

Review

Postmortem Interval Estimation: New Approaches by the Analysis of Human Tissues and Microbial Communities' Changes

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Abstract: There are several methodologies available to estimate time since death based on different changes that a corpse undergoes after death. However, these methods are imprecise due to the decomposition process being affected by several factors, principally temperature and humidity. Current trends for the determination of the Postmortem Interval (PMI) attempt to estimate the PMI in a quantifiable manner, based on chemical changes on and in the body, summarized in the field of “thanatochemistry”. Although these methodologies have improved PMI estimates, additional research has been developed to increase the accuracy and precision of this determination. As a result, the fields of “thanatobiology” and “thanatomicrobiome” have emerged. Thanatobiology is based on the estimation of the PMI from DNA/RNA degradation, signaling pathways of cell death, and protein analysis. Thanatomicrobiome refers to changes in the bacterial communities as a consequence of the decomposition process. Although both approaches seem to improve PMI estimates, applications of thanatobiology methodologies are more appropriate in the first phases of decomposition, while thanatomicrobiome analyses are applicable in advanced stages. Further research is needed in these new fields in order to establish their applicability in forensic cases. This is a review of the current state-of-the-art methodology in these two subfields.

Keywords: postmortem interval; DNA; RNA; protein analysis; putrefaction; next generation sequencing technologies; bacterial communities



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1. Introduction

In forensic science, the accurate determination of the post-mortem interval (PMI), or time since death, is crucial for elucidating possible criminal acts and determining appropriate civil repercussions [1].

The analysis of the PMI is based on changes that a corpse undergoes after death including physical processes (body cooling and hypostasis), metabolic processes (supravital reactions), autolysis (loss of selective membrane permeability, diffusion), physicochemical processes (rigor mortis), bacterial processes (putrefaction), and the effect of insect activity [2–9]. Additionally, the environment in which the body is decomposing and the potential for vertebrate scavenging activity may impact changes in the corpse and, therefore, the estimation of time since death [10].

Traditional methods for estimating PMI are based on changes undergone by a corpse after death, including early changes involving physical processes (e.g., body cooling and hypostasis), metabolic processes (supravital reactions), autolysis (loss of selective membrane permeability, diffusion), and physicochemical processes (rigor mortis) [11].

They produce relatively reliable estimates of time since death for the early post-mortem interval (EPMI), typically only within the first 24 h after physiological death. After this stage, the focus shifts almost exclusively to loss of soft tissue and, later, to changes to the skeletal structures [12]. These methods provide only very rough estimates of PMI, particularly after EPMI. Additionally, these methods are imprecise, due to several factors which may affect their progression, i.e., changes in room temperature (like air conditioning) could alter the algor mortis, and as a result, the time of death could be erroneously determined.

Forensic entomological analyses of insect activity on a corpse are utilized to estimate longer PMIs, in ranges of days or first few months after death, depending on the method (larval development or insect succession). These methods also depend on whether insects had access to the body or were ecologically active at the time of death and deposition [13].

Recent research attempting to improve PMI estimates have considered more predictable and quantifiable biological markers and processes associated with human decomposition, particularly from the field of thanatochemistry, which focuses on post-mortem chemical changes exhibited by the decomposing body [14–16]. This field is mostly based on the quantification of volatile byproducts of different organic decay processes [17,18]. Thanatochemistry considers influencing factors, such as temperature and humidity [19–23].

In addition to the above methods, recent trends, based on the process of autolysis, attempt to determine the PMI using molecular biology by analyzing DNA/RNA degradation, signaling pathways of cell death, and proteins. Collectively, these approaches may be called “thanatobiology” [24–26].

Bacterial community changes throughout the putrefaction process have also been proposed to estimate the PMI.

During life, the human microbiome, that is, the native microorganisms that colonize the human body, coexists in an overall balance under healthy conditions.

There are five major sites of microbial colonization in the human body: the gastrointestinal tract (representing 29% of the total number of bacterial cells in the human microbiome), the oral cavity (representing 26%), the skin (representing 21%), the airways (representing 14%), and the urogenital tract (representing 9%). The other 1% of the total number of bacterial cells in the human microbiome can be found in blood [27].

Upon death, the physical and immunological barriers of the human body start to break down, giving the microorganisms contained in the human microbiome the opportunity to spread and colonize anatomical sites that they could not access during life. After death, and throughout the decomposition process, microbial communities change in a specific sequence. The analysis of these communities has opened a new field known as “thanatomicrobiome” [28].

