MICROBIAL ECOLOGY

OF

MOSQUITOS AND TICKS

by

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TABLE OF CONTENTS

1. BACKGROUND 1

The Host Associated Microbiome 1
Next Generation Sequencing 2
Microbial Community of Arthropods 2
Intercellular Endosymbiosis 3
Gut Symbiosis 3
Tick Background 5
Tick Ecology 5
Tick-Borne Disease 6
Tick-Microbe Symbiosis 7

2. MICROBIAL DIVERSITY OF CULICIDAE 10

Abstract 10
Introduction 10
Materials and Methods 13
Mosquito Collection 13
DNA Extraction and 16S rRNA Gene Sequencing 16
Data Processing 16
Results 18
Validation of Sequence Data 30
Discussion 30
Conclusion 32

3. MICROBIAL ECOLOGY OF NEOTROPICAL TICKS 33

Introduction 33
Methods 35
Tick Collection 35
16S rRNA Gene Amplification and Sequencing 38
Data Processing 38
Statistical Analyses 39
Results 40
Discussion 46
CLE, *A. platys*, and FLE 46
Interactions between dominant microbiome members 48
Conclusion 49

REFERENCES CITED 50

LIST OF TABLES

Table Page

2.1 West Nile virus risk score 26

3.1 BLAST results of the most common OTUs..41

3.2 Iteration between dominant OTUs in *R. sanguineus* 43

LIST OF FIGURES

Figure Page

2.1 Trap locations....................15

2.2 Phylum level classification....................19

2.3 Alpha diversity by species....................21

2.4 Ordination by taxonomic classification....................24

2.5 Ordination by West Nile virus risk....................27

2.6 Cluster analysis....................29

3.1 Tick body measurements....................37

3.2 Taxonomic makeup of microbiome....................41

3.3 Negative interactions in the microbiome....................43

3.4 Beta-diversity of *R. sanguineus* microbiome....................45

ABSTRACT

Host evolutionary history has been shown to select for distinct host associated microbial communities over large evolutionary time scales. The microbiomes of disease vector have been shown to alter the capacity of their host to vector pathogens. Much remains to be understood about how the microbiome of mosquitos and ticks assemble *in situ*. We conducted a large-scale investigation of microbiome composition between mosquito species as well as a second investigation of microbiomes of brown dog ticks collected in Iquitos, Peru. Intraspecific and interspecific bacterial community diversity was compared across 26 species of mosquitoes collected in Montana. Previous studies of lab reared mosquitoes report greater variation in microbial communities between species than within. Using 16S rRNA sequencing we observed a large amount of intraspecific variation in microbiomes, as well as different species hosting very similar microbiomes. The tick microbiome was found to be dominated by a few select community members that were seen at an extremely high abundance and resembled intracellular tick-borne pathogens. It is common for ticks to host endosymbionts that closely to human pathogens but are not pathogenic themselves. Negative interactions were seen between the most abundant organism observed in the ticks.

BACKGROUND

The Host Associated Microbiome

The ecology of Earth is dependent on the actions of microorganisms. For three billion years all life on the planet was microbial. In that time microbes have adapted to the most challenging and demanding environments the earth has to offer in terms of temperature, pressure, pH, and salinity. They not only survive but thrive in environments ranging from acidic hot springs, to the bottom of the Marianas trench, and to the stratosphere. Virtually no environment on earth is devoid of microbial life. Therefore, it is no surprise that many microbes have the evolved the capability to colonize multicellular organisms.

When a microorganism colonizes a multicellular organism, it is said to be a member of the host-associated microbiome. Many different bacteria, eukaryotes, archaea and viruses have evolved to exploit niches in host-associated microbiome. From plants, to arthropods, to mammals there is not a single organism that does not play host to a microbiome in some fashion[1]. The microbiome can aid in host nutrient acquisition, immune system regulation and can even influence host behavior and development[1]. The microbiome can carry out metabolic reactions critical for host survival[2]. The microbiome can affect the host's susceptibility to disease[3]. Microbiomes have even been shown to modulate host hormone activities[4].

Next Generation Sequencing

High throughput DNA sequencing has fundamentally changed biology, through expanding understanding of global microbial diversity. In just over a decade, by being able to look beyond cultivable organisms, microbial diversity estimates for all of earth have increased from millions of species[5] to possibly trillions[6].

Now it possible examine the relationship between host and the entire microbiome rather than just the microbes that can be grown in a lab. Extensive research is taking place currently to characterize the members and function of the human microbiome. The Human Microbiome took five years from its commencement in 2007 to publish its first initial findings using 16S ribosomal sequencing[7,8]. Since then, it has become clear the human microbiome is both a highly diverse and very complex system[9]. Now microbiomes can be analyzed in weeks, rather than years by using streamlined techniques and data processing pipelines that have been developed in the last few years. This has allowed for the publication of hundreds of non-human microbiome studies[1].

The Microbiome of Arthropods

Arthropods are the most diverse and abundant groups of animals on earth[10]. Many Arthropods have evolved an intimate relationship with their microbiome. These symbionts provide a variety of services to the host, and are often important for digestion and metabolism. If they are not vertically transmitted from mother to egg, they establish themselves in the gut through feeding[11].

Intercellular Endosymbiosis

The majority of the arthropod's microbiome members are commensals or parasites, however some have been found to be beneficial for the host[11]. Specific obligate endosymbionts are needed for host survival. One of the best examples of an obligate symbiosis is between Pea aphids and the bacteria *Buchnera.* The aphid contains large specialized bacteriocyte cells that house the bacteria. These bacteria have lost many of the genes essential to survive outside of the bacteriocyte. In exchange for the aphid providing the bacteriocyte, *Buchnera* synthesizes essential amino acids. Genomic analysis has shown there are no redundant amino acid synthesis pathways between the two symbionts[12], and neither is capable of living independently[13].

Obligate endosymbionts are by no means confined to only the pea aphid. Similar cases are seen cicadas, cockroaches, mealybugs, leafhoppers, and whiteflies. Interestingly the genomes of insect endosymbionts share many characteristics with mitochondria in terms of size and function, therefore blurring the line between endosymbiont and organelle[14]. Obligate endosymbionts are particularly common in blood-sucking insects, such as tsetse flies and body lice. It is thought blood-sucking insects are dependent on endosymbionts to synthesize vitamins not present in vertebrate blood[15,16].

Gut Symbiosis

Maintaining mutualistic relationship through time is challenging. The most stable mode for persistence through host generations is vertical transition. However, some insects have also evolved a dependence on horizontally transferred gut microbiota to

enhance their nutrient poor diets. It is not surprising that this is seen in predominantly social insect where multiple generations are in physical contact with each other.

Herbivorous turtle ants are reliant on specialized bacteria to enrich their nutrient poor diet. A survey across ant species found the gut of herbivorous ants to be colonized by nitrogen fixing *Rhizobiales* bacteria that were absent from the gut of predaceous relatives. Even more fascinating is phylogenetic analysis indicates this mutualistic relationship between nitrogen fixing bacteria and herbivorous ants has likely evolved on five separate occasions, indicating the importance of gut microbiota in defining the host niche[17].

The gut is both dramatically richer and more dynamic than the intracellular environment. Gut residents are subject to a constant influx of ingested food matter and transient microbes. A recent investigation in the mechanism behind *Rhizobiales* population maintenance in the Sonoran Desert turtle ant, *Cephalotes rohweri,* found the ant employs a 0.2 µm micro-pore filter[18]. The filter functions to protect the gut inhabitants from large partials and bacteria but still allows for the passage of dissolved nutrients, a literal ecological filter. In order to inoculate their gut, freshly emerged sterile worker ants almost immediately begin to consume the rectal fluid of nest mates[18,19]. This behavior maybe particularly important in insects that employ a physical filter, as the filter selects for small microbes and by bombarding it with symbionts for another host greatly increases the likelihood the microbiome of the new host is founded by the same symbionts as the rest of the colony.

Tick Background

Tick Ecology

There is global increase in instances of tick-borne disease[20]. Ticks are obligatory, hematophagous (i.e. blood feeding) ectoparasites. They can be both vectors and reservoirs of a diverse group of pathogenic fungi, protozoa, viruses, and bacteria. Ticks are second only to mosquitoes with respect to their impact on global human health[21]. For example, Lyme disease, caused by *Borrelia burgdorferi,* began emerging 35 years ago[22] has now become the most prevalent vector-borne disease in the Northern Hemisphere[23]. Over 36,000 cases of probable and confirmed Lyme disease have been reported in the United States in 2013, but this is thought to be only a fraction of the actual number which is estimated to be closer to 300,000 cases[24].

Similarly, new over ten new subspecies of *Rickettsia rickettsii,* the bacterium responsible for Rocky mountain spotted fever also known as tick-borne rickettsioses, have emerged[25] within the last couple decades. Climate, habitat availability, and host densities all affect the distribution and density of tick populations[26]. As the climate continues to change, interactions between humans, livestock, and ticks is predicted to increase[27,28]. As the climate warms, there are more degree days throughout the year thus increasing the amount of time that ticks are active in temperate climates. Also, colder regions that were not as conducive to ticks are warming. As a result, tick ranges have expanded both geographically and temporally throughout the year[28,29].

Tick host seeking behavior is affected by changes in climate. For example, the brown dog tick, *Rhipicephalus sanguineus*, traditionally was thought to have a low

preference for human hosts, requiring canine hosts to maintain its population. This preference has been especially well documented in temperate climates[30,31]. Non-canine hosts were thought to be fed on only when in close contact with canines. However, *R. sanguineus* ticks kept at warmer temperatures readily switched to non-canine hosts, suggesting that climate change may have a significant impact on host preference[30].

Tick-Borne Disease

Ticks can acquire human pathogens in three ways. They can be transmitted vertically between generations, or horizontally acquired from the environment, or horizontally from feeding from an infected host. To introduce two distinct life-history strategies used by tick-borne pathogens we will compare *Rickettsia rickettsii,* the bacterium responsible for Rocky mountain spotted fever also known as tick-borne rickettsioses and *Borrelia burgdorferi* the agent of Lyme disease.

B. burgdorferi is a motile spirochete[32]. Its natural reservoir is small mammals where infection does not lead to disease[33]. The population is maintained in an enzootic cycle. The pathogen has not been shown to be vertically transmitted, instead *B. burgdorferi* is reliant colonizing a tick larvae or nymph that is feeding on an infected host. An adult is a dead end for the pathogen because it will not quest for another host in its lifetime. Larvae or nymphs however, will drop off the host, molt and quest for another host. When the tick begins feeding on a new host, the blood meal triggers replication, after which some of the pathogen escape from the midgut and make it to the salivary glands, and ultimately into the new host[34,35]. Interestingly adult ticks, which were colonized as a nymph or larvae are a less competent vector of *B. burgdorferi*[35].

B. burgdorferi and *R. rickettsii* are vectored by the same species of ticks, but they employ vastly different life history strategies. *R. rickettsii* is an obligate intracellular bacterium[36]. Ticks serve as both reservoir and vector of *R. rickettsii*[37]. *R. rickettsii* populations replicate in the salivary glands and ovaries, facilitating vertical transmission[38]. Horizontal transmission also occurs between ticks feeding on the same host.

Tick-Microbe Symbiosis

Ten genera of vertically transmitted bacteria have been reported to colonize ticks[39]. Notable tissue symbionts observed in this study *Francisella* and *Coxiella* have been shown to have the capacity to evolve pathogenic and non-pathogenic life strategies, making them candidates of interest when predicting sources of novel emergent infections[39]. Both *Francisella*-like endosymbionts and *Coxiella*-like endosymbionts have been found in multiple tick species. Even though both *Francisella*-like endosymbionts and *Coxiella* have been found in the same tick species previous studies have not observed co-occurrence[40,41].

Most tick endosymbionts occupy the cytoplasm of host cells and have reduced genomes suggesting they have been occupying this intracellular niche for considerable time. Ovaries and malpighian tubules appear to house high densities of endosymbionts closely related to either *Francisella tularensis* or *Coxiella burnetii* indicating a stable route for vertical transmission[40].

Many of the non-pathogenic microorganisms hosted by ticks closely resemble human pathogens[42,43]. These organisms are thought to be endosymbionts of the tick, that

is to say an organism that resides within in a host's cells or tissue to the mutual benefit of both organisms. The functions of many of tick-associated bacteria are not known; we referred to these organism as endosymbionts because they are closely related to organisms that have been found to colonized the inside of the host's cells.

