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THE POST-MORTEM MICROBIOME: A CRITICAL REVIEW OF MICROBIAL SUCCESSION FOR ESTIMATING POST-MORTEM INTERVAL AND ELUCIDATING CAUSE OF DEATH

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ABSTRACT

Advances in sequencing technologies have seen an increase of interest in the field of forensic microbiology as applied to post-mortem interval estimation and the nature of microorganisms that may contribute to cause and manner of death. In the present review, we discuss recent progress and gaps in our knowledge of the microbial succession on and in bodies following death (thanatomicrobiome and epinecrotic microbiome) including their dynamic interactions with environmental microbial communities. The conserved trends over successional development of these communities provide a robust, albeit complex, approach to forensic science that shows potential in furthering post-mortem interval estimates and also perhaps identifying elemental indicators of value for forensics. Although progress has been made complex confounding factors (e.g. environmental variables and host-specific characteristics) hamper such applications from forensic microbiome analysis with regard to forensic credibility and courtroom feasibility. In this critical review, we summarize current findings and map out the methodological workflows, major applications, main limitations as well as future prospects of merging microbial ecology with forensics. This inter-disciplinary field integrates microbiology, ecology and forensic science toward a goal of revolutionizing estimation of time since death (and other forensic parameters) through identification of new methods that will replace existing ones.

Keywords: Forensic microbiology, Post-mortem interval, Thanato-microbiome, Microbial succession.

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INTRODUCTION

Traditional techniques for estimating the time of death, including algor mortis, livor mortis and rigor mortis don't provide precise time estimation and lose accuracy after 72 h post-mortem, these methods also can be influenced by environmental factors as well as individual anatomical variations. As a result, the scientific community has begun to explore the post-mortem microbiome to provide broader, more reliable intervals for PMI estimation [**1**]. This novel scientific area of forensic microbiomes, focused on successional shifts across microbial taxa- including bacteria, fungi, viruses, and archaea, during cadaver colonization and post- mortem decay. This process can be attributed to an endogenous host microbiota as well as to the exogenous microorganisms from the surrounding environment, which together constitute dynamic microbial ecosystems. The dynamic development of the microbiome through a combination of host-intrinsic determinants and environmental factors as highlighted across recent exploratory studies, suggest a robust, complementary biological clock for forensic investigations with likely advantages over previously described room temperature postmortem microbial successional methods with respect to time span and concerning PMI estimation [**2**]. The phylogenetic and metabolic heterogeneity of these microbial communities means that they can

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provide fine-grained information about post-mortem processes, which stands in stark contrast to with former approaches that were largely limited to early post-mortem physical changes. In addition, the recent introduction of multi-omics approaches, such as metagenomics, transcriptomics, proteomics and metabolomics has greatly increased the resolution at which these postmortem microbial dynamics can be studied enabling a better understanding of how metabolic capabilities are harnessed and shifted during decomposition [**3**]. More specifically, these tools support deep taxonomic and functional characterization of microbial communities, including shifts in dominant taxa such as Proteobacteria and Firmicutes associated with the progression of decomposition that may be used as biomarkers for PMI prediction [**4**].

Defining the Post-mortem Ecosystem

The necrobiome is a highly dynamic ecosystem where distinct microbial communities interact concurrently to drive the decomposition process. This complex habitat includes the thanatomicrobiome (endomicrobes), the epinecrotic microbiome (epinecrobes), and the grave soil niche (edaphon), which collectively contribute to postmortem decay **[5]**. Specifically, the thanatomicrobiome describes the microbial communities present in internal organs and tissues after death, whereas the epinecrotic microbiome represents organisms inhabiting the external surfaces of the decaying corpse. In contrast, the grave soil niche includes microorganisms inhabiting soil in direct contact with or associated with decomposing corpses, displaying distinct successional dynamics driven by cadaveric exudates **[5].**

Thanatomicrobiome

The thanatomicrobiome is derived from the host's native microbiota, particularly that of the gut, which undergoes translocation to several body tissues following death and contribute to internal decay. With immune surveillance and physiological homeostasis suddenly no longer able to control microbial proliferation, these organisms rapidly turn invasive within host tissues, driving changes in microbial community composition (structure) and functions [**6**]. These changes in the indigenous microbiota are frequently associated with fluctuations in the dominance of certain bacterial phyla, for example, a strong positive correlation between actinomycetota and cumulative precipitation, as well as gammaproteobacteria and initial mass of the cadaver. In addition, the thanatomicrobiome varies substantially by organs system, each tissue harbors a distinct microbial assemblage and this diversity predictably changes as a function of the hours elapsed since death [**7**].

