Postmortem succession of gut microbial communities in human cadavers

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The human microbiome has demonstrated importance for health and functioning in living individuals. However the fate of the microbiome after death is poorly understood. In addition to a better understanding of microbe-mediated decomposition processes, postmortem succession of human-associated microbial communities has been suggested as a possible forensic tool for estimating time since death, or postmortem interval (PMI). The objective of our study was to document postmortem changes in human gut bacterial communities. Gut microflora were repeatedly sampled from the caeca of cadavers as they decayed under natural environmental conditions. 16S rRNA gene amplicon sequencing revealed that over time, bacterial richness significantly increased ($r_s = 0.449$) while diversity decreased ($r_s = -0.701$). The composition of gut bacterial communities changed in a similar manner over time towards a common decay community. OTUs belonging to Bacteroidales (Bacteroides, Parabacteroides) significantly declined while Clostridiales (Clostridium, Anaerosphaera) and the fly-associated Gammaproteobacteria Ignatzschineria and Wohlfahrtiimonas increased. A best fit multiple regression model, which included five OTUs, improved the ability to predict PMI ($R^2 = 0.824; p < 0.001$). Our examination of human caeca microflora in decomposing cadavers adds to the growing literature on postmortem microbial communities, which will ultimately contribute to a better understanding of decomposition processes.
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Abstract (200 words)

The human microbiome has demonstrated importance for health and functioning in living individuals. However the fate of the microbiome after death is poorly understood. In addition to a better understanding of microbe-mediated decomposition processes, postmortem succession of human-associated microbial communities has been suggested as a possible forensic tool for estimating time since death, or postmortem interval (PMI). The objective of our study was to document postmortem changes in human gut bacterial communities. Gut microflora were repeatedly sampled from the caeca of cadavers as they decayed under natural environmental conditions. 16S rRNA gene amplicon sequencing revealed that over time, bacterial richness significantly increased ($r_s = 0.449$) while diversity decreased ($r_s = -0.701$). The composition of gut bacterial communities changed in a similar manner over time towards a common decay
community. OTUs belonging to Bacteroidales (*Bacteroides, Parabacteroides*) significantly declined while Clostridiales (*Clostridium, Anaerosphaera*) and the fly-associated Gammaproteobacteria *Ignatzschineria* and *Wohlfahrtiimonas* increased. A best fit multiple regression model, which included five OTUs, improved the ability to predict PMI ($R^2 = 0.824; p < 0.001$). Our examination of human caeca microflora in decomposing cadavers adds to the growing literature on postmortem microbial communities, which will ultimately contribute to a better understanding of decomposition processes.

**Introduction**

Decomposition of human or vertebrate mortalities is driven by microbial activity. Following death, a lack of oxygen in the body results in cell autolysis, releasing macromolecules. The body’s resident microbes, particularly those concentrated in the GI tract, metabolize these cellular products in the process of putrefaction. The fermentative activities of these microbes cause bloating as gasses build up inside the cadaver or carcass, and ultimately liquefaction of tissues. While it is well accepted the human microbiome plays an important role in tissue decomposition, the composition and dynamics of these microbial communities postmortem have been poorly documented.

Understanding the processes and dynamics of decomposition has application in forensic science, particularly with respect to developing robust estimates of time since death, or postmortem interval (PMI). All methods have limitations, thus using a combination of approaches typically provides the best estimates of PMI (1). Therefore, there is an ongoing need to develop and validate new PMI estimation methods. There has been recent interest in the use of microbial
communities in the decomposition environment as markers of PMI: If the postmortem succession
of microbial communities is repeatable and predictable, then it may be possible to use the
communities as forensic indicators, similar to the approach taken by forensic entomology (2).
Recent studies have begun describing human postmortem microbial communities associated with
a variety of habitats, including skin (3), mouth and rectum (4), ear and nasal canals (5), internal
organs (6, 7), bones (8) and soils below the cadavers (3, 9). Other studies have also reported
postmortem changes associated with decomposing animal carcasses (10-14). Collectively these
studies are beginning to reveal general patterns of microbial succession, which include a shift
towards a higher relative abundance of anaerobic taxa. Attempts to relate taxa abundances or
community patterns to PMI have demonstrated that bacterial community composition may be
able to predict PMI with an accuracy of a few days (3, 5).

