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Analysis of bacterial dynamics and biochemical markers in *postmortem* interval estimation

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Analysis of bacterial dynamics and biochemical markers in *postmortem* interval estimation

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Resumo:

A determinação do intervalo *postmortem* (PMI) é fundamental na investigação forense, mas continua desafiante devido a múltiplos fatores, incluindo a causa de morte e as condições ambientais. Indicadores tradicionais, como *algor mortis*, *rigor mortis* e *livor mortis*, são úteis apenas nas fases iniciais. Abordagens complementares da entomologia, botânica e microbiologia mostram-se promissoras, mas variam na precisão. Avanços em sequenciação de alto rendimento destacaram o potencial da microbiologia *postmortem*. Contudo, apesar de dominar o campo, a sequenciação não substitui abordagens baseadas em culturômica, cruciais para resolução ao nível da espécie, assim como métodos bioquímicos, ainda pouco explorados no estudo do PMI.

Este estudo teve como objetivo analisar microbiológica- e bioquimicamente diferentes órgãos (pulmões, coração, rins, fígado e cérebro) em seis PMI (0-96 horas) em murganhos machos da linhagem C57BL/6J, desprovidos de patógenos específicos (n=3/ponto temporal). Foram quantificadas a carga bacteriana total e a distribuição de *Escherichia coli* e *Enterococcus faecalis*. Fezes e órgãos foram homogeneizados em água peptonada tamponada e cultivados em meios não seletivos (agar sangue) e seletivos (MacConkey e Slanetz-Bartley). As unidades formadoras de colônias (UFC) por grama de tecido foram determinadas após incubação aeróbica. Para a análise bioquímica, homogeneizados dos órgãos em tampão fosfato (pH 7,4) foram processados no autoanalisador Accent MC240 (Cormay®). Os marcadores incluíram a ureia, o lactato, o ácido úrico, a glicose, as proteínas totais, o magnésio, o etanol e o ferro. Para desenvolver um modelo matemático para estimativa do PMI, os diferentes marcadores bioquímicos foram normalizados para o magnésio, e apenas aqueles com uma tendência clara e consistente de aumento ou diminuição, com um coeficiente de determinação (R^2) >0.900, foram selecionados.

A dinâmica do microbioma *postmortem* dependeu fortemente do tecido e do tempo, com cargas bacterianas aeróbicas totais atingindo o pico às 72 horas na maioria dos órgãos, exceto no cérebro (96 horas), provavelmente devido à proteção anatômica. Não foi detectada *E. coli* no fígado, no coração e no cérebro. *E. faecalis* apresentou uma colonização consistente, particularmente nos rins, nos pulmões e no cérebro, com invasão inicial às 24 horas e pico entre 72-96 horas. Estes resultados realçam o valor da culturômica na detecção de padrões bacterianos relevantes para a estimativa do PMI.

Os marcadores bioquímicos mostraram tendências distintas dependentes de tecido e tempo. O lactato aumentou precocemente, atingindo o pico entre as 24-48 horas, mantendo-

se elevado no cérebro, provavelmente devido ao metabolismo anaeróbico. O ácido úrico aumentou progressivamente, especialmente nos rins, no coração e no cérebro, refletindo uma provável degradação das purinas e/ou autólise. A ureia apresentou um forte aumento em fases tardias, enquanto o ferro aumentou continuamente nos rins e no coração, atingindo o pico mais precocemente no fígado. Entre todos os parâmetros analisados, o lactato foi o mais informativo nas fases iniciais do PMI, enquanto o ácido úrico e ureia se correlacionaram com intervalos posteriores.

Estes resultados