-124	99.60%

Pseudomonas sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MT631989.1	Pseudomonas poae strain XJX-5 16S ribosomal RNA gene, partial sequence
MT626824.1	Pseudomonas putida strain SeaQual_P_B75 16S ribosomal RNA gene, partial sequence
MN629076.1	Pseudomonas lactis strain B35 16S ribosomal RNA gene, partial sequence

***Pseudomonas* 2** continued	no. reads	Best E-value	Percent identity
1QZJ7_03830_01056	7251	3.00E-126	100.00%

Pseudomonas sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MN192431.1	Pseudomonas lactis strain SM0110A 16S ribosomal RNA gene, partial sequence
MK373701.1	Pseudomonas sp. strain RL17-333-BIF-B 16S ribosomal RNA gene, partial sequence

KM146571.1	Uncultured bacterium clone LNH_12_1_11_Water.263785 16S ribosomal RNA gene, partial sequence

***Pseudomonas* 3** continued	no. reads	Best E-value	Percent identity
1QZJ7_03830_01056	6645	3.00E-126	100.00%

Pseudomonas sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MT631985.1	Pseudomonas brenneri strain XJC-6 16S ribosomal RNA gene, partial sequence
MT611297.1	Pseudomonas sp. strain T776 16S ribosomal RNA gene, partial sequence
MT605327.1	Pseudomonas sp. strain MB320 16S ribosomal RNA gene, partial sequence
MT605325.1	Pseudomonas sp. strain Ts1 16S ribosomal RNA gene, partial sequence
MT641229.1	Pseudomonas extremaustralis strain WS-1 16S ribosomal RNA gene, partial sequence
MN826583.1	Pseudomonas extremaustralis strain cqsV14 16S ribosomal RNA gene, partial sequence
MT583077.1	Pseudomonas marginalis strain PMK1 16S ribosomal RNA gene, partial sequence

Ehrlichia	no. reads	Best E-value	Percent identity
1QZJ7_04769_02992	61613	3.00E-126	100.00%
1QZJ7_00834_07143	11104	5.00E-124	99.60%

Ehrlichia sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MN463729.1	Uncultured Ehrlichia sp. clone Hainan 16S ribosomal RNA gene, partial sequence
MH500005.1	Ehrlichia minasensis isolate E-2650 16S ribosomal RNA gene, partial sequence
KY046297.1	Uncultured Ehrlichia sp. clone VKAA024 16S ribosomal RNA gene, partial sequence

Asinibacterium	no. reads	Best E-value	Percent identity
1QZJ7_01761_01844	19756	3.00E-126	100.00%
1QZJ7_03261_05084	8541	9.00E-127	100.00%

Asinibacterium sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

KP301113.1	Asinibacterium sp. ZJ6106 16S ribosomal RNA gene, partial sequence

NR_132297.1	Asinibacterium lactis strain LCJ02 16S ribosomal RNA, partial sequence
JN999927.1	Hydrotalea flava strain FJLY16 16S ribosomal RNA gene, partial sequence

Streptococcus	no. reads	Best E-value	Percent identity
1QZJ7_01552_06658	7561	3.00E-126	100.00%

Streptococcus sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

LR809138.1	Streptococcus sp. Marseille-Q2617 partial 16S rRNA gene, strain Marseille-Q2617
MT626073.1	Streptococcus infantis strain 1795 16S ribosomal RNA gene, partial sequence
MT613549.1	Streptococcus pseudopneumoniae strain 3203 16S ribosomal RNA gene, partial sequence

uncult. Caulobacteraceae	no. reads	Best E-value	Percent identity
1QZJ7_04448_04756	6475	3.00E-126	100.00%

uncultured Caulobacteraceae sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MN628471.1	Uncultured bacterium clone ASBud30 16S ribosomal RNA gene, partial sequence
MG858982.1	Uncultured bacterium clone OTU1190_Y_1_A_1139273 16S ribosomal RNA gene, partial sequence
KX350860.1	Uncultured Phenylobacterium sp. clone Z3AcetMagB29 16S ribosomal RNA gene, partial sequence

uncultured Clostridiales	no. reads	Best E-value	Percent identity
1QZJ7_03110_02047	5648	3.00E-126	100.00%

Uncultured Clostridiales sequence matches: GenBank accession nos. and names for set of identical best E-value matches to sequences obtained

MN646967.1	Clostridioides mangenotii strain CBA7501 16S ribosomal RNA gene, partial sequence
MN203748.1	Uncultured bacterium clone OTU12 16S ribosomal RNA gene, partial sequence
LR588760.1	uncultured bacterium partial 16S rRNA gene, clone A22
MH532195.1	Uncultured bacterium clone OTU_8547 16S ribosomal RNA gene, partial sequence

Table S3.4. Phylotypes and number of reads in the extraction control sample.

	extraction control	total dataset
Pseudomonas	11489	418592
Escherichia-Shigella	7272	12846
Francisella	3038	3807726
Burkholderiaceae_unclassified	1525	7490
Lactobacillus	1248	3874
Enterobacteriaceae_unclassified	1239	6472
Finegoldia	874	1218
Staphylococcus	855	11849
Bdellovibrio	751	983
Polyangiaceae_unclassified	731	731
Flavobacterium	720	1280
*Parcubacteria*_unclassified	665	1104
Halomonas	623	2125
Sphingomonas	618	12176
*Corynebacterium*_1	571	7368
Rothia	505	6782
A4b_ge	496	510
Delftia	492	740
Polaromonas	446	451
*Omnitrophicaeota*_ge	398	443
Williamsia	395	565
uncultured	392	536
IMCC26256_ge	377	394
Rhizobiaceae_unclassified	374	2536
SJA-28_ge	363	369
Micrococcaceae_unclassified	306	2182
Renibacterium	256	303
Lawsonella	243	2473
uncultured	230	230
Bacteria_unclassified	175	3356
Candidatus_*Rhabdochlamydia*	138	138
Sphingomonadaceae_unclassified	136	2443
Anoxybacillus	129	5208
Haliangium	123	210
Francisellaceae_unclassified	120	154929
Pseudomonadaceae_unclassified	104	4842
Rhodobacteraceae_unclassified	98	1447

Paracoccus	91	522
Curtobacterium	76	455
Stenotrophomonas	70	9639
Halomonadaceae_unclassified	67	525
Corynebacteriaceae_unclassified	48	13523
Microbacteriaceae_unclassified	45	2040
Gammaproteobacteria_unclassified	40	10739
Methylomonaceae_unclassified	34	51
Candidatus_*Kaiserbacteria*_ge	32	42
Cyclobacteriaceae_unclassified	25	27
Cytophagales_unclassified	24	109
Bacillaceae_unclassified	22	1665
Candidatus_*Moranbacteria*_ge	21	43
Lactobacillaceae_unclassified	18	112
Corynebacteriales_unclassified	15	1086
Rickettsia	14	23
uncultured_ge	12	116
Xenophilus	11	12
Staphylococcaceae_unclassified	7	419
Tibeticola	7	7
Chlamydiales_unclassified	6	49
Bacillales_unclassified	6	12082
Acidovorax	6	27
Nocardiaceae_unclassified	5	28
Candidatus_Nomurabacteria_ge	5	70
Rhodoferax	5	13
Polyangiaceae_ge	4	4
Betaproteobacteriales_unclassified	4	1028
Enterobacter	4	17
Micrococcales_unclassified	3	413
Flavobacteriaceae_unclassified	3	147
Stakelama	3	7
Aquabacterium	3	23
Legionella	3	275
Bacteroidia_unclassified	2	367
SBR1031_unclassified	2	146
Lactobacillales_unclassified	2	8383
1174-901-12	2	357
Aurantimicrobium	1	9
Cutibacterium	1	24

uncultured_ge	1	441
Sediminibacterium	1	59
Ignavibacteria_unclassified	1	1
Simkaniaceae_unclassified	1	1
A4b_unclassified	1	1
*Chloroflexi*_unclassified	1	62
Paenibacillus	1	5081
*Bacilli*_unclassified	1	127
*Gracilibacteria*_ge	1	193
Gemmataceae_unclassified	1	9
Geobacter	1	114
Polynucleobacter	1	19
Neisseria	1	817
Klebsiella	1	27
Acinetobacter	1	34314

Table S3.5. Ticks sampled from the same elk individuals, arranged by elk, showing number of subsampled reads for 7 major bacterial phylotypes.

	Tick _stage	Tick _sex	elk_ sampled	*Fran-cisella*	*Pseudo-monas*	*Ehrli-chia*	Francis. _unclas	*Asiniba-cterium*	*Acinet-obacter*	*Strepto-coccus*
YFF5	adult	female	OR41	23189	0	0	1280	8	24	4
YFM18	adult	male	OR41	10788	0	0	309	382	367	12
OFF12	adult	female	OR56	20649	0	2795	1497	0	0	15
OFM24	adult	male	OR56	20003	0	1	691	0	57	118
OFM21	adult	male	OR81	21212	0	114	702	156	91	26
OFN31	nymph	na	OR81	20360	0	0	974	955	0	1048
YFF3	adult	female	OR83	22309	0	0	1133	6	10	7
YFN29	nymph	na	OR83	16066	487	0	1	105	817	668
YFN34	nymph	na	OR83	2471	0	126	2	0	1906	1258
YFF2	adult	female	OR89	23165	0	0	1690	17	0	33
YFM13	adult	male	OR89	12541	0	10248	1418	104	19	147
YFF1	adult	female	OR94	23583	0	0	1293	6	0	61
YFN26	nymph	na	OR94	20359	0	0	506	174	219	78
YFF4	adult	female	OR97	14529	0	8808	1485	3	20	32
YFM16	adult	male	OR97	19160	0	513	55	178	609	439
OFF10	adult	female	OR99	13729	9473	0	592	132	169	3
OFM23	adult	male	OR99	21708	0	0	918	14	16	14
OFF9	adult	female	OR99	23939	0	0	657	76	1	11
OFM20	adult	male	OR99	8960	10338	0	298	163	314	89
OFM22	adult	male	YL112	10795	3504	0	2989	102	1222	306
OFN33	nymph	na	YL112	23561	0	0	756	0	25	207
OFF11	adult	female	YL167	23147	0	2	1582	0	2	2
OFF7	adult	female	YL167	20608	0	0	3302	0	49	39
OFM19	adult	male	YL167	23231	0	0	735	180	26	12

Table S3.6. Generalized Linear Model output for A) nymph, B) female and C) male as reference category. Significant P values are bolded.

A. nymph as reference	Estimate	Std. Error	P-value
H0, phylotype richness (log-link)			
tick class (female)	0.0117	0.2065	0.9550
tick class (male)	0.5271	0.2007	**0.0086**
engorgement (flat)	0.2589	0.1658	0.1185
input DNA	0.0002	0.0038	0.9578
number of sequences	0.0000	0.0000	0.1851
Pielou phylotype evenness (log-link)			
tick class (female)	-0.6684	0.1907	**0.0005**
tick class (male)	0.0115	0.1572	0.9418
engorgement (flat)	0.3573	0.1483	**0.0160**
input DNA	0.0028	0.0028	0.3050
H1, expShannon (log-link)			
tick class (female)	-0.8030	0.3094	**0.0140**
tick class (male)	0.2258	0.3227	0.4889
engorgement (flat)	0.4226	0.2636	0.1184
input DNA	0.0046	0.0059	0.4412

B. female as reference	Estimate	Std. Error	P-value
H0, phylotype richness (log-link)			
tick class (male)	0.5155	0.2211	**0.0197**
tick class (nymph)	-0.0117	0.2065	0.9550
engorgement (flat)	0.2589	0.1658	0.1185
input DNA	0.0002	0.0038	0.9578
number of sequences	0.0000	0.0000	0.1851
Pielou phylotype evenness (log-link)			
tick class (male)	0.6799	0.2064	**0.0010**
tick class (nymph)	0.6684	0.1907	**0.0005**
engorgement (flat)	0.3573	0.1483	**0.0160**
input DNA	0.0028	0.0028	0.3050
H1, expShannon (log-link)			
tick class (male)	1.0288	0.3416	**0.0050**
tick class (nymph)	0.8030	0.3094	**0.0140**
engorgement (flat)	0.4226	0.2636	0.1184

input DNA	0.0046	0.0059	0.4412

C. male as reference	Estimate	Std. Error	P-value
H0, phylotype richness (log-link)			
tick class (nymph)	-0.5271	0.2007	0.0086
tick class (female)	-0.5155	0.2211	**0.0197**
engorgement (flat)	0.2589	0.1658	0.1185
input DNA	0.0002	0.0038	0.9578
number of sequences	0.0000	0.0000	0.1851
Pielou phylotype evenness (log-link)			
tick class (nymph)	-0.0115	0.1572	0.9418
tick class (female)	-0.6799	0.2064	**0.0010**
engorgement (flat)	0.3573	0.1483	**0.0160**
input DNA	0.0028	0.0028	0.3050
H1, expShannon (log-link)			
tick class (nymph)	-0.2258	0.3227	0.4889
tick class (female)	-1.0288	0.3416	**0.0050**
engorgement (flat)	0.4226	0.2636	0.1184
input DNA	0.0046	0.0059	0.4412

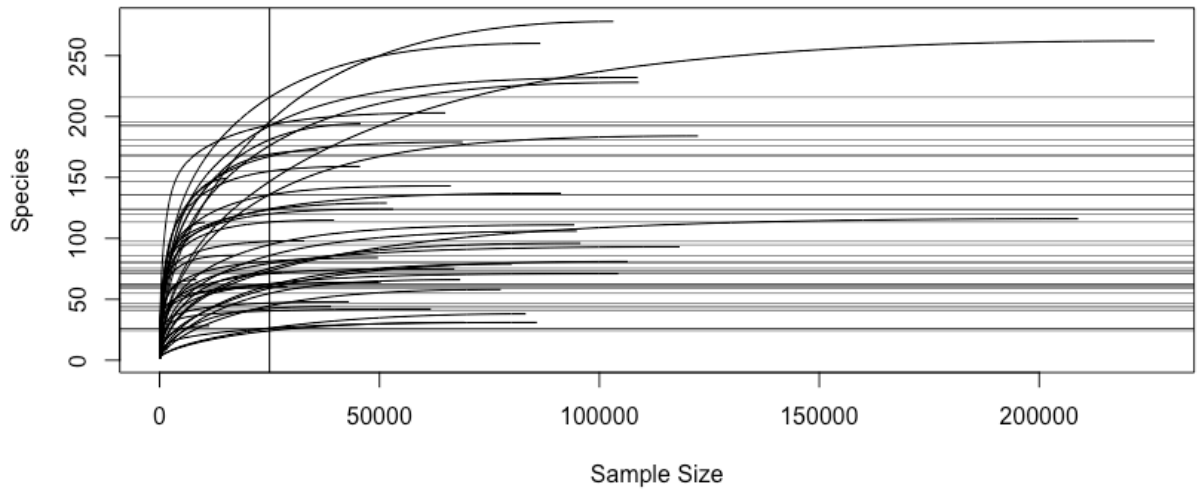

Figure S3.1. Rarefaction curves for 43 tick samples. Horizontal lines represent rarefied species richness at 25000 reads, the rarefaction threshold.

