Title
Contribution of Host and Environmental Microbes in the Decomposition Process

Author
Kahalehili, Heather M

Publication Date
2021

Peer reviewed|Thesis/dissertation

Contribution of Host and Environmental Microbes in the Decomposition Process

By

HEATHER MALIA-ANN KAHALEHILI
THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Forensic Science

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____
Allison Ehrlich, Chair

_____
Edward Imwinkelried

_____
Joanne Emerson

Committee in Charge

2021

Abstract

The postmortem interval (PMI) is one of the most challenging pieces of evidence to obtain in the field of forensic science. In recent years, a novel approach for estimating PMI has been developed based on predictable patterns in microbial community progression found in both mouse models and human cadavers. However, it is unknown if factors that contribute to the composition of the host microbiome (e.g., drug and antibiotic use, immune deficiency, and diet) impact this model's predictive ability. In the current study, we utilized specific pathogen free (SPF), and germ-free (GF) mice buried in non-sterile and sterile graves to identify sources of variability in microbial community progression. Intestinal contents were collected over the course of a 21-day decomposition period, and bacterial communities were then identified by 16S rRNA sequencing. We found that GF mice remained sterile over the study period, regardless of soil sterility. In contrast, soil sterility had an impact on microbial community dynamics in SPF mice. These data suggest that microbial communities at the time of death influence the entry of environmental microbes and microbial progression. Together, these results show that differences in the host microbiome at the time of death can significantly impact the predictive power of microbial succession in calculating PMI and should be taken into consideration when developing future models.

Introduction

Postmortem Interval (PMI) - In forensic investigations, the postmortem interval, or time elapsed since death, is used as a tool to help narrow the timeline of events that led up to death. This information can also provide insight into potential suspects or the ruling out of certain suspects (by establishing or negating alibis), the cause (COD) and manner (MOD) of death, and the corroboration of witness statements. Determining the PMI, however, remains a great challenge for forensic teams, since current methods have a range of shortcomings and are prone to errors (Giannelli, Imwinkelried et al, Scientific Evidence § 19.08 (6th ed. 2020)). Because of these challenges, most methods are applicable only under specific circumstances (e.g. whether or not insects are present) (Pittner, Bugelli et al. 2020). Some of the most common methods for estimating PMI include assessment of body core temperature, assessment of morphological changes during decomposition (by total body score), and forensic entomology (§§ 19.08[2][c][ix]-[x] of the Giannelli treatise). More recent methods for estimating PMI that have emerged include chemical methods that can account for factors such as body weight and ambient temperature (Cordeiro, Ordóñez-Mayán et al. 2019) as well as microbial methods. Since the analysis of PMI can be so complex and challenging, it is important for the investigator to consider the combination of available methods that would best fit the given circumstances to arrive at the most accurate estimation possible.

Body Temperature - One of the most widely used methods for estimating PMI is measuring body cooling by rectal temperature (Cordeiro, Ordóñez-Mayán et al. 2019). The accuracy of this method can be affected by several factors, including internal (e.g., body mass and microbiome) and external conditions (e.g., ambient temperature). Although this method has a high error rate, recent

studies have shown that a compound method based on the electrical and mechanical excitability of skeletal muscle can reduce the margin of error. To further increase the accuracy of this method, conditional probability distributions for time since death should be included (Madea 2016). Several different mathematical equations have been proposed to account for some of the factors that influence PMI, notably including body weight and hyper- and hypothermia antemortem conditions (Cordeiro, Ordóñez-Mayán et al. 2019).

Decomposition Score - Morphological changes during decomposition are typically scored by using Total Body Score (TBS), a scale frequently used in taphonomic studies (i.e., studies of how organisms decay) and forensic anthropology. This method focuses on the visual changes that occur at different stages of decomposition from the fresh stage to active decay to skeletonization. The body is divided into three different sections, (--the head and neck, trunk, and limbs) that are scored based on the presence or absence of listed criteria. Some common criteria include signs of discoloration, purging of fluids, and bloating. One factor that can affect microbial motility during decomposition is the availability of moisture in the soil. According to Carter et. al, moisture can be the dominant environmental factor govern decomposition in soil (Carter, Yellowlees et al. 2010). However, the limitations of this method include the uncertainty about how to score scavenged remains. Moreover, the scoring can vary between different forensic technicians, depending largely on their level of experience (Metcalf, Wegener Parfrey et al. 2013).

Forensic Entomology - Forensic entomology, the study of insects to aid in legal investigations, has commonly been used to estimate PMI and is most effective within the early postmortem period (<30 days) (Carter, Yellowlees et al. 2010). By studying insect populations in and around a corpse

as well as any developing larval stages, forensic scientists can estimate not only the PMI, but also form opinions about any changes in body position and even the cause of death (COD). Some limitations of this method include the fact that types of insects may be absent during certain seasons. Furthermore, there are region-specific insect communities, and the investigator may err by applying a study conducted in one region to a fact situation in another region. Given such limitations, this method has an error rate that varies from days to months (Metcalf, Wegener Parfrey et al. 2013).

Chemical Analysis - Chemical methods include the analysis of chemicals in the body after death. In recent years, analysis of the vitreous humor (VH) by determining levels of potassium and hypoxanthine has been shown to aid in estimating PMI. As the concentration of potassium and hypoxanthine increase, the PMI also increases. Several factors affect this methodology, including possible errors in the extraction of VH, ambient temperature, and body weight. Simple models that integrate the analysis of the VH with ambient temperature, rectal temperature, and body weight have been designed to help reduce the error rate in estimating PMI (Cordeiro, Ordóñez-Mayán et al. 2019).

Microbial Studies - Microbial studies has been of particular interest to forensics teams because they can eliminate some of the limitations of the previously listed methods. Tracking the progression of microbial communities throughout the decomposition is possible with high-throughput DNA sequencing and has been shown to accurately estimate PMI within 3 days with a mouse model system (Metcalf, Wegener Parfrey et al. 2013). Several studies have shown that bacterial communities follow a consistent trajectory throughout the stages of decomposition

(Metcalf, Wegener Parfrey et al. 2013),(Pechal, Crippen et al. 2014), making this method helpful in predicting PMI.

Stages of Decomposition

The five basic stages of decomposition are: fresh, bloat, active decay, advanced decay, and dry/skeletonization.

Immediately after death, autolysis or self-digestion begins, as lysosomes and bacteria present before death release their digestive enzymes to break down tissues in the body. Cessation of any cardiac activity, blood circulation and respiration occur at death, resulting in an excess supply of carbon dioxide. As an acidic environment forms from carbon dioxide buildup, the muscles in the body begin to stiffen (in a process called rigor mortis)(§ 19.08[2][b][iii] in Giannelli) and the body temperature begins to cool and equalize to the surrounding environment (in a process called algor mortis) (§ 19.08[2][b][iv] in Giannelli),(Almulhim, Menezes 2021). Several factors can affect the speed of this process, including the surface area of the body, the body weight, and the temperature gradient between the body and its environment (Nelson 2000). Approximately two hours after death, the blood begins to pool to the lowest point in the body in a process called livor mortis (§ 19.08[2][b][ii] in Giannelli).

Bloat or putrefaction is characterized by the production of gases such as sulfur dioxide, ammonia, and methane that give the body a swollen or bloated appearance (Can, Javan et al. 2014). This bloating is often accompanied by an odor of decaying flesh and light skin discoloration as a result of sulfur-containing compounds released by bacteria.

Active decay is most recognizable by loss in mass or a collapse of the body due to the escape of gases and fluids. Skin discoloration darkens to black during this stage, and the odor grows stronger. In an outdoor setting, this is the stage in which most insects are present to feed on the decomposing body. Advanced decay occurs when most of the soft tissue has been processed by bacterial and/or insect activity. The cadaver then begins to dry out, and a strong odor from butyric acid is present (Nafte 2009).

