MICROBIOME OF BONE MARROW FOR POSTMORTEM INTERVAL

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Mary Nichole Ruble

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ABSTRACT

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When determining time since death estimations are limited in their accuracy by a number of external factors such as the decomposition of soft tissues, presence of scavengers, and environmental influences. Therefore current research focuses on means to increase this accuracy. By taking advantage of the longevity of bones as well as their nutrient rich inner matrix it may be possible to limit some of these factors such as the rapid decomposition time and contaminants introduced by scavengers, the burial environment, and the weather. Over the course of 202 days from summer to winter 2016, the bone marrow of three cadavers was sampled at the Southeast Texas Forensic Science Facility in Huntsville, Texas. Samples were sent out for PCR amplification and sequencing of the 16S rRNA gene in order to identify members of the bacterial community at the time of sampling. While preliminary, our data demonstrate that there are consistent shifts in the microbial taxa over time within and among cadavers. Actinobacteria and Firmicute genera decreased over time while Proteobacteria increased both in abundance and class diversity. Bacteroidetes genera also increased over time. Taken together, these data suggest that the microbial composition of bone marrow may be a potential aid for estimating PMI with increased accuracy.

KEY WORDS: Postmortem interval, Forensic science, Metagenomics, Sam Houston State University, Graduate school, Texas

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Last but certainly not least this project, like most forensic research, would be impossible without the generous donation of cadavers. To the body donors and their families I would like to extend my most sincere thanks.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

Introduction

Forensic investigations rely on numerous sources of information to form conclusions about the circumstances associated with an individual's death. These sources are incredibly varied and examples include scavenger activity, burial site chemistries, and cadaver chemistries (e.g. Beck, Ostericher, Sollish, & De León, 2015; Petersen & Luxton, 1982; Schoenen & Schoenen, 2013). Derived from these sources of information is an estimate of time since death or postmortem interval (PMI) (e.g. Megyesi, Nawrocki, & Haskell, 2005; Metcalf et al., 2013; Moffatt, Simmons, & Lynch-Aird, 2016).

Decomposition

The human body is not unchanged after death and has been extensively studied. While the stages of decomposition may vary in name and number depending on the study, the process of decomposition remains the same, first outlined by Mégnin and further studied and summarized by individuals such as Reed, Gill-King, and Vass (Gill-King, 1997; Mégnin, 1894; Reed, 1958; Vass, 2001). Decomposition begins immediately with the degradation of individual cells and progresses over the course of months-to-years to the diagenesis of skeletal elements (Gill-King, 1997; Reed, 1958; Vass, 2001). As oxygen sources within the body are depleted ATP production within cells is reduced, leading to the failure of ATP-driven processes and causing cell swelling and autolysis. The lack of oxygen also prompts a switch to fermentative metabolism for ATP synthesis among still-living cells. Fermentation ultimately leads to the build-up of lactic acid and a resultant acidosis. This acidosis in turn further hastens cell degradation. Soon after, rigor mortis and liquefaction of whole organs occurs as they necrotize and liquefy. This begins

with the digestive and cardiovascular tissues and progresses to the respiratory, endocrine, and nervous tissues. Skeletal muscles and connective tissues are the last to liquefy (Gill-King, 1997; Perper, 2006; Reed, 1958; Vass, 2001).

Autolysis and liquefaction provides an ideal environment for microorganisms subsequent to the lowered pH and anaerobic conditions. There is a rapid expansion of the microbial communities within a corpse which ultimately results in the putrefaction and bloat stages of decomposition. As bacterial communities persist gaseous waste is excreted from the cells and fills the body cavity until bursting, an event known as purge. Following purge liquefied tissues may exit the body from numerous exit sites and slough off the external body in the case of skin and muscle tissues.

At this point decomposition is driven less by processes of dying human cells and more by microbial and scavenger activity. Both will pick at soft tissues and bones as available until only hard tissues (skeletal elements) remain. Eventually chemical weathering and diagenesis will completely decompose the bones as well (Gill-King, 1997; Perper, 2006; Reed, 1958; Vass, 2001). These stages of decomposition have clear, observable characteristics that forensic investigators rely upon for their investigations; however, said observations are often subjective and not ideal for consistency.

Forensic Investigations and Time since Death

While PMI is based on the characteristic stages of decomposition these stages themselves are influenced by factors both biotic and abiotic which can alter the decomposition rate and therefore the accuracy of such an estimate. Examples of these factors include burial site geography, climate, and other constantly changing conditions such as season, temperature, weather, and the availability of insects and scavengers

(Mann, Bass, & Meadows, 1990). The recent trend in forensic science studies has been to supplement decomposition-stage-based PMI with additional observations in the hopes of creating a more accurate estimate.

Some studies focus on more accurately observing the physical aspects of decomposition in the presence of differing factors. Two such studies are those completed by Parks, Suckling and colleagues, both of which observe PMI based on physical changes in cadavers as it occurs specifically in central Texas. Parks' research confirms that decomposition of human remains in the area match other decomposition timetables from other regions of the southwest (Parks, 2011). Suckling and colleagues' research emphasizes the need to better tailor existing equations to the region and climate influencing decomposition as PMI and adjusted degree days (ADD) calculations were found to significantly differ from the observed time since death (Suckling et al., 2016). Both studies reinforce that ecoregions differ in the exact time frame for decomposition and provide data for future studies on the topic (Parks, 2011; Suckling et al., 2016).

Other studies focus on gaining more data from the environment to better predict PMI. Studies such as Campobasso's utilize the presence of different insect species (in this case those of the order Diptera) to indicate the stage of decomposition (Campobasso, Di Vella, & Introna, 2001). However, while Diptera species may be found worldwide their colonization of cadavers and development is still influenced by environmental factors (Campobasso et al., 2001). Numerous studies have investigated changes in soil, soil chemistry, and soil microbe composition at burial sites including those by Vass, Meyers, Howard, and Hopkins. These studies have taken place over multiple regions and with multiple soil types, giving investigators a better idea of how soil type, environment, and

climate all interact to affect the rate of decomposition (e.g. Hopkins, Wiltshire, & Turner, 2000; Howard, Duos, & Watson-Horzelski, 2010; Meyers & Foran, 2008; A. Vass, Bass, Wolt, Foss, & Ammons, 1992). These studies, while informative about specific regions and soil types, again confirm the variable influence of environment on decomposition rather than showcase a consistent element for PMI estimates to focus on. Still other studies focus on specific conditions or scenarios such as Dickson's study on marine decomposition which gives investigators a basis for estimating the time of marine submersion of a body but is not informative for PMI estimates outside of this unique circumstance (Dickson, Poulter, Maas, Probert, & Kieser, 2011). As Vass argues, for PMI to be most useful it should be able to be applied reliably across environments, climates, and circumstances, requiring the use of elements that are consistently present at corpse locations (Vass et al., 1992).

A third area of forensic research has been within the cadaver itself. While most of this research centers on toxicology and the detectability of various drugs after death, such as Butzbach's study on Risperidone and Paliperidon degradation in the bloodstream postmortem, some studies have turned to serology and blood chemistry for better insight into the decomposition process (Butzbach et al., 2013). Early work on this subject is reviewed in-depth by Coe; but to briefly summarize: numerous chemicals and molecules within the blood including glucose, lactic acid, creatinine, and various lipids, enzymes, and hormones all experience changes in concentration postmortem. Depending on the chemical or molecule in question values may be expected to increase or decrease at levels that are generally predictable; or to remain the same (Coe, 1974). However it has also been noted that the starting values for some of these may change depending on the

medical history of the individual (Coe, 1974; Hamilton-Paterson & Johnson, 1940; Hill, 1941). Serology has also been a benefit as studies completed by Robinson, Brazinsky, and Kellenberger and verified by McCormick found that IgG, IgM, and IgA concentrations postmortem, as well as a number of antigens, reflect values in the body antemortem (Brazinsky & Kellenberger, 1970; McCormick, 1972; Robinson & Kellenberger, 1962). Blood is not the only fluid with potential to be studied in this way; McCormick also reviews studies focusing on cerebral spinal fluid and vitreous humor as potential postmortem chemistry resources (McCormick, 1972). While these routes show potential, there is still much to be studied. Again preliminary data indicates that lifestyle and history influences these values, making abundance of elements within bodily fluids less than ideal for a generalized model.

A current difficulty with PMI determination is the dry stage of decomposition in which most or all soft tissues have been removed from the cadaver and the skeletal elements remain. While decomposition in the form of diagenesis still occurs at this stage, it is difficult to discern from sight alone (Gill-King, 1997; Vass, 2001). Various studies have tried to improve PMI at this stage. Introna, Di Vella, and Campobasso determined that analysis of chemiluminescence of blood remnants in powdered bone produced via the luminol test, performed on mid-shaft femur bone powder, can provide a range of time since death. Even better, their method of filming the luminol reaction to observe its intensity and distribution provides for a rare quantitative means of establishing PMI (Introna et al., 1999). In another investigation, citing the consistent concentration of citrate within bone across individual age and sex during life, Schwarcz, Agur, and Jantz explored the use of decreasing citrate content in bone as a measure of PMI. While the

original consistency of citrate is promising for a model that is independent of the factors of age and sex, Schwarcz and colleagues found that the storage conditions of the cadaver do affect the rate of citrate loss postmortem (Schwarcz, Agur, & Jantz, 2010). Unfortunately most methodologies that focus on bone including the two mentioned here rely on destructive sampling of the bone which risks destroying other forensic evidence (e.g. Damann, Williams, & Layton, 2015; Introna et al., 1999; Schwarcz et al., 2010).

Metagenomics and Forensic Science

While studies have attempted to utilize microbes both on and around cadavers for PMI estimates, whether via identification of the microbes through culturing or sequencing or via chemical evidence of their presence and growth (e.g. Tuomisto, Karhunen, Vuento, Aittoniemi, & Pessi, 2013; Vass et al., 1992), these studies find difficulty in recording a full account of microbes present for numerous reasons. As Vass states, such studies are difficult due to the number of bacterial species present both on the cadaver itself and the environment in which it is placed (Vass, 2001). Additionally older studies relied on the ability of researchers to culture the microbes in a lab setting which is commonly known to be difficult if not impossible for a large number of bacterial species (Tuomisto et al., 2013; Vass, 2001). However, recent advances in gene amplification and sequencing via polymerase chain reaction (PCR) and high-throughput sequencing have made it not only easy to identify the members of a microbial community but also fast and affordable. Metagenomics, the study of microbial genes within a specific sample, uses these advances to better characterize microbiomes.

In studies utilizing metagenomics such as the one completed by Shade and colleagues, DNA is extracted from collected samples and a gene – frequently the 16S

rRNA gene – is amplified via PCR before sequencing. Sequencing methodologies vary depending on the study and samples used. These sequences are then compared to a bank of known sequences and matched within a certain degree of similarity (Shade, Caporaso, Handelsman Knight & Fierer, 2013). With metagenomics-based software packages it is possible to identify and quantify bacterial communities, allowing for diversities among and between samples to be compared and analyzed with statistical tests (Caporaso et al., 2010). While metagenomics is useful in collecting a large amount of data about a sample and the circumstances in which it was obtained, it is not without its weaknesses, which are debated in reviews such as those by Abram, Gonzalez, Hedlund, Wooley and colleagues. The field is relatively new and there is still debate concerning some of its methodologies. Additionally, while metagenomics skips the necessity of culturing samples in the lab for identification, the gene databases it uses are built from such lab-cultured specimens and are therefore incomplete (Abram, 2015; Gonzalez et al., 2012; Hedlund, Dodsworth, & Staley, 2015; Wooley, Godzik, & Friedberg, 2010).

More recent microbiology-focused forensic studies have utilized metagenomics to better understand their observations. Many of these studies, such as those completed by Metcalf, Hyde, and colleagues, concern the application of microbial community changes during decomposition as an indicator of PMI. While their individual results varied depending on the circumstances of corpse placement and sampling method, some general trends remained common throughout. A dominance of Proteobacteria was found throughout the studies with Alphaproteobacteria increasing in abundance and Gammaproteobacteria decreasing over time. Firmicutes generally decreased over time while Bacteroidetes increased. Acidobacteria and Actinobacteria organisms were present

throughout these previous studies in small amounts though the patterns fluctuated too much between studies to give an overall trend (Damann et al., 2015; Metcalf et al., 2013; Pechal et al., 2014).