Perhaps the best example of the similarity between tick-borne pathogens and endosymbionts, is found in the *Coxiella* genus. *Coxiella burnetii* is a unique intracellular pathogen. Unlikely most intracellular pathogens, *C. burnetii* has evolved to be resilient to a wide range of adverse conditions. Its stability outside the host cell and low infectious dose make it a dangerous human pathogen[44,45]. Most human *Coxiella burnetii* infections are the result of contact with contaminated aerosols[44]. However, endosymbionts similar to *Coxiella* infect at least two thirds of tick species[46], *Coxiella*-like endosymbionts have been observed in tick salivary glands and ovaries[47].

Other notable endosymbionts resemble *Francisella tularensis,* a gram-negative coccobacillus, also an intracellular pathogen which causes tularemia in humans[48]. Similar to *Coxiella*-like endosymbionts, *Francisella* like endosymbionts have been observed in female reproductive tissues[49]. Vertical transmission of *Francisella tularensis* was demonstrated in early experiments however more recent experiments failed to confirm these findings[50].

A single *Coxiella*-like endosymbiont was selected for genome sequencing, the endosymbiont had a highly reduced (656,901 bp) in comparison to *C. burnetii* (1,995,281 bp). The endosymbiont encodes most major vitamin and cofactor biosynthesis pathways suggesting the endosymbiont may provide the tick host with vitamins. The *Coxiella* like

endosymbiont used for this genome analysis did not have recognizable virulence genes[47]. Indicating that although symbionts are closely related to pathogens, they are functionally very different.

Further comparison using complete genome sequence data between a selected *Coxiella*-like endosymbiont and *C. burnetii* indicate *C. burnetii* likely evolved to be virulent from a non-pathogenic ancestor. Whereas the *Francisella*-like endosymbiont sequenced had a genome similar in size to *Francisella tularensis,* but the *Francisella*-like endosymbiont contained a large number inactivating mutations in protein-coding genes. This indicates the *Francisella*-like endosymbiont likely evolved from a virulent ancestor[51]. The evolution of virulence from a non-pathogenic ancestor highlights the importance of understanding non-pathogenic tick endosymbionts.

MICROBIAL DIVERSITY OF CULICIDAE

Introduction

Vector borne human pathogens and are responsible for more than one million deaths per year[52]. With the exception of Antarctica, Mosquitoes inhabit every continent on earth and are vectors of a wide variety of human pathogens. Mosquito control is crucial to the health of the millions of people living in areas where mosquito-borne infectious diseases, including malaria, yellow fever, dengue, West Nile, and chikungunya, are endemic[53].

Three interacting organisms play important roles in the transmission cycle of a mosquito vectored pathogen; namely the pathogen, the mosquito, and a susceptible host. Each interaction between organisms in the transmission cycle is a point where possible control mechanisms could be implemented. In order to successfully vector a pathogen, the mosquito must either acquire it horizontally when the mosquito takes a blood meal from an infected host, or in the case of certain viral pathogens, there is a possibility of vertical transmission from the mother to its offspring. In the event of horizontal transmission most pathogens must successfully colonize the mosquito midgut tissue[54]. Here, pathogens interact with mosquito cells and other members of the mosquito gut microbiome. For example, plasmodium establishes itself in the gut where it will either replicate or mature[55,56]. After replication, most pathogens must translocate to the mosquito salivary glands in order to be transmitted to another susceptible host during a subsequent feeding[54].

Flaviviruses, including yellow fever, dengue, West Nile virus, and Zika may be vertically transmitted at low rates. Typically less than 1% of the progeny of an infected mosquito becomes infected in this way[57]. However, even low rates of vertical transmission are epidemiologically important and allow pathogens to persist during adverse conditions.

It is possible that the mosquito microbiome influences the mosquito's ability to transmit pathogens (i.e., vector competence)[58-60]. For this to occur, deleterious pathogen-microbiome interactions may limit pathogen replication, survival and/or translocation within mosquito tissues[61,62]. Beside these direct effects, the microbiome may also influence mosquito fitness by controlling the digestion of refractory food sources and the nutritional value of the diet[63]. The microbiome can also aid in protecting mosquitoes against mosquito pathogens[64]. Therefore, a better understanding of pathogen-microbiome interactions within mosquitoes could hypothetically reduce human disease.

Empirical evidence supporting this hypothesis comes from a study of *Anopheles gambiae*, an important mosquito vector of *Plasmodium falciparum*, which is a eukaryotic parasite responsible for causing human malaria. Reintroduction of select bacteria isolated from adult *A. gambiae* into antibiotic-treated females resulted in upregulation of immune genes and a substantial reduction of the *P. falciparum* abundance compared to antibiotic-treated females that did not have bacteria reintroduced[61]. Similarly, multiple studies have found that the presence of bacteria from the *Wolbachia* genus inhibited the ability of mosquitoes to transmit *Flaviviruses*[58,65,66]. Despite these examples, the mosquito microbiome has not been fully investigated across different mosquito species. A better

understanding of the mosquito microbiome may lead to exciting new pathogen-microbiome dynamics and new targets for interventions that limit human infection.

Closely related mosquito species can differ greatly in their capacity to transmit disease. For example, *Aedes aegypti* is one of the most common vectors of dengue fever, chikungunya, Zika, and yellow fever, whereas the capacity to vector these diseases by other *Aedes* mosquitoes varies greatly. Zika virus is capable of colonizing one of the most common mosquitoes in Montana, *Aedes vexans*, but the virus is less proficient at translocating throughout the body compared to *A. aegypti*[67].

A recent survey of mosquito microbiomes found a surprising amount of diversity between individuals of the same mosquito species[68]. This diversity was so great, in fact, that mosquitoes belonging to different species hosted very similar microbiomes[68]. This study is somewhat in contrast with studies of laboratory-reared mosquitoes that had a more uniform and consistent microbiome diversity between individuals within the same species[69]. Another study of the microbiome of wild-caught mosquitoes in Canada showed that the identification of mosquito species was the largest driver of microbiome membership. However, this finding was attributed to the presence or absence of *Wolbachia*[70]. Based on these studies, there is currently little consensus in the field as to microbiome diversity within and between mosquito species, and that more work is needed to address this issue.

To add to the growing literature on mosquito microbiomes, and to focus on co-occurring mosquito species, we collected and analyzed the microbiome of mosquitoes from Montana, USA. Although mosquitoes in Montana do not vector many diseases of

human and animal importance, West Nile virus emerged in 2002 and since then in the state there have been 559 documented human cases and 12 human deaths associated with this disease as of 2015[71,72]. It is maintained in an enzootic cycle between *Culex* mosquitoes and birds, which are the amplifying hosts, whereas humans are a dead-end, or spillover host[73].

In addition to causing human disease, mosquito parasitism adversely affects wild mammals, birds, and livestock. Mosquito parasitism decreases the productivity of ranch cattle, a primary source of agricultural revenue in the state, by causing cattle to bunch together and disrupting grazing activity[74]. Tourism is adversely affected when swarming mosquitoes discourage people from participating outdoor activities. Of growing concern, the range and life histories of many mosquito species is expected to change as the climate of the earth warms and urbanization continues[75,76]. Therefore, this study was designed to characterize the mosquito microbiome currently endemic to this state so that future studies can examine mosquito responses to climate change and the potential for human and animal pathogen transmission.

Materials and Methods

Mosquito Collection

Mosquitoes were collected from seven locations across Montana in the summer of 2013 from 2013 from June 6th to August 13th using battery-operated CDC light traps baited with CO_2 to attract female mosquitoes (Figure 2.1). Species were identified using the *Identification and Geographical Distribution of the Mosquitoes of North America*

Guide[77] at the Veterinary Entomology Lab in Bozeman Montana, and stored at -20 °C until DNA extraction.

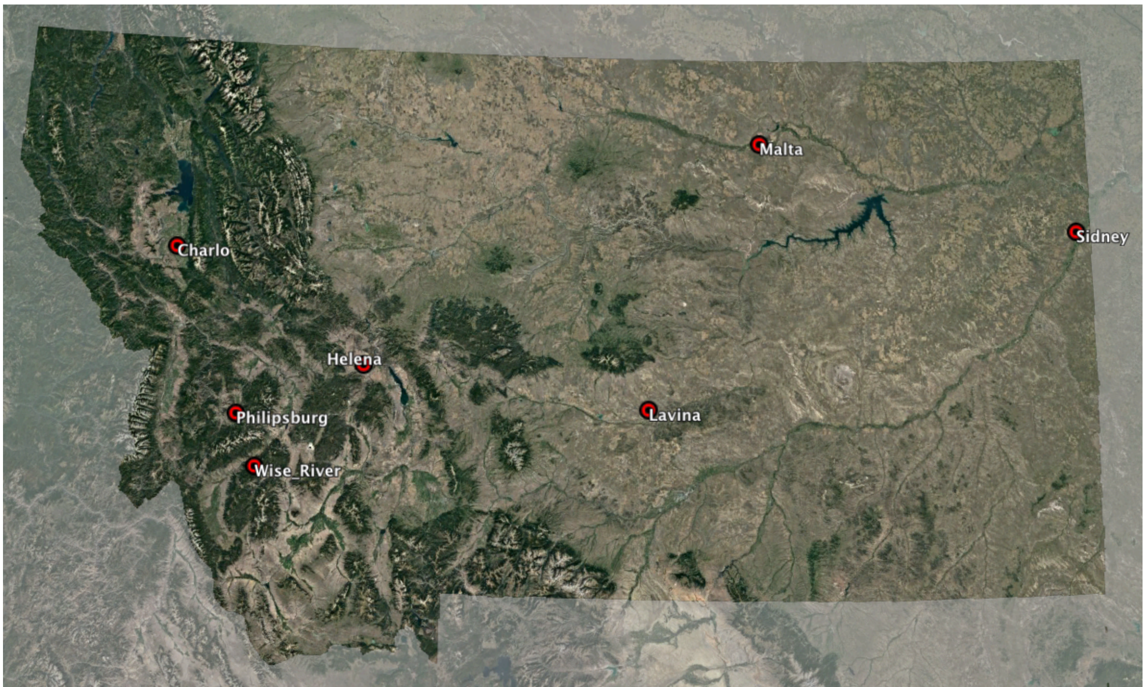


Figure 2.1 Trapping locations.

DNA Extraction and 16S rRNA Gene Sequencing

Across all sites, five representative mosquitoes from the 26 most common species (n=130 total) were selected for microbiome analysis. DNA was extracted from the entire insect using the MoBio PowerSoil 96-well DNA isolation kit (Carlsbad, CA) with a bead beating step. 16S rRNA sequences were generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. Variable region 4 (V4) of the bacterial 16S rRNA gene (515F-806R) was amplified with barcoded primers. Each sample was amplified in triplicate in a 25 µL PCR reaction, consisting of 12µL of M Bio PCR Water (Certified DNA-Free), 10 µL 5 Prime HotMasterMix (2.5x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. PCR amplification was done under the following conditions: 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90s; final extension 10 min at 72 °C. Amplicon molarity was quantified using PicoGreen (Invitrogen) and a plate reader (Infinite 200 PRO, Tecan). Each amplicon library was represented in equimolar amounts after PCR products were pooled.

Data Processing

16S reads were processed using mothur v.1.37.5[78]. Low quality bases were removed following the mothur MiSeq SOP[79] (access date 1 December 2016). Assembled sequences < 275 bp as well as any reads with ambiguous bases or homopolymers > 8 bp were discarded. Identical sequences were combined. Remaining sequences were aligned against the SILVA reference database (version 128) that was bioinformatically trimmed

to the V4 region. A pre-cluster step was used to combine rare contigs with 3 or less nucleotide difference to more abundant members in the dataset. Chimeras were identified and removed in mothur with UCHIME [80]. Sequences sharing 97% similarity were binned into Operational taxonomic units (OTUs) using VSEARCH [81]. OTUs containing less than 100 sequences in the dataset were removed to guard against spurious sequencing artifacts. Representative sequences from each OTU were taxonomically classified using software at the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/) [82]. OTU classification was corroborated using BLAST against the GenBank database (http://blast.ncbi.nlm.nih.gov) [83].

Within mothur, the Ribosomal Database Project classifier (train set 10) was used to assign taxonomy to OTUs with an 80% confidence threshold (RDP; http://rdp. cme.msu.edu/). After sequencing data clean-up ~2,400,000 of the ~3,500,000 raw sequences remained. All mosquito microbiomes were rarefied to 2500 randomly selected quality DNA sequences per sample. the size threshold was set to keep 90% of the individuals within the dataset. Individuals with less than 2500 quality DNA sequences were excluded from further analysis. After quality filtering 113 microbiome of the initial 130 were included in the final analysis. All downstream analysis was done in R with “Vegan”, “Labdsv”, “cluster”, and “optpart” packages. Dissimilarity of microbiomes between insects (beta diversity) was quantified using Bray-Curtis Dissimilarity.