The dry/skeletonization stage is reached when only bone and potentially hair remain. There are multiple factors that contribute to the rate of the decomposition process, including age, body weight, general health, and drug use. Environmental conditions play a large role in the variability seen across estimated postmortem intervals because weather and temperature can greatly affect the rate of chemical reactions, and the metabolism of microbes (Wescott 2018). Dry and hot environments tend to speed up the process, while wet and cold environments tend to slow the process.

Forensic entomology is one of the most used methods for analyzing the decomposition process because the physical changes to a cadaver attract insects to feed on the decaying flesh. Particularly for bodies or corpses found in outdoor settings, identifying insects present at the time of discovery and the succession of these insects can help in estimating PMI (Madea 2016). Although there are several factors that may affect the decomposition process, the general stages remain the same; - and the biotic signatures associated with each stage such as the development rate of blow fly larvae can aid in estimating PMI (Metcalf, Wegener Parfrey et al. 2013). Furthermore, understanding the stages of decomposition may provide information on the position of the body in the hours after

death by evaluating lividity, or discoloration patterns on the body due to pooling of blood in the lower regions of the body.

Thanatomicrobiome

The human microbiome is a diverse collective of all microbiota that exists on and within a human being. Within this collective are distinct microbial communities that form on different locations in the body. From the time of birth, the phylogenetic diversity of these microbial communities increases significantly and linearly with time (Ursell, Metcalf et al. 2012), (Koenig, Spor et al. 2011). The microbiome interacts with many other microorganisms, and its composition may shift dramatically depending on a person's diet, the use of drugs and antibiotics, and whether the person has any current illnesses (Pechal, Crippen et al. 2014), *(*Koenig, Spor et al. 2011). Some of these microbial communities form symbiotic relationships with their hosts or other microorganisms as they are continuously in contact with the surrounding environment. One common type of symbiotic relationship that microbiota engage in is mutualism, in which two species benefit from each other. For example, a human host benefits from the gut microbiota to consume food for energy and survival, while the gut microbiota gains habitat and nutrition from the host. Each individual has their own unique set of microbiota that can provide information regarding an individual's DNA fingerprint, general health, and behavioral patterns (Clarke, Gomez et al. 2017).

The postmortem microbiome is comprised of two components: the thanatomicrobiome and the epinecrotic microbial community (Oliveira and Amorim 2018). The thanatomicrobiome consists of the microbes colonizing the internal organs and orifices after death while the epinecrotic microbial community consists of microeukaryotes residing in and/or moving on the surface of

decomposing remains (Javan, Finley et al. 2016). The postmortem microbiome has been recognized as a useful microbial biomarker of both the time and location of host death because of significant variation observed at the same body site across different stages of the decomposition process. Additionally, environmental microbial communities vary across geographic location and can help provide information about the origin of a sample or piece of evidence (Zhou and Bian 2018). The environmental microbiome acts the same as the human microbiome in that it encounters thousands of other microorganisms to form a unique DNA fingerprint of that region or area. Studies have shown that unique microbial community profiles exist for certain areas of a city, suggesting that this information could be used in determining the origin of an item from a crime scene (Robinson, Pasternak et al. 2020).

Collectively, analyzing these microbiomes can aid in providing information about potential suspects, the cause of death (COD), the time of death (TOD), and the location of death (Oliveira and Amorim 2018). Recent advances in next generation sequencing (NGS) technologies have provided an exponential increase in available sequencing data useful for forensic investigations and have allowed researchers to profile thousands of DNA samples simultaneously (Clarke, Gomez et al. 2017). Various researchers have employed different approaches to NGS, such as sequencing the whole genome of a particular organism or sequencing the entirety of the microbial species present in a sample (Oliveira and Amorim 2018). NGS of both human and environmental microbiomes has demonstrated that microbial DNA can be used to aid in identifying a single individual in a population, define biogeographical patterns from different objects, and estimate time since death (TOD) (Clarke, Gomez et al. 2017).

Microbial Forensics

Using 16S rRNA gene sequencing, forensic scientists have been able to reliably estimate PMIs by following microbial community progression. Studies have specifically looked at levels of diversity and abundance in microbes colonizing the internal organs and orifices after death (thanatomicrobiome) as well as their interactions with environmental microbes. Documenting and identifying differences in these communities along different time points of the decomposition process have been shown to provide key information in predicting physiological time since death (Pechal, Crippen et al. 2014). Using both a mouse model and human cadavers, Metcalf, et al., were able to identify a predictable "microbial clock" that could accurately estimate PMI within 3 days. They studied the composition of microbial communities by using high-throughput sequencing, (both 16S rRNA gene sequencing and 18S rRNA gene sequencing) and found changes that were significant and consistent across models. The microbial communities within the corpse influenced those in the soil and vice versa. By studying the interaction between internal and external environments, investigators can gain insight into how microbes facilitate the decomposition process and identify additional patterns that might be useful to forensic investigations (Metcalf, Wegener Parfrey et al. 2013).

In a separate study looking at microbial community assembly and metabolic function during mammalian corpse decomposition, Metcalf, et al., reported that microbial succession was predictable across soil types, seasons, and host species (Metcalf, Xu et al. 2016). Mice were decomposed on three different types of grave soil (i.e., desert, shortgrass, and forest), and similar succession patterns were found regardless of soil type. In their human experiments, Metcalf's team saw similar succession of microbes across bodies within the same season (e.g., winter or spring).

The team found that the most abundant bacteria at the end of the decomposition process originated from the soil and that the soil was more likely to be the source of the decomposer microbial communities. The team also surmised that the bacteria and archaea found in the soil likely play a larger role in the decomposition process than any internal microbiota (Metcalf, Xu et al. 2016). One limitation of their model is that it does not account for differences in the host microbiome before death. More specifically, differences in dietary preference, antibiotic use, and environmental exposure may affect the composition of the host microbiome (Oliveira and Amorim 2018). Therefore, in this project we aim to further explore the contribution of host and environmental microbes in the decomposition process and estimating PMI. Gaining a better understanding of how these microbes contribute to the decomposition process can aid in having them be helpful resources to solve a case.

Microbial studies may offer an improved approach to estimating postmortem intervals because microbial succession can be predictable and reproducible. Although there is no universal method of estimating PMI due to the vast amount of circumstances in any single forensic case, innovative techniques associated with NGS can be used in combination with classical PMI methods for a more accurate estimation. Knowledge gaps currently revolve around understanding how variation in the host microbiome before death affects the microbial community dynamics during the subsequent decomposition process. In the current study, we aimed to learn the contribution of host and environmental microbes in the decomposition process by determining the abundance of predictive bacterial taxa (i.e. identifying "decomposers"), and by identifying a predictable pattern of microbial community progression (previously reported by Metcalf et. al.). Collectively, we

found that the GF mice remained sterile- regardless of soil sterility, and that the predictable pattern of microbial community progression found in SPF mice depend on soil microbes.

Materials and Methods

Experimental Design and Sample Collection

Mice were humanely sacrificed by using carbon dioxide gas and then transported from Mouse Biology Program to Meyer Hall on campus. Mice (non-obese diabetic wildtype, NOD WT) ranged in age from 5-7 weeks and were randomly assigned to groups. Mice were placed on their right side on top of approximately 100 g of soil in an autoclaved pipette tip box. These "graves" were then placed in a HEPA filtered container inside a fume hood.

Four conditions were set up with two types of mice (--germ-free (GF) mice and specific-pathogen free (SPF) mice) and two types of soil (--autoclaved potting soil (sterile) and normal potting soil (non-sterile)). In this study, the expressions "sterile samples" and "non-sterile samples" refer to samples buried on autoclaved or non-autoclaved soil. Destructive host sampling was performed on days 1, 3, 7, 14, and 21 to collect the intestinal contents of each mouse. In addition, a soil sample from underneath each carcass was collected at the time of destructive sampling. Photographs of the mice were taken at several points: before dissection, after opening the skin layer, and again after laying out intestines. Intestinal contents were scraped out and collected in sterile 1.5 ml Eppendorf tubes.

