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Preliminary Examination of the Bacterial Diversity Harbored by the Pill Bug (Armadillidium Vulgare)

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PRELIMINARY EXAMINATION OF THE BACTERIAL DIVERSITY HARBORED BY THE PILL BUG (*ARMADILLIDIUM VULGARE*)

by

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Pill bugs (*Armadillidium vulgare*) were examined at 4 time points during 2015- 2016. The time points corresponded to summer, fall, winter, and spring. At each time point, 2 to 10 pill bugs were collected from each of three sites. All sites were located in San Antonio, Texas. The first site was in central San Antonio, but due to difficulties in collecting specimens the first site was only utilized at the first time point. The remaining localities, Bonilla Science Hall on the campus of the University of the Incarnate Word and a private residence in northwest San Antonio, were utilized for the remaining three time points.

After collection, pill bugs were washed. The recovered supernatant was serially diluted and plated on nutrient agar plates and incubated overnight at 37^{o} C. After incubation, colonies were scored for morphological characteristics and subjected to a variety of biochemical tests. The results of these analyses revealed 65 unique isolates, four of which could not be sequenced.

The 16s rRNA gene was sequenced for each of these isolates in order to classify each to genus and species. Based upon DNA analysis, 61 isolates generated DNA data that were found to represent 15 described genera of bacteria. These 61 isolates were further identified to 30 unique species of which 16 were described species.

The most common bacterial genus identified was *Bacillus*. At least one species of

Bacillus was identified at time points and in all localities. Overall, the most common species identified was *B. cereus*. This species was found at all localities and at all time points. There was no other genera or species identified at more than two localities or time points—although several were found at two time points or sites. In general, other than *B. cereus*, there was no genus or species consistently found at a single site. These data suggest that there is a unique bacterial fauna at each site and each time point—which could be correlated to climatological conditions, such as temperature, humidity and/or rainfall.

These data reveal that there are pathogens associated with pill bugs at each site and during each season. The highest incidence of pathogenic bacteria occurs during time points associated with summer and spring. These are times of the year when mammals, particularly humans, could be in contact with pill bugs and suggest that there is the potential for pathogens to be transferred from pill bugs to humans. Overall, this study suggests that there is a diverse bacterial fauna associated with pill bugs.

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Background Information for a Study of the Pill Bug

Arthropods are considered the most versatile and successful species on earth. One primary reason for this is that arthropods have the capacity to survive in a wide variety of ecological habitats. As a result, arthropods exercise a large impact on human society where they either help to support human life or they compete with humans for food, space, and natural resources. One of the major groups of arthropods is insects. Examples of insects that can have a dramatic impact on humans include mosquitoes, lice, flies, and cockroaches. Another important aspect of the relationship between humans and insects is the role insects play as vectors for human disease. There are many types of insects that are known to carry bacteria that could be harmful to humans. For example, flies are potential vectors that have been shown to carry and transmit bacteria to humans during their life cycles (Leclerque & Kleespies, 2012). This results from the fact that these organisms frequently congregate around animal feces and rotten foods that are the site of many diverse types of bacteria. Likewise, mosquitoes have also been known to be carriers of various disease causing agents. They can transmit a plethora of diseases many of which are fatal to humans (e.g. Malaria, Yellow Fever, and West Nile Virus). These vector-borne diseases are epidemiologically related to the changes in the climate by permitting the vectors to grow during certain seasons of the year (Anderson & May, 1981). It is useful to point out that while they can be harmful to humans; many bacteria have evolved beneficial roles in the ecosystem such as being decomposers or assisting in nitrogen fixation in the environment.

Endosymbiotic interactions between arthropods and microorganisms are varied and range from pathogenic to mutualistic. They may be either obligate or facultative from both the perspectives of host and microbes. The nature of relationship between an arthropod host and bacterial endosymbiont is complex; for instance, an endosymbiosis microbial community can

induce reproductive alterations or under certain circumstances it may be a pathogen for the host (Leclerque & Kleespies, 2012).

The gut microbiota of arthropods can contain members of the bacterial, fungal, protozoan, and archaeal groups. These organisms are reported to assist in various physiological functions. The functions that can be linked to gut microbes include the digestion of food and nutrient acquisition, nitrogen fixation, and pheromone production (Hongoh, 2010). In addition to having a gut microbiota, arthropods can possess an external microbiota. For example, studies examining cockroaches and house flies have documented a wide variety of potentially pathogenic bacteria, e.g., *E. coli*, *K. pneumonia*, and *E. cloacae*, in both urban and suburban environments (Chaichanawong et al., 2004; Tatfeng, et al., 2005). Therefore, arthropods have the potential to carry pathogenic bacteria both internally and externally that could have wide ranging impacts on human health.

Pill Bugs

Another arthropod that humans commonly encounter is the pill bug (*Armadillidium vulgare*). Pill bugs are classified as Isopods. Pill bugs' preferred habitats are damp, protected places most of which are found under flowerpots, boards, and rocks. Pill bugs cannot survive and will die quickly in a warm, dry environment. Pill bugs feed on the fungi and bacteria that invade rotting vegetation, manure, and compost. They can harm plants by injuring seedlings by cutting roots and girdling young stems. Although they do not perform surface damage to vegetables or a fruit themselves, if another pest has injured a fruit, pill bugs will gain entry thereby feeding on the plant and producing ecological impact through the process of decomposition and returning nutritive properties to the soil (Ware, 1988).

Pill bugs are native to the Mediterranean region, but have been found worldwide. The

population sizes of this organism are thought to be high, particularly in temperate climates. Apart from other isopods, pill bugs have an exceptional ability of conglobation, in other words, the capacity to roll up into a ball often under unfavorable or threatening conditions (Smigel & Gibbs, 2008).

Anatomy

Armadillidium vulgare are usually oval in shape and their dorsal plane is moderately flattened. They have a cephalic shield that is considered to be less durable compared to the fully fused carapace found in other crustaceans. Their body consists of three parts: the head, thorax, and abdomen. The head bears their antennae. The antennae are used as sensory organs that enable them to respond to stimuli as well as locate food. The oxygen acquired by Isopods is primarily through a thickened cuticle. The cuticle is made up of a fibrous matrix filled with calcium carbonate allowing the diffusion of gas and acts as a water conservation mechanism. The pleon on the first two ventral segments supports two pairs of respiratory structures known as pleopods. They are hypothesized to have originated from what was once a pair of appendages.

The pleopods function by trapping air using pseudo-tracheae which are sponge-like structures with the appearance of a white color. The white color by pseudo-tracheae appears to be different from that on the dorsal calcium carbonate plates formed during the molting stage known as pre- ecdysis (Beauché & Richard, 2013; Metegnier et al., 2015).

