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USING A CULTURABLE APPROACH AND METAGENOMIC ANALYSIS TO UNCOVER GUT BACTERIAL DIVERSITY OF GRAY BATS IN KANSAS

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master in Science

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USING A CULTURABLE APPROACH AND METAGENOMIC ANALYSIS TO UNCOVER GUT BACTERIAL DIVERSITY OF GRAY BATS IN LOCALITIES OF SOUTHEAST KANSAS

An Abstract of the Thesis by
Bobbi Monroe

Humans have historically had an ambivalent relationship with bats. In one hand, bats perform an important service to humans by reducing populations of many insect pests. On the other hand, they act as reservoirs of disease as highlighted by the recent Coronavirus pandemic. In the United States, many bat populations have been threatened by white nose syndrome caused by the fungus *Pseudogymnoascus destructans.* This study was designed to characterize the bacterial diversity associated with the gray bat (*Myotis grisescens*) in Southeast Kansas. In addition, this study aimed to shed light on the presence of bacterial pathogens, if any, among this bat population. A total of 32 guano samples were collected and bacterial isolates with different colony morphology were recovered on tryptic soy agar media after enrichment. The majority (21/32, 65%) of isolates were Gram positive. All isolates were tested for growth on selective and differential media. Sugar fermentation profiles showed that 78% (25 of all isolates) fermented all four sugars, 9% (3/32) fermented three sugars, another 9% (3/32) fermented two sugars, and one isolate (1/32) 3% fermented only one sugar. Urea was hydrolyzed by seven (21%) isolates while one isolate (3%) was positive for indole production. After bacterial isolation results were shortlisted to removed possible repeats, additional tests yielded that 52% (13/25) were one step nitrate reducers, 68% (17/25) were MR positive while 32% (8/25) were VP positive, 52% (13/25) were oxidase positive and only 0.04% (1/25) was positive for phenyl alanine. All isolates were tested for their susceptibility to

multiple antibiotics. In addition to biochemical characterization, the isolates were identified using molecular techniques. Pooled samples were sequenced using an Illumina mini sequencer. A total of 2,909,555 reads were completed. The most common Gram positive genera being *Bacillus* (17.01%) and *Lysinibacillus* (19.93%) while the most common Gram negative genera were *Serratia* (26.36%) and *Achromobacter* (20.17%). Male and Female samples were sequenced using the same Illumina mini sequencer. For males a total of 5,408,935 reads were completed and for females 9,645,398 reads were completed. The most common species in male Gray Bats were *Bacillus pakistanensis* (17%) followed by *Enterococcus faecalis* (7.17%) and *Jeotgalicoccus pinnipedialis* (2.70%). The most common species in female Gray Bats were *Romboustia clostridium* (11%), followed by *Enterrococcus faecalis* (7.9%), *Bacillus pakinstanensis* (3.90%). The identification of both known and novel bacteria in bats is important for prevention of possible disease spread and in preserving the bat species further.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

Bats are a diverse group of species that use a multitude of habitats (e.g., caves, forests, waterbodies). Bats are classified in the order *Chiroptera,* which contains over 1,100 known species (Li, et al., 2010). Bats hold an important place in the ecosystem, as they function in pollinating plants, dispersing seeds, and control of both agricultural pests and human disease vectors. Without the flowers being pollinated by bats or their ability to disperse seeds, many ecosystems would be negatively affected and could possibly collapse if bats become rare or absent (U.S. Fish and Wildlife, 2021). However, not all aspects of bat biology are positive, as they are reservoirs to many viruses that pose risks to humans via zoonoses and several other animal species (e.g., insects, etc.). A zoonosis is a disease that has the capability to jump from the host to a human. A pathogen that is considered zoonotic can be bacterial, viral, or parasitic (Zoonoses, 2023). Due to the clustering nature of bat colonies, disease spread between them rapidly and abundantly **(Fig. 1).** Bats will cluster for warmth during their hibernation months and will also groom each other socially (Carter & Leffer, 2015).

Figure 1. Tri-colored bats *Perimyotis subflavus* exhibit clustering behavior during hibernation. (Photo by Bobbi Monroe)

Some may even spread through unconventional methods, such as spreading via direct contact, through water or the environment. There have been pathogens of similar genetic makeup shared between bats and honeybees. Aphis lethal virus (ALPV) and Big Sioux River Virus (BSRV) have been found to be pathogenic to honeybees (Zana, et al., 2018). The presence of these viruses has been found in bat guano samples. This suggests that viruses can be circulated in the ecosystem by bats. On an average bats will fly between 15 km (about 9.32 mi) and 28 km (about 17.4 mi) each night as they forage (Oleksy et al., 2015). This is ample time for guano and urine droppings to fall as they fly. Other animals, insects or humans may then encounter the excrement and as a result

bacteria or zoonotic diseases may spread. Bats have become more widespread in urban areas, due to habitat loss and human encroachment; this puts them in closer contact with humans and thereby raising the risk of disease transmission (Allocati et al., 2016). There is also some supporting evidence that bats carry mites or parasites, specifically belonging to the genus *Plasmodium*. These fall into a clade of rodent malaria parasites and move between several different mammalian hosts (Schaer et al., 2013). Bat flies are common hematophagous ectoparasites among bats. These flies encourage bacterial spreading among bat colonies and can contain bacteria known to be infectious to humans. There may be some parasites that live symbiotically with bats however, it is important to note that they can also be mediators of zoonotic diseases (Lee et al., 2021). The parasites that both live and feed off bats are a choice food source for many other insects such as ants and spiders as well as species such as chickens, turkeys, ground birds, etc. Through the food chain those insects are then eaten by larger organisms who are also eaten by even larger organisms. Throughout this process any number of zoonotic diseases can jump from one organism to another. It is important to understand what can be carried and how it may spread (Benyedem et al., 2022).

While bats are a diverse group that provide many important ecosystem functions, studies of bat microbiomes are quite limited. Microbiomes are the combination of all the microbiota that reside on or within an organism’s tissues and associated biofluids. Some examples include microbiomes of the skin, mammary glands, seminal fluid, uterus, lung, saliva, oral mucosa, gastrointestinal tract, etc. (Federici et al. 2022). The microbiome is an essential part of a species' development, nutrition, and immunity. Although some microbes are pathogenic, not all microbial species that live within an animal are harmful.

Many may function as beneficial colonizers rather than harmful invaders. A dysfunction of the microbiome within humans is known to cause autoimmune diseases like diabetes, rheumatoid arthritis, multiple sclerosis, fibromyalgia and muscular dystrophy, and these diseases are predominantly tied to the gut microbiome. However, bats do not rely on gut bacteria in the same way humans do, due to the fact that their digestive tracts are shorter in length compared to humans given the demands and constraints associated with flying (Ingala, et al., 2018). Bats carry less food and less intestinal tissue as a result. So, for bats, their microbiome is built more from the environments that they live in rather than from any evolutionary predispositions to host specific bacteria. Fecal samples were the most noninvasive collection method for examining the gut microbiota as opposed to others such as mucosal biopsies, intestinal fluid, etc. (Tang et al., 2020). Besides, for those individuals without rabies vaccinations fecal samples were the easiest to collect. There is still some risk associated with the fecal bacteria not being fully separated from the intestinal floral bacteria resulting in possible contamination so the collections will need to be repeated to account for variability and for accuracy.

Pathogen outbreaks are common among bats living in close quarters, so it is important and necessary to learn about gut bacterial diversity. The recent pandemic has resulted in more focus on bats as carriers of many diseases that may affect humans. Other scientists have performed research into the viral genomes of various bat species and in different locations (Bergner, et al., 2019). Additionally, there has been an increase in White Nose Syndrome affecting bat populations and resulting in high mortality. White nose syndrome was first documented in New York in the winter of 2006 –2007 and has since spread across the Eastern US and Canada and has been found even as far South as

Mississippi, Southern Texas and in Western states, affecting hibernating bats. Infected bats will fly outside during the cold months, (Coleman, 2014) and have been found both sick and dying in extremely high numbers in and around caves and mines. Statistical records show that more than 5.5 million bats in the Northeastern U.S. and Canada have been affected and in some locations the death rate is between 90 and 100%. More than 47 bat species in the US and Canada must hibernate to survive the winter putting them at risk. Currently, 12 species, including 2 endangered and 1 threatened, have been confirmed with White nose syndrome in North America. (Frick, et al., 2010). As such, more information on bat microbiomes has become desperately needed. It is important to study bat species from a variety of different areas to learn what microbes they carry individually and how that varies across space. This will be particularly useful in attempting to prevent further spread.

The bats used for this research were found to be roosting in stormwater systems in Pittsburg, Kansas **(Fig. 2).** Typically, Gray Bats prefer to inhabit karst systems near water and dense floodplain vegetation (Decher, 1989). However, they can be opportunistic and have been found occasionally using storm drains in other locations.

Figure 2. Stormwater system near Evergy in Pittsburg, KS (Photo by Haley Price, PSU).

It is believed that the dark and humid environment of the sewer tunnels appeals to the Gray Bats. These bats prefer temperatures between 6 and 11º C (42-52ºF) for winter hibernation and between 14 and 25ºC (57-77ºF) for summer roosting and will seek out the most humid location in these temperature ranges (Kessler, 2022). Unfortunately, white nose fungus prefers the same cold and humid conditions. While most Gray Bats are one of the few North American bats that inhabit caves year-round, due to human encroachment the hibernating and roosting locations have begun to change. One of the biggest dangers to Gray Bats is human disturbance during hibernation or roosting (Kessler, 2022).

Figure 3. Gray Bat *Myotis grisescens* (Photo by Bobbi Monroe)

The number of Gray Bats had previously been on a decline however their numbers are increasing in Kansas and the surrounding areas (**Fig. 3**). It is likely they will be removed from the endangered list in the coming years (Gray bat, 2022). In 1984 the number of Gray Bat colonies that hibernated in caves in Missouri, Oklahoma, and Kansas was approximately 100,000 (Harvey, 1984). In other locations across the U.S. Gray Bat populations, as a result of protections, have been able to hold steady and even increase slightly. Currently in Kansas, however, the population is still considered as a species in need of conservation and in Missouri it is vulnerable (Nature Serve, 2022). Missouri currently contains approximately 20 percent of the Gray Bat population. The locations in which they typically reside are found south of the Missouri River, particularly around the Ozarks (Gray myotis, 2022).