Visual Scoring by Total Body Score

Three people scored blinded photographs of mice. The scores were averaged and plotted using GraphPad Prism. Visual body scores were recorded for each carcass by using the following key adapted from Megyesi et al. (2005) (Megyesi, Nawrocki et al. 2005) and Metcalf et al. (2013) (Metcalf, Wegener Parfrey et al. 2013):

----Fresh: no discoloration (1 point);

----Active Decay (early decomposition): discoloration (2 points), purging of decomposition fluids out of eyes, nose, or mouth (3 points), bloating (any location on carcass) (4 points); and

----Advanced Decay: drying (5 points), sagging, or sinking of flesh (6 points), caving in of flesh (7 points), mummification (8 points).

DNA Extraction, PCR amplification, and 16S rRNA Gene Sequencing

Intestinal mouse content and soil DNA extractions were performed by using the QIAGEN PowerSoil Pro DNA Kit and the QIAGEN PowerFecal DNA Kit (Qiagen Inc., Germantown, MD, USA), following protocols specified in the manufacturer's instructions. The DNA concentrations were determined by using Qubit 4 Fluorometer Broad Range DNA assay (Invitrogen by ThermoFisher Scientific, Hanover Park, IL, USA). The extracted DNA samples were stored in a freezer at -20°C until further use.

16S rRNA gene sequencing was conducted as previously described in Kahalehili, Newman et al. 2020. Total genomic DNA was subjected to PCR amplification targeting the 16S rRNA hypervariable region 4 (V4) by using bacterial primer set NOD_AhR. The primers 319F and 806R were used to amplify the V3-V4 domain of the 16S rRNA gene by using a two-step PCR procedure.

In step one of the amplification procedure, both forward and reverse primers contained an Illumina tag sequence (bold), a variable length spacer (no spacer, C, TC, or ATC for 319F; no spacer, G, TG, ATG for 806R) to increase diversity and improve the quality of the sequencing run, a linker sequence (italicized), and the 16S rRNA gene target sequence (underlined) In step two, each sample was barcoded with a unique forward and reverse barcode combination by using forward primers

(**AATGATACGGCGACCACCGAGATCTACAC***NNNNNNNN*TCGTCGGCAGCGTC) with an Illumina P5 adapter sequence (bold), a unique 8 nt barcode (N), a partial matching sequence of the forward adapter used in step one (underlined), and reverse primers (**CAAGCAGAAGACGGCATACGAGAT***NNNNNNNN*GTCTCGTGGGCTCGG) with an Illumina P7 adapter sequence (bold), unique 8 nt barcode (N), and a partial matching sequence of the reverse adapter used in step one (underlined). The final product was quantified on the Qubit instrument by using the Qubit Broad Range DNA kit (Invitrogen), and individual amplicons were pooled in equal concentrations. The pooled library was cleaned by utilizing Ampure XP beads (Beckman Coulter). Then the band of interest was further subjected to isolation *via* gel electrophoresis on a 1.5% Blue Pippin HT gel (Sage Science). The library was quantified *via* qPCR followed by 300-bp paired-end sequencing using an Illumina MiSeq instrument in the Genome Center DNA Technologies Core, University of California, Davis. The QIIME 2 (30) bioinformatics pipeline (v. 2018.8.0) was used to demultiplex and quality filter the forward-end fastq files. Denoising was performed by using DADA2 (31). The raw data can be accessed at NCBI Sequence Read Archive (SRA) (Accession #PRJNA679964).

Statistical Analyses

Statistical analyses were performed by using GraphPad Prism and Microsoft Excel. For comparing two groups, a student's t-test was performed. For multiple comparisons, one-way ANOVA with Tukey's test was used. Following the common convention, $P < 0.05$ was considered statistically significant. All plotted data points represent an individual mouse.

Results

Visual decomposition score was not predominantly driven by the host microbiome.

To begin to determine the dynamic relationship between internal and external microbes and their contributions in the decomposition process, we designed a study using GF and SPF mice buried on sterile or non-sterile soil (depicted in Figure 1A). Soil and intestinal content samples were collected on days 1, 3, 7, 14, and 21 (Figure 1B). To assess the progression of decomposition, we used the Megyesi visual key assigning a score based on physical changes seen across the known stages of the decomposition process. Mice buried under different conditions generally followed a similar pattern; there was a large jump in score around days 7-14 and then plateauing between day 14 and day 21 (Figure 1C). Over the duration of 21 days, mice progressed through the major stages of decomposition up until the active decay stage, but not the advanced decay process. We saw a trend in decomposition scores, particularly on day 7; the SPF mice buried on non-sterile soil had higher scores than the mice in other housing conditions (Figure 1D).

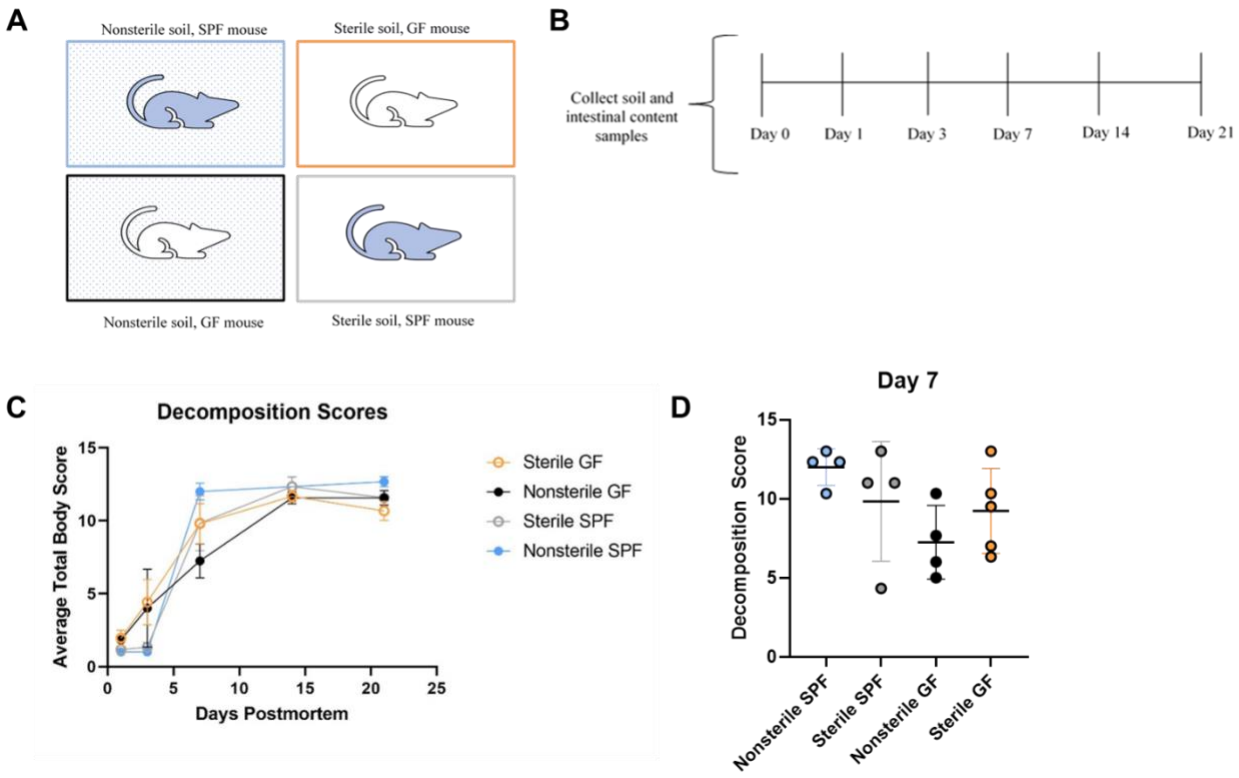


Figure 1. Visual decomposition score was not predominantly driven by the host microbiome. (A) Schematic showing the four different conditions used in this experiment. Two types of soil: sterile (autoclaved) and non-sterile and two types of mice: GF and SPF. (B) Timeline of sample collection time points for both soil and mouse intestinal content. (C) Average total body scores (Megyesi key) plotted across the sampling time points. (D) Decomposition scores for individual mice on Day 7. DN= 4-5 mice/timepoint/group.