Anatomy and Decomposition of Bones

A pitfall with many current methods of PMI estimation is that they rely on elements that remain only as long as the soft tissue on the cadaver. A different approach must be taken to discern PMI during the dry stage, the longest stage of decomposition. Skeletal elements are present at the time of death and persist longer than any other body components as observed in numerous studies of decomposition (e.g. Brain, 1981; Galloway, Willey, & Snyder, 1997; Toots, 1965). These elements consist of a hard, inorganic hydroxyapatite exterior that protects an organic center from external drivers of decomposition such as weather and scavengers (Child, 1995; Shultz, 1997; van Doorn, Wilson, Willerslev, & Gilbert, 2011). Bones' persistence postmortem as well as their structure makes them ideal for forensic investigations if a proper means of studying them can be found.

Long bones are of particular forensic interest as they remain on-scene longer than any other skeletal elements (Haglund, Reay, & Swindler 1988; Hill, 1979). Both the femur and humerus are considered in this category. Such bones possess a dense cortical outer layer that is 90% calcified. These bones are divided into four major sections: the epiphysis, the end of the bone from the growth plate to the articular cartilage, the physis, the region of growth in children, the diaphysis, or shaft, which is the middle cylindrical portion of the bone, and the metaphysis which lies between the physis and the diaphysis (Bartl & Bartl, 2016; Gray, 1910).

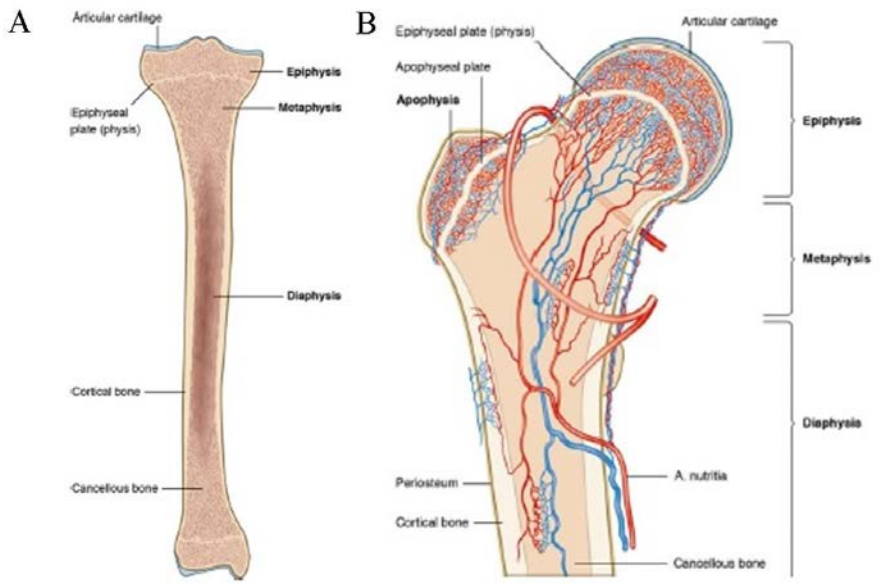

Figure 1. Anatomy of long bones. *Figure 1A:* General anatomy of long bones. *Figure 1B:* A more detailed anatomy of the growth regions of long bones. Taken from Bone Disorders: Biology, Diagnosis, Prevention, Therapy by Bartl, Christoph; Bartl, Reiner. Reproduced with permission of Springer International Publishing in the format Thesis/Dissertation via Copyright Clearance Center.

The diaphysis contains not only the dense cortical layer outside but an inner medullary cavity containing fatty yellow bone marrow. The intracellular matrix of these long bones is comprised of approximately 25 percent organic material (predominately collagen but also proteins such as osteopectin, osteocalcin, osteopontin, and glycans such as glycosaminoglycans and proteoglycans) and 50 percent inorganic matter. The inorganic mineral matter consists of phosphate, calcium, carbonate, and trace amounts of citrate, nitrate, sodium, magnesium, fluoride, and strontium. These minerals form the hydroxyapatite crystals that give bone its strength. The remaining 25 percent of bone intracellular matrix is water, which surrounds the hydroxyapatite crystals (Bartl & Bartl,

2016; Peate & Nair, 2016; Shultz, 1997). All of these components may be utilized as nutrients for a variety of bacteria.

Another bone of interest is the pelvis, specifically its ilium. While not a long bone the pelvis is also made up of a cortical bone outer layer and a cancellous bone inner layer. Where the pelvis differs from long bones is that within its center lies red bone marrow rather than fatty yellow, leading to a different availability of nutrients (Peate & Nair, 2016; Shultz, 1997). Also differing is the biochemical makeup of the pelvis (iliac crest) matrix. In a study that compared both pelvis and femur matrices it was found that the femur possessed an increased amount of sialic and uronic acids, hexoses, and a smaller bone matrix. Tested pelvis materials were found to have a larger matrix and larger sialic acid, uronic acid, and hexose to collagen ratios (Mbuyi-Muamba & Dequeker, 1987). These differences in available nutrients may lead to differences in the microbiome across bones.

Bones are not static elements within the human body and much variance in size and structure is seen between individuals which may also impact microbial colonization. However, there are also characteristics that remain constant. Bone begins as bone-shaped cartilage at the beginning of life that is replaced by osseous tissue over time. Osseous centers, such as the epiphysis and ilium crest growth plates, produce more cartilage as an individual ages and grows. This cartilage is converted to osseous tissue once additional growth has relocated it to the metaphysis. When the bone reaches the diaphysis it is resorbed and remodeled to add to the dense shaft. As an individual’s growth begins to slow, epiphyseal closure is experienced in which the epiphyseal growth plate itself is replaced by osseous tissue and fuses with the diaphysis. This usually occurs around the

ages of 12-14 years old (Jowsey, 1960; Krogman & İşcan, 2013). The same fusing can be seen with the iliac crest at the ages of 18-23 years old along with changes in the pubic symphyses (Krogman & İşcan, 2013; Todd, 1920). Even after the closing of the growth plates, osseous tissue is replaced with newer tissue over time by the process of remodeling. This process also increases the number of osteons within a particular bone, making osteon accumulation a good indicator of age with adult bones (Krogman & İşcan, 2013).

Age is not the only determinant in differences within bones. Sex can also lead to variance. Males generally have larger and thicker bones than females do; however, this is not where the differences end. Morphologically, the femur is the most similar between sexes of the three bones considered here, with only differences in head diameter and shaft circumference (Black, 1978; DiBennardo & Taylor, 1979; Dwight 1905; Krogman & İşcan, 2013). The humerus has far more differences. The trochlear in males is less pinched and is asymmetric at the end while females have a more pinched, but symmetrical, structure. The olecranon fossa is triangular in males but ovular in females, and the medial epicondyle is horizontal in males but angled in females (Krogman & İşcan, 2013; Rogers, 1999). The pelvis exhibits the most dimorphism with numerous morphological differences observed. The most notable is the existence of the ventral arc on female pubic bones which is not found on males. Other dimorphisms include the subpubic shape and angle (convex and acute in males, concave and obtuse in females), the size of the sciatic notch (smaller in males, wider in females), and the position of the ilium (high and vertical in males, low and laterally divergent in females) (Bruzek, 2002;

Krogman & İşcan, 2013; Phenice, TW; Washburn, 1948). These differences in age and sex may impact the microbial community progression during decomposition.

Objectives and Hypotheses

In order to increase the accuracy of PMI estimates an observed character must be highly variable over time while still being consistent across individuals. Ideally this character should also persist over a long period of time through skeletonization and diagenesis. The objectives of this thesis are to explore the feasibility of using bone marrow to accurately estimate PMI via microbial community succession. This thesis is intended to be a pilot study for similar future studies, determining whether or not there is a change in bone microbiomes over time as seen from sampling the bone marrow and whether this change is consistent enough between individuals to use as a marker. Additionally this study will look at differences between various bones to determine which may be better for sampling in future studies and will provide a methodology for future studies. While the lack of previous data and small sample size prevent a PMI model from being created in this pilot study the information gathered here may be added to future studies for a greater sample pool.

Bone marrow contains nutrients that will encourage bacterial growth postmortem and the outer bone will protect the inner matrix from contamination (Child, 1995; Introna et al., 1999; Shultz, 1997; van Doorn et al., 2011). Utilization of a bone marrow biopsy tool and water-proof sealant will minimize destruction of the bone itself and protect entry sites from outside contamination, allowing for the resampling of bones multiple times over the course of a study. The following hypotheses were tested over the course of 202

days by sampling the left and right femur, humerus, and pelvis of three different cadavers:

$H_0$1: There will be no significant change in the microbial communities observed within the individual bones (femur, pelvis, and humerus) over time.

H1: There will be significant change in the microbial communities observed within the individual bones (femur, pelvis, and humerus) over time.

$H_0$2: The microbial communities found within the left bones (femur, pelvis, and humerus) will not be significantly different from their right side counterparts from the same individual.

H2: The microbial communities found within the left bones (femur, pelvis, and humerus) will be significantly different from their right side counterparts from the same individual.

$H_0$3: The microbial communities found within the bones (femur, pelvis, and humerus) of one individual will not be significantly different from the microbial communities found within the corresponding bones on the other two study individuals.

H3: The microbial communities found within the bones (femur, pelvis, and humerus) of one individual will be significantly different from the microbial communities found within the corresponding bones on the other two study individuals.

$H_0$4: A sample taken from a reused, sterilized biopsy needle or a culture swab will not be significantly different from a sample taken at the same time using a brand new biopsy needle.

H4: A sample taken from a reused, sterilized biopsy needle or a culture swab will be significantly different from a sample taken at the same time using a brand new biopsy needle.

CHAPTER II

Materials and Methods

The following materials and methods were adapted from Fakhri et al., 2017.

Placement

Three human cadavers, two male and one female, were placed at the Southeast Texas Forensic Science (STAFS) facility on May 11, 2016 following approval of the project by STAFS research council and STAFS Director Dr. Joan Bytheway. Cadavers were placed unclothed in a supine position with all heads to the east and feet to the west. Cadavers were individually covered with a wire and wood enclosure to prevent scavenger activity.

Figure 2. Cadaver setup at STAFS.

Sample Collection

Prior to all samples being taken the sample area of each bone was cleaned of any body tissues or debris using a sanitized blade and sanitized via 70% alcohol wipe to prevent contamination. In order to penetrate the dense long bones a drill was used to

create an appropriately sized hole (between 2.38 and 4.76 mm) with the drill bit cleaned between bones with a fresh 70% alcohol wipe. Using new sterile T-Lok bone marrow biopsy needles (Figure 3), marrow samples were taken from the left femur, pelvis, and humerus, of all three cadavers on the day of placement, recorded as Day 0. Following placement samples from all three cadavers were taken from the left femur and pelvis every two days and from the left humerus every four. The right side of each cadaver, considered the control side, was sampled every ten days for the femur and pelvis and every eight for the humerus to compare to the left side samples for possible contamination.

Figure 3. T-Lok bone marrow biopsy needle pack.

Sampling of the Long Bones. Femoral and humeral samples were taken starting at the most proximal end of each bone's shaft, just below the lesser trochanter and proceeded linearly down the shaft until reaching the epicondyle. Once this most distal

region was reached bones were rotated and the process restarted, beginning again just below the lesser trochanter.

Figure 4. Drilling into femur.

Sampling of the Pelvic Ilium. Sampling of the ilium began at the iliac crest, starting at the most anterior region and proceeding towards the posterior. Due to the limited surface area of the iliac crest (compared to the shafts of the long bones) sampling eventually proceeded medially on the anterior ilium spines.

Figure 5. Sampling the iliac spine.

Following sampling each hole was sealed using a combination of plumber's putty, superglue, and Elmer's Pro-Bond Glue to prevent contamination of the inner bone post-sampling. In the event that bone marrow was not able to be acquired using a biopsy needle, a Culture Swab EZ sterile swab was used to swab the inside of the bone instead after drilling a hole using the aforementioned procedure. Sampling continued on this schedule through September where the schedule was changed to every four days for the left pelvis and left femur, every eight days for the left humerus, every twenty days for the right pelvis and femur, and every sixteen days for the right humerus. Sampling continued until November 29, 2016 for a total of 202 days.