Here we expanded on this body of work to include bacterial communities of the proximal large
intestine (caecum); a habitat not previously investigated using high throughput sequencing (to
our knowledge). In a previous study, we sampled gut microflora of the caeca of six cadavers
repeatedly following death, until tissues were too compromised to distinguish (15). Using qPCR,
we found populations of Bacteroides and Lactobacillus declined exponentially as decay
progressed. This suggested that the microbial communities change in structure over time, and led
to the hypothesis that other populations of bacteria may be useful as biomarkers of time since
death. Our previous study was limited to examining only three microbial populations using
targeted qPCR. Therefore, the objective of this study was to characterize and quantify the entire
microbial communities of the human gut following death as decomposition progresses. We
hypothesized the communities would change in structure with time, with a decline in
Bacteroidetes, and increase in more robust taxa such as Clostridia and Proteobacteria. The
changes in intestinal microflora have been examined in mice up to 24h postmortem (12). Here we present the postmortem changes in gut microflora of humans, up to 30 days following death.

Materials and Methods

Cadaver decomposition and sampling. Four cadavers were placed at the University of Tennessee, Knoxville, Anthropology Research Facility (ARF) in the summer of 2011, as part of a larger study described previously (15). The cadavers were donations to the University of Tennessee, Knoxville, Forensic Anthropology Center (FAC) for the W. M. Bass Donated Skeletal Collection (http://web.utk.edu/~fac/collection.html). As no living human subjects were involved, this work was exempt from review by the University of Tennessee Institutional Review Board. No preference was employed for sex, age, ancestry, weight, etc. FAC standard protocol for accepting donors ensured the individuals did not have communicable diseases. The bodies were not autopsied or embalmed; they were immediately refrigerated after death and placed at the ARF within three days. The four cadavers (referred to as A6, B6, C5, D6) ranged in age at death from 62 to 67 years and weights from 56 to 77 kg, and all died of natural causes. A small incision was made in the abdomen and sterile swabs were used to collect gut material from the caecum. The incision was sealed with tape and re-sampled daily until tissues were too decayed to identify. Sampling times were converted to cumulative degree hours (CDH) based on hourly measurements of air temperature from a local meteorological station as previously described (15).

DNA Sequencing and Amplicon Library Analyses. DNA was extracted using a MoBio PowerSoil DNA extraction kit. 16S rRNA gene libraries (V4 region) were built and sequenced
on the Illumina MiSeq platform at the Hudson Alpha Genomic Service Lab (Huntsville, AL)
using primers and conditions described in (16). Sequence QC and analysis was done in Mothur
v.1.37.0 (17) as previously described (9). Briefly, forward and reverse reads were joined; then,
sequences containing ambiguous bases, homopolymers longer than eight nucleotides, and
unreasonable amplicon lengths were removed. Sequences were aligned to the Silva reference
alignment, preclustered, and screened for chimeras using UChime. Sequences that were
taxonomically classified as something other than bacteria were removed. OTUs were determined
based on phylotype; sequences were taxonomically classified against the RDP database (>80%
similarity) and classified by genus. Prior to alpha-diversity calculations, libraries were
subsampled to 25,082 reads per library. Data analysis and statistics were conducted in Mothur,
Primer v6 (18), and R (19). Across all samples, 734 unique OTUs were identified. Raw
sequences were deposited in the NCBI Short Read Archive (BioProject PRJNA369241).

Influence of sampling. The sampling method, which involved introduction of a sterile swab into
the abdominal cavity, undoubtedly introduced some oxygen to the gut, which would have
normally been anaerobic. We attempted to limit this introduction as much as possible and
included several bodies as controls to determine if the sampling had an effect. The method and
controls are discussed in our previous study (15). The six control bodies were only sampled once
at various PMIs, and analyzed along with six test bodies. We found no difference in population
relative abundances between control and test bodies for all but the rare populations (results
provided in (15)). Thus, based on our research questions, we concluded that our sampling
method did not have an influential effect on the dominant gut bacterial populations.