<u>The gut of GF mice remained sterile during decomposition regardless of the presence of soil microbes.</u>

To assess whether microbial communities from the soil impact the microbial community dynamics of the host, we measured the DNA concentrations of 40 GF and 50 SPF mice with Qubit broad range DNA assays and calculated the amount of DNA per gram of intestinal content. We found that apart from day 1, the gut of the GF mice remained sterile throughout the decomposition process with DNA concentrations too low to detect (Figures A-E). The DNA readings recorded for GF mice on day 1 were confirmed to not be of bacteria origin by qPCR (Figure 1A).

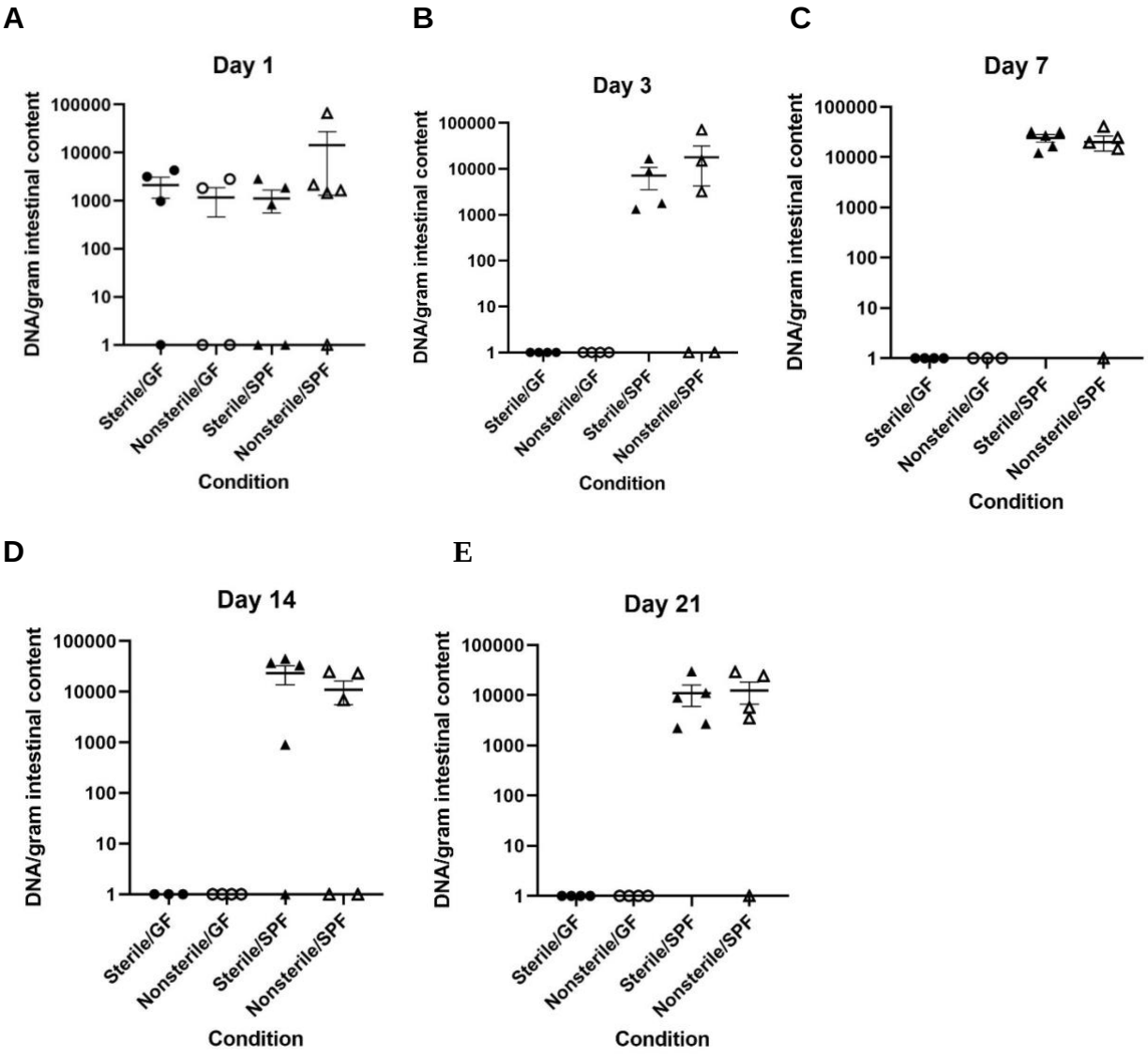
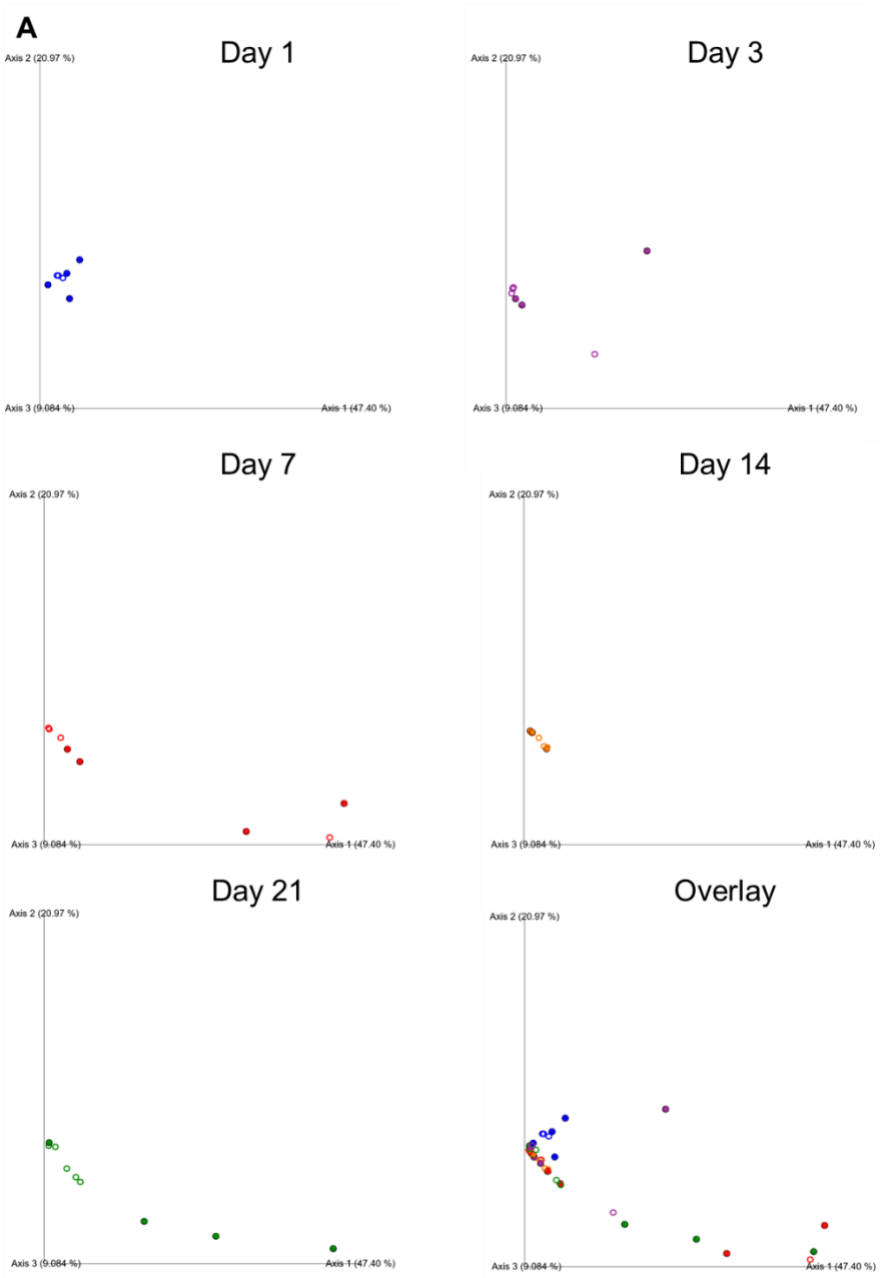


Figure 2. The gut of GF mice remained sterile during decomposition regardless of the presence of soil microbes. (A-E) DNA concentration per gram of intestinal content plotted per time point per condition. GF mice remained sterile from days 3-21.