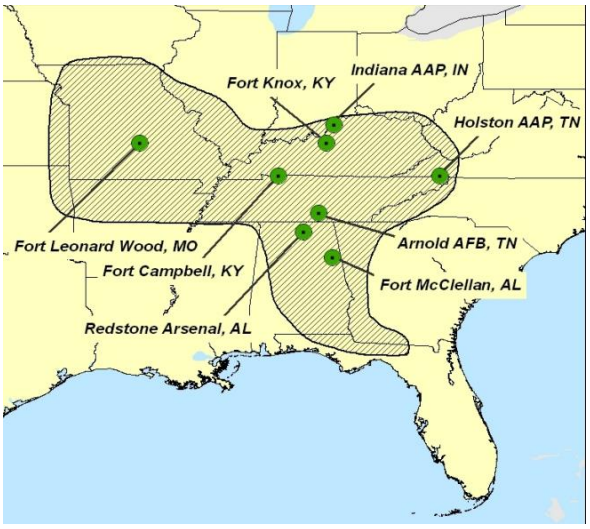

Figure 4. Approximate Gray Bat range based off the location of military installation population documentation (Martin, 2007).

The purpose of this research was to describe the gut bacterial diversity of the Gray Bat colonies in Southeast Kansas using a culturable approach and metagenomic analysis, which was important to understand disease transmission and help preserve bat species.

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF CULTURABLE ISOLATES FROM GRAY BAT GUANO

INTRODUCTION

As stated in the previous chapter, bats perform an important service to humans by reducing populations of many insect pests. However, on the other hand, they act as reservoirs of diseases as highlighted by the recent Coronavirus pandemic. In this chapter, bat guano samples were collected and bacterial isolates with different colony morphology were recovered on tryptic soy agar media after enrichment. These isolates were further characterized using biochemical tests and identified using molecular techniques. The purpose of this part of the study was to identify the dominant culturable microbes that were shed in the guano and that would be at a higher risk of contraction to people and other animals coming in contact.

METHODS

Collection Procedure and Phenotypic Analysis

A total of 25 bat guano samples were collected from individual Gray Bats from two different storm water systems in Pittsburg, Kansas in Crawford and Cherokee counties (**Fig. 5A and 5B**). These collections were performed by previous undergraduate researchers and preserved in individual enrichment vials. Samples were collected from the years 2017 and 2018 (**Fig. 6**). The enrichment vials were made using one gram of guano inoculated in 10 ml (about 0.34 oz) brain heart infusion broth and incubated up to 24 hours at 30° C. These vials were preserved at 4°C refrigerator until ready for bacterial isolation. Bacterial isolates with different colony morphology were then recovered on tryptic soy agar media (TSA) after streaking enrichment. Colony morphology was noted individually.

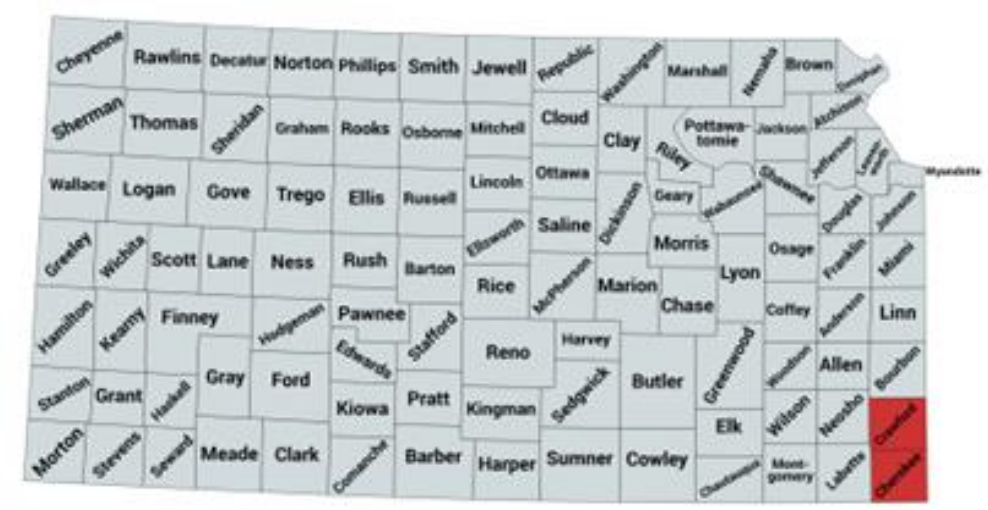

Figure 5A. Gray Bat colony sites in Kansas

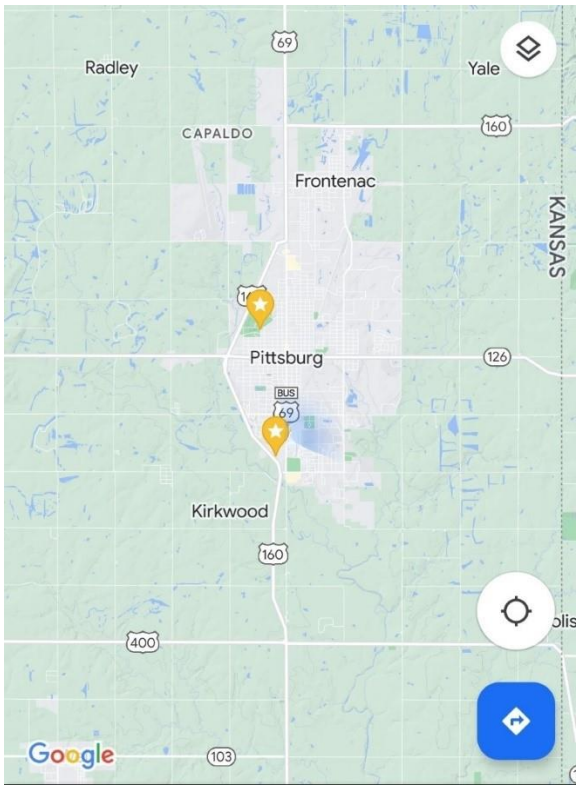

Figure 5B. Collection sites for this study in Pittsburg, Kansas

Gram staining was performed using glass slides for heat-fixing bacterial samples and flooded with Gram's crystal violet, iodine and 95% ethanol and rinsed. Safranin was used as the counterstain. The slides were viewed under an oil immersion microscope to determine gram-positive or gram-negative results. All staining procedures were followed according to Microbiology Laboratory Theory & Application- Brief/Edition 2 (Leoboffe & Peirce, 2016).

Figure 6. Preserved individual enrichment vials used for bacterial revival.

Biochemical Tests

All isolates were checked for their growth on selective and differential media: Eosin Methylene Blue (EMB), MacConkey agar (MAC), and Mannitol Salt agar (MSA) to confirm the Gram stain profile. All isolates were tested for presence of catalase, nitrate reduction, oxidase production, presence of gelatinase on gelatin agar, and presence of amylase on starch agar using specific reagents.

Each isolate was spotted on Blood agar to test for hemolysin production. Additionally, fermentation of carbohydrates including lactose, glucose, maltose, and sucrose were tested as well as Methyl Red-Voges Proskauer (MRVP) for determining mixed acid fermentation or stable acid end products (Fig. 7). Phenylalanine deamination, also known as phenylpyruvic acid test (PPA) for deaminase production (Fig. 8), urea broth was used to indicate urease production, and tryptone broth was used to indicate indole production. All biochemical experimental procedures were followed according to Microbiology Laboratory Theory & Application- Brief/Edition 2 (Leoboffe & Peirce, 2016).

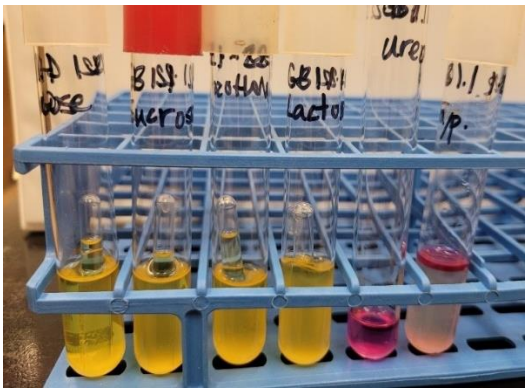

Figure 7. Selected isolate inoculated in (left to right) sugar fermentation tubes containing glucose, sucrose, maltose, lactose, urea broth and tryptone broth.

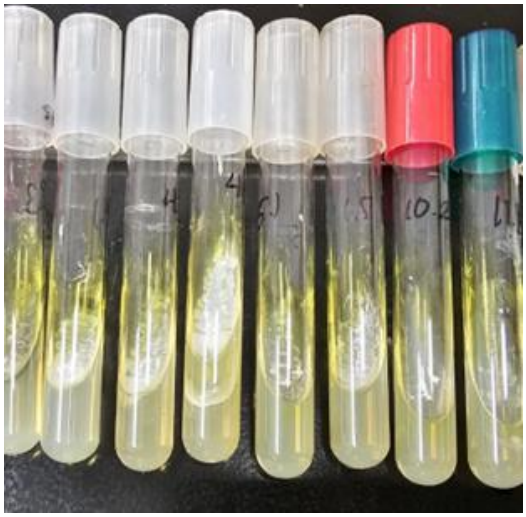

Figure 8. Selected isolates inoculated in Phenylalanine slants and reagent added

Biofilm

In three separate sterile 96-well plates Brain Heart Infusion broth (BHI) was added as follows: all blanks were filled with 300 µl of BHI and the remaining wells were filled with 270 µl of BHI. The controls and bacterial isolates were pipetted into the wells in the amount of 30 µl each (Fig. 9). The positive control used was *Pseudomonas aeruginosa* while the negative control used was *Staphylococcus aureus.* See Biofilm protocol (Appendix A).

Figure 9. Inoculated 96 well plate stained with crystal violet to test for biofilm formation

Antibiotic Susceptibility Testing

Antibiotic susceptibility was tested by Kirby-Bauer disc diffusion method on Mueller-Hinton agar with following classes of antibiotics: β-lactams, aminoglycosides, tetracyclines, quinolones, macrolides, cephalosporins, glycopeptides, and carbapenem. Each isolate was spread plated on Mueller-Hinton agar and antibiotic discs were placed throughout. Zone of clearance was measured to determine resistance (Fig. 10).

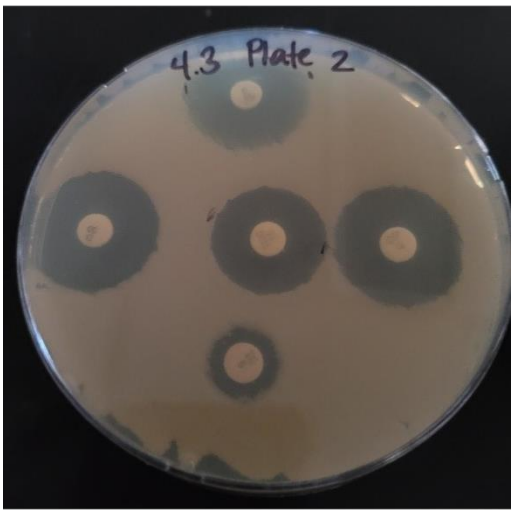

Figure 10: Antibiotic resistance profile on Mueller-Hinton agar displaying zones of clearance.