Epinecrotic Microbiome

In contrast to the endogenous thanatomicrobiome, microorganisms that colonize the external surfaces of decomposing remains that are frequently derived from environmental exposure or the skin microbiota of the cadaver represents the epinecrotic community. This external community is of central importance in the initial steps of decomposition, with its species composition strongly influenced by ambient temperature, relative humidity and scavenging activity. **[1-3]**. The bacterial and fungal succession on the external surfaces adds a temporal dimension that could be used to estimate PMI, particularly during outdoor decomposition [**8**]. Pechal et al. defined this epinecrotic community as the organisms present on or moving across the surface of decaying organic matter, including prokaryotes, protists and fungi. The diversity and number of these external microbial communities are often affected by exogenous factors which include seasonal variations as well as surrounding environmental microbiomes. This epinecrotic community experiences regular successional processes where specific microbial taxa increase or decrease in abundance over time, aiding investigators in refining the post-mortem interval (PMI) [**9**].

Bone Microbiome

In this respect, the community structure of bone microbiome, which evolves from a gut-associated bacterial profile in fresh remains to a soil-like one as time since death and decomposition progresses is an exciting approach for PMI prediction. In particular, studies have noted genera such as *Phyllobacterium* and *Devasias* being more frequently present at higher accumulated degree days for useful insights in the ecology of decomposed bone across time [**10**]. In addition, microbial “plastisphere” communities associated with plastic debris suggest that individual substrates can impose a selective pressure favoring unique bacterial attachments over the long term, an outcome analogous to how bone chemistry could influence colonization and succession by microbes. This selective pressure leads to distinct microbial communities on all materials, highlighting the importance of a substrate-specific profiling in forensic investigation [**11**].

The Grave Soil Niche

The grave soil is an ecological niche, separate from the internal and external cadaveric microbiomes which includes diverse soil microbial communities that live immediately adjacent to and are in close interaction with decaying remains and provide a unique interface that enables the exchange of nutrients [**12**].These communities experience drastic compositional and functional dynamics such as influxes of fluids and tissues, vie nutrients rich substrates, and develop unique microbial successional patterns that diverge from the

undisturbed soil. This can enrich particular microbial taxa like those responsible for decomposition and nutrient cycling that may leave unique fingerprints in the soil indicative of the presence and extent of human decomposition [**13**].

Post-Mortem Microbial Succession Basics

Post-mortem microbial succession is a very complex and dynamic process with an observable change of composition and function in microbial communities over time. These successional dynamics are determined by a concert of endogenous factors and exogenous environmental influences resulting in an interrelated series of ecological relationships that modulate the thanatomicrobiome, epinecrotic microbiome, and grave soil niche [**2**]. Continued microbial succession, from self-regeneration to organismal colonization and interaction with the environment, thus becomes a theoretical foundation for using "micro-organisms" as evidence in forensic science. This predictable nature of the dynamics that operate on microbial communities following death makes it possible to create model-based predictions for PMI estimation and even crime scene scenarios. For instance, such successional changes could be exploited as a "microbial clock" to predict PMI and some particular microbial cues for cause/manner of death can even be obtained through this type of investigation [**2**]. The passage from a living host to a putrefying corpse triggers, in an approximately stereotypic pattern, microbial community shifts inside the cadaver and around it; together known as the "thanatomicrobiome," that gives rise to new forensic clues. These ecological processes that are governed by nutrient availability and interspecific competition can be formally tested using designed experiments with low diversity systems to address particular ecological hypotheses [**12**]. This succession is especially well represented in buried remains, where the compositional patterns of known microbial taxa involved in decomposition form a predictable sequence over time and can therefore serve as an informative framework for PMI determination. Moreover, certain bacterial families appear to accumulate at specific PMIs and total taxon richness was reported to decrease throughout the decomposition process.

