The effects of temperature on the microbiome of the ectoparasite *Amblyomma americanum*

THESIS

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20c15(1-5)	20°C/15-days/sample number
20c30(1-5)	20°C/30-days/sample number
33c15(1-5)	33°C/15-days/sample number
33c30(1-5)	40°C/30-days/sample number
40c15(1-5)	40°C/15-days/sample number
40c30(1-5)	40°C/30-days/sample number
$(NH_4)_2SO_4$	ammonium sulfate
BL	Baseline
bp	basepair
BSA	bovine serum albumin
°C	degrees Celsius
DNA	deoxyribonucleic acid
IACUC	Institutional Animal Care and Use Committee
KCl	potassium chloride
K_2SO_4	potassium sulfate
µl	microliter
µM	micromolar
mM	millimolar
ml	milliliter
mm	millimeter

NPMANOVA	non-parametric multivariate analysis of variance
pM	picomolar
PCR	polymerase chain reaction
SD	standard deviation
TAE	tris (hydroxymethyl) aminomethane, acetic acid, ethylenediamine tetraacetic acid
rRNA	ribosomal ribonucleic acid
UV	ultraviolet

Abstract

Ticks are the second leading vector of disease transmission to humans. Though observations of the bacteria carried by ticks started almost a century ago there are still unanswered questions, not least among those being 'what external factors shape the composition of bacteria within the tick'. The answer to this question is integral to our understanding of the viability and virulence of the diseases ticks carry, and may have implications on how to control them and limit their transmission. There are many instances where distribution of diseases carried by ectoparasites do not mimic the vector's habitat range, and given that there has been little research looking specifically at external effects on the microbiome of ticks, we proposed to investigate if temperature influenced the microbiome composition of the ectoparasite *A. americanum* under controlled laboratory conditions. We hypothesized that there would be statistically significant differences among the bacterial community compositions within the microbiomes of *A. americanum* based on the incubation temperature. In order to test this, colony-reared ticks were exposed to environments with several different incubation temperatures for 15 or 30-days. DNA was then collected and sequenced from these ticks and subsequent metagenomic analysis was conducted to investigate the bacterial composition of their microbiomes. Results indicated that there was no significant difference in microbial communities with respect to temperature, but there was in terms of length of incubation.

Introduction

Tick biology

Ticks are obligate, bloodsucking ectoparasites that feed on mammals, reptiles, birds, and amphibians around the world; they are nonpermanent and their life cycle does not depend on the longevity of its host. Ticks are classified in the subclass Acari, which also contains mites. Acari is a subclass of Arachnida, which both ultimately reside in the phylum Arthropoda (Ehle, 2003a). Given these classifications it appears that ticks are more closely related to spiders, mites, and scorpions than they are to insects ("Tick biology," 2011). Ticks are further placed into the order Ixodida which is separated into two main family groups: "hard ticks" (Ixodidae) and "soft tick" (Argasidae). Of the two, hard ticks are much more prevalent ("Tick biology," 2011).

The life cycle of each family are fairly similar. Since this research utilizes an Ixodidae tick, background on Argasidae will be limited. Hard ticks have great uniformity within the family. Many Ixodid ticks have a 3-host-life cycle. All Ixodid ticks have one nymphal stage. In a 3-host-life cycle, a hungry 6-legged larva hatches from an egg and quests for a host. Questing is a behavior exhibited by Ixodid ticks where they climb a blade of grass or similar structure and wait, with their front legs outstretched, for a suitable host to walk by, at which point they latch on (Leonovich, 2015). Once finding a host the tick attaches and begins to feed. During the process of attachment Ixodid ticks secrete a cement-like material from their salivary glands, which aides in a tight attachment. This is important because Ixodid ticks feed slowly and stay with the host for an extended time ranging from a few days to several weeks (Sonenshine, 1992). Once engorged, often resulting in expansion of the tick to three times its original size, the larva detaches from the host, and undergoes ecdysis thus molts into an 8-legged nymph. The process of questing, attaching, feeding, engorging, detaching, and ecdysis repeats with each new host.

Completion of the process for a third time results in the nymphal tick molting into an adult tick. The new adult tick also quests for a host and attaches. While attached to a host adult, Ixodid ticks mate (they only mate while attached to a host). Once mating is complete, females will feed rapidly and detach. While males will generally feed and stay on the host in attempts to mate with another female. The mated females will seek sheltered sites, usually under leaf litter, where they can commence oviposition. Females die shortly after laying their eggs. (Ehle, 2003b). The duration of time spent on the host is variable. It has been reported that during the 2 to 6 year life span of an Ixodid tick, some species spend upwards of 90% of their life on various hosts. (Needham & Teel, 1986).

By contrast to Ixodid ticks, Argasid or soft ticks exhibit immense diversity in their life cycle development. Development is not uniform throughout the family, with each species varying widely in number of nymphal phases, or nymphal instars (Ehle, 2003c). Argasid ticks have a multi-host life cycle. In this the development is gradual and there are often multiple nymphal instars prior to reaching the adult from.

Tick morphology

Ticks lack a distinct head. Their structure is composed of two parts fused together: the capitulum and the body (Figure). The capitulum is where what are considered the mouth parts of the tick are located. The mouth parts are composed of the readily mobile palps; between which are two chelicerae, wherein lies the hypostome. The palps are two, 4-segmented structures position the capitulum for feeding (Ghosh & Misra, 2012) but do not enter the wound of the host. The hypostome is the part of the tick that is actively inserted into the wound of the host. The feeding channel that the hypostome generates is used for the intake of host fluids and injection of

the tick's saliva to the host (Anderson & Magnarelli, 2008). The hypostome have relatively large backward facing *teeth* that help latch the tick to the organism. In addition, most Ixodid ticks produce a cement-like substance that is secreted by the salivary glands ensure attachment to the host. Ixodid ticks have a hard shield, known as the scutum, on the dorsal body surface ("Lone star tick - Amblyomma americanum [Linnaeus]," 2016). The body is where the legs are attached; larval ticks have six legs, while nymphal and adult ticks have eight legs. Also associated to the body is the midgut of the tick, an internal structure constructed of numerous diverticula which are used to digest the blood meal (Yuan, 2010). Most species of ticks exhibit sexual dimorphism, but it is only notable in the adult stage ("Lone star tick - Amblyomma americanum [Linnaeus]," 2016). One common sexual dimorphism trait among Ixodid ticks is size, with the female being larger than the male. Unfed ticks can range in length from 2mm to 20mm. Blood engorged females can vary in length from 25mm to 30mm and weigh up to 100 times their pre-engorged weight (Anderson & Magnarelli, 2008). Male and female ticks can also exhibit different markings. For example, in *Amblyomma americanum*, males have white streaks along the margins of their dorsal body, while females have a lone white spot located centrally on the posterior of the scutum, giving rise to their more common name, the lone star tick.

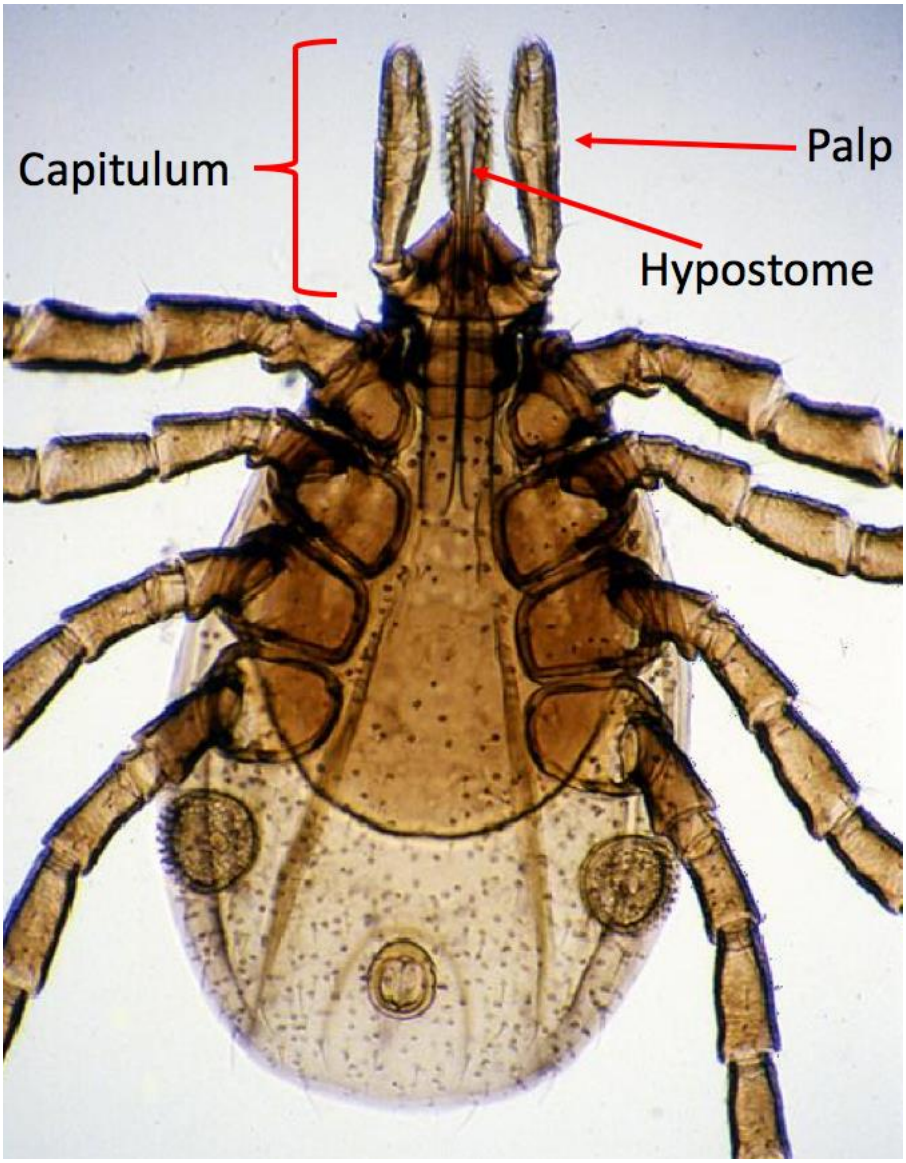


Figure 1. Hard tick structure ("Appearance," 2004)

Ticks and disease

Ranking second only to mosquitos, ticks serve as a supremely effective vector in disease transmission to humans and animals (Yuan, 2010). Ticks carry a wide array of human pathogens, including bacterial pathogens such as the causative agent of Lyme disease, human monocytic ehrlichiosis, Rocky Mountain spotted fever, and tularemia; protozoan pathogens including babesiosis and cytauxzoonosis; and viral infections such as Powassan encephalitis and Colorado tick fever. Ticks can also cause toxic conditions such as toxicosis and paralysis, as well as elicit an allergic reaction and irritation at the bite site.

Ticks also transmit a plethora of diseases to livestock, companion animals, and wild life which are subject to the conditions seen in humans, including allergic reactions, toxicosis, and tick paralysis. Ticks are also a huge concern as pests, as severe tick infestations can lead to livestock skin injuries that may lead to open wounds that can become infected. Animals subjected to heavy tick burdens can also present with weight loss, be more prone to having abortions, and demonstrate decreased milk production (Norval, Sutherst, Jorgensen, & Kerr, 1997). Without investments in major tick control, livestock production is almost impossible in some areas; it has been estimated in places such as Tanzania that $384 million USD is lost annually for example, due to tick-borne diseases of livestock (Kivaria, 2006).