The budget did not allow for new biopsy needles for every individual sample, resulting in the reuse of needles. Following sampling, each needle was thoroughly scrubbed with soap and water before being immersed in a mixture of 70-90% ethanol for 24 hours. This mixture was placed on a lab rocker to better facilitate the removal of

remnant tissues and DNA. After the 24 hour period, needles were rinsed with MilliQ water, sealed into aluminum foil, and sterilized via autoclave.

Sample storage and selection

All samples were stored in a -20 ℃ freezer at the Sam Houston State University campus until they were sent off for sequencing. Not all samples could be sequenced due to financial constraints therefore an effort was made to ensure that the samples that were sent off were representative of the overall sample collection period. For any given day and bone, samples from all three cadavers were sent off. When selecting right side (control) samples for sequencing, coinciding left side (study) samples were also selected.

Amplification and sequencing of the 16S rRNA gene

At the end of the sample collection period samples were sent to the Baylor College of Medicine in Houston, Texas for PCR amplification of the 16S rRNA gene and Illumina sequencing following the protocols listed by the Human Microbiome Project (Fakhri et al., 2017).

Processing and analysis of sequenced data

Following sequencing of the 16S rRNA gene data was processed and analyzed using QIIME software version 1.9.1 (Caporaso et al., 2010). Taxonomic classification was assigned to each OTU using the 97% identity fasta and taxonomy files from the Greengenes database. OTUs that made up less than 0.25% of the total observation count were filtered from the data, as were samples with less than 654 reads and reads that were classified as chloroplast genes. The resulting OTU table was split by STAFS identification number, bone element, bone and side of the body, as well as STAFS ID and bone and side to be used for all further analyses.

Beta diversity for each individual bone, bone type, and each STAFS cadaver was calculated with an evenness value of 654. Principal coordinate analyses with an enforced time axis were created from the diversity analyses of the complete sample pool, those of each STAFS subject's bone type, the complete sample pool of each STAFS subject, and all samples belonging to the same bone type and side of the body. Taxonomy summaries for each STAFS subject's individual bones and for each bone with samples sorted by time in days since placement were also created from these. Alpha diversities for the full dataset were calculated using the Shannon Index with a minimum rarefaction depth of 4, a maximum depth of 654, increasing in increments of 65 with five replicates per depth.

CHAPTER III

Results and Discussion

Results

Observation of STAFS subjects. Three human cadavers were placed at STAFS for this study. STAFS 2015-109 was a heavy-set elderly female with scar tissue on the thoracic region . STAFS 2016-027 was an underweight male with what appeared to be ports for tracheal and gastrointestinal tubes. STAFS 2016-033 was another male of average height and weight with no visible indications of past poor health.

Sample summary. Overall a total of 730 samples were collected from the three cadavers. Of these, 213 samples were sent to Baylor for PCR amplification and sequencing. A summary of the samples sent out for sequencing may be seen in Table 1, while a summary of all samples taken from May 11 to November 29, 2016 may be found in Appendix B.

Table 1

Summary of samples sequenced by bone source and month taken

Totals by Bone	May	June	July	Aug	Sept	Oct	Nov	Total
L Femur	9	12	12	9	6	6	6	60
R Femur	3	3	0	3	0	0	3	12
L Pelvis	9	12	12	9	6	6	6	60
R Pelvis	3	3	0	3	0	0	3	12
L Humerus	9	11	11	9	6	6	6	58
R Humerus	3	3	0	3	0	0	3	12
Total	36	44	35	36	18	18	27	214

Taxonomy summaries. Taxonomy summaries were generated for each bone overall using OTU tables sorted by days since placement. Summaries were generated to the genus level in all cases. An overall shift from Proteobacteria (primarily Gammaproteobacteria), and Actinobacteria genera to Alpha and Betaproteobacteria was observed. Firmicute genera also decreased with Actinobacteria. While the individual STAFS subjects did vary somewhat in taxa abundance and composition the inclusion of samples from all three cadavers in a specific bone's taxonomy summary did not significantly disrupt the visible trends indicating an overall similarity in abundance shifts over time. Composite taxonomy summaries at the class level for each bone may be seen on the following pages. Composite samples were created using Qiime's collapse_samples.py command. Samples were collapsed by days since placement and taxa counts were summed. Additional summaries at the genus level, featuring each individual sample may be found in Appendix C.

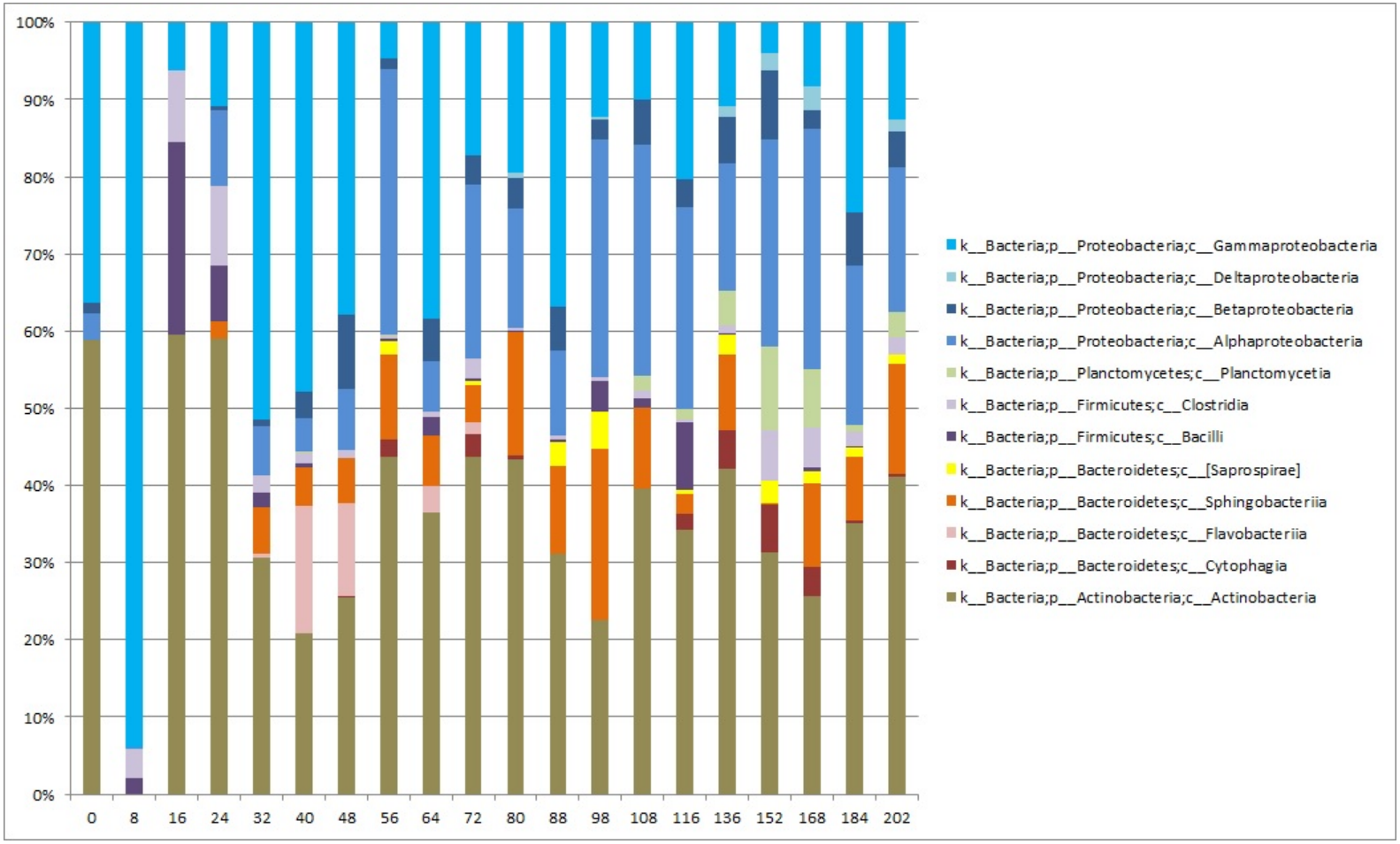

Figure 6. Left Femur Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced left femur samples from all STAFS subjects. Samples are labelled by days since placement.

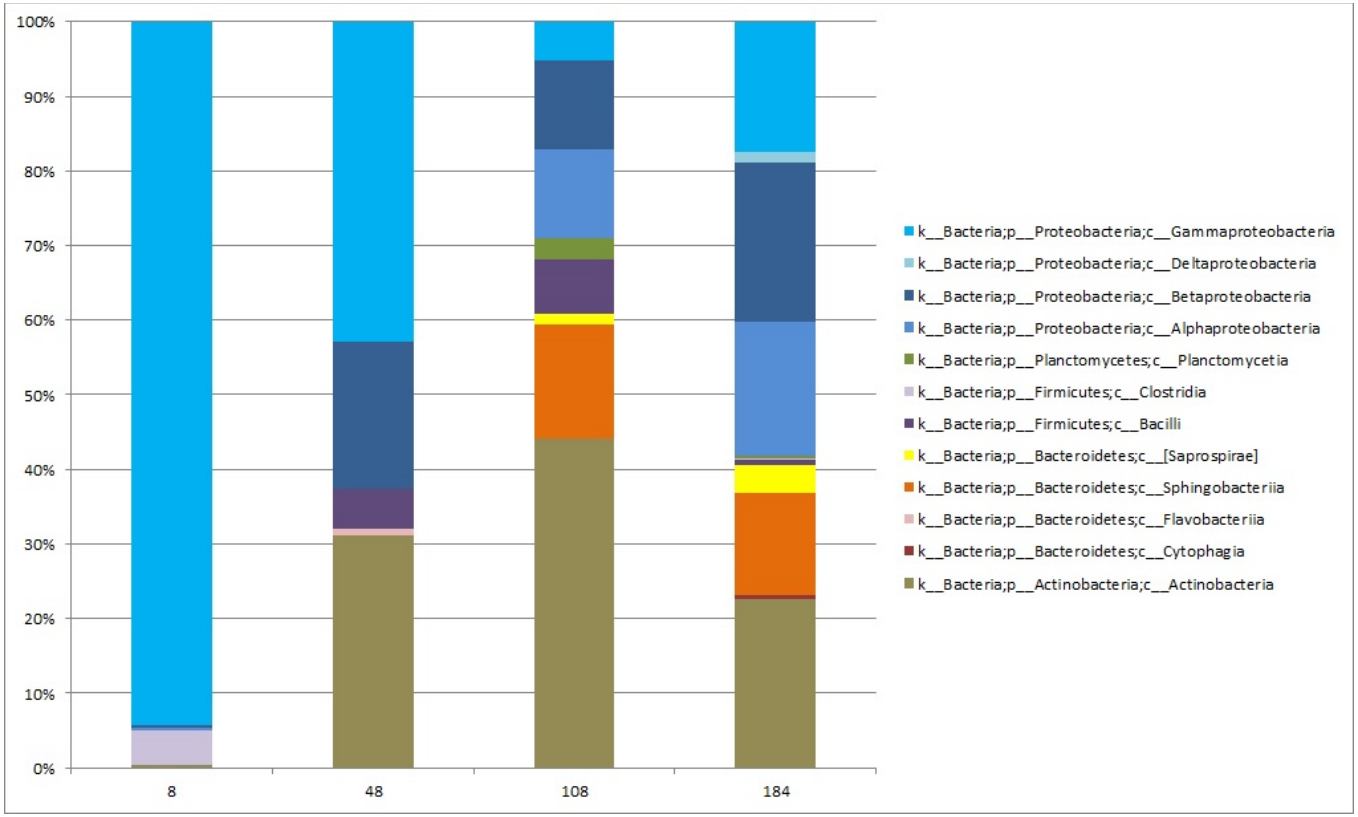

Figure 7. Right Femur Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced right femur samples from all STAFS subjects. Samples are labelled by days since placement.