Molecular Characterization

The 16S rRNA gene was targeted for PCR amplification using universal primers 27F and 1492R. Individual bacterial isolates were selected from TSA with a sterile toothpick and resuspended in 50 µl of sterile water. These samples were gently mixed with a miniRoto Fisher Scientific Vortex Mixer and placed in Bio-Rad C 1000 Touch Thermal Cycler to heat denature the genomic DNA at 95°C for ten minutes.

A random combination of 15 isolates (1 µl from each denatured suspension) were pooled into a 1.5 ml (about 0.05 oz) microcentrifuge tube. A total of 15 µl of isolate DNA template was combined with 485 µl of dH_2O. Three microcentrifuge tubes were prepared, and all culturable isolates were covered in this random selection. A total of 20 µl PCR reaction mixture included: one µl of bacterial DNA as the template from the pooled tube, 0.5 µl of each primer (20 pmole), both forward and reverse, 10 µl of PCR Master Mix (which company?) and the rest was sterile water. PCR protocol was as follows: denaturation at 95°C for three minutes prior to 29 cycles of the following: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for one minute. Finally, the last extension step was at 72°C for 5 minutes. The infinite hold was set at 4°C.

Agarose Gel Electrophoresis

Gel was prepared using one-percent agarose and 1X Tris base, acetic acid and EDTA buffer (TAE) and ~2.5 µl (recommended 1 µl per 20 ml (about 0.68 oz) gel) iNtRON Redsafe Nucleic Acid Staining Solution (20,000x). The gel was poured into a Fisher Biotech Electrophoresis Mini Horizontal Unite and left to solidify. The gel was run using 1X TAE buffer as tank buffer.

PCR sample preparation for loading the gels as follows: four µl of loading buffer (MIDSCI) was added to each 20 µl PCR reaction and mixed by pipetting up and down before loading twenty µl into each well. A Bullseye 100 base pair (bp) DNA ladder (MIDSCI) was pipetted into the first well in the amount of five µl direct. The samples were run at 150 volts for ~75 minutes and the gel was then removed from the buffer

solution to visualize under ultraviolet light Electrophoresis Systems 312 Transilluminator (Fisher Scientific).

Quantification of DNA

Nanodrop Lite (Thermo Scientific) was used to take readings for 27 isolates in order to record 260/280 ratios and ng/µl (Appendix B)

DNA Sequencing

The isolates were identified at the genus and species level after PCR amplification and 16S rRNA gene sequencing using an Illumina minisequener. Sequencing identification was performed using Illumina miniseq and BaseSpace Sequence Hub website. The sequencing workflow included library preparation, cluster generation, sequencing, and data analysis. Library prep is a critical step for successful sequencing. The aim of the prep was to obtain nucleic acid fragments with adapters attached to both ends. To do this, identification barcode markers are attached to P5 and P7 binding regions. The P5 and P7 regions were included in the indices used to prep the samples. The steps for cluster generation were: Denature and dilution (cluster densities of ~160-220 K clusters/mm2 were desired). To ensure that NaOH was fully neutralized in the final solution, 200 mM Tris-HCl (pH 7.2) was used. PhiX 1.4 pM was diluted and then mixed with library samples to result in a 1% PhiX control volume ratio. A total of 0.5 ml of the denatured and diluted sample was used in the reagent cartridge. The cartridge was thawed, inverted 5 times to mix thoroughly and then loaded with custom index 1 and 2 index primers and custom read primers information. The sequencing run performed by

the MiniSeq set up was as follows: Cluster generation = 90 minutes, sequencing cycle = 4 minutes per cycle, paired end turnaround = 60 minutes and post run wash = 60 minutes.

Basespace Analysis

Sequences were obtained and after creating fasta files were uploaded to MEGA11 program in order to create traditional, circular, radial or cladogram phylogenetic trees. Neighbor-joining trees were also created using aligned sequences in MEGA11 software.

RESULTS AND DISCUSSION

Each sample was enriched via one gram of guano inoculated in BHI broth and incubated for 24 hours at 30°C. These enrichment broths were preserved in the refrigerator and further spread plated on Tryptic Soy Agar (TSA) for isolation and selection of individual bacterial colonies. Once isolated further preservation was done placing each sample in a glycerol mix and placed in -20°C for keeping. Finally, a total of 32 isolates were preserved.

Gram Staining and Biochemical Characterization

Gram stain profile showed that out of all isolates 21/32 (65%) were Gram positive and 11/32 (35%) were Gram negative. The shape of bacterial cells were predominately cocci followed by bacilli. Gram stain results can be helpful indicators when performing antibiotic resistance tests (Lagier et al. 2012).

Growth on selective and differential media (Eosin Methylene Blue, MacConkey, Mannitol Salt Agar) mostly corroborated with their Gram status, except a few (5/32) that

did not grow on any selective media at all (Fig. 16). Sugar fermentation profiles of lactose, sucrose, maltose and glucose showed that 78% (25 of all isolates) fermented all four sugars, 9% (3/32) fermented three sugars, another 9% (3/32) fermented two sugars, and one isolate (3%) fermented only one sugar (Fig. 11).

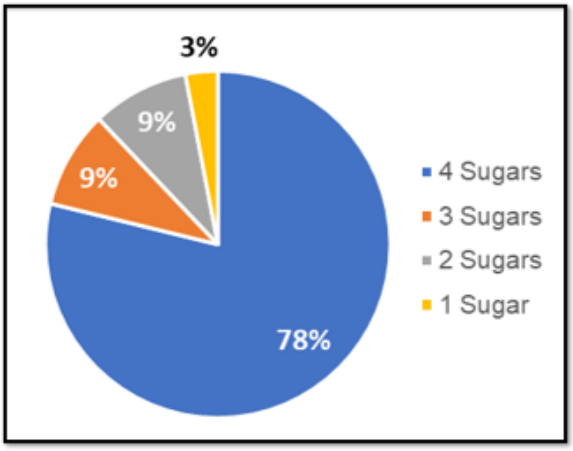

Figure 11. Carbohydrate fermentation profile

Several other biochemical tests yielded varying results. Add a line for starch and gelatin (Fig. 15 and 16). Notably, urea was hydrolyzed by seven (21%) isolates while one isolate (3%) was positive for indole production. The presence of gelatinase was more common compared to amylase. Methyl red-Voges Proskaur (MRVP) indicated 64% (16/25) following mixed acid fermentation of glucose and the remaining 32% (8/25) were negative indicating the utilization of the butanediol fermentation pathway for glucose (Fig. 13A and 13B). Only one isolate (0.04%) was positive for phenylalanine deamination or phenylpyruvic acid test (PPA) (Fig. 12). PPA positive is interesting because this means the bacteria oxidatively deaminate phenylalanine to phenylpyruvic acid and can help differentiate among urea positive Gram- negative bacilli. The biochemical characterization of both Gram-positive and Gram-negative isolated indicated

diverse metabolic potential. This is important to note as it helps determine viability of a particular bacterial type once it is excreted via guano in different environments.

Figure 12. The left tube displays a dark hunter green ring near the top of the slant indicating a positive result in Phenylalanine deamination test

Figure 13A. Methyl Red-Voges Proskauer (MRVP): Red indicates positive for mixed acid fermentation. Yellow indicates a negative result.

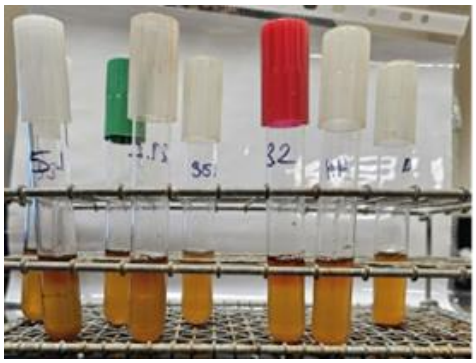

Figure 13B. The rusty red ring at the top of the tubes indicates a positive result of deaminase production for Voges Proskauer portion of MRVP test.

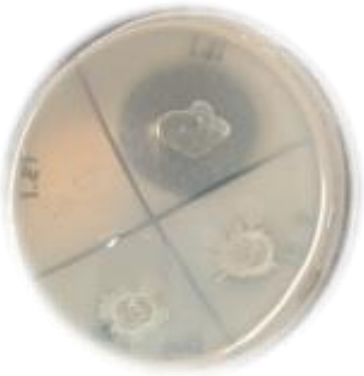

Figure 14: Zone of clearance indicates a positive result of gelatinase production

Figure 15: Zone of clearance indicates positive production of Amylase.

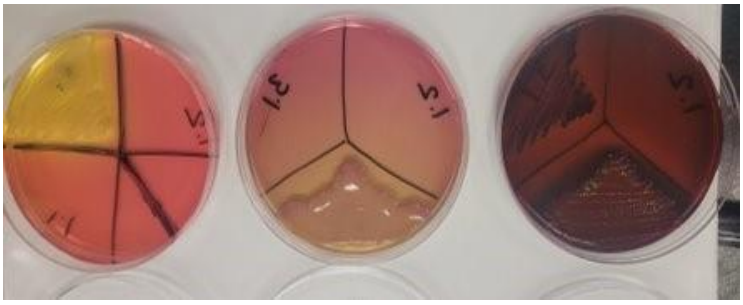

Figure 16: From left to right: Growth on MacConkey, Mannitol Salt and Eosin Methylene Blue agar.