It has been hypothesized that ticks are successful vectors of disease because their midgut is a less hostile environment as compared to other blood-sucking vectors (Anderson, 2002). One reason for this is the nearly absolute absence of intraluminal proteolytic enzymes, as digestion in ticks is almost completely intracellular (Sonenshine, Hynes, Ceraul, Mitchell, & Benzine; 2005). In addition, the digestion of a blood meal occurs over an extended period of time, enabling pathogens to survive and multiply.

Amblyomma americanum

A. americanum, more commonly referred to as the lone star tick, is a three host Ixodid tick and was the first tick species to be characterized in the United States (Childs & Paddock, 2003). These ticks have very aggressive feeding patterns and appear to lack discrete host preferences, readily feeding on humans, bovids, cervids, canids, and rodents (Bishopp & Trembley, 1945). They are also a very abundant tick, a 10-year study of human tick bites found that 53% of all ticks recovered from humans in Mississippi were *A. americanum* (Goddard, 2002), and a study in Georgia and South Carolina found that 758 (83%) of 913 ticks removed from 460 persons were lone star ticks (Felz, Durden, & Oliver, 1996). Their abundances is also felt by animal populations, a study out of Oklahoma State University show entomologists indicated that up to 57% of all new white-tailed deer fawns within certain areas of the Ozark region die each year due to heavy lone star tick infestations (Goddard & Varela-Stokes, 2009). They are known vectors for pathogens such as *Francisella tularensis* and *Ehrlichia chaffeensis,* which cause tularemia and ehrlichiosis, respectively. In addition, they can transmit *Borrelia lonestari*, which has been suggested as the causative agent of southern tick-associated rash illness ([STARI]; James et al., 2001).

A. americanum have a large range in North America, covering a vast amount of the Eastern and Southeastern United States. They range as far south as the tips of Texas and Florida, and north to the coastlines of Maine. One study has shown that behaviorally *A. americanum* tend to be dormant in the winter and active in the spring and summer months; however, in southern and southeastern states their presence can be noted year round (Menchaca et al., 2013). Curiously, diseases the lone star ticks are able to transmit do not mimic their geographical

distribution, a pattern that is evident in other tick species as well. For example, according to the CDC, tularemia is mostly reported in Kansas, Missouri, Arkansas, and Oklahoma (CDC, 2015a), though the *A. americanum* vector has a much broader distribution. Given that all of the necessary components for transmission are present in a large portion of the United States, logically the disease should be present evenly across the tick range. That this is not true may be attributed to environmental factors such as temperature differences across the range of *A. americanum* having a direct or indirect impact on the bacterial microbiomes of these organisms.

Microbiome

The term microbiome was first used by Joshua Lederberg, Vence, Staff, and Grens (2001) "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share [the same] space". The microbiome of an organism is a dynamic environment that can be changed by various factors, including the host feeding, immune system, transient community members, host lifestyle, and external environment such as temperature and humidity (Turnbaugh et al., 2007). A review by Narasimhan and Fikrig (2014) presents data from multiple studies that have characterized the microbiomes of various tick species, illustrating that though ticks are small they certainly do not lack a complex microbiome. One study presented in the review researchers dissected tick, separated out various organelles, and tested each individually and found that each had specific microbiomes with predominant bacterial make-ups (Narasimhan et al., 2014). Also described in the review, data reported from Mixson and colleagues (2006), which shows ticks can be co-infected with more than one pathogen and can host various endosymbiotic bacteria (Mixson et al., 2006). Interestingly reported data shows that directly manipulating the tick microbiome can lead to

changes in their biological functions, supported by a study that showed after curing *Amblyomma americanum* of *Coxiella*, a known endosymbiont, the ticks presented with a reduction in reproductive fitness (Zhong, Jasinskas, & Barbour, 2007). Lastly, data reported from a study illustrated the that the microbiomes of ticks appear to have plasticity given changes in their immediate environments, age, and fed status (Menchaca et al., 2013). These data are promising for future studies as they suggest that microbial populations within the tick are dynamic.

16S Ribosomal RNA metagenomic analysis

The microbiome is most often studied via metagenomic analysis. Metagenomic analysis is a sequence-based approach that allows for the genetic material of a set of microbes to be analyzed without needing to grow the various organisms (Santamaria et al., 2012). This means that organisms can be processed from their natural environments, such as the microbiome from a tick, and can be extracted directly from the sample itself with no middle cultivating step. This technique also allows for the determination of the relative abundance of various microbes within one sample.

The leading approach in metagenomic analysis is utilizing the 16S ribosomal RNA (rRNA) gene (Figure 2) a gene that codes for a component of the small ribosomal subunit in bacteria and archaeal species. The ribosome is almost universally distributed among all microorganisms (Woese et al., 1975). Because 16S rRNA has an essential role in the structure and proper functioning of the ribosome, evolution has stringently preserved the gene sequence among bacterial and archaeal species, thus making it a prime target when examining said populations. Among the highly conserved regions in the 16S rRNA gene there are nine hypervariable regions with a considerable amount of sequence diversity that can be used to

differentiate between bacterial taxa. Work with *E. coli* indicates the nine variable regions are located at nucleotides 69-99, 137-242, 433-497, 576-682, 822- 879, 986-1043, 1117-1173, 1243-1294, and 1435-1465 respectively within the gene for V1 to V9 (Illumina Inc, 2012).

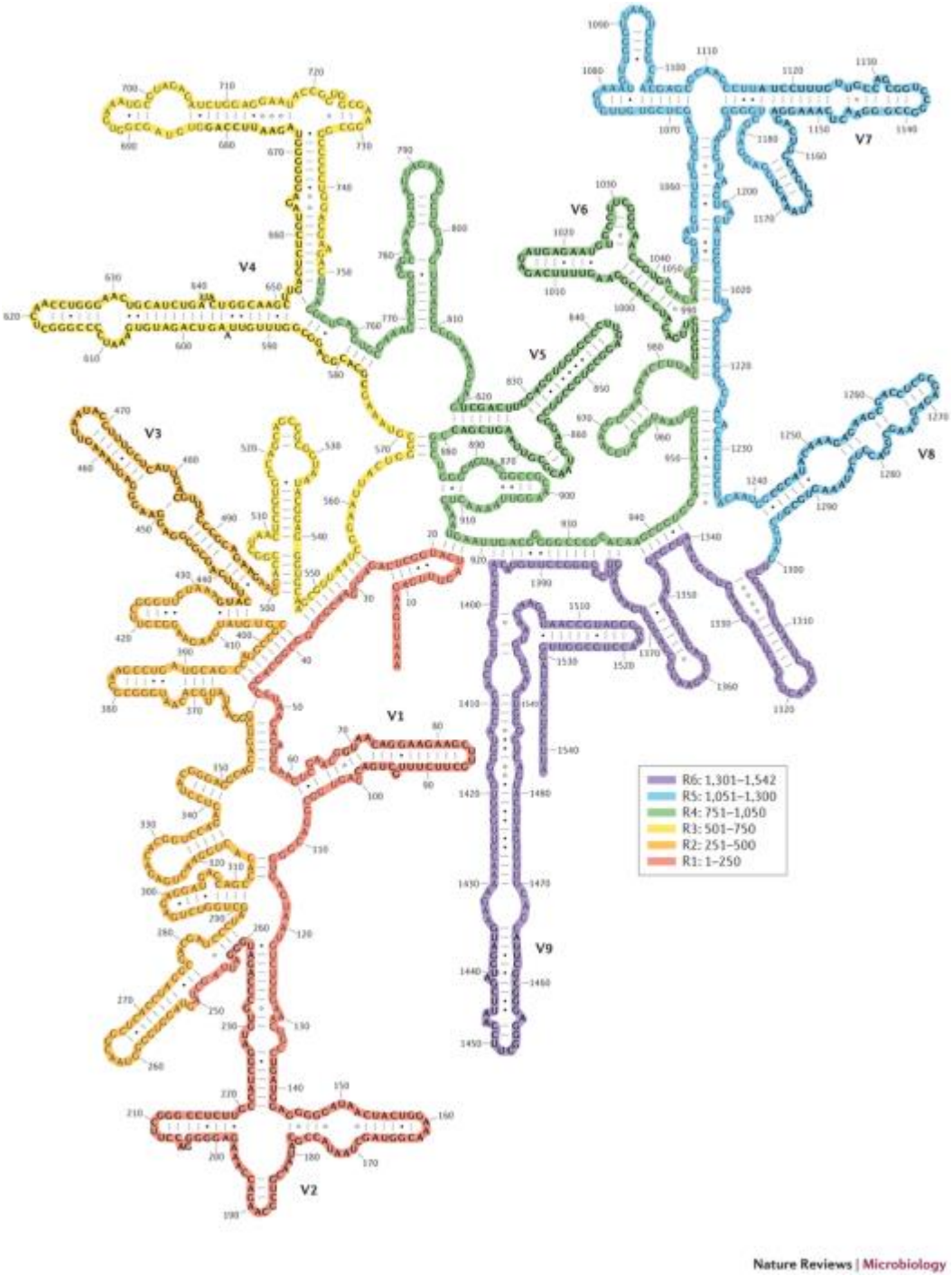


Figure 2. 16S rRNA gene (Yarza et al., 2014)

While 16S analysis has made an everlasting impact in the field of metagenomics, it is not without limitations (Benbow, Tomberlin, & Tarone, 2015); including, but not limited to, DNA extraction kit differences, PCR amplification bias and errors, DNA copy number, and primer design. A study by Stach and colleagues (2001) tested the first limitation listed by taking a single soil sample and processing it using five different DNA extraction kits. Results showed a wide array of purity of samples based on which kit was used, which suggested this was a limiting factor in true analysis of population diversity.

The physiological characteristics of bacteria present in a population also have a commanding role in extraction efficacy of a given DNA extraction kit. Gram-positive bacteria are not only more sturdy than gram-negative bacteria, but some also have the ability to form spores, which requires more thorough lysing step in the extraction process. This can result in one of two outcomes . (1) If a kit with standard lysing protocol is used there can be an insufficient capture of all bacteria present secondary to not lysing the spores or the sturdier gram-positive organisms. (2) If an increased lysing protocol is used it can lead to the shearing of the genomic DNA of organisms that are more readily lysed (Jiang et al., 2011). One of the most problematic PCR amplification errors is the generation of sequence artifacts. Examples of such artifacts are chimeras, novel sequences arising from the combination of two or more parental DNA strands that are inappropriately annealed during PCR. These molecules are considered misleading because researchers may mistakenly identify them as novel organisms (Hugenholtz & Huber, 2003). PCR bias can result in representative skewing of PCR products within a sample, secondary to unequal amplification, which can arise from amplification difference in the DNA templates themselves or inhibition present in the samples being analyzed (Polz & Cavanaugh, 1998). Bias from the templates themselves can include copy number bias, which is the false

appearance of relative abundance of a given organism in a sample. This occurs as a consequence of organisms having differences in the number of the 16S rRNA genes present. For example, if bacteria A has four copies of the 16S rRNA gene as opposed to organism B that only has one copy, organism A will appear to have a greater relative abundance (Kembel, Wu, Eisen, & Green, 2012). Lastly, primer design can affect the bacterial species that will and will not be amplified. Not only is there not one universal primer than can be used in amplification of all organism, but each primer has intrinsic binding affinity, thus possibly resulting in the accidental missing of some bacterial species that are present (Bergmann et al., 2011).

Materials and Methods

Temperature selection

Temperature selection was determined by studying maps denoting the geographic distribution of *A. americanum* (Figure 3) and maps denoting the geographic distribution of diseases they carry were studied (CDC, 2015b). Subsequently, general geographical areas that possessed the same hosts and vectors, but with differing rates of infection, were identified. The average summer and winter temperatures of these areas were identified using U.S. climate data (NOAA, 2015). Based on the data observed and feasibility in a laboratory environment, incubation temperatures of 4°C, 20°C, 33°C, and 40°C were chosen. This method of temperature selection is not present in literature, rather it was implemented for its logical application.