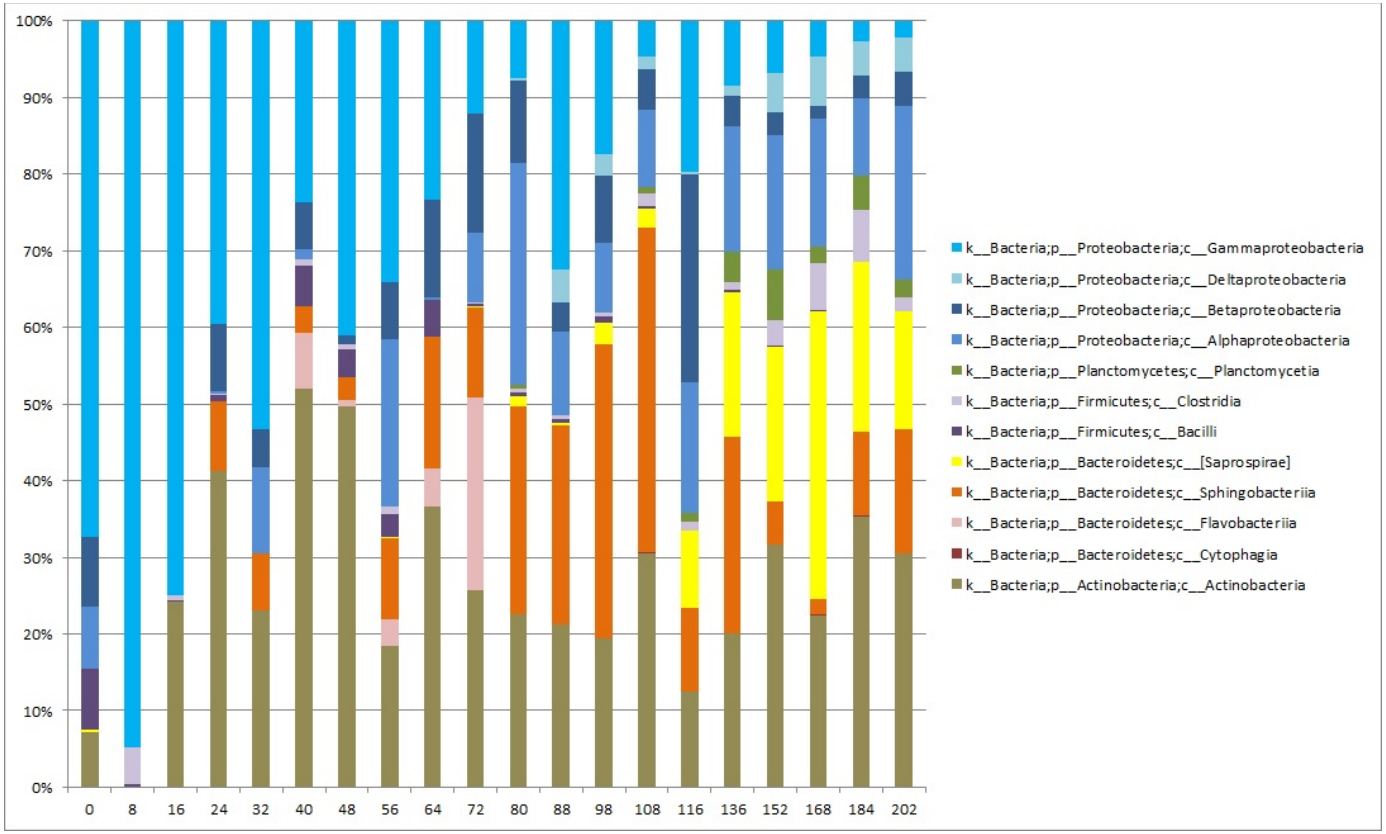

Figure 8. Left Pelvis Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced left pelvis samples from all STAFS subjects. Samples are labelled by days since placement.

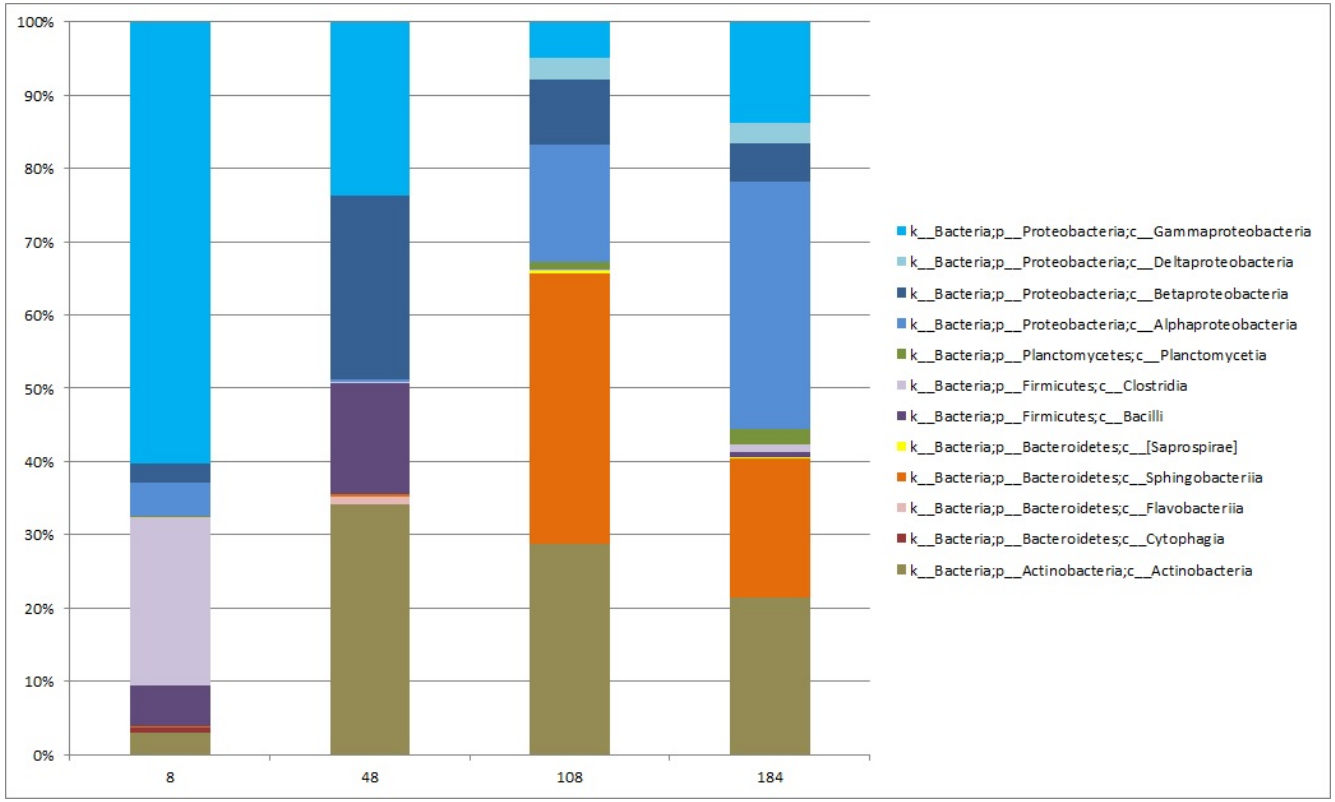

Figure 9. Right Pelvis Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced right pelvis samples from all STAFS subjects. Samples are labelled by days since placement.

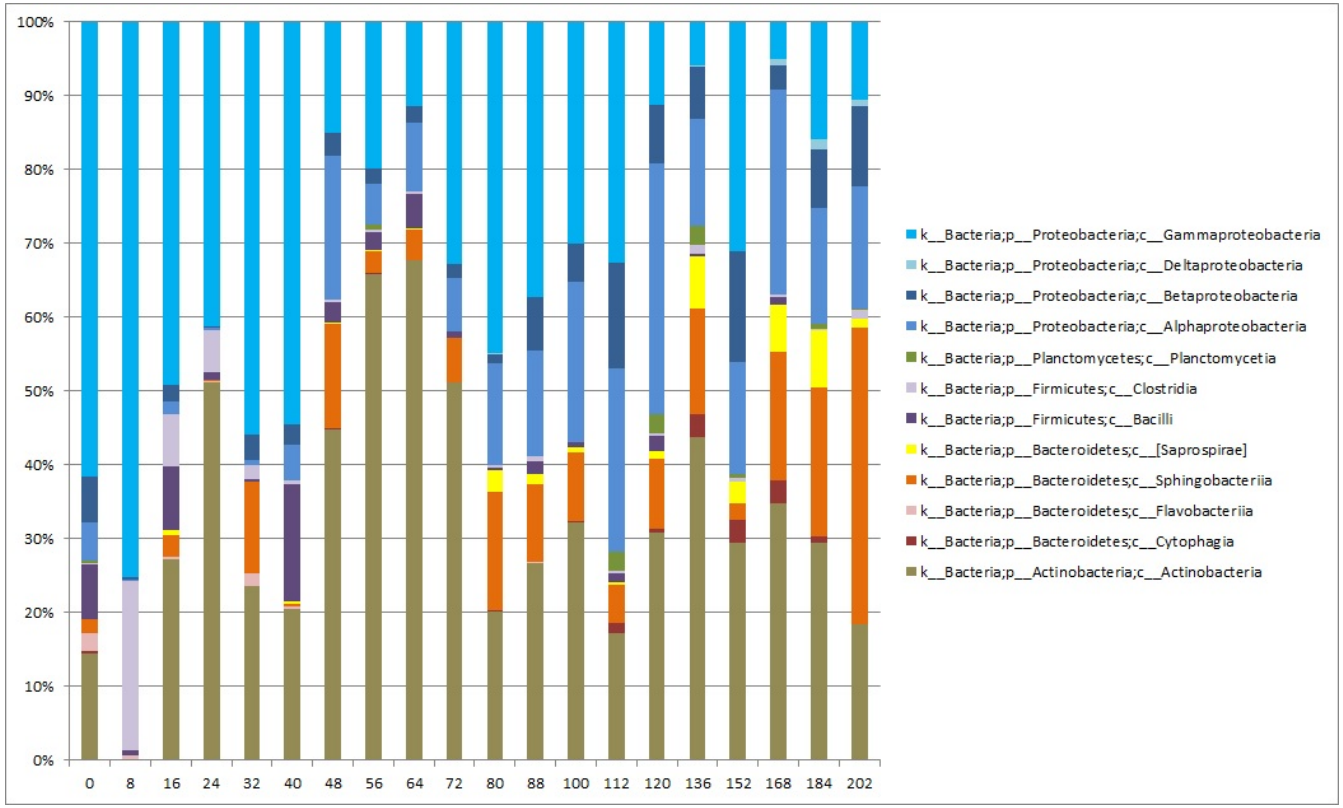

Figure 10. Left Humerus Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced left humerus samples from all STAFS subjects. Samples are labelled by days since placement.

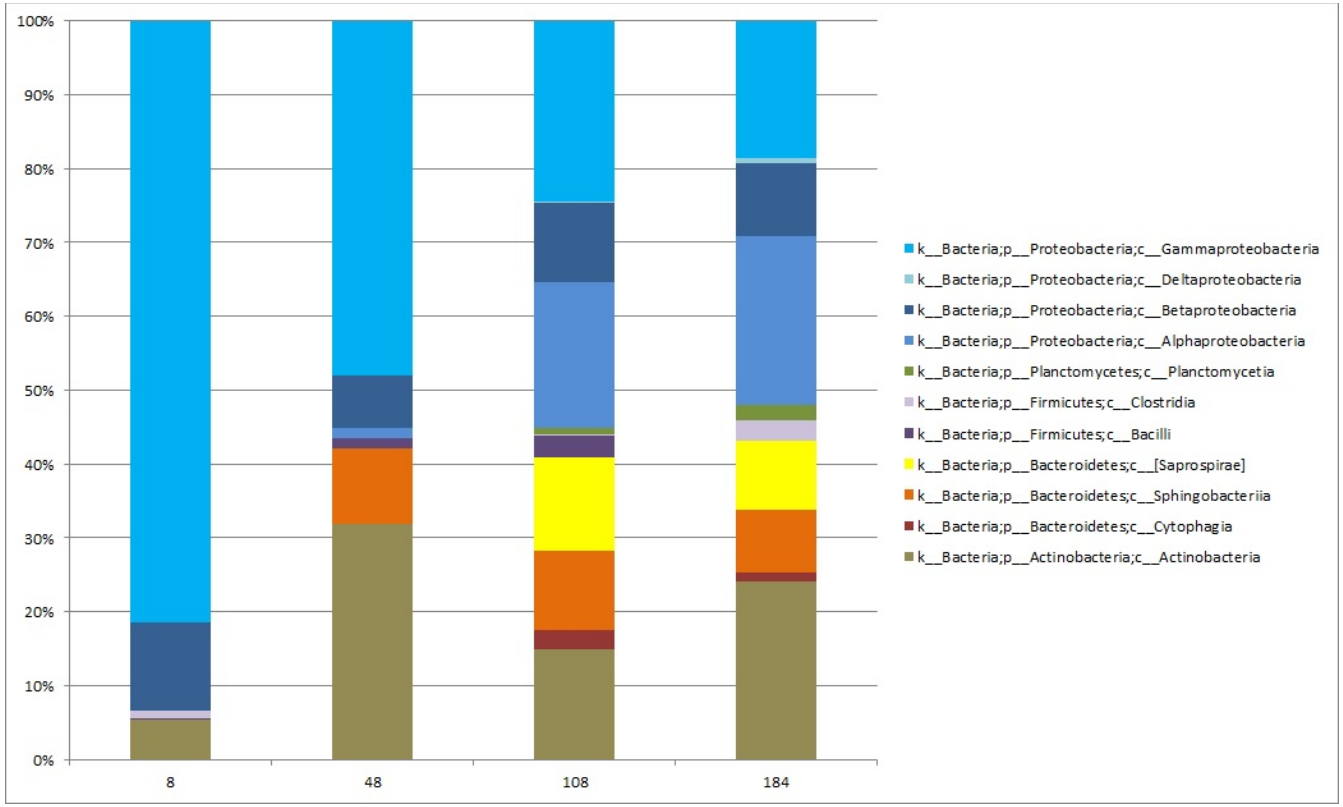

Figure 11. Right Humerus Taxonomy Summaries. Percent abundance of bacteria at the class level for all sequenced right humerus samples from all STAFS subjects. Samples are labelled by days since placement.

