Title

Above or Below: Examining the Contribution of Host and Environmental Microbes in the Decomposition Process

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Above or Below: Examining the Contribution of Host and Environmental Microbes in the Decomposition Process

By

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Abstract

Understanding human decomposition has the potential to significantly improve estimations of post-mortem intervals (PMI), a key component of forensic investigations (1). The post-mortem interval is one of the most challenging pieces of evidence to obtain in the field of forensic science. In recent years, a novel approach for PMI calculations has emerged through the use of a microbial clock, an estimation tool based on microbial community data (2). This method, based on predictable patterns in microbial community progression, demonstrates that microbes provide an accurate clock starting at death and relies on the ecological changes in the microbial communities in the body and surrounding environment. However, there is not much known about how interactions between soil and the host microbiome influence PMI estimation. 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 and soil contents were collected over the course of a 21-day decomposition period and bacterial communities were identified by 16S sequencing. We found that GF mice remained sterile over the study period, regardless of soil sterility. In contrast, soil sterility did have an impact on microbial community dynamics in SPF mice. The data demonstrates that microbial communities at the time of death influence the entry of environmental microbes and microbial progression. Together, these results suggest 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

I. PMI/Decomposition

Forensic science is concerned with identifying and interpreting physical evidence. Common and reliable forms of physical evidence include fingerprints, bloodstains, hairs, fibers, soils, and DNA (2). However, physical evidence can take many forms as long as it provides reliable insight into the activities associated with the crime or scene of death (2). Physical evidence is critical to a criminal investigation since testimonial evidence, the statements provided by victims, suspects, and witnesses, is often inaccurate or subject to bias (2).

The post-mortem interval, describing the amount of time between death and corpse discovery, is a form of physical evidence that is very difficult to determine. The PMI offers vital information within death investigations. It can aid in the identification of the deceased, cause and manner of death, and the possible validation of witness statements and suspect alibis. However, due to multiple variables involved in corpse decomposition, PMI is difficult to establish and remains a great challenge for forensic teams as the current methods have a range of shortcomings and are prone to errors (3). Common methods for estimating PMI include assessment of body core temperature, assessment of morphological changes during decomposition (by total body score), and forensic entomology. However, these methods may only be useful in cases with a short PMI or under specific circumstances (e.g. whether or not insects are present) (4). More recent emerging methods for estimating PMI include chemical methods that can account for factors such as body weight and ambient temperature (5) and microbial methods that incorporate an ecological perspective.

PMI also proves to be difficult to establish due to the relatively poor understanding of corpse decomposition, a continuous process influenced by multiple factors. Decomposition takes place in three overall stages – (1) fresh stage, (2) active decay stage (bloating and rupture occur), and (3) advanced decay (dry/skeletonization then occurs) (2). 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. Bloat or putrefaction is characterized by the production of gases such as sulfur dioxide, ammonia, and methane that result in a swollen or bloated appearance of the body (5). This is often accompanied with the odor of decaying flesh and light skin discoloration as a result of sulfur-containing compounds released by bacteria. Advanced decay occurs when most of the soft tissue has been processed by bacterial and/or insect activity. The cadaver begins to dry out and a strong odor from butyric acid is present. The dry/skeletonization stage is reached when only bone and potentially hair remain. Microbial communities within a corpse influence those in the soil when a corpse has ruptured by releasing fluids rich with ammonia into the soil causing significant effects on the concentration of nitrogen and the pH of soil (2). This abundance of nutrients and changes to soil chemistry initiate a clear succession of soil microbial organisms (6).

Environmental conditions also play an important role in the variability seen across estimated post-mortem intervals because season and temperature can greatly affect the rate of the decomposition process. Dry and hot environments tend to speed up the process, while wet and cold environments tend to slow down the process. A combination of the available methods best fit to the specific given circumstances may give us a better understanding of corpse decomposition and arrive at the most accurate estimation possible.

II. 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 post-mortem period (<30 days) (7). It is one of the methods most closely linked to the decomposition process, as the physical changes to a cadaver attract insects to feed on the decaying flesh. Identifying insects present at the time of discovery and the succession of these insects can help in estimating PMI particularly for bodies or corpses found in outdoor settings (8). 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 any changes in body position and potentially the cause of death. With several limitations, this method has an error rate that varies from days to months (2). Some limitations of this method include lack of insects during certain seasons and region-specific insect communities. Although there are several factors that may affect the decomposition process, the general stages remain the same and biotic signatures associated with each stage such as the development rate of blow fly larvae can help in estimating PMI (9). Furthermore, understanding the stages of decomposition may also provide information on the position of the body in the hours after death by looking at lividity or discoloration patterns on the body due to pooling of blood.