<u>Microbial diversity changed throughout decomposition and was dependent on soil microbes.</u>

To identify any patterns in microbial diversity over time, we used QIIME Emperor Ordination to create PCoA plots (Figure 3A). Each circle represents a single mouse. Samples from mice buried on sterile soil are represented with an open circle while those buried on non-sterile soil are represented by a solid circle. Over time, day 1 (blue), day 3 (purple), day 7 (red), day 14 (orange), and day 21(green) samples separate out on Axis 1, creating a gradual shift. The majority of the

samples buried on sterile soil remained on the left, while samples buried on non-sterile soil progressively made their way to the right, indicating shift in diversity across these conditions over time. To identify any patterns, similarities, or correlations between our soil conditions and our time points, we plotted parallel coordinate plots by using QIIME Emperor Ordination. We found that soil separates predominantly on axis 1 (Figure 3B) while time separates primarily on axes 2 and 3 (Figure 3C).


A
Day 1
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)
Day 3
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)
Day 7
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)
Day 14
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)
Day 21
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)
Overlay
Axis 2 (20.97 %)
Axis 3 (9.084 %)
Axis 1 (47.40 %)

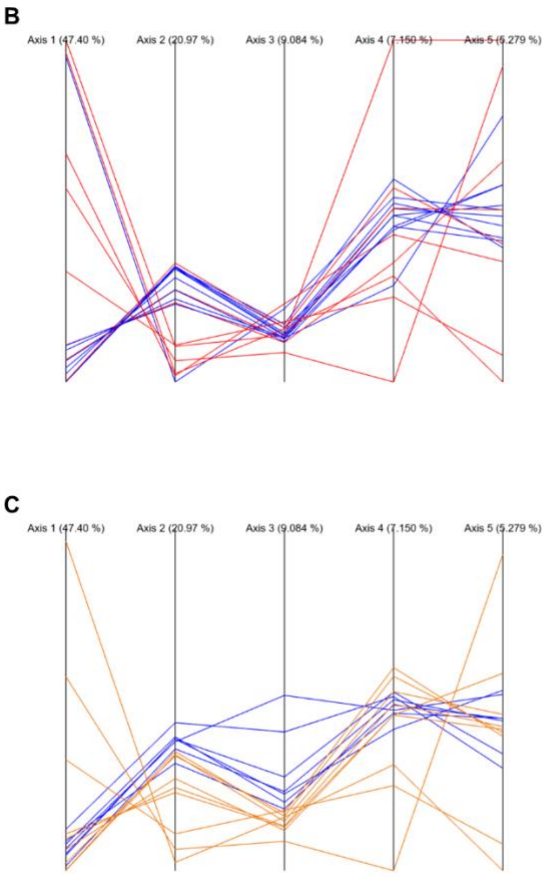


Figure 3. Microbial diversity changed throughout decomposition and was dependent on soil microbes. (A) Principal coordinate analysis (PCoA) plots based on Bray-Curtis distance displaying microbial community change over time. (B) Parallel PCoA plot of sterile (red) and non-sterile (blue) soil shows soil separates predominantly on axis 1. (C) Parallel PCoA plot of day 1 (blue) and day 21 (orange) shows time separates predominantly on axis 1 and 2.

Microbes that significantly increased from day 1 to day 21 are primarily from the Firmicutes and Actinobacteriota phyla.

To identify the microbes that increase from day 1 to day 21 (i.e. the bacteria that are considered "decomposers"), we sorted the data by different taxonomic levels. We identified the genus *Enterococcus* and species *Lactobacillus johnsonii* within the Firmicutes phylum and the species *Bifidobacterium choerinum* within the Actinobacteriota as the microbes that increased

significantly over time. *Bifidobacterium choerinum* and *Lactobacillus johnsonii* showed a significant difference between day 1 and day 21 in samples from sterile soil while *Enterococcus* showed a significant difference in samples from non-sterile soil.

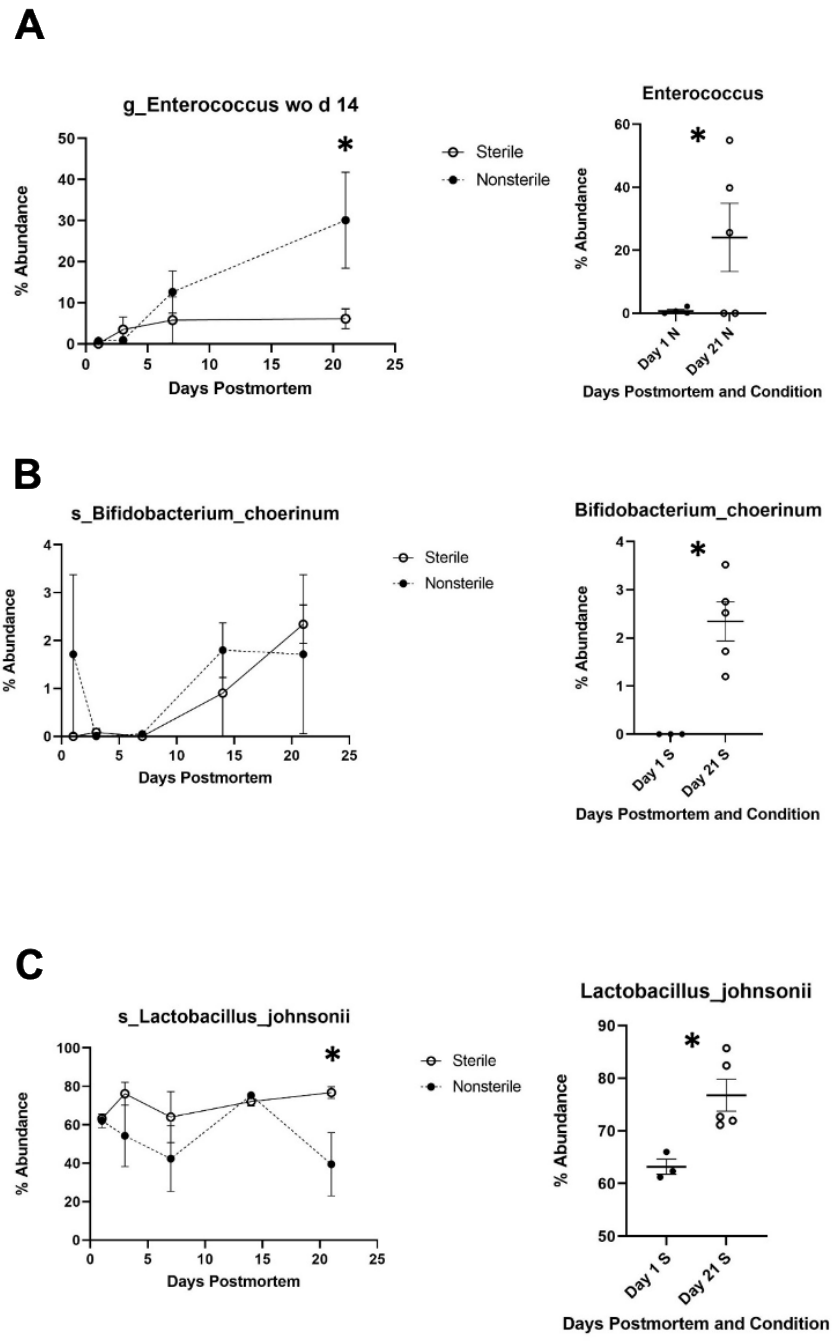
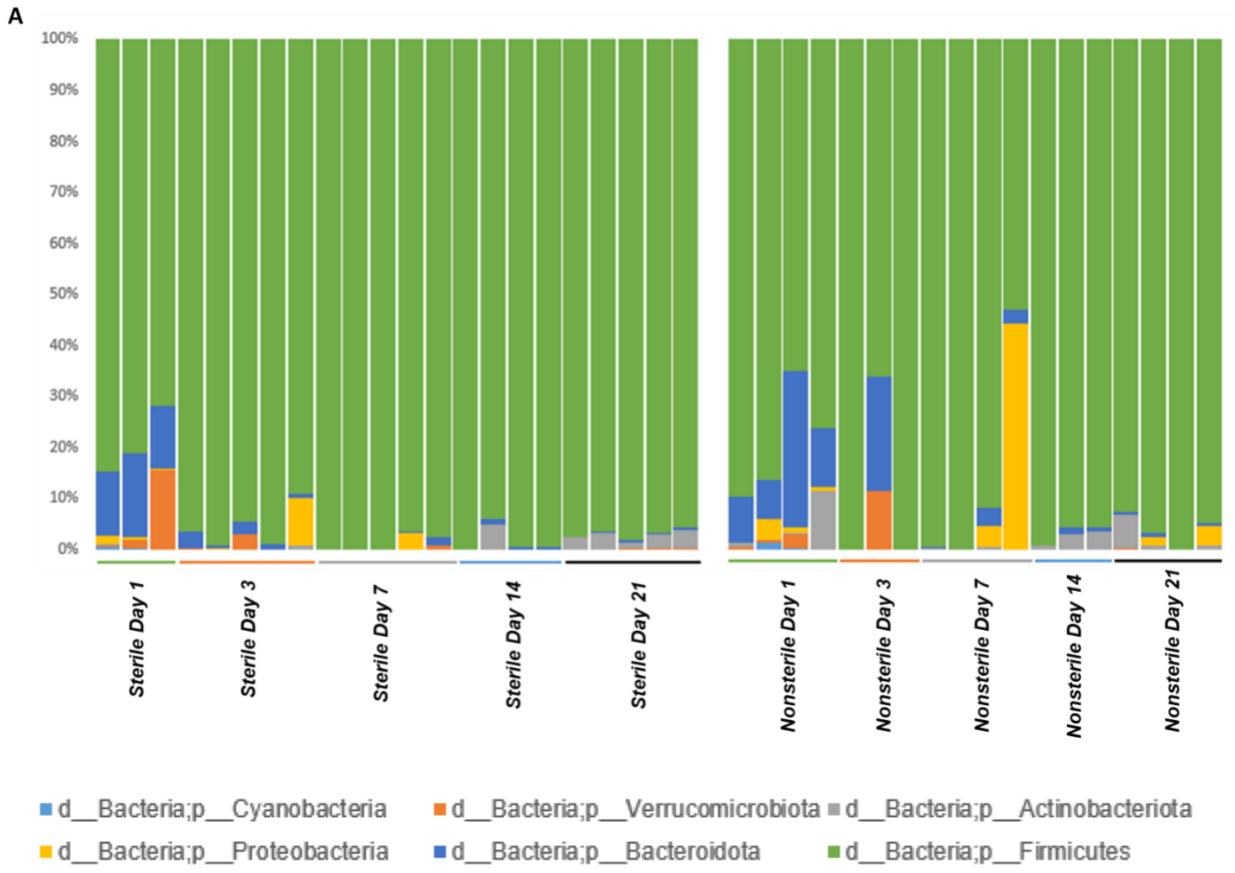