Reproduction

Mating pill bug pairs can hypothetically form up to seven days before the female's receptive period. However, males have more attraction to the females with noticeable calcium plates, which relate to their higher generative receptivity period (Holland & Roles,

2016). Isopods usually mate in spring. However, warmer conditions often lead to earlier reproduction. In regions with mild winters, especially Mediterranean climates, pill bugs can remain sexually active throughout the whole year. Mating is brief, finished within a few seconds, and synchronized with the start of the molting cycle of the female. Females can store sperm from several males, who abandon the female once mating is over and are free to mate again. Sperm from a single mating incident can be stored for a year for use in the ensuing broods. Sperm bundles that are older, normally take precedence over more recent genetic material during the laying process of the broods. After mating, the females enter into a 'refractory period'. Therefore, there are no truly permanent mating pairs (Holland & Roles, 2016). During this time, any further male attempts by the males to mate are rejected (Hornung, 2011).

Bacteria

In general, bacteria are among the simplest prokaryotes. Bacteria can be found in a diverse variety of habitats including water, soil, as well as animal and human intestines. Bacteria are incredibly important in nutrient recycling and can flourish in a wide range of environments. For example, in hydrothermal vents or seeps, bacteria recycle nutrients such as hydrogen sulphide and methane allowing other organisms to thrive (Bakker, 1961).

Classification of bacteria is often based on morphology via gram staining. Bacteria can either be gram-positive or gram-negative based on variation in their cell wall and shaped as rod, cocci, or spirochetes. Gram-positive bacteria possess cell walls with a basic structural constituent comprised primarily of peptidoglycan. In gram-negative bacteria, there is slight variation in the form of additional phospholipid outer membrane that exists along with the peptidoglycan layer (Whitman, et al. 1998; Beveridge, 1990). The basic difference in gram

stain is due to how the cell wall stains when exposed to crystal violet and safranin.

Insects may act as vectors for the transmission of the pathogenic bacteria. Pill bugs have been shown to be associated with a wide variety of bacteria e.g., *Photorhabdus luminescens*.

After entry into an isopod host, this entomo-pathogenic bacterium rapidly distributes a toxic protein into the blood. The protein causes a rapid reduction in the concentration of hemocytes (Sicard et al., 2014). Finally, the protein causes rapid death of the pill bugs. *Rickettsiella* is another bacteria that can occur in a wide range of hosts including arachnids, crustaceans and insects (Cordaux, 2007). The prevalence of this bacterium within arthropods is >60%. This bacterium is known to have a mutualistic and/or ecological role in these groups. For instance, a mutualistic relationship has been hypothesized between *Rickettsiella* and the hard tick (*Ixodes woodi)*. This relationship suggests that these bacteria could be transmitted to mammals (Leclerque & Kleespies, 2012). In addition, *Rickettsiella* can live in soil, and as they move within the soil, they aerate the soil. The aerated soil is paramount for plants and crop growth, but this could lead to *Rickettsiella* being transmitted to these organism with subsequent transmission to mammals.

Lifecycle of Bacteria

The lifecycle of typical bacteria has several phases: Lag, Log, Stationary, and Death. In the Lag phase, the bacteria adapt to the growth conditions and the surrounding environment. The bacteria will not divide during this phase, but instead prepare for cellular division. In lag phase, the synthesis of enzymes, RNA, growth factors and all the molecules needed for growth begin. The Log phase is characterized by the logarithmic pattern of growth. The rate of growth depends on factors such as the availability of nutrients, pH, and other environmental factors

present in the growing environment. The Stationary phase begins as growth-limiting factors such as the depletion of vital nutrients, and/or the formation of an inhibitory product - such as an organic acid, are formed in the media. The number of new bacteria will match the rate of cell death; as a result, you will see “smooth,” horizontal linear portion of the growth curve during the Stationary phase. The Death phase is triggered as nutrients are finally depleted, or temperature, pH, or other environmental conditions change dramatically (Whitman, et al. 1998; Woese, et al. 1990).

Rationale

This project uses an interdisciplinary approach combining genetics, microbiology, and entomology. Overall, this project is designed to investigate the association of pill bugs with bacteria and, thereby, their potential impact on human health. The project’s ultimate goal is to determine whether, or not, the pill bugs harbor bacteria that have either a beneficial ecological role or a pathogenic association with humans. The first stage of this project will be to isolate bacteria from pill bugs. This will be followed by a determination of the bacterial load carried by each pill bug. After this step is accomplished, the identification of bacteria will be completed using multiple approaches i.e. gram staining, biochemical tests, differential growth media and DNA analyses.

In addition, I want to determine whether, or not, pill bugs carry the same bacterial load/diversity at different locations. These findings could have dramatic impacts on humans, particularly if discrepancies exist in bacterial loads and species. Finally, the DNA sequence of each unique isolate will be determined in order to trace the genetic similarities or differences among the isolated bacterial species. For this purpose, the 16S ribosomal region of the DNA will be examined. This will be of particular importance as this provides evidence of the

evolutionary aspect and the rate of mutation in the resident bacteria in the host.

Expected Outcomes

The overall expectation is to find a variety of bacteria during this study. The primary bacteria expected are the ubiquitous soil genera e.g., *Bacillus* and *Pseudomonas*. Moreover, it would be expected that the other common bacteria, such as *Escherichia* might also be found. It is also hypothesized that there would be a difference in bacterial load between the seasons, with higher loads present in the warmer months. In terms of load, we would hypothesize that there would be a lower bacterial load at Location #2 – Bonilla Science Hall. The primary reason for this is that there is a higher likelihood that this site is managed and will have had chemicals added to the soil that could serve to reduce the number and type of bacteria present.

Materials and Methods

Specimen Collection

Pill bugs were collected from 3 localities. The first locality, 238 Funston Place, will be designated as Locality #1 (latitude 29.4573 and longitude 98.4655), the second locality, Bonilla Science Hall, will be designated as Locality #2 (latitude 29.4661 and longitude 98.4658), and third locality, 3727 Ticonderoga Drive, will be designated Locality #3 (latitude 29.5336 and longitude 98.5578). Live pill bugs were collected in modified potato traps for a minimum of one to two weeks per collection time point. At each point during collection, the goal was to collect a minimum of 2 to10 pill bugs. Collections took place at 4 points throughout the calendar year - time periods corresponded to the four seasons (winter, spring, summer, and fall). Adult specimens were identified to species using standard taxonomic keys. All samples were frozen at -20^{o} C for DNA isolation after bacterial isolation.