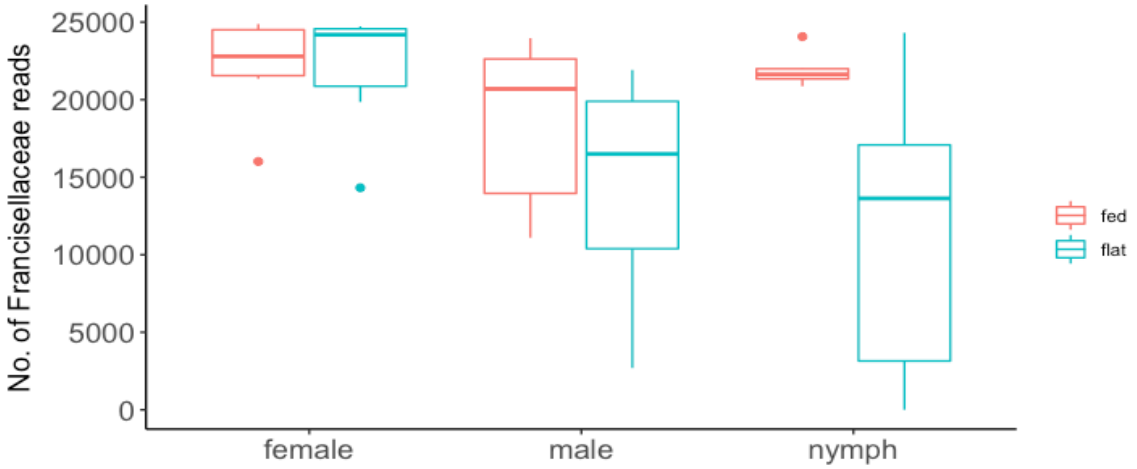

Figure S3.2. Number of reads assigned to Francisellaceae for female, male and nymphs separated by feeding status.

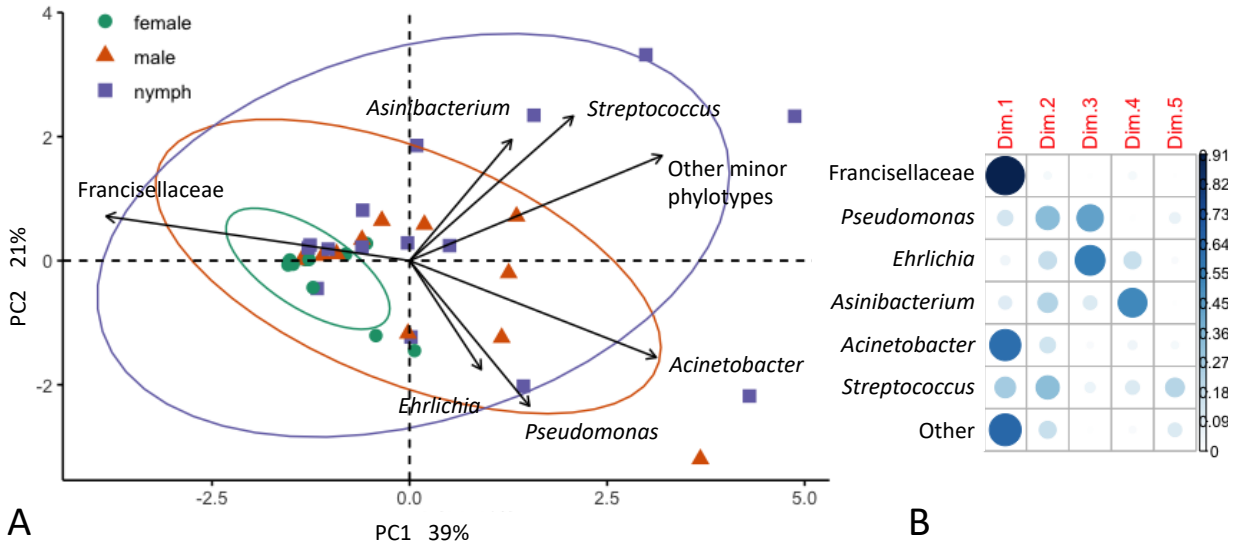

Figure S3.3. Principal components analysis (PCA) biplot of PC1 and PC2 (A), and Cos2 plot (B), of seven bacterial taxonomic categories associated with 38 *D. albipictus* ticks. Francisellacae includes reads assigned to *Francisella* plus Francisellaceae_unclassified. Minor bacterial phylotypes comprising ≤ 1% in the total dataset are combined as "Other" phylotypes.

CHAPTER 4

Microbiome composition and *Borrelia* detection in *Ixodes scapularis* ticks at the northwestern edge of their range

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4.1 Introduction

Accurate characterization of the microbial communities that are associated with ticks is essential to understanding both the ticks and the diseases they transmit (Bonnet et al., 2017; Gondard et al., 2017; Yuan et al., 2020). The most diverse and well-studied components of tick microbiomes are the bacteria (Greay et al., 2018), with different bacteria often interacting by interspecific and strain-level competitive displacement (Dib et al., 2008; Galletti et al., 2009; Genné et al., 2019). The assembly of such a microbial community can be affected by the immune responses of a tick to previously acquired microbes (Martins et al., 2017) and host-associated responses to tick salivary proteins (Gomes-Solecki et al., 2020). The impacts of these bacteria on ticks are varied and can include positive effects such as increased reproductive output (Greay et al., 2018; Nováková and Šmajs, 2019), overwintering success (Herrmann and Gern, 2010; Neelakanta et al., 2010), or egg survival (Machado-Ferreira et al., 2015). Although nutritional endosymbionts are the most abundant bacteria found in ticks, soil-associated bacteria are usually the most diverse (Andreotti et al., 2011; Carpi et al., 2011), and even minor microbiome members can be critical during off-host periods in the lifecycle of ticks (Burtis et al., 2019).

Since ticks often carry bacteria that are pathogenic to their vertebrate hosts, characterization of their microbial assemblages has historically focussed on only a few pathogens. This perspective changed with the advent of high throughput sequencing (Greay et al., 2018; Pollet et al., 2020) and with recognition that the same bacteria that act as pathogens of mammals will sometimes also boost tick survival (Herrmann and Gern, 2010; Neelakanta et al., 2010). Ticks can also transmit more than one pathogen in a single bite, and thus it is informative to consider the interactions that

contribute to a pathobiome (Cabezas-Cruz et al., 2018; Vayssier-Taussat et al., 2015). Taxonomic profiling of bacteria is now generally based on PCR with universal primers for a conserved marker gene, followed by high-throughput sequencing of the amplicon. Although amplicon sequencing has biases, it is cost-effective, is supported by well-curated databases, and has a large foundation of previous studies for comparisons (Cabezas-Cruz et al., 2018; Greay et al., 2018). On the other hand, targeted identification of particular bacteria using specific primers and quantitative real time PCR (qPCR) can provide a fast and accurate assay of the target bacteria (Modarelli et al., 2019; Sanchez-Vicente et al., 2019), although it provides little information about microbial community composition. Both approaches are multistep processes affected by variation in sample storage, extraction methods, primer specificity, number of PCR cycles, and data analysis. In a public health laboratory, all of these factors can cause significant constraints, and thus it can be helpful to apply both approaches to profiling microbiomes (Janda and Abbott, 2007; Jian et al., 2020).

Ixodes ticks have recently expanded their range in Canada (Bouchard et al., 2019; Leighton et al., 2012), which is a serious concern because they transmit *Borrelia* species that cause Lyme disease, the most common arthropod-vectored disease in North America (Bouchard et al., 2019; Lloyd and Hawkins, 2018; Ogden et al., 2019). The main vector of Lyme disease in North America is the blacklegged tick, *Ixodes scapularis*, the microbiome of which is dominated by *Rickettsia* in established populations, while more than 50% of ticks may also be infected with *Borrelia* in some areas (Brinkerhoff et al., 2020; Tokarz et al., 2019). Few microbiome studies of *I. scapularis* are available from areas where the tick is expanding its range or is adventitious, such as Alberta (Fitzgerald, 2012; Government of Alberta, 2018). Adventitious ticks are defined in Canada as being found sporadically in an area that does not have multiyear reproducing populations ("Consensus conference on Lyme disease," 1991). Currently, *I. scapularis* ticks are considered to be established in five provinces but not in Alberta (Government of Canada, 2020), and local public health messaging is influenced by whether ticks are regarded as being adventitious or from an established population (Ogden et al., 2014). Infection status of ticks as *Borrelia*-positive or negative is also critical to medical diagnoses (Hatchette et al., 2014). Concern over exposure to Lyme disease has led to several surveys of *Borrelia burgdorferi* in *Ixodes* ticks (Gasmi et al., 2018; Kulkarni et al., 2018), including a surveillance program established by Alberta in 2007 (Government of Alberta, 2015). In Alberta, *Ixodes* ticks have been evaluated using qPCR for *B. burgdorferi*, with a yearly range of 10−19% testing positive during 2013−2018 (Government of

Alberta, 2020). However, little else is published about the microbiome of the ticks that potentially vector Lyme disease in western Canada.

Our study has three aims: (1) to characterize the bacterial microbiome of *Ixodes scapularis* ticks that are potential vectors of Lyme disease in Alberta, (2) to determine whether the composition of the microbiome of *Ixodes* ticks sampled in Alberta depends on their *Borrelia* infection status, and (3) to compare the performance of qPCR with 16S rRNA amplicon sequencing for detection of *Borrelia*. We used DNA extracts from a previous qPCR-targeted study on Lyme disease (Government of Alberta, 2020) and found that the microbiome of *I. scapularis* from Alberta was typical of that described for other regions. We also showed greater microbiome diversity in *Borrelia*-positive ticks, and strong correspondence between methods for detection of *Borrelia*. Our results expand understanding of tick-borne pathogens and establish a basis for future comparisons of *Ixodes* microbial assemblages in an area where *I. scapularis* is not yet established, but putatively adventitious ticks are commonly found.

4.2 Material and methods

4.2.1 Tick specimens

We sampled the microbiome of 20 female *Ixodes* ticks, including 18 *Ixodes scapularis* and 2 identified as *Ixodes* sp. (Table 4.1), which were submitted during January to May 2016 to the Alberta Tick Surveillance Program (ATSP) of Alberta Agriculture and Forestry (ATSP)(Government of Alberta, 2020). These ticks were removed from dogs or cats, then identified to life stage and species using the keys of Kierans and Clifford(Keirans and Clifford, 1978). The two unidentified *Ixodes* sp. could not be identified to species level due to morphological damage to the samples. DNA was extracted from ticks using a QIAmp DNA Mini Kit (catalog no. 51304) without prior washing of the exterior of the tick. Extracts were stored at −80 °C in ATSP freezers until transfer to −70 °C at the University of Alberta.

4.2.2 Control samples

To provide context and calibration for our tick samples, we analyzed 3 additional control samples at the same time. First, we used a mock community (MOC) to estimate the lower limit of detection and to assess primer amplification biases. The MOC was provided by BEI Resources (Manassas, VA, USA; staggered low concentration HM783D, lot 60304010), with varied numbers

of 20 bacterial strains in 17 different bacterial genera at concentrations ranging from 10^3 to 10^6 operons (= 16S rRNA sequence copies) per bacterial strain (Table S4.1). An aliquot of 2 µL of the total 35 µL was used in the initial 16S rRNA amplification of the MOC. Second, a sham extraction control (SEC) was used to estimate the amount and identity of bacteria associated with the extraction kit as well as the potential for aerosol contamination during sample handling. This SEC used the QIAmp DNA Minikit and followed all steps for extraction of a tick sample without using an input specimen, thus sampling DNA from extraction reagents and aerosols produced during the sham extraction process (Table S4.2). Third, a no-template PCR control (NTC) from the 16S rRNA amplifications was included to detect potential contaminants arising solely during the 16S rRNA amplification process (Table S4.3).