III. Succession of Microbial Communities

Given the limitations of current PMI estimation methods, the succession of microbes and their utility in estimating PMI has garnered great interest in the forensic community (10). Although comparisons of the gut microbiome and soil microbiome have been studied, there is not much known information on how microbes in the soil are affected by the host microbiome. Metcalf et

al. have used high-throughput sequencing to study the bacteria in dead and decomposing mice, and also in the soil beneath them, over the course of 48 days. Using 16S sequencing, forensic scientists have been able to identify PMIs by following microbial community progression. The changes were significant and consistent across the corpses, with the microbial communities in the corpses influencing those in the soil, and vice versa (6). Metcalf et al. also showed that these measurements could be useful for post-mortem interval estimation within approximately 3 days, which suggests that the work could have applications in forensic science.

Although a universal method of estimating PMI ceases to exist, microbial studies combined with innovative techniques associated with Next-Generation Sequencing can offer an improved approach to the estimation of post-mortem intervals. Using microbial community change to track the progression of decomposition may circumvent many of these limitations because microbes are ubiquitous in the environment, located on humans before death, and can be reliably quantified using high-throughput DNA sequencing (6).

IV. The Internal and External Microbiome

Our bodies—especially skin, saliva, the lining of the mouth, and gastrointestinal tract—are home to a diverse collection of bacteria and other microorganisms called the microbiome (6). The human microbiome is a diverse aggregate of all microbiota that exists on and within a human being. Within this collection are distinct microbial communities that form across different anatomical sites and the phylogenetic diversity of these microbial communities increase significantly and linearly from the time of birth (11). The microbiome interacts with many other microorganisms and its composition may shift dramatically depending on a person's diet, use of drugs and antibiotics, and any current illnesses (12). Each individual has their own

unique set of microbiota that can provide information regarding an individual's DNA fingerprint, general health, and behavioral patterns (13). The post-mortem microbiome consists of two components: the thanatomicrobiome and the epinecrotic microbial community (14). 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 (15). The post-mortem microbiome is 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.

The environmental microbiome acts the same as the human microbiome in that it encounters thousands of 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 helpful in determining the origin of an item from a crime scene (16). Collectively, analyzing these microbes can aid in providing information about potential suspects, cause of death, time of death and location of death (14). This perspective focuses on how environmental microbes and the progression of the microbial community contribute and/or drive decomposition. Most microbial community studies have focused on live animals, so little is known about what happens to the microbiome after its host dies. The microbiology of corpse decomposition can be investigated in detail by utilizing sequencing advances that enable entire communities to be characterized across the timeline of decomposition. This data will not only allow us to understand the underlying microbial ecology of corpse decomposition, but also the feasibility of using microbes as evidence (6). Together, the data found allowed us to: (i) determine and understand the relationship between soil and the host microbiome, (ii) identify and

assess the different communities derived from the soil microbiome that are involved in host decomposition, and (iii) test whether changes in microbial communities are predictable over the timeline of decomposition, which is crucial for assessing whether microbes can be used as a 'clock' to estimate PMI.

Materials and Methods

Experimental Design and Sample Collection

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 (nonsterile). Mice (non-obese diabetic wildtype, NOD WT) ranged in age from 5-7 weeks and were grouped randomly. Mice were humanely sacrificed using carbon dioxide gas and allowed to decompose in soil graves. Mice were placed on their right side on top of approximately 100 g of soil in autoclaved pipette tip boxes that were then placed in a HEPA filtered containers inside a fume hood. Destructive host sampling was performed on days 1, 7, 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. Photos of the mice were taken before dissection, after opening the skin layer, and after laying out intestines. Intestinal contents were scraped out and collected in sterile 1.5 ml Eppendorf tubes.

DNA Extraction

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

PCR Amplification and 16s rRNA Gene Sequencing

For 16s rRNA gene sequencing, total genomic DNA was subjected to PCR amplification targeting the 16s rRNA hypervariable region 4 (V4) using bacterial primer set NOD_AhR. The primers 319F and 806R were used to amplify the V3-V4 domain of the 16S rRNA using a two-step PCR procedure. In step one of the amplification procedure, both forward and reverse primers contained an Illumina tag sequence and 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 target sequence (underlined). In step two, each sample was barcoded with a unique forward and reverse barcode combination 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 using the Qubit Broad Range DNA kit (Invitrogen) and individual amplicons were pooled in equal concentrations. The pooled library was cleaned 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 bioinformatics pipeline (v. 2018.8.0) was used to demultiplex and quality filter

the forward-end fastq files. Denoising was performed using DADA2 (17). The raw data can be accessed at NCBI Sequence Read Archive (SRA) (Accession #PRJNA679964).

Visual Scoring by Total Body Score

Photographs of mice were scored in a randomized order and scores from three reviewers were averaged and plotted using GraphPad Prism. Visual body scores were recorded for each carcass using the following key adapted from Megyesi et al. (2005) (18) and Metcalf et al. (2013) (2): 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); Advanced Decay: drying (5 points), sagging or sinking of flesh (6 points), caving in of flesh (7 points), mummification (8 points).

Statistical Analyses

Statistical analyses were performed 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. $P < 0.05$ was considered statistically significant. All plotted data points represent an individual mouse.