This article will provide an overview of “thanatobiology” and “thanatomicrobiome” relating to approaches of postmortem interval estimation.

2. Thanatobiology

Decomposition begins approximately 4 min after death, with a process called autolysis. During autolysis, cells are progressively destroyed, and there is a consequential release and damage of cellular components and metabolites [1].

2.1. DNA

Based on this initial process, some studies have analyzed postmortem DNA degradation in relation to the time since death [29,30]. Cina [29] determined the relationship between the rate of DNA degradation and the time elapsed since death in spleen tissue harvested from a series of autopsies with known PMIs. Subsequently, Di Nunno et al. [30,31] studied samples taken from a deceased person’s spleen, liver, and blood to determine the relationship between degradation of DNA and time since death using flow cytome-

try. The authors found the best correlation with hepatic tissue. A more recent study by Williams et al. [32] compared brain and spleen tissue samples, finding lower rates of DNA degradation in brain tissue.

Other studies based on DNA degradation have applied the comet assay technique [33]. Johnson and Ferris found an increase in DNA fragmentation correlated with the PMI (from 0–56 h) [34]. Lin et al. [35] found a similar linear relationship in rat liver cells during the early postmortem interval. Chen et al. [36] found the same results in heart, liver, and kidney cells, reporting rapid degradation in the first 6 h after death. These results were consistent with those of other groups who used the same comet assay technique [37] in other types of cells [38]. This was extended to mouse brain and dental pulp cells and prolonged PMIs, up to 72 h, finding a high linear correlation between the comet assay parameters and PMI [39].

2.2. RNA

Initial studies on the correlation of RNA expression and the PMI were based on the degradation of this molecule by ribonucleases present in the cell and/or originating from bacteria or other environmental contamination [40]. Studies have shown that, depending on the tissue, RNA can be either more or less stable. Finger et al. found a rapid degradation of RNA in the pancreas and liver due to an increase in ribonucleases [41]. The highest stability was reported in human and rat brain tissue [42–45].

Partemi et al. [46] analyzed RNA degradation in human heart tissue from individuals with short and long postmortem intervals based on the expression of four genes: beta-glucuronidase (*GUSB*), Nitric Oxide Synthase 3 (*NOS3*), Collagen 1 (*COL1A1*), and Collagen 3 (*COL3A1*). Although they found differences in RNA quality between short and long PMIs, quantitative polymerase chain reaction (q-PCR) of these genes only indicated a negative correlation between *NOS3* expression and the PMI. The rest of the analyzed genes were not affected by the PMI.

Following this line of research, and using the same qPCR technique, Li et al. [47], analyzed the expression of 18S-rRNA and microRNAs (miRNA) in rat heart tissues with PMIs between 0 and 168 h. The authors found a strong parabolic relationship between the postmortem period and Ct values for 18S-rRNA. In qPCR, Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample. miRNA showed the same type of relationship; however, the correlation was lower.

Lv et al. [48] developed a mathematical model, based on qPCR expression of Glyceraldehyde-3-Phosphate-Dehydrogenase (*GAPDH*) and β -actin (*ACTB*) in both lungs and muscles samples collected from rats from 0 to 6 days after death and in analogous samples collected from human autopsies up to 24 h after death, taking temperature into account. During the validation of these models, they obtained low error rates in both subjects.

In a similar study, also applying qPCR, Tu et al. [49], analyzed the expression levels of housekeeping genes, miRNA, and circular RNAs (circRNAs) in mouse heart, liver, and skeletal muscle tissues with PMIs between 0 and 8 days. They normalized the expression of the housekeeping genes with respect to miRNAs. Taking this into account, they created different formulas for tissues based on the target/housekeeping genes (*U6*, *Rps18*, and β -actin) and their correlation with the PMI.

Recently, Tao et al. [50] also analyzed the PMI in rat cardiac tissue, with time since death between 0 and 36 h. In this case, they used a microarray to screen 217 mRNA markers and applied qPCR to validate the candidate markers with highest correlations. Among all of the tested markers, cell division cycle 25 homologue B (*Cdc25b*) had the best correlation coefficient with early PMI, creating a model with a cubic equation. In addition, *Rpl27* seemed to be suitable for use as an endogenous control for the technique.