Results

DNA was extracted from a total of 130 mosquitoes, from which 3,394,338 sequences, averaging 25,715 ± 1,596.99 (standard error) per mosquito, were generated. Following OTU binning and taxonomic identification, each microbiome was dominated by only a few bacterial phyla (Figure. 2.2), the majority of which were *Acidobacteria* (29%), *Proteobacteria* (27%), and *Verrucomicrobia* (11%). At the lowest level of bacterial taxonomy, only a single species, a member of the *Bradyrhizobium* genus (operationally defined as ≥ 97% 16S rRNA sequence identity; see methods section), was shared between all mosquitoes.

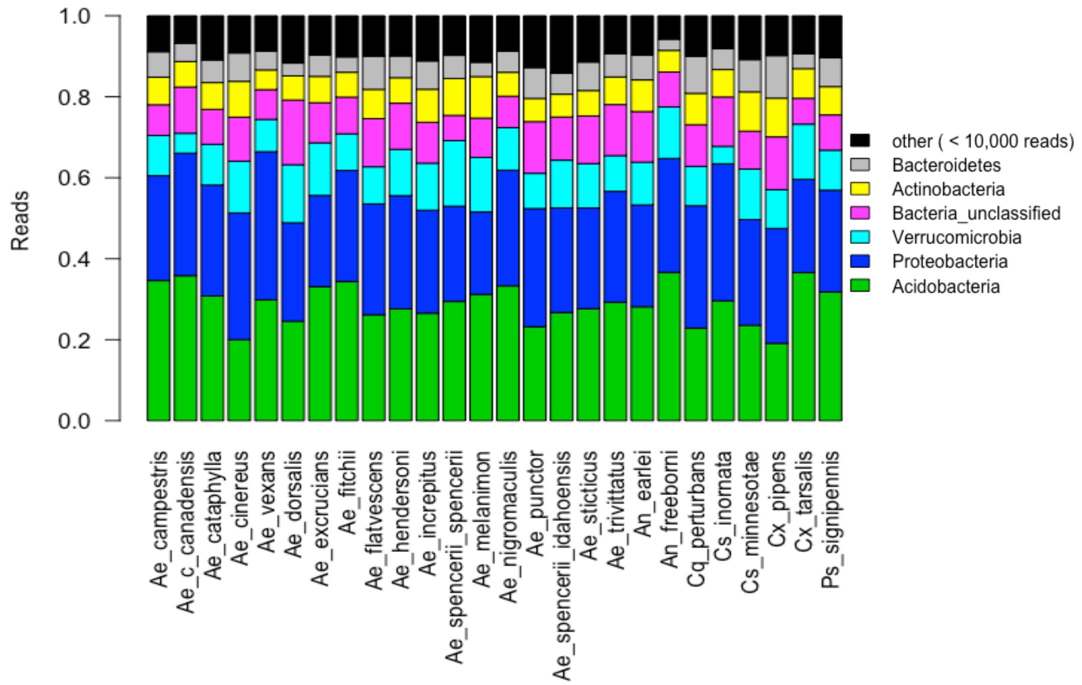


Figure 2.2 Classification microbiome members for each mosquito species
Proportional abundance of microbiome members classified to the phylum level within each mosquito species.

OTU richness and evenness was highly variable between mosquitoes of the same species (Figure 2.3). For example, there was an average of 480.3 OTUs per mosquito, but the standard deviation was 124.77. We found significant difference in alpha diversity between species based on inverse Simpson index (ANOVA f = 1.7524, p = 0.03172). Pairwise t tests were used to determine which species were significantly different, results are reported in figure 2.3. When adjustments were made for multiple comparisons none were found to be significant.

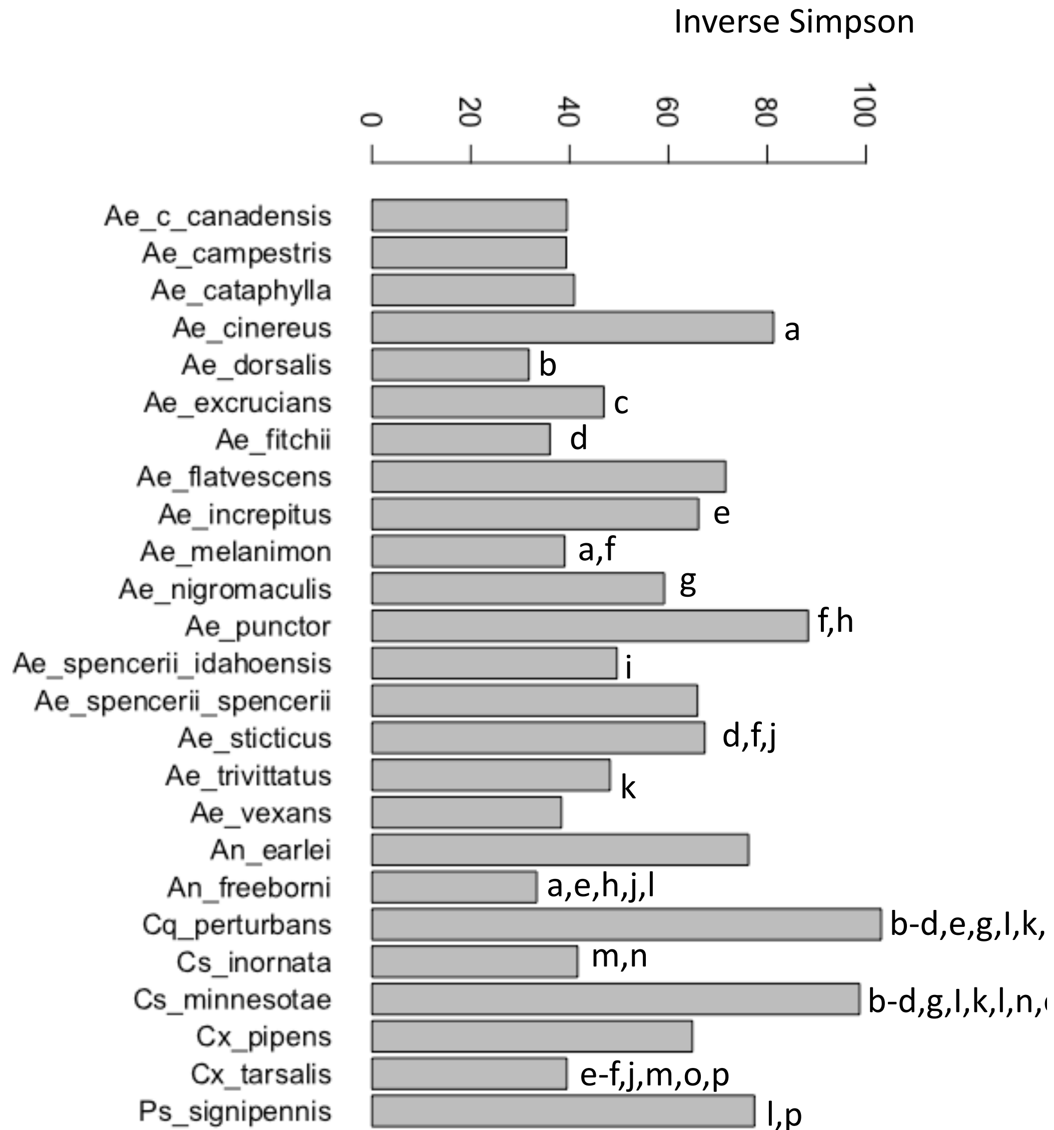


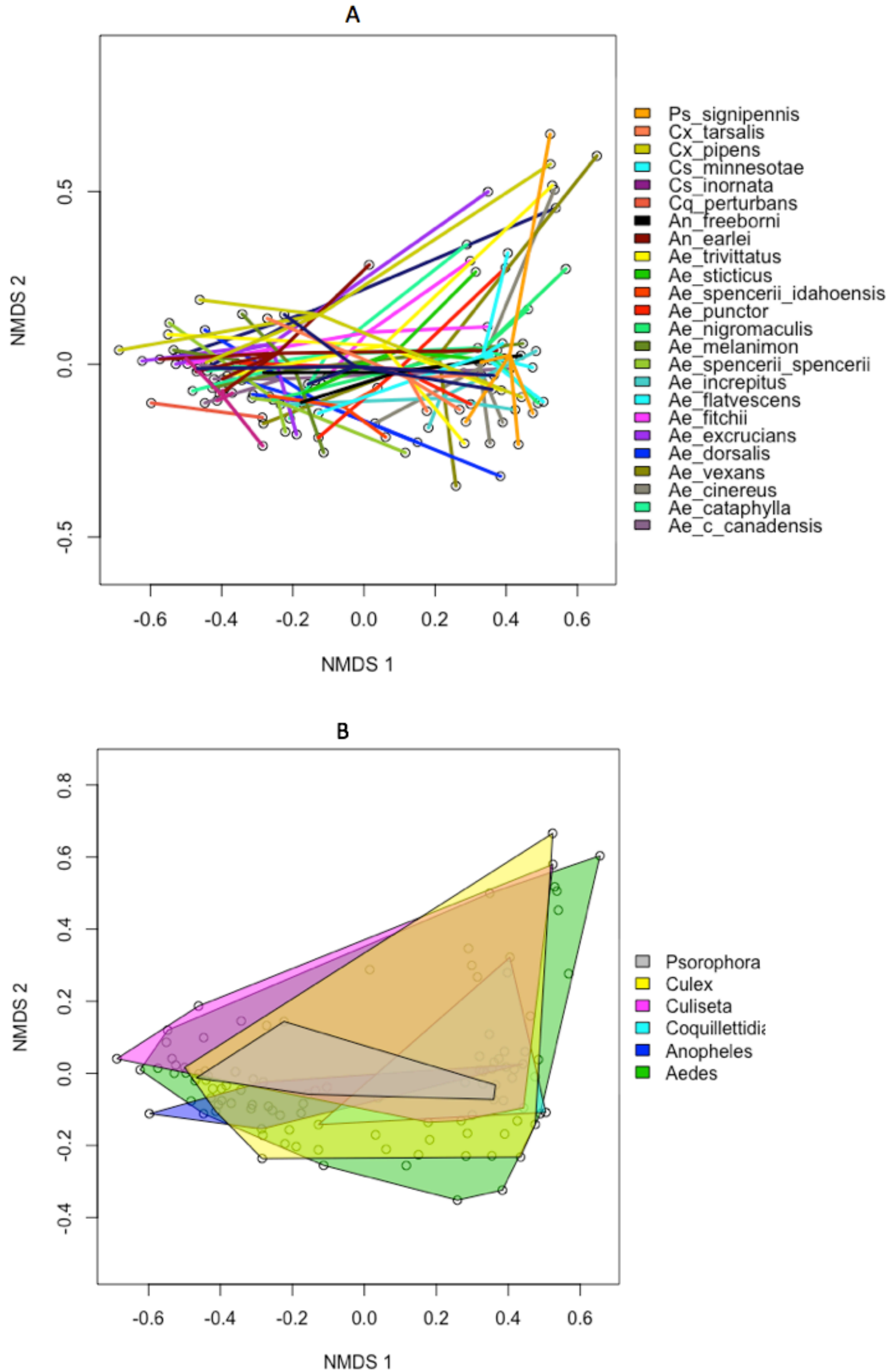
Figure 2.3 Microbiome richness and evenness within mosquito species as measured by the Inverse Simpson index. The large amount of variation may be the result of mosquito age or feeding history. Letters indicate significantly different ($p < 0.05$) pairwise comparisons.

Dissimilarity between microbiomes was quantified using the Bray-Curtis dissimilarity index. The dissimilarity matrix was partitioned by comparisons between microbiomes belonging to the same species (intraspecific) between species in the same genus (interspecific). Median intraspecific Bray-Curtis distance was 0.6796, whereas the median interspecific distance was 0.704. Intraspecific distances were slightly lower than interspecific distances. We used the nonparametric Mann–Whitney to test the likelihood a randomly selected intraspecific distance will be less than a randomly selected intraspecific distance and found that indeed microbiomes within a species were more likely to be similar (p-value = 0.01042). The small difference between intraspecific distance and interspecific variation in microbiome diversity shows that it is possible for closely related mosquitoes (within species) to not necessarily harbor more similar microbiomes compared to more distantly related mosquitoes.