Results

Decomposition score is not predominantly driven by the host and soil microbiomes.

GF and SPF mice buried on sterile or nonsterile soil (depicted in Figure 1A) to determine the dynamics between internal and external microbes and their contributions in the decomposition process. Soil and intestinal content samples were collected on days 1, 7, and 21 (Figure 1B). To assess the progression of decomposition, we used the Megyesi visual key to assign a score based on physical changes seen across the stages of decomposition. Mice buried under different conditions generally followed a similar pattern, with a large jump in score around days 7-14 and then plateauing between day 14 and day 21 (Figure 1C). Consistent with the previous findings of Metcalf et al., mice progressed through the major stages of decomposition up until the active decay stage, but not the advanced decay process (2).

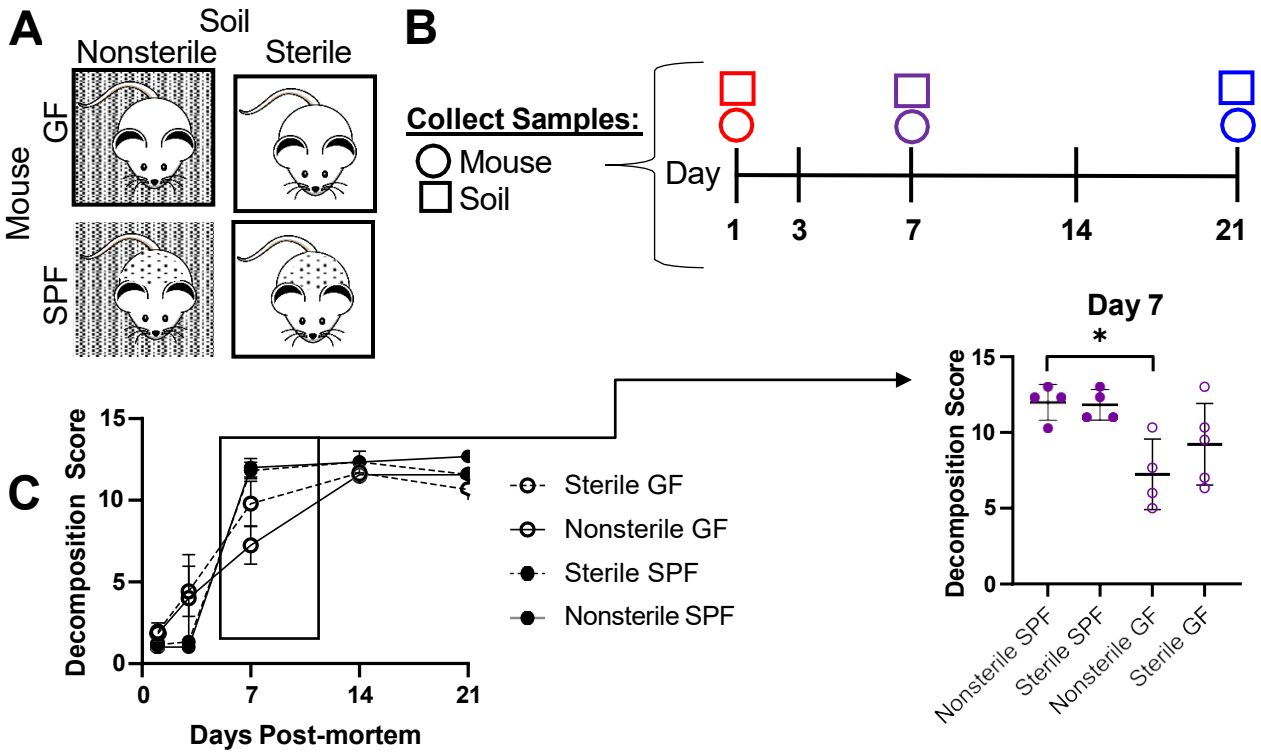

Figure 1. Decomposition score is not predominantly driven by the microbiome. (A) Schematic showing the four different conditions used in this experiment. Two types of soil: sterile (autoclaved) and nonsterile and two types of mice: GF and SPF. (B) Timeline of sample collection time points for both soil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

GF mice and sterile soil remained bacteria-free during the decomposition process.

To determine if the interaction of the soil and host microbial communities influenced bacteria abundance, DNA was measured from each sample on days 1, 7 and 21. Quantifiable DNA was isolated from SPF mice and nonsterile soil, while samples collected from GF mice on days 7 and 21 and sterile soil had DNA concentrations below the limit of detection. On day 1, DNA was isolated from the gut of GF mice, although this was likely of mammalian origin. To differentiate between bacterial and nonbacterial DNA, we conducted qPCR using universal bacteria primers. We found that the gut of the GF mice remained sterile throughout the decomposition process with no amplification of bacterial DNA by qPCR (Figure 2A). Likewise, sterile soil remained sterile throughout the decomposition process, regardless of the presence of SPF or GF mice (Figure 2B). In SPF mice, bacterial DNA increased over time, consistent with previous reports that bacteria increase in abundance over the decomposition process (19). Conversely, bacterial DNA abundance in the soil remained relatively stable throughout the decomposition process.