4.2.3 *Borrelia qPCR*

Testing at ATSP established that 10 extracted tick samples were *Borrelia*-positive (B +) and 10 were *Borrelia*-negative (B−). The assay was based on a modified qPCR protocol that amplified the outer surface protein A (OspA) gene of *B. burgdorferi* directly without first screening for *B. burgdorferi* using the 23S marker, but was otherwise comparable to the method used by the National Microbiology Lab of Canada (Dibernardo, 2010) with the following changes: the qPCR amplification was set for 50 cycles of 95 °C for 5 s and 61 °C for 10 s and 72 °C for 15 s. In 2016, a total of 215 *Ixodes* were tested from companion animals, of which 18% were positive for *B. burgdorferi* (Government of Alberta, 2020). Positivity was based on qPCR using primers specific for *B. burgdorferi* OspA, including OspA forward: CTGGGGAAGTTTCAGTTGAAC, OspA reverse: TTGGTGCCATTTGAGTCGTA and OspA Probe:FAM-MGB CTGCAGCTTGGAATTCAGGCACTT (Dibernardo, 2010).

4.2.4 16S rRNA sequencing

End point PCR amplification of the 16S rRNA gene encompassed 6 variable regions separately: V2, V3, V4, V6-7, V8, and V9 (Sperling et al., 2017). Amplification was conducted in 2 multiplex pools using the Ion 16S Metagenomics kit (Ion Torrent, 2015) with 25 amplification cycles of 95 °C for 30 s and 58 °C for 30 s. The first pool included V2, V4, and V8 and the second pool was V3, V6-7, and V9. Estimated length of amplicons ranged from 215 to 295 bp, depending on the region. Amplicons were visualized on an agarose gel to confirm amplification and

quantified using Qubit High Sensitivity Assay. Aggregate amplicons for each of the 10 B+ tick samples and 10 B- samples were used for library preparation with 400 bp chemistry. Library concentrations were assessed with Agilent High Sensitivity Bioanalyzer and diluted to 26 pM before pooling. Sequencing used Ion Personal Genome Machine PGM™ with a 318C chip. To assess the repeatability of microbiome community proportions, we performed a second library preparation and sequencing run for the 5 qPCR B+ samples that had the lowest read counts in the first sequencing run. Sequences are deposited in National Center for Biotechnology Information NCBI's Sequence Read Archive as BioProject Accession Number PRJNA668181.

4.2.5 Sequence Data Processing

Reads were processed using Ion Reporter Software 5.14.1.0, with sequences being retained for taxonomic assignment if both forward and reverse primers were present. A retention threshold of 10 identical copies per sequence was the default used in Ion Reporter and is intended to filter out reads with sequencing error or index switching. Taxonomic assignment was performed first by NCBI megaBLAST searches of the curated MicroSeq Reference Library v2013, followed by a second stage BLAST search of the curated Greengenes v13.5 database for all sequences that had an E-value greater than 0.01 in searches against the MicroSeq database.

Counts for each taxon were additive for each variable region, e.g., a taxon detected by primers for V2 was also counted when detected in region V3. However, taxa could be classified at the level of family-only for some variable regions and the level of genus in the same or other variable regions. Designation as family-only could be due to the diagnostic limitations of a given variable region (Chakravorty et al., 2007) or may result from natural variation or sequencing error in the sequences used to compare to a particular database. A single genus, for example *Streptococcus*, may be represented at the family-only level for some reads in region V6-7 and genus level for regions V2, V3, V4, V6-7, and V8. The total number of reads for *Streptococcus* species in the MOC sample were therefore spread over the level of Streptococcaceae family-only (15 reads) and the genus level of *Streptococcus* (9450 reads) (Table S4.4).

Some variation in names is due to similarity of sequences to different databases that use different names for the same bacteria. For example, multistage BLAST searches for *Escherichia* sequence in the mock community resulted in most reads being assigned as *Escherichia/Shigella* while a minority of sequences were named *Escherichia*, on the basis of e-values larger than 0.01

when queried against the MicroSeq database but with similarity to the second stage Greengenes database at an e-value of less than 0.01 (Tables S4.1 and S4.4). The proportion of sequences expected for each bacterial genus is based on the number of operons provided in the mock community.

Once reads were assigned taxonomically, the community matrix of sequence identifications produced by Ion Reporter was imported into an Excel spreadsheet, where reads present in the SEC sample or NTC sample were subtracted from the number of reads for each taxon. For taxa found in both SEC and NTC, the greater number of reads in either the SEC or NTC was subtracted from the sample numbers. Negative numbers were reset to zero. The community matrix was next imported into vegan 2.5−6 (Oksanen et al., 2019) and rarefied to 33,000 reads, which was the size of the smallest sample, rounded down (Figure S4.1).

4.2.6 Diversity measures and associations with tick variables

We used Pielou evenness and the Hill Number series H0-H3 as microbiome diversity measures. H0 is richness (number of species/taxa), while H1 corresponds to the exponential of Shannon entropy, and H2 is the inverse of Simpson's index, with successive Hill numbers putting decreasing emphasis on rare members of a community. To assess beta diversity, we calculated Bray Curtis Distance measures and metaMDS using vegan 2.5-6.

A series of generalized linear models (GLM) were used to test the significance of associations between diversity measures (H0-H3, Pielou evenness) and several tick variables (*Borrelia* OspA status, host animal, date collected, travel history outside the province in the previous 2 weeks). Pielou evenness was modelled using a beta distribution as values are bounded between 0-1. For Pielou evenness, we used B+ status and host animal as model variables. H0 (richness expressed as count data) and H1-H3 (continuous integers) were modelled using negative binomial and gamma distributions, respectively, each with a log-link function. Categorical variables were *Borrelia* OspA status (negative/positive), host animal (dog or cat), and travel history outside province in last 2 weeks (yes/no/not available), while date collected (expressed as Julian date) was continuous. To compare the 2 sequencing runs, we ran a series of GLMs using only the run number as a variable. Beta distribution was used for Pielou evenness, negative binomial for H0, and gamma distributions for H1-H3.

4.3 Results

4.3.1 *Ixodes scapularis microbiome characteristics*

We sampled the microbiome of 20 female ticks that were previously PCR-tested as *Borrelia*-positive (B+) or *Borrelia*-negative (B-) (Table 4.1). The 20 Alberta *Ixodes* ticks gave a mean of 112,676 (Standard Error = SE ± 5742) total raw 16S rRNA reads before filtering per tick in the first sequencing run (Table 4.1), with a mean of 64,377 (SE ± 3058) remaining after filtering out all reads that were not represented by at least 10 identical sequence copies. We sampled five B+ ticks to greater depth in a second 16S rRNA sequencing run. Finally, we sequenced three controls, a mock bacterial community, a sham extraction, and a no-template control.

We showed the proportions of bacterial reads assigned to each bacterial taxon for B- and B+ ticks (Figure 4.1). Bacterial taxa that comprised more than 1% of the total reads in at least one tick are shown with all taxa consistently representing less than 1% of reads grouped as "Other". *Rickettsia* were generally the most common bacteria in both B- and B+ tick samples (Figure 4.1), comprising a mean of 82% in the B- and 62% in the B+ groups. *Rickettsia* comprised > 50% of reads in 8 of 10 B- and 7 of 10 B+ ticks, with proportions varying from 100% to 0.05%. Only two B- ticks had less than 99% *Rickettsia.* For these two ticks*, Pseudomonas* comprised 76% (tick Ix4) and 45% (Ix17) of the total reads. The Ix17 tick also contained 53% Enterobacteriaceae (including 36% *Erwinia*).

We related the presence of reads corresponding to *Borrelia* to the results of the qPCR test. No *Borrelia* reads were found in any B- ticks, whereas *Borrelia* comprised the second most common bacterial taxon in B+ ticks (Figure 4.1). *Borrelia* proportions in B+ ticks ranged from 93% to 0%, after reads with less than 10 identical copies were filtered out. One qPCR-positive tick (Ix20) had 0% *Borrelia* reads for 16S rRNA testing when filtered at a 10-copy threshold, but nonetheless had 21 *Borrelia* reads when a single copy filtering threshold was used. Ix20 also had small numbers of other Spirochaetaceae reads at the single copy threshold (15 *Spirochaeta*, 70 *Treponema*, and 1 family level only, totaling 107 Spirochaetaceae reads with the 21 *Borrelia*) that were not found in any other tick samples. This Ix20 tick had the largest number of bacterial genera (28 total in 10-copy filtered sequences). After *Borrelia*, the most frequent bacterial taxon was Xanthomonadaceae, which was only represented by about 10% of the reads in a single tick (Ix11, a B+ tick).

To assess the repeatability of microbiome surveys of B+ ticks, we sequenced five B+ samples with the lowest number of reads again after a second library preparation that gave an increase in reads (mean = 318,783 raw reads per sample; 216,558 reads after 10-copy filtering). The proportions of reads for major bacterial groups remained essentially identical after the second preparation (Figure 4.1). Sample Ix20 produced 98 *Borrelia* raw reads in the second run, which became 12 reads after filtering at the 10-copy threshold. This tick also had correspondingly greater numbers of other Spirochaetaceae. These additional Spirochaetaceae taxa remained undetected in the four other samples included in the second run.

4.3.2 Comparison of microbiomes with or without Borrelia

To assess the diversity of the microbiomes of B+ and B- ticks, we represented the number of taxa by Hill numbers (Figure 4.2). The B+ ticks had noticeably greater overall bacterial diversity than ticks without *Borrelia*. In terms of taxon richness (H0), B+ ticks were twice as diverse, with an average of 6.3 ± 2.5 bacterial taxa detected compared to B- ticks that only had an average of 3.1 ± 0.75 ($p < 0.014$, Table S4.5). This trend continued for H1-H3, with B+ ticks having 1.8 to 1.5 "effective taxa" (in this case mainly genera) compared to B- ticks with 1.3-1.2.

We visualized the differences in community composition between B+ and B- ticks using nonmetric multi-dimensional scaling (NMDS) of Bray–Curtis distance dissimilarity measures. An ordination plot shows that the diversity of 8 of 10 B- ticks was contained within the greater range of values for B+ ticks (Figure S4.2A). The two B- tick exceptions (Ix4 and Ix17) had very low *Rickettsia* proportions. The data were well represented in two dimensions with stress values less than 0.01 and R^2 of 1 (Figure S4.2B).

To test for associations between diversity and sample characteristics, we used generalized linear models (GLMs). Several variables did not have significant associations with any diversity measures, including the mammalian host of the tick (dog vs. cat), collection date, and travel history in the previous 2 weeks (Table S4.5). However, B+/B- status was significantly associated with all diversity measures, including Pielou evenness and the Hill numbers H0-H3 (Table S4.6).

To determine whether microbiome sequencing depth changes the proportions of taxa identified, we compared the data from the first and second sequencing runs. Although the second sequencing run of five tick samples provided 3.4 times more reads than the first, there were no significant differences in diversity between the two sequencing runs (Figure 4.1, Table S4.6). The

number of bacterial taxa detected (H0) in the second run was numerically greater (16.4 ± 10.4 effective number of taxa) when compared to the same five samples in the first run (9.4 ± 4.7), but the difference was not statistically significant. Since H1-H3 have greater weighting on the most abundant taxa in samples, these diversity measures are even less likely to demonstrate differences. No measures were significantly different between the two runs, and hence assessment of tick microbiomes was robust to sequencing depth (Figure 4.2, Table S4.6).

4.3.4 *Borrelia detection: 16S rRNA versus qPCR*

To assess the sensitivity of the qPCR test compared to microbiome sequencing, we examined the sequences that corresponded to *Borrelia*. Characterization of the microbiome of ticks using 16S rRNA surveys was congruent with the prior determination of *Borrelia* status on the basis of qPCR. Sequences from the first 16S library preparation had no *Borrelia* in any of the 10 qPCR B- samples, while showing *Borrelia* in 9 of the 10 B+ samples. This first set of sequences showed no *Borrelia burgdorferi* in one B+ tick (Ix20) and only 13 *Borrelia* reads in a second sample (Ix10). These two samples were among the five samples in the second library preparation, which had an increase by a factor of 3.4X in the average number of reads both before and after reads were filtered and taxonomically classified. The presence of *Borrelia* in the tick sample was more evident in the second run, confirming the qPCR results for Ix20. Across all samples that were *Borrelia*-positive, four of the six 16S rRNA variable regions produced at least some reads (Table S4.7). Within each sample, the largest number of *Borrelia* reads per sample was produced by primers for variable region V4 (7 of 15 samples) or region V6-7 (5 of 15), while regions V8 and V9 were consistently the worst or had no reads at all. In addition to confirming the qPCR-based *Borrelia* status of all ticks, 16S rRNA-based methods showed that all 20 tick specimens contained *Rickettsia*, a bacterial genus with potential pathogenicity to mammals that in this case may represent a nutritional symbiont of the tick (Kurtti et al., 2015). No other bacterial pathogens of mammals vectored by ticks in Canada (Bouchard et al., 2019), including *Ehrlichia*, *Anaplasma*, or *Francisella,* were detected in our samples.

To estimate the limits of detection of different bacterial taxa, we included three control samples in our microbiome sequencing. To assess the efficacy of our primers for amplifying different taxa of bacteria, we sequenced a mock bacterial community (MOC) composed of known bacteria in known proportions. MOC results showed both overestimates (e.g.,

Escherichia/Shigella) and underestimates (e.g., *Staphylococcus*) of the true proportions of reads for different taxa in the mock community, compared to the expected number of reads (Table S4.1). We detected all members of the mock community that had greater than 10^4 operons (16S copies) per bacterial strain in the mock community, with the sole exception of *Listeria*. Utilizing a minimum filtering threshold of 10 identical sequences and both primers, we detected no members of the MOC with fewer than 10^3 operons. To examine the efficacy of our primers to detect different taxa, we assigned reads to each 16S rRNA variable region that was amplified (Table S4.4).