Bray-Curtis distances were further explored using ordination analyses. Non-metric multidimensional scaling plots (NMDS plots) confirmed that microbiome composition was highly variable between mosquitoes of the same species, and no grouping by mosquito taxonomy was observed. Differences in microbiome diversity based on mosquito taxonomy was evaluated using PERMANOVA, which is a multivariate adaptation of ANOVA (Figure 2.4). In this analysis, Bray-Curtis distances were partitioned by mosquito taxonomy in order to test for meaningful differences in centroids and dispersion. Based on this analysis, taxonomy was not a significant at any taxonomic level tested (species p = 0.061; subgenus p = 0.260; genus p = 0.547). We repeated an ANOSIM analysis used in a previous study on mosquito microbiome[68].

Similar to analyses above, no significant difference was observed between twelve mosquito species (R= 0.05622, p = 0.068931).

Figure 2.4 NMDS ordination by taxonomic classification
(A) spider plot drawn and colored by connect microbiomes from the same species, variation between species was not significant, PERMANOVA: $f = 1.191$, $p = 0.061$**.**
(B) plot grouped and colored by genera, variation between species was not significant; PERMANOVA: $f < 1$, $p = 0.547$.



Since there are multiple species of mosquitoes in Montana capable of transmitting West Nile virus, we tested whether mosquitoes the potential to vector West Nile was correlated with similar microbiome diversity. A scale, ranging from one to five, was used based on the population size, range, and feeding behavior[84] (Table 2.1, Figure 2.5). Based on ANOSIM, a significant correlation between the potential to vector West Nile and microbiome diversity was found ($R = 0.09532$, $p = 0.031$), suggesting that the microbiome may play a role in West Nile vector capacity. However, the very small R statistic (below 0.25) associated with this test means that the effect size is small, and may not be biologically meaningful[85,86].

Table 2.1 West Nile virus risk score

Mosquito species	West Nile virus risk score
Aedes campestris	1
Aedes canadensis canadensis	1
Aedes cataphylla	1
Aedes cinereus	1
Aedes dorsalis	3
Aedes excrucians	1
Aedes fitchii	1
Aedes flavescens	1
Aedes hendersoni	1
Aedes increpitus	1
Aedes melanimon	3
Aedes nigromaculis	2
Aedes punctor	1
Aedes spencerii idahoensis	1
Aedes spencerii spencerii	1
Aedes sticticus	1
Aedes trivittatus	1
Aedes vexans	3
Anopheles earlei	1
Anopheles freeborni	1
Anopheles punctipennis	1
Coquillettidia perturbans	3
Culex pipiens	5
Culex tarsalis	5
Culiseta inornata	2
Culiseta minnesotae	1
Psorophora signipennis	1

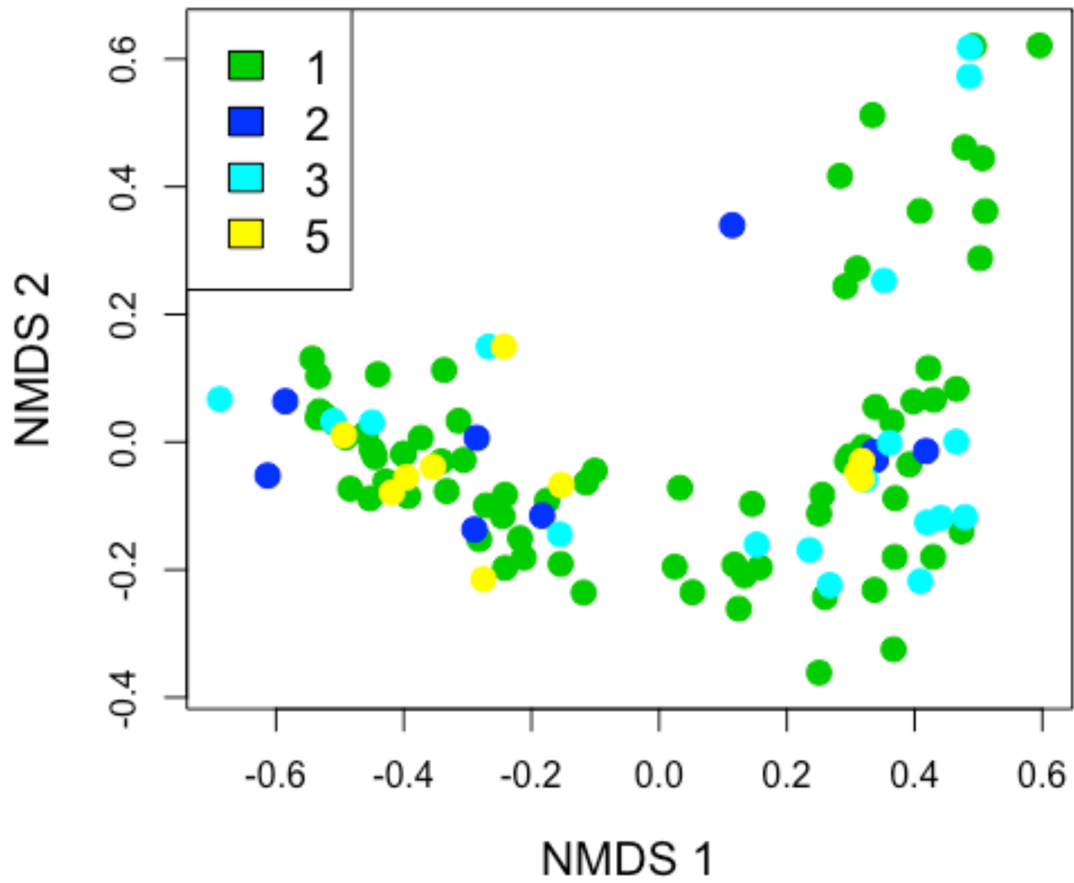


Figure 2.5 NMDS ordination by West Nile virus risk score. ANOSIM revealed significant but low correlation between mosquito species risk score and microbiome diversity (R = 0.09532, p = 0.031).

Finally, cluster analysis was used to create discrete clusters of mosquitoes based on microbiome composition (Figure 2.6). This analysis showed that cluster membership was not random ($p = 0.02849$, Fisher exact test, 2000 replicates). However, each mosquito species appeared in multiple clusters, which again demonstrated that mosquitoes in Montana are capable of hosting a wide range of bacteria.

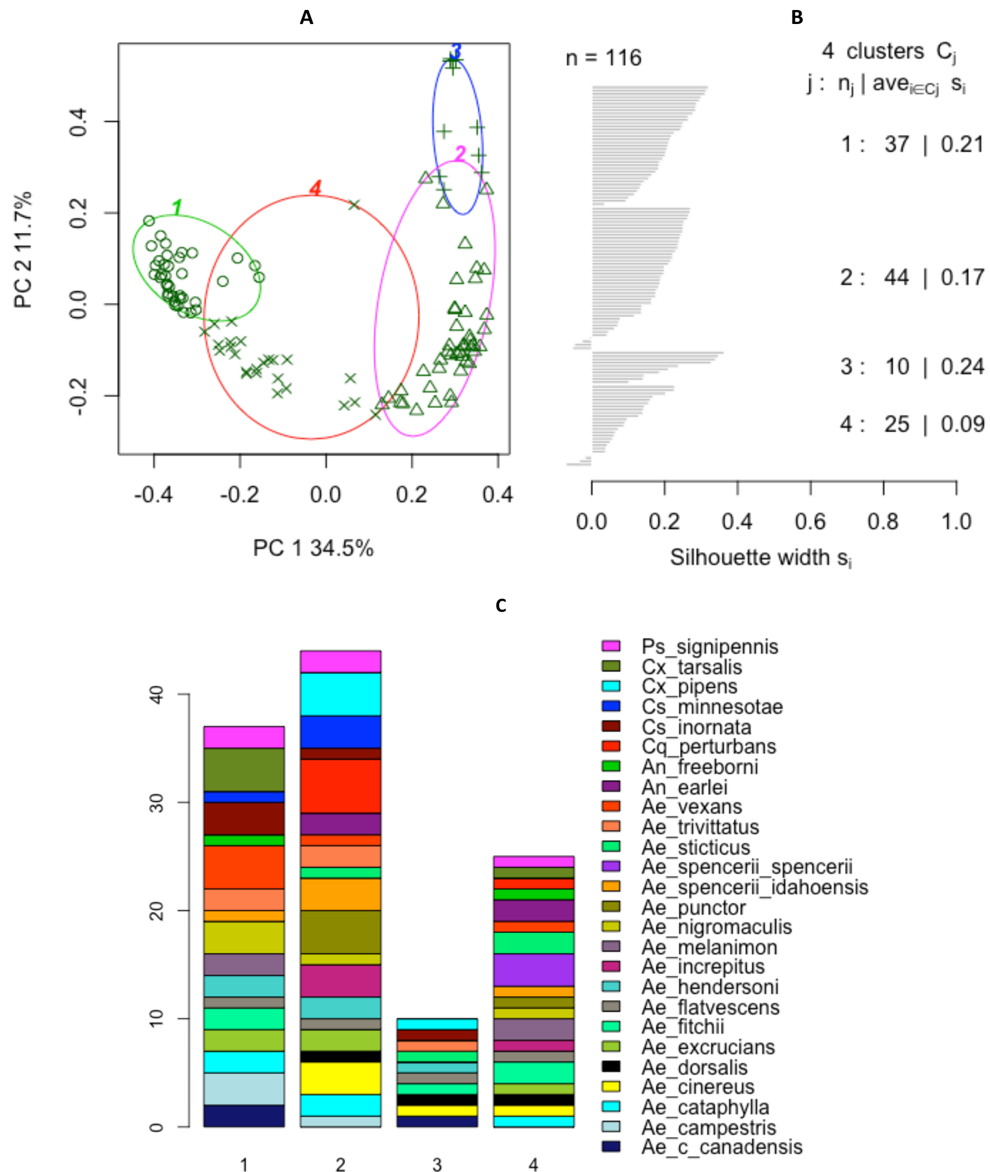


Figure 2.6 Cluster analysis (A) Microbiome cluster membership as a function of partitioning around four medoids using Bray-Curtis dissimilarity. All microbiomes were grounded into four discrete clusters to optimize within cluster homogeneity and heterogeneity among clusters. Final clustering was done by fixed-cluster used partitioning around medoids[87]. (B) Silhouette plot showing the effectiveness of clustering. Horizontal bars indicate the similarity of a microbiome to the medoid of cluster it was assigned to compared to other clusters. It was determined that four clusters achieved the highest silhouette width. Average silhouette length = 0.17. j indicates the cluster a microbiome was assigned: n_j number of microbiomes within a cluster ave $_{i \in Cj}$ $_{Si}$ is the average silhouette width of each cluster. (C) Counts of the of microbiomes in the four clusters color coded by mosquito species.

None of the environmental variables (location, elevation, temperature, and precipitation) were significant predictors of microbiome diversity. Precipitation was also found to be significant in univariate analysis (p= 0.01419, ANOVA), but this association did not hold after adjustment for multiple comparisons (p = 0.09933).

Validation of Sequences Data

We were surprised by the seemingly small role of host taxonomy on microbiome diversity. To test the reproducibility of our findings, a second round of sequencing was done, using different primers targeting a different 16S variable region (V3-V4), a different MiSeq sequencer (at MSU), and 20 additional mosquitoes (10 *Aedes* and 10 *Anopheles*). Unfortunately, this run yielded low-quality sequences (~20% of sequences passed the quality filter), and so the data were not used.

All 16S contigs that did not align to the Silva database were selected, preclustered, and chimeras were removed. Contigs were then binned into OTUs at a 99% similarity cutoff, and a representative sequence for each OTU (n=864) was compared to *Culicidae* and *Midichloriaceae* genomes using BLAST. No significant sequence similarity was found to these genomes, suggesting that little, if any, mosquito DNA was present in the 16S datasets.

Discussion

This study revealed a large amount of microbiome variability between individual mosquitoes of the same species. A similar result was found in at least two other

studies[68,88]. In contrast, another study found large differences in Beta diversity between insect species within a family[88], suggesting that differences arise over longer evolutionary timescales. Thus, our results add to the growing body of evidence that different mosquito species share large components of their microbiomes[68,88,89]. Studies of lab reared mosquitoes determined microbiome composition to be associated with mosquito taxa[69]. The inconsistency between microbiome studies of lab-reared mosquito and wild caught mosquitoes suggests that important factors present in the environment are not captured in the laboratory[90]. These factors are capable of overpowering the effect of mosquito taxonomy in the environment.