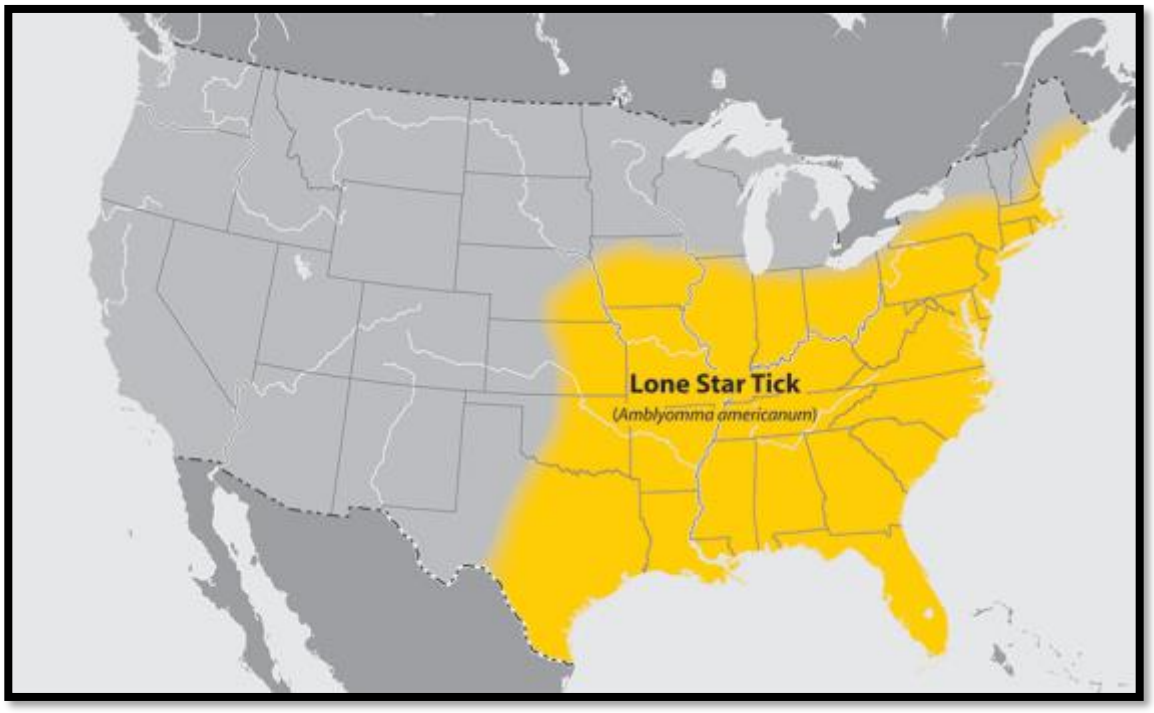


Figure 3. Geographic distribution of ticks that bite humans. CDC.

Tick collection

Currently, there is no Institutional Animal Care and Use Committee (IACUC) protocol for invertebrate animal sampling, handling, or processing (this includes ticks). Live adult *A. americanum* were obtained from a colony maintained at the Tick Research Laboratory, Texas AgriLife Research, at Texas A&M University, College Station, TX. There domestic chickens (*Gallus gallus)* are used as hosts for blood meals, which is approved under Animal Use Protocol No. 2011-213 (Menchaca et al., 2013). The samples were sent in glass containers with damp paper towels as a source of humidity for the ticks.

Separation and incubation

Upon receiving the live ticks from Texas AgriLife Research, individuals were randomly placed into four separate autoclaved glass mason jars, which served as their housing for the duration of the study. The glass mason jars were meshed covered to allow the conditions in the jar to equilibrate with conditions of the environmental chambers in which they were placed. Mesh material was also placed inside the mason jar to give the ticks material to climb on and hide within (Figure 4). Each jar held five males and five females, for a total of 10 ticks per jar. The only preference in selection, with later extraction in mind, was a visually larger size, though this still fluctuated. The incubation times covered two periods, with half of the ticks from each mason jar being removed after 15-days of incubation and the other half being removed after 30-days of incubation. The males were not processed in this study in order to keep confounding variables at a minimum.

The literature indicates that ticks survive best in environments where the relative humidity (RH) is kept around 80% to 90% (Rodgers, Zolnik, & Mather, 2007), which was

achieved through including vessels containing concentrated salt solutions in the different environmental chambers. All temperatures and humidities were monitored using 3 Onset HOBO Data Loggers (ONSET Cape Cod, Massachusetts, USA). Appropriate salt solutions for maintenance of humidity at given temperatures were indicated by Winston and Bates (1960): ammonium sulfate [$(NH_4)_2SO_4$] was used for 4°C, potassium chloride (KCl) was used for 20°C and 33°C, and potassium sulfate (K_2SO_4) was used for 40°C. The solution mixture was formulated using the MiTeGen guide of saturated solutions of salt (Thorne, 2005).

Temperature control of 33°C and 40°C was done utilizing two separate incubators. The mason jars containing the ticks, as well as the vessels containing the saturated salt solutions, were placed in Styrofoam boxes wrapped with cellophane within the incubators, because the incubators had inherent RH regulators, which distorted the desired RH (Figure 5).

Temperature control of 20°C was done by keeping the mason jars and saturated salt solution vessel is a large plastic container within the lab, which was kept at 20°C. The tub was also wrapped with cellophane to prevent leaking of internal conditions, which occurred prior to the addition of the cellophane (Figure 6).

Temperature control of 4°C was done by using a drawer from the lab refrigerator. The mason jar containing the ticks and the vessel containing the saturated salt solution were placed in the drawer. The drawer was wrapped in cellophane and placed in the refrigerator (Figure 7).