Bacterial Isolation and Bacterial Load

Collected pill bugs were washed with sterile H_2O and transferred to a sterile 1.5 ml tube containing 500 ml of buffered saline (0.9%). The individual tubes were vortexed for approximately four minutes in order to remove bacteria residing on the surface of each pill bug. Liquid samples were then serially diluted and 0.1-0.2 ml of each suspension was plated onto nutrient agar. All the samples were plated in triplicate. All inoculated plates were incubated overnight at 37^{o}C. The following day the number of colonies growing on each plate was manually counted. Plates containing colonies ranging between 30-300 were used to determine the overall bacterial load for each sample (Sieuwerts et al., 2008). The number of bacteria (Bacterial load) was determined using the following formula: Colony Forming Units/Sample Volume = Original Bacterial Density. For each sample, the average of three samples for the chosen dilution was used to determine the overall bacterial load for that specimen. After the determination of bacterial load, each plate was analyzed to determine the number and type of colonies present. Each unique colony was screened using biochemical tests, differential media, and DNA analysis to determine the genus and species identity of that colony. In addition, glycerol stocks were prepared from each type of the unique colonies identified. These stocks are stored at -80^{o}C.

Bacterial Identification

A variety of different methods were employed for the identification of bacterial species. For each bacterial isolates that could be cultured, gram staining, differential media, and biochemical tests were performed.

Gram Staining

In order to differentiate isolates on the basis of cell wall component as either gram-

positive or gram-negative bacteria, gram staining was performed. Initially, a smear was prepared from a direct sample. The sample was allowed to air dry followed by fixation with gentle heat or by alcohol. Next, the slide was completely flooded with crystal violet and allowed to sit for approximately 60 seconds. Next, the slide was washed with water for five seconds. Next, the slide was immersed in iodine and allowed to sit for 60 seconds and rinsed with water for five seconds. Next, the decolorizing agent was added, and the slide was rinsed for five seconds with water. Finally, the counterstain, i.e. safranin, was applied for 60 seconds and the slides were blotted and allowed to air dry (Beveridge, 1990). Gram-positive bacteria will retain the primary dye i.e. crystal violet and remain blue-violet in appearance. Gram-negative bacteria, however, appear pink due to the presence of the counter dye.

Biochemical Test and Differential Media

The first test to be conducted was the Catalase test. The basic procedure of the Catalase test involves mixing hydrogen peroxide H_2O_2 with the unidentified bacteria. If bubbles are produced, this indicates a positive test, whereas no bubbles indicate a negative test. Aerobic bacteria and facultative aerobes are expected to give positive results as they contain the enzyme, *Catalase*. For example, *Staphylococci* will give Catalase-positive results whereas other bacteria, such as *Streptococci* will produce a Catalase-negative test. Different types of selective media will be used including MacConkey (MAC), Eosin methylene blue agar (EMB) and Mannitol Salt agar (MSA). The individual agar plates can be used to identify specific types of bacteria due to the specific growth requirements of each bacterial species.

MAC agar is a selective and differential media used for the isolation of gram-negative enteric bacteria based on the ability to ferment lactose. If the bacteria have the enzymes to ferment lactose, it will grow on this media. If it does not have the enzymes, no

growth will appear. The lactose fermenting bacteria such as *Escherichia* and *Klebseilla* appeared pink on MAC agar (Allen, 2005). EMB will differentiate gram-negative bacilli based on the colony color. Colored colonies are the lactose fermenter, and colorless colonies appeared as non-lactose fermenters such as genera *Salmonella* and *Shigella*. MSA is a selective and differential medium based on the ability to ferment mannitol for the isolation of *Staphylococcus* species. Mannitol- fermenting bacteria produce yellow colonies such as *S. aureus* whereas non-fermenters, *S. epidermidis,* produce white colonies (Shields & Tsang, 2006).

16S rRNA Sequencing

Overnight cultures for each unique colony were grown at 37^{o}C in nutrient agar broth. After 16-18 hours the broth was transferred to a sterile 1.5 ml tube. Each tube was centrifuged at 9,000 RPM to pellet bacteria. Cells were re-suspended in 175 ml of Quick Extract DNA Extraction Solution (Epicentre). The suspensions were incubated for 20 minutes at 65^{o}C. This incubation was followed by an additional incubation at 100^{o}C for 20 minutes. After incubation, samples were subjected to gel electrophoresis and DNA quality was assessed. To determine the identity of each unique isolate, the 16s rRNA gene was examined using the primers, 337F and 805R (Weidner & Puhler, 1996). PCR reactions were run for 35 cycles under the following conditions: 95^{o}C for 30 seconds, 53^{o}C for one minute and 72^{o}C for one minute and 30 seconds. Sequencing was conducted using standard protocols at the UTHSCSA DNA Core Facility. Once sequences were generated, they were screened via GenBank to identify each bacterial isolate to genus and species (where possible).

Results

A total of 75 pill bugs (*Armadillidium vulgare*) were collected from three sites in San

Antonio, Texas, during the summer, fall, winter, and spring of 2015/2016. The first site examined was a residential neighborhood in central San Antonio. This site was only examined in the summer of 2015 due to difficulty in isolating pill bugs during the remaining time points. The second site, the University of Incarnate Word campus, outside of Bonilla Science Hall was examined at all four time points. The third site was a residential neighborhood in northwest San Antonio. This site was only utilized for three collections, fall, winter, and spring.

Determination of Bacterial Load

At each site, between two and ten pill bugs were examined in order to assess the bacterial load carried by each pill bug. These examinations involved serial dilutions between 1:2 and 1:100, depending upon the site with three replicates plated per sample to ensure validity. The results of these tests are presented in Table 1 as Correlations or R^2 values for number and size of the pill bug examined. Results are shown only for pill bugs used in DNA analyses. These results indicate that Sites 1 & 2 show little, to no, correlation between the size of the pill bug and bacterial load. However Site 3 does appear to have a positive correlation between size and load for two of the three time points examined. Third time point could not be accurately assessed as only three pill bugs were collected.

Biochemical Tests

Biochemical tests were conducted on all morphologically distinct isolates. However, there were several instances in which the colony produced no growth. These samples were eliminated from all further analyses. The results of the Biochemical tests are presented in Tables 2 to 9. The majority of the results obtained in these analyses agree with results expected for each genera, based upon published data. The only instance in which our results differed from the expected, were several instances in which gram staining showed an ambiguous result.

Generic Diversity at Each Site

Figure 1 illustrates the overall generic diversity present at each locality. There was only a single bacterial species common across all seasons and localities, *B. cereus*. There were several other bacteria present at multiple sites, or time points, including *B. subtilis* locality 1, summer and 3, winter, *B. pumilis* locality 3, fall and locality 2 winter, *A. hydrophila* localities 1 and 2, summer, and locality 2, spring, *R. ornitholytica* locality 1, summer and locality 2, winter and spring, *Arthrobacter* sp1 locality 3, winter and locality 2, spring. Remaining bacteria were present at a single site and time point.