The anatomy of the insect gut is important for selecting certain types of bacteria. Although both the foregut and hindgut can provide a stable surface for colonization, the majority of the microbes colonizing the mosquito are found in the midgut[91]. An important component of the midgut is the peritrophic matrix; a complex of chitin, proteins and glycoproteins separating the gut lumen and the epithelium. Mosquitoes secrete a peritrophic matrix (type I) after a blood meal[92]. This is thought to protect their epithelial cells from the oxidative stresses associated with blood catabolism[93]. Formation of the peritrophic matrix confines bacteria to the gut lumen, stopping them from penetrating the midgut lining and colonizing other tissues[93,94]. A subset of bacteria in the gut bloom when a mosquito takes a blood meal, resulting in a reduction in overall diversity of the microbiome[93]. As such, determining whether or not and how recently a mosquito has taken a blood meal is a confounding factor in mosquito microbiome studies, including this study.

Other factors may also confound insect microbiome studies. For example, different taxa could occupy the same niche based on their environmental abundance throughout the year or geographic (site-to-site) distribution.

Mosquitoes are colonized by the bacteria present in their aquatic, larval habitat[95], and so it is possible that fluctuations in bacterial diversity of the aquatic habitat determine microbiome diversity in the adult stage. Studies of laboratory reared *A. aegypti*, *A. albopictus*, and *A. gambiae* have found that some bacteria persist in the mosquito gut through the first instar stage to adulthood[90,96]. Since multiple mosquito species can co-occur in a single body of water[97], it seems reasonable to hypothesize that the aquatic, larval habitat is a primary factor in structuring the mosquito microbiome.

Conclusion

We characterized the microbiome of 26 of the most common mosquito species in Montana. The microbiomes of different mosquito species in Montana were not significantly different. There was a large amount of variation in microbiome of individuals of the same mosquito species, and many instances of different species host very similar microbiomes. This appears to be an emerging theme from similar studies in the field.

MICROBIAL ECOLOGY OF NEOTROPICAL TICKS

Introduction

Ticks host the greatest variety of microorganisms of any of the blood-feeding arthropods[21]. All microorganisms colonizing a host are members of an ecological community, referred to as a microbiome. Select bacteria within insect microbiomes have also been referred to as endosymbionts because they reside within tick cells or body cavity, and they live together, in symbiosis, with their tick host[98,99]. Symbiotic relationships between ticks and their microbiome range between being beneficial to both host and microbe to being detrimental to one or the other[40]. Decades of mechanistic endosymbiont and microbiome research have shown that these symbiotic relationships operate by affecting host nutrition, physiology, metabolism and immune function[100,101]. Thus, the microbiome can be thought of as an organ that is additive to and not confined by the genetic potential of the host. Some members of the tick microbiome are pathogenic to vertebrates and can be transmitted to humans and animals of agricultural importance. Thus, a better understanding of tick-microbiome interactions may provide novel strategies for the prevention of tick-borne diseases.

Only a small percentage (~10%) of the almost 900 identified tick species are competent vectors of vertebrate pathogens[21,102]. Within this subset, it is common for the same pathogen to be vectored by several tick species, as well as for single tick species to vector multiple pathogens[103,104]. For example, the brown dog tick, *Rhipicephalus sanguineus,* vectors canine ehrlichiosis (*Ehrlichia canis*), canine babesiosis (*Babesia*

canis), human Mediterranean spotted fever (*Rickettsia conorii*), human Rocky Mountain spotted fever (*Rickettsia rickettsii*), and human Anaplasmosis (*Anaplasma phagocytophilum* or *Anaplasma platys*)[102,105,106].

Certain attributes of the microbiome of disease-vectoring ticks seems to correlate with the likelihood of pathogen transmission (i.e. vector competency)[107,108]. Laboratory investigation of the deer tick (also called blacklegged tick), *Ixodes scapularis*, found greater microbiome species richness was negatively correlated with how well the causative agent of Lyme disease, *Borrelia burgdorferi*, was able to colonize. Larvae of the sterile, laboratory-reared ticks showed decreased expression of peritrophin, a primary component of the peritrophic matrix in the gut[107]. Since the peritrophic matrix is a mucus-like layer separating the tick gut lumen from the epithelial cell lining, this result supports the hypothesis by Hegedus et. al., that the presence of the peritrophic matrix directly influences the abundance of pathogens and thus the tick's ability to transmit disease to susceptible hosts[109].

The microbiome was also found to limit colonization of *I. scapularis* by *A. phagocytophilum*. However, ticks colonized with *A. phagocytophilum* increased production of *I. scapularis* antifreeze glycoprotein (IAFGP) in the gut. IAFGP was shown to bind the terminal D-alanine residue of bacterial peptidoglycan, which reduced the capacity of microbiome bacteria to form biofilms. This, in turn, led to reduced peritrophic matrix integrity and increased gut permeability. All of these conditions were favorable for further colonization by *A. phagocytophilum*[108]. Besides these studies with *I.*

scapularis, information regarding the role of the tick microbiome using next generation sequencing methods in other tick species is lacking[110].

There are a number of important tick-microbiome questions to be answered, including whether human pathogens negatively interact and exclude each other in ticks and if human pathogens are affected by the rest of the tick microbiome. To begin to address these questions; to generate relevant tick-microbiome information, we characterized the microbiome of the world's most widespread tick[105], *R. sanguineus*. Given the species-specific nature of insect microbiomes in general[88], we hypothesized that the *R. sanguineus* microbiome would be noticeably distinct from other arthropod microbiomes. Our results support this hypothesis, and also suggest that there are strong negative symbiotic interactions between the bacterial genera to which important human pathogens belong.

Methods

Tick Collection

Brown dog ticks were collected in Iquitos, Peru in March of 2015. Actively feeding ticks were removed from 14 dogs using forceps and 5 ticks were donated by a local veterinarian. All ticks were immediately stored in 70% ethanol. Ticks collected from the same animal were stored in 1.5 mL tubes, and no more than 20 ticks were placed into the same tube. Ticks collected from different dogs were stored in different tubes to avoid possible cross-contamination between dogs. Tubes were stored at 4°C after collection while in Peru (between March 5 and March 20, 2015) and brought back at

ambient temperature to Montana State University on March 24, 2015. Ticks were stored in ethanol at -20°C until processing took place.

Ticks were morphologically identified to the species level and sexed with a dissection microscope using the "Ticks of Veterinary Importance" key published by the USDA[111]. Photos of the dorsal and ventral side of each tick were taken under the dissection microscope to address any discrepancies in tick identification (pictures can be made available upon request). In addition to tick sex, weight, and dimensions, (Figure 3.1), the life stage was also determined. These variables, along with collection location, were used considered as environmental variables in microbiome analysis described below and in the results section.

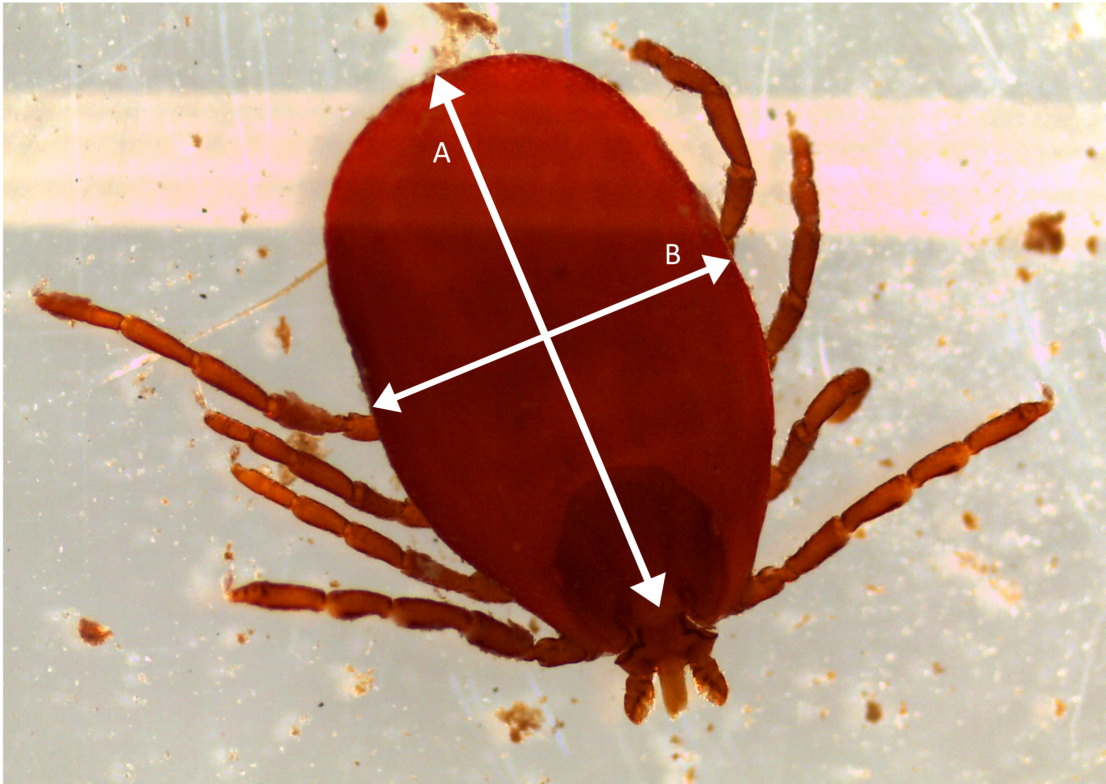


Figure 3.1 Tick body measurements (A) length was the distance between the posterior end of the basis capituli and the posterior of the abdomen. (B) width was quantified using the width of the abdomen at its widest point.

To minimize the contribution of microorganisms on the exoskeleton, ticks were washed (30 seconds) in a 10% bleach solution and rinsed twice with nuclease free water prior to DNA extraction[112,113]. Ticks were physically quartered and DNA was extracted from entire body using the MoBio Powersoil 96 well kit (Carlsbad CA) with silica glass bead-beating.

16S rRNA Gene Amplification and Sequencing

16S rRNA sequences were generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. Variable region 4 (V4) of the bacterial 16S rRNA gene (515F-806R) was amplified with barcoded primers. Each sample was amplified in triplicate in a 25 µL PCR reaction, consisting of 12µL of molecular grade water (Certified DNA-Free), 10 µL 5 Prime HotMasterMix (2.5x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. PCR amplification was done under the following conditions: 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; final extension 10 min at 72 °C. Amplicon molarity was quantified using PicoGreen (Invitrogen) and a plate reader (Infinite 200 PRO, Tecan). Each amplicon library was represented in equimolar amounts after PCR products were pooled.

Data Processing

16S reads were processed using mothur v.1.37.5[78]. Low quality bases were removed following the mothur MiSeq SOP[79] (accessed 1 December 2016). Assembled

sequences < 275 bp or containing any reads with ambiguous bases or homopolymers >8 bp were discarded[114]. Identical sequences were combined. Remaining sequences were aligned against the SILVA reference database (version 128) that was bioinformatically trimmed to only the V4 region. A pre-cluster step was used to combine rare contigs with 3 or less nucleotide differences to more abundant members in the dataset. Chimeras were identified and removed in mothur with UCHIME [80]. Sequences sharing 97% similarity were binned into operational taxonomic units (OTUs) using VSEARCH[81]. OTUs containing less than 100 sequences in the dataset were removed to guard against spurious sequencing artifacts. Representative sequences from each OTU were taxonomically classified using software at the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/) [82]. OTU classification was corroborated using BLAST against the GenBank database (http://blast.ncbi.nlm.nih.gov) [83]. Approximately 16.5% of the total sequences in the dataset were removed with the data processing steps. The final dataset was composed of 1,033,619 out of 1,237,185 original assembled contigs. Prior to statistical analyses, OTUs in each microbiome were rarefied to 1000 reads. When considering the “rare microbiome” (see text in results section), we changed the level of rarefaction to 250 reads. The same technique was recently used to evaluate the role of *Wolbachia* in mosquito microbiome analyses[115].

Statistical Analyses

All downstream analysis was done in R with “Vegan” and “Labdsv” packages. Dissimilarity of microbiomes between insects (beta diversity) was quantified using Bray-Curtis Dissimilarity. ANOSIM and PERMANOVA test were used to test the role of

environmental variables on microbiome diversity. Pearson correlation coefficient was calculated to test for significant interactions between OTUs.