Figure 4. Mason jar containing ticks

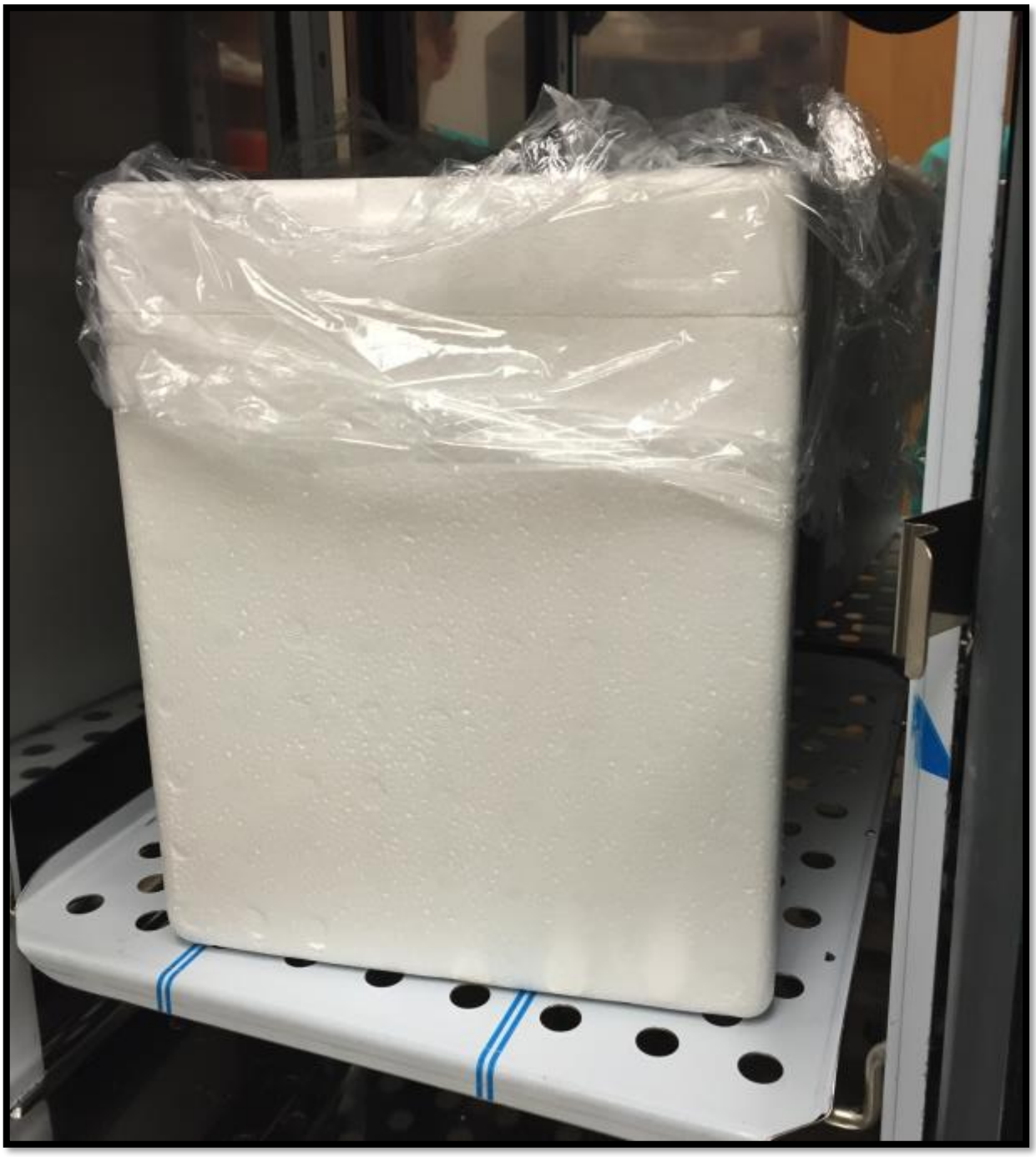


Figure 5. 33°C and 40°C housing

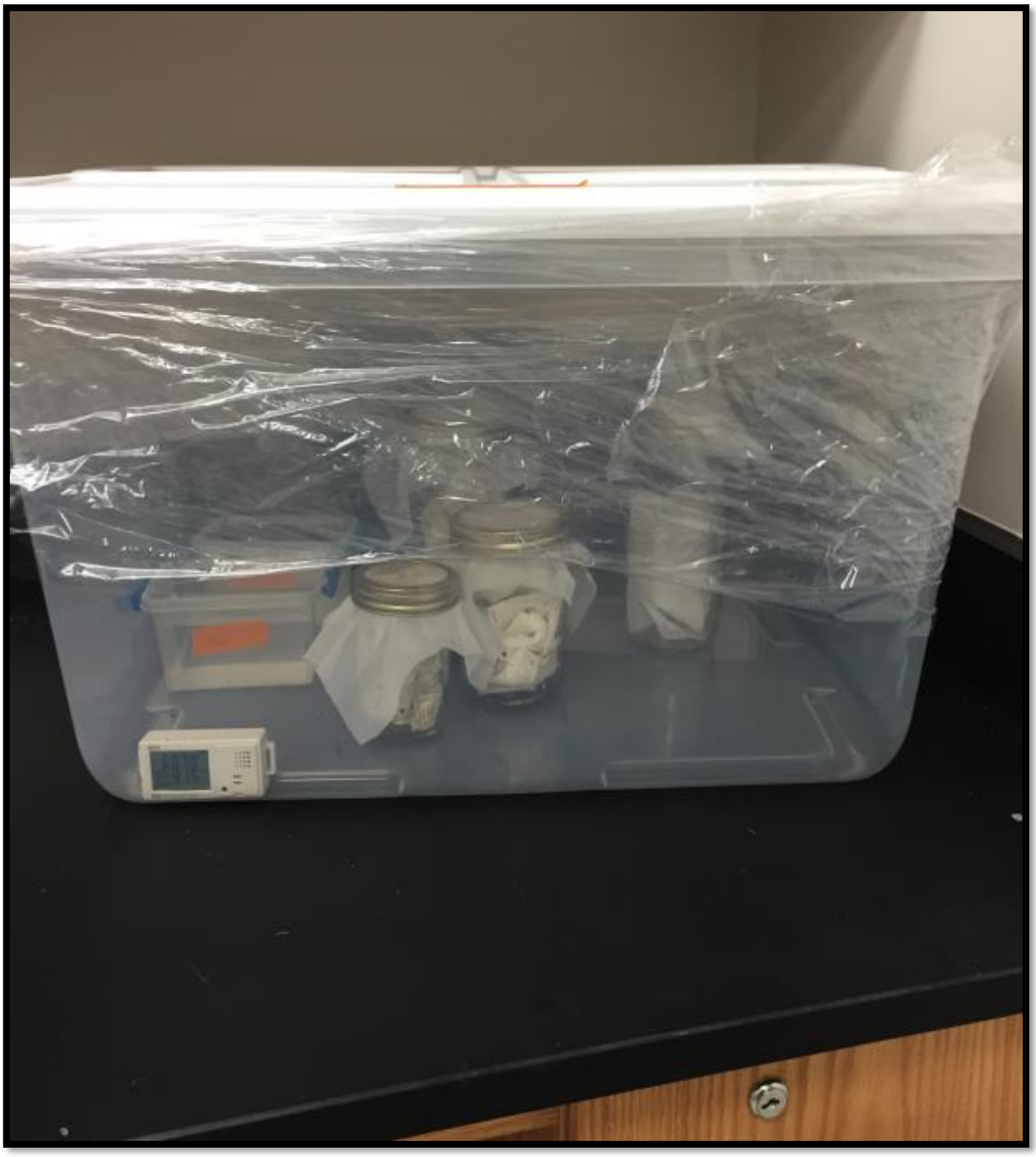


Figure 6. 20°C housing

Figure 7. 4°C housing

Extraction

Ticks were removed from conditions on 2 separate days, 15-days after incubation and 30-days after incubation. Prior to extraction, five female ticks were removed from each mason jar, placed in a 6-1/2 in. x 5-7/8 in sandwich bag, and immediately placed in the freezer (-18°C) which is how euthanization occurred. Ticks were labeled with their temperature, incubation time, and number one to five as an identifier. For example, the first tick being processed at day 15 from 4°C was labeled 4c151; this notation was used for the duration of the experiment. The other non-degree label is BL which represents the baseline ticks, those that were euthanized right when they were received, and which the rest of the ticks were compared against during analysis.

Once euthanization was complete ticks were then processed for DNA extraction using the E.Z.N.A® Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA) with minor modifications that have been optimized from previous work in our lab. For surface sterilization ticks were dipped in a 10% bleach solution followed by a dip in molecular grade water and, after which they were allowed to dry. Once dry each tick was dissected into eight different sections with a sterile scalpel and the entire tick was placed into a screw-capped 2ml FastPrep tube (MP Biomedicals, LLC., Santa Ana, CA, USA) containing 350µL ML1 Buffer and 25µL Proteinase K Solution and sterile 2.8mm ceramic beads (MoBio Laboratories Inc., Carlsbad, CA, USA). The tubes were then placed in the FastPrep-24™ 5G Instrument (MP Biomedicals, LLC. Santa Ana, CA, USA) for homogenization; it works by utilizing multidirectional, simultaneous impaction of tissue with the aforementioned beads. The FastPrep-24™ 5G Instrument was set to three cycles at a speed of 7m/sec for 1 minute, with a pause time of 1 minute between each sample. Tubes were then placed on a heatblock at 60°C for a minimum of 60 minutes. Three hundred fifty microliters of cholofrom:isoamyl alcohol (24:1) was used for liquid-liquid extraction. Once vortexed the top

aqueous layer was transferred to a new 1.5mL microcentrifuge tube, being careful to avoid the contaminant and inhibitor-rich middle milky interface layer. Amounts transferred ranged from 250µl to 320µl, with the majority of samples being at 300µl. Based on the amount transferred into the new 1.5mL microcentrifuge tube, an equivalent volume of the MBL buffer was added (i.e., if 300µl of sample was transferred then 300µl MBL was used). To this 10µl RNase A was added, an enzyme that results in the breakdown of ribonucleic acid (RNA) into oligonucleotides and smaller molecules. This mixture was vortexed and incubated on a heating block at 70°C for 10 minutes, after which one volume of 100% ethanol was added and mixed thoroughly.

The mixture was transferred 750µl at a time into a HiBind® DNA Mini Column placed inside a collection tube and vortexed to discard filtrate, this was repeated until all of the sample was successfully transferred, at which point a new collection tube was used. The HiBind® DNA Mini Column was prepared by adding 100µl of 3M NaOH to the tube and centrifuging it at max speed for 60 seconds, the filtrate was subsequently discarded (this step is optional, but was followed for column equilibration).

Five hundred microliters of HBC buffer was placed into the HiBind® DNA Mini Column, vortexed, and the filtrate discarded. The sample was washed twice with a DNA wash. Finally, the HiBind® DNA Mini Column was transferred to a clean 1.5mL microcentrifuge tube for the final collection of sample. Fifty microliters of the elution buffer was added, the tube was vortexed, these steps were repeated for a final elution volume of 100µl; this amount has been optimized from previous work with these samples.

DNA amplification

AccuPrime™ *Taq* DNA Polymerase high fidelity system (ThermoFisher Scientific, Waltham, MA) was used for amplification of all samples. In all cases a master mix was used in preparation of groups of samples. Amplification was performed in 0.2ml PCR tubes containing a standard master mix solution of approximately 25µl composed of: 5µl of 10X AccuPrime PCR Buffer, 0.5µl of forward primer (10µM), 0.5µl of reverse primer (10µM,) 2.5µl of 10X BSA (1mg/µl), 1µl of extra magnesium, 0.3µl of AccuPrime Taq High Fidelity (5U/µl), 10µl of DNA, and 5.2µl of molecular grade water. An appropriate positive control, 10µl of known *Escherichia coli* DNA, and an appropriate negative, 10µl of molecular grade water, were created the same way for every master mix created.

Variable region V4 of the prokaryotic 16S ribosomal RNA gene (16S rRNA) was targeted for amplification. 16S Amplicon PCR Forward Primer: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA

16S Amplicon PCR Reverse Primer: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT (Illumina Inc, San Diego, CA).

Amplification was carried out in a BioRad C1000TM thermal cycler (Bio-Rad, Hercules, CA). PCR cycling parameters were as follows: an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles consisting of denaturation at 94°C for 15 seconds, primer annealing at 55°C for 15 seconds, and extension at 68°C for 20 seconds, with a final extension step at 68°C for 5 minutes and a 4°C indefinite hold.

Gel electrophoresis

Amplicons were separated via gel electrophoresis using a 1.5% ethidium bromide TAE agarose gel with 1X TAE buffer. Reaction products were visualized under UV light with an EC3 transilluminator imaging system (UVP BioImaging Systems, Upland, CA).

Sequencing using Illumina

One of the leading high throughput, or more commonly called next-generation sequencing, approaches is sequencing by synthesis, a proprietary process created by Illumina, Inc (Illumina, Inc., San Diego, CA, USA). Sequencing with this technology starts with tagging extracted and purified DNA. In doing so the target DNA is flanked with sequencing primers, indices, and regions that are complementary to an oligo connected to the flow cell, a specialized glass slide. The flow cell is a glass apparatus with lanes coated with two types of oligos that bind to complementary regions added to the DNA. This allows for DNA to hybridize and undergo clonal amplification via bridge amplification; which occurs simultaneously for millions of clusters. Once bridge amplification is completed the reverse strands are washed away leaving behind only the forward strands.

Sequencing begins with the extension of the sequencing primer bound to the template DNA. Four fluorescently-tagged and terminator-capped nucleotides compete for attachment to the growing chain, and only one is incorporated based on the sequence of the template strand; while the rest are washed away. After the addition of a single nucleotide, the clusters are excited by a light source and a characteristic fluorescent signal is produced indicating the base was successfully incorporated. Both the fluorescent molecule and terminator cap are then washed away and the process is repeated. This occurs in parallel with hundreds of thousands of clusters at a time and happens first for the forward strand and subsequently for the reverse strand. Cycle

number determines the length of the read. Once complete, sequences of pooled sample libraries are separated in silico by the index regions added to the DNA during preparation. Data can then be compared to the reference genome if doing a whole genome study, or the resulting amplicon sequences can be identified using a third party database (Chakravorty, Helb, Burday, Connell, & Alland, 2007).

For this research, in all cases, pre- and post-PCR handling was done in separate lab spaces to prevent contamination. PCR assays were done in duplicate for samples that had strong amplification and in triplicate for samples that had weak amplification (Table 1).

For samples that had strong initial amplification, a total of two PCRs per sample were was carried out using a master mix solution containing: 7.7µl of molecular grade water, 2.5µl of 10X AccuPrime PCR Buffer, 0.5µl of forward primer (10µM), 0.5µl of reverse primer (10µM,) 2.5µl of 10X BSA (1mg/µl), 1µl of extra magnesium, 0.3µl of AccuPrime Taq High Fidelity (5U/µl), and 10µl of DNA (Table 2).

For samples that weakly amplified during the initial PCR, triplicate PCRs were carried out using a master mix solution of: 2.5µl of 10X AccuPrime PCR Buffer, 0.5µl of forward primer (10µM), 0.5µl of reverse primer (10µM,) 2.5µl of 10X BSA (1mg/µl), 1µl of extra magnesium, 0.3µl of AccuPrime Taq High Fidelity (5U/µl), and 17.7µl of DNA (Table 2).

Sequencing preparation was done according to the Illumina guide "16S Metagenomic Sequencing Library Preparation-*preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*" (Illumina Inc, San Diego, CA). Upon completion of all PCRs, all like samples were combined, (i.e., all BL1 samples were put into a single 0.2ml PCR tube). Twenty-five microliters of each of the newly combined samples was then transferred into a 96 well MIDI plate for the first round of PCR clean up. The clean-up step used AMPure XP beads (Beckman

Coulter, Inc, Brea, CA) for purification by magnetic separation to rid the samples of free primers and primer dimers. A single channel pipet was used and the protocol was followed with no changes. At the completion of clean-up he MIDI plate containing the samples was covered with a Microseal "A" adhesive seal and stored at -20°C.

Table 1. Table showing which samples were run in PCR duplicate and triplicate. PCR assays were done in duplicate for samples that had strong amplification and in triplicate for samples that had weak amplification

Duplicate	4c151, 4c152, 4c301, 20c151, 20c152, 20c154, 20c302, 20c303, 20c305, 33c15, 33c154, 33c301, 33c302, 33c303, 33c304, 33c305, 40c151, 40c152, 40c153, 40c302, 40c303, 40c305
Triplicate	BL1, BL2, BL3, 4c153, 4c154, 4c155, 4c304, 4c305, 20c153, 20c155, 33c155

Table 2. Table showing DNA volume used for PCR amplification. Dashes indicate no sample in that category.