METHODOLOGICAL PIPELINE: FROM SAMPLING TO STATISTICAL MODELING

The rigrous use of microbial forensics requires a shared methodological pipeline that includes well-defined sampling, stringent DNA extraction and sequencing procedures in laboratory, as well as advanced bioinformatic tools for data treatment and interpretation. This pipeline enables the production of high-quality microbial community data which is essential to achieve reliable models for estimation of PMI and to interpret deaths cape. This end to end strategy, which begins with collecting a sample and ends with considering data, will help pave the way to converting microbial ecology knowledge into forensic useable intelligence. The recent evolution of next-generation sequencing technologies and bioinformatics has greatly broadened the range and resolution at which samples can be characterized for site-specific microbiome analysis in forensic settings. These advancements have enabled detailed profiling of complex microbial communities that yield new information on post-mortem changes at different taxa depth. For example, 16S rRNA gene sequencing for bacterial identification and ITS sequencing for fungal analysis are now routinely employed, allowing profiling of both the bacterial and fungal population in forensic samples. These molecular methodologies including amplification by universal primers i.e. 338F and 806R for bacterial community from bacteria of 16S rDNA gene and ITS1F and ITS2R for fungi from region of fungal ITS, are useful for large scale analysis in the diversity and structure of microbial communities. The resultant amplicon sequencing data is processed through strict quality filtering, clustering and phylogenetic analysis in order to identify and quantify microbial taxa. This enables the establishment of microbial profiles that can be statistically examined with the aim of associating somehow modifications in bacterial communities with post-mortem interval or other forensic parameters. For example, the DADA2 pipeline is commonly used to denoise sequencing reads, filter out chimeras and produce amplicon sequence variants that can be taxonomically annotated using databases such as ribosomal database project (RDP). In addition, metagenomic sequencing provides more complete information by sequencing all the DNA in a sample to provide species-level representation and functional profiling at greater expense of cost and computational resources than amplicon sequencing 81. The high throughput sequencing data of these methods require some advanced analytical tools, and artificial intelligence serves as an excellent tool to dynamically interpret complex multi-omics data for the post-mortem interval estimation with precision and revealing microbial mechanism. However, the application of these next-generation methodologies, especially most amplicon sequencing methods, usually employs short-read technologies that may impair species level identification accuracy and require to quantify full-length DNA in order to more accurately annotate in a taxonomic manner.

Microbiome for Post-mortem Interval Estimation

Microbial succession pattern analysis provides a prospective path to estimate PMI more accurately, particularly in the advanced decomposition stages that constrain traditional methods. This methodology leverages the recurrent variation of microbial communities over time, enabling the construction of predictive models that associate

changes in microbial community structure with PMI. Progress in sequencing platforms and computational pipelines has greatly improved our ability to explore these highly complex microbial communities, making microbiomics increasingly useful for PMI estimation **[3].** 16S rRNA gene sequencing has been broadly used for characterizing microbial communities in PMI estimation and enables the detection of bacterial taxa across different post-mortem stages. However, 16S rRNA sequencing may be limited in its ability to achieve species-level resolution, leading to the investigation of metagenomic sequencing as an alternative approach for species-specific and functional profiling. This is particularly important because amplicon sequencing of true community members can be misleading and biased. Hence, combining multi-omics methodologies including metagenomics, transcriptomics, and metabolomics with novel artificial intelligence approaches is likely to improve the accuracy and reliability of PMI inferences **[10]**. In fact, machine learning algorithms and artificial intelligence models are actively used to analyze these comprehensive datasets, offering the possibility of identifying subtle microbial fingerprints and creating models capable of predicting PMI more accurately. Yet, the hypervariable (V) region of the 16S rRNA gene chosen for primer design can severely impact the operational taxonomic units (OTUs) obtained, altering the perceived microbial diversity and requiring careful consideration of methodological choices. The ability to integrate microbial data with other PMI-influencing factors and post-processing changes is likely to refine PMI estimates even further through the development of multimodal models **[14].** Although progress has been made in analyzing the microbiota for PMI estimation, current methods including 16S rRNA sequencing—still present obstacles given divergent technical and interpretative biases resulting from environmental and cadaveric variables, demonstrating that there is not yet a single gold-standard technique for PMI assessment. Moreover, additional endeavors are needed to address these challenges in future studies and establish well-standardized protocols for the robustness and reproducibility of microbiome-based PMI estimation across different forensic environments **[10].** For instance, the implementation of machine learning techniques such as Random Forest models has proven to be a highly promising approach for processing high-dimensional microbiome data by examining patterns across the entire spectrum of microbial communities rather than focusing on individual taxa, thereby effectively predicting PMI **[15].**