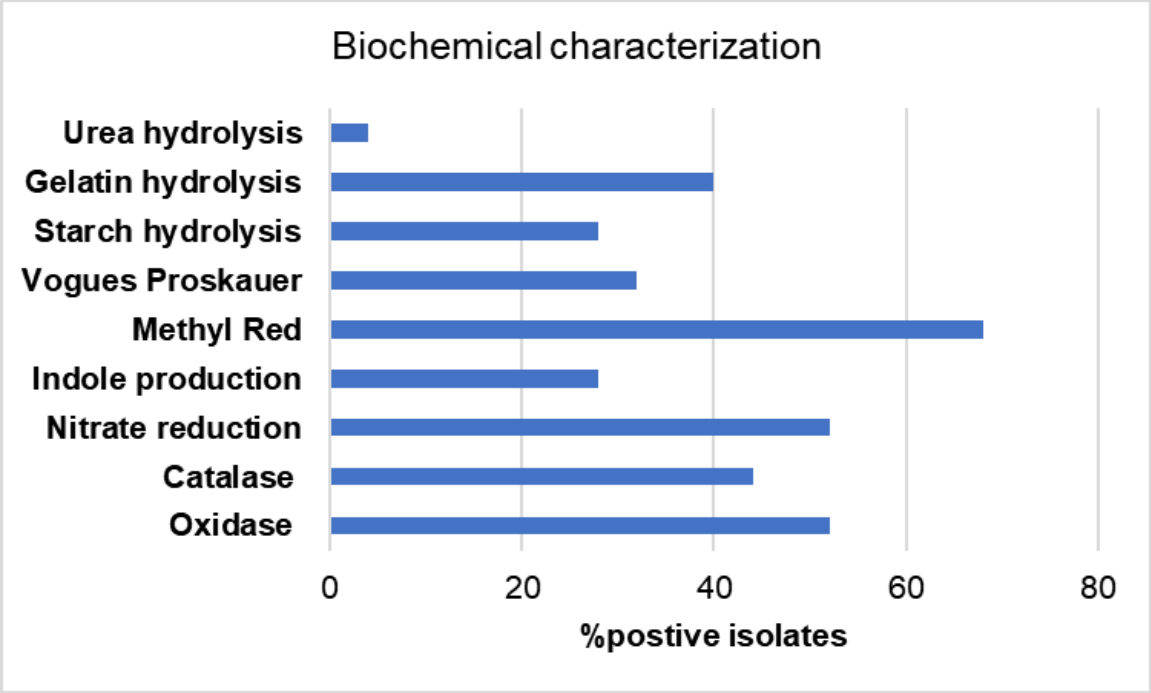

Figure 17: Overall biochemical profile (n=25)

Antibiotic Resistance Profile

The Gram-positive isolates were frequently resistant to vancomycin and tetracycline while mostly susceptible to gentamycin and tigecycline. The Gram-negative isolates showed frequent resistance to meropenem and susceptibility to imipenem (Fig. 18A and 18B). Frequent resistance to vancomycin is unlikely and therefore needs to be further confirmed. Total resistance to meropenem is also unlikely and needs to be further confirmed. The data for meropenem was not conclusive and excluded from the graph.

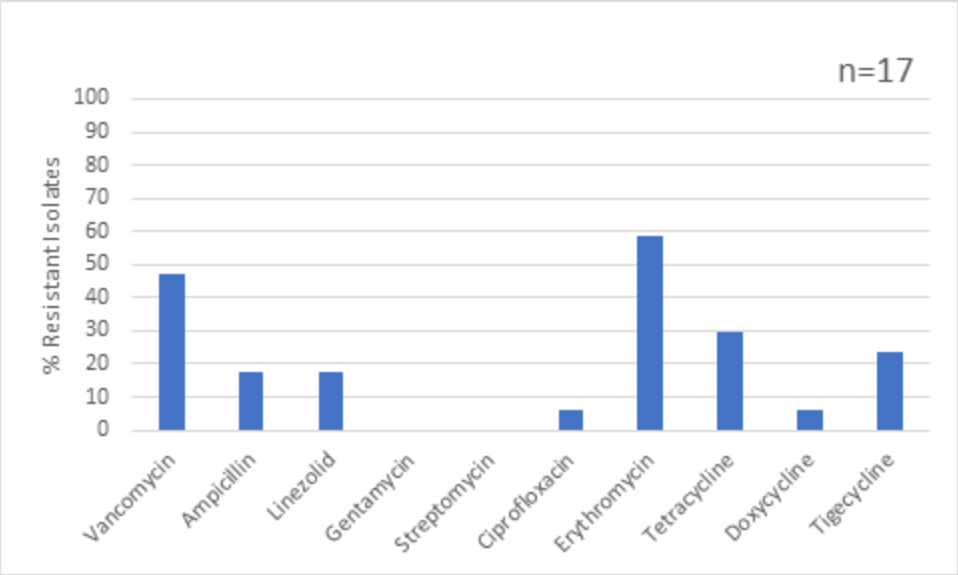

Figure 18A: Antibiotic resistance profile for Gram positive isolates: Pip/Taz: Piperacillin/Tazobactum; Tic/Clav: Ticaracillin/Clavulonic acid; Sulfa: Sulfamethoxazole

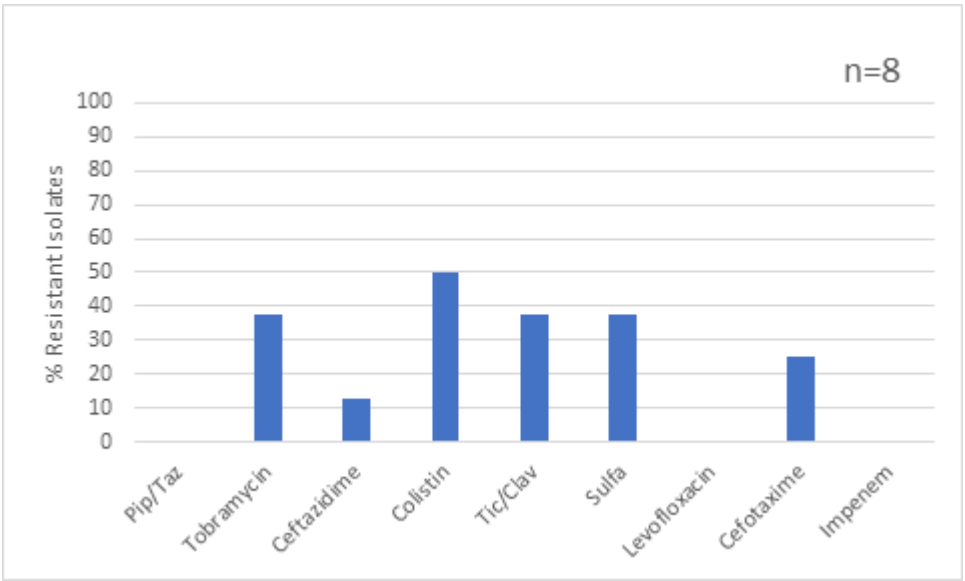

Figure 18B: Antibiotic resistance profile for Gram-negative isolates: Pip/Taz: Piperacillin/Tazobactum; Tic/Clav: Ticaracillin/Clavulonic acid; Sulfa: Sulfamethoxazole

Biofilm Formation Capability

Biofilm results indicate that 92% (23/25) of the isolates were biofilm formers with the majority being high formers (Fig. 19). Production of biofilm could indicate possible resistance to treatments due to their abilities to grow and persist on both living and non-living surfaces.

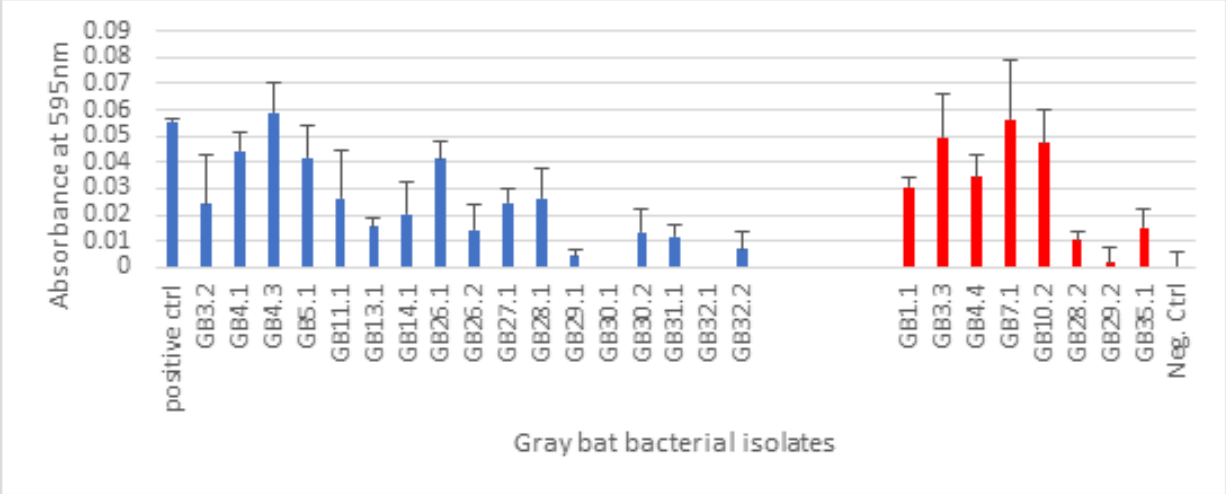

Figure 19. Extent of biofilm formation. Blue bars indicate Gram positive isolates and red bars indicate Gram negative isolates. Any isolate giving results of at least 50% of the positive control (>0.027) are considered biofilm positive.

Molecular Characterization and DNA Sequencing

The most abundant genera within the pooled Gray Bats were *Serratia* followed by *Achromobacter*, *Lysinibacillus*, *Bacillus*, *Acinetobacter*, *Enterrococcus*, *Paenibacillus*, *Staphylococcus*, *Sporobacter* and *Mycoplasma* (Fig. 20).