Based on the process of cell death signaling, C. Zapico et al. [1] analyzed the early PMI (0–8 h) in the gastrocnemius muscle of rats. Using qPCR, this study analyzed the mRNA expression of Fas Ligand (*FasL*), one of the triggers of apoptotic cell death, and phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*), a regulator of apoptosis. This study indicated an increase in the mRNA levels of both genes up until 6 h after death, finding a strong positive linear correlation.

This study prompted other works based on gene expression, in the field known as “thanatotranscriptome”. Javan et al. [24] applied an apoptosis PCR array on human liver samples with PMIs between 6 and 48 h. Their findings indicated that pro-apoptotic genes such as caspases were upregulated with the PMI, while anti-apoptotic genes such as *BCL2* and *BAG3* were downregulated.

The same group [51] recently analyzed the transcriptome from prostate tissues with PMIs between 24 and 120 h. They again applied an apoptosis PCR array, finding an increase in the expression of pro-apoptotic genes, along with that of a few anti-apoptotic genes such as *BAG1*, *BCL2*, and *XIAP*. In contrast, pro-apoptotic genes such as *TP53* and *TNFSF10* did not show an upregulation.

Aiming to provide a method of estimating the PMI in drowning cases, Cho et al. [52] analyzed the expression of the Receptor of Advanced Glycation Endproducts (RAGE) in lung tissue from drowned rats. qPCR and Western blot were used to measure protein expression in rats with PMIs ranging between 1 and 7 days. Degradation over time was found. These results were confirmed with immunostaining analysis.

A recent publication from Martinez et al. [53] analyzed the expression of autophagy genes during the early PMI (0–8 h) in rat gastrocnemius muscle. Autophagy is an essential process that maintains cellular homeostasis via the degradation and recycling of long-lived proteins, intracellular aggregates, and damaged organelles [54]. Their results found that the expression of these genes was in agreement with the sequence of events triggering autophagy signaling. *LC3*, which acts in the first steps, showed an increase at 2 h and a decrease after that. *Beclin-1*, *ATG7*, and *ATG12*, which act in the latest steps, showed the highest increase at 4 h and a decrease after that. In addition, an increase in free radicals (reactive oxygen species) correlated with melatonin receptor expression, with the highest level of both at 4 h and a decrease after that. At 8 h, the level of free radicals increased again, likely due to the processes of death. Considering the previous results with apoptosis signaling, it seems likely that shortly after death, there is an initial increase in autophagy signaling, attempting to mitigate stress and save the cell. However, due to the process itself, there is then a switch to apoptosis signaling.

Based on these previous studies, the current trends are focused on screening the expression of several genes and correlating it with the PMI. Hunter et al. in this regard developed one of the first studies in 2017 [55]. They recovered samples from zebrafish with PMIs from 0 to 96 h and brain and liver samples from mice with PMIs from 0 to 48 h. They applied a modification of a microarray, a “Gene Meter” approach, to analyze expression changes with the PMI. The results allowed them to create different models based on 11 genes for zebrafish and 7 genes for brain and liver samples in mice to predict the PMI, with correlations around 0.9.

In this line of research, based on the human transcriptomics data from post-mortem samples obtaining from the Genotype Tissue Expression Database (GTEx), Zhu et al. [56] demonstrated a change in the expression of 266 genes with short-time and long-time PMIs and also pointed out that RNA degradation is tissue-specific. This was supported by the study by Ferreira et al. [57], describing that these expression changes correlated with the PMI are faster in some tissues than in others, i.e., the digestive tract showed earlier changes, while the central nervous system remained more stable in the first hours after death.

2.3. Proteins

Javan et al. [25] analyzed the expression of autophagy genes in human brain and heart tissues with PMIs between 6 and 58 h (“thanatophagy”). The authors chose to use the Western Blot technique to analyze the proteins. In heart tissue, LC3 II, p62, Beclin-1, and Atg7 levels increased in a time-dependent manner. In contrast, BNIP3 level in cardiac tissue decreased with the PMI. In cerebral tissues, LC3, p62, and Atg7 levels increased with the PMI, while BNIP3 and Beclin-1 levels decreased with time elapsed.

The same group [58] used Western blot to analyze other biomarkers in human brain tissue with PMIs between 0 and 72 h. Among the studied proteins, they found a decrease in the expression of Talin-1 with the PMI. This protein is involved in connecting the integrin family of cell adhesion molecules to the actin cytoskeleton at the plasma membrane.