Results

Of the 161 *R. sanguineus* ticks originally collected, 128 were eventually processed, and 16S sequences of sufficient quality and quantity were obtained from 118 individuals. In total, 72 unique OTUs represented by 100 reads or more were identified and included in downstream analyses. The median number of reads per tick was 6,257 (6,707 interquartile range, IQR). Following normalization, (i.e. the relative OTU abundance in each microbiome was rarefied using 1,000 sub-sampled reads), OTUs were taxonomically classified and quantified (Figure 3.2, Table 3.1). Somewhat surprisingly, all microbiomes were almost completely dominated by bacteria belonging to the *Proteobacteria* phylum (~98% of all microbiomes). The three most abundant OTUs in the dataset (all *Proteobacteria*), were at least an order of magnitude more abundant than any other OTU. Otu01 was present in all ticks, and shared 100% sequence identity with a *Coxiella*-like endosymbiont (CLE). Otu02 was present in approximately 20% of ticks, and shared 100% identity with the anaplasmosis pathogen, *A. platys*. Otu03 was present in about 10% of ticks, and shared 99% identity with a *Francisella*-like endosymbiont (FLE). All other OTUs were exceedingly rare and variably present-absent in ticks.

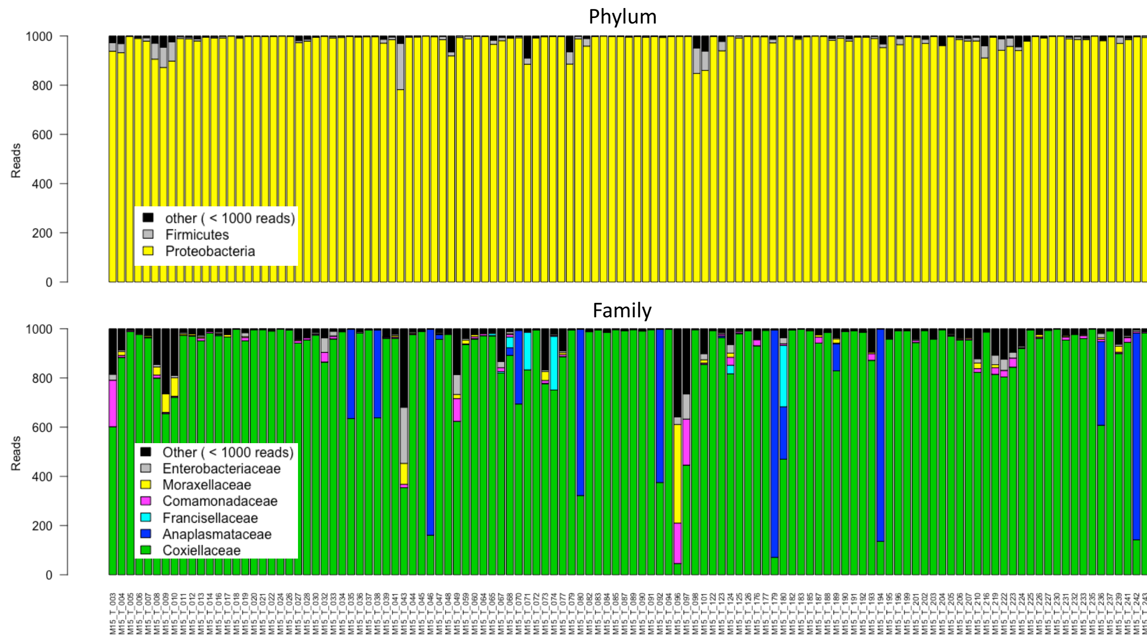


Figure 3.2 Classification of microbiome members for each tick at the phylum (top panel) and family (bottom panel) level. Each bar is an individual tick. The number of reads contained in each bar is shown on the y-axis, and tick sample names are shown along the x-axis.

Table 3.1 BLAST results of the most common OTUs

OTU	Organism	Prevalence	E value	% identity
Otu01	CLE	118/118	1e-127	100
Otu02	*A. platys*	25/118	1e-127	100
Otu03	FLE	13/118	3e-124	99

A second member of the *Coxiella* genus, Otu08 (96% identical to Otu01), was present in 36% of ticks, and its consensus sequence shared 98% identity (e value = 6 e-121). Since Otu01 and Otu08 were by definition >3% different and each shared greater similarity to different CLE in public sequence databases, it is likely that they represent biologically distinct bacteria. To understand whether they negatively interacted with one another, the co-occurrence of both OTUs was tested against a random distribution using the Pearson correlation coefficient (two-sided). A negative correlation was found ($R = -0.30$, $p = 0.04$, $n=118$), suggesting that these two OTUs were not likely to co-occur in the same tick.

To identify possible interactions between the other dominant tick microbiome members, we grouped both CLE OTUs together (Otu01 and Otu08), and tested for significant pairwise co-occurrences with *A. platys* (Otu02), and/or FLE (Otu03). Negative interactions were observed between the CLE OTUs and *A. platys* (Figure 3.3). There was also an indication that CLE and FLE interacted negatively (Table 3.2), although this interaction did not reach statistical significance.

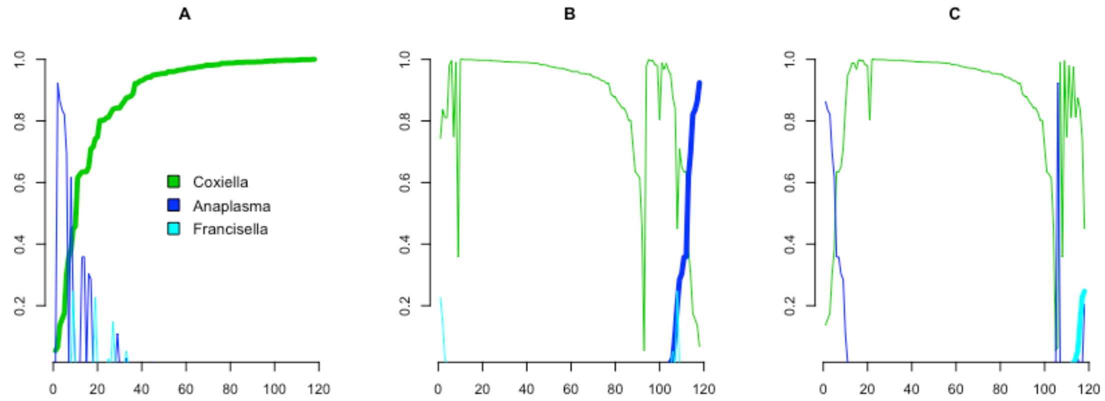


Figure 3.3 Negative interactions between dominant OTUs in *R. sanguineus*. The frequency (relative abundance) of dominant members were ranked according *Coxiella* (A), *Anaplasma* (B), or *Francisella* (C). The co-occurrence of *Coxiella* and *Anaplasma* were not random. Instead, the one was more likely to be observed when the other was at a low abundance or absent. The same trend was observed between *Coxiella* and *Francisella* (see Table 3.2).

Table 3.2 Interactions between dominant OTUs in *R. sanguineus*.

Comparison	Correlation	p-value
CLE and *A. platys*	-0.778	< 2.2e-16
CLE and FLE	-0.164	0.07512
A. platys and FLE	0.018	0.8452

The less abundant members of the microbiome (i.e. rare microbiome) were examined by removing all CLE, *A. platys*, and FLE reads and re-normalizing the overall dataset (i.e. rarifying to 250 reads). Only 39 (33%) of the microbiomes contained 250 or more reads after removing CLE, *A. platys*, and FLE. Ordinations Principal component analysis (PCA) of both microbiome datasets (with and without CLE, *A. platys*, and FLE) were generated for comparison (Figure 3.4). The rare microbiome had a much more even distribution of OTUs, which belonged to at least six bacterial phyla. Due to their low abundance and present-absent nature across ticks, however, statistical associations between members could not be tested.

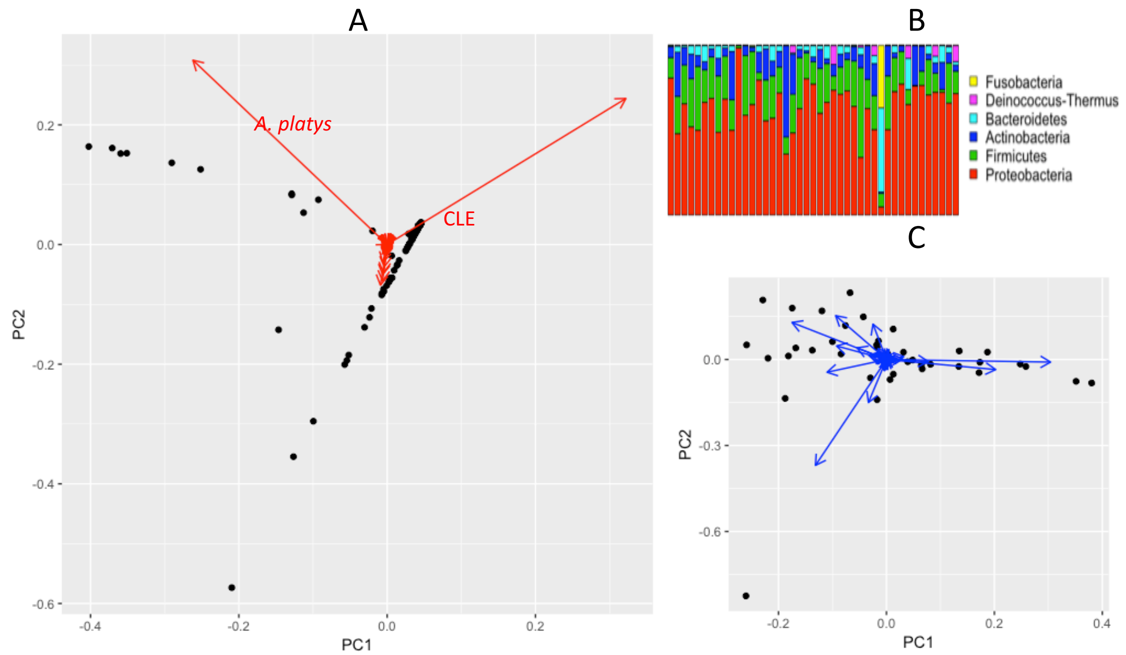


Figure 3.4 Beta-diversity of *R. sanguineus* microbiome. PCA of the entire microbiome dataset (A). Black dots represent individual microbiomes and the distance between dots represents microbiome similarity. Classification at the phylum level of the rare microbiome (after the removal of CLE, *A. platys*, and FLE reads) and re-normalization (B). PCA of the rare microbiome (C). The cloud-like distribution of dots suggested that ticks carried a broad diversity of OTUs in addition to CLE, A. platys, and FLE.

Discussion

To our knowledge, this was the first investigation of the *R. sanguineus* microbiome to use next generation sequencing. We found the microbiome of *R. sanguineus* to be extremely uneven and dominated by a single OTU belonging to either *Coxiella*, *Anaplasma,* or *Francisella.* Many other studies have documented the association between these genera and ticks[116-121], however to our knowledge, this is the first documentation of negative interactions between them (esp. CLE and *A. platys*).

Because DNA was extracted from the whole tick, it is impossible to know the exact transmission routes of microbiome members found in the ticks using only 16S sequence data. Only 39 communities had enough rare OTUs to be used in secondary community analysis. This may have biased our results in favor of richer and more even communities. While external sterilization of ticks was used to remove exoskeleton-associated bacteria, it is possible they may play an important role in shaping the internal community. Infection exclusion of different *Anaplasma marginale* strains has been reported in the tick *Dermacentor variabilis*[122]. This phenomenon may be more common in ticks than our findings indicate because the binning of sequences into OTUs masks strain level population dynamics.

CLE, *A. platys*, and FLE

Three of the four dominant OTUs, the two CLEs and FLE, shared > 99% sequence identity with previously characterized tick endosymbionts and one of the three with a tick-borne pathogen[116-121]. The observation of low alpha diversity in every tick is

consistent with previous findings; primarily because most ticks host endosymbionts, and many endosymbiont hosted by ticks closely resemble human pathogens[40]. The most common OTU in the microbiome of *R. sanguineus* was a CLE, which is not surprising as non-pathogenic CLEs are found in most tick species[46]. The consensus sequence of Otu01 shared 100% identity with a *Coxiella*-like endosymbiont (CLE) recently reported in *R. sanguineus* ticks collected in Australia[118] and *Rhipicephalus turanicus* ticks collected in Israel[119]. The consensus sequence Otu08 of another CLE was also found in *Ornithodoros occidentalis* ticks collected in Northeast Africa[123].