10µl of DNA	**17.7µl of DNA**
_____	BL1 BL2 BL3
4c151 4c152	4c153 4c154 4c155
20c151 20c152 20c154	20c153 20c155
33c151 33c153	33c155
40c151 40c152 40c153	_____
4c301	4c304 4c305
20c302 20c303 20c304 20c305	_____
33c301 33c302 33c303 33c304 33c305	_____
40c302 40c303 40c305	_____

Next, samples were prepared for index PCR, which attached dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. Indices were added because these samples were multiplexed with others from another study. As previously, a master mix solution was generated with 10X AccuPrime PCR Buffer and AccuPrime Taq High Fidelity were used in place of the 2x KAPA HiFi HotStart Ready Mix, second to optimization from previous work in the lab; the protocol was otherwise followed stringently. Each TruSeq well on the Index Plate Fixture included 5µl 10X AccuPrime PCR Buffer, 5µl Nextera XT Index Primer 1 (N7xx), 5µl Nextera XT Index Primer 2 (S5xx), 0.2µl AccuPrime Taq High Fidelity (5U/µl), 5µl DNA, and 29.8µl of molecular grade water; totaling to 45µl. Once set up was complete the TruSeq Index Plate Fixture was placed in a BioRad C1000TM thermal cycler, with cycling perimeters set for an initial denaturation step at 94°C for 3 minutes, followed by 8 cycles consisting of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 68°C for 30 seconds, followed by a final extension step at 68°C for 5 minutes and a 4°C indefinite hold.

After index PCR the samples went through a second clean up, purifying the final library as much as possible before quantification. As with the first clean up step, the second used AMPure XP beads and a 96 well MIDI plate. Both a multichannel and single channel pipet were used and the protocol was followed exactly. The MIDI plate containing the samples was covered with a Microseal "A" adhesive seal and stored at -20°C.

Samples were quantified using a modified PicoGreen protocol generated by our lab and using 96-well plate. A PicoGreen working solution was generated (1:200 dilution) with 148µl of solution was pipetted into each well to be used. Subsequently, 2µl of DNA was added to a corresponding well and mixed, resulting in a 150µl volume for every well. This procedure was done to generate a working standard in two of the well columns. After incubation at room

temperature, fluorescence was measured in the Synergy 2 Multi-Mode Reader (BioTek Instruments, Winooski, VT). Concentrations were computed by the Gen5 Microplate Reader and Imager Software (BioTek Instruments, Winooski, VT). For this study the MiSeq was set to 500 cycles, therefore the average library size was 500. This was done for every sample. Samples were then diluted with 10mM Tris pH=8.5 to 4 nM.

Next was library denaturing and MiSeq loading. First, denaturing of DNA was accomplished by following the protocol: 5µl of 4 nM DNA and 0.2µl nM NaOH were combined, centrifuged, and incubated for 5 minutes at room temperature to denature DNA to single strands. Nin hundred ninety microliters of HT1 was added, resulting in a 20pM denatured library in 1mM NaOH. The 20pM denatured library was then diluted to a 12pM denatured library by adding together 300µl of the 20pM denatured library and 240µl of HT1.

Per the protocol, a 5% solution of PhiX DNA had to be created for the run to serve as an internal control for low diversity libraries. This was generated by first diluting the PhiX library to 4nM. 2µl of 10nM PhiX library was diluted with 3µl of 10nM Tris pH 8.5. The 4nM PhiX was then denatured with 5µl of 0.2N NaOH. It was then vortexed and incubated at room temperature for 5 minutes to denature DNA to single strands. 10µl of the denatured PhiX library was then mixed with 990µl of HT1, generating a 2-pM PhiX library. The denatured 20pM PhiX library was then diluted to a 12pM denatured PhiX library in the same way the amplicon library was created.

The amplicon library and PhiX control were combined, adding 30µl of the denatured/diluted PhiX control and 570µl denatured/diluted amplicon library together into a microcentrifuge tube. The combined libraries were then heat denatured at 96°C for 2 minutes, inverted a five to six times for mixing, and placed in an ice water bath for 5 minutes.

Immediately after the 5 minute ice bath the library was loaded into the MiSeq V2 reagent cartridge and loaded onto the MiSeq.

Once the run was complete the data were uploaded into BaseSpace, a program Illumina offers to facilitate data sharing and processing through apps. The raw data was processed using the QIIME software package (Caporaso et al., 2010). To begin this processes, QIIME preprocessing requires a mapping file that can be generated with Google Docs Spreadsheet (Google, Mountain View, CA; [Appendix 3]). Processing with QIIME has two steps, the initial step uses the QIIME preprocessing application and the second uses the QIIME visualization application. The preprocessing of 16S rRNA raw sequences includes de-multiplexing, quality filtering by removing ambiguous or low-quality reads, and identifying operational taxonomic units (OTUs) present in the samples at 97% identity, which were determined and classified according to the taxonomy of the Greengenes database (DeSantis et al., 2006; Lecuit et al., 2004; Ley, Peterson, & Gordon, 2006; Schloss & Handelsman, 2006). Once the raw data is preprocessed, a file is generated that can be opened using the QIIME visualization app. This app then generates various visualization aids, and output files that can be loaded into various programs for further analysis

Statistical analysis

Statistical analyses were performed using Microsoft Excel for Mac, version 15.22 (Microsoft Corporation, Redmond, WA, USA), and R Studio (http://www.r-project.org), with which the vegan package was used; an R program designed for analysis of microbiome data (Oksanen et al., 2016). Sample data were manually reduced to contain only bacterial genera that had made up at least 1% of the total raw data, for refining purposes. Upon completion there were

16 bacterial genera to compare. All statistical tests were performed using relative frequencies of bacterial species within a sample.

Results

Microbiome

An overall total of 5,259,325 sequence reads were analyzed with a mean of 159,373.50 per sample (standard deviation [SD], 262,574.96), which corresponded to 16 operational taxonomic units (OTUs) at the genus level with a mean of 14.15 OTUs (SD, 2.55) per sample, at a cutoff of 97% identity level. A heat map was generated for an initial observation of the bacterial communities (Figure 8). To evaluate any clustering of samples, a dendrogram was generated ([Figure 9] Oksanen et al., 2016) that illustrates the relationships of similarity among the groups. Upon examination of the dendrogram a slight clustering based on length of incubation can be seen. All baseline (BL) samples are localized in a single clade (highlighted) indicating greatest similarity, and a deviation from this clade can be noted in all other samples with moderate, but still evident, clusters of 15-day and 30-day samples (15-days in blue, 30-days in orange). Contrary to the prediction, there did not appear to be any clustering based on incubation temperature. Based on these findings, relative abundance graphs were generated for comparison of baseline samples to 15-day samples, and baseline samples to 30-day samples (Figure 10, Figure 11).

Figure 8. Relative abundance of major genera per sample.
Each column represents a specific bacterial genus. Each row represents a specific sample. See text for sample name nomenclature.

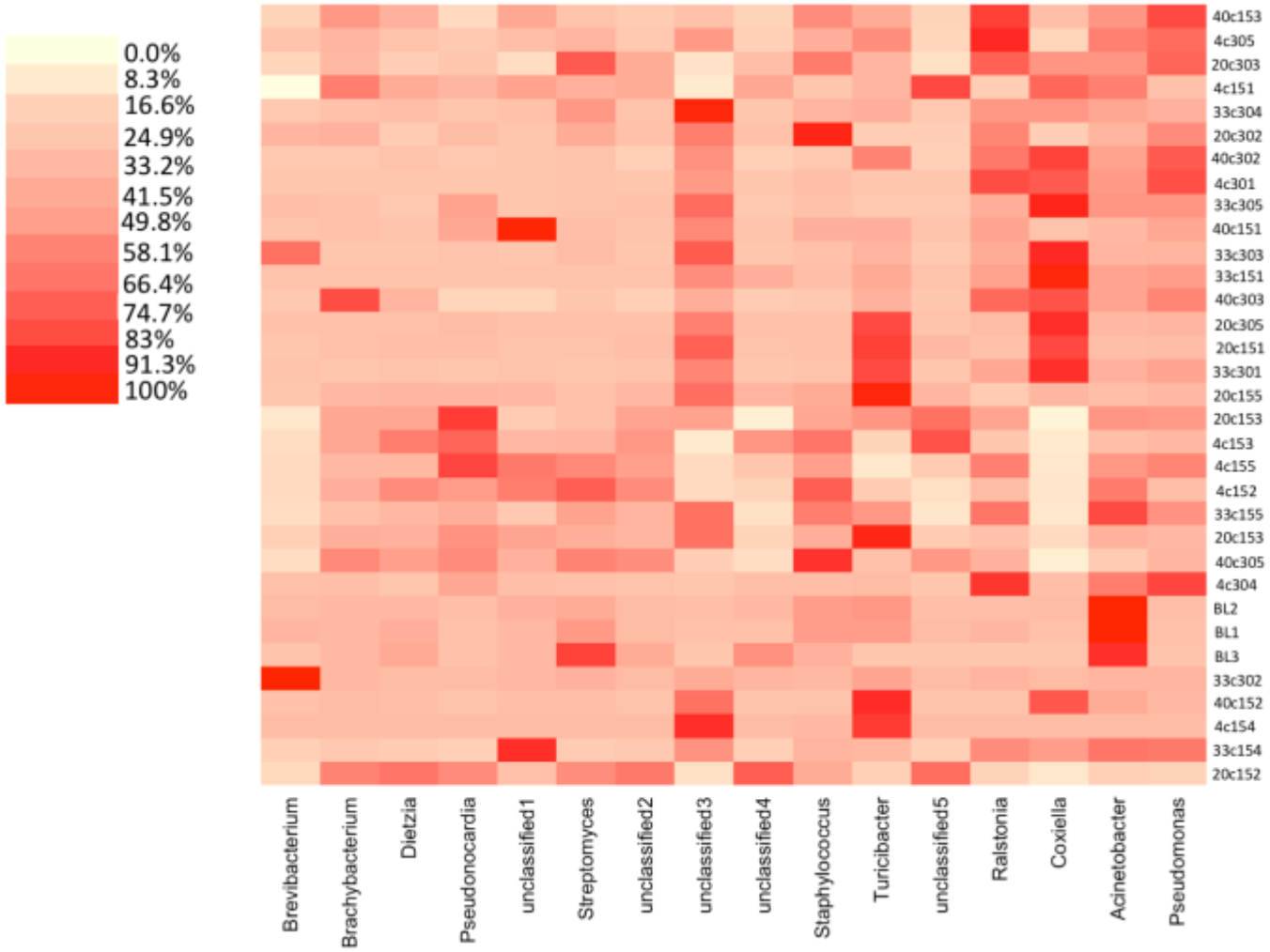


Figure 9. Clustering dendrogram of beta-diversities
The height of each branch point indicates how similar or dissimilar entities are from each other: the greater the height, the more dissimilar. 15-day samples are in blue, 30-day samples are in orange, and baseline samples are highlighted.