Results
After processing to remove erroneous sequences, 8,756,105 sequences remained in our dataset. The sample with the least number of sequences contained 25,082, so all other samples were randomly subsampled to this size. At 25,082 sequences per sample, mean coverage for all samples was 0.998. Replicate libraries were made for a random selection of four samples to assess technical error. Duplicates were more similar to each other than to other libraries, with Bray Curtis similarities of community composition ranging from 92.06 to 93.52 at the OTU level and 97.55 to 98.77 at the phylum level. Initial screening of the sequence libraries revealed that one cadaver (A6) had very different postmortem gut microbial communities compared to the other three cadavers. Given this individual had been on a feeding tube prior to death, we felt these differences would override any time-based differences we might detect with the other three. Thus, we removed A6 and performed the remaining analyses on the other three cadavers (B6, C5, D6).

Over time, the richness of the bacterial communities increased, while the diversity significantly decreased, indicating a decrease in evenness (Figure 1). The number of OTUs increased with time (Spearman’s rho ($r_s$) = 0.449, $p = 0.003$). In contrast, diversity (Simpson’s Index) showed a significant decrease with time ($r_s = -0.701$, $p < 0.001$). An NMDS analysis of Bray-Curtis similarities revealed that the microbial communities in these bodies changed over time (Figure 2). Over the course of the study, the mean similarities of the communities within each body were low (44.74 to 50.31%). This analysis revealed that while there was considerable variation between cadavers, there was a change along a similar trajectory for all three. A hierarchical group average clustering of communities revealed a distinct shift from ‘early’ communities to ‘late’ communities (Figure 2). This shift happened in the middle of the traditionally defined ‘bloat’ stage (sensu Payne (20)) around the same time for the three cadavers: after days five,
four, and seven for cadavers C5, D6, and B6, respectively. When corrected for environmental temperature differences, these time periods represent cumulative degree hours (CDH) 77-173, 67-91, 155-184, respectively.

The ‘early’ microbial communities had a mean of 135 ± 17 OTUs. The diversity was high, with a mean inverse Simpson’s Index of 12.93 ± 3.91. They had an average Bray-Curtis similarity of 59.42%. Major phyla in these communities were Firmicutes and Bacteroidetes, characteristic of human gut communities (Figure 1). A SIMPER analysis was used to determine which OTUs contributed most to the difference between early and late microbial communities (Figure 3). This analysis revealed early communities had significantly higher abundances of *Bacteroides* and *Parabacteroides* (Phylum: Bacteroidetes), and the Firmicutes *Faecalibacterium*, *Phascolarctobacterium*, *Blautia*, *Lachnospiraceae incertae sedis*.

The ‘late’ microbial communities had a higher richness but lower diversity than the early microbial communities: the late communities had a mean of 186 ± 78 OTUs, which was significantly higher than the early communities (two-tailed T test, t=-3.215, p=0.003). These late microbial communities were also more variable compared to the early microbial communities, with a mean Bray Curtis similarity of 49.45%. The inverse of the Simpson’s Diversity Index was 6.608 ± 3.78, indicating significantly lower diversity than the early communities (t = 5.232, p < 0.001). At the phylum level, Firmicutes still dominate, but we observed reduced relative abundances of Bacteroidetes (Figure 1). These communities were significantly enriched in OTUs belonging to order Clostridiales within phylum Firmicutes (*Clostridium*, *Peptostreptococcus*, and *Anaerosphaera*), and Gammaproteobacteria OTUs (*Wohlfahrtiimonas*, *Ignatzschineria*, *Acinetobacter* and *Providencia*) (Figure 3).
While there was a clear change in the postmortem gut microbial community with time, there were some differences between the three cadavers. For example, we observed increases in Proteobacteria in two of the cadavers, and an increase in Synergistetes in the third (Figure 1). At the OTU level, we observed some secondary clustering within the ‘late’ cluster: the later C5 and B6 samples clustered together while D6 had slightly different community structures, largely due to the proliferation of Proteobacteria and increase in Ignatzschineria (Figure 4C).

To determine the utility of using individual OTUs as a predictor of time since death, or postmortem interval (PMI), we examined the relationships between OTUs and CDH. Table 1 lists OTUs with a significant correlation to CDH, positive or negative. The strongest individual predictors of PMI were two Bacteroidales OTUs (Figure 4A) which declined with time and two Clostridiales OTUs that increased over time (Figure 4B). Multiple regression using all possible combinations of the top 30 most abundant OTUs was used to determine the combination of OTUs that would best predict time since death. We found the OTUs consistently included in the best models were OTUs 004 (Bacteroides), 012 (Faecalibacterium), 013 (Cloacibacillus), 015 (Wohlfahrtimonas), and 016 (Clostridium). The multiple regression using these five OTUs as predictors yields a $R^2 = 0.824$ ($p < 0.001$) (Model: $CDH=-50.1-57.0OTU004-49.5OTU012-52.7OTU013-61.0OTU015+74.4OTU016$). Inclusion of other OTUs in the model improves the fit only slightly: the best models using eight OTUs as predictors yield an $R^2 = 0.880$.