To assess the reads arising from the sequencing environment, we sequenced a sham extraction control. The sham extraction control (SEC) produced 71,920 reads, of which 30,830 reads had primers detected on each end and were mapped to the taxonomic database (Table S4.2). Finally, we included a no template control (NTC) to assess the reads arising from the amplification steps. The NTC control gave a total of 44,610 reads, of which 19,515 reads had both ends and were mapped to the taxonomic database (Table S4.3). Taxa associated with the SEC and NTC were primarily taxa described as being associated with PCR and extraction kit reagents (e.g., *Pseudomonas* (Lejal et al., 2020; Salter et al., 2014)); however, there were 18 reads associated with *Rickettsia* in the kit control. Using a threshold value of a single copy, we detected 25 *Rickettsia* reads and 2 *Borrelia* reads in the MOC. This is within the range expected for index switching (0.167%) with PGM (Palmer et al., 2018).

4.4 Discussion

The microbiome of *Ixodes scapularis* ticks in Alberta is dominated by *Rickettsia* endosymbionts, with *Pseudomonas* more common in some individuals, making it similar to the microbiome composition reported for these ticks in established areas of their range (Brinkerhoff et al., 2020; Tokarz et al., 2019; Van Treuren et al., 2015). Alberta ticks that carried *Borrelia* had significantly greater microbiome diversity than ticks that had no *Borrelia*, a finding that suggests an altered microbiome ecology in *Borrelia*-positive ticks, as well as the potential to use microbiome diversity as an indicator of elevated risk of infection by *Borrelia*. Depending on filtering thresholds, 16S rRNA marker gene amplification and scanning was less sensitive for detection of *Borrelia* than was qPCR, leading us to suggest that qPCR is the preferred method of detection when there are known pathogens in a region. However, the generally elevated bacterial diversity found in *Borrelia*-positive ticks suggests that the current focus on a single

predetermined pathogen could lead to significant biases in understanding the interactions and disease potential of bacteria associated with *I. scapularis* in Alberta.

4.4.1 Components of the microbiome

Our finding that the microbiome of *I. scapularis* in Alberta resembles the microbiome of this species elsewhere in the range of the tick gives a starting point for better identification of its components. Ogden et al., (2011) found the *Borrelia* sequence type in a single positive tick from Alberta was most closely related to *Borrelia* found in Manitoba and the midwestern USA. *Rickettsia* sequences in Alberta ticks likely represent a known *I. scapularis* endosymbiont, *Rickettsia buchneri,* which was described from an isolate in Minnesota, USA (Kurtti et al., 2015). However, it is not feasible to reliably distinguish among *Rickettsia* species in our data (Trout Fryxell et al., 2015), as the short 16S rRNA fragments that are widely used to survey bacteria do not accurately identify bacteria to the species level (Kim et al., 2020; Mignard and Flandrois, 2006). Standard gene-based identifications at the species level in *Rickettsia* require four protein coding genes (gltA, ompA, ompB, and gene D) in combination with 16S rRNA gene sequences (Fournier et al., 2003). Since other *Rickettsia* species such as *R. rickettsii* that are pathogenic to mammals may also be found in *I. scapularis* (Bonnet et al., 2017), it would be appropriate to test for these *Rickettsia* using nested PCR or qPCR with species-specific primers.

Although *Rickettsia* were the most abundant bacteria in most of the ticks we sampled, some ticks were dominated by *Pseudomonas*, various Enterobacteriaceae, or *Borrelia*. Two *Borrelia*-negative ticks had large proportions of *Pseudomonas*, which has also been found in blacklegged ticks in Ontario, Canada (Clow et al., 2018; Sperling et al., 2017), and eastern USA (Thapa et al., 2019; Van Treuren et al., 2015). The role of *Pseudomonas* in the microbiome of ticks remains to be clarified, although a study focused on *Pseudomonas* from healthy vertebrates in Spain recovered *Pseudomonas fluorescens* (11 isolates) and *Pseudomonas gessardii* (6 isolates*)* from two ticks that were included in their sampling (Ruiz-Roldán et al., 2020). *Pseudomonas fluorescens* can have antibiotic and probiotic properties and is part of a healthy soil community (Haas and Keel, 2003), while *P. gessardii* is a member of the same *P. fluorescens* group but was isolated from mineral waters rather than soil (Verhille et al., 1999). Thus, the presence of these bacteria in ticks is most likely an indication of the ubiquity of *Pseudomonas* in a variety of habitats (Palleroni, 2015). The abundance of *Pseudomonas* relative to *Rickettsia* in our 16S rRNA dataset

may also partly reflect the greater number of copies of 16S rRNA in *Pseudomonas.* This genus has an average of 4.9 copies of the 16S rRNA gene per genome (range 1−9, mode = 4), while *Rickettsia* averages 2.1 (range 1-6, mode = 1: rrnDB version 5.6 (Stoddard et al., 2015)). However, as recommended by Louca et al. (2018), in order to retain comparability among studies, we made no corrections for 16S rRNA gene copy number to our dataset.

One of the two *Borrelia*-negative ticks with few *Rickettsia* (Ix17) had 36% of its reads assigned to *Erwinia,* a member of the Enterobacteriaceae. The presence of large amounts of *Erwinia*, a genus best known for plant pathogens like fireblight (Palacio-Bielsa et al., 2012), also suggests the possibility of a tick pathogen. *Erwinia aphidicola* is a pathogen of the pea aphid (Harada et al., 1997), while other species of *Erwinia* are associated with diverse arthropods such as bark beetles (Skrodenytė-Arbačiauskienė et al., 2012) and thrips (de Vries et al., 2001), although their roles in these insects are unknown. In addition to *Erwinia*, 15% of the sequences assigned to Ix17 could only be identified as Enterobacteriaceae at the family level. Undescribed Enterobacteriaceae were the most common bacteria found in *Ixodes scapularis* in North Carolina and may be an important commensal of arthropods (Van Treuren et al., 2015). In addition to being present in ticks in our current study, bacteria that could only be identified as bacterial family Enterobacteriaceae have been reported in *I. scapularis* from Ontario and *Ixodes angustus* in British Columbia (BC) (Sperling et al., 2017).

Tick species are often distinguished from each other by characteristic assemblages of bacteria (Greay et al., 2018; Pollet et al., 2020; Ross et al., 2018). This phenomenon can be used to support identification of a tick that is no longer morphologically intact or whose DNA has been used up, as was the case for our samples. On this basis, the most likely species identity of the two ticks labelled as *Ixodes* sp. (Ix20, Ix23) is *I. scapularis*. In addition to *I. scapularis*, two other *Ixodes* are plausible for samples in the Alberta ATSP survey: *Ixodes angustus* and *Ixodes pacificus*. The microbiome of *I. angustus* is normally dominated by *Francisella*-like endosymbionts (Sperling et al., 2017), which were not found in the two *Ixodes* sp. samples. Alternatively, these samples could have been *I. pacificus*, but this species is typically only found in Alberta on animals that have recently travelled to BC, which was not true of these two ticks. Further surveys of geographic variation in the composition of the microbiome of *Ixodes* ticks, both between and within species, would be highly informative in establishing the potential source

regions of ticks in Alberta, especially since *I. scapularis* is currently regarded as being solely of adventitious origin in this region (Fitzgerald, 2012; Lindquist et al., 2016).

4.4.2 Borrelia infection status and tick microbiome diversity

We found that *Borrelia*-positive adult female ticks had significantly greater overall bacterial diversity than those that were *Borrelia*-negative. This result is consistent with the finding by Narasimhan et al. (Narasimhan et al., 2014) that laboratory-reared *I. scapularis* ticks with greater microbiome diversity were more likely to be successfully colonized by *B. burgdorferi.* These ticks also had more intact and thicker peritrophic membranes, which may protect *B. burgdorferi* from digestion by the tick (Kurokawa et al., 2020; Narasimhan et al., 2014). In the Narasimhan et al. (2014) study, tick dysbiosis (= altered microbiome composition), rather than absolute bacterial diversity, was considered to be the key factor in colonization success. In fact, the microbiome diversity of the gut of ticks may be quite low, according to work by Ross et al. (2018), and microbiome diversity can differ greatly between specific tissues and whole ticks (Pollet et al., 2020).

In contrast to our results, three recent studies have shown no significant difference in bacterial diversity associated with *Borrelia* infection status (Chauhan et al., 2020; Kwan et al., 2017; Thapa et al., 2019), and a fourth study was ambiguous (Brinkerhoff et al., 2020). However, one of these studies reported results for a mix of females and males (Chauhan et al., 2020), which would have increased the variance in an already noisy system (Pollet et al., 2020). A second study (Kwan et al., 2017) analyzed nymphs of *Ixodes pacificu*s and found no significant differences in richness or evenness. A third study (Thapa et al., 2019) distinguished males from females and found no significant differences in bacterial community composition between B+ and B- ticks, on the basis of unweighted UniFrac distance; however, they did not address differences in numbers of bacterial taxa for B+ and B- ticks. Finally, the fourth study (Brinkerhoff et al., 2020) gave mixed results. Richness in B+ ticks was significantly higher when all life stages were considered; however, there was no difference in diversity between B+ and B- nymphal ticks alone (Brinkerhoff et al., 2020). This variability in results underscores the need to control for life history stages when evaluating the significance of patterns relating to *Borrelia* status.

One life history factor in ticks that is associated with *Borrelia* infection is overwintering success. In Switzerland, *Ixodes ricinus* ticks infected by *Borrelia* are more likely to overwinter

successfully (Herrmann and Gern, 2010). If such ticks also have increased bacterial diversity, then this may provide increased opportunities for functional adaptations, in keeping with observations in other organisms that microbiome diversity is generally beneficial (Reese and Dunn, 2018).

The source of the blood meal of a tick is another ecological factor that can impact its microbiome diversity. For example, deer blood has bacteriolytic effects on *Borrelia* in *I. scapularis* (Ullmann et al., 2003). In fact, Brinkerhoff et al. (2020) have proposed that this phenomenon should cause reduced overall bacterial diversity across the microbiome of fed ticks. Swei and Kwan (2016) also reported reduced microbiome diversity of *I. pacificus* after feeding on western fence lizards, which are known to be refractory to *Borrelia* (Kuo et al., 2000; Lane and Quistad, 1998). However, the *I. scapularis* ticks sampled in our study were all feeding on dogs and cats, which are not considered refractory to *Borrelia.* Furthermore, Thapa et al. (2019) reported that ticks feeding on dogs had increased bacterial diversity compared to unfed ticks, although *Borrelia* was not detected in any of their ticks. Thus, our finding of increased bacterial diversity in the ticks that are infected with *Borrelia* will need to be reexamined with larger sample sizes, ticks of both sexes and all life stages, and in ticks with a range of feeding statuses. However, if this result is confirmed, it would raise the question of whether there is an elevated risk of co-transmission of other bacterial pathogens by ticks that are carrying *Borrelia*.

A variety of studies have shown that the infection status of *Ixodes* ticks by *B. burgdorferi* is affected by the presence of other co-circulating pathogens (Dunn et al., 2014; Nieto and Foley, 2009; Pokutnaya et al., 2020). Positive associations have been found between *B. burgdorferi* and *Babesia microti* (Dunn et al., 2014; Pokutnaya et al., 2020), while mixed positive and negative associations are reported for *Anaplasma phagocytophilum* (Nieto and Foley, 2009; Pokutnaya et al., 2020). This may be explained by differences in the role of the tick peritrophic membrane in facilitating colonization by different bacteria. In contrast to *B. burgdorferi*, which benefits from the protection provided by an intact peritrophic membrane (Narasimhan et al., 2014), *A. phagocytophilum* is better able to colonize ticks with a more permeable peritrophic membrane (Abraham et al., 2017). This contrast suggests that *B. burgdorferi* and *A. phagocytophilum* should rarely be found as co-infections in the same tick. However, this expectation is contradicted by the study of Pokutnaya et al. (2020), who reported that female ticks were more likely to be infected with *B. burgdorferi* if they are also infected with *A. phagocytophilum*. The interactions among these bacteria are still poorly understood, and it will ultimately also be necessary to determine

whether such co-infected ticks have a reduced probability of actually transmitting either *A. phagocytophilum* or *B. burgdorferi*.

Although no *A. phagocytophilum* was detected in the 20 ticks examined in our study, this sample size is insufficient to conclude that *A. phagocytophilum* is not present in Alberta. On the basis of increasing detection of *A. phagocytophilum* in ticks in Ontario (Nelder et al., 2019), we recommend including surveillance of *A. phagocytophilum* in tick surveys in Alberta. At least one human case with no history of travel has been detected in Alberta, which suggests that *A. phagocytophilium*-infected ticks may be found in Alberta sporadically (Parkins et al., 2009). A multiplex qPCR protocol for both *B. burgdorferi* and *A. phagocytophilium* has already been developed at the Canadian National Microbiology Laboratory and could be easily incorporated into the Alberta tick surveillance program (Dibernardo, 2010).

4.4.3 *Effectiveness of 16S rRNA surveys*

Our study found that *B. burgdorferi* was detected consistently in the same tick samples using both qPCR and 16S rRNA end point detection, although this finding was dependent on 16S rRNA sequencing depth at the lowest levels. Targeted qPCR detection of specific pathogens can be more cost-efficient than broad-range primers, and qPCR primers can be designed for higher annealing temperatures and therefore give greater specificity than general PCR primers that may include degenerate bases (Parada et al., 2016). Competition for template and GC bias can also mask low levels of a pathogen to a greater degree in general 16S rRNA amplification than with qPCR (Kralik and Ricchi, 2017). In our study, the PCR annealing temperatures for qPCR and 16S rRNA end point detection differed by only 2 °C, and thus competition for template and the degenerate primers used in 16S rRNA amplification are more likely explanations for the more decisive detection by qPCR.