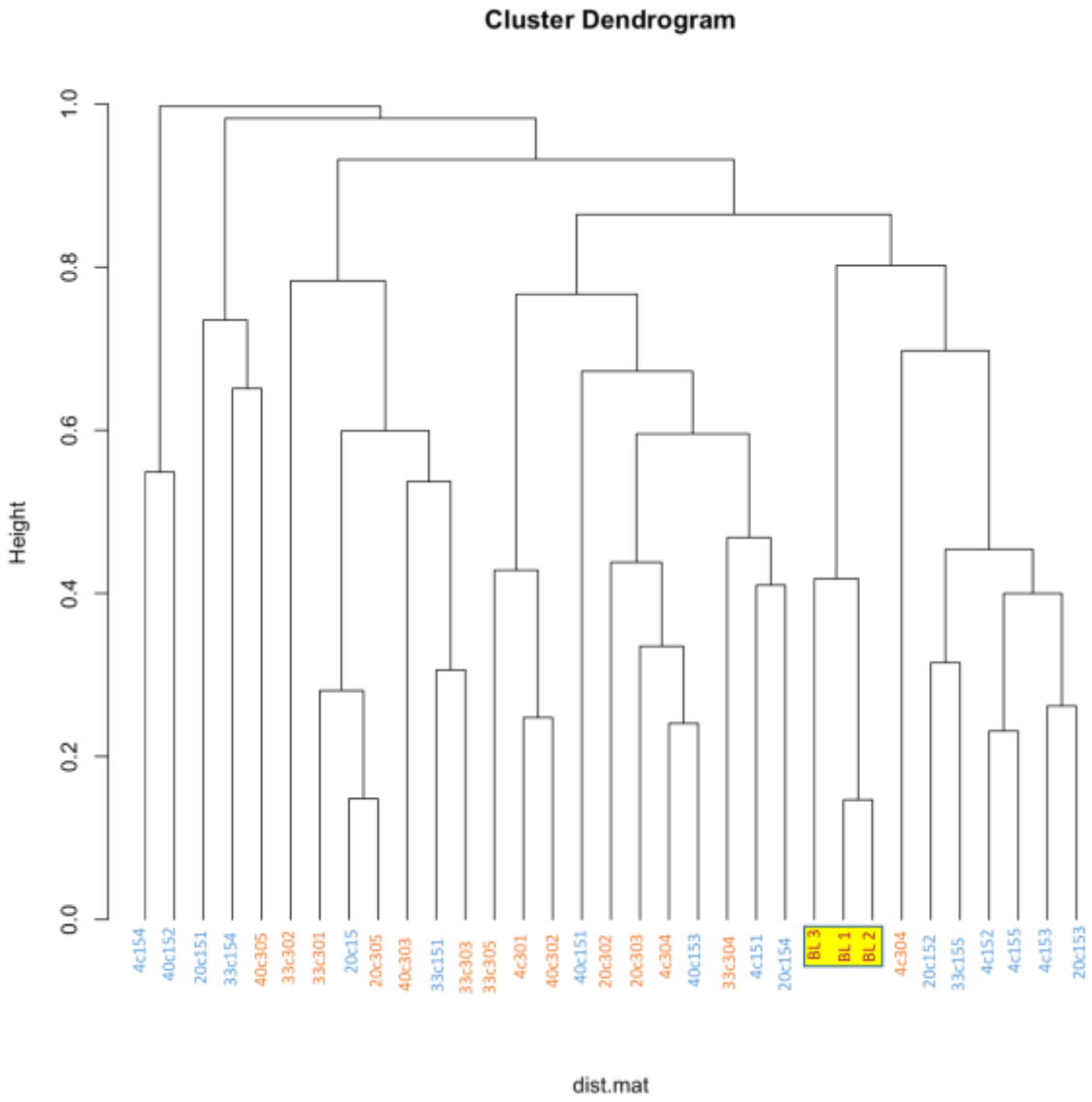


Figure 10. % Relative abundance of bacterial genera at baseline and 15-days for the different incubation temperatures

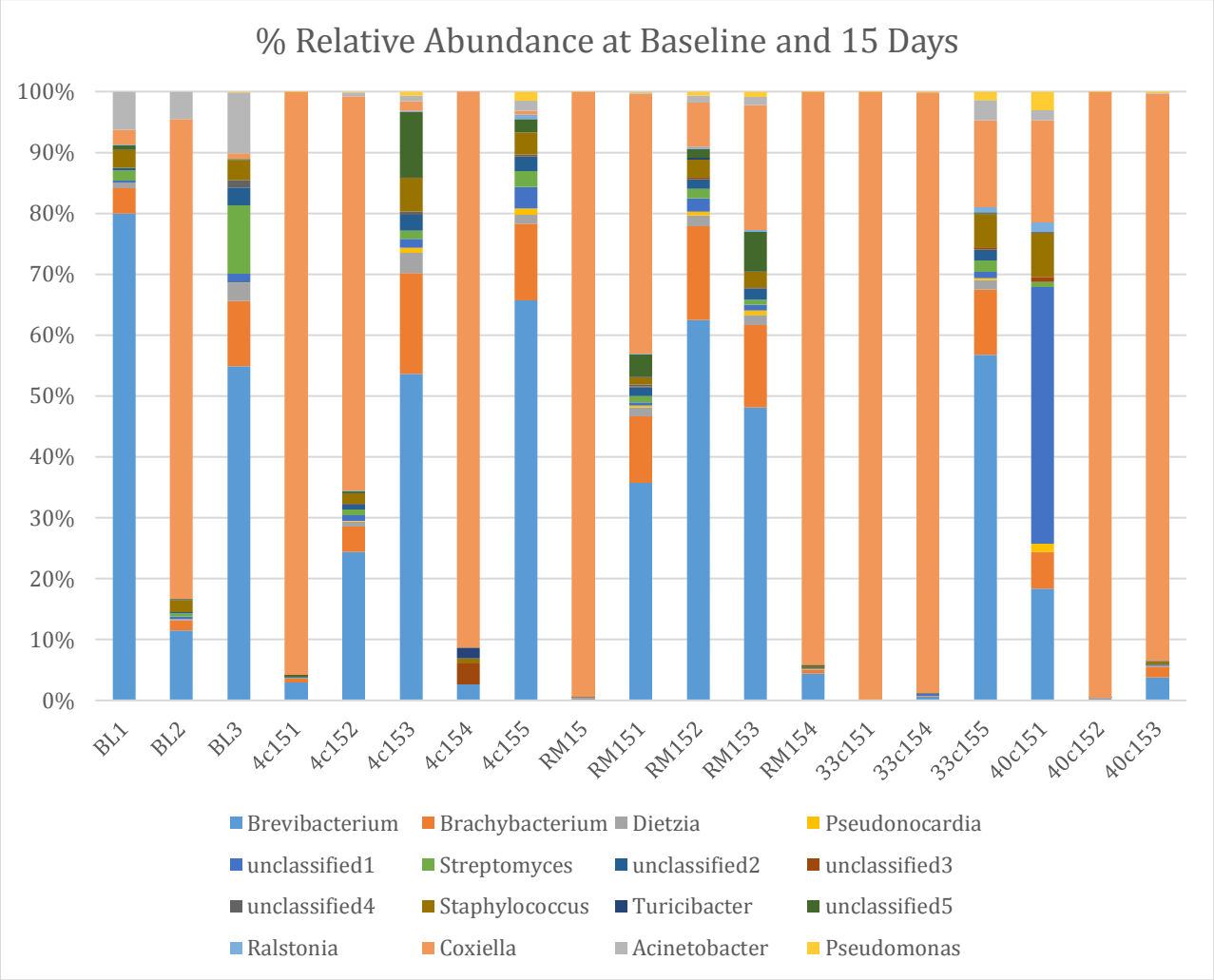
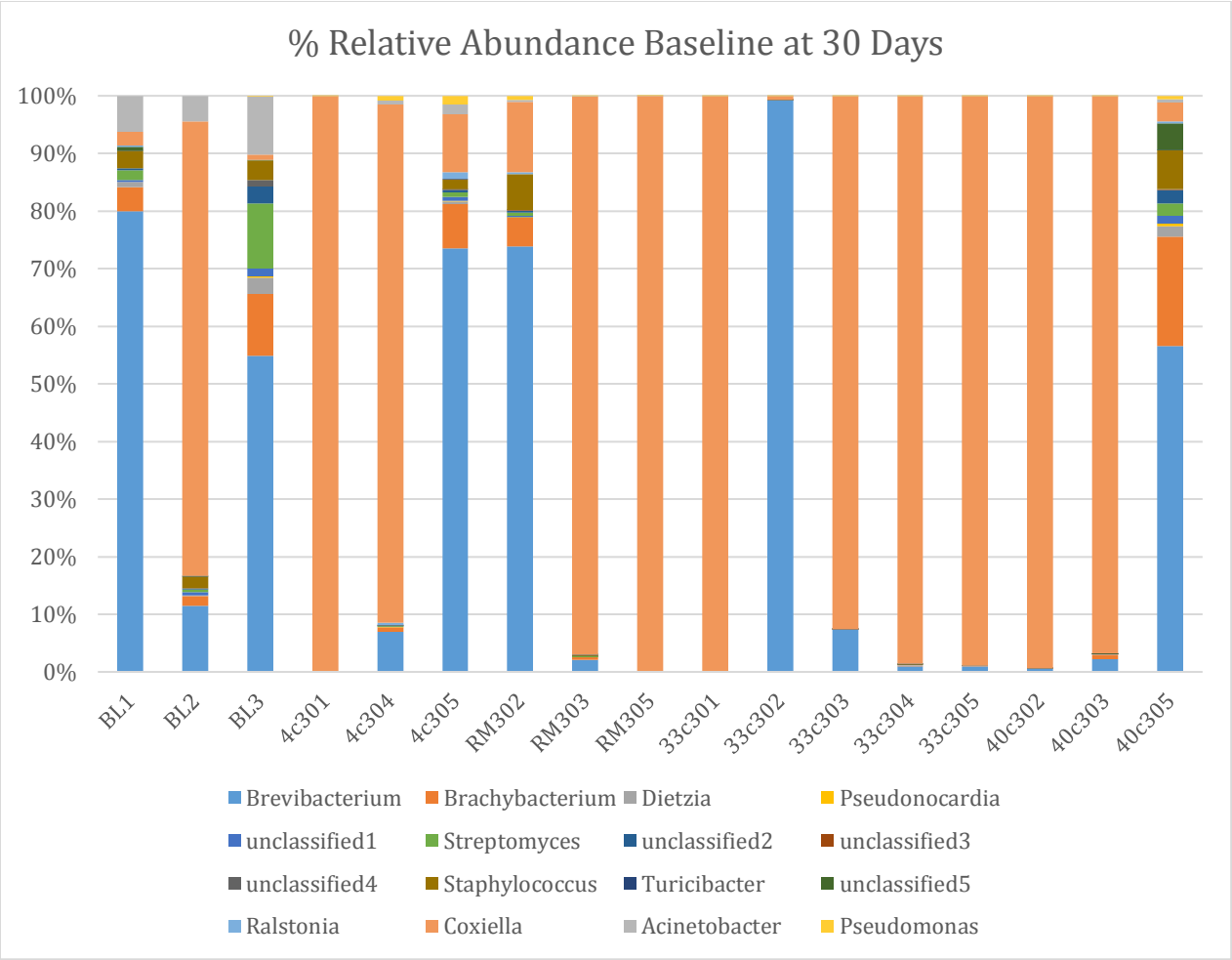