Taxonomy of STAFS 2015-109. Overall all six sampled bones belonging to STAFS 2015-109 exhibited the same trends. Early samples were dominated by Gammaproteobacteria though specific genera varied from Enterobacteriaceae *Proteus* in the femur and pelvis to Pseudomonadaceae *Pseudomonas* and Xanthomonadaceae *Ignaztschineria* in the humerus. Later samples saw a shift in dominance from these Proteobacteria to Alpha and Betaproteobacteria genera which diversified as time continued though no one genus gained dominance over the others in these categories. The right pelvis saw an increase in Deltaproteobacteria *Myxococcus* abundance in the late sampling period as well. Actinobacteria orders fluctuated in their presence throughout the sampling period but the overall Actinobacteria abundance was driven by *Corynebacterium* species. Bacteroidetes and Firmicute genera fluctuated in abundance throughout the sampling period and were dominated by Sphingobacteraceae, Clostridia, and Bacillii genera. *Planctomyces* was present in trace amounts in all bones during late sampling.

Taxonomy of STAFS 2016-027. STAFS 2016-027 saw similar trends in microbial abundance as STAFS 2015-109. Actinobacteria genera were present in highly abundant amounts at the start of the sampling period with the large fluctuations in abundance driven by *Corynebacterium* species. Proteobacteria, specifically Gammaproteobacteria genera *Ignatzscheria, Pseudomonas, Proteus,* and *Morganella* remained the dominant phylum, with shifts to diverse Alpha and Betaproteobacteria genera during late sampling. Bacteroidetes genera increased in abundance over time with the main drivers of this transition being *Sphingobacterium.* Firmicute microbes, specifically Clostridium and Bacilli classes, were in high abundance during early

sampling and decreased over time. Once again, *Planctomyces* was present in trace amounts.

Taxonomy of STAFS 2016-033. Proteobacteria within STAFS 2016-033 samples saw the overall trending shift from Gammaproteobacteria to Alpha and Betaproteobacteria. Common genera in early sampling included those belonging to the orders Enterobacteriales, Pseudomonadales, and Xanthomonadales. Actinobacteria genera diversified earlier when compared to the other two subjects though abundance was still driven by *Corynebacterium*. Bacteroidetes genera saw an increase in abundance over time with *Sphingobacterium* being the main driver. Firmicutes were predominantly from class Bacilli though Clostridia genera were seen on occasion. *Planctomyces* was present in later samples in trace amounts.

Principal Coordinate Analysis. PCoA plots were generated for the complete data set, each bone overall, and each bone per STAFS subject. While some data points were placed apart from the majority, in general the PCoA plots exhibited a clear trend line throughout the sampling period. The variance in position of samples on the plot over time indicates a shift in the community composition of the samples at the microbial level while the closeness of samples from the same day indicates a similarity between the microbial DNA in these samples. This indicates that there is in fact a shift in the microbial community within decomposing bones and that this shift is mostly consistent between bodies and bones.

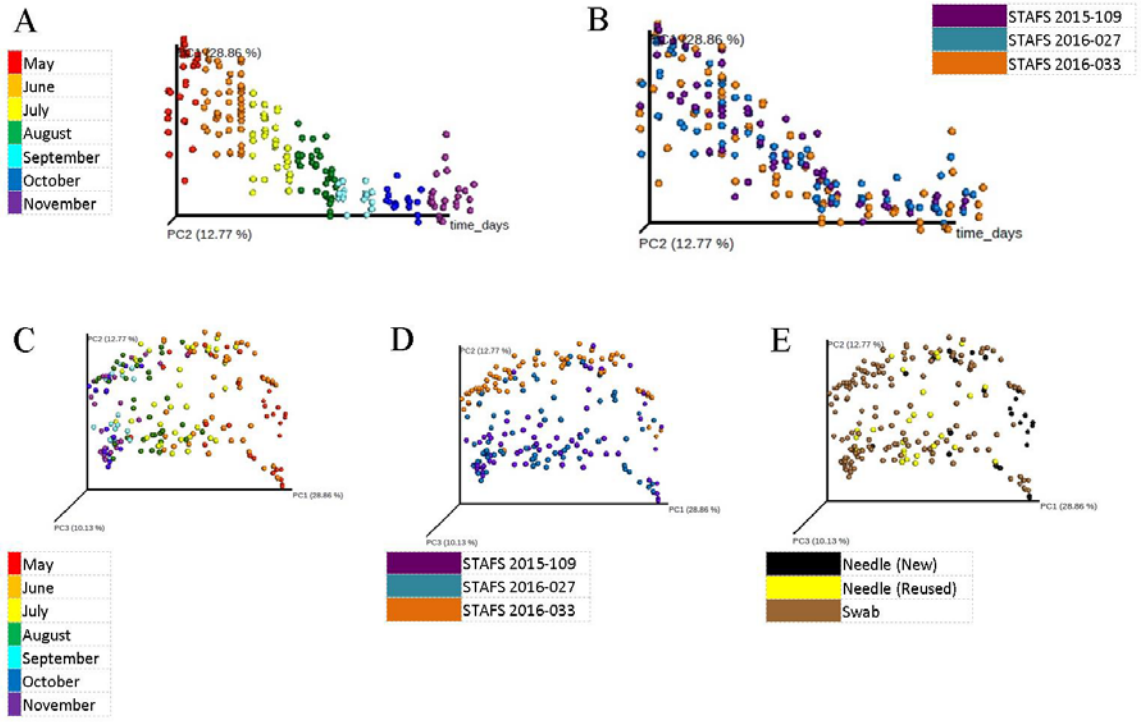

Figure 12. Principal Coordinate Analyses of all sequenced samples. *Figure 12A* Time-enforced PCoA labeled by month sample was taken in. *Figure 12B* Time-enforced PCoA labeled by STAFS subject. *Figure 12C* Unenforced PCoA labeled by month sample was taken in. *Figure 12D* Unenforced PCoA labeled by STAFS subject. *Figure 12D* Unenforced PCoA labeled by sample method.

Overall PCoA and PCoA by sampling method. The primary concern of this study was to determine whether or not bone marrow microbial community trends are consistent enough over individuals and bones to warrant further studies; as well as to determine whether or not the bone marrow sampling method was a viable means of producing uncontaminated samples. To this end it was important to examine the full dataset at once via principal coordinate analysis. Figure 12 is a comparative figure showing the full sequenced dataset with various criteria. From the analyses of all samples on a time enforced axis it can be seen that community composition of the samples clearly changed from month to month while the individual communities of the three STAFS subjects grew more similar over time.

Due to the interest in overall similarity of samples the remaining three PCoA plots of Figure 12 were not created with an enforced time axis to allow for maximum clustering based solely on genetic similarity. Two distinct trend lines form with the uppermost line containing almost exclusively STAFS 2016-033 samples and the bottom line containing a mixture of STAFS 2015-109 and STAFS 2016-027. Samples found between these lines belong to STAFS 2015-109 and STAFS 2016-027 as well. There is a similar division of samples by STAFS ID seen on the enforced time axis-all samples PCoA though the division is on the z axis rather than the y.

Labelling samples by bone origin reveal that there is a mixture of bones throughout with no one bone clustering in an area. The center mixed samples are however predominately left humerus samples. A clear mix of all three sample methods can be seen, though new needles tended to cluster to the right side of the chart. Recoloring the figure by month sampled reveals a right to left trend by time with May

samples being found on the farthest right and November samples being found on the farthest left. As new needles were predominately used in the earlier sampling months, the grouping of new needles on the PCoA plot may be due to the natural shift in community diversity over time rather than any faults in the sampling method.

PCoA of individual bones. Along with concerns about overall sample trends the trends between the same bones of different individuals and the trends between different sides of the same individual must be observed. The following is an analysis of the femur, pelvis, and humerus sample subgroups.

Femur subgroup PCoA. Femur samples followed the same trends as the overall dataset, shifting in diversity over time as well as increasing in sample similarity. This trend remained true both between the same bones of all three STAFS subjects as well as between the right and left sides of the same STAFS subject. PCoA plots for the femur subgroup may be found in Figure 13.

Pelvis subgroup PCoA. The samples of the pelvis subgroup also followed the overall trend, though compared to the femur subgroup similarity greater over time. An exception to this was STAFS 2016-033, which had several data points that did not fall within the general trend line. These will be further discussed in the next section. Pelvis subgroup plots may be seen in Figure 14.

Humerus subgroup PCoA. The humerus subgroup was much like the femur subgroup in terms of similarity between samples. While overall similarity increased over time the later samples exhibited less similarity between individuals than seen in the femur and pelvis samples for the left bone. These trends can be seen in Figure 15.

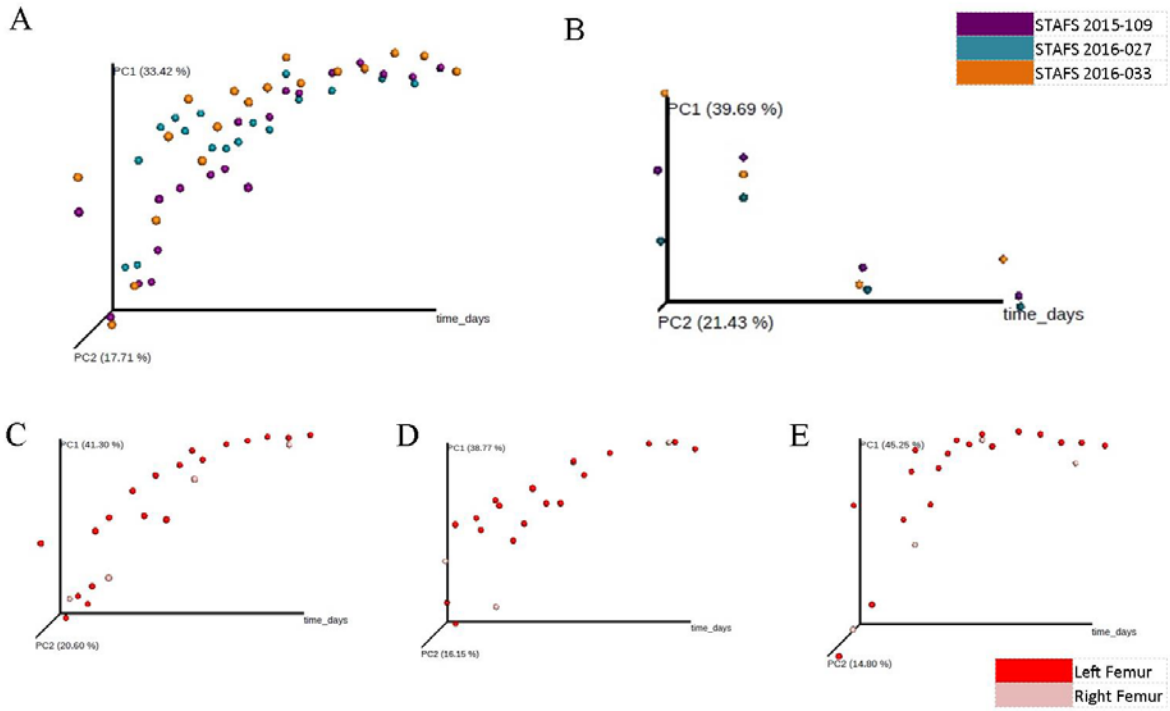

Figure 13. Femur PCoAs. Principal coordinate analyses of the femur subgroups with a time enforced axis. *Figure 13A:* Left femur subgroup colored by STAFS subject. *Figure 13B:* Right femur subgroup colored by STAFS subject. *Figure 13C-E*: Femur subgroups of STAFS 2015-109, 2016-027, and 2016-033, respectively, colored by side of body sampled.