Some groups applied proteomics to estimate the PMI. Procopio et al. [26] analyzed changes in protein expression in pig bones with PMIs between 1 and 6 months, using liquid chromatography–tandem mass spectrometry (LC–MS/MS). They found a decrease of specific plasma and muscle proteins with increasing PMIs, as well as an increased deamidation of biglycan, a protein with a role in modulating bone growth and mineralization. The same group analyzed the possibility of estimating the PMI in aquatic environments by applying a proteomics approach [59].

In this line of research, Nolan et al. [60] studied the decomposition fluid from domestic pig (as a human model) in winter and summer and analyzed the peptides produced, applying a nano HPLC system coupled with a TripleTOF mass spectrometer. They found common peptides in both seasons, suggesting that quantitative peptide analysis may be useful in estimating the PMI, independently of the temperature and environmental conditions.

Using femur bones from human cadavers, with PMIs ranging from 5 to 20 years, Prieto-Bonete et al. [61] also analyzed the proteome using LC–MS/MS. The authors found 32 proteins that could discriminate between a PMI of 5–12 years and a PMI of 13–20 years. Of these proteins, 10 participate in biological processes of the bone, 4 have a molecular function, and 18 are cellular components.

Applying the same technique of LC–MS/MS, Choi et al. [62] analyzed skeletal muscle proteins from rat and mouse with PMIs between 0 and 96 h. They found a consistent pattern of degradation for the two proteins eEF1A2 and GAPDH, correlated with the PMI. In addition, they validated their results with Western blot on muscle samples from rat and human autopsy cases.

A recent article from Mickleburgh et al. [63] assessed the impact of intrinsic and extrinsic variables on human proteome analysis in a decomposition scenario, finding that inter-individual and inter-skeletal differences in bone mineral density affect the survival of proteins. Additionally, they identified new potential biomarkers for PMI estimation based on proteome analysis.

Another recent trend is the analysis of metabolites [64] produced as a consequence of the death process. Wu et al. [65] studied the early PMI (0–72 h) using rat cardiac blood, detecting endogenous metabolites through GC–MS. Based on these metabolites, they created a statistical model to predict the PMI. Du et al. [66] used femoral muscle and extended PMIs (3–168 h) to determine the time since death based on metabolomic analysis through LC–MS. They applied Principal Component Analysis (PCA) and Projection to Latent Structures–Discriminant Analysis (PLS–DA) statistics to elucidate the formulas for PMI prediction.

Table 1 presents a summary of the thanatobiology methods discussed in this section.

Table 1. Summary of thanatobiology approaches, applied techniques, and assessed tissues.

Approach	Technique	Tissues
DNA Degradation	Flow Cytometry	Blood, Spleen, Brain, Liver
	Comet Assay	Liver, Heart, Kidney, Brain, Dental Pulp
RNA Degradation	Q-PCR: NOS3, 18S rRNA, miRNA, circRNAs	Heart, Liver, Skeletal Muscle
	Q-PCR-mRNA microarray	Cardiac Tissue
	Q-PCR RAGE/Western Blot/Immunostaining	Lung Tissue in drowning cases
Thanatotranscriptome (Signaling pathways)	Q-PCR Autophagy/Apoptosis signaling pathways	Gastrocnemius Muscle
	Apoptosis microarray	Liver, Prostate
	Microarrays	Brain, Liver
	Transcriptomic analysis	Several Tissues
Proteins	Western Blot	Brain
	HPLC-TOF	Decomposition Fluid
	LC-MS/MS	Bones, Skeletal Muscle
Metabolomics	GC-MS	Cardiac Blood
	LC-MS	Femoral Muscle

3. Thanatomicrobiome

Microbes inhabit the human body during life and after death. In life, the human microbiome is an integral component for the maintenance of health [27,67] and its changes play an important role in disease. Moreover, microbes have a key role after death, being the driving force of putrefaction. The decomposition of the body begins with cellular autolysis by hydrolytic enzymes resulting in the release of carbohydrates, proteins, minerals, and fat from the body tissues; these products are then utilized by bacteria in the putrefaction process [68,69].

The postmortem microbiome encompasses the thanatomicrobiome (microorganisms found in internal organs and cavities upon death) and epinecrotic microbial communities (the microbiome on surfaces of decaying remains) [28].