Figure 2 illustrates the representation of generic and species diversity recovered in this study. Overall, the most common genus recovered was *Bacillus*. This genus represented approximately 47% of the generic diversity recovered. Among the remaining genera that were identified, there were none that were present at greater than 8% of the total. Among the identified species, *Bacillus* contributed the largest species diversity at nine, of 30, identified species. The next highest percentage was *Aeromonas* with three species.

The generic representation at each site appears to differ from season to season (Figures 1, 2, and Table 11). For example at Site 3, there were six species representing three genera in the fall. In contrast, there were six species representing four in the winter and four species representing three genera in the spring. In each, the genus and species common to each time point was *B. cereus*. Similar results can be observed at the remaining sites and across the seasons (Table 11).

Generic Diversity per Individual Pill Bug

Tables 10 to 16 illustrate the divergent colony morphologies that were present at each site during each time point for the eight pill bugs used in DNA analyses (data for pill bug #26 is not

presented). Overall, there was a wide variety in identified morphologies. In several instances, colonies were identified with a distinct morphology, but when subject to DNA analysis was determined to be the same genus and species of bacteria. For example, *B. cereus* identified from Locality 2, winter 2016 were medium in size, convex in elevation, presented irregular margins, and were irregular in shape. In contrast, *B. cereus* identified from Locality 2, summer 2015 were medium in size, flat in elevation, presented erose margins, and were filamentous in shape. Tables 12 to 19 illustrate additional examples in which morphological description was in contrast to results obtained via DNA analyses.

16s Sequencing

Eight individual samples were sequenced in this study. Primers 337f-804r were used to amplify a 410–440bp region of the rRNA gene, *16s*, in each sample. This region was chosen because it amplifies the II–IV region of the *16s* rRNA gene, a region that has been shown to be phylogenetically informative in the majority of bacteria examined to date. There were several instances where this primer set was unable to amplify the *16s* gene; these samples were removed from our analyses. Overall, the genetic diversity found within genera with multiple species varied significantly. However, there were instances where no genetic diversity was identified. For example, *B. cereus* was found at all sites and at all time points, but there was no genetic diversity present within any of these samples. In contrast, the samples identified as *Paenibacillus* were 9.2% divergent from one another (the highest level of diversity identified within a genus in the present study). The next highest levels of divergence were present among samples identified as *Aeromonas*. The three samples identified as *A. hydrophila* were identical, but differ by 3.2%–4.4% from two unidentified samples that were 1.4% divergent from each other. In addition, two samples identified as *Arthrobacter* were 1.2% divergent from one another and our samples

identified as *Serratia* were 3.5% divergent from one another. Lastly the samples identified as *Microbacterium* and *Citrobacter* exhibited 1.3% and 1.5% sequence divergence, respectively. The remaining genera were represented by a single example and could not be assessed for genetic diversity (Table 17). The overall diversity and pathogenic status was identified where possible. There were bacteria identified as potential pathogens at each time and at each locality (Tables 17 and 18).

Discussion

Bacterial growth is dependent on the appropriate nutrients being abundant in the environment. In addition, the environmental conditions must facilitate bacterial growth. The abundance of nutrients will be affected by various factors including temperature, rainfall, and humidity and as a result the abundance of bacteria can change as these resources are altered. This would suggest that there could be seasonal variation in any environment where bacteria are present. The primary focus of the present study was to investigate whether, or not, the number and types of bacteria change as these climatological factors change. In order to assess these factors and their importance in determining the abundance of bacteria, we chose to examine four time points representing summer, fall, winter and spring. These are time points in which the above factors should be altered in order to determine their impact on the bacterial fauna associated with pill bugs in south central Texas.

Determination of Bacterial Load

The load carried by any individual pill bug regardless of locality or season seemed to have little correlation to the size of the pill bug i.e., there were small bugs with large bacterial loads and large bugs with small bacterial loads (Table 1). This would seem to indicate some factor(s) other than size is important in determining the bacterial load for a given pill bug. There

did appear to be a higher correlation, albeit a weak one, between Site 3 and bacterial load when compared to Site 2. This could be used to support the hypothesis that a more natural site would have a higher correlation between size and load given that a managed site, like Site 2, would be influenced more by anthropogenic impacts and result in a more altered ecosystem, which could be reflected as the lack of correlation evident at this site. Site 1, another non managed site, could not be assessed as pill bugs were only collected at a single time point, but this single time point does support the data recovered from Site 3.

Biochemical Tests, Generic Diversity at Each Site and per Individual Pill Bug

The tests chosen for this study are standard biochemical tests used to identify common bacteria. For the most part, we were able to generate a hypothesis regarding the generic identification of each sample based upon these tests. However, there were instances where the tests were inconclusive and positive identifications could not be made – particularly regarding gram stain results (Tables 2 to 9). As result, there were a significant number of samples were generic identifications were not possible. Therefore, while useful, these tests were not optimal for the identification of bacteria associated with pill bugs and as such are probably of limited utility in analyses such these.

The generic diversity recovered at each site does support the hypothesis that there are seasonal differences between the bacterial fauna present at these localities. With the exceptions of *B. cereus* (all sites and all time points), *B. subtilis*, *B. pumilis*, and *Arthrobacter sp1*, (found at two locations), and *R. ornithinolytica*, *B. thuringiensis*, *A. hydrophila*, and *B. amyloliquefaciens*, which were found at the same site during multiple time points, all bacteria were present at only a single time point and/or locality (Tables 17 and 18). This suggests that these localities do differ throughout a calendar year and we can expect that the bacterial fauna present at a given site will

change from season to season. This could be of importance to humans particularly if a higher number of potentially pathogenic bacteria are present during seasons where humans are more likely to come in contact with pill bugs. If these data are examined, they indicate that the highest number of potentially pathogenic bacteria is associated with pill bugs in the summer and spring. This could be used to suggest there is some risk of passing pathogens to humans as, in Texas, these are months when humans are outside for large portions of the day and are more likely to come in contact with pill bugs. Overall, these data suggest that, although the generic and specific composition do differ, the time points do harbor similar numbers of genera at time point, again suggesting that as the environmental conditions change so does the bacterial fauna.

There was a surprising amount of diversity present on pill bugs from different sites (data not shown as all samples were not sequenced). In addition, the colony morphology was in some cases altered for samples of the same genus There were several instances in which individuals of the same species exhibited different morphologies suggesting that morphological analyses alone are not sufficient to identify bacteria to genus and species (Tables 10 to 16). In total, these results of these analyses provide useful data in the identification of bacteria, but these results suggest they should not be the sole means by which to identify bacteria.