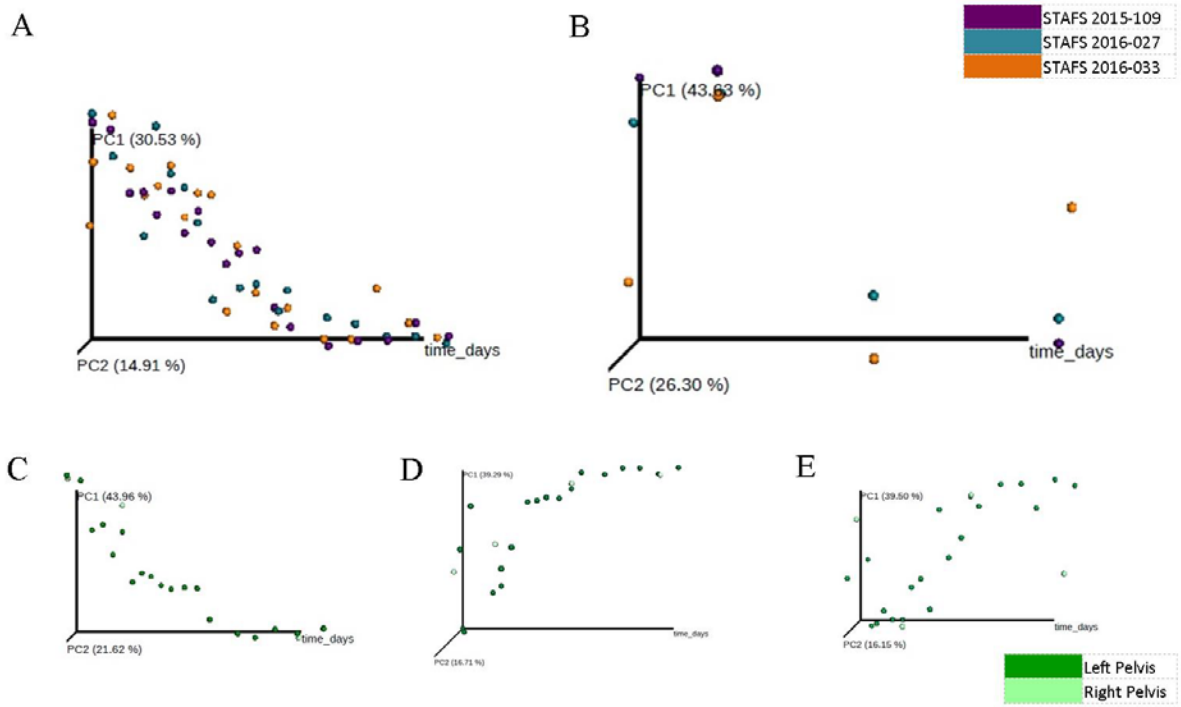

Figure 14. Pelvis PCoAs. Principal coordinate analyses of the pelvis subgroups with a time enforced axis. *Figure 14A:* Left pelvis subgroup colored by STAFS subject. *Figure 14B:* Right pelvis subgroup colored by STAFS subject. *Figure 14C-E*: Pelvis subgroups of STAFS 2015-109, 2016-027, and 2016-033, respectively, colored by side of body sampled.

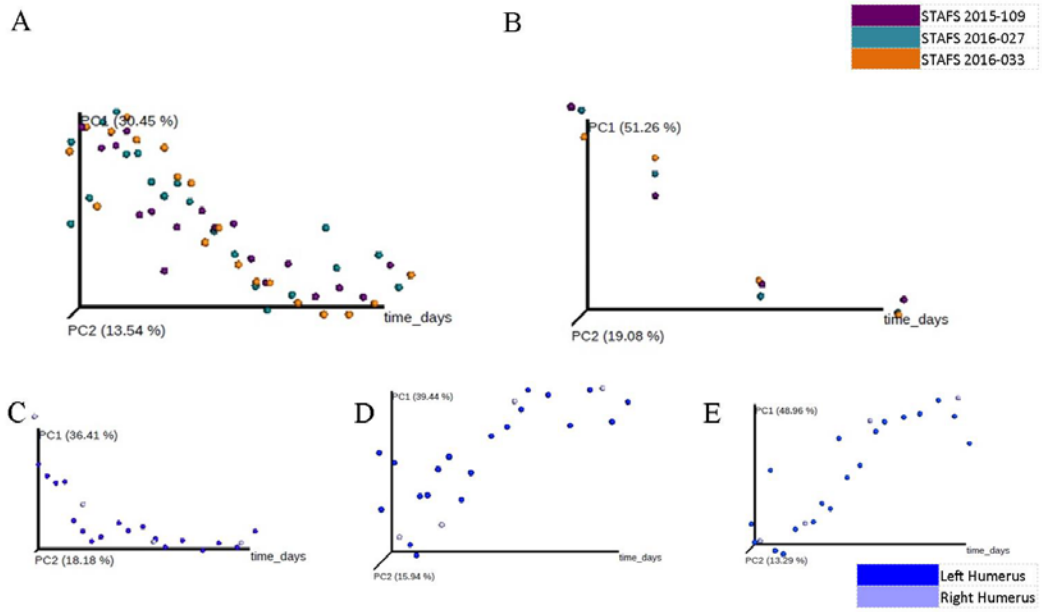

Figure 15. Humerus PCoAs. Principal coordinate analyses of the humerus subgroups with a time enforced axis. *Figure 15A:* Left humerus subgroup colored by STAFS subject. *Figure 15B:* Right humerus subgroup colored by STAFS subject. *Figure 15C-E*: Humerus subgroups of STAFS 2015-109, 2016-027, and 2016-033, respectively, colored by side of body sampled.

Data points falling outside the trends. Though most data points followed the trend lines on the PCoA plots, there were some that did not. With a sample size of three, it is impossible to know whether these points are true outliers however their differences are still worth acknowledging. STAFS 2016-027 had two such points. Samples taken from both the right and left femur on day 48 were drastically different. Reviewing the taxonomy summaries of these two samples revealed a lack of Alphaproteobacteria within the right femur sample, leading to a sample composition that was closer to early sampling than mid-sampling. Re-plotting the femur dataset for STAFS 2016-027 without an enforced time axis seems to support this explanation as the right femur sample clustered closer to samples from earlier in the study while the left femur sample clustered with other samples from its sample time period.

The second set of points included two samples labeled 2016.027.0.5N and 2016.027.0.5N.original. The labels indicate that these two samples should be exactly the same, the left humerus of STAFS 2016-027 taken on day 0, with the former being a second attempt at amplification and sequencing due to questionable results from the latter. Reviewing the taxonomy summaries for these two samples revealed that 05.N exhibited a large difference in Firmicutes abundance (35% compared to 05.N.original's 3.7%) as well as Bacilli and Clostridia class abundances. Overall, sample 05.N showed greater diversity and was closer to the trends of the rest of the STAFS 2016-027 humerus samples supporting the conclusion that the original PCR product (05.N.original) was not amplified enough to give an accurate estimate of overall community diversity and composition.

STAFS 2016-033 also contained deviating sets such as the samples taken on days 8 and 48 from the left and right femurs. In both of these cases, differences were likely caused due to changes of abundance within the observed phyla. Day 8 showed an abundance of Proteobacteria around 99.85% in the right femur which was not shared in the left. Additionally the right femur was mainly inhabited by Enterobacteriaceae rather than Xanthomonadaceae genera as the left femur was. Likewise, day 48 showed a low abundance in Bacteroidetes and Firmicutes genera within the right femur while the left showed much more diverse taxa.

Alpha diversity. Alpha diversity was calculated using the Shannon Index for the full dataset. A rarefaction plot generated by this calculation is featured in Figure 16 labeled by individual sample. While the number of sequences necessary varied and occasionally fluctuated within a sample the majority of sample sets did reach a threshold in the number of novel OTUs detected indicating that the samples taken were large enough to sufficiently represent the full diversity of the community. Figure 17 shows the same alpha diversity analysis labelled by month sampled. As can be seen in this plot, diversity within samples increased over time with later months yielding a greater, but more similar, level of diversity.

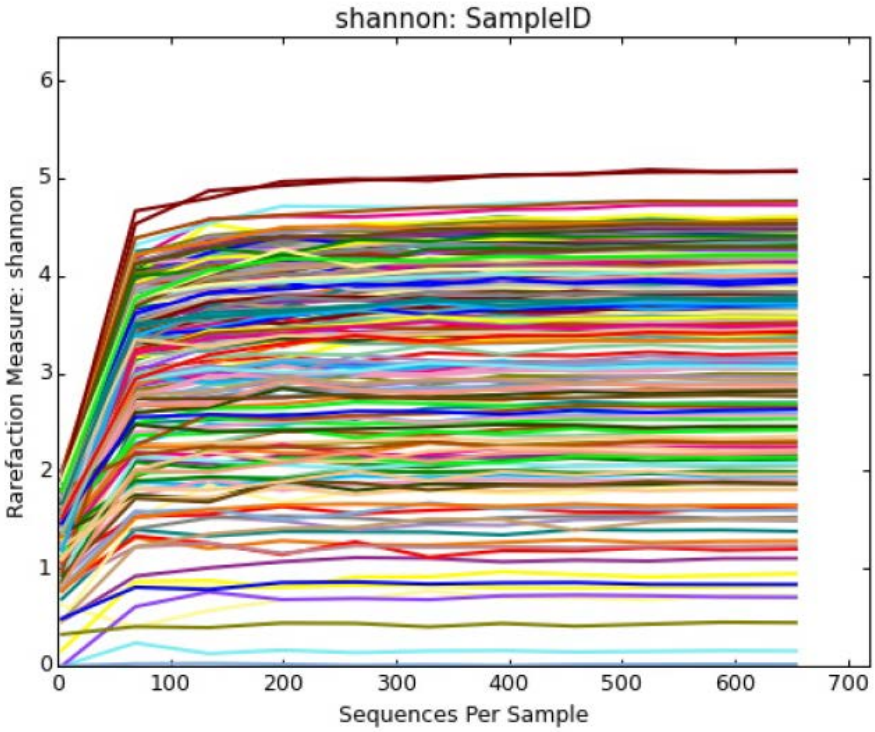

Figure 16. Rarefaction curves of all datasets by sample ID.

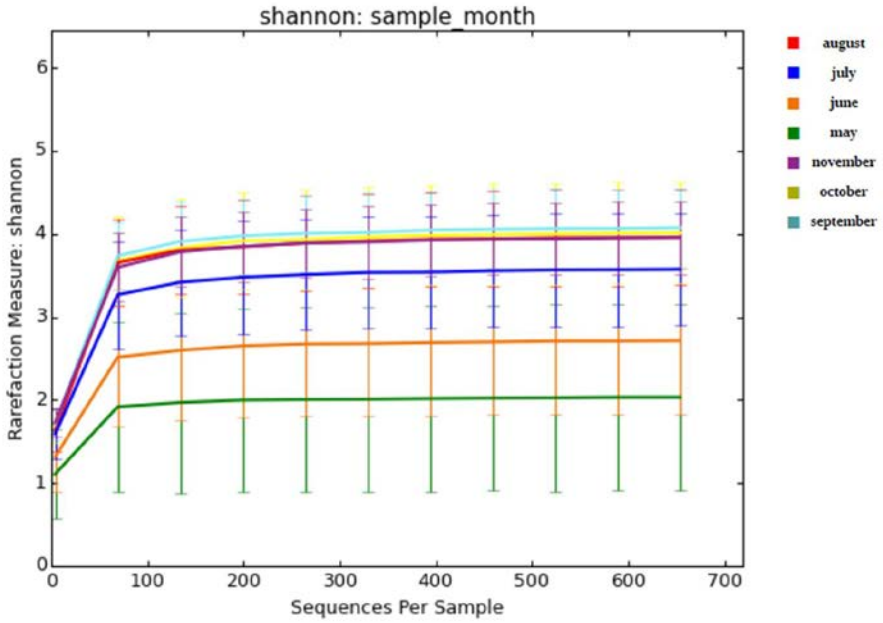

Figure 17. Rarefaction curves of all samples by month.