Some studies use a more generalized term for thanatomicrobiome, defining it as the postmortem microbial community of the human body, which involves a successional process where trillions of microbes inhabit, proliferate, and die internally and externally throughout the dead body, resulting in temporal shifts in community composition over time [69–72].

The vast majority of the studies analyze remains decomposing on the surface; however, some research has been performed in buried remains [73,74], demonstrating that microbial successional changes can be potentially utilized to estimate the PMI despite the specific environment where the corpse is decomposing.

3.1. Analysis of the Thanatomicrobiome

Postmortem microbial studies have used non-human (swine and rodents) [75–80] and human research models. Swine are the preferred non-human model as their decomposition is similar to that of humans [81]. Human cadavers used for postmortem microbial studies are donated bodies from willed donation programs to outdoor decomposition research facilities [69,71,82–86] or autopsied cadavers in a medical examiner/coroner's office [70,72,87,88].

Different studies propose collecting the samples by swabbing the area of study or dissecting the tissue. According to thanatobiome studies, swabbing provides higher microbial diversity than dissecting the tissue [88]. The double-swab technique is advised due to its efficiency in collecting biological samples. The first swab is immersed in sterile distilled water, rolled over the sampling surface using moderate pressure and a circular motion, and air-dried. The second swab is rolled over the same area using similar pressure and motion to absorb all of the moisture left by the first swab and then air-dried. Swabs from the same site are generally pooled together [69,89,90].

Different studies have explored different body sites, organs, and tissues to collect samples from. The body site for collecting the sample should be taken into account, especially when comparing microbial community changes. The different body sites, organs, and tissues present signature species in their indigenous bacterial communities. Additionally, different anatomical sites will undergo different decomposition changes at different rates, and this will create different microenvironments that will determine the species that find their niche according to the changing environmental conditions (such as access to oxygen) [91].

The application of postmortem microbial studies to estimate time since death relies on changes in the composition of the microbial communities during the decomposition process. Therefore, the goal of these studies is to determine which OTUs (Operational Taxonomic Unit) are present and their abundance throughout the different stages of decomposition.

Microbiome composition can be investigated through culture-dependent and culture-independent methods.

Culture-dependent methods consist in the isolation or culture of living microbes sampled from a decomposing body or a substrate around and/or beneath the corpse, in order to characterize the microbes that grow in culture. Although this method allows an accurate identification of the different OTUs, an important disadvantage is the bias imposed by the culture conditions [92,93]. Not all microorganisms from the environment will grow in a laboratory culture medium, since it is impossible to reproduce the exact environmental conditions in a microbial culture; these unculturable bacteria have certain requirements that are not replicated in the laboratory culture. It is estimated that over 50% of human-associated microbes are considered unculturable [94]. For this reason, it is difficult, if not impossible, to infer the abundance of the cultured microbes in their original community [92]. Presently, this limitation is addressed by using culture-independent methods to study microbial diversity in forensic samples.

Culture-independent methods consist in the extraction and analysis of the genetic materials of the microorganisms present in a sample. This approach represents a more realistic, comprehensive, and high-throughput description of the microbial community [92,95].

The most widely used culture-independent methodology for the investigation of the microbial diversity of a decomposing body sample is DNA sequencing. The first step is microbial DNA extraction.

Microbial DNA can be collected from samples using classical methods of DNA extraction, such as the phenol/chloroform method. However, when dealing with samples containing soil, soil DNA extraction kits are highly recommended to mitigate PCR inhibition by the humic acid present in the soil [70,71,81,82].

Once DNA is extracted, gene amplification must be performed to multiply and study the DNA present in the sample; the different OTUs are then identified by their gene sequence. Typically, the 16S rRNA genes for bacteria and archaea and the Internal Transcribed Spacer (ITS) for fungi are transcribed. An alternative approach is sequencing the whole genome using whole-genome shotgun (WGS) sequencing.

Sequencing techniques have evolved significantly over the last decade with a wave of new and improved technologies leading sequencing into a high-throughput or next-generation era [96]. High-throughput sequencing techniques offer the most accurate results to determine microbial diversity. Next-generation sequencing is being implemented in research, and its use will only increase as technology, bioinformatics, and resources evolve

to address its current limitations, improve the quality of the results, and increase the number of potential applications [97].