16s rRNA Sequencing

Of the colonies identified as unique using morphological analyses, there were only two that could not be sequenced. There were 65 individual colonies identified after morphological analyses. After DNA analyses, these 65 unique morphological isolates were found to represent 15 distinct genera and 30 species (four samples identified in morphological analyses could not be sequenced). Sixteen of these unique isolates were identified to species, whereas 14 that could not be identified to species (Tables 17 and 18, Figure 1). There are additional genes, or additional

regions of 16s, that could be utilized to identify these individuals, but these analyses were not conducted in the present study. In the majority of cases, sequencing of the 16s rRNA gene was sufficient to identify the unique colony to genus and species (Figures 1 and 2). In instances were multiple species were identified within a genus, the levels of genetic divergence were moderate to high—between 1.2% and 9.2%. While we would not advocate that a specific percent divergence be used to classify these unknown bacteria, these data do suggest that we are likely dealing with bacteria that represent novel species. For example, we examined several of the species identified as *Bacillus* and found the overall sequence divergence to range from 3.0% to 6.5% (data not shown). The levels of genetic diversity present among several samples with unidentified species, *Paenibacillus, Aeromonas,* and *Serratia,* are similar to these levels and could be used to suggest these samples represent unknown species within these genera. However, additional sequencing could reveal these isolates represent previously described species. Studies will be conducted in the future to determine, if possible, the correct identity of these unknown specimens.

Overall, we identified 65 total isolates using colony morphology. Of these 65 isolates, we were able to sequence 61. Of these, 30 were found to be unique isolates after DNA analyses. These unique isolates were identified to 15 described genera (Table 17). Nine of the 30 isolates were identified as *Bacillus* – all were identified to species. Seven additional isolates were also identified to genus and species. Of the 16 isolates identified to genus and species, three are known pathogens, four are not known to be pathogens, and for the remaining nine the pathogen status is unknown. The remaining 14 unique isolates were identified to genus, but could not be identified to species, nor could their pathogen status be assessed.

Conclusion

Overall, this study reinforces our hypothesis that environmental factors can have a significant impact on bacterial fauna and that morphological and DNA analyses are both useful in recovering the levels of diversity in a given locality. Overall, this study revealed that *Bacillus* is the most common genus of bacteria associated with pill bugs in the localities examined. Furthermore, the species, *B. cereus*, is the most common and widespread bacterium found in association with pill bugs. This study also revealed that there is no correlation between size and bacterial load. In addition, the present study determined that the overall generic diversity is quite variable throughout the year—the genus and species composition does change, although there are a few bacteria that can be found at multiple times throughout the year. Moreover, the present study identified that pill bugs do harbor bacteria that are potential pathogens to mammals and that these pathogens are at their highest concentration when pill bugs have the highest potential for direct contact with mammals, including humans.

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Tables and Figures

Table 1

Correlation or R^2 for Bacterial Load

		Correlation	R^2
Summer	L1	0.295	0.087
	L2	-0.102	0.047
Fall	L2	-0.819	0.027
	L3	0.714	0.509
Winter	L2*	N/A	N/A
	L3	0.519	0.342
Spring	L2*	N/A	N/A
	L3*	1	1

Note. Data determined for each site and for each season. Locality designations are the same as indicated previously.
* indicates a site where the number of pill bugs collected could not be used to accurately determine the Bacterial Load.

Table 2

Biochemical tests conducted on bacteria collected from pill bug specimen #22 at locality #2 during the summer of 2015

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
22A	***B. cereus***	+	+	-	-	+	+
22B	*B. acidiceler*	+	-	-	-	+	+
22C	*Achromobacter*	-	-	+	-	+	+
22D	*Serratia sp1*	-	-	+	+	+	+
22E	*Aeromonas sp2*	-	-	+	-	+	-
22F	*A. hydrophila*	-	-	+	-	+	-
22G	***B. cereus***	+	-	-	-	+	+

Note. Abbreviations for Biochemical Tests follow those in text. Highlighted text indicates identifications where the Biochemical tests differ in isolates that were identified as the same genus (and species) using *16s* rRNA analyses.

Table 3

Biochemical tests conducted on bacteria collected from pill bug specimen #26 at locality #1 during the summer of 2015 Abbreviations for Biochemical Tests follow those in text.

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
26A	*Enterobacter sp1*	-	-	+	-	+	+
26B	*Rhodococcus sp1*	+	-	-	-	+	+
26C	*Rhodococcus sp1*	+	-	-	-	+	+
26D	*Rhodococcus sp1*	+	-	-	-	+	+
26E	*Enterobacter sp1*	-	-	+	-	+	-
26F	*B. subtilis*	+	Yellow	-	-	+	+
26G	*R. ornithinolytica*	-	-	+	-	+	-
26H	*Citrobacter sp1*	-	-	+	+	+	-
26I	*Achromobacter sp1*	-	-	-	-	+	-
26J	*B. cereus*	+	-	-	-	+	+
26K	*Enterobacter Sp1*	-	-	+	+	+	+
26L	*A. hydrophila*	-	-	-	-	+	-

Note. Abbreviations for Biochemical Tests follow those in text.

Table 4

Biochemical tests conducted on bacteria collected from pill bug specimen #57 at locality #2 during the fall of 2015

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
57A	DNG	-	-	-	-	-	-
57B	*B. cereus*	+	-	-	-	+	+
57C	*B. cereus*	+	-	-	-	+	+
57D	*Paenibacillus sp1*	+	-	-	-	+	+
57E	*Paenibacillus Sp1*	+	-	-	-	+	+
57F	*B. simplex*	+	-	-	-	+	+
57G	DNG	-	-	-	-	-	-

Note. Abbreviations for Biochemical Tests follow those in text. The abbreviation DNG = Did Not Grow

Table 5

Biochemical tests conducted on bacteria collected from pill bug specimen #71 at locality #3 during the fall of 2015

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
71A	*B. cereus*	+	-	-	-	+	+
71B	*P. pocheonensis*	+	-	-	-	+	+
71C	***B. amyloliquefaciens***	+	-	-	Black, pink	+	+
71D	*B. megaterium*	+	-	-	Black	+	+
71E	*B. cereus*	+	-	-	-	+	+
71F	*B. thuringiensis*	+	-	-	Black	+	+
71G	*L. sphaericus*	+	-	-	Black	+	+
71H	*B. megaterium*	+	-	-	Black	+	+
71I	*B. pumilus*	+	-	-	-	+	+
71J	DNS	-	-	-	Black, pink	+	+
71K	***B. amyloliquefaciens***	+	-	-	-	+	+

Note. Abbreviations for Biochemical Tests follow those in text. Bold text indicates identifications where the Biochemical tests differ in isolates that were identified as the same genus (and species) using *16s* rRNA analyses. The abbreviation DNS represents specimens in which the colony Did Not Sequence.