MICROBIOME AS EVIDENCE OF CAUSATION AND MANNER OF DEATH

In addition to being used for estimations of post-mortem interval, the post-mortem microbiome may also have implications as a potential test for cause and manner of death so that it represents a new forensic tool investigating the circumstances around the time of death. For example, certain microbial signatures or the presence of particular pathogens in specific tissue types may infer ante-mortem infection, sepsis, or toxicological exposure. The general use of artificial intelligence and deep learning, experienced in accomplishing tasks utilizing massive datasets and can be expected to revolutionize PMI estimation as well as for interpreting forensic microbiome data by revealing subtle microbial patterns related to cause/manner of death [**2**]. In addition, distinctive microbial signatures from isolated organs/tissues may be associated with specific types of trauma or toxic ingestion and thus serve as important adjunct evidence in forensic cases. There may be poison some specific bacterial toxin, or a shift in gut microbiome; there may be cause of death: drowning (due to water microorganisms in lung) or pneumonia etc. In addition, the different abundance of types of microorganisms on various anatomical locations could also indicate the development process of disease or influence from factors such as environment applying to the dead before death [**16**]. However, despite these promising applications a lack of real-world application is still present, with many microbiome-based PMI prediction models underpinned by small sample sizes and limited coverage of body sites analyzed, highlighting the necessity for more in-depth investigations at larger scales [**17**].

Major Confounding Factor 1- Environment

Decomposition of the remains and metabolic activity of microbial communities are in turn, affected by the external environment (temperature, humidity, soil type and presence/absence of insects or scavengers). These environmental factors have significant influence on the succession of microbes, resulting in site-specific microbiome profiles which can complicate general estimates of PMI [**2**]. For example, it is known that substrate features (e.g., surface roughness and hydrophobicity) greatly influence microbial colonization, although environmental factors can often still have a pivotal impact on microbial association patterns. The influence of the environment even goes so far as to specify substrate conditions for microbial colonization with substantial difference in community patterns shaped by environmental factors such as salinity, temperature and nutrient concentrations. As a result, the variability of estimates of PMI can be highly and unpredictably variable in different environmental settings [**18**]. This inaccuracy can however lead to error rates for advanced stages of decomposition on the order of 5-7 days through these confounding variables [**49**]. This variation underscores the need for regional studies to develop solid base line data on microbial

succession under different environmental conditions, including within buried remains where soil microbiology is a crucial factor in decay. For example, the discovery of particular mammalian-associated taxa such as Bacteroides spp. in soil under the body indicate the presence of cadaveric material for months following PMI, but in general the structure of both gross and fine scale microbial communities is greatly influenced by fluctuations in temperature, moisture content, vegetation and insect activity [**2**]. Together, these environmental conditions determine the pathway of microbial decomposition and not only the rate at which biomass is degraded, but also the identity and metabolic activity of participating microbes. In addition, it is known that the organic matter content, moisture and ph of the burial soil could have a significant influence on how microbial communities develop and interact with decomposing remains, further affecting the precision of PMI estimation [**18**]. For instance, differences in nutrient content in aquatic habitats have been found to drive the specificity of bacterial assemblages colonizing microplastics and this principle can also be extended to broader decay contexts. Release of a variety of adipose tissues, volatile and organic acids, nitrogenous compounds from the cadavers during decomposition exert drastic changes on soil microbial communities surrounding these dead bodies with which PMI can be predicted [**19**]. This complex interplay highlights the importance of environmental context in deciphering forensic applications of microbiome data. To increase the applicability of this data in forensic science, future experiments should include a broad array of soil microbial colonization under differing soil conditions and from diverse geographic regions, which can be incorporated to build better baselines for future testing other dissimilar environments that may further supplement or validate more generalizable predictive models [**20**].