Figure 4. Microbes that significantly increased from day 1 to day 21 are primarily from the Firmicutes and Actinobateriota phyla. (A-C) Percent abundance of identified "decomposers" increased from day 1 to day 21. S= sterile soil, N= non-sterile soil. * = $p<0.05$.

Increased abundance and diversity in microbial communities were more evident in mice decomposed on non-sterile soil compared to those decomposed sterile soil.

To identify any differences between the soil conditions, we sorted the data by different taxonomic levels. We found that sorting by phylum revealed the most distinct differences. Increased abundance and diversity of microbes were more evident in mice decomposed on non-sterile soil compared to those decomposed on sterile soil (Figure 5A). To pinpoint the key decomposers, we identified any major phylum that significantly changed in abundance over time. Actinobacteriota and Firmicutes were the primary decomposers, while the other four phyla decreased significantly over time (Figures 5B-G).



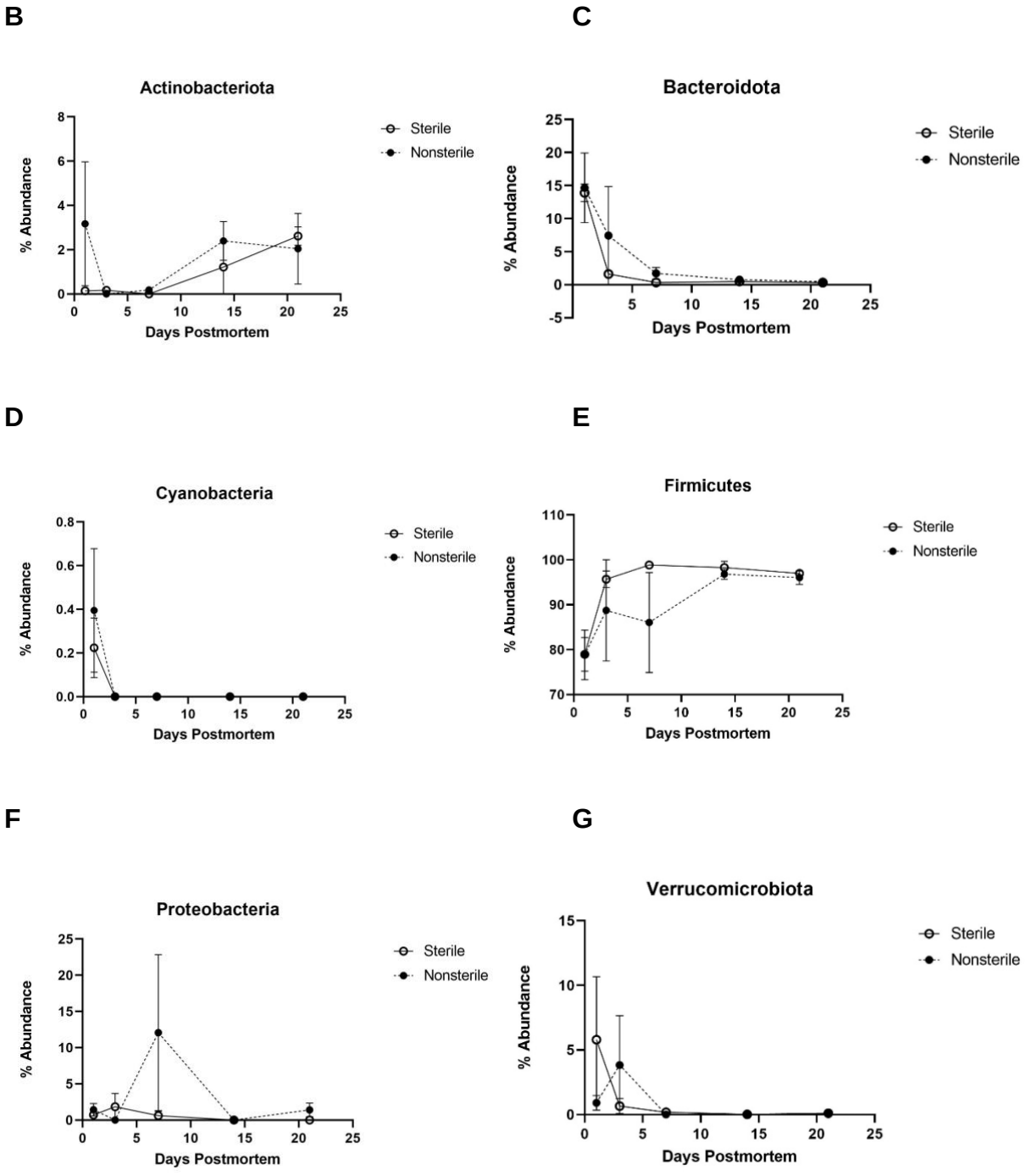
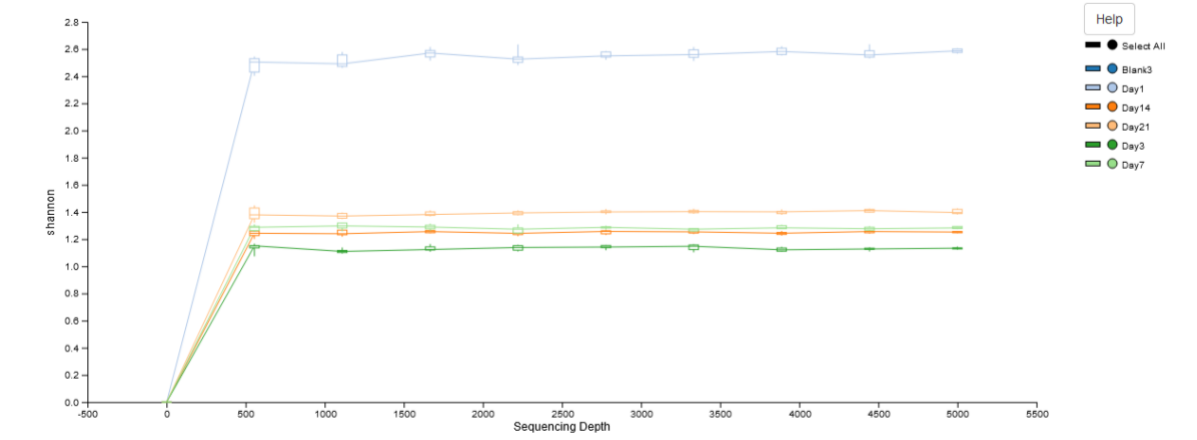