Table 6

Biochemical tests conducted on bacteria collected from pill bug specimen #75 at locality #3 during the winter of 2016

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
75A	*Arthrobacter sp1*	+	-	-	-	+	+
75B	*A. phenanthrenivorans*	-	-	-	+	+	+
75C	DNS	-	-	-	+	+	+
75D	*B. thuringiensis*	+	Yellow	-	-	+	+
75E	*B. cereus*	+	-	-	-	+	+
75F	*B. subtillis*	+	Yellow	-	-	+	+
75G	*B. megaterium*	+	Yellow	-	-	+	+
75H	*B. subtillis*	+	Yellow	-	-	+	+
75I	*Microbacterium sp1*	+	-	-	+	+	+

Note. Abbreviations for Biochemical Tests follow those in text. The abbreviation DNS represents specimens in which the colony Did Not Sequence.

Table 7

Biochemical tests conducted on bacteria collected from pill bug specimen #76 at locality #2 during the winter of 2016

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
76A	*B. cereus*	+	Yellow	-	-	+	+
76B	*B. niancini*	+	-	-	-	+	+
76C	*B. pumilis*	+	Yellow	-	-	+	+
76D	*R. ornithinolytica*	-	-	+	+	+	-
76E	*C. dublinensis*	-	-	+	+	+	+
76F	*C. hominis*	+	-	-	-	+	+
76G	*Microbacterium sp2*	+	-	-	-	+	+
76H	*B. niancini*	+	-	-	-	+	+

Note. Abbreviations for Biochemical Tests follow those in text.

Table 8

Biochemical tests conducted on bacteria collected from pill bug specimen #80 at locality #2 during the spring of 2016

Colony	Genus	Staining	MSA	MAC	EMB	NA	Catalase
80B	*A. hydrophila*	-	-	Light brown	-	+	-
80C	*Arthobacter sp1*	+	-	-	-	+	+
80E	*B. cereus*	+	-	-	Yellow	+	+
80G	*R. ornithinolytica*	-	-	Light pink	Dark purple	+	-
80J	*B. flexus*	+	-	Light brown	-	+	+
80K	*Acinetobacter sp1*	-	-	Light brown	Pink	+	+

Note. Abbreviations for Biochemical Tests follow those in text.

Table 9

Biochemical tests conducted on bacteria collected from pill bug specimen #81 at locality #3 during the spring of 2016

Colony	Genus	Gram	MSA	MAC	EMB	NA	Catalase
81B	*Pseudomonas sp1*	-	-	-	-	+	-
81D	*B. megaterium*	+	Yellow	-	+	+	+
81F	*B. flexus*	+	Yellow	-	+	+	+
81H	*B. cereus*	+	-	-	-	+	+
81K	*B. amyloliquefaciens*	+	Yellow	-	-	+	+
81M	*Pseudomonas sp1*	-	-	-	-	+	-
81N	*Serratia sp2*	-	-	Light pink	Purple	+	+

Note. Abbreviations for Biochemical Tests follow those in text.

Table 10

Morphology and colony count from bacteria collected from specimen #22 at locality #2 during the summer of 2015

Colony	Size	Elevation	Margin	Form	Number	Genus
22 A	Medium	Flat	Erose	Filamentous	2	***B. cereus***
22 B	Medium	Flat	Rhizoid	Filamentous	1	*B. acidiceler*
22 C	Big	Umbonate	Smooth	Round	10	*Achromobacter sp1*
22 D	Medium	Umbonate	Smooth	Round	10	*Serratia sp1*
22 E	Medium	Umbonate	Smooth	Round	8	*Aeromonas sp2*
22 F	Small	Umbonate	Smooth	Round	26	*A. hydrophila*
22 G	Medium	Flat	Rhizoid	Filamentous	1	***B. cereus***
Total					58	

Note. The specimens diameter was 4.7 mm. Bacterial load was determined using a 1:10 dilution and 3 replicates. For this specimen, bacterial load was determined to be 3,867. Bolded taxa represent instances where colony morphologies were different for bacteria identified as the same genus and species.

Table 11

Morphology and colony count from bacteria collected from specimen #57 at locality #2 during the Fall of 2015

Colony	Size	Elevation	Margin	Form	Number	Genus
57 A	Tiny	Flat	Smooth	Round	1	DNG
57 B	Big	Flat	Irregular	Irregular	6	***B. cereus***
57 C	Medium	Flat	Irregular	Irregular	4	***B. cereus***
57 D	Small	Flat	Irregular	Irregular	13	***Paenibacillus sp1***
57 E	Small	Flat	Smooth	Round	10	***Paenibacillus sp1***
57 F	Big	Flat	Smooth	Round	14	*B. simplex*
57 G	Medium	Flat	Smooth	Round	11	DNG
Total					59	

Note. The specimens diameter was 4.8 mm. Bacterial load was determined using a 1:20 dilution and 3 replicates. For this specimen, bacterial load was determined to be 7,867. Bolded taxa represent instances where colony morphologies were different for bacteria identified as the same genus and species.

Table 12

Morphology and colony count from bacteria collected from specimen #71 at locality #3 during the Fall of 2015

Colony	Size	Elevation	Margin	Form	Number	Genus
71 A	Medium	Flat	Irregular	Irregular	2	***B. cereus***
71 B	Tiny	Flat	Smooth	Round	5	*P. pocheonensis*
71 C	Medium	Flat	Irregular	Irregular	4	***B. amyloliquefaciens***
71 D	Big	Flat	Smooth	Round	7	***B. megaterium***
71 E	Small	Flat	Smooth	Round	9	***B. cereus***
71 F	Medium	Flat	Smooth	Round with filamentous	6	*B. thuringiensis*
71 G	Medium	Flat	Lobate	Irregular	1	*L. sphaericus*
71 H	Medium	Flat	Irregular	Irregular	4	***B. megaterium***
71 I	Medium	Flat	Irregular	Irregular	2	*B. pumilus*
71 J	Medium	Flat	Irregular	Irregular	1	DNS
71 K	Small	Flat	Smooth	Round	15	***B. amyloliquefaciens***
Total					56	

Note. The specimens diameter was 3.7 mm. Bacterial load was determined using a 1:1 dilution and 3 replicates. For this specimen, bacterial load was determined to be 745. Bolded taxa represent instances where colony morphologies were different for bacteria identified as the same genus and species.