**Discussion**

In this study we documented the shifts in human gut microflora postmortem, as cadavers decayed in an outdoor environment. To our knowledge, this was the first examination of human
gut microbial communities postmortem focusing on the proximal large intestine (caecum). The bacterial communities in these decomposing cadavers had a distinct compositional shift in the middle of the bloat phase of decomposition, between days four and seven in this study. Prior to this point in decomposition, the communities were relatively diverse and similar between the three cadavers, with bacterial assemblages appearing typical of human gut communities (21). Mid-way through bloat, the communities underwent a distinct shift. The timing of this shift may correspond to a change in the physical environment (e.g. liquefaction of surrounding cells and structures), chemical environment (e.g. buildup of putrefaction byproducts) and/or biological competition (e.g. sensitive members of the human gut flora die off while more resilient members proliferate; and/or invasion of external microbes).

Over time, the bacterial communities exhibited decreased diversity. As richness was not affected, this indicates a reduction of evenness, as the communities become dominated by a few dominant genera. This is typical of either a disturbance response or a bloom event. In a decomposing body, likely both scenarios occurred: the rapidly shifting environmental conditions and production of products of putrefaction likely stressed some members of the community, while others were more tolerant and proliferated on the newly available substrates. It was notable the variation between cadavers increased in the late communities; that is, bacterial communities were more similar between individuals in the beginning. External factors such as temperature, soils, and arthropod communities were relatively similar between the cadavers, as they were all placed in the same environment around the same time. However, internal differences that we could not control for (e.g. body fat content, presence of drugs or drug metabolites, etc.) may have influenced the trajectory of the decomposer communities. It should also be recognized that decomposition, because of its highly dynamic nature, is not entirely deterministic and would be
subject to stochasticity that would result in slightly different trajectories of the bacterial communities between individuals.

Despite the inter-individual variation, we documented several patterns consistent between all three cadavers. The changes from ‘early’ to ‘late’ communities were driven in part by pronounced changes in OTUs classifying as Clostridiales. *Clostridium* spp. are normal members of the human gut microflora, and have been reported as a prominent members of postmortem microbial communities, including both internal organs and external sites (6, 7, 9, 11, 22).

Another Clostridiales, *Anaerosphaera* spp., have been previously isolated from animal waste reactors and identified as aminolytic anaerobes, fermenting amino acids into volatile fatty acids (23), implicating them as members of the putrefactive consortia as well. Mouse model studies examining translocation of bacteria postmortem have demonstrated the migration and proliferation of *Clostridium* and other anaerobic taxa in the internal organs with increasing postmortem intervals (12, 13); an increase in Clostridium in human internal organs postmortem has also been reported (6, 7). Clostridia are known putrefactive organisms, so their observed increase in relative abundance may be due to an increase in vegetative growth, where they gained energy through the fermentation of cellular products. Alternatively, but less likely, their ability to form endospores may have allowed them to withstand the stressful conditions within the decomposing body, while other taxa were reduced in abundance.

The other groups significantly enriched in the late communities included several Gammaproteobacteria. The increase in OTUs classifying as an *Ignatzschineria* and to a lesser extent, *Wohlfahrtiimonas*, was intriguing. *Ignatzschineria* spp. have been identified in flesh flies (Diptera: Sarcophagidae) (24-26) and blow flies (Diptera: Calliphoridae) (27). *Wohlfahrtiimonas* spp. have also been associated with fly larvae (26, 28, 29). Thus, it is likely that flies visiting the
bodies introduced these bacteria. Other studies of postmortem bacterial communities have also
documented the presence of *Ignatzschineria* and *Wohlfahrtiimonas* (4, 9). Their dominance in
the overall bacterial community and significant increase in relative abundance over the
postmortem interval (PMI) suggests they are not just simply introduced, but may have thrived in
the gut communities, implicating them as participants in decomposition.