Figure 5. Increased abundance and diversity were more evident in mice decomposed on non-sterile soil compared to sterile soil. (A) Relative abundance of six major phylum groups shows increased abundance and diversity within the samples using non-sterile soil. (B-G) Data separated by phylum show that Actinobacteriota and Firmicutes are the primary decomposers, showing their abundance increasing the most within the 21-day decomposition period.

Alpha diversity decreased over time and is lower in mice decomposed on sterile soil.

To assess the richness (number of taxonomic groups) and evenness of our data (distribution of abundances of the groups), we compared our data via alpha diversity and alpha rarefaction. Rarefaction is a method that adjusts for differences across samples to aid in alpha diversity (Willis 2019). Over time, alpha diversity decreases with the highest diversity occurring on day 1 (Figure 6A). Across soil conditions, alpha diversity is lower in mice decomposed on sterile soil (Figure 6B).

A



B

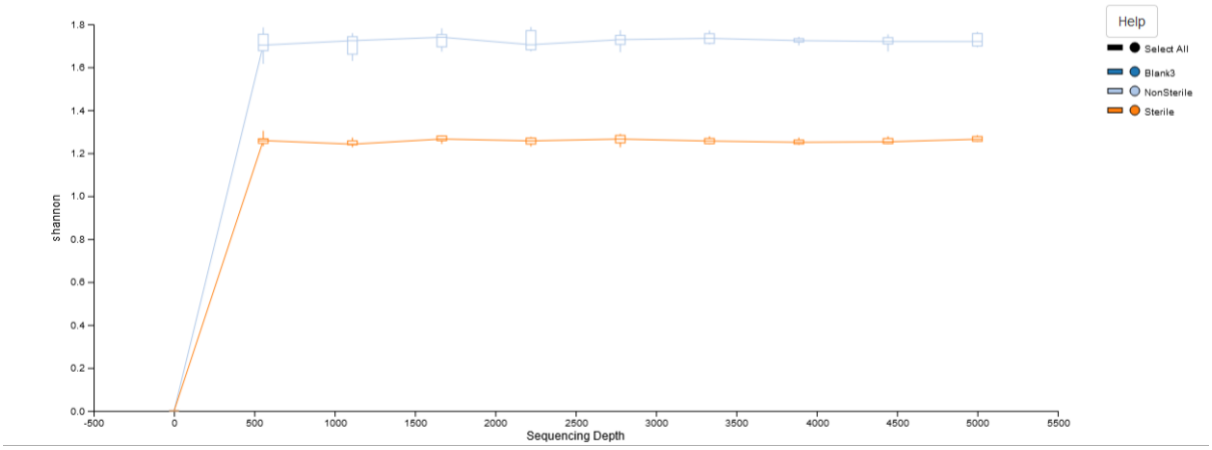


Figure 6. Alpha diversity decreased over time (A) and is lower in mice decomposed on sterile soil (B).

Discussion

Since estimating PMI is one of the greatest challenges in the field of forensic science, improving the methodology for estimating PMI is of great importance. In recent years, tracking microbial community progression has proven to be successful in accurately estimating PMI within 3 days. This method has been found to be predictable and reproducible by Metcalf et. al. However, studies focusing on how a compromised host microbiome might affect this predictable pattern have yet to be done. Here, we aimed to identify any differences in the microbial communities of mice with and without a host microbiome and how those differences affect the decomposition process. Gaining insight into how differences across host microbiomes can potentially contribute to the predictable pattern may help in modifying current methodologies to account for these differences.

This project revealed several findings that may be useful to the forensic community. First, without a host microbiome (or with a compromised host microbiome), the predictable microbial pattern shown in previous studies will most likely be compromised as well. Apart from day 1, the gut of GF mice remained sterile regardless of the presence of soil microbes. All GF samples were too low in DNA concentration to detect on the Qubit and were also too low to generate amplicon targets for sequencing. Using universal bacterial primers, we found no amplification of bacterial DNA by qPCR. Therefore, the DNA detected in GF mice on day 1 (Figure 2A) were confirmed not to be bacterial by qPCR. One possible explanation for the presence of DNA found in day 1 GF samples could be that eukaryotic DNA was also extracted while using the Qiagen PowerFecal DNA kit and may have degraded over time.

In contrast, the analysis of the gut microbes in SPF mice showed a time course progression across the timeline of the experiment, with a progressive separation between sterile and non-sterile soil conditions. Interestingly, the primary decomposers we identified from the Actinobacteriota and Firmicutes phyla differed from previous studies. Metcalf et. al. identified Alphaproteobacteria (mostly Rhizobiales) as the most abundant in post-rupture stage soil samples (Metcalf, Wegener Parfrey et al. 2013). Some differences between our experimental designs might explain this discrepancy. The mouse strain they used was B6 in contrast to the NOD WT strain we used. In addition, we used basic potting soil while Metcalf et. al. used soil collected from the organic layer of a dry creek bed in Eldorado Creek near Boulder, CO. We would expect the soil they used to have a higher variety of microbes, including fungi and nematodes. Additionally, they drilled small holes into the sides of their plastic Tupperware-like containers to prevent anaerobic conditions. Conversely, we did not control for humidity levels or anaerobic conditions. Maintaining moisture levels may have resulted in more microbial activity, and consequently, the decomposition process may have proceeded at a different rate (Carter, Yellowlees et al. 2010).

It has been noted in previous studies that the most informative sampling area in terms of displaying the predictable community progression is the soil, even more so than the skin or abdomen. In this study, by focusing on the gut microbiome, we endeavored to explore how the differences in host microbiome impact these microbial communities. Our data suggests that the presence of the host microbiome is critical in initiating the decomposition process, especially during the early stages when bloating, purging and rupture occur. During bloat (~6-9 days), endogenous and facultative anaerobes such as Firmicutes and Bacteroidetes increase in the abdominal cavity. After rupture (~9 days), those decrease; and there is a strong shift from anaerobic to aerobic bacteria

(Alphaproteobacteria) (Metcalf, Wegener Parfrey et al. 2013). With mice that are essentially germ free, no predictable microbial progression pattern could be identified, despite microbes present in non-sterile soil conditions.