Microbiome scans using 16S rRNA are widely regarded as producing inherently noisy datasets, in part due to sequencing platforms with different error profiles (Auer et al., 2017; Greay et al., 2018). For example, Ion PGM sequencing has a greater number of indel errors than Illumina sequencing (Marine et al., 2020; Song et al., 2017). To reduce any sequencing error, a threshold number of identical sequences is considered necessary, on the assumption that errors will be random along the sequence and deeper coverage will allow sequencing errors to be distinguished from biological variation (Ebbert et al., 2016). In order to filter such errors, Ion PGM software

uses a default number of 10 identical sequences for taxonomic profiling; if fewer than 10 copies of a specific sequence are found then these all remain unclassified. A threshold of 10 reads may generally provide a workable balance between accuracy and inclusion, but it risks overlooking rare variants. For example, when sample Ix20 data was analyzed with a threshold level of a single read, the first sequencing run resulted in 107 sequences matching Spirochaetaceae at the family level. However, no particular single sequence was found to have more than 10 copies and therefore the sample was considered to be *Borrelia*-negative in the initial analysis. Smaller thresholds for read filtering may be more appropriate for Illumina sequencing (Auer et al., 2017), but the most appropriate copy number for filtering has not yet been established for any high throughput sequencing platform (Pollock et al., 2018).

Control samples provide a crucial test of the likelihood of technical artifacts in the detection of taxa reported in microbiome surveys, and their use is now expected in microbiome studies [92,93]. Predefined "mock communities" are one such positive control for PCR amplification in 16S rRNA scans [93]. Our sequencing results for the staggered mock community show that different genera are detected at differing abundances. This may be due to biases in primers, GC content affecting gene amplification and sequencing as well as classification (Laursen et al., 2017; Pollock et al., 2018). In our MOC sample, no taxa were detected that had an expected relative abundance of 0.02%, while five of six taxa were detected at 0.22% and all taxa comprising at least 2% of the total sample were found (Table S4.1). Detection of low abundance bacteria (0.22% or below) was not related to GC content. Extrapolating from the mock community to our tick samples, we should have detected most of the taxa present in our tick samples if they comprised at least 0.22% of the 16S rRNA sequences in the total sample.

Acting as a complement to the MOC, extraction controls (SEC) and no-template controls (NTC) commonly identify a number of taxa that may be erroneously attributed to a biological sample (Lejal et al., 2020). The number and diversity of reads that we found in both the SEC and NTC indicates that much of the contamination in 16S rRNA sequencing occurred at the stage of DNA extraction. This is most clearly demonstrated by the presence of 18 reads attributed to *Rickettsia* in the SEC and no reads attributed to *Rickettsia* in the NTC. The positive (MOC) and negative (SEC, NTC) standardized controls that we included in our experimental design allowed us to determine where possible contamination arises as well as to pinpoint reagent-associated

contaminants for subtraction from biological samples. This should also facilitate comparisons to similar studies in other labs.

4.4.4 Variation in tick microbiomes as a tool for monitoring disease risk

The microbiome of ticks can have substantial geographic variation (Van Treuren et al., 2015). *Ixodes scapularis* apparently does not have established, reproducing populations in Alberta (Fitzgerald, 2012; Lindquist et al., 2016)[, and thus the ticks found in this region are assumed to have been derived via recent, continuing dispersal from populations of ticks that are established in Manitoba, eastern Canada, or adjacent areas of the United States south to Mexico (Lindquist et al., 2016). This raises the question of whether microbiome variation in *I. scapularis* could be used to identify the geographic origins of the *I. scapularis* ticks that are found in Alberta.

The presence of *I. scapularis* in Alberta is most likely due to tick attachments to migratory birds, and potentially also to deer and other mobile mammals, which allow dispersal well beyond the established range of the tick (Lindquist et al., 2016; Scott et al., 2018). Such dispersing ticks are expected to bring an assemblage of bacteria that resembles their original habitats, in addition to maternally transmitted bacteria associated with the tick species and bacteria picked up from exposure to earlier hosts. It may therefore be possible to explain the presence and origin of potential pathogens in a geographic region—such as Alberta—by building a database of the bacteria associated with ticks from different areas outside the region of interest. Such a catalog of bacteria would also advance our understanding of the spread of tick-borne pathogens, expand our understanding of tick biology and provide an opportunity to clarify hypotheses relating to microbial interactions of the tick microbiome. Our study provides a starting point for monitoring incursions of blacklegged ticks and their associated bacteria in concert with environmental changes.

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Table 4.1. Tick collection data and control samples. All ticks were engorged adult females. Control samples: MOC (mock community), SEC (sample extract control), NTC (no-template control). *Outside Alberta* travel applies when host animals were known to have travelled outside Alberta within two weeks of collection. *Bb qPCR* status is the assay for *Borrelia burgdorferi* OspA gene. 16S rRNA reads are the numbers of raw reads before filtering and taxonomic classification.

Tick ID	Date Collected	Outside Alberta	Tick species	Host animal	Bb qPCR	16S reads - 1st prep.	16S reads – 2nd prep.
Ix1	2016-05-06	Yes	*I. scapularis*	Dog	Neg.	91,317	-
Ix2	2016-05-02	No	*I. scapularis*	Cat	Neg.	71,209	-
Ix3	2016-05-13	Unkn.	*I. scapularis*	Cat	Neg.	134,766	-
Ix4	2016-05-17	No	*I. scapularis*	Dog	Neg.	109,864	-
Ix5	2016-05-18	No	*I. scapularis*	Cat	Neg.	145,698	-
Ix6	2016-05-19	No	*I. scapularis*	Dog	Neg.	81,204	-
Ix7	2016-05-20	No	*I. scapularis*	Dog	Neg.	62,927	-
Ix8	2016-05-25	No	*I. scapularis*	Dog	Neg.	104,139	-
Ix9	2016-05-26	Unkn.	*I. scapularis*	Dog	Neg.	110,758	-
Ix10	2016-01-17	Yes	*I. scapularis*	Dog	Pos.	118,427	289,781
Ix11	2016-04-11	No	*I. scapularis*	Dog	Pos.	130,881	339,121
Ix12	2016-04-18	No	*I. scapularis*	Dog	Pos.	100,333	321,740
Ix13	2016-04-27	No	*I. scapularis*	Dog	Pos.	104,545	-
Ix17	2016-05-17	No	*I. scapularis*	Dog	Neg.	174,362	-
Ix18	2016-05-08	No	*I. scapularis*	Cat	Pos.	120,645	-
Ix19	2016-05-13	No	*I. scapularis*	Dog	Pos.	123,266	-
Ix20	2016-05-31	No	*Ixodes* spp.	Dog	Pos.	126,995	347,373
Ix21	2016-04-28	No	*I. scapularis*	Dog	Pos.	98,205	-
Ix22	2016-05-20	No	*I. scapularis*	Dog	Pos.	127,989	-
Ix23	2016-05-04	Unkn.	*Ixodes* spp.	Dog	Pos.	115,995	295,902
MOC	n/a	n/a	n/a	n/a	n/a	95,023	-
SEC	n/a	n/a	n/a	n/a	n/a	71,920	-
NTC	n/a	n/a	n/a	n/a	n/a	44,610	-
Ticks $\bar{x}$						112,676.3	318,783.4
Ticks SE						5,741.7	11,411.4

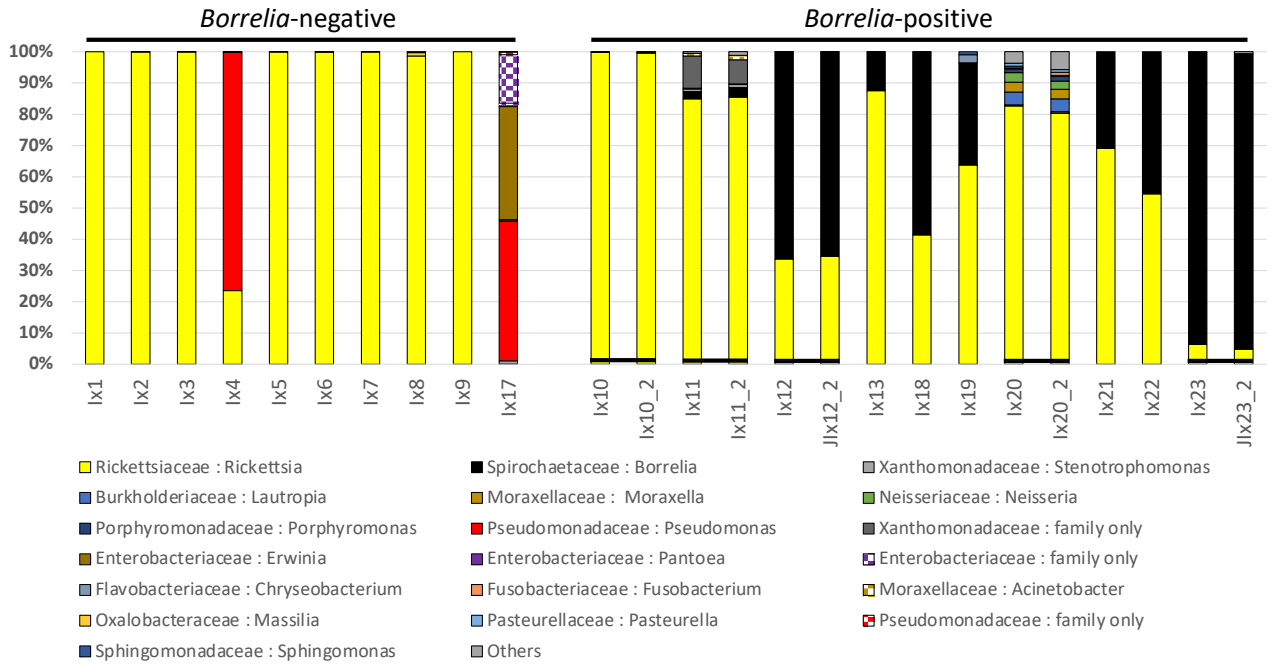

Figure 4.1. Proportions of bacterial reads detected for 10 *Borrelia*-negative and 10 *Borrelia*-positive ticks. Bacterial taxa found at greater than 1% of any single tick are distinguished; the remaining lower frequency taxa are grouped as 'Other'. Repeated sampling of 5 ticks is shown with black bars joining the same samples.

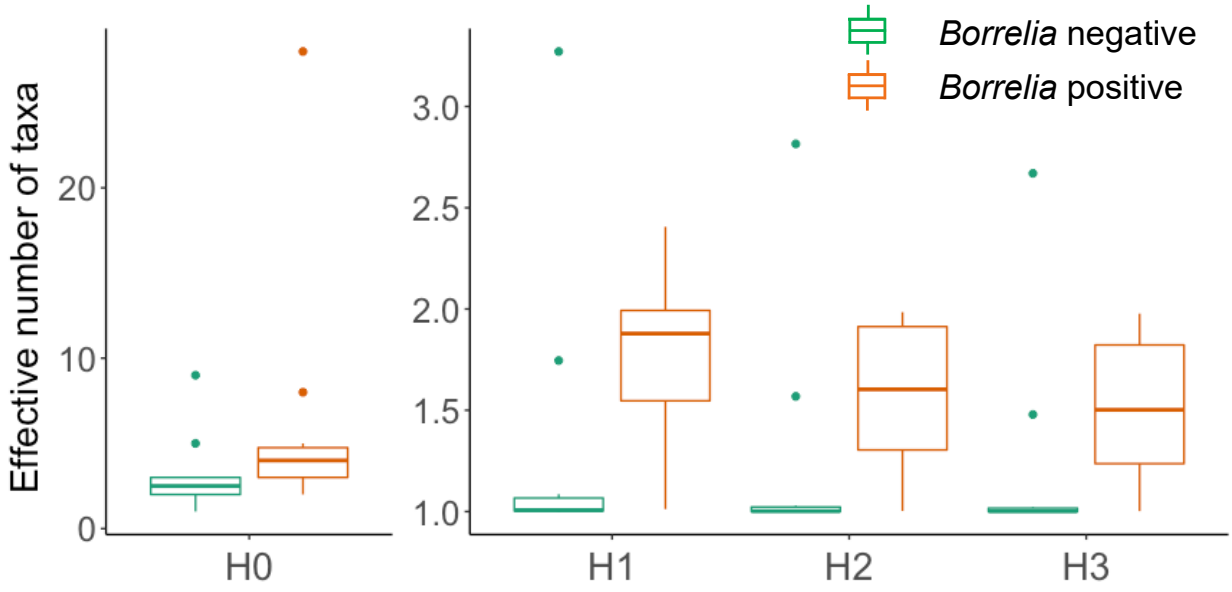

Figure 4.2. Diversity of bacteria in *Borrelia*-negative and *Borrelia*-positive ticks represented by Hill numbers: H0 (richness), H1 (exp. Shannon), H2 (inv. Simpson) and H3.

Table S4.1. Composition of expected and observed bacterial taxa in the mock community (MOC). GC content of the 20 bacterial strains is from NCBI genome (https://www. ncbi.nlm.nih.gov/genome/).