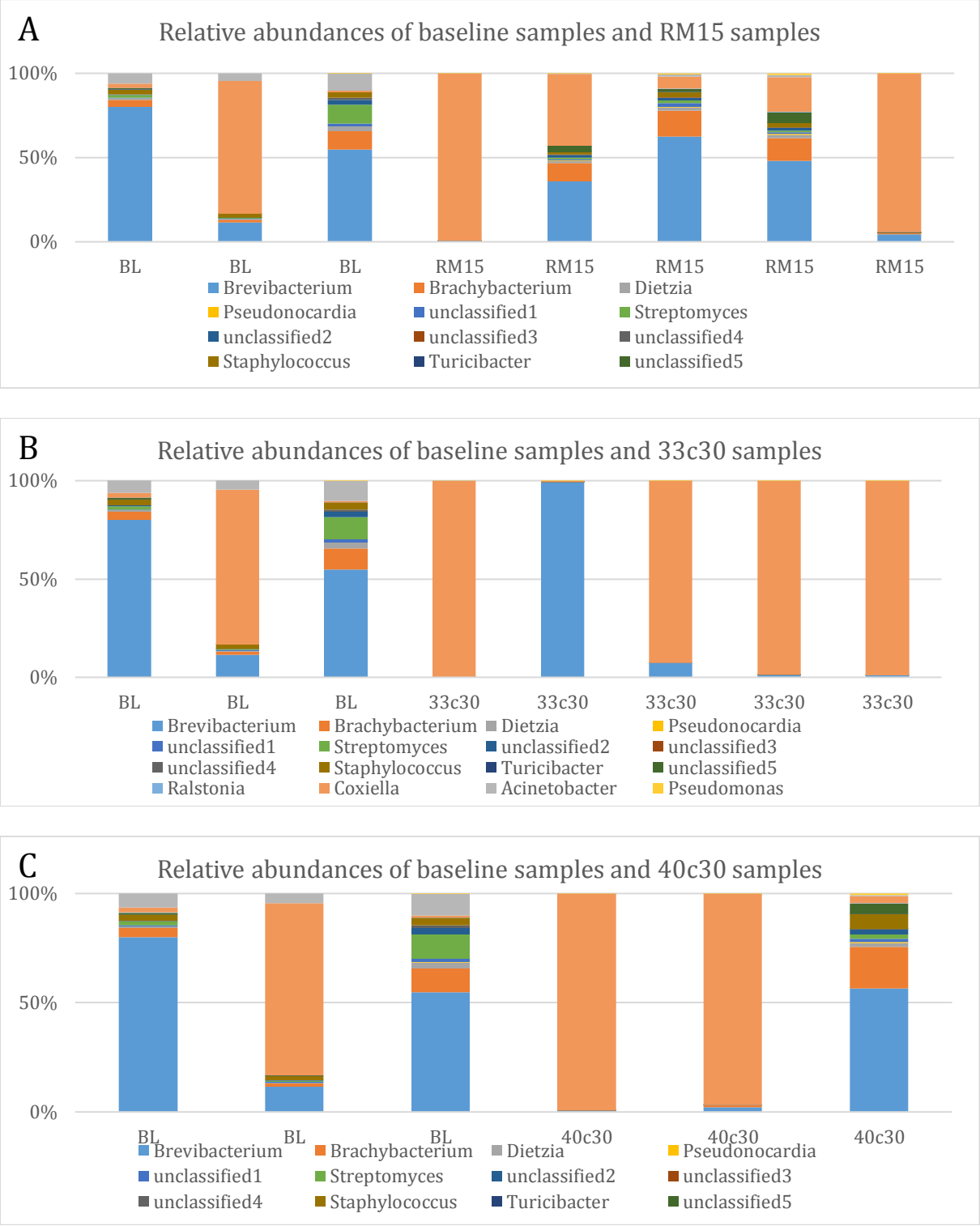
Figure 11. % Relative abundance of bacterial genera at baseline and 30-days for the different incubation temperatures



Because the data in this study do not fit a normal distribution, non-parametric multivariate analysis of variance (NPMANOVA) with a Bray-Curtis beta-diversity metric were used to compare samples (Oksanen et al., 2016); measuring the dissimilarity between samples—a significant value indicating significant dissimilarity. An initial NPMANOVA was conducted comparing samples across temperature. No significant differences were identified ([Adonis statistic, F=0.874]; Anderson, 2008). Subsequent NPMANOVA's were conducted to compare baseline samples to 15-day incubation samples ([Adonis statistic, F=0.005), and baseline samples to 30-day incubation samples (Adonis statistic, F=0.002).

Comparison of 15- and 30-day samples with baseline samples both resulted in significant values indicating statistically significant dissimilarity from baseline. The NPMANOVAs were conducted comparing each experimental condition's samples to baseline samples as a quasi *posteriori* testing (NPMANOVA," 2016). Three experimental conditions were found to significantly deviate from the baseline samples, RM15 ([Adonis statistic, F=0.019], Figure 12, A), 33c30 ([Adonis statistic, F=0.012], Figure 12, B), and 40c30 ([Adonis statistic, F=0.016], Figure 12, C)

Figure 12. Relative abundance graphs comparing baseline samples and experimental conditions that differed significantly from baseline. Relative abundances of baseline samples and A) RM15 samples, B) 33c30 samples, and C) 40c30 samples.



Of the total OTUs, the most prevalent genera were *Coxiella* (86.01%) and

Brevibacterium (12.48%). The remaining genera each represented less than 1% of the overall total bacteria (Figure 10, Figure 11). Relative abundance of greater than 50% occurred for *Brevibacterium* in 10 samples (30.3%), and with *Coxiella* in 20 samples (60.6%). When examining the relative abundance graph, it appeared that the presence of each bacteria in the samples was almost exclusive (i.e, samples were dominated by one or the other bacteria). A Pearson's Correlation was performed in Excel using the 'CORREL' function, which indicated a negative correlation between the two groups of bacteria. ([Perasons coefficient r = -0.94]; Figure 13).

This study also evaluated the alpha diversities of the samples. Alpha diversity is the diversity within a single population. Shannon indices were generated for all samples using Excel (Figure 14). Two-sample t-tests assuming unequal variances were used to compare baseline Shannon values to each experiential condition's Shannon values. Only the 33c30 condition showed a significant difference from base line samples (P one-tail= 0.027, P two-tail= 0.054).

Figure 13. Scatter plot of relative abundance of *Brevibacterium* versus that of *Coxiella* shows a negative correlation between the two groups. Perasons coefficient r = -0.94

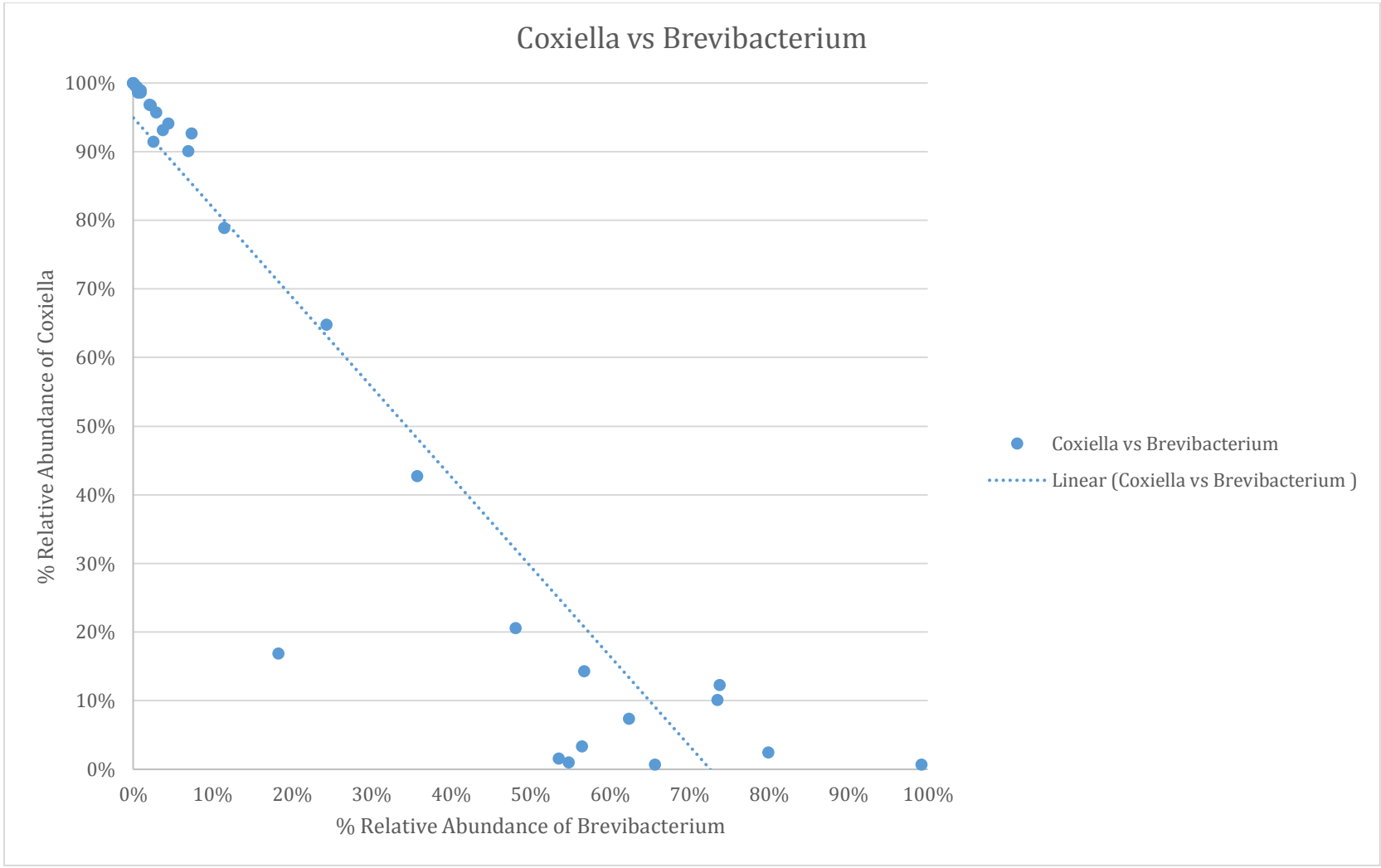
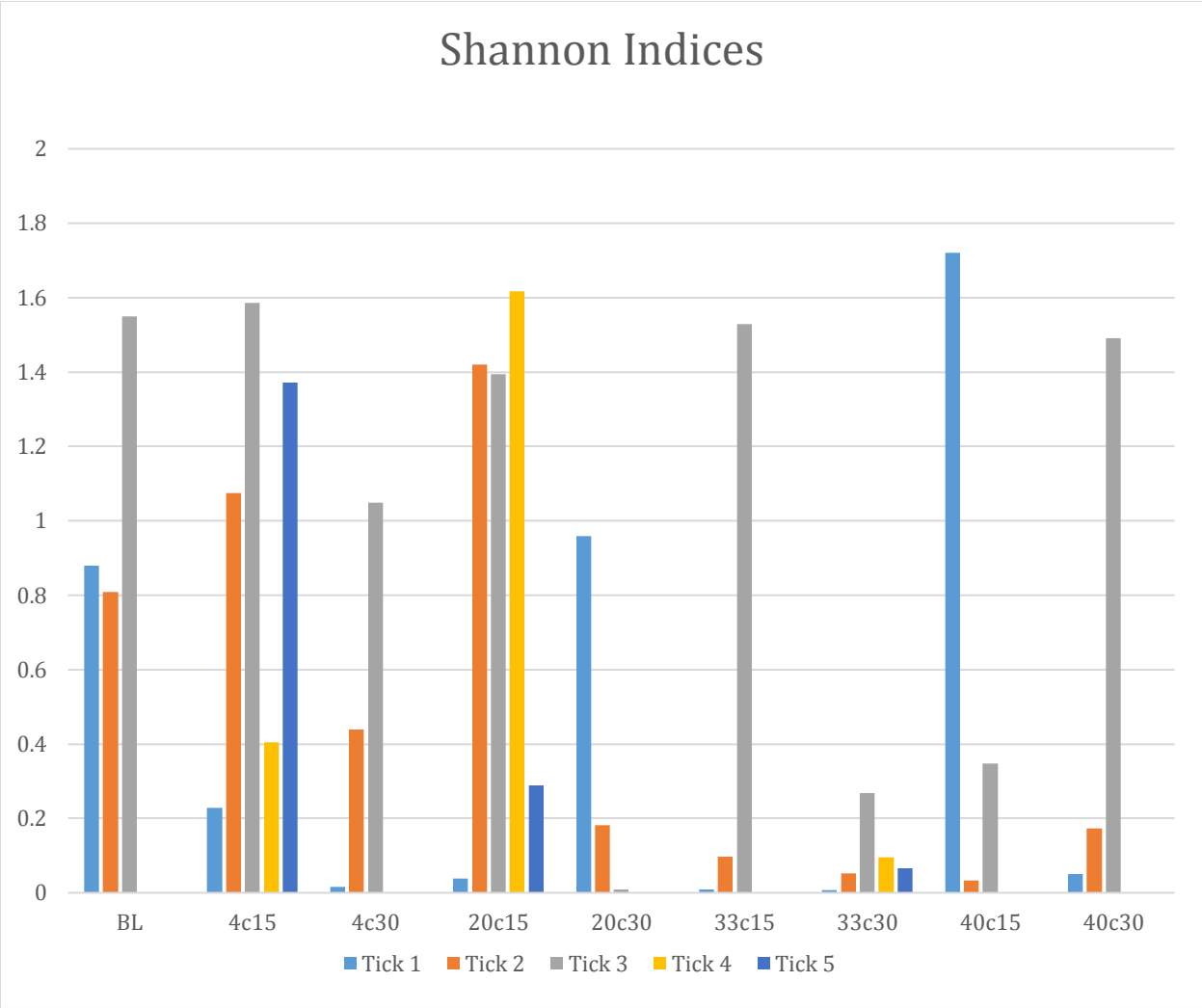