Discussion

The taxonomy summaries generated by this study identified trends in the microbiome composition of bone marrow. While individual genera differed between bones, the phyla observed displayed these trends clearly. Actinobacteria was shown to decrease over time along with Firmicute abundance while Bacteroidetes and Proteobacteria increased in abundance. Within Proteobacteria the Gammaproteobacteria class was shown to at first increase, becoming the dominant taxa, then decrease over time as it was replaced in dominance by Alpha and Betaproteobacteria. Between these two classes, Alphaproteobacteria often achieved dominance in abundance even over Betaproteobacteria.

These trends share both similarities and differences with previous studies of their kind. Metcalf et al.'s study of the abdominal cavity, soil, and head and stomach skin during human decomposition revealed similar trends for soil and skin – especially the skin. Dominant families within these studies (Xanthomonadaceae, Sphingobacteriaceae, and Pseudomonadaceae) also shared similar trends in abundance over time (2013). Damann et al.'s study of bone differed in that an increase of Actinobacteria was observed rather than an overall decrease but otherwise exhibited the same trends (2015). The greatest difference is seen when comparing Pechal et al.'s swine skin study to the data collected here where all trends were reversed (2014). Another great difference between previous studies and this study was the presence of Acidobacteria and Planctomycetes phyla. Only Metcalf's study found traces of Planctomycetes within their samples and even then the traces were only seen in two out of the four locations sampled (soil and the skin of the head). In the cited previous studies all but Pechal's detected the presence of

Acidobacteria over time in at least one sample site. However this study found no traces of Acidobacteria in any of the 202 samples sequenced. This can be considered a positive as Acidobacteria consist of predominately soil bacteria whose presence within bone would be a possible indicator of contamination (Damann et al., 2015; Metcalf et al., 2013; Pechal et al., 2014). If nothing else this shows that bone marrow is not as quickly overtaken by environmental microbes as other sites.

The PCoA plots of the complete dataset and its subgroups demonstrated a trend of increasing similarity over time when comparing samples from different individuals and sides. While this may indicate that bone marrow is not ideal for PMI analysis during early stages of decomposition it also implies that this medium could be ideal for the later stages when most known methods of PMI via metagenomics fail due to lack of tissues to sample. Furthermore the PCoA plots demonstrate a clear shift in community composition over time even in the later stages of decomposition which is crucial for the construction of a PMI model. They also indicate that the chosen sampling method likely did not affect the microbial composition of the samples taken during this experiment.

Conclusion

The purpose of this study was to examine the feasibility of utilizing bone marrow from human cadavers as an indicator of PMI by observing the progression of microbial communities within. Overall a consistent trend in the genomic composition of samples can be seen both between bones within a single individual and between bones of different individuals. Similarity of microbial community composition between individuals increased over time, particularly in the later sampling months after purge had occurred and the cadavers transitioned to the dry stage. While there are some deviations these

could be easily explained by a number of events and circumstances, including variation between individuals and contamination of samples. True statistical significance in these trends cannot be determined at this time due to the extremely small sample size. Nonetheless the data shown here does demonstrate that further research into this field to better understand these trends would be a fruitful endeavor, especially in the interest of utilizing this data to form a more accurate PMI.

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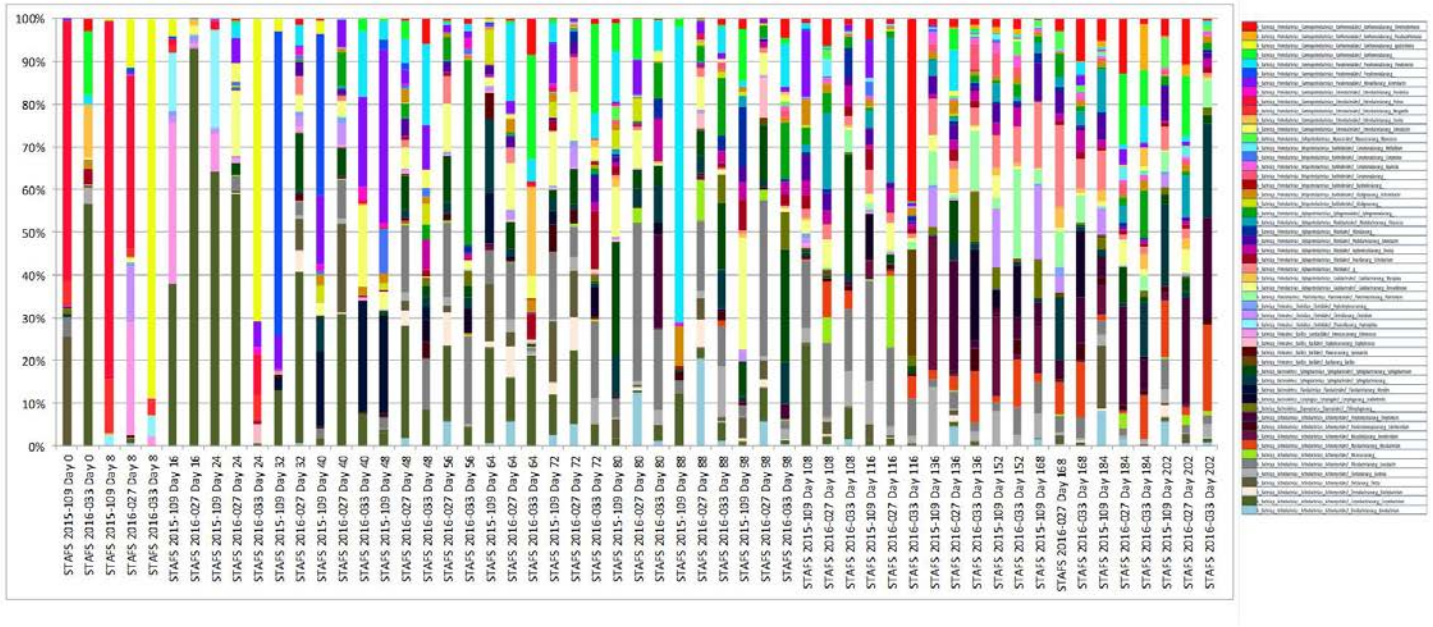
APPENDIX A

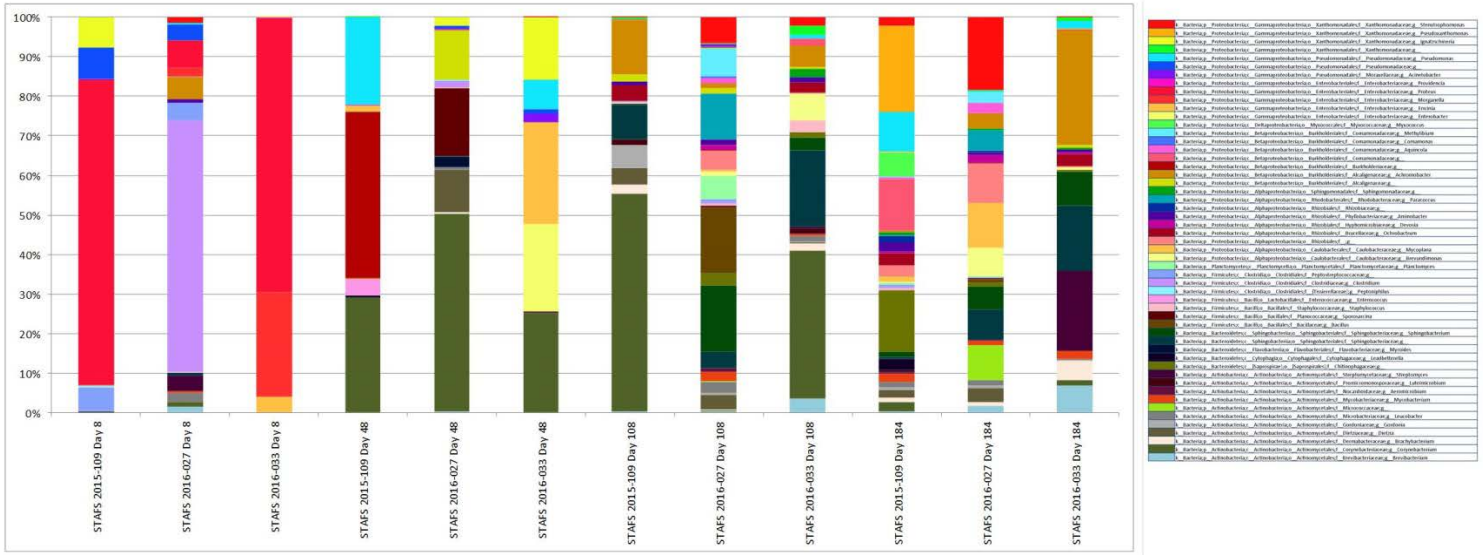
	May	June	July	Aug	Sep	Oct	Nov
L. Femur	33	45	45	50	17	24	21
R. Femur	6	9	9	11	0	6	3
L. Pelvis	33	45	46	48	18	24	21
R. Pelvis	6	9	9	9	0	6	3
L. Humerus	18	21	23	22	10	12	12
R. Humerus	3	15	12	12	2	6	6
Total	99	144	144	152	47	78	66

Total number of samples collected, divided by bone and month.