Family	bacterial 'strains' in MOC, input by BEI Resources	GC content	MOC abundance	genus-level taxa detected by 16S	genus reads	family reads	expected abundance	observed abundance
Staphylococcaceae	*Staphylococcus aureus*	33%	2.19%	*Staphylococcus*	5734	0	24.10%	15.4%
Staphylococcaceae	*Staphylococcus epidermidis*	32%	21.91%	*Staphylococcus* (incl. above)	-	-	-	-
Streptococcaceae	*Streptococcus agalactiae*	35%	2.19%	*Streptococcus*	9450	15	24.12%	25.4%
Streptococcaceae	*Streptococcus mutans*	37%	21.91%	*Streptococcus* (incl. above)	-	-	-	-
Streptococcaceae	*Streptococcus pneumoniae*	40%	0.02%	*Streptococcus* (incl. above)	-	-	-	-
Enterobacteriaceae	*Escherichia coli*	51%	21.91%	*Escherichia*/*Shigella* (MicroSeq db)	434	13249	21.91%	37.0%
				Escherichia (Greengenes db)	97	0	-	-
Rhodobacteraceae	*Rhodobacter sphaeroides*	69%	21.91%	*Rhodobacter*	6031	0	21.91%	16.2%
Bacillaceae	*Bacillus cereus*	35%	2.19%	*Bacillus*	404	0	2.19%	1.1%
Clostridiaceae	*Clostridium beijerinckii*	30%	2.19%	*Clostridium*	432	0	2.19%	1.2%
Pseudomonadaceae	*Pseudomonas aeruginosa*	66%	2.19%	*Pseudomonas*	532	624	2.19%	3.1%
Helicobacteraceae	*Helicobacter pylori*	39%	0.22%	*Helicobacter*	75	0	0.22%	0.2%
Lactobacillaceae	*Lactobacillus gasseri*	35%	0.22%	*Lactobacillus*	28	0	0.22%	0.1%
Moraxellaceae	*Acinetobacter baumannii*	39%	0.22%	*Acinetobacter*	46	0	0.22%	0.1%
Proprionibacteriaceae	*Propionibacterium acnes*	60%	0.22%	*Propionibacterium*	39	0	0.22%	0.1%
Neisseriaceae	*Neisseria meningitidis*	52%	0.22%	*Neisseria*	85	0	0.22%	0.2%
Listeriaceae	*Listeria monocytogenes*	38%	0.22%	*Listeria*	0	0	0.22%	0.0%
Actinomycetaceae	*Actinomyces odontolyticus*	65%	0.02%	*Actinomyces*	0	0	0.02%	0.0%
Bacteroidaceae	*Bacteroides vulgatus*	42%	0.02%	*Bacteroides*	0	0	0.02%	0.0%
Deinococcaceae	*Deinococcus radiodurans*	67%	0.02%	*Deinococcus*	0	0	0.02%	0.0%
Enterococcaceae	*Enterococcus faecalis*	37%	0.02%	*Enterococcus*	0	0	0.02%	0.0%
			100.00%	total reads	23387	13888	100.00%	100.0%

Table S4.2. Identity of bacterial taxa detected in the sham extraction (SEC) control. A total of 30,830 reads were identified, and are arranged by decreasing abundance.

Bacterial family	Genus	Count
Pseudomonadaceae	*Pseudomonas*	6142
Propionibacteriaceae	*Propionibacterium*	3219
Staphylococcaceae	*Staphylococcus*	1388
Comamonadaceae	(family level ID only)	1103
Prevotellaceae	*Prevotella*	983
Comamonadaceae	*Delftia*	982
Moraxellaceae	*Acinetobacter*	915
Corynebacteriaceae	*Corynebacterium*	905
Moraxellaceae	(family level ID only)	812
Bifidobacteriaceae	*Gardnerella*	784
Nakamurellaceae	*Nakamurella*	645
Streptococcaceae	*Streptococcus*	563
Sanguibacteraceae	*Sanguibacter*	450
Lachnospiraceae	(family level ID only)	445
Rhizobiaceae	(family level ID only)	443
Clostridiales Family XI. Incertae Sedis	*Finegoldia*	410
Enterobacteriaceae	(family level ID only)	368
Sphingomonadaceae	(family level ID only)	359
Nocardioidaceae	(family level ID only)	342
Rhodobacteraceae	*Paracoccus*	340
Peptoniphilaceae	*Anaerococcus*	322
Flavobacteriaceae	*Flavobacterium*	315
Bacillaceae	*Bacillus*	305
Coriobacteriaceae	*Slackia*	270
Micrococcaceae	*Kocuria*	266
Nostocaceae	(family level ID only)	260
Legionellaceae	*Legionella*	249
Brevibacteriaceae	*Brevibacterium*	239
Sphingomonadaceae	*Sphingomonas*	231
Bartonellaceae	*Bartonella*	220
Dermacoccaceae	(family level ID only)	219
Comamonadaceae	*Comamonas*	219
Legionellaceae	(family level ID only)	191
Clostridiales Family XI. Incertae Sedis	*Peptoniphilus*	190
Caulobacteraceae	(family level ID only)	186
Alicyclobacillaceae	(family level ID only)	178

Geodermatophilaceae	(family level ID only)	172
Carnobacteriaceae	*Alloiococcus*	162
Clostridiales Family XIII. Incertae Sedis	*Mogibacterium*	159
Thermaceae	(family level ID only)	155
Oxalobacteraceae	*Herbaspirillum*	155
Peptoniphilaceae	(family level ID only)	152
Carnobacteriaceae	*Dolosigranulum*	146
Rhizobiaceae	*Shinella*	145
Clostridiales Family XI. Incertae Sedis	*Anaerococcus*	142
Bradyrhizobiaceae	*Bradyrhizobium*	141
Micrococcaceae	*Nesterenkonia*	130
Moraxellaceae	*Enhydrobacter*	129
unclassified Bifidobacteriales	*Turicella*	126
Solirubrobacteraceae	(family level ID only)	125
Caulobacteraceae	*Brevundimonas*	125
Rubrobacteraceae	(family level ID only)	121
Sphingobacteriaceae	*Pedobacter*	119
Microbacteriaceae	*Frondihabitans*	118
Veillonellaceae	*Dialister*	118
Peptoniphilaceae	*Peptoniphilus*	104
Solirubrobacteraceae	*Solirubrobacter*	103
Acetobacteraceae	(family level ID only)	103
Micrococcaceae	(family level ID only)	99
Comamonadaceae	*Xenophilus*	95
Hyphomicrobiaceae	*Hyphomicrobium*	93
unclassified Burkholderiales	(family level ID only)	92
Caulobacteraceae	*Caulobacter*	91
Lachnospiraceae	*Lachnoclostridium*	90
Geodermatophilaceae	*Geodermatophilus*	87
Micrococcaceae	*Arthrobacter*	85
Clostridiales Family XIII. Incertae Sedis	(family level ID only)	82
Burkholderiaceae	*Burkholderia*	76
Neisseriaceae	*Conchiformibius*	76
Hyphomicrobiaceae	(family level ID only)	75
Enterobacteriaceae	*Klebsiella*	75
Rhodospirillaceae	(family level ID only)	69
Micrococcaceae	*Micrococcus*	63
Phyllobacteriaceae	*Mesorhizobium*	63
Veillonellaceae	*Veillonella*	60
Peptoniphilaceae	*Finegoldia*	55

Pasteurellaceae	*Haemophilus*	53
Comamonadaceae	*Schlegelella*	52
Sinobacteraceae	*Hydrocarboniphaga*	51
Pasteurellaceae	*Otariodibacter*	49
Iamiaceae	(family level ID only)	44
Actinomycetaceae	*Actinomyces*	44
Pseudomonadaceae	(family level ID only)	44
Methylococcaceae	(family level ID only)	43
Microbacteriaceae	*Candidatus Aquiluna*	41
Flavobacteriaceae	*Cloacibacterium*	40
Oxalobacteraceae	*Janthinobacterium*	39
Microbacteriaceae	(family level ID only)	36
Enterobacteriaceae	*Pantoea*	35
Thermoactinomycetaceae	(family level ID only)	34
Carnobacteriaceae	*Carnobacterium*	31
Nitrosomonadaceae	(family level ID only)	30
Lactobacillaceae	*Lactobacillus*	29
Staphylococcaceae	*Macrococcus*	25
Lachnospiraceae	*Sporobacterium*	25
Dermabacteraceae	(family level ID only)	23
Alcaligenaceae	*Achromobacter*	21
Comamonadaceae	*Acidovorax*	21
Listeriaceae	(family level ID only)	19
Rickettsiaceae	*Rickettsia*	18
Cardiobacteriaceae	*Cardiobacterium*	18
Ruminococcaceae	(family level ID only)	17
Enterobacteriaceae	*Erwinia*	17
Flavobacteriaceae	(family level ID only)	14
Thermoanaerobacteraceae	(family level ID only)	14
unclassified Burkholderiales	*Aquabacterium*	13
Aeromonadaceae	*Aeromonas*	13
Micrococcaceae	*Rothia*	12
Sphingomonadaceae	*Sphingopyxis*	11
Cytophagaceae	*Arcicella*	10
Veillonellaceae	(family level ID only)	10

Table S4.3. Identity of bacterial taxa detected in the no template PCR (NTC) control. A total of 19,515 reads were identified, and are arranged by decreasing abundance.

Bacterial family	Genus	Count
Pseudomonadaceae	*Pseudomonas*	3381
Bifidobacteriaceae	*Gardnerella*	2375
Clostridiales Family XI. Incertae Sedis	*Peptoniphilus*	1808
Propionibacteriaceae	*Propionibacterium*	1683
Comamonadaceae	(family level ID only)	1131
Corynebacteriaceae	*Corynebacterium*	969
Clostridiales Family XI. Incertae Sedis	*Finegoldia*	871
Comamonadaceae	*Delftia*	819
Clostridiales Family XI. Incertae Sedis	*Anaerococcus*	797
Prevotellaceae	*Prevotella*	774
Lachnospiraceae	*Sporobacterium*	586
Veillonellaceae	*Dialister*	435
Moraxellaceae	*Acinetobacter*	291
Brevibacteriaceae	*Brevibacterium*	230
Enterobacteriaceae	(family level ID only)	225
Methylobacteriaceae	*Methylobacterium*	223
Streptococcaceae	*Streptococcus*	186
Bradyrhizobiaceae	(slash calls)	161
Lachnospiraceae	(family level ID only)	159
Alicyclobacillaceae	(family level ID only)	152
Campylobacteraceae	*Campylobacter*	141
Sphingobacteriaceae	*Pedobacter*	138
Microbacteriaceae	*Microbacterium*	130
Peptoniphilaceae	*Peptoniphilus*	121
Peptoniphilaceae	*Finegoldia*	120
Phyllobacteriaceae	*Aquamicrobium*	117
Sphingomonadaceae	*Sphingomonas*	101
Micrococcaceae	*Kocuria*	97
Sphingomonadaceae	*Sphingobium*	97
Gaiellaceae	(family level ID only)	94
Enterobacteriaceae	*Erwinia*	91
Clostridiales Family XIII. Incertae Sedis	(family level ID only)	83
Desulfovibrionaceae	(family level ID only)	81
Dermabacteraceae	*Dermabacter*	79
Peptoniphilaceae	*Anaerococcus*	79
Carnobacteriaceae	*Dolosigranulum*	65

Flavobacteriaceae	*Bergeyella*	61
Flavobacteriaceae	*Cloacibacterium*	58
Ruminococcaceae	(family level ID only)	46
Rhodobacteraceae	(family level ID only)	46
Sphingomonadaceae	(family level ID only)	44
Caulobacteraceae	*Caulobacter*	42
Aerococcaceae	(family level ID only)	41
Comamonadaceae	*Diaphorobacter*	41
Flavobacteriaceae	*Flavobacterium*	39
Gloeobacteraceae	(family level ID only)	39
unclassified Burkholderiales		37
Peptococcaceae	(family level ID only)	34
Mycoplasmataceae	(family level ID only)	26
Flavobacteriaceae	*Capnocytophaga*	14
Staphylococcaceae	*Staphylococcus*	14
Clostridiaceae	(family level ID only)	11
Bacteriovoracaceae	(family level ID only)	11
Moraxellaceae	*Moraxella*	11
Veillonellaceae	(family level ID only)	10

Table S4.4. Bacterial taxa detected in the mock community (MOC), subdivided by the number of reads for each 16S rRNA variable region.

Bacterial taxa assigned	genus-level total reads	family-only total reads	V2 reads	V3 reads	V4 reads	V67 reads	V8 reads	V9 reads
Staphylococcus sp.	5734	0	452	2549	1102	1588	43	0
Streptococcus sp.	9450	-	77	3499	1992	1424	2458	0
Streptococcaceae, family-only	-	15	0	0	0	15	0	0
Escherichia/*Shigella* (MicroSeq)	434	-	0	0	0	125	309	0
Escherichia (Greengenes db)	97	0	35	0	0	52	10	0
Enterobacteriaceae, family-only	-	13249	360	2796	1137	1756	2305	4895
Rhodobacter	6031	0	270	1679	715	671	2696	0
Bacillus	404	0	17	191	76	120	0	0
Clostridium	432	0	0	199	51	182	0	0
Pseudomonas	532	-	105	304	123	0	0	0
Pseudomonadaceae, family-only	-	624	0	11	0	169	444	0
Helicobacter	75	0	15	44	0	16	0	0
Lactobacillus	28	0	0	17	11	0	0	0
Acinetobacter	46	0	0	23	0	0	0	23
Propionibacterium	39	0	0	0	0	10	29	0
Neisseria	85	0	0	23	30	0	32	0
Listeria	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0
Bacteroides	0	0	0	0	0	0	0	0
Deinococcus	0	0	0	0	0	0	0	0
Enterococcus	0	0	0	0	0	0	0	0
total reads	23387	13888	1331	11335	5237	6128	8326	4918

Table S4.5. GLM model outputs for associations among five bacterial diversity measures and five tick variables.