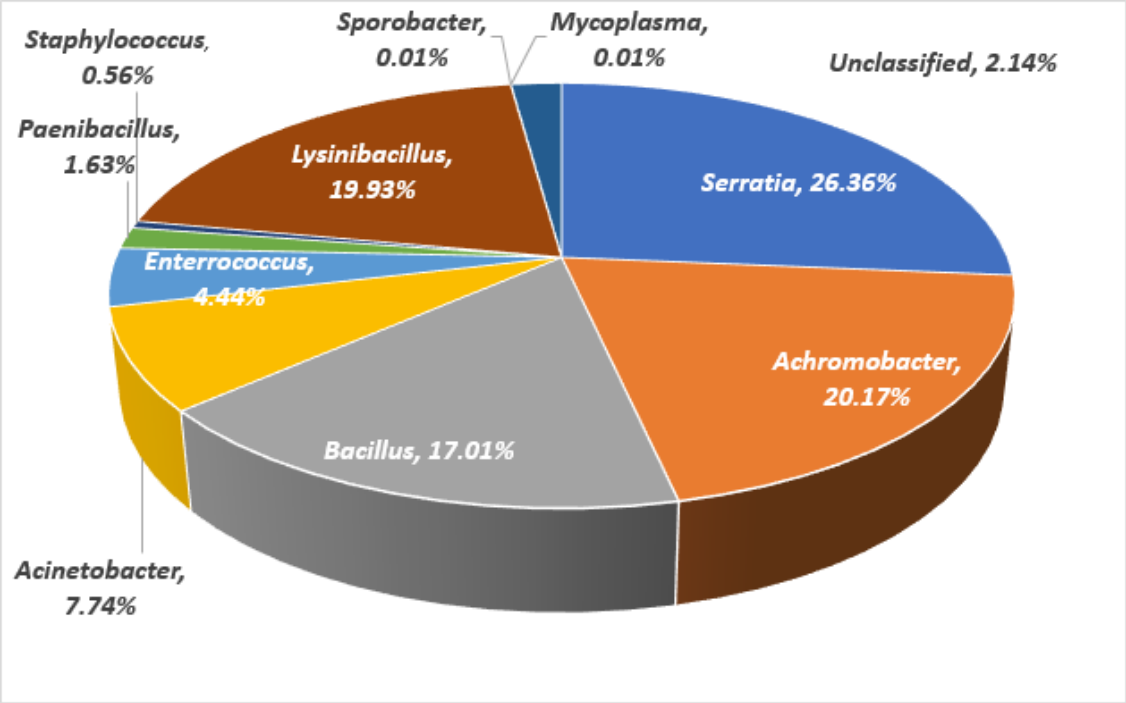

Figure 20. Most abundant genera of pooled Gray Bat samples

Serratia is a genus known to be a Gram negative, rod-shaped facultative anaerobe. It was originally considered nonpathogenic and used in medical experiments but has since been found to harbor multi drug resistance and can complicate treatment decisions (Mahlen, 2011). This genus can infect humans, insects and plants and has been found in water and soil as well. Bats potentially house this bacterium through the food chain and environmental interactions and so can potentially spread this along to other species by extension.

Interestingly, *Mycoplasma* is a genus of bacteria that, like the other members of the class *Mollicutes*, does not possess a cell wall around their cell membranes. Peptidoglycan is absent. This characteristic makes them naturally resistant to antibiotics that target cell wall synthesis. Due to these characteristics *Mycoplasma* can be parasitic or saprotrophic.

Achromobacter is a genus of bacteria, included in the family *Alcaligenaceae* in the order *Burkholderiales*. These cells are Gram-negative straight rods and are motile by using anywhere from 1 to 20 peritrichous (meaning around the mouth or body) flagella. They are strictly aerobic and are found in soils and water.

The presence of *Sporobacter* is also an intriguing discovery. This bacterium is known to be an obligate anaerobe. It was able to grow in an environment that contained oxygen, which should be noted. The species *Sporobacter termitidis* is relatively new and has been found in the guts of termites (Grech-Mora et al., 1996). The most common mammalian predator of termites is bats. Bats are insect eaters and are highly skilled termite-hunters. These bats feed on termites that swarm at dusk and during the nighttime hours. Bats have been found darting toward groups of swarming termites with unique precision.

It is known that an imbalance of the microbiome in humans can lead to several different issues. A study found that in patients with hypertension there was an abundance of *Catabacter*, *Robinsoleilla*, *Serratia*, *Enterobacteriaceae*, *Ruminococcus torques*, *Parasutterella*, *Escherichia*, *Shigella*, and *Klebsiella*, while a decreased abundance of *Sporobacter*, *Roseburia hominis*, *Romboutsia spp.*, and *Roseburia* (Naik et al.,2022). While these may serve different functions in bat species the significance is relevant. Bats that reside in different locations around the globe likely house contrasting bacterial types. This may be due to the environment in which they feed and dietary habits (Cláudio et al., 2018). Not all bacterium within a bat's microbiome are dangerous but they are known to house many bacteria that can jump from host to host and become pathogenic. It is

important to note these in order to prevent disease spread while maintaining preservation of bat species.

This study focused on the Gray Bat (*Myotis griscescens*) colonies in Pittsburg, Kansas. An encouraging future direction for this research might include different bat species in Pittsburg, Kansas and comparing the two. Additionally, this study could be conducted at different times of the year but also extend out to bats around the world and include fungal and more viral studies.

CHAPTER III

COMPARISON OF MALE AND FEMALE BAT GUT BACTERIA

INTRODUCTION

The first half of this study concentrated on characterization of culturable bacterial isolates from a bat population followed by identification of dominant bacterial genera. The subsequent part of this research aimed at comparison of gut bacterial diversity between male and female bats using sequencing approach. It is possible that male and female bats house different bacteria due to variable foraging behaviors (Leven et al., 2013).

METHODS

Field Sample Collection

Sterile equipment was taken to two different collection sites. These locations were chosen based on previously known roosting places of Gray Bats. These sites were a storm water system near Evergy Electric Company (A) and a stormwater system in Lincoln

Park (B), both in Pittsburg, KS. These are the same collection sites referenced in Chapter 2 (Fig. 5B).

Guano samples were collected in September of 2021, July of 2022, and September of 2022 (Table 1).

Table 1. Collection details of male and female bat guano samples using Harp trap

Chapter 2. Culturable method: Preservation vials for individual bats	10 from 2017 (pooled)	21 from 2018 (pooled)	-	-
Chapter 3. 16S metagenomics	Males	Females	Pooled	Dominant bat type
July 15, 2021	0	0	1 vial (From mat)	Adults
September 15, 2021	2	1	1 vial (moderate yield)	Adults
July 15, 2022	9	4	0	Juveniles
September 28-29, 2022	10	7	1 vial (good yield)	Adults

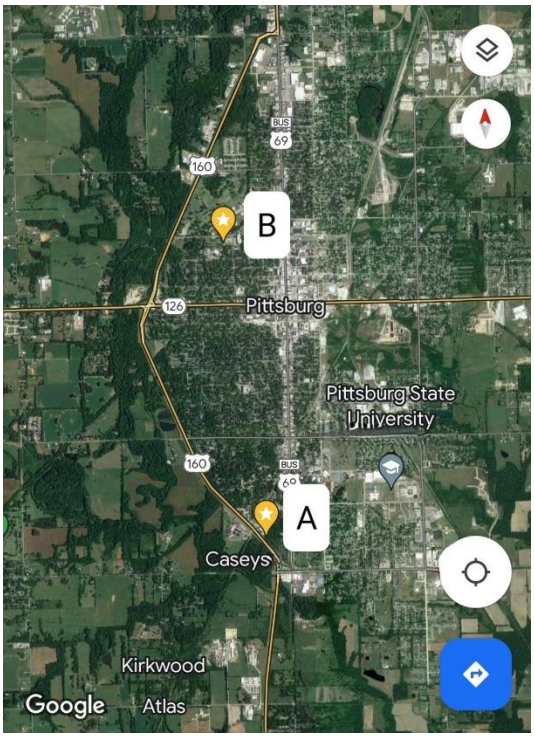

Figure 5B. Gray Bat guano collection site map

Gray Bat guano samples were collected aseptically using sterile equipment: a plastic tube with a scoop attached to the lid, latex gloves, paper bags, and recording materials. A harp trap was set up at the entrance of the stormwater system at each location (Fig. 20) shortly before dusk. After several hours, once the Gray Bats had time to forage and return, they were removed from the trap and individually placed in paper bags (Fig. 21A). They remained in the bags for approximately 30 minutes. After the allotted time frame, each bat was removed from the bag, the sex was noted and each bag was inspected for fecal material. Any droppings were collected via the sterile scoop and labeled appropriately (Fig. 22B). Each bat was then released unharmed.

Figure 21. Harp trap set up at location B (Photo by Bobbi Monroe).

Figures 22A. Gray Bats in paper bags during collection (left panel); **22B**. Labeled fecal collection tube (right panel). (Photo by Bobbi Monroe)

Sequencing Identification

DNA Extraction and quantitation

The male samples totaled 21 and the female samples totaled 12 (Fig. 23). The male samples were divided into three groups of 7 while the females were divided into two groups of 6. Extraction was completed using FastDNA SPIN Kit for Feces (MP Biomedicals) protocol (Appendix C). The quality of DNA was checked using a Nanodrop Lite (Thermo Scientific) (Appendix B).

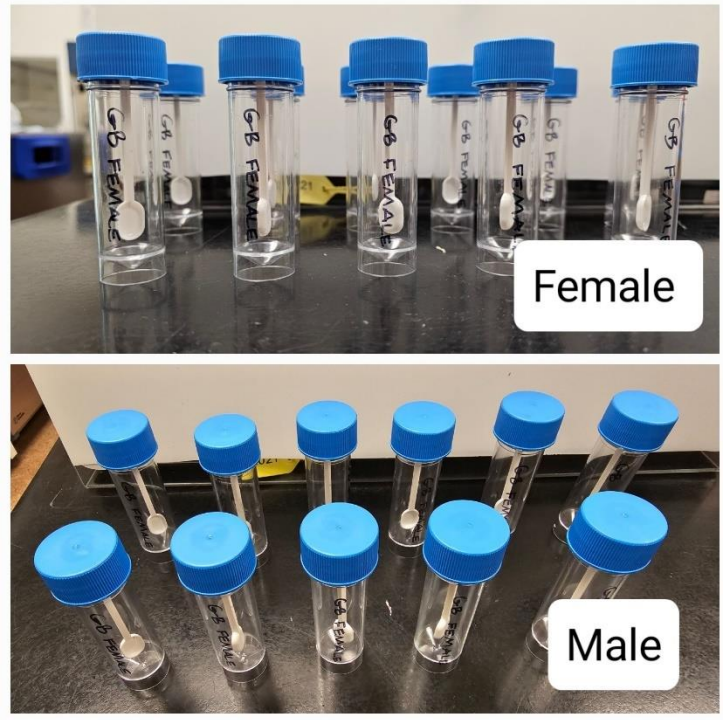

Figure 23. Distribution of male and female bat guano samples

PCR amplification and agarose gel electrophoresis

The entire 16S rRNA gene sequence length is 1.5kb (1500 bp) and the primers were selected for PCR amplification of the full length of the gene (Fig. 24). The PCR protocol followed was: denaturation at 95°C for 5 minutes prior to 29 cycles of the following: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for one minute. The last extension step was at 72°C for 10 minutes.

The infinite hold was set at 4°C. The gel prepared and run the same way as described in Chapter 2. Preparation of loading samples as well as visualization of agarose gel was also mentioned in the methods section in Chapter 2.