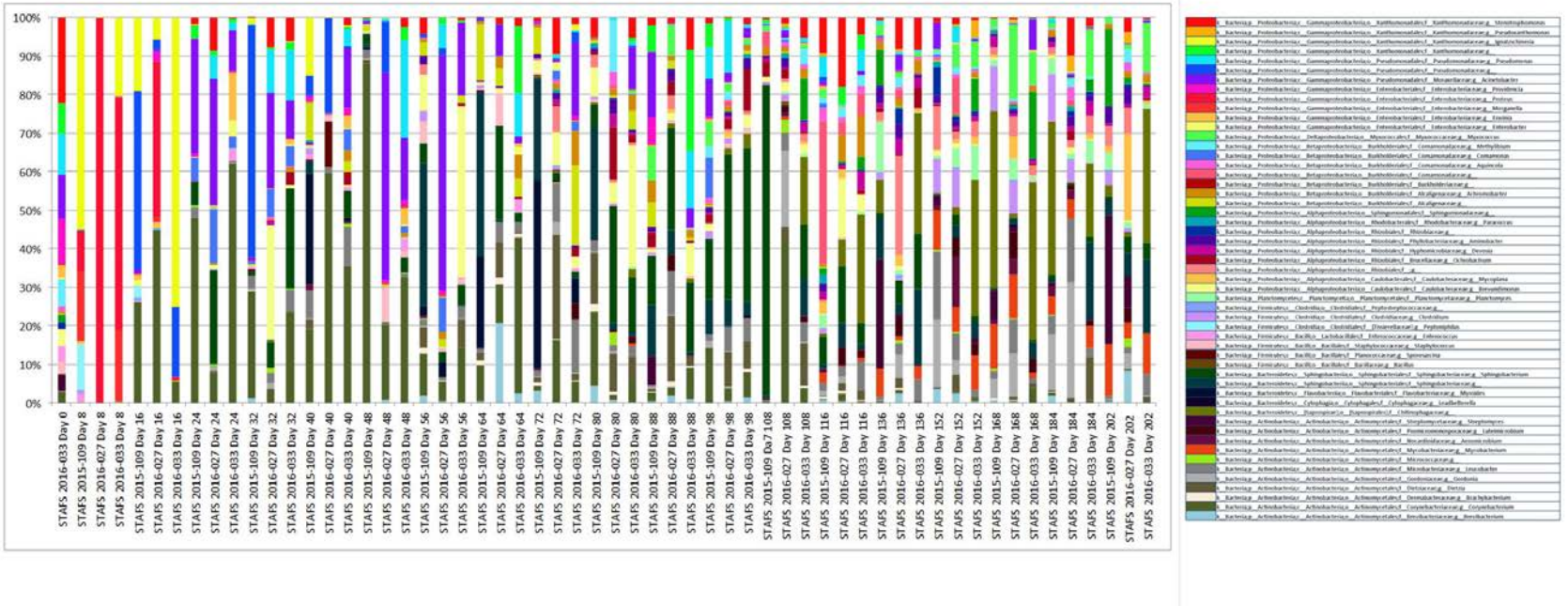
APPENDIX B



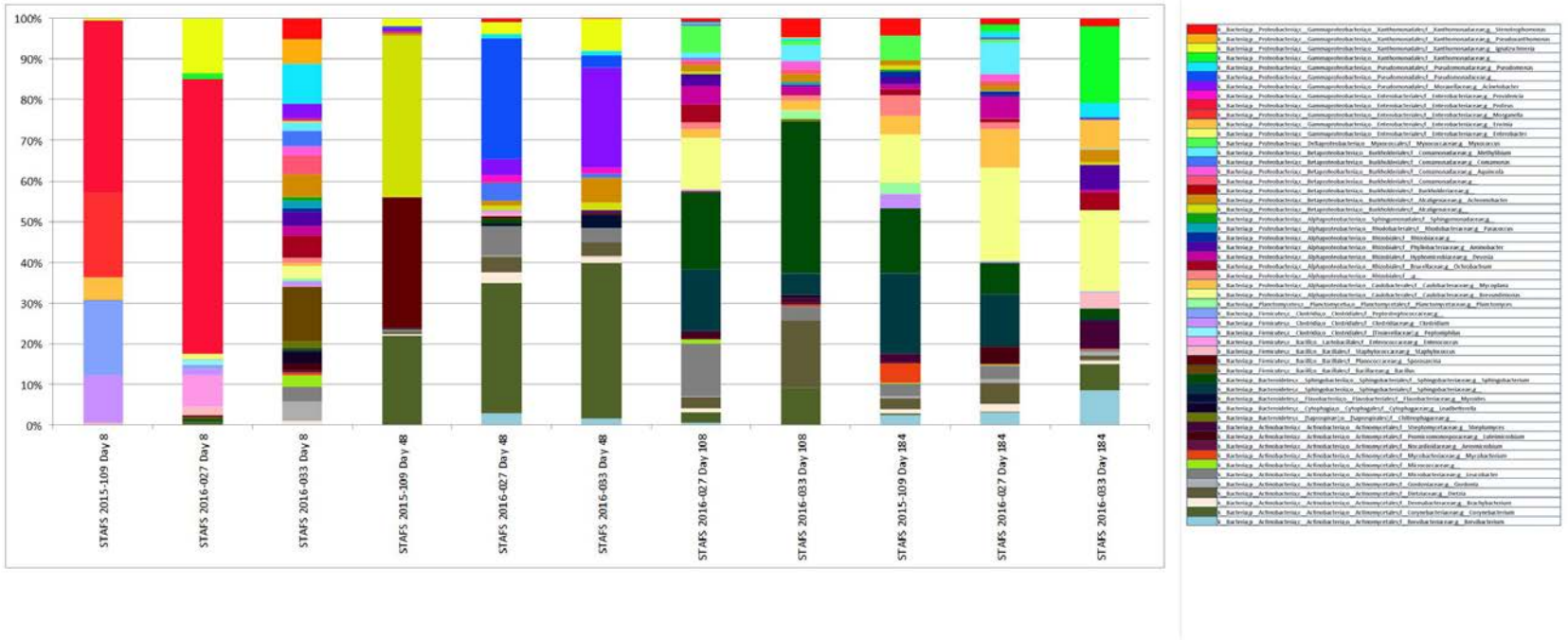
Taxonomy summary of all left femur samples at the genus level. Labelled by STAFS ID and days since placement.



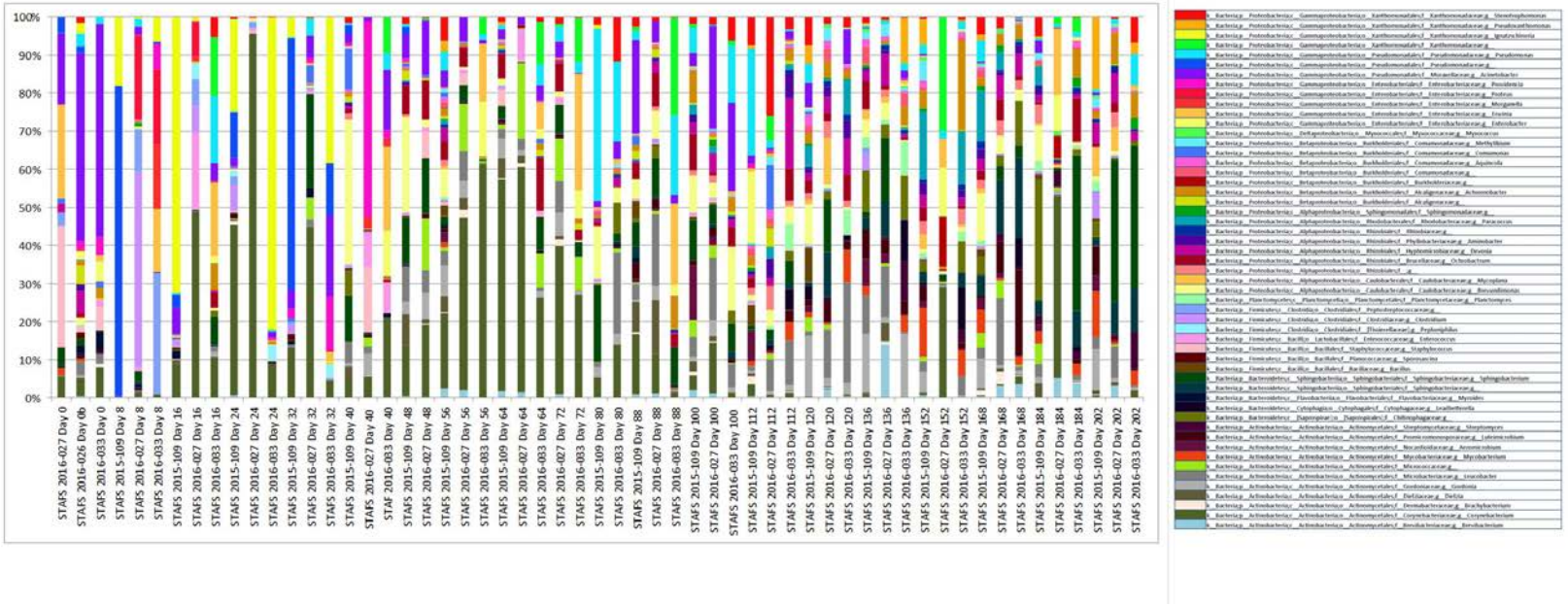
Taxonomy summary of all right femur samples at the genus level. Labelled by STAFS ID and days since placement.



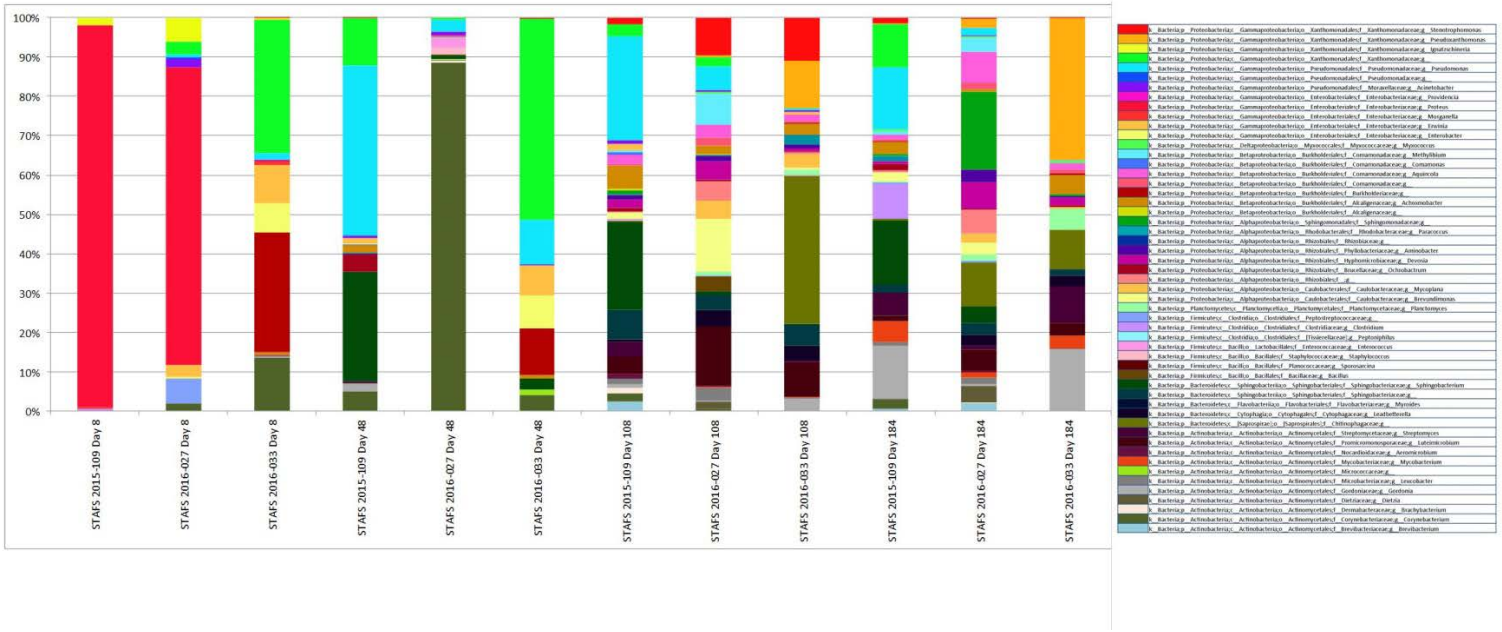
Taxonomy summary of all left pelvis samples at the genus level. Labelled by STAFS ID and days since placement.



Taxonomy summary of all right pelvis samples at the genus level. Labelled by STAFS ID and days since placement.



Taxonomy summary of all left humerus samples at the genus level. Labelled by STAFS ID and days since placement.



Taxonomy summary of all right humerus samples at the genus level. Labelled by STAFS ID and days since placement.

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VITA

M. NICHOLE RUBLE

EDUCATION

Master of Science student in Biology at Sam Houston State University, August 2015 – present. "Microbiome of bone marrow for postmortem interval."

Bachelor of Science (December 2014) in Biology. Texas A&M University, College Station, Texas.

ACADEMIC EMPLOYMENT

Lab Instructor: Human Anatomy and Physiology II, Department of Biological Sciences, Sam Houston State University, January 2018 – present. Responsibilities include: Instructing and guiding students through weekly anatomy and physiology labs and dissections, administering regular lab practicals, and grading.

Graduate Teaching Assistant: Human Physiology, Department of Biological Sciences, Sam Houston State University, September 2017 –December 2017. Responsibilities include: Instructing and guiding students through weekly physiology labs and grading.

Graduate Teaching Assistant: Applied Microbiology, Department of Biological Sciences, Sam Houston State University, June 2016 – August 2017. Responsibilities included: Instructing and guiding students through weekly microbiology labs, preparing weekly labs, and grading.

Graduate Teaching Assistant: Freshmen Botany, Department of Biological Sciences, Sam Houston State University, January 2016 – May 2016. Responsibilities included: Instructing and guiding students through weekly botany labs, assisting professor in lab preparation, and grading.

PUBLICATIONS

Fakhri, C., Rudie, L., Baker, S., Mann, M., Bivens, S., Spoonire, L., and Ruble, N.M. (2017). A methodology for extracting bone marrow from cadavers. *Aisthesis, 8.*

PRESENTATIONS AT PROFESSIONAL MEETINGS

Ruble, M. N., Lewis, P., and Lynne, A. Microbiome succession within bone marrow during human decomposition.10th Annual Meeting of the Texas Association of Biological Anthropologists, University of Texas, Austin, Texas, 19 November 2016.

Ruble, M. N., Lewis, P., and Lynne, A. Succession of microbial communities within bone marrow over the course of human decomposition. 120th Annual Meeting of the Texas Academy of Science, University of Mary Hardin-Baylor, Belton, Texas, 4 March 2017.

Ruble, N., Lewis, P., and Lynne, A. Microbiome of bone marrow during human decomposition. 86th Annual Meeting of the American Association of Physical Anthropologists, New Orleans, Louisiana, 22 April 2017.