Major Confounding Factor II- The Host

In addition to environmental factors, the intrinsic host elements such as age, sex, nutritional state or ante-mortem diseases also have a profound impact on the postmortem microbial colonization and decomposition process. For example, the hosts' physiological status, such as tissue constitution and internal microbiota, are likely to affect the way where decay proceeds with consequences on which type and number of microbes are implicated in the process [**18**]. For example, the post-mortem microbiome may be influenced by diet (sometimes different to that pre-mortem), medication and lifestyle of an individual in life, factors which will play a role in determining the first microbial colonizers, as well as controlling degradability and stability within carcasses. Additionally, the host immune response and underlying pathologies can influence the rate/time course of decomposition making inferences on microbial data for PMI estimation complex. For example, cause of death is a factor that can significantly influence the cadaveric microbiome, but such important determinant is often missing from available literature, thus restricting potential relation between these variables and predictive models in PMI estimation [**2**]. This omission constitutes a major limitation given that the initial microbial load, as well as the types of specific pathogens at death, may dramatically influence the earliest stages of decomposition and overall microbial succession. Second, the complicated relationship between host specific factors and environmental microbiome introduces huge meta-analysis challenges to identify general microbial signatures from those of individual decomposition events. These analyses are necessary for building powerful, translatable forensic microbiome models that address the influences of both host variation and environment. This would include thorough characterization of the antemortem host microbiome, complete medical history and cause of death in order to disentangle their contribution to the post-mortem microbial profile [**21**].

Big Confusion - Sample Infection and PM Translocation

Sampling contamination and migration of microbes after death add further complexity that directly affects the validity of forensic microbiome data. This requires rigor in sampling and development of sophisticated bioinformatic methods to distinguish between exogenous contaminants versus endogenous microbial community members pertinent to the decomposition dynamics. For example, microbial translocation (triggered, in large part, by the compromise of anatomical barriers after death) may be accompanied by dissemination of microbes from their primary sites (e.g, gut), to other body compartments and surfaces [**22**]. This internal movement confounds the accurate estimation of PMI and cause of death, as it can mask early microbial signatures occurring around the time of death. This inner translocation should be carefully considered when explaining changes in microbial communities, given that not all post-mortem changes might represent environmental exposures and the movements of existing host-associated microbes. Furthermore, environmental contamination from soil, water or sampling device may lead to foreign microbial species thereby confounding the analysis aiming to detect a genuinely cadaveric microbial signature [**2**]. Such contaminants may confound useful indicators of microbial activity, and can be erroneously interpreted as being those associated with decomposition, resulting in misinterpretations of PMI or even COD. Accordingly, sterile technique during sample collection and stringent methods of computational contaminant identification are

essential to the reliability and forensic defensibility of microbiome analyses. Consequently, it is of utmost importance to define standardized procedures with regard to sample procurement, storage and processing in order to prevent bacteria contamination from outside source and for ensure analytical validity of the obtained data [**23**].