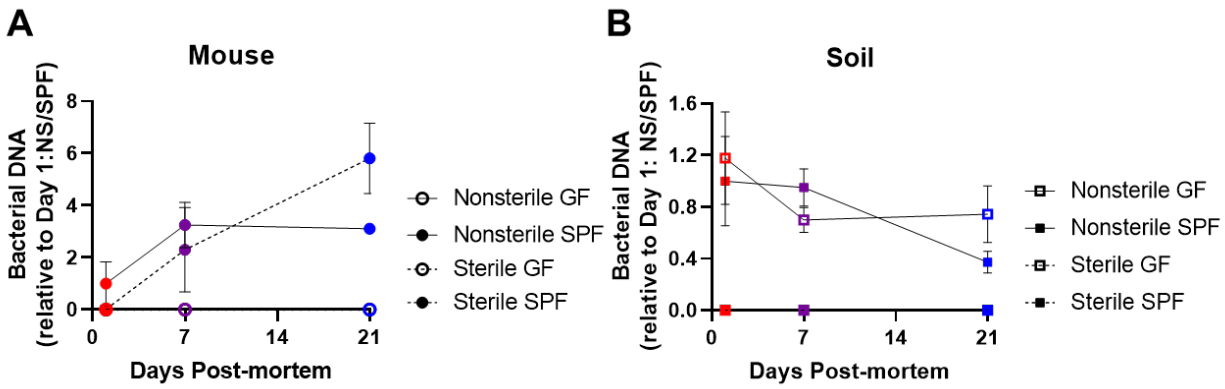

Figure 2. The gut of GF mice and autoclaved soil remain sterile during decomposition regardless of the presence of microbes in soil or mice, respectively. (A-B) Bacterial DNA was measured by qPCR and normalized to samples from GF mice on nonsterile soil from Day 1 (A) or samples from nonsterile soil under SPF mice on Day 1 (B).

Microbial diversity changes throughout decomposition and is dependent on soil microbes.

To identify any time-dependent patterns in microbial diversity in samples from SPF mice and non-sterile soil, we performed 16S sequencing and used QIIME Emperor Ordination to assess variance (Figure 3A-C). Variation in soil diversity was represented across axis 3, whereas variation in mouse diversity was represented by axis 4. While there was a trend toward increased diversity across axis 3 from soil samples under SPF mice, compared to GF mice, the data was not statistically significant (Figure 3B). Furthermore, there was a difference in variance between samples from SPF mice buried on sterile soil compared to non-sterile soil, as well as a difference between Day 7 and 21 compared to Day 1 (Figure 3C).