A. platys is a small rickettsiae found in the platelets of infected dogs and is the causative agent of anaplasmosis[124]. *R. sanguineus* has been previously suggested as the vector of *A. platys*[125]. Although *A. platys* primarily causes disease in canines, recently two Venezuelan women presenting with headaches and muscle pains were reported to carry *A. platys*. Both patients reported exposure to *R. sanguineus*, one had received a tick bite, the second had acquired a tick infested dog[126].

The difference between pathogens and endosymbionts may not be clear cut. Comparisons between tick endosymbionts and their pathogenic relatives has shown that the evolution of virulence is a two-way street. For instance, *Coxiella burnetii* likely evolved from a non-pathogenic CLE[51,127],whereas certain lineages of pathogenic *Francisella* evolved to become non-pathogenic FLEs[51]. It is possible that *A. platys* is in a position where it is poised to evolve into a tick endosymbiont, if it has not already. Co-infection of *A. platys* and other endosymbionts within a tick may create a dangerous

environment where horizontal gene transfer between pathogens and endosymbionts is possible and give rise to novel zoonotic pathogens.

Similar to known endosymbionts *Francisella* and the *Coxiella*, some *Anaplasma* bacteria are able to synthesize all major vitamins, so they may be beneficial to host ticks[128]. A second similarity between *A. platys* and confirmed tick endosymbionts is that both reside within a host's cells[129]. Both the *Francisella* and the *Coxiella* genera have been described as vertically transmitted endosymbionts[46]. Whereas vertical transmission of *A. platys* has not been documented, it has been observed in the closely related pathogenic bacteria *A. phagocytophilum*[130]. If *A. platys* is also vertically transmitted, this could facilitate evolution of a mutualistic symbiosis between *A. platys* and *R. sanguineus*.

<u>Interaction between dominant microbiome members</u>

Negative interactions between symbionts are not a unique finding. For example, the presence of *Rickettsia* has been observed to reduce the *Buchnera* population in pea aphids[131]and *Spiroplasma* reduces *Wolbachia* populations in fruit flies[132]. Negative interactions among co-occurring bacterial lineages may have important implications for the likelihood of pathogen transmission. Infection exclusion between different members of the *Rickettsia* genus has been documented in ticks[122].

Both the unevenness and the negative interaction observed between CLE and *A. platys* are evidence of competitive exclusion and support the hypothesis that vertically transmitted endosymbionts may act as mutualists either by providing the tick with nutrients or through exclusion of other more virulent horizontally transmitted parasites or both[133]. Although the mechanisms by which CLE and *A. platys* interfere with each other

remains unknown, there are many kinds of interactions between microbes and the tick host which can influence or be influenced by colonization of different bacterial species.

Analysis of communities containing other confirmed pathogens may determine if the presence of certain endosymbionts within *R. sanguineus* decreases the likelihood of acquiring certain pathogenic microbes. The strong negative interaction between CLE and *A. platys* indicates that promoting the success of endosymbionts could be an avenue for disease control. The co-occurrence of endosymbionts and pathogens may be a public health risk and warrant further evaluation.

Conclusion

This study provides evidence that tick endosymbionts have the capacity to interact negatively with pathogens. Ticks feeding from the same host shared endosymbionts. Future studies could compare genomes of endosymbionts and pathogens isolated from the same tick or population of ticks to define the niche each endosymbiont occupies in the tick and how similar it is to that of the pathogen. This would also help in understanding the function of endosymbionts and pathogens within the tick.

REFERENCES CITED

1 Colston, T. J. & Jackson, C. R. Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. *Molecular ecology* **25**, 3776-3800 (2016).

2 Bäckhed, F. *et al.* The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 15718-15723 (2004).

3 Shreiner, A. B., Kao, J. Y. & Young, V. B. The gut microbiome in health and in disease. *Current opinion in gastroenterology* **31**, 69 (2015).

4 Markle, J. G. *et al.* Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* **339**, 1084-1088 (2013).

5 Schloss, P. D. & Handelsman, J. Status of the microbial census. *Microbiology and Molecular Biology Reviews* **68**, 686-691 (2004).

6 Locey, K. J. & Lennon, J. T. Scaling laws predict global microbial diversity. *Proceedings of the National Academy of Sciences* **113**, 5970-5975, doi:10.1073/pnas.1521291113 (2016).

7 Consortium, H. M. P. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207-214 (2012).

8 Turnbaugh, P. J. *et al.* The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* **449**, 804 (2007).

9 Bahrndorff, S., Alemu, T., Alemneh, T. & Lund Nielsen, J. The microbiome of animals: implications for conservation biology. *International journal of genomics* **2016** (2016).

10 May, R. M. How many species are there on earth? *Science(Washington)* **241**, 1441-1449 (1988).

11 Krishnan, M. *et al.* Insect gut microbiome–An unexploited reserve for biotechnological application. *Asian Pacific journal of tropical biomedicine* **4**, S16-S21 (2014).

12 Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. & Ishikawa, H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp.* APS. *Nature* **407**, 81-86 (2000).

13 Ishikawa, H. Biochemical and molecular aspects of endosymbiosis in insects. *International review of cytology* **116**, 1-45 (1989).

14 Campbell, M. A. *et al.* Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. *Proceedings of the National Academy of Sciences* **112**, 10192-10199 (2015).

15 Kirkness, E. F. *et al.* Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle. *Proceedings of the National Academy of Sciences* **107**, 12168-12173 (2010).

16 Akman, L. *et al.* Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature genetics* **32**, 402-407 (2002).

17 Russell, J. A. *et al.* Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences* **106**, 21236-21241 (2009).

18 Lanan, M. C., Rodrigues, P. A. P., Agellon, A., Jansma, P. & Wheeler, D. E. A bacterial filter protects and structures the gut microbiome of an insect. *The ISME journal* **10**, 1866-1876 (2016).

19 Wheeler, D. E. Behavior of the ant, Procryptocerus scabriusculus (Hymenoptera: *Formicidae*), with comparisons to other *Cephalotines*. *Psyche* **91**, 171-192 (1984).

20 Dantas-Torres, F., Chomel, B. B. & Otranto, D. Ticks and tick-borne diseases: a One Health perspective. *Trends in parasitology* **28**, 437-446 (2012).

21 Jongejan, F. & Uilenberg, G. The global importance of ticks. *Parasitology* **129**, S3-S14 (2004).

22 Wormser, G. P. *et al.* Association of specific subtypes of *Borrelia burgdorferi* with hematogenous dissemination in early Lyme disease. *The Journal of infectious diseases* **180**, 720-725 (1999).

23 Swei, A. & Kwan, J. Y. Tick microbiome and pathogen acquisition altered by host blood meal. *The ISME journal* **11**, 813-816 (2017).

24 Mead, P. S. Epidemiology of Lyme disease. *Infectious disease clinics of North America* **29**, 187-210 (2015).

25 Parola, P., Paddock, C. D. & Raoult, D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clinical microbiology reviews* **18**, 719-756 (2005).

26 Estrada-Peña, A. n. Forecasting habitat suitability for ticks and prevention of tick-borne diseases. *Veterinary parasitology* **98**, 111-132 (2001).

27 Ogden, N. H., Lindsay, L. R., Morshed, M., Sockett, P. N. & Artsob, H. The emergence of Lyme disease in Canada. *Canadian Medical Association Journal* **180**, 1221-1224 (2009).

28 Gabriele-Rivet, V. *et al.* Different ecological niches for ticks of public health significance in Canada. *PloS one* **10**, e0131282 (2015).

29 Süss, J., Klaus, C., Gerstengarbe, F. W. & Werner, P. C. What makes ticks tick? Climate change, ticks, and tick-borne diseases. *Journal of travel medicine* **15**, 39-45 (2008).

30 Socolovschi, C., Raoult, D. & Parola, P. Influence of temperature on the attachment of *Rhipicephalus sanguineus* ticks on rabbits. *Clinical Microbiology and Infection* **15**, 326-327 (2009).

31 Parola, P. *et al.* Warmer weather linked to tick attack and emergence of severe rickettsioses. *PLoS neglected tropical diseases* **2**, e338 (2008).

32 Motaleb, M. A. *et al. Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. *Proceedings of the National Academy of Sciences* **97**, 10899-10904 (2000).

33 Tilly, K., Rosa, P. A. & Stewart, P. E. Biology of infection with *Borrelia burgdorferi*. *Infectious disease clinics of North America* **22**, 217-234 (2008).

34 Brisson, D., Drecktrah, D., Eggers, C. H. & Samuels, D. S. Genetics of *Borrelia burgdorferi. Annual review of genetics* **46**, 515-536 (2012).

35 Re III, L., Occi, J. L. & Macgregor, R. R. Identifying the vector of Lyme disease. *American family physician* **69** (2004).

36 Dantas-Torres, F. Rocky Mountain spotted fever. *The Lancet infectious diseases* **7**, 724-732 (2007).

37 Parola, P. & Raoult, D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clinical infectious diseases* **32**, 897-928 (2001).

38 Rehácěk, J. Rickettsia slovaca, the organism and its ecology. (1984).

39 Duron, O. *et al.* Evolutionary changes in symbiont community structure in ticks. *Molecular Ecology* **26**, 2905-2921 (2017).

40 Narasimhan, S. & Fikrig, E. Tick microbiome: the force within. *Trends in parasitology* **31**, 315-323 (2015).

41 Scoles, G. A. Phylogenetic analysis of the *Francisella*-like endosymbionts of Dermacentor ticks. *Journal of medical entomology* **41**, 277-286 (2004).

42 Noda, H., Munderloh, U. G. & Kurtti, T. J. Endosymbionts of ticks and their relationship to *Wolbachia spp.* and tick-borne pathogens of humans and animals. *Applied and environmental microbiology* **63**, 3926-3932 (1997).

43 Niebylski, M. L. *et al. Rickettsia peacockii* sp. nov., a new species infecting wood ticks, Dermacentor andersoni, in western Montana. *International Journal of Systematic and Evolutionary Microbiology* **47**, 446-452 (1997).

44 Voth, D. E. & Heinzen, R. A. Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii. Cellular microbiology* **9**, 829-840 (2007).

45 Moos, A. & Hackstadt, T. Comparative virulence of intra-and interstrain lipopolysaccharide variants of *Coxiella burnetii* in the guinea pig model. *Infection and immunity* **55**, 1144-1150 (1987).

46 Bonnet, S. I., Binetruy, F., Hernández-Jarguín, A. M. & Duron, O. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. *Frontiers in Cellular and Infection Microbiology* **7**, 236 (2017).

47 Smith, T. A., Driscoll, T., Gillespie, J. J. & Raghavan, R. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star tick. *Genome biology and evolution* **7**, 831-838 (2015).

48 Abd, H., Johansson, T., Golovliov, I., Sandström, G. & Forsman, M. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Applied and environmental microbiology* **69**, 600-606 (2003).

49 Niebylski, M. L., Peacock, M. G., Fischer, E. R., Porcella, S. F. & Schwan, T. G. Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. *Applied and Environmental Microbiology* **63**, 3933-3940 (1997).

50 Petersen, J. M., Mead, P. S. & Schriefer, M. E. *Francisella tularensis*: an arthropod-borne pathogen. *Veterinary research* **40**, 1 (2009).

51 Gerhart, J. G., Moses, A. S. & Raghavan, R. A *Francisella*-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. *Scientific reports* **6** (2016).

52 Organization, W. H. A global brief on vector-borne diseases. (2014).

53 Benelli, G. Research in mosquito control: current challenges for a brighter future. *Parasitology research* **114**, 2801-2805 (2015).

54 Sim, S., Jupatanakul, N. & Dimopoulos, G. Mosquito immunity against arboviruses. *Viruses* **6**, 4479-4504 (2014).

55 Cowman, A. F., Berry, D. & Baum, J. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol* **198**, 961-971 (2012).

56 Florens, L. *et al.* A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* **419**, 520-526 (2002).

57 Thangamani, S., Huang, J., Hart, C. E., Guzman, H. & Tesh, R. B. Vertical transmission of Zika virus in *Aedes aegypti* mosquitoes. *The American journal of tropical medicine and hygiene* **95**, 1169-1173 (2016).

58 Dutra, H. L. C. *et al. Wolbachia* blocks currently circulating Zika virus isolates in Brazilian Aedes aegypti mosquitoes. *Cell host & microbe* **19**, 771-774 (2016).

59 Ramirez, J. L. *et al.* Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS neglected tropical diseases* **6**, e1561 (2012).