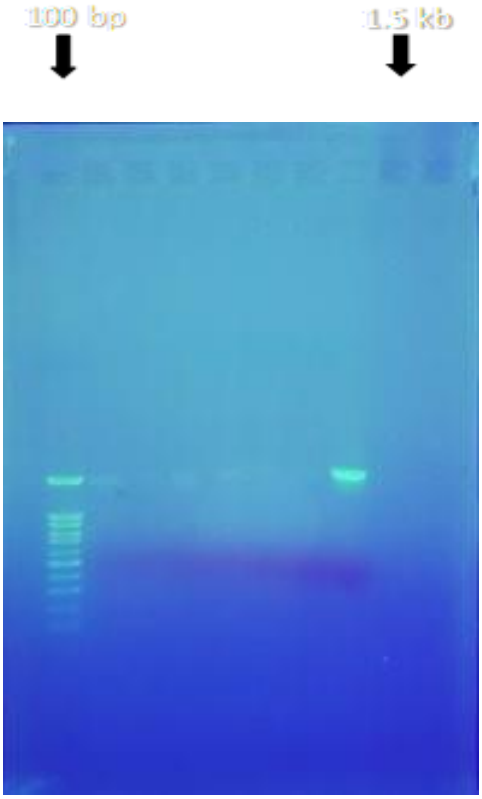

Figure 24. Agarose gel showing PCR amplification of full length 16S rRNA gene.

Quantification of DNA before sequencing

Nanodrop Lite (Thermo Scientific) was used to take readings for all 5 samples in order to record 260/280 ratios and ng/µl. Readings for each sample were also taken using a Qubit fluorometer 4 (Appendix D). (Table 2)

Table 2. Nanodrop and Qubit Quantification of PCR amplicon

	Method of quantification	
Sample ID.	**Nanodrop**	**Qubit Fluorimeter**
M1 (Male 1)	A260 (10mm):1.133 A260/A280: 1.81 56.7ng/µl	70.8 ng/µl
M2 (Male 2)	A260 (10mm):1.070 A260/A280: 1.82 53.5 ng/µl	42.0 ng/µl
M3 (Male 3)	A260 (10mm):1.257 A260/A280: 1.84 62.9 ng/µl	41.8 ng/µl
F4 (Female 4)	A260 (10mm):1.740 A260/A280: 1.82 87.0 ng/µl	76.6 ng/µl
F5 (Female 5)	A260 (10mm):2.828 A260/A280: 1.83 141.4 ng/µl	106 ng/µl

16S Metagenomic Sequencing Library Prep

Library prep for sequencing was performed using the proper protocols beginning with 16S Library preparation workflow (Appendix E), followed by First round of sequencing PCR protocol (Appendix F), Clean-up protocol of first round of sequencing PCR (Appendix G), Index PCR protocol – second round (Appendix H), Clean-up

protocol of index PCR (Appendix I), and finally, library quantification, normalization, and pooling (Appendix J).

RESULTS AND DISCUSSION

The most abundant phyla between male and female bats was Firmicutes. In males it was 48.29% and in females it was at 42.49%. These are fairly close in number. Next was Actinobacteria at 31.85% in males and 20.28% in females. Following that, females contain bacteria in the phyla Bacteroidetes at 18.25% while males are only at 4.10%. The phyla between male and female Gray Bats seem to be very similar in type and somewhat similar in distribution.

The phyla Firmicutes are a gram-positive bacteria that have a cell wall. In humans, high amounts of Firmicutes in the gut have been linked to depression and IBS (Huang et al., 2018). In mice, a correlation between depressive behavior and Firmicutes has also been found. Firmicutes are a gut bacterium that make contributions towards protecting the stomach lining. It was not unexpected to find Firmicutes present, however, the excess or lack of this bacteria could have health implications.

Actinobacteria are one of the largest phyla in bacteria and are classified as a gram-positive bacteria with a high guanine and cytosine content. They possess a wide variety of morphologies as well as different behaviors. They can be pathogens, soil inhabitants, plant commensals or gastrointestinal. They can be found both aquatically and terrestrially (Ventura et al., 2007).

Bacteroidetes are another phyla that are diverse. Typically, gram-negative but can be either anaerobic or aerobic depending on the environment. They are non-motile and

can be found in many different ecological niches including soil, ocean water, freshwater and the GI tract of animals. They are involved in the breakdown of food that is eaten and do well handling pH changes, nutrients and oxygen availability (Thomas et al., 2011). Having Bacteroidetes in the gut is normal to a certain degree. When there is an excess, as with most other bacterial imbalances, problems may ensue. Due to their opportunistic behaviors in functioning as an anaerobe and the gram-negativity they are considered to be potentially dangerous pathogens.

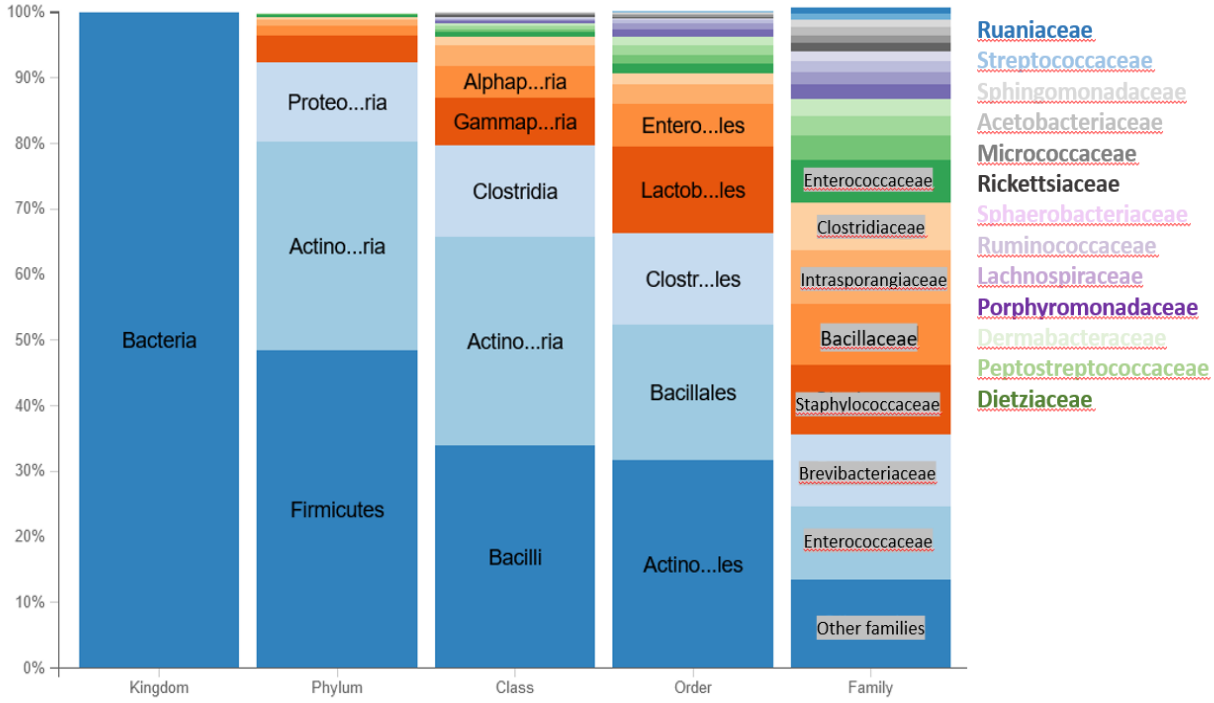

Figure 25A. Phyla classification of male Gray Bats gut bacterial isolates

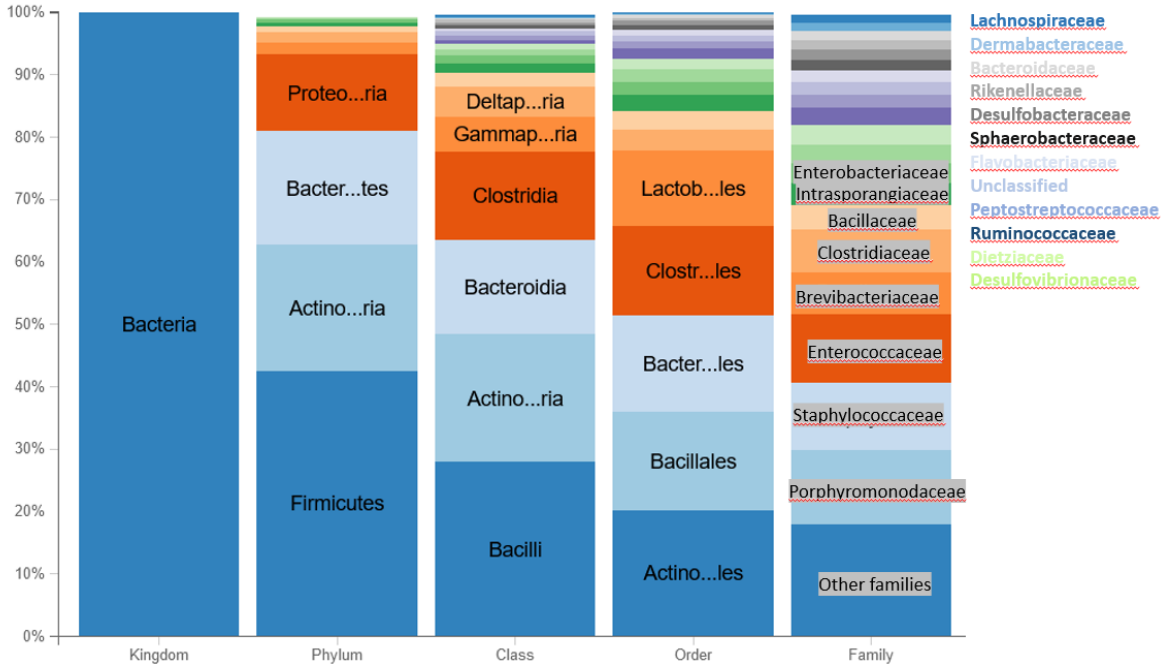

Figure 25B. Phyla classification of female Gray Bats gut bacterial isolates

The most abundant species in female Gray Bats was *Romboustia clostridium*, followed by *Enterrococcus faecalis*, *Bacillus pakinstanensis*, *Clostridium sensu stricto/Clostridium perfringens*, *Frederiksenia pastueraliacaeae bacterium*, *Clostridium bifermentans*, *Clostridum colinum*, *Dysgonomonas hofstadii*, *Dysgonomonas gadei*, P*lesiomonas shigelloides* and *Dietzia timorensis* (Fig. 26).

The most abundant species found in male Gray Bats was *Bacillus pakistanensis* followed by *Enterococcus faecalis, Jeotgalicoccus pinnipedialis, Ornithinimicrobium murale, Actinomyces bowdenii, Dietzia timorensis, Morganella morganii, Roumboustia clostridium sp., Coenonia anatina, Enterobacer tabaci, Pleisiomonas shigelloides, Corynebacterium frankenfortense, Isobaculum melis, Enterrococcus casseliflavus, Rosenbergillia nectarea,* and *Staphylococcus cohnii* (Fig. 27).