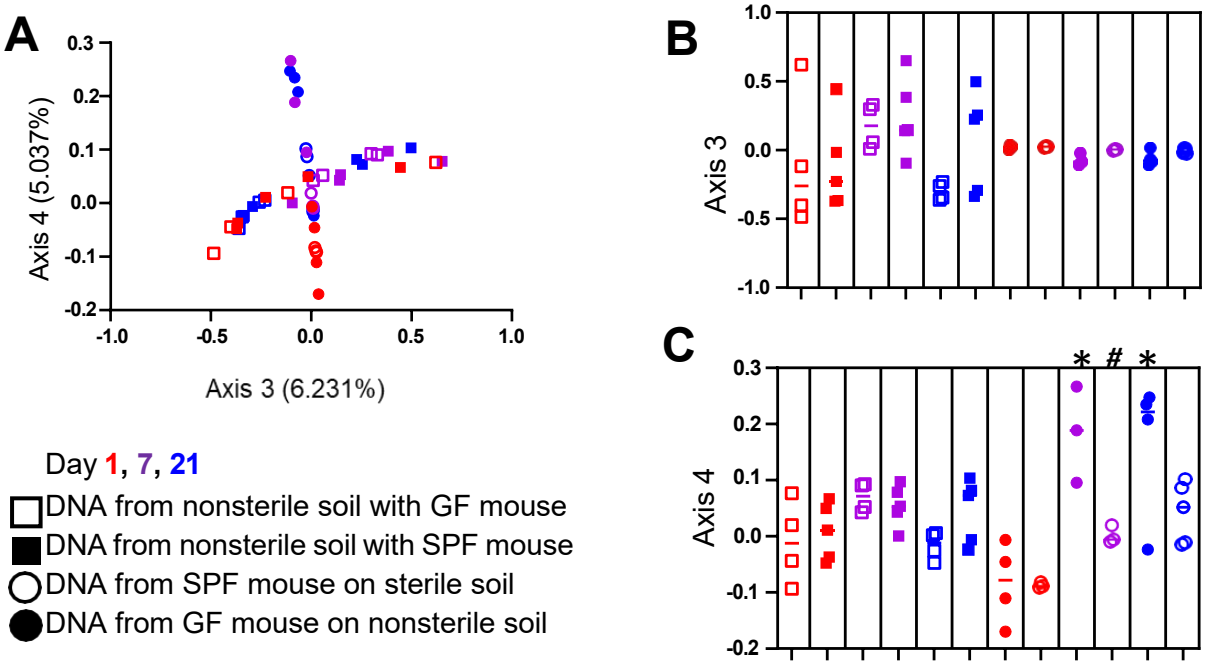

Figure 3. Microbial diversity changes throughout decomposition and is dependent on soil microbes. (A-C) Principal component analysis plots based on Bray-Curtis distance displaying microbial community change over time. The asterisk represents the significant difference compared to Day 1 under the same condition. The pound represents a significant difference between sterile and nonsterile at the same time point. Each symbol represents a single mouse. Samples from SPF mice buried on sterile soil are represented with an open circle while GF mice buried on nonsterile soil are represented by a solid circle.

Diversity in mouse intestinal contents decreases during decomposition.

In this study, the Shannon Diversity Index operated as a statistical representation of biodiversity in which both the species richness and the abundance evenness are considered. Shannon Diversity within the mouse intestinal contents decreased between Day 1 and Day 7, as well as Day 1 and Day 21, although this did not reach statistical significance. Likewise, there was a trend toward decreased diversity in mice buried on non-sterile soil compared to mice buried on sterile soil on Days 7 and 21. Shannon Diversity within the soil remained constant through the duration of the study, Day 1 – Day 21.

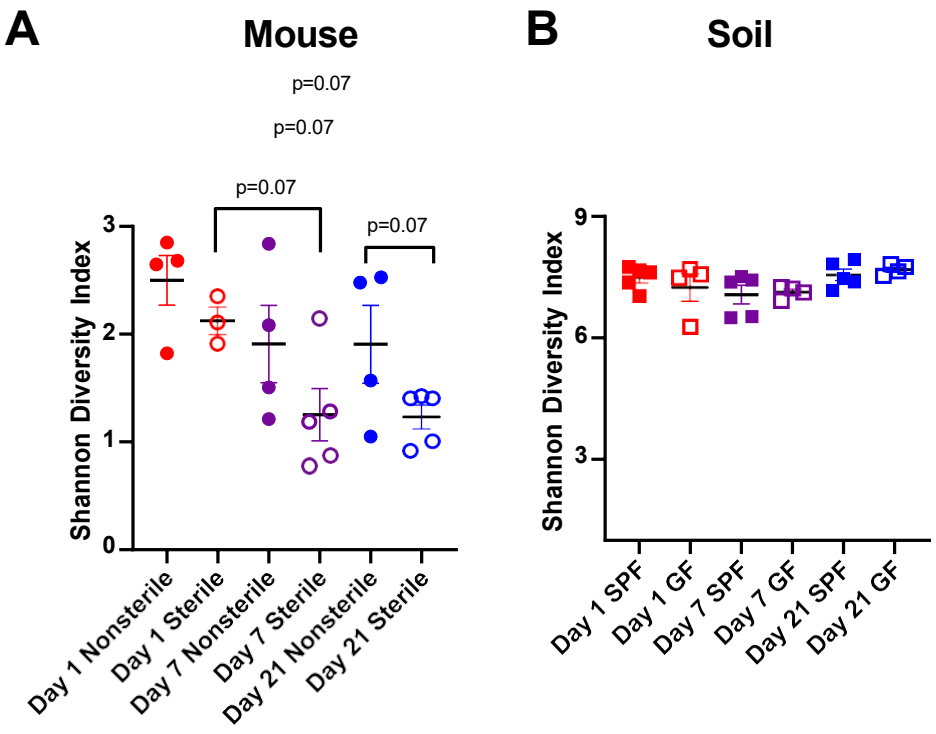

Figure 4. Shannon Diversity in mouse intestinal contents decreases during decomposition. (A) Shannon Diversity within the mouse intestinal contents significantly decreased between Day 1 and Day 7, as well as Day 1 and Day 21. (B) Shannon Diversity within the soil remained constant during the duration of the time points.

Actinobacteriota and Firmicutes are the primary decomposers in mice decomposed on nonsterile soil.

To determine any potential primary decomposers in the microbial communities, we sequenced 16S rRNA amplicons from the collected samples. To identify any differences between the soil conditions, we sorted the data by different taxonomic levels. We found that sorting by phylum displayed the most distinct differences. Increased abundance and diversity of microbes were more evident in mice decomposed on nonsterile soil compared to the sterile soil (Figure 5). In order to pinpoint the key decomposers, major phylum that significantly changed in abundance over time were identified. Actinobacteriota and Firmicutes increased in abundance over time, while the other four phyla decreased significantly over time (Figures 5). Actinobacteriota displays a decrease in abundance in the early stages of decomposition and then increases from Day 7 to Day 21, which is characterized as the bloat and rupture stage. Firmicutes shows a drastic increase in abundance on both sterile and nonsterile soil from Day 1 to Day 21.