Figure 14. Visual representation of Shannon Indices of Diversity.
Shannon indices show alpha diversity (diversity within a sample). Typical values range from 1.5-4, higher values indicating more diverse populations (Kerkhoff, 2010). Each bar color represents an individual tick in that sample population



Discussion and Conclusion

This study tested the hypothesis that ticks incubated at different temperatures would result in significant alterations to the bacterial compositions of their microbiomes (4°C, 20°C, 33°C, and 40°C). The experimental design included five ticks per each temperature condition and exposed for a period of either 15 or 30 days. Somewhat unexpected and despite previous supporting research, this prediction was not supported by the statistical analysis of our results. However, there was a change in bacterial diversity with respect to length of incubation. Several reasons could give rise to these results. First, since tick processing was conducted at two different time points, the amount of blood present could be a potential confounding variable. One study showed that blood feeding drastically changes the composition of the microbiome (Zhang et al., 2014). It takes about two to three weeks for a complete blood meal to digest (Sojka et al., 2013). Being that this research was conducted in a laboratory, results may reflect certain laboratory based limitations such as no diurnal temperature change or day and night light cycles. The constant conditions to which the ticks were exposed to do not reflect real life conditions.

When comparing the results of this study to data published by Menchaca and colleagues, a study that obtained ticks form the same research facility as this study did, some interesting similarities and differences were identified. There was similar community domination by *Coxiella*, but there was no evidence of the family *Brevibacteriaceae*, where *Brevibacterium* resides. This difference may be a result of each study targeting different hypervariable regions of the 16S rRNA gene for phylogenetic identification, using different sequencing platforms, or the differing environmental conditions. However, *Brevibacterium* in the microbiome of *A. americanum* is not a novel finding. A study by Heise and colleagues, who compared the microbiomes of colony reared *A. americanum* to wild caught *A. americanum*, revealed a

microbiome pattern consistent with that of this study (i.e. *Coxiella,* 89%; *Brevibacterium*,11%) in colony reared ticks,. The also reporteda complete absence of *Brevibacterium* in wild caught ticks, which they discussed could possibly be secondary to the colony rearing process or the increased presence on infectious bacteria such as *Rickettsia* species (Heise, Elshahed, & Little, 2010). In that study, their colony reared ticks were fed on disease-free New Zealand White Rabbits and American Beagles, and their wild-caught ticks were captured in Oklahoma and Georgia.

The almost ubiquitous domination of samples by *Coxiella* in this and other studies discussed is not surprising, for this organism has been well documented as an endosymbiont of *A. americanum* (Zhong, Jasinskas, & Barbour, 2007). High concentrations of *Coxiella* have been identified particularly in the ovaries of *A. americanum* (Klyachko, Stein, Grindle, Clay, & Fuqua, 2007). However, that there are still some female ticks that do not show an overwhelming amount of Coxiella may imply there are underlying, inter- individual differences. This inherent variability, particularly in the base line ticks, may have obscured other changes in the microbiome in this study.

Though there is ongoing tick research there is still a lot to be discovered. For example, it is unknown if there is a common or core microbiome, or exactly what factors modulate the microbiome composition.

Potential limitations of this study include the relatively small number of samples in each temperature group, using a tick that has a known endosymbiont, using only females, and using colony reared ticks. A sample size of 5 ticks per condition was chosen for feasibility. However, this proved to be quite limiting for analytical purposes. Most notably, the baseline samples, which all other conditions were compared to, had extensive inter-individual variation in the

dominant *Coxiella* endosymbiont levels, and since there were only three ticks in the baseline condition, any variation was heavily weighted. Future studies using sample sizes of 100 or greater would allow for more accurate results. The fact that a tick with a known endosymbiont whose numbers could dominate total bacterial population was used may be limiting because there could be underlying mechanisms within the ticks themselves that ensure the survival and proliferation of *Coxiella*. As one study showed, *A. americanum* demonstrated reduced reproductive fitness when cured of *Coxiella*. This study chose to utilize only females in order to minimize variables, however it potentially could have benefitted from using males alternatively or as well, since there is such a high prevalence of *Coxiella* in ovarian tissues of some female individuals. And finally, since the ticks were raised and maintained in a colony, the application of results determined here to wild caught ticks could be limited.

In conclusion, there was a change in bacterial diversity in the microbiome of *A. americanum* with respect to length of incubation, but not specifically with incubation temperature over the length of time tested. The primary hypothesis of this study, therefore, was not supported. Further studies with larger sample sizes, males ticks, or both are needed to reveal subtler effects. None of the ticks in this study possessed bacterial species pathogenic to humans. Future studies with experimentally infected ticks may lead to other insights on changes in the relative abundance of pathogenic species, which may behave differently than commensal bacteria. Also, seeing survival or death of pathogens in samples could lead to a better understanding of conditions necessary for pathogen maintenance in wild populations.

Appendices

Appendix 1. Arrangement of samples for Synergy 2 Multi-Mode Reader. Generated by Gen5 Microplate Reader and Imager Software

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK	STD1 667	STD1 667	BL1	BL1	RM151	RM151	40151	40151	RM305	RM305	40305	Well ID
													Conc/Dil
B	BLK	STD2 335.5	STD2 335.5	BL2	BL2	RM152	RM152	40152	40152	33301	33301	40305	Well ID
													Conc/Dil
C		STD3 167.5	STD3 167.5	BL3	BL3	RM153	RM153	40153	40153	33302	33302		Well ID
													Conc/Dil
D		STD4 83.87	STD4 83.87	4c151	4c151	RM154	RM154	40301	40301	33303	33303		Well ID
													Conc/Dil
E		STD5 41.93	STD5 41.93	4c152	4c152	RM155	RM155	40304	40304	33304	33304		Well ID
													Conc/Dil
F		STD6 20.96	STD6 20.96	4c153	4c153	33151	33151	40305	40305	33305	33305		Well ID
													Conc/Dil
G		STD7 10.48	STD7 10.48	4c154	4c154	33154	33154	RM302	RM302	40302	40302		Well ID
													Conc/Dil
H		STD8 0	STD8 0	4c155	4c155	33155	33155	RM303	RM303	40303	40303		Well ID

Appendix 2. DNA concentrations generated by Synergy 2 Multi-Mode Reader. Generated by Gen5 Microplate Reader and Imager Software. 485/20 = 485 nm excitation filter with 20 nm range. 528/20 = 528 nm emission filter with 20 nm range. Concentration is presented expressed in ng/µL.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	99	48860	48160	17019	25793	8377	3940	30764	15583	2471	2472	6945	485/
	3	48764	48064	16923	25697	8281	3844	30668	15487	2375	2376	6849	Blan 485/
	3.556	678.962	669.266	237.921	359.453	118.217	56.759	428.308	218.03	36.411	36.425	98.382	[Cor
B	93	23325	23001	16706	18358	700	591	44537	36416	2190	1438	5579	485/
	-3	23229	22905	16610	18262	604	495	44441	36320	2094	1342	5483	Blan 485/
	3.473	325.267	320.78	233.585	256.468	11.881	10.371	619.082	506.596	32.519	22.103	79.461	[Cor
C		11717	11552	21415	16427	44465	36044	28134	15963	872	1021		485/
		11621	11456	21319	16331	44369	35948	28038	15867	776	925		Blan 485/
		164.481	162.195	298.811	229.721	618.085	501.443	391.879	223.294	14.263	16.327		[Cor
D		5874	5897	30451	21215	18554	6549	11916	6874	635	691		485/
		5778	5801	30355	21119	18458	6453	11820	6778	539	595		Blan 485/
		83.547	83.866	423.972	296.041	259.183	92.897	167.237	97.399	10.98	11.756		[Cor
E		2947	3000	28985	13343	37430	22638	3223	3019	24424	17040		485/
		2851	2904	28889	13247	37334	22542	3127	2923	24328	16944		Blan 485/
		43.005	43.739	403.666	187.003	520.641	315.752	46.828	44.002	340.49	238.212		[Cor
F		1575	1533	40385	11626	2115	2420	27245	24250	8990	6297		485/
		1479	1437	40289	11530	2019	2324	27149	24154	8894	6201		Blan 485/
		24	23.419	561.572	163.22	31.48	35.705	379.565	338.08	126.708	89.407		[Cor
G		819	843	17635	18174	2164	2600	48118	34824	8590	3204		485/
		723	747	17539	18078	2068	2504	48022	34728	8494	3108		Blan 485/
		13.529	13.861	246.453	253.919	32.159	38.198	668.684	484.544	121.168	46.564		[Cor
H		7	7	10773	16778	5664	5196	14045	13230	4266	3858		485/
		-89	-89	10677	16682	5568	5100	13949	13134	4170	3762		Blan 485/
		2.282	2.282	151.405	234.583	80.639	74.156	196.727	185.438	61.274	55.623		[Cor

Appendix 3. Google Doc Spreadsheet required for data processing via QIIME in BaseSpace. First column is the sample that was processed and the last column was the sample identifier for the researcher.

SampleID	BarcodeSequence	LinkerPrimerSequence	Description
BL1	ATCTCAGGCTCTCTAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	BL1
BL2	ATCTCAGGTATCCTCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	BL2
BL3	ATCTCAGGGTAAGGAG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	BL3
4c151	ATCTCAGGACTGCATA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c151
4c152	ATCTCAGGAAGGAGTA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c152
4c153	ATCTCAGGCTAAGCCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c153
4c154	ATCTCAGGCGTCTAAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c154
4c155	ATCTCAGGTCTCTCCG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c155
20c151	GCTCATGACTCTCTAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c151
20c152	GCTCATGATATCCTCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c152
20c153	GCTCATGAGTAAGGAG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c153
20c154	GCTCATGAACTGCATA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c154
20c15	GCTCATGAAAGGAGTA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c15
33c151	GCTCATGACTAAGCCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c151
33c154	GCTCATGACGTCTAAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c154
33c155	GCTCATGATCTCTCCG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c155
40c151	GTAGAGGACTCTCTAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	40c151
40c152	GTAGAGGATATCCTCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	40c152
40c153	GTAGAGGAGTAAGG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGG	40c153

	AG	TGCCAGCMGCCGCGGTAA	
4c301	GTAGAGGAACTGCATA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c301
4c304	GTAGAGGAAAGGAGTA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c304
4c305	GTAGAGGACTAAGCCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	4c305
20c302	GTAGAGGACGTCTAAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c302
20c303	GTAGAGGATCTCTCCG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c303
20c305	TAAGGCGAACTGCATA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	20c305
33c301	TAAGGCGAAAGGAGTA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c301
33c302	TAAGGCGACTAAGCCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c302
33c303	TAAGGCGACGTCTAAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c303
33c304	TAAGGCGATCTCTCCG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c304
33c305	CGTACTAGACTGCATA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	33c305
40c302	CGTACTAGAAGGAGTA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	40c302
40c303	CGTACTAGCTAAGCCT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	40c303
40c305	CGTACTAGCGTCTAAT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	40c305

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