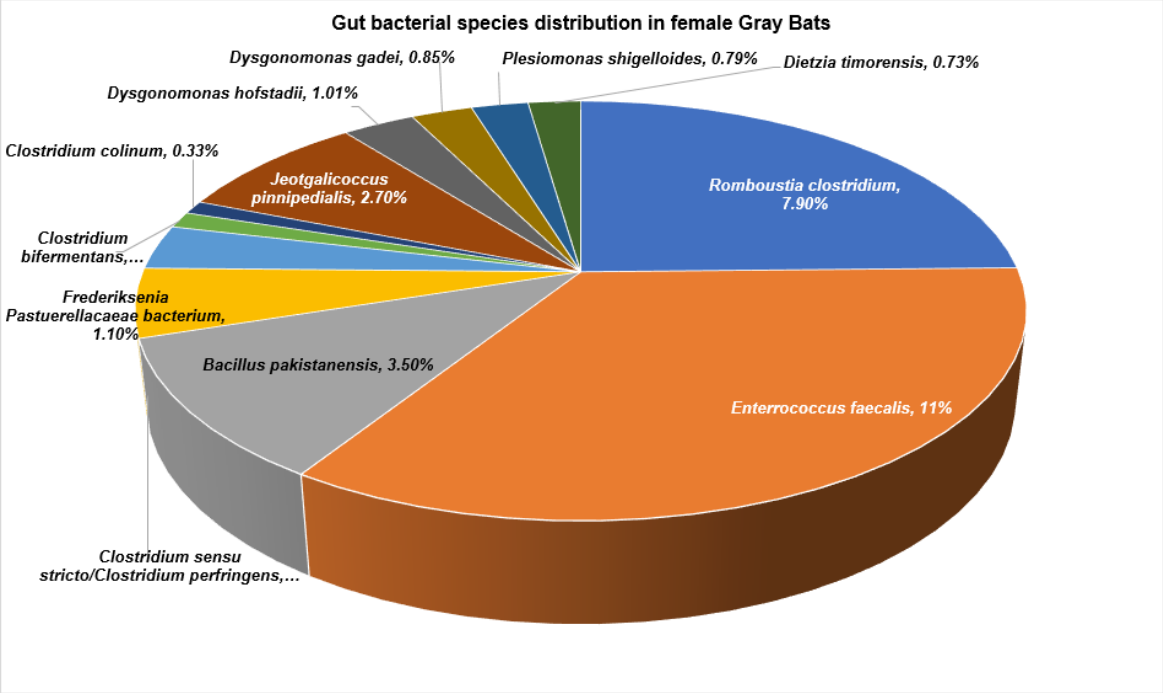

Figure 26. Chart representing the most abundant species of gut bacterial species in female Gray Bats.

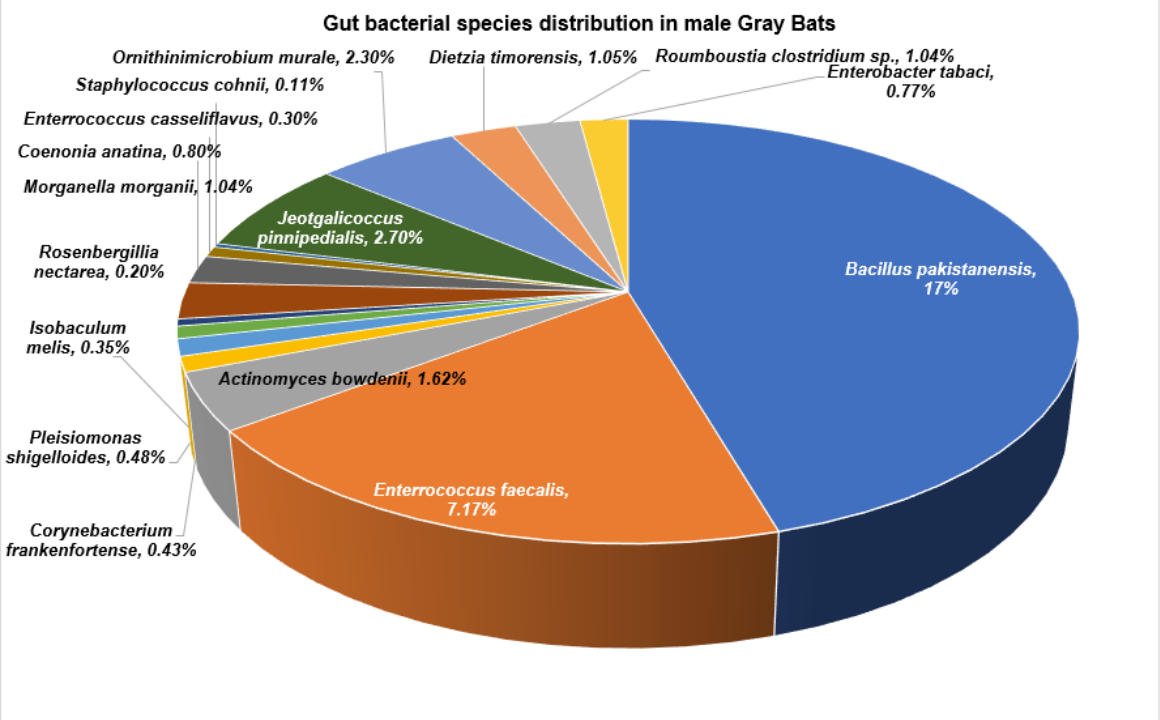

Figure 27. Chart representing the most abundant species of gut bacterial species in male Gray Bats.

Bacillus pakinstanensis was the most abundant in males at and was also among the top few of female results. This bacterium is halo resistant, Gram positive and endospore forming. The halo resistance could contribute to survivability in different environments.

Dietzia timorensis spp. are closely related to *Rhodococcus equi* phenotypically. R. equi, in humans, is an opportunistic pathogen and has been associated with severe immunodeficiency as well as pneumonia (Pilares et al., 2010).

Male and female Gray Bats were shown to contain the same bacterial types but in different concentrations. For example, in the males *Bacillus pakinstanensis* was at 17% but at 3.50% in females. In addition, in males house *Enterococcus faecalis* at 7.17% while females were at 11%. For males, *Romboustia clostridium* was 1.04% and for females 7.90%. These differences are stark in comparison.

The initial expectation was that the female and male bats would house different bacteria. Upon closer evaluation they are shown to carry mostly the same bacteria but in differing concentrations. This may be due to how females feed at different times and amounts than males especially if they are lactating. Female bats will consume higher amounts of protein during lactation, and this may account for the percentage differences between the sexes (Li et al., 2021). Further testing could be done between males and lactating females of different species and locations in this area to additionally confirm bacterial differences and details.

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APPENDIX

Appendix A. Biofilm protocol

Appendix B. Table showing nanodrop data before sequencing procedure

Appendix C. FastDNA SPIN Kit for Feces (MP Biomedicals) protocol

Appendix D. Qubit fluorometer 4 parameters and quantitation data

Appendix E. 16S Library preparation workflow

Appendix F. First round of sequencing PCR protocol

Appendix G. Clean-up protocol of first round of sequencing PCR

Appendix H. Index PCR protocol (second round of sequencing PCR)

Appendix I. Clean-up protocol of index PCR

Appendix J. Protocol of library quantification, normalization, and pooling

Appendix A

Biofilm Assay Procedure

Inoculate BHI broths with bacteria and incubate 24-48 hours at 30 or 37°C degree C, or until broth is turbid with bacterial growth

Pipetting into 96-Well Plate (round bottom)

Procedure:

1. Pipette 270 µL of BHI broth into each well using multichannel pipettor (pour broth from autoclaved bottle onto Petri plate)

2. In wells B2-B6 pipette 30 µL of the positive control (Pseudomonas aeruginosa) (5 replicates)

3. In wells C2-C6 pipette 30 µL of the first sample (5 replicates)

4. Repeat step 3 for other samples through G2-G6, then continue pipetting samples in wells B7-B11 down through F7-F11 (5 replicates)

5. In wells G7-G11, pipette 30 µL of the negative control (Staphylococcus aureus) (5 replicates)

6. Incubate plate for 24-48 hours at 37°C.

Washing the Plate

Procedure:

1. Dump the liquid in the plate into the sink (use a flicking/throwing motion).

2. With 200 µL of phosphate-buffered saline (PBS), rinse each well using a multichannel pipette using an up and down motion (5 times) of the liquid

3. Repeat step 2.

4. Dry in inverted position without the lid (leave it for at least 48 hours, it can be more than that if needed)

5. Stain using 300 µL of 1% Gram's Crystal Violet for 15 minutes. (prepare the stain beforehand and filter using coffee filter, work with stain near the sink)

6. Dump the CV out. Wait for 10 min.

7. Wash with 200 µL of 80:20 ethanol-acetone (prepare solvent beforehand) using the same technique as in step 2.

8. Transfer the extracted stain into new 96-well plate.

Reading the Plate

Use Epoch Microplate Spectrophotometer (SN 1703014) downstairs in common equipment room to observe optical density. The plates need to be scanned at 495 nm wavelength. See machine manual for directions.

Appendix B

Nanodrop Quality Data Before Sequencing

Nanodrop quality	M1 (Male 1)	A260 (10mm):1.133 A260/A280: 1.81 56.7ng/µl
Nanodrop quality	M2 (Male 2)	A260 (10mm):1.070 A260/A280: 1.82 53.5 ng/µl
Nanodrop quality	M3 (Male 3)	A260 (10mm):1.257 A260/A280: 1.84 62.9 ng/µl
Nanodrop quality	F4 (Female 4)	A260 (10mm):1.740 A260/A280: 1.82 87.0 ng/µl
Nanodrop quality	F5 (Female 5)	A260 (10mm):2.828 A260/A280: 1.83 141.4 ng/µl

Appendix C

FastDNA SPIN KIT for Feces (MP Biomedicals) protocol

5.2 FastDNA™ SPIN Kit for Feces Detailed Protocol NOTE: See section 3 for other important guidelines

1. In a 2 mL Lysing Matrix E tube, add 500 mg feces sample, 825 µL Sodium Phosphate Buffer, and 275 µL of PLS solution. Shake to mix. Vortex 10-15 seconds

2. Centrifuge samples at 14,000 x g for 5 minutes and decant supernatant.

3. Add 978 µL Sodium Phosphate Buffer and 122 µL MT Buffer. Shake vigorously or vortex briefly to mix.

4. Homogenize samples in the FastPrep 24 instrument at setting 6.0m/s for 40 seconds.

5. Centrifuge samples at 14,000 x g for 5 minutes. NOTE: Extending centrifugation to 15 minutes can enhance elimination of excessive debris from large samples, or from cells with complex walls.