Table 13

Morphology and colony count from bacteria collected from specimen #75 at locality #3 during the Winter of 2016

Colony	Size	Elevation	Margin	Form	Number	Genus
75 A	Tiny	Flat	Smooth	Round	50	*Arthrobacter sp1*
75 B	Small	Flat	Smooth	Round	20	*A. phenanthrenivorans*
75 C	Medium	Flat	Smooth	Round	19	DNS
75 D	Big	Flat	Smooth	Round	35	*B. thuringiensis*
75 E	Medium	Convex	Irregular	Irregular	4	*B. cereus*
75 F	Big	Convex	Smooth	Round	1	***B. subtillis***
75 G	Medium	Flat	Irregular	Filamentous	1	*B. megaterium*
75 H	Medium	Flat	Smooth	Round	1	***B. subtillis***
75 I	Medium	Flat	Irregular	Irregular	2	*Microbacterium sp1*
Total					133	

Note. The specimens diameter was 4.5 mm. Bacterial load was determined using a 1:1 dilution and 3 replicates. For this specimen, bacterial load was= 887. Bolded taxa represent instances where colony morphologies were different for bacteria identified as the same genus and species.

Table 14

Morphology and colony count from bacteria collected from specimen #76 at locality #2 during the winter of 2016

Colony	Size	Elevation	Margin	Smooth	Number	Genus
76 A	Medium	Convex	Irregular	Irregular	8	*B. cereus*
76 B	Medium	Flat	Smooth	Round	2	***B. niancini***
76 C	Medium	Flat	Smooth	Round	5	*B. pumilis*
76 D	Small	Flat	Smooth	Round	10	*R. ornithinolytica*
76 E	Medium	Flat	Smooth	Round	1	*C. dublinensis*
76 F	Big	Flat	Smooth	Round	4	*C. hominis*
76 G	Tiny	Flat	Smooth	Round	6	*Microbacterium sp2*
76 H	Medium	Flat	Smooth	Round	1	***B. niancini***
Total					37	

Note. The specimens diameter was 3.8 mm. Bacterial load was determined using a 1:5 dilution and 3 replicates. For this specimen, bacterial load was 4,933. Bolded taxa represent instances where colony morphologies were different for bacteria identified as the same genus and species.

Table 15

Morphology and Colony Count From Bacteria Collected From Specimen # 80 at Locality #2 During the Spring of 2016

Colony	Size	Elevation	Margin	Form	Number	Genus
80 B	Medium	Convex	Smooth	Round	14	*A. hydrophila*
80 C	Tiny	Convex	Smooth	Round	38	*Arthobacter sp1*
80 E	Medium	Flat	Irregular	Filamentous	2	*B. cereus*
80 G	Medium	Convex	Smooth	Round	24	*R. ornithinolytica*
80 J	Medium	Flat	Smooth	Round	1	*B. flexus*
80 K	Medium	Flat	Irregular	Irregular	3	*Acinetobacter sp1*
Total					82	

Note. The specimens diameter was 4.1 mm. Bacterial load was determined using a 1:20 dilution and 3 replicates. For this specimen, bacterial load was = 10,933

Table 16

Morphology and colony count from bacteria collected from specimen #81 at locality #3 during the spring of 2016

Colony	Size	Elevation	Margin	Form	Number	Genus
81 B	Tiny	Convex	Smooth	Round	64	*Pseudomonas sp1*
81 D	Big	Convex	Smooth	Round	4	*B. megaterium*
81 F	Medium	Flat	Smooth	Round	7	*B. flexus*
81 H	Medium	Flat	Irregular	Irregular	3	*B. cereus*
81 K	Medium	Convex	Irregular	Irregular	1	*B. amyloliquefaciens*
81 L	Medium	Flat	Irregular	Irregular	2	
81 M	Small	Convex	Smooth	Round	33	*Pseudomonas sp1*
81 N	Medium	Convex	Smooth	Round	13	*Serratia sp2*
Total					127	

Note. The specimens diameter was 3.8 mm. Bacterial load was determined using a 1:5 dilution and 3 replicates. For this specimen, bacterial load was=4,233.

Table 17

A Summary of the Unique Isolates Recovered in the Present Study. Included is the Overall % Diversity Among Identified Genera or Species.

Genus	Species	Isolates	Time Points	L1	L 2	L 3	% Diveristy	Pathogen
Bacillus	*cereus*	11	Summer	X	X		0.0	A
			Fall		X	X		
			Winter		X	X		
			Spring		X	X		
	subtilis	3	Summer	X			0.0	B
			Winter			X		
	niancini	2	Winter		X		0.0	C
	thuringiensis	2	Fall			X	0.0	B
			Winter			X		
	amyloliquefaciens	2	Fall			X	0/0	B
			Spring			X		
	simplex	2	Fall		X		0.0	C
	acidiceler	1	Summer		X		N/A	C
	pumilis	2	Fall			X	0.0	C
			Winter		X			
	flexus	1	Spring		X		N/A	C
Paenibacillus	*pocheonensis*	1	Fall			X	9.2	C
	sp.1	2	Fall		X			
Lysinibacillus	*sphaericus*	1	Fall			X	N/A	C

(Table 17 continues next page.)

Genus	Species	Isolates	Time Points	L1	L 2	L 3	% Diveristy	Pathogen
Aeromonas	*hydrophila*	3	Summer	X	X		0-4.4	A
			Spring		X			
	sp.1	1	Summer		X			?
	sp.2	1	Summer		X			?
Arthrobacter	*phenanthrenivorens*	1	Winter			X	0-1.2	B
	sp.1	2	Winter			X		?
			Spring		X			
Raoultella	*ornithinolytica*	4	Summer	X			0.0	C
			Winter		X			
			Spring		X			
Rhodococcus	*sp.1*	3	Summer	X			0.0	B
Microbacterium	*sp.1*	1	Winter			X	1.3	C
	sp.2	1	Winter		X			?
Pseudomonas	*sp.1*	2	Spring			X	0.0	A
Serratia	*sp.1*	1	Summer		X			A
	sp.2	1	Spring			X		A
Citrobacter	*sp.1*	1	Summer	X				B
	sp.2	1	Summer		X			?
Enterobacter	*sp.1*	4	Summer	X			0	A
Cellulomonas	*hominis*	1	Winter		X		N/A	C
Chronobacter	*dublinensis*	1	Winter		X		N/A	A
Acinetobacter	*sp.1*	1	Spring		X		N/A	A

Note. In addition, the pathogenicity of each genus or species is identified. The "?" represents isolates where the pathogen status is not known

Table 18

A summary of the bacterial isolates broken down by locality and season. Bacteria that are potential pathogens are highlighted in yellow