Pielou Evenness model: beta distribution	Estimate	Std. Error	P value
Borrelia status (positive)	2.68328	0.90212	**0.01**
Host (dog)	0.40683	1.18883	0.737
Julian date	0.01496	0.01851	0.432
Travel history (within Alberta)	-1.19393	0.94893	0.229
Travel history (unknown)	-1.75137	1.16679	0.156

Richness model (H0): neg. binomial (log link)	Estimate	Std. Error	P value
Borrelia status (positive)	0.939108	0.382552	**0.0141**
Host (dog)	0.232634	0.49309	0.6371
Julian date	0.007126	0.007307	0.3295
Travel history (within Alberta)	-0.559235	0.466006	0.2301
Travel history (unknown)	-0.387324	0.472275	0.4121

Exponential of Shannon model (H1): gamma distribution (log link)	Estimate	Std. Error	P value
Borrelia status (positive)	0.449218	0.210121	**0.0506**
Host (dog)	0.123831	0.251459	0.63
Julian date	0.004547	0.003779	0.2488
Travel history (within Alberta)	-0.16673	0.251704	0.5185
Travel history (unknown)	-0.244885	0.248095	0.3404

Inverse of Simpson model (H2): gamma distribution (log link)	Estimate	Std. Error	P value
Borrelia status (positive)	0.354409	0.190374	**0.0838**
Host (dog)	0.057966	0.227827	0.8029
Julian date	0.003367	0.003424	0.3422
Travel history (within Alberta)	-0.173633	0.228048	0.4591
Travel history (unknown)	-0.199355	0.224779	0.3901

Hill No. H3 model: gamma distr. (log link)	Estimate	Std. Error	P value
Borrelia status (positive)	0.331772	0.182679	**0.0908**
Host (dog)	0.04041	0.218618	0.856
Julian date	0.003147	0.003285	0.3544
Travel history (within Alberta)	-0.176795	0.21883	0.4327
Travel history (unknown)	-0.181482	0.215693	0.4143

Table S4.6. GLM outputs for tests of association within diversity measures for five B+ tick microbiomes sequenced at low read depth (mean = 64,377 after filtering) and higher read depth (mean = 216,558 after filtering). All comparisons use data rarefied to 33,000 reads.

	Estimate	Std. Error	P value
Pielou Evenness model: Beta distribution	0.01183	0.60975	0.9845
Richness model (H0): negative binomial (log link)	0.5566	0.608	0.36
Exponential of Shannon model (H1): negative binomial	0.05625	0.22812	0.8114
Inverse of Simpson model (H2): gamma distrib. (log link)	0.01212	0.14948	0.9374
Hill No. H3 model: gamma distribution (log link)	0.009793	0.142945	0.9471

Table S4.7. Number of reads assigned to *Borrelia* (A) and ranking (B), by 16S rRNA variable region with threshold number of 5 copies.

A. *Borrelia*	**Ix10**	**Ix10_2**	**Ix11**	**Ix11_2**	**Ix12**	**Ix12_2**	**Ix13**	**Ix15**	**Ix18**	**Ix19**	**Ix20**	**Ix20_2**	**Ix22**	**Ix23**	**Ix23_2**
V2	6	68	208	1903	3882	27463	842	677	6324	3667	0	18	7897	14366	58956
V3	15	7	332	875	8815	26223	1901	1491	8458	3696	0	6	3180	13448	44792
V4	15	41	443	1701	9108	29661	1839	1775	14614	8657	0	20	17098	24040	51788
V67	5	21	655	2572	12212	42275	2787	1368	10636	6463	0	6	3934	14091	48111
V8	0	0	0	22	1934	19015	31	34	431	122	0	0	2648	856	2137

B. *Borrelia* V-region ranking (ties ranked equally)															**sum**
V2 rank	3	1	4	2	4	3	4	4	4	4	2	2	2	1	40
V3 rank	1	4	3	4	3	4	2	2	3	3	3	4	4	4	44
V4 rank	1	2	2	3	2	2	3	1	1	1	1	1	1	2	23
V67 rank	4	3	1	1	1	1	1	3	2	2	3	3	3	3	31
V8 rank	5	5	5	5	5	5	5	5	5	5	5	5	5	5	70

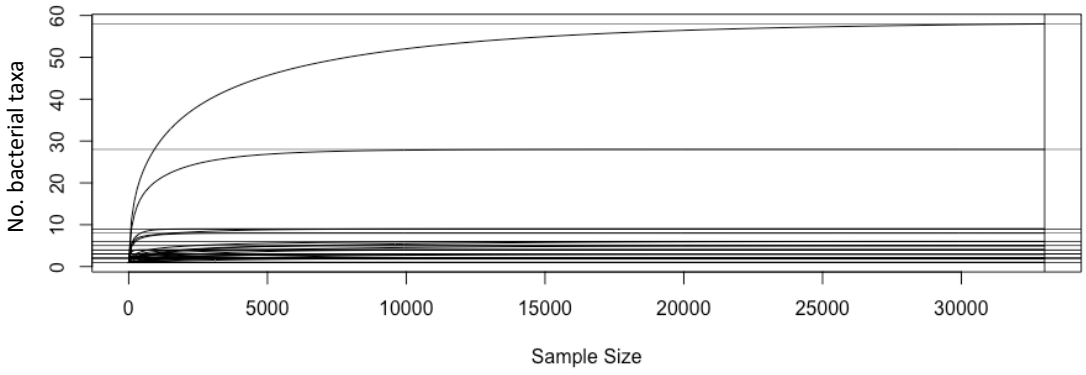

Figure S4.1. Rarefaction curves for the bacterial microbiome of 20 *Ixodes* ticks. Vertical line is at 33000 reads.

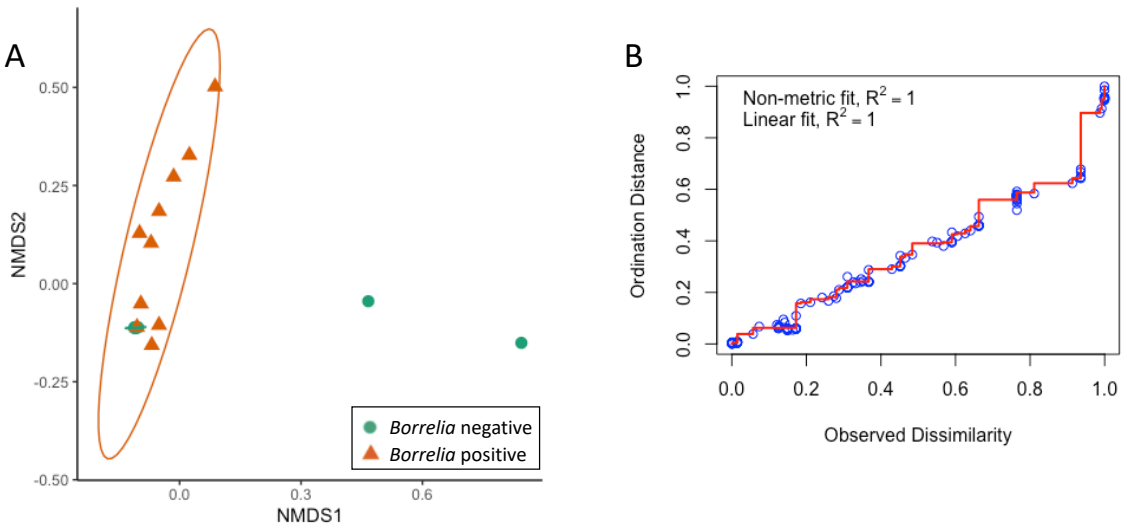

Figure S4.2. NMDS ordination (A) and 2-dimensional stress plot (B) of Bray Curtis dissimilarities among microbiome communities of *Borrelia*-negative and *Borrelia*-positive ticks. Ellipse in A is a multivariate t- distribution.

CHAPTER 5

Conclusions

5.1 Research summary

The primary aim of this thesis has been to examine the reproducibility and breadth of use of 16S rRNA amplicon sequences for identifying and comparing bacterial assemblages associated with ticks. Accurate documentation of the presence of bacteria in ticks is important for surveillance of human and animal pathogens as well as in expanding our understanding of tick microbiomes, including the bacteria that affect the health of ticks. I found that surveys of bacteria associated with ticks are influenced by the choice of primers and variable region of the 16S rRNA marker gene, and these choices influence the diversity estimates that are obtained. I also found that the major bacterial component of a tick microbiome can vary within tick species and life stage, but it was usually the known endosymbiont for that tick species (Ahantarig et al. 2013, Bonnet et al. 2017, Novakova et al. 2018, Papa et al. 2017). In *Ixodes scapularis*, a *Rickettsia* tended to dominate, whereas a *Francisella*-like endosymbiont was the most frequent bacterial taxon for *Ixodes angustus* and *Dermacentor albipictus*. Previous knowledge of the bacteria associated with ticks in Canada has usually been based on qPCR assays of specific pathogens, whereas my microbiome surveys with the 16S rRNA marker gene have extended our knowledge of the diversity and identity of the bacterial community associated with ticks. For example, *Ixodes* ticks containing *Borrelia* also had a greater diversity of other bacteria. Such 16S rRNA surveys do not allow species-level identifications of pathogens, but they can support investigation of the ecological context of pathogen presence, as well as help to guide subsequent targeting of pathogens.

The first goal of my thesis was to understand the limitations of different 16S rRNA variable regions for detection of diverse bacteria. It is essential to maximize the information content of sequencing effort while minimizing cost in surveillance programs. If a single 16S rRNA region were sufficient, we could decrease cost with minimal loss of information. In Chapter 2, I show that bacterial taxa are differentially detected by separate 16S rRNA primer sets and 16S rRNA variable regions, and a single region is not enough to comprehensively survey the bacteria associated with ticks. Variation among studies in the choice of primers for estimating

tick microbiomes therefore contributes to observed differences in OTU richness, as described in Chapter 1. Such differences in detection probability, particularly of low-abundance bacteria, should be considered regardless of whether a single 16S rRNA variable region is used or they are combined. For example, if the common tick endosymbiont is preferentially amplified in one selected region, this could overwhelm abundance measures of the endosymbiont even more heavily when the data for multiple regions is combined. However, using primers for multiple regions of 16S rRNA also increases the probability of detection of rare bacteria in at least one of the regions, which is the most critical goal of surveillance studies for pathogens in ticks.

This work also led me to ask whether rare sequences in observed bacterial assemblages are real, and therefore potentially meaningful, or are methodological artifacts. Rare sequences may show random associations, but low frequency taxa can still be important members of a microbiome, with the apparent randomness of their associations being due to the incomplete sampling expected of any rare member of a population. Since detection of bacteria in ticks is also tissue dependent (Pollet et al., 2020), rarity in one tissue does not necessarily correspond to rarity in the entire tick. Rarity may also be determined by the timing of when the sample was selected. For example, titres of *Rickettsia rickettsii* are known to oscillate in the salivary gland of ticks over time, with no clear association with titres in the haemolymph (Levin et al. 2020). Furthermore, the detection limit for qPCR of *R. rickettsii* in skin biopsies was expected to be 160-180 DNA copies per skin biopsy sample (Levin et al., 2020) which was not sufficient to definitively declare that a biopsy sample was negative, even when the sample did not return a positive qPCR result. Even though *R. rickettsia* DNA was not always detected in the skin biopsies investigated by Levine et al (2020), clinically significant disease was noted in the majority of cases, demonstrating that tick-borne pathogens may be found at a such a low level that they are undetectable in a sample but may still be sufficiently frequent to cause disease.

Ecological and life history variables potentially introduce a substantial amount of noise into an already noisy system, making it difficult to detect true biological signal stemming from specific factors (Hornung et al., 2019; Pollock et al., 2018). Thus, variation in detection of bacteria in ticks may be due to the nature and circumstances of the initial selection of samples that are used. In Chapter 2, I chose samples to represent a broad cross section of geographic regions. The results of this Canada-wide sampling led me to question the influence of geography on the variability of microbiome composition, a factor also described by Van Treuren et al.

(2015). At a local geographic scale, host blood meal and tick developmental stages are additional factors known be a source of variation in tick microbiome composition (Swei and Kwan, 2017) and this is also true for tick developmental stages (Zolnik et al., 2016). Therefore, in the next study (Chapter 3), I controlled for these factors by keeping location and host constant, and explicitly comparing tick life stages. I also built on lessons learned in Chapter 2, by limiting the study in Chapter 3 to a single 16S rRNA region, V4, that simplified comparisons within the study as well as with published data from other studies.

My study in Chapter 3 was motivated by the fact that ticks are associated with hundreds of kinds of bacteria (Greay et al., 2018), but some researchers (Couper et al., 2019; Ross et al., 2018; Tokarz et al., 2019) have recently argued that this diversity is an artefact due to overamplification of surface bacteria or contaminants from the DNA extraction process. I examined variability in the bacterial communities associated with the one-host winter tick, *Dermacentor albipictus,* on elk, *Cervus canadensis*, in the Ya Ha Tinda area of Alberta. By restricting my focus to one tick species with a simplified life cycle on a single host species and sampling from a single location, I expected to find lower diversity than for three-host ticks sampled from more than one population. If overall bacterial diversity is due primarily to external contaminants, then lower diversity should be found in a simplified system than has been reported for ticks exposed to more diverse conditions. I examined bacterial diversity on ticks feeding on elk of differing ages, sexes and migratory status, as well as on ticks of differing life stages and sexes. I found extensive variability in bacterial diversity, with males and nymphs having more diversity than females, but I found no significant differences among ticks on different elk groups (migratory status or elk sex). Using Ion Torrent PGM technology, bacterial diversity measures for a low-variability tick system revealed remarkably high microbial diversity among individual ticks that was comparable to the diversity found in three-host ticks (Table 1.1). These diversity measures also demonstrated that single ticks, even when removed from the same host at the same time, can show different assemblages of pathogens. Therefore, a single tick sample is insufficient to convincingly state that all pathogens in a given location have been sampled.