FORENSIC VALIDATION AND ADMISSIBILITY

In order for the microbiome to develop from a promising research tool into an acceptable form of evidence for forensic applications, validation studies are required that can support its reliability and accuracy or generalizability across a range of case scenarios. This will require standardization of methods, creation of strong statistical models that will survive legal challenges this is the current dividing 44 ATTIA barrier between academic research and forensic use [**24**]. Of course, this also relies on such validation considering the natural variability of post mortem microbiome-compositions (which are determined by various factors including environmental settings, host phenotypes and actually sampled anatomical places. Additionally, it is important to create large distributed reference databases including different post-mortem conditions and geographic origin for interpretation of casework. In addition, application of reliable statistical procedures to compensate for various confounding factors is necessary in order to produce valid; values are expressed as mean ± standard deviation significant and forensically accepted results [**25**]. In this sense, those validation efforts should also favors to a degree blind testing (i.e., laboratories test samples without knowing the origin) to evaluate objectively how constant and reproducible images are. Last, research on microbiome biobanks with focus for forensic purposes is vital to further standardize reference data and enable comparison across laboratories (to alleviate the small sample size and variations in sample population observed here. These non-clinical biobanks, although not specifically for clinical use, would allow for a more robust characterization of community level microbial dynamics across multiple forensic contexts (e.g., different decomposition environments and host demographics) [**26**].

FUTURE DIRECTIONS AND INTEGRATIVE APPROACHES

Thus, for developing forensic microbiology as a discipline practitioners are called on to work inter disciplinarily and to combine various associated technologies in order to exploit its potential. Such an integration would mean the further development of molecular tools like metagenomics and meta transcriptomics and associated bioinformatics to begin providing a more holistic view of microbial communities at work in decomposition. These advances are essential for understanding the specific ways in which microbial mixed cultures influence the rate and pattern of decomposition and it passes beyond community taxonomic profiling to functional insights [**27**]. Moreover, future studies should focus on the development of new soil management technologies (i.e., microbial inoculants and bio stimulants) to manipulate post-mortem soil microbiomes for improved accuracy in PMI estimations. This requires more in-depth insight into the intricate relationship between terrestrial microbial diversity and ecosystem processes, especially how ecological mechanisms are influenced by changes in soil microbial community composition. For example, this knowledge can be drawn from the study of simplified microbial consortia that provide a controlled environment to understand the complex interplay between species diversity and function [**2**]. Furthermore, the use of multi-omics strategies combining genomic, transcriptomic, proteomic and metabolomic data will present new insights into metabolic activities and interactions of thanatomicrobiome and improve our interpretation of microbial changes in a forensic context. Further, on-site studies in (unprocessed) soils using whole cadavers and common plant litters at different times of the year are required to fully explore subsurface postmortem necrobiome communities [**28**]. Such all-encompassing approaches extending beyond prokaryotic to eukaryote systems will offer a more comprehensive insight into microbial succession in distinct ecological microenvironments within the forensic taphonomic continuum. Analysis of the "core" genera and their relative abundances, before and after death, could be reliable indicators of post-mortem interval. Additionally, such tools, as well as advanced analytical methods (e.g., machine learning and co-occurrence network analysis), will be critical for elucidating complex microbial community interactions during decomposition, and potential key species that consistently associate closely with various decomposition states representing different stages of the process [**29**].

CONCLUSION

Because post-mortem microbial communities are both dynamic and complex, this can offer huge potential for forensic science but also major difficulties in terms of how results are correctly interpreted and validated. Although the "microbial witness" has the potential to provide invaluable information regarding PMI and or to establish the cause of death with accuracy never available before, scientists must exercise extreme caution if it is to be used responsibly thereby avoiding travesties of justice. As such, ongoing interdisciplinary research and the establishment of consensus guidelines are needed to translate promising results into forensic applicability that will withstand legal challenge. Ongoing developments in genomic and proteomic approaches, together with big data analysis are

extending the value of microbial profiling even further; notably for PMI estimation and cause of death inference. Nevertheless, it must be recognized that despite these promising progresses there are still problems to overcome, especially in relation to the influences of environmental factors and seasonal variations on microbial decomposition drivers that should be investigated better as a way to improve PMI estimations. Furthermore, integrated procedure of microbial profiling along with advanced imaging analyses like 3D scanning or dental impressions and chemical investigation is envisage to enhance forensic evidences and forensically relevant applications related precision. Future studies must equally aim at understanding the precise functional contributions of microbial communities during different stages of decomposition to produce more reliable and generalizable predictive models.

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