3.2. Postmortem Changes in Microbial Communities

Microorganisms native to the body start the putrefaction process. Once the immune system ceases its function, the bacterial communities inhabiting the different sites of the human body are able to transmigrate and colonize previously uninhabitable areas of the corpse [69–72].

As decomposition progresses, environmental microorganisms colonize the corpse. Qualitative and quantitative changes (changes in the number of the different group representatives and changes in the community composition) take place during the decomposition process [69–72].

As stated, bacterial communities native to the body develop in the early stages of decomposition. However, as the decomposition process advances, bacterial communities change according to the newly available environmental conditions, and opportunistic bacteria take advantage when the microenvironmental conditions meet their requirements. Oxygen availability seems to be the major driver for these changes. Thus, the temporal aerobic/anaerobic conditions of different body sites at different stages of decomposition strongly determine bacterial colonization and, as a consequence, bacterial diversity [69].

Firmicutes and Bacteroides are the most abundant phyla throughout the decomposition process [88]. Firmicutes appear to play a critical role in cadaver decomposition, as they are present at the onset of the cadaver breakdown as well as in advanced stages of decomposition. However, these two Firmicutes communities are not the same. The initial Firmicutes community is represented by bacteria native to the human body, whilst the Firmicutes community present in advanced decay correspond to Clostridiales, which take advantage of the newly available ecological conditions [82] (Figure 1).

In a 2017 study conducted by Adserias-Garriga et al., changes in bacterial communities throughout the decomposition process were analyzed in three donated individuals. The stages of decomposition were assessed visually according to Payne [98]. Study individuals 1 and 3 displayed all six stages of decomposition, whilst the bloat stage was not observed in individual 2. Despite the absence of the bloat stage in individual 2, the overall changes in the bacterial communities were similar in all three donated individuals [69]. Changes in quantity and composition of the bacterial communities may provide more accurate estimates of the time since death. However, it must be noted that the timing of different microbial changes is highly dependent on environmental conditions at the location of body decomposition. Accordingly, the local environmental condition must always be considered when these methods are used for PMI estimation. Attempts should be made to set standards for the times at which certain microbial changes take place in particular environments.

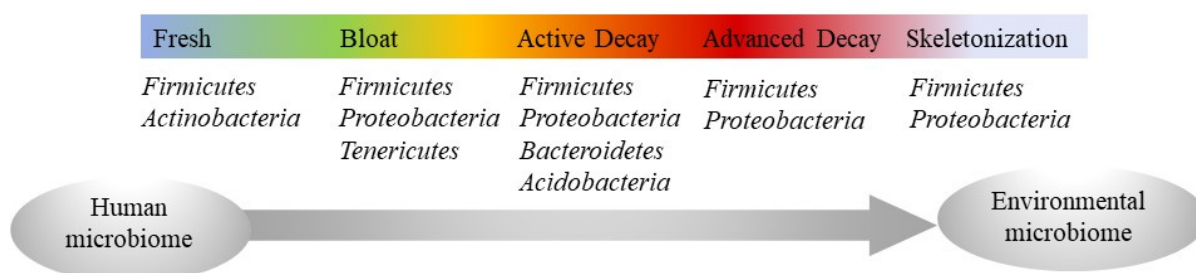


Figure 1. Main bacterial taxa representatives in the different stages of decomposition of the human body, when samples are taken from the oral cavity. Note that Firmicutes present in the fresh stage include bacterial groups from the human microbiome, while Firmicutes present at skeletonization include bacterial groups from the surrounding environment.

The concepts of thanatobiome and epinecrotic microbial communities encompass the complete aggregation of microorganisms found in distinct locations of a decomposing body. While the majority of studies are focused on bacteria, some research reports postmortem fungal community changes over the decomposition process.

4. Conclusions

Current studies are seeking more precise and accurate approaches for the determination of the PMI in a quantifiable manner. Recent trends point to the utility of molecular biology techniques to estimate the time since death from two points of view: decomposed tissues and bacterial communities. Thanatobiology approaches appear most suitable in the first phases of decomposition, while thanatobiome analyses, applying NGS technologies, seem to have greater utility in advanced stages. As molecular biology techniques advance, more research in both areas will be developed, fostering studies in transcriptomics and proteomics, as well as more in deep research on metagenomics and functional genomics related to thanatobiome, improving both approaches for PMI estimation and leading to their potential applicability to forensic cases.

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