Summer	L1	L2
	B. cereus	***B. cereus***
	B. subtilis	*B. acidceler*
	A. hydrophila	*A. hydrophila*
	R. ornitholytica	*Aeromonas sp2*
	Rhodococcus sp1	*Serratia sp1*
	Citrobacter sp1	
	Enterobacter sp1	

Fall	L2	L3
	B. cereus	***B. cereus***
	B. simplex	*B. thuringiensis*
	Paenibacillus sp 1	*B. amyloliquefaciens*
		B. pumilis
		L. sphaericus
		P. pocheonensis

Winter	L2	*L3*
	B. cereus	***B. cereus***
	B. niancini	*B. subtilis*
	B. pumilis	*B. thuringiensis*
	R. ornithinolytica	*A. phenanthrenivorens*
	Microbacterium sp2	*Arthrobacter sp1*
	C. hominis	*Microbacterium sp1*
	C. dublinensis	

Spring	L2	L3
	B. cereus	***B. cereus***
	B. flexus	*B. amyloliquefaciens*
	A. hydrophila	*Pseudomonas sp1*
	Arthrobacter sp1	*Serratia sp2*
	R. ornithinolytica	
	Acinetobacter sp1	

Note. Bolded taxa were found at all time points.

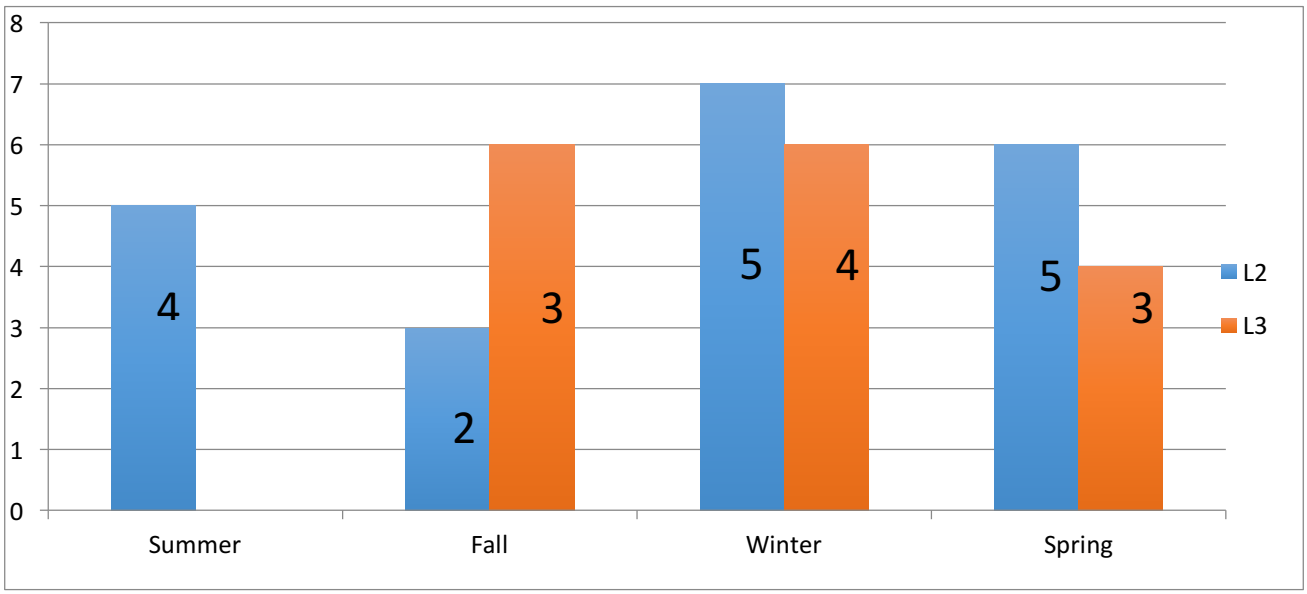

Figure 1. The distribution of genera and species at each site during each time point recovered in the present study. Locality 1 is excluded from this analysis as there were no samples taken after the first time point, summer. Locality 3 is represented by samples from fall, winter, spring, with no sample taken in the summer. The numbers inside each bar represent the unique genera recovered at that time point.

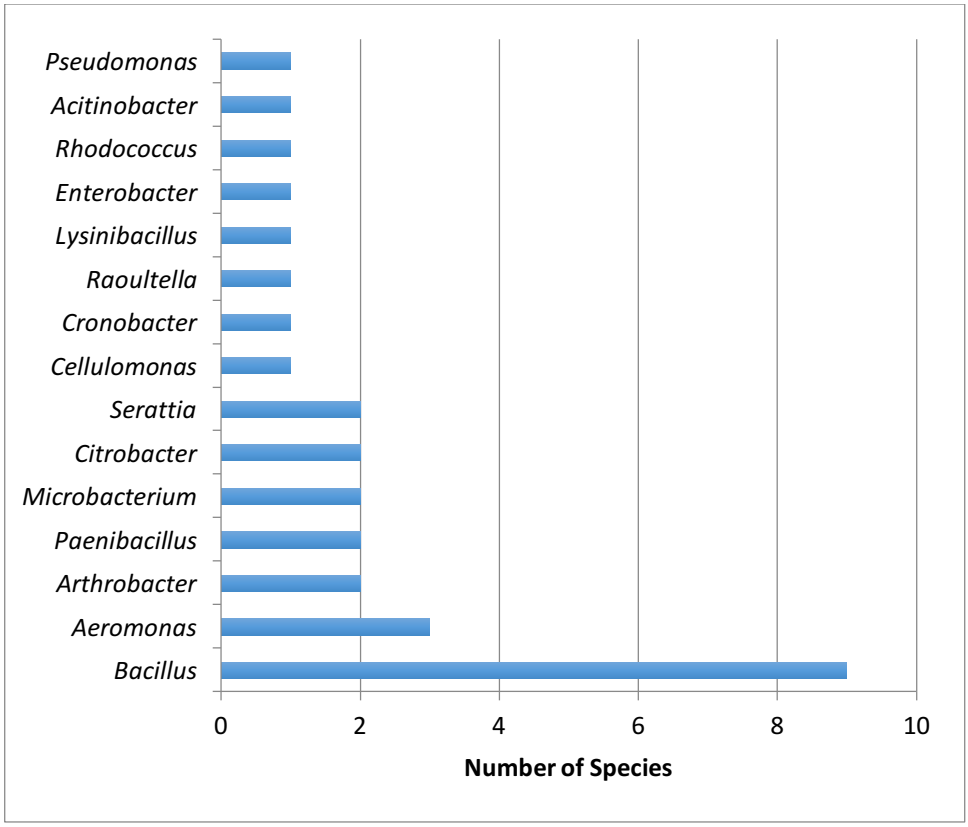

Figure 2. The number of species identified within each genus.