My third major project (Chapter 4) applied taxonomic profiling of multiple regions of 16S rRNA to evaluate whether this approach gave results that were consistent with qPCR tests for *Borrelia*. We used the same methods as in Chapter 2, to maximize the probability of detection of rare bacteria by amplification with different primer sets. In this case, differential

amplification was seen as a strength since increased breadth of sampling was the goal. We used only female ticks, since this was the most frequently collected life stage, and relied on samples that were previously tested as part of a regional tick surveillance program that was designed to monitor the presence of Lyme disease in *Ixodes* ticks in Alberta (Government of Alberta, 2015). Using ticks submitted by the public to this surveillance program, we demonstrated the utility of 16S scans for detection of *Borrelia* that are potentially vectored by the ticks. Such 16S rRNA scans also provide a means to not only monitor *Borrelia* but other potential tick-borne pathogens as they are introduced into Alberta.

Each *Ixodes* tick submitted to the Alberta surveillance program that I used in Chapter 4 was removed from either a cat or a dog, with the underlying assumption that the tick originated from outside Alberta (if the host had recent travel history) or from a source population that was recently founded from outside the province. This assumption relies on a particular definition of what constitutes an 'established population'. For *Ixodes scapularis* in Canada, all three life stages (larva, nymph, adult) must be found in a given locality in at least two consecutive years before this designation is applied (Paulson, 1991). Since the Alberta program depends on passive surveillance of ticks submitted by veterinarians, only ticks large enough to be detected by a pet owner or vet and then removed and submitted to the program are counted. The much smaller larval and nymphal stages may be present in an area but are likely to remain undetected by passive surveillance. More recent work recognises the limitations of this approach and recommends a less restricted definition of endemic areas (Clow et al., 2019).

My survey of the microbiome of *Ixodes* ticks from the Alberta surveillance program showed diverse and varied assemblages that were consistent with the assumption of transport to Alberta from varied geographic regions (Chapter 4). However, with more precise species and strain-level identification and cataloging of the pathogens that these ticks are carrying, it may become possible to determine the geographic source of ticks as well as whether an individual tick is likely to be from a local population in Alberta or came from outside Alberta. If the established population versus adventitious status of *Borrelia*-vectoring *Ixodes* in Alberta can be determined more definitively, this could in turn support more cost-effective diagnoses. In addition, documentation of tick-borne diseases that co-infect as multiple pathogens from a single tick could provide important information to clinicians, since co-infected patients may be sicker and targeting more than one bacterial pathogen simultaneously or in sequence may influence the

choice of antimicrobials (Cameron et al., 2014). Consequently, microbiome surveys of ticks in Alberta would have the potential to reduce suffering by patients as well as burden on our healthcare system.

However, I also found that the 16S rRNA amplicon sequencing approach to microbiome analysis has limitations. These were the limited resolution of bacterial species using short sequences, and the lower sensitivity of endpoint PCR compared to qPCR for detection of targeted bacterial taxa. Increased resolution of bacterial species is likely using full length amplicon sequencing of the 16S rRNA gene and lower sensitivity of 16S rRNA amplicon sequencing could be mitigated by using a blocking primer for the dominant bacterial endosymbiont. Further refinements and improvements in sequencing technologies are discussed below.

5.2 Future Use of DNA Technologies

Sequencing technology continues to evolve and includes improvements in read length, especially for long-read sequencers such as PacBio's SMRT technology (Ardui et al., 2018) and Oxford nanopore technologies (Bowden et al., 2019). However these longer read technologies require different extraction techniques to retain the integrity of longer DNA (Mayjonade et al., 2016). For example, bead beating, currently recommended for tick extractions due to the highly sclerotized exoskeleton (Ammazzalorso et al., 2015) would shear longer strands of DNA. Furthermore, other logistical, technical and computational challenges remain, although the PromethION platform and improved base calling and read mapping algorithms are promising (Bowden et al., 2019). Long read technology should allow phasing of the different variable regions of the 16S rRNA gene, leading to better understanding of the 16S rRNA taxonomic profiles of bacteria found in ticks, but current constraints include labour intensive DNA extraction and increased cost for producing the long read sequences as well as data storage (Amarasinghe et al., 2020).

Whole genome sequencing (WGS), which does not rely on an initial PCR for a marker gene, is likely to show its strengths using long read technologies. Current limitations of WGS are found in assembling short reads, especially in regions with repetitive elements (Gao and Smith, 2017). Assembled metagenomes contain sequence data for multiple taxa from all domains of life, many of which may be understudied and therefore contain genes that are not found in reference

databases. Assembly and binning may also create artificial hybrid genomes (Dong and Strous, 2019) equivalent to chimeras in PCR reactions. In contrast to 16S rRNA amplicon sequencing, where taxonomic profiling is then used to estimate the functional gene families contributing to a metagenome (e.g. PICRUST Langille et al., 2013), annotation of the genes found in a complex metagenomic sample is focussed on functional potential of the entire set of genes rather than taxonomic profiling of these same genes. This collection of sequences will include metagenome-assembled-genomes (MAGS) of varying quality, as well as unbinned metagenomic contigs and potentially millions of unassembled reads (Dong and Strous, 2019). Even using software pipelines such as MetaErg (Dong and Strous, 2019), with state-of-the-art algorithms, taxonomic classification of genes by similarity remains misleading because of uneven representation of taxa in databases with biases towards highly sampled taxa (Kunin et al., 2008).

When applied to tick microbiomes, WGS suffers from the fact that the largest amount of DNA being sequenced may be from the tick itself or the blood meal if the tick has been fed. The proportions of DNA relating to the tick versus host blood meal versus microbiome components remains to be determined but even unfed ticks are likely to have >90% tick DNA relative to DNA contributed by bacteria or other components of the microbiome. Pereira-Marques et al. (2019) showed that both high proportions of host DNA (>90%) and low sequencing depth (<5 million reads) interferes with sensitivity of microbiome profiling in WGS datasets. Unless tick DNA can be depleted prior to sequencing, amplicon sequencing remains the technique of choice.

Highly abundant sequences can interfere with amplicon based or WGS detection of rarer members of a bacterial assemblage (Gonzalez et al., 2012). Blocking primers can be designed to prevent overamplification of these abundant sequences by inhibiting annealing or by inhibiting extension between the two primers (Boessenkool et al., 2012). Gofton et al. (2015) designed an endosymbiont annealing blocking primer for Australian and European *Ixodes* ticks which by blocking the major endosymbiont significantly increased detection of bacterial diversity for these samples. A similar blocking primer could be designed for the endosymbionts of North American ticks (e.g. *Francisella*-like endosymbiont or *Rickettsia buchneri*) to permit greater detection of rare members of the bacterial assemblages associated with ticks. Using an endosymbiont blocker will also allow more effective detection of pathogens that may be much less common than the endosymbiont.

An alternative approach to enriching sequences of interest is to use biotinylated target enrichment. Campana et al. (2016) have developed target enrichment probes for identifying tick species using the cytochrome C oxidase subunit 1 (CO1) barcode gene simultaneous with blood meal and pathogens. Pathogens in the study of Campana et al. (2016) were limited to 12 pathogens and included several vector species and multiple host blood targets. A shortcoming of this approach was that blood from less commonly studied host animals was misclassified as the more commonly studied equivalent (e.g. wild canids were misclassified as domestic dogs). However, this approach of simultaneous detection of tick species, host and pathogen promises to be of interest for expanding our understanding of host-vector associations. Furthermore, it relies on an established baseline for which probes can be developed.

Targeted qPCR for multiple pathogens can also identify known pathogens. Using this approach, bacteria as well as viruses can be targeted. However most viruses in ticks are RNA viruses with the exception of *Asfivirus* (Park et al., 2020), so extraction of RNA in addition to DNA is required to detect these viruses. Another approach is to use bulk RNA sequencing and subsequent identification of sequences by similarity searches for viral sequences (Gondard et al., 2020). Extensive diversity of viruses in ticks has recently been described (Gondard et al., 2020; Harvey et al., 2019; Pettersson et al., 2017). In any case, it is clear that continued updating of testing methods, through application of the most recently available technologies, is essential to timely progress in pre-emptively understanding any changes in the presence of microbes in ticks as well as their interactions and environmental context.

5.3 Historical perspective on tickborne illnesses and social context

Tick-borne diseases have impacted human history for millennia, with the earliest records dating back to the ancient Egyptians in 1500 BC (Arthur, 1965). Since the late 19th century, the inevitability of the emergence of new vectored diseases and the importance of surveillance for these diseases has been foundational to the study of medical and veterinary entomology (Fontenille et al., 2017). Since 1932, when an outbreak of epidemic tick-borne relapsing fever was declared in British Columbia (Palmer and Crawford, 1933), and 1935, when the first case of Rocky Mountain Spotted Fever was reported in Alberta (Bow and Brown, 1945), the number of tick-borne diseases endemic to Canada has expanded to 7 bacterial diseases, 1 piroplasm and 3 viruses (Bouchard et al., 2019).

The sociopolitical implications of tick-borne diseases in North America date at least to 1844, when the introduction of relapsing fever to North America was blamed on Irish immigrants (Palmer and Crawford, 1933). However, the distinction between louse vectored *Borrelia recurrentis* and tick vectored *Borrelia hermsii* was not made in the 1933 report of Palmer and Crawford and these Irish immigrants were more likely to have suffered from *B. recurrentis* (Dworkin et al., 2008). Although this example shows that disinformation about tick-borne disease has existed for a long time, Lyme disease provides a more recent series of examples of how science and politics have been intertwined for decades, a pattern that continues into the internet era (Government of Canada, 2017; Levesque and Klohn, 2019; Sperling et al., 2012; Sperling and Sperling, 2009).

Confusion about Lyme disease risk is demonstrated in Alberta by reports of tick infection rates based on aggregate totals that include untested ticks. Specifically, the Alberta tick surveillance program only tests tick species that are considered to competently vector *Borrelia*, which in Alberta includes only *Ixodes scapularis* and *I. pacificus* (Government of Alberta, 2020). *Dermacentor* species make up the great majority (about 90%) of ticks submitted to the surveillance program but these ticks are not tested because they have not been shown to vector Lyme disease (Government of Alberta, 2020). However, some media statements report the rate at which ticks are infected by *Borrelia* relative to the total number of ticks submitted, rather than the number of ticks that are actually tested (e.g. CBC news, 2017). For example, in 2017 in Alberta 54 *Ixodes* ticks were infected out of 297 ticks that were tested, but a total of 1941 ticks were submitted to the program, of which 1644 were not tested because they were not *I. scapularis* or *I. pacificus*. Consequently, the frequency of *Borrelia*-infected ticks was reported as ten times lower than the rate for the known vectors of *Borrelia* (less than 2% versus the documented 18% for *Ixodes* in 2017). Although it is understandable that the lower number would be reported if infection rates were considered to be equal across all ticks, it is actually quite easy to distinguish *Ixodes* and *Dermacentor* ticks based on a photo (NCCEH, 2020). A person with an embedded tick can readily be given a much more informative estimate of the probability that the tick was infected, since identification of the genus of tick places the probability of infection as either 18% or 0%, not <2%. The most responsible course of action for a medical practitioner would therefore be to report infection rates in the context of tick genus rather than within an uninformative or misleading aggregate of all ticks (CBC news, 2017).

The increased diversity of bacteria that I found in *Borrelia*-positive ticks raises an additional issue and opportunity. Although my microbiome surveys of 20 *Ixodes* ticks in Alberta did not detect any co-infections by other known pathogens, my surveys of the microbiome of *Dermacentor* ticks on elk in Alberta showed at least 15 of 43 ticks with *Ehrlichia*, a known pathogen of both animals and humans (Dumler 2015). Thus 16S rRNA amplicon sequencing can detect other pathogens, and the simplest explanation for the lack of other pathogens in the *Ixodes* that I surveyed is that the rates of co-infection were low in these samples. This is consistent with findings in other areas like Manitoba, where *Anaplasma* has been found in 6% of *Ixodes* (Krakowetz et al., 2014) while 17-41% contain *Borrelia* (Government of Manitoba, 2018). The ready availability of methods for efficiently surveying bacterial communities raises the opportunity to focus efforts on multiple tick-borne pathogens at the same time. This would give an early warning system that could be used to alert public health officials to the possibility of exposure to pathogens that might otherwise be overlooked. Since diagnosis of tick-borne illnesses in areas with low physician familiarity is complicated by inadequate serological tests (Lloyd and Hawkins, 2018) there is a greater risk of a missed diagnosis in areas with sporadic tick encounters. Providing knowledge of the identity of bacteria in Canadian ticks allows targeted testing for pathogens in humans, their companion animals or livestock when exposed to tick bites, and should also improve clinical diagnoses. It additionally provides the opportunity to contribute to understanding the complexity of symptoms suffered by patients with illnesses like Lyme disease that have extremely varied symptoms (Cameron et al., 2014).

The research completed as part of this PhD thesis has also had an important outreach component. By working with the public, I have established a better bridge between evidence-based researchers and the people who are most at risk of exposure. Knowledge of ticks found in Canada, as well as their associated microbes, supports better diagnostics for tick borne diseases among pets, wildlife and the people alike. An action item arising from this work is to persuade the provincial government to reinstate the recently-paused Alberta tick surveillance program using microbiome surveillance in addition to qPCR, and potentially also with the added value of including surveillance for viruses as well as bacteria.

The importance of understanding tick-borne illness in Alberta has been acknowledged since 1936, when J.D. Gregson defended his M.Sc. thesis on tick feeding and tick paralysis at the University of Alberta. The 1938-1943 Rocky Mountain Spotted Fever survey led by Jack Brown

(M.Sc. 1942) was initiated due to further concerns over human disease (Humphreys and Campbell, 1947). Now almost a century later, we have finally arrived at an opportunity to deploy a cost-effective early warning system for tick-borne pathogens that would position Alberta to be a leader in providing strategic advice to public health officials. It remains to be seen whether we step up to the plate.

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