6. Transfer the supernatant to a clean 2.0 mL centrifuge tube.

7. Add 250 µL of PPS solution, shake vigorously to mix, and incubate at 4°C for 10 minutes. Do not vortex! Centrifuge samples at 14,000 x g for 2 minutes.

8. While samples are centrifuging, add 1 mL of Binding Matrix Solution to a clean 15 mL conical tube (not supplied).

9. Transfer supernatant to the Binding Matrix Solution in the 15 mL conical. Shake gently by hand to mix, then place on a shaker/rocker for 3-5 minutes.

10. Centrifuge samples at 14,000 x g for 2 minutes. Decant the supernatant.

11. Wash the binding mixture pellet by gently resuspending with 1 mL Wash Buffer #1.

12. The following step will require two spins. First, transfer approximately 600 µL of the binding mixture to a SPIN Filter tube and centrifuge at 14,000 x g for 1 minute. Empty the catch tube. Add the remaining binding mixture to the SPIN Filter tube and centrifuge as before. Empty the catch tube again.

13. Add 500 µL of prepared Wash Buffer #2 to the SPIN Filter tube and gently resuspend using the force of the liquid from the pipette tip to resuspend the pellet. Do not vortex. NOTE: Ensure that ethanol has been added to the Wash Buffer #2. See section 3.1.

14. Centrifuge samples at 14,000 x g for 2 minutes. Discard the flow-through.

15. Centrifuge the sample again for 2 minutes to extract residual ethanol from the binding matrix and dry the sample.

16. Transfer the SPIN Filter bucket to a clean 1.9 mL Catch Tube. Add 60-100 µL TES. Flick the tube or stir the matrix with a pipette tip to resuspend the pellet. Do not vortex.

17. Centrifuge samples at 14,000 x g for 2 minutes to elute purified DNA into the clean catch tube. Discard the SPIN filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or 4°C until use.

Appendix D

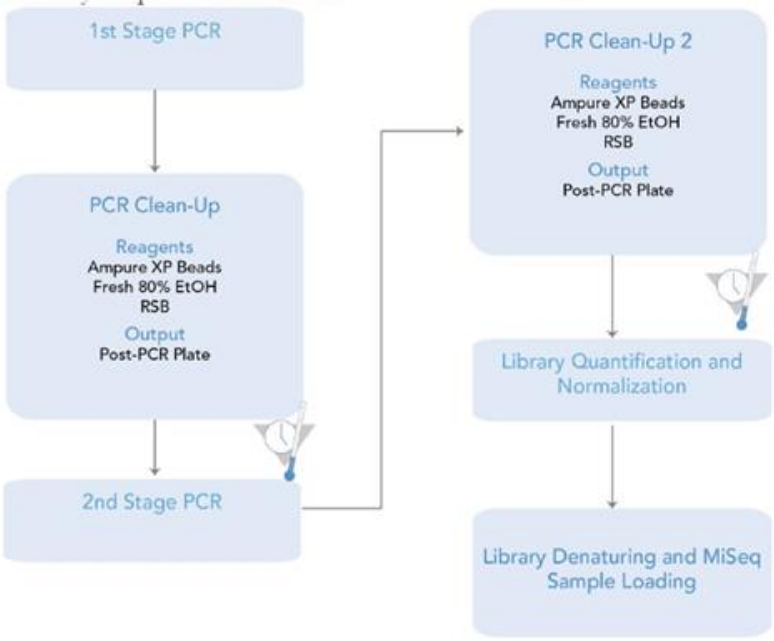
Qubit Flourometer 4 Parameters and Quantitation Data

Qubit quality	M1 (Male 1)	70.8 ng/µl
Qubit quality	M2 (Male 2)	42.0 ng/µl
Qubit quality	M3 (Male 3)	41.8 ng/µl
Qubit quality	F4 (Female 4)	76.6 ng/µl
Qubit quality	F5 (Female 5)	106 ng/µl

Appendix E

16S Library Preparation Workflow

Figure 2 16S Library Preparation Workflow



Appendix F

First Round of Sequencing Protocol

Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached. For more information on primer sequences, see Amplicon Primers, on page 3.

Consumables

NOTE
For more information on consumables and equipment for this protocol see Consumables and Equipment, on page 21.

Item	Quantity	Storage
Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5)	2.5 µl per sample	-15° to -25°C
Amplicon PCR Reverse Primer (1 µM)	5 µl per sample	-15° to -25°C
Amplicon PCR Forward Primer (1 µM)	5 µl per sample	-15° to -25°C
2x KAPA HiFi HotStart ReadyMix	12.5 µl per sample	-15° to -25°C
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	
[Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504)		

Procedure

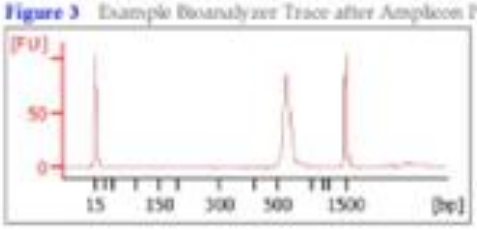
1 Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:

	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
2x KAPA HiFi HotStart ReadyMix	12.5 µl
Total	25 µl

2 Seal plate and perform PCR in a thermal cycler using the following program:
- 95°C for 3 minutes
- 25 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

3 **[Optional]** Run 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace after the Amplicon PCR step is ~550 bp.

Figure 3 Example Bioanalyzer Trace after Amplicon PCR Step



Appendix G

Clean-up Protocol of First Round of Sequencing PCR

PCR Clean-Up

This step uses AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	52.5 µl per sample	-15° to -25°C
AMPure XP beads	20 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Preparation

- Bring the AMPure XP beads to room temperature.

Procedure

1. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
2. **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 25 µl, transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples.

   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
3. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.
4. Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.
5. Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.

9 With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   b Incubate the plate on the magnetic stand for 30 seconds.
   c Carefully remove and discard the supernatant.

10 With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:
   a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   b Incubate the plate on the magnetic stand for 30 seconds.
   c Carefully remove and discard the supernatant.
   d Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

11 With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

12 Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

13 Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

16 Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

SAFE STOPPING POINT
If you do not immediately proceed to *Index PCR*, seal plate with Microseal "B" adhesive seal and store it at -15° to -25°C for up to a week.

Appendix H

Index PCR Protocol (second round of sequencing PCR)

Index PCR

This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit.

Consumables

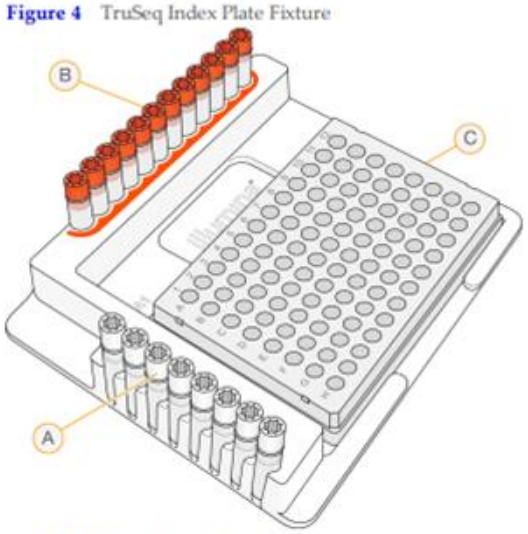
Item	Quantity	Storage
2x KAPA HiFi HotStart ReadyMix	25 µl per sample	-15° to -25°C
Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
PCR Grade Water	10 µl per sample	
TruSeq Index Plate Fixture (FC-130-1005)	1	
96-well 0.2 ml PCR plate	1 plate	
Microseal 'A' film	1	

Procedure

1. Using a multichannel pipette, transfer 5 µl from each well to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored for other uses.
2. Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture) using the following arrangements as needed:
   a. Arrange Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
   b. Arrange Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

   For more information on index selection, see Dual Indexing Principle, on page 23.

Figure 4 TruSeq Index Plate Fixture



A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate

3 Place the 96-well PCR plate with the 5 µl of resuspended PCR product DNA in the TruSeq Index Plate Fixture.

4 Set up the following reaction of DNA, Index 1 and 2 primers, 2x KAPA HiFi HotStart ReadyMix, and PCR Grade water:

	Volume
DNA	5 µl
Nextera XT Index Primer 1 (N7xx)	5 µl
Nextera XT Index Primer 2 (S5xx)	5 µl
2x KAPA HiFi HotStart ReadyMix	25 µl
PCR Grade water	10 µl
Total	50 µl

5 Gently pipette up and down 10 times to mix.

6 Cover the plate with Microseal 'A'.

7 Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

8 Perform PCR on a thermal cycler using the following program:

- 95°C for 3 minutes
- 8 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

Appendix I

Clean-up Protocol of Index PCR

PCR Clean-Up 2

This step uses AMPure XP beads to clean up the final library before quantification.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	27.5 µl per sample	-15° to -25°C
AMPure XP beads	56 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Procedure

1. Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
2. **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 50 µl, transfer the entire Index PCR product from the PCR plate to the MIDI plate. Change tips between samples.

   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
3. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.
4. Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.
5. Gently pipette mix up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
9. With the Index PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   b. Incubate the plate on the magnetic stand for 30 seconds.
   c. Carefully remove and discard the supernatant.

10 With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:
   a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   b Incubate the plate on the magnetic stand for 30 seconds.
   c Carefully remove and discard the supernatant.
   d Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

11 With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

12 Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of 10 mM Tris pH 8.5 to each well of the Index PCR plate.

13 If using a 96-well PCR plate, gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column. If using a MIDI plate, seal plate and shake at 1800 rpm for 2 minutes.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

16 Using a multichannel pipette, carefully transfer 25 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

SAFE STOPPING POINT
If you do not plan to proceed to *Library Quantification, Normalization, and Pooling, on page 16*, seal the plate with Microseal "B" adhesive seal. Store the plate at -15° to -25°C for up to a week.

Appendix J

Protocol of Library Quantification, Normalization, and Pooling

Library Quantification, Normalization, and Pooling

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes.

Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace:

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol × average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{\text{15 ng/µl}}{\text{(660 g/mol × 500)}} \times 10^6 = \text{45 nM}$$

Dilute concentrated final library using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM. Aliquot 5 µl of diluted DNA from each library and mix aliquots for pooling libraries with unique indices. Depending on coverage needs, up to 96 libraries can be pooled for one MiSeq run.

For metagenomics samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads allows for sample pooling to the maximum level of 96 libraries, given the MiSeq output of > 20 million reads.