There were limitations to our study that could have impacted our results and interpretations. First, this experiment was conducted in a controlled setting in a fume hood where carcasses were not exposed to any environmental factors; those factors not only speed up the decomposition process, but also introduce many of the prominent microbial decomposers seen throughout the decomposition process. The microbes in the non-sterile soil alone did not penetrate the internal barrier of GF mice, and therefore, the concentrations of DNA in GF mice were too low to quantify. One possible explanation for the lack of data from GF mice is that the strain of mice used (non-obese diabetic mice) has a genetic makeup that spontaneously acquires type 1 diabetes. The mice collected for the study were around 5-7 weeks of age, when they first can develop insulitis. The genetic makeup of these mice may have contributed to the differences between this study and experiments conducted with C57BL/6 mice. Comparison studies could be conducted to test the role of this strain on postmortem microbial progression.

Second, we focused on intestinal contents of the mouse along with the soil beneath each carcass. Additional sampling sites, including swabbing of the skin surface in contact with the soil, may have resulted in additional data in GF mice.

The third limitation was that the last time point in our study was day 21. It is likely that including a later timepoint (day 40) might have led to stronger patterns and correlations.

Nevertheless, this study has yielded new information that may help improve the state of the art of estimating TOD. We showed that if the host microbiome is compromised (particularly by a genetic background involving immunodeficiency), the ability of identifying the predictable microbial progression pattern seen in previous studies may be diminished. *Therefore, the microbial progression associated with decomposition is significantly influenced by the host microbiome*. Future studies should focus on how other factors that alter the microbiome such as the use of antibiotics or drugs, other immunodeficiencies, or health conditions, could affect the pattern.

We also identified a time course progression in SPF mice across sterile and non-sterile soil conditions. Over time, the mice decomposed on non-sterile soil had an increased abundance and diversity of microbes. However, the most abundant primary decomposers differed from previous studies. We identified bacteria from the Firmicutes and Actinobacteriota phyla to be the primary decomposers, as they were most abundant by the end of the study period. Therefore, despite having SPF mice with host microbes present, we were unable to replicate the predictable microbial progression pattern. Moving forward, focusing on ways to refine current methodology for estimating PMI will be of great benefit to the forensic science community.

References

Can, I., G. T. Javan, A. E. Pozhitkov and P. A. Noble (2014). "Distinctive thanatomicrobiome signatures found in the blood and internal organs of humans." J Microbiol Methods **106**: 1-7.

Carter, D. O., D. Yellowlees and M. Tibbett (2010). "Moisture can be the dominant environmental parameter governing cadaver decomposition in soil." Forensic Sci Int **200**(1-3): 60-66.

Clarke, T. H., A. Gomez, H. Singh, K. E. Nelson and L. M. Brinkac (2017). "Integrating the microbiome as a resource in the forensics toolkit." Forensic Sci Int Genet **30**: 141-147.

Cordeiro, C., L. Ordóñez-Mayán, E. Lendoiro, M. Febrero-Bande, D. N. Vieira and J. I. Muñoz-Barús (2019). "A reliable method for estimating the postmortem interval from the biochemistry of the vitreous humor, temperature and body weight." Forensic Sci Int **295**: 157-168.

Javan, G. T., S. J. Finley, Z. Abidin and J. G. Mulle (2016). "The Thanatomicrobiome: A Missing Piece of the Microbial Puzzle of Death." Front Microbiol **7**: 225.

Kahalehili, H. M., N. K. Newman, J. M. Pennington, S. K. Kolluri, N. I. Kerkvliet, N. Shulzhenko, A. Morgun and A. K. Ehrlich (2020). "Dietary Indole-3-Carbinol Activates AhR in the Gut, Alters Th17-Microbe Interactions, and Exacerbates Insulitis in NOD Mice." Front Immunol **11**: 606441.

Kaszubinski, S. F., J. L. Pechal, C. J. Schmidt, H. R. Jordan, M. E. Benbow and M. H. Meek (2020). "Evaluating Bioinformatic Pipeline Performance for Forensic Microbiome Analysis." J Forensic Sci **65**(2): 513-525.

Koenig, J. E., A. Spor, N. Scalfone, A. D. Fricker, J. Stombaugh, R. Knight, L. T. Angenent and R. E. Ley (2011). "Succession of microbial consortia in the developing infant gut microbiome." Proc Natl Acad Sci U S A **108 Suppl 1**: 4578-4585.

Madea, B. (2016). "Methods for determining time of death." Forensic Sci Med Pathol **12**(4): 451-485.

Megyesi, M. S., S. P. Nawrocki and N. H. Haskell (2005). "Using accumulated degree-days to estimate the postmortem interval from decomposed human remains." J Forensic Sci **50**(3): 618-626.

Metcalf, J. L., L. Wegener Parfrey, A. Gonzalez, C. L. Lauber, D. Knights, G. Ackermann, G. C. Humphrey, M. J. Gebert, W. Van Treuren, D. Berg-Lyons, K. Keepers, Y. Guo, J. Bullard, N. Fierer, D. O. Carter and R. Knight (2013). "A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system." Elife **2**: e01104.

Metcalf, J. L., Z. Z. Xu, S. Weiss, S. Lax, W. Van Treuren, E. R. Hyde, S. J. Song, A. Amir, P. Larsen, N. Sangwan, D. Haarmann, G. C. Humphrey, G. Ackermann, L. R. Thompson, C.

Lauber, A. Bibat, C. Nicholas, M. J. Gebert, J. F. Petrosino, S. C. Reed, J. A. Gilbert, A. M. Lynne, S. R. Bucheli, D. O. Carter and R. Knight (2016). "Microbial community assembly and metabolic function during mammalian corpse decomposition." Science **351**(6269): 158-162.

Nelson, E. L. (2000). "Estimation of short-term postmortem interval utilizing core body temperature: a new algorithm." Forensic Sci Int **109**(1): 31-38.

Oliveira, M. and A. Amorim (2018). "Microbial forensics: new breakthroughs and future prospects." Appl Microbiol Biotechnol **102**(24): 10377-10391.

Pechal, J. L., T. L. Crippen, M. E. Benbow, A. M. Tarone, S. Dowd and J. K. Tomberlin (2014). "The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing." Int J Legal Med **128**(1): 193-205.

Pechal, J. L., C. J. Schmidt, H. R. Jordan and M. E. Benbow (2018). "A large-scale survey of the postmortem human microbiome, and its potential to provide insight into the living health condition." Sci Rep **8**(1): 5724.

Pittner, S., V. Bugelli, K. Weitgasser, A. Zissler, S. Sanit, L. Lutz, F. Monticelli, C. P. Campobasso, P. Steinbacher and J. Amendt (2020). "A field study to evaluate PMI estimation methods for advanced decomposition stages." Int J Legal Med **134**(4): 1361-1373.

Robinson, J. M., Z. Pasternak, C. E. Mason and E. Elhaik (2020). "Forensic Applications of Microbiomics: A Review." Front Microbiol **11**: 608101.

Ursell, L. K., J. L. Metcalf, L. W. Parfrey and R. Knight (2012). "Defining the human microbiome." Nutr Rev **70 Suppl 1**: S38-44.

Wescott, D. J. (2018). "Recent advances in forensic anthropology: decomposition research." Forensic Sci Res **3**(4): 327-342.

Willis, A. D. (2019). "Rarefaction, Alpha Diversity, and Statistics." Front Microbiol **10**: 2407.
Zhou, W. and Y. Bian (2018). "Thanatomicrobiome composition profiling as a tool for forensic investigation." Forensic Sci Res **3**(2): 105-110.

Henssge, C., Madea, B.(2007). Estimation of the time of death. *Forensic Science International* 165 (**2-3)**: 182-184.

Almulhim, A.M., Menezes, R.G. Evaluation of Postmortem Changes. [Updated 2021 May 7]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK554464/

Nafte, M. *Flesh and Bone: An Introduction to Forensic Anthropology, 2nd Edition.* (2009).

Paul C. Giannelli, Edward J. Imwinkelried, Andrea Roth, Jane Campbell Moriarty & Valena Beety, Scientific Evidence § 19.08 (6th ed. 2020).