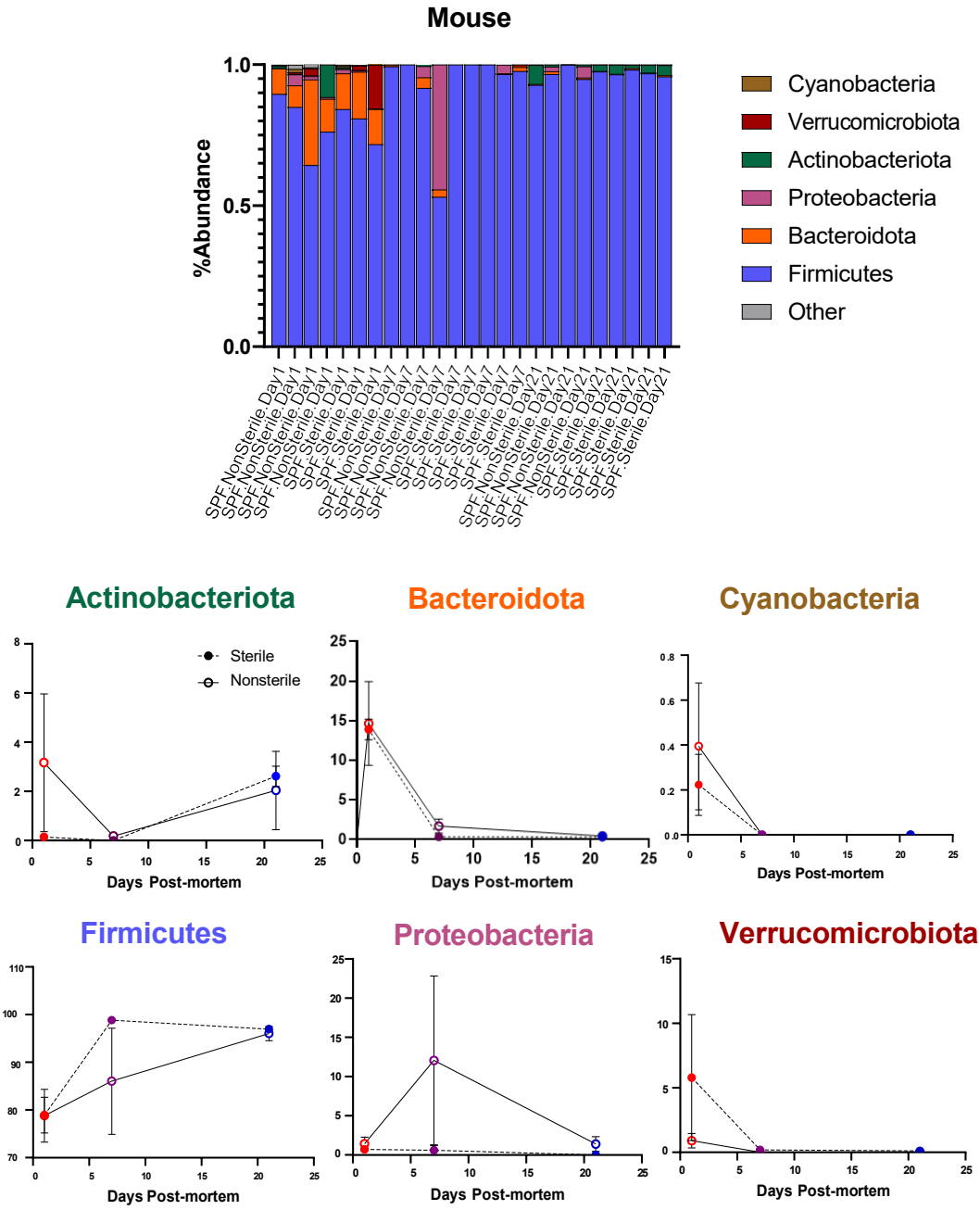

Figure 5: Actinobacteriota and Firmicutes are the primary decomposers in mice decomposed on nonsterile soil. Relative abundance of six major phylum groups show increased abundance and diversity within the samples using nonsterile soil. Data separated by phylum show that Actinobacteriota and Firmicutes are the primary decomposers.

Actinobacteriota and Proteobacteria are the most abundant in nonsterile soil.

To identify any distinct differences between microbes in the soil underneath GF and SPF mice, we sorted the data by different taxonomic levels. Within the soil, Actinobacteriota and Proteobacteria made up the majority of the bacteria, although all phyla remained relatively stable over time (Figure 6). Although Actinobacteriota displays a decrease in abundance from Day 7 to Day 21, it remained the most abundant of the six major phyla (Figure 6). Proteobacteria maintained increased abundance in both SPF and GF mice from Day 7 to Day 21 (Figure 6). In general, soil microbes remained consistent and were not influenced by the presence of a mouse corpse, regardless of bacterial colonization status.

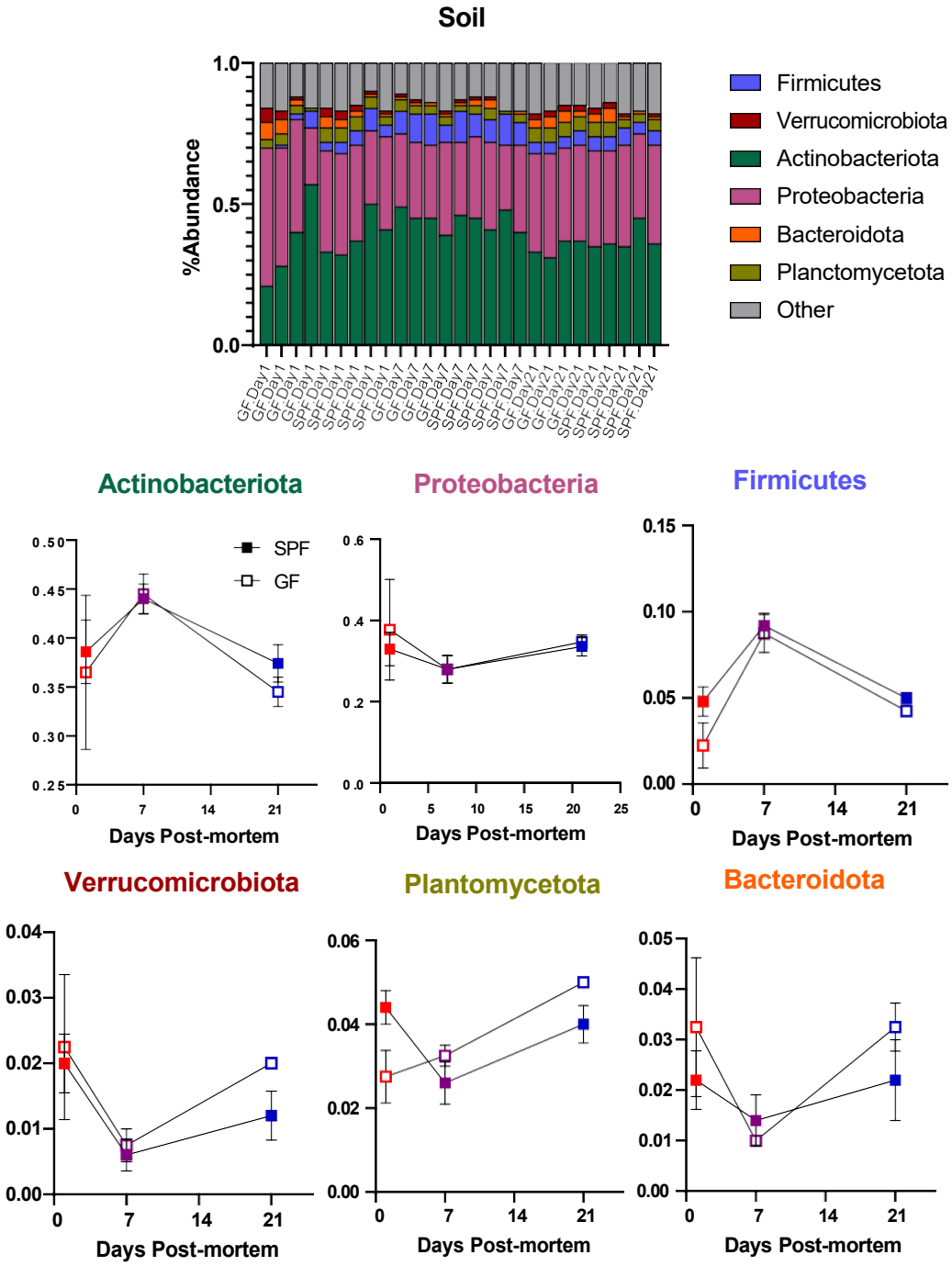

Figure 6. Actinobacteriota and Proteobacteria are the most abundant in nonsterile soil. Relative abundance of six major phylum groups show increased abundance and diversity within the samples on nonsterile soil. Data separated by phylum show that Actinobacteriota and Proteobacteria are the most abundant.

Microbes from the soil contribute to mouse diversity over time and vice versa.

In 16s analysis, reads are clustered with a 97% sequence identity to obtain taxonomically different groups called operational taxonomic units (OTUs), which correspond to the bacterial species (19). The number of observed OTUs represents the community's species richness (also known as α diversity) and the number of 16S reads approximates the abundance of the OTU (19). A Euler diagram (20) was created to assess the changes at the number of operational taxonomic groups in order to determine if microbes from the soil contribute to mouse diversity over time (Figure 7). For nonsterile microbial communities, alpha diversity increased at each time point between Day 1 and Day 21 (Fresh to Advanced Decay stages). For SPF mice, alpha diversity decreased across the intestinal sample site between Day 1 and Day 21 (Fresh to Advanced Decay stages).

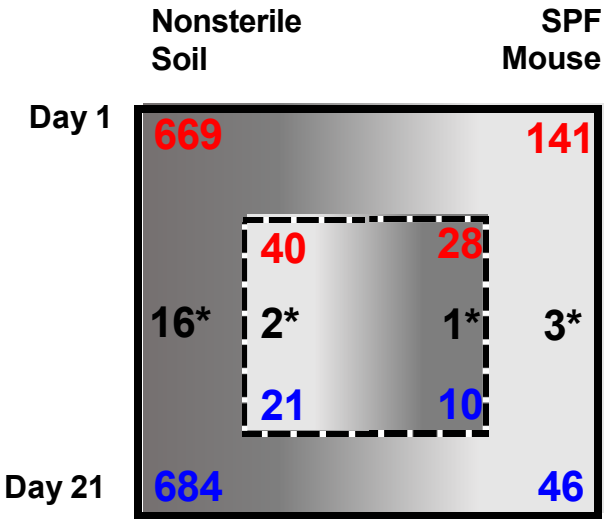

Figure 7. Microbes from the soil contribute to mouse diversity over time and vice versa. Alpha diversity decreases in the intestinal content of the mouse over time. Alpha diversity increased at each time point for nonsterile microbial communities, contributing to mouse diversity over time. The dashed inset shows OTUs that are found in both mouse and soil. Numbers in red represent Day 1 samples. Numbers in blue represent Day 21 samples. Asterisk represents significant difference.

Discussion

Improving the methodology for estimating PMI is of great importance, as PMI is one of the most challenging pieces of evidence to obtain in the field of forensic science. In recent years, tracking microbial community progression has proven to be successful in accurately estimating PMI within 3 days. This method, by Metcalf et al., has been found to be predictable and reproducible. However, studies focusing on how a compromised host microbiome might affect this predictable pattern have yet to be done. This study 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 alter or contribute to the known 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. 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 also too low to generate amplicon targets during qPCR sequencing. On the other hand, the gut microbes in SPF mice were able to show a time course progression across the timeline of the experiment, with a progressive separation between sterile and nonsterile soil conditions.

The primary decomposers were predominated by Actinobacteriota, Firmicutes, and Proteobacteria phyla. These decomposers differed from previous studies where Metcalf et al. identified

Alphaproteobacteria (mostly Rhizobiales) as the most abundant in post-rupture stage soil samples (2). Aside from half of the mice being GF, a few differences between our experimental designs may explain the discrepancy. Metcalf et al. used the C57BL6 mouse strain as compared to the NOD WT strain we used. In this experiment, basic potting soil was used whereas Metcalf et al. used soil collected from the organic layer of a dry creek bed in Eldorado Creek near Boulder, CO. That soil is expected to have a higher variety of microbes, including fungi and nematodes. Additionally, small holes were drilled into the sides of their plastic Tupperware-like containers where their mice were left to decompose, to prevent anaerobic conditions. We did not control for humidity levels or anaerobic conditions. Maintaining moisture levels may have resulted in more microbial activity and potentially, the carcasses may have moved further along in the decomposition process (8).

It has been noted in previous studies that the soil tends to be more informative than the skin or abdomen in terms of displaying the predictable community progression. The interaction between the host and soil microbiome was explored through identifying and assessing the different communities derived from the soil and host microbiome that are involved in host decomposition. The data suggests that the presence of the host microbiome is critical in initiating the decomposition process, especially the early stages where bloating, purging, and rupture occur. During the bloating stage (~6-9 days), endogenous and facultative anaerobes such as Firmicutes and Bacteriodetes increase in the abdominal cavity. After rupture (~9 days), those decrease and there is a strong shift from anaerobic to aerobic bacteria (Alphaproteobacteria) (2).

With mice that are germ free, no predictable microbial progression pattern could be identified, despite microbes being present in nonsterile soil conditions.

There were some limitations to this study that could have impacted our results and interpretations. Only the intestinal contents of the mouse along with the soil beneath each carcass were sampled. Additional sampling sites, including swabbing of the torso and head surface that was in direct contact with the soil, might result in more informative data. Another limitation was the length of the study – it is likely that including later time points, spanning up to day 48, would have led to stronger patterns and correlations.

Despite several limitations, new information has been provided by this study. It has been shown that if the host microbiome is compromised, the chances of identifying the predictable microbial progression pattern seen in previous studies, are slim. Therefore, the microbial progression associated with decomposition is significantly influenced by the host microbiome. Future studies would need to focus on how other factors that alter the microbiome such as the use of antibiotics or drugs and other immunodeficiencies or health conditions would affect the pattern as well. A time course progression in SPF mice across sterile and nonsterile soil conditions was also identified. Over time, the mice that had decomposed on nonsterile soil had an increased abundance and diversity of microbes. However, the most abundant primary decomposers differed from previous studies. Therefore, despite having SPF mice with host microbes present, we were unable to replicate the predictable microbial progression pattern. Moving forward, understanding the relationship between the host and soil microbiome and focusing on ways to refine

current methodology for future PMI estimation models will be of great benefit to the forensic science community.

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