60 Apte-Deshpande, A., Paingankar, M., Gokhale, M. D. & Deobagkar, D. N. *Serratia odorifera* a midgut inhabitant of *Aedes aegypti* mosquito enhances its susceptibility to dengue-2 virus. *PLoS One* **7**, e40401 (2012).

61 Dong, Y., Manfredini, F. & Dimopoulos, G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS pathogens* **5**, e1000423 (2009).

62 Bennett, K. E. *et al.* Variation in vector competence for dengue 2 virus among 24 collections of Aedes aegypti from Mexico and the United States. *The American Journal of Tropical Medicine and Hygiene* **67**, 85-92 (2002).

63 Douglas, A. E. Lessons from studying insect symbioses. *Cell host & microbe* **10**, 359-367 (2011).

64 Parker, B. J., Spragg, C. J., Altincicek, B. & Gerardo, N. M. Symbiont-mediated protection against fungal pathogens in pea aphids: a role for pathogen specificity? *Applied and environmental microbiology* **79**, 2455-2458 (2013).

65 Moreira, L. A. *et al.* A Wolbachia symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* **139**, 1268-1278 (2009).

66 Hoffmann, A. *et al.* Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* **476**, 454 (2011).

67 O'Donnell, K. L., Bixby, M. A., Morin, K. J., Bradley, D. S. & Vaughan, J. A. Potential of a Northern Population of *Aedes vexans* (Diptera: *Culicidae*) to Transmit Zika Virus. *Journal of medical entomology* (2017).

68 Muturi, E. J., Ramirez, J. L., Rooney, A. P. & Kim, C.-H. Comparative analysis of gut microbiota of mosquito communities in central Illinois. *PLoS neglected tropical diseases* **11**, e0005377 (2017).

69 Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J. & Bordenstein, S. R. Phylosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. *PLoS biology* **14**, e2000225 (2016).

70 Novakova, E. *et al.* Mosquito Microbiome Dynamics, a Background for Prevalence and Seasonality of West Nile Virus. *Frontiers in Microbiology* **8**, doi:10.3389/fmicb.2017.00526 (2017).

71 Prevention, C. f. D. C. a. West Nile Virus Disease Cases and Presumptive Viremic Blood Donors by State – United States, 2016 (as of January 17, 2017). (2016).

72 Program, M. D. C. D. E. West Nile Virus. *Montana.gov* (2015).

73 Go, Y. Y., Balasuriya, U. B. & Lee, C.-k. Zoonotic encephalitides caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. *Clinical and experimental vaccine research* **3**, 58-77 (2014).

74 Marra, P. P. *et al.* West Nile virus and wildlife. *BioScience* **54**, 393-402 (2004).

75 Reiter, P. Climate change and mosquito-borne disease. *Environmental health perspectives* **109**, 141 (2001).

76 Rochlin, I., Ninivaggi, D. V., Hutchinson, M. L. & Farajollahi, A. Climate change and range expansion of the Asian tiger mosquito (*Aedes albopictus*) in Northeastern USA: implications for public health practitioners. *PloS one* **8**, e60874 (2013).

77 Darsie Jr, R. F. & Ward, R. A. Identification and geographical distribution of the mosquitoes of North America, north of Mexico. (WALTER REED ARMY INST OF RESEARCH WASHINGTON DC, 1981).

78 Schloss, P. D. *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* **75**, 7537-7541 (2009).

79 Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology* **79**, 5112-5120 (2013).

80 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200 (2011).

81 Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584 (2016).

82 Cole, J. R. *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids research* **42**, D633-D642 (2013).

83 Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research* **25**, 3389-3402 (1997).

84 Johnson, G. Montana Mosquito Surveillance - Unpublished Data. *Montana Mosquito and Vector Surveillance Program* (2017).

85 Clarke, K. R. & Gorley, R. N. *PRIMER V6: user manual-tutorial*. (Plymouth Marine Laboratory, 2006).

86 Ramette, A. Multivariate analyses in microbial ecology. *FEMS microbiology ecology* **62**, 142-160 (2007).

87 Kaufman, L. & Rousseeuw, P. J. Partitioning around medoids (program pam). *Finding groups in data: an introduction to cluster analysis*, 68-125 (1990).

88 Jones, R. T., Sanchez, L. G. & Fierer, N. A cross-taxon analysis of insect-associated bacterial diversity. *PLoS one* **8**, e61218 (2013).

89 Osei-Poku, J., Mbogo, C., Palmer, W. & Jiggins, F. Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular ecology* **21**, 5138-5150 (2012).

90 Duguma, D. *et al.* Developmental succession of the microbiome of Culex mosquitoes. *BMC microbiology* **15**, 140 (2015).

91 Engel, P. & Moran, N. A. The gut microbiota of insects–diversity in structure and function. *FEMS microbiology reviews* **37**, 699-735 (2013).

92 Lehane, M. Peritrophic matrix structure and function. *Annual review of entomology* **42**, 525-550 (1997).

93 Wang, Y., Gilbreath III, T. M., Kukutla, P., Yan, G. & Xu, J. Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PloS one* **6**, e24767 (2011).

94 Rodgers, F. H., Gendrin, M., Wyer, C. A. & Christophides, G. K. Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. *PLoS Pathogens* **13**, e1006391 (2017).

95 Boissière, A. *et al.* Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS pathogens* **8**, e1002742 (2012).

96 Coon, K. L., Vogel, K. J., Brown, M. R. & Strand, M. R. Mosquitoes rely on their gut microbiota for development. *Molecular ecology* **23**, 2727-2739 (2014).

97 Rueda, L. M. Global diversity of mosquitoes (Insecta: Diptera: *Culicidae*) in freshwater. *Hydrobiologia* **595**, 477-487 (2008).

98 Taylor, M., Mediannikov, O., Raoult, D. & Greub, G. Endosymbiotic bacteria associated with nematodes, ticks and amoebae. *FEMS Immunology & Medical Microbiology* **64**, 21-31 (2012).

99 Papa, A., Tsioka, K., Kontana, A., Papadopoulos, C. & Giadinis, N. Bacterial pathogens and endosymbionts in ticks. *Ticks and tick-borne diseases* **8**, 31-35 (2017).

100 Weiss, B. & Aksoy, S. Microbiome influences on insect host vector competence. *Trends in parasitology* **27**, 514-522 (2011).

101 Round, J. L., O'Connell, R. M. & Mazmanian, S. K. Coordination of tolerogenic immune responses by the commensal microbiota. *Journal of autoimmunity* **34**, J220-J225 (2010).

102 Dantas-Torres, F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806)(Acari: Ixodidae): from taxonomy to control. *Veterinary parasitology* **152**, 173-185 (2008).

103 Schouls, L. M., Van De Pol, I., Rijpkema, S. G. & Schot, C. S. Detection and Identification of *Ehrlichia, Borrelia burgdorferi* Sensu Lato, and *Bartonella* Species in Dutch Ixodes ricinusTicks. *Journal of clinical microbiology* **37**, 2215-2222 (1999).

104 Mixson, T. R. *et al.* Prevalence of *Ehrlichia, Borrelia,* and *Rickettsial* agents in *Amblyomma americanum* (Acari: *Ixodidae*) collected from nine states. *Journal of medical entomology* **43**, 1261-1268 (2006).

105 Dantas-Torres, F. Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. *Parasites & Vectors* **3**, 26 (2010).

106 Ramos, R. A. N. *et al.* Detection of *Anaplasma platys* in dogs and *Rhipicephalus sanguineus* group ticks by a quantitative real-time PCR. *Veterinary parasitology* **205**, 285-288 (2014).

107 Narasimhan, S. *et al.* Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. *Cell host & microbe* **15**, 58-71 (2014).

108 Abraham, N. M. *et al.* Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proceedings of the National Academy of Sciences*, 201613422 (2017).

109 Hegedus, D., Erlandson, M., Gillott, C. & Toprak, U. New insights into peritrophic matrix synthesis, architecture, and function. *Annual review of entomology* **54**, 285-302 (2009).

110 Ahantarig, A., Trinachartvanit, W., Baimai, V. & Grubhoffer, L. Hard ticks and their bacterial endosymbionts (or would be pathogens). *Folia microbiologica* **58**, 419 (2013).

111 Robert K. Strickland, R. R. G., James & L. Hourrigan, a. G. O. S. in *AGRICULTURE HANDBOOK NO. 48 5* (ed UNITED STATES DEPARTMENT OF AGRICULTURE) (1965).

112 Hammer, T. J., Dickerson, J. C. & Fierer, N. Evidence-based recommendations on storing and handling specimens for analyses of insect microbiota. *PeerJ* **3**, e1190 (2015).

113 Hammer, T. J., McMillan, W. O. & Fierer, N. Metamorphosis of a butterfly-associated bacterial community. *PLoS One* **9**, e86995 (2014).

114 Tito, R. Y. *et al.* Insights from characterizing extinct human gut microbiomes. *PloS one* **7**, e51146 (2012).

115 Novakova, E. *et al.* Mosquito Microbiome Dynamics, a Background for Prevalence and Seasonality of West Nile Virus. *Frontiers in microbiology* **8** (2017).

116 Harrus, S. *et al.* Molecular detection of *Ehrlichia canis, Anaplasma bovis, Anaplasma platys, Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. *Clinical Microbiology and Infection* **17**, 459-463 (2011).

117 Ahantarig, A., Trinachartvanit, W., Baimai, V. & Grubhoffer, L. Hard ticks and their bacterial endosymbionts (or would be pathogens). *Folia microbiologica* **58**, 419-428 (2013).

118 Oskam, C. L. *et al.* Molecular investigation into the presence of a *Coxiella sp.* in *Rhipicephalus sanguineus* ticks in Australia. *Veterinary Microbiology* **201**, 141-145 (2017).

119 Gottlieb, Y., Lalzar, I. & Klasson, L. Distinctive genome reduction rates revealed by genomic analyses of two *Coxiella*-like endosymbionts in ticks. *Genome biology and evolution* **7**, 1779-1796 (2015).

120 Sainz, Á. *et al.* Guideline for veterinary practitioners on *canine ehrlichiosis* and *anaplasmosis* in Europe. *Parasites & vectors* **8**, 75 (2015).

121 Mokhtar, A., Lim, S. & Tay, S. Research Note Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. *Trop Biomed* **30**, 345-348 (2013).

122 de la Fuente, J., Blouin, E. F. & Kocan, K. M. Infection exclusion of the rickettsial pathogen *Anaplasma marginale* in the tick vector *Dermacentor variabilis*. *Clinical and diagnostic laboratory immunology* **10**, 182-184 (2003).

123 Duron, O. *et al.* The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. *PLoS pathogens* **11**, e1004892 (2015).

124 Arraga-Alvarado, C., Palmar, M., Parra, O. & Salas, P. *Ehrlichia platys* (*Anaplasma platys*) in dogs from Maracaibo, Venezuela: an ultrastructural study of experimental and natural infections. *Veterinary pathology* **40**, 149-156 (2003).

125 Sanogo, Y. *et al.* First evidence of *Anaplasma platys* in *Rhipicephalus sanguineus* (Acari: Ixodida) collected from dogs in Africa. (2003).

126 Arraga-Alvarado, C. M. *et al.* Molecular evidence of *Anaplasma platys* infection in two women from Venezuela. *The American journal of tropical medicine and hygiene* **91**, 1161-1165 (2014).

127 McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. *Nature reviews. Microbiology* **10**, 13 (2012).

128 Hotopp, J. C. D. *et al.* Comparative genomics of emerging human ehrlichiosis agents. *PLoS genetics* **2**, e21 (2006).

129 Rikihisa, Y. Mechanisms to create a safe haven by members of the family *Anaplasmataceae*. *Annals of the New York Academy of Sciences* **990**, 548-555 (2003).

130 Baldridge, G. D. *et al.* Transovarial transmission of *Francisella*-like endosymbionts and *Anaplasma phagocytophilum* variants in *Dermacentor albipictus* (Acari: *Ixodidae*). *Journal of medical entomology* **46**, 625-632 (2009).

131 Gurfield, N., Grewal, S., Cua, L. S., Torres, P. J. & Kelley, S. T. Endosymbiont interference and microbial diversity of the Pacific coast tick, *Dermacentor occidentalis*, in San Diego County, California. *PeerJ* **5**, e3202 (2017).

132 Goto, S., Anbutsu, H. & Fukatsu, T. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Applied and environmental microbiology* **72**, 4805-4810 (2006).

133 Lively, C. M., Clay, K., Wade, M. J. & Fuqua, C. Competitive co-existence of vertically and horizontally transmitted parasites. *Evolutionary Ecology Research* **7**, 1183-1190 (2005).