There has been increasing interest in determining if patterns in postmortem microbial
communities may be useful in a forensic context. To this end, we determined the taxa most
correlated to time since death, or postmortem interval (PMI). These analyses revealed both single
taxa with strong correlations to PMI, as well predictive combinations of taxa using multiple
regression. Single taxa include *Bacteroides* and *Parabacteroides*, which declined over time and
were significantly inversely correlated to PMI. This corroborates our previous study with these
and other cadavers, in which we quantified *Bacteroides* using qPCR and demonstrated a
significant, quantifiable decay relationship in the relative abundances of these organisms with
increasing PMI (15). *Clostridium* was the strongest positive predictor of PMI. A multiple
regression model that included *Bacteroides*, *Clostridium* and three other OTUs
(*Faecalibacterium*, *Cloacibacillus*, and *Wohlfahrtiimonas*), improved the predictive ability. This
supports the use of multiple taxa in to improve PMI estimates, as has been done in other studies
of postmortem microbial communities (3, 5).

Our examination of human gut microflora of the caecum in decomposing cadavers adds to the
growing literature on postmortem microbial communities. In revealing patterns of microbial
succession, this work will ultimately contribute to a better understanding of decomposition
processes and potential tools for postmortem interval estimation.
Acknowledgements

The authors thank all of the donors and their families who make this research possible. Funding for this work came from the William M. Bass Endowment, the University of Tennessee Undergraduate Summer Internship program, the University of Tennessee Microbiology Across Campuses Research and Educational Venture and NSF Award 1549726. We gratefully acknowledge the staff and students of the Forensic Anthropology Center who assisted in sampling; J. Smith, R. Taylor, Y. Jeong, B. Dudzik, and D. Mercer along with K. Cobaugh who assisted with DNA libraries.

References


Table 1. Pearson’s correlation coefficients (r) between log transformed OTU relative abundance and postmortem interval (as cumulative degree hours, CDH) for OTUs that were significantly correlated (α < 0.01) to CDH.

<table>
<thead>
<tr>
<th>OTU #</th>
<th>Phylum</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>r</th>
</tr>
</thead>
<tbody>
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<td>4</td>
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<td>Bacteroidaceae</td>
<td>Bacteroides</td>
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</tr>
<tr>
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<td></td>
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<td>Parabacteroides</td>
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</tr>
<tr>
<td>16</td>
<td></td>
<td>Clostridia</td>
<td>Clostridiaceae</td>
<td>Clostridium</td>
<td>0.717</td>
</tr>
<tr>
<td>27</td>
<td>Firmicutes</td>
<td>Clostridales</td>
<td>Incertae_Sedis_XI</td>
<td>Anaerosphaera</td>
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</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
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<td>Blautia</td>
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<tr>
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<td>-0.607</td>
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</tr>
<tr>
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<td>Xanthomonadales</td>
<td>Xanthomonadaceae</td>
<td>Ignatzschineria</td>
<td>0.406</td>
</tr>
</tbody>
</table>
Figure 1. Relative abundance of phyla in the bacterial communities as a function of time since death (postmortem interval). Red dashed lines differentiate the ‘early’ from the ‘late’ communities as determined by hierarchical cluster analysis. White diamonds show the community diversity, as estimated by the inverse Simpson’s Index (1/D).
Figure 2. NMDS of Bray Curtis distances revealed the changes in cadaver gut bacterial communities over time. Symbols represent the three cadavers: B6 (diamonds), C5 (triangles), D6 (squares) and numbers refer to the day postmortem. Overlay shows the results of hierarchical group average clustering: red lines indicate 37% similarity which divided the communities into ‘early’ and ‘late’ groups; yellow lines show samples with 50% similarity.
Figure 3. Relative abundances (square root transformed) of OTUs that contributed the most to the differences between ‘early’ and ‘late’ communities, as identified by SIMPER analysis.
**Figure 4.** Relative abundance of OTUs as a function of time since death (cumulative degree hours). A) Bacteroidales genera *Bacteroides* and *Parabacteroides*; B) Clostridiales genera *Clostridium* and *Anaeropshera*; C) Gammaproteobacteria *Ignatzschineria* for each of the three cadavers showing the inter-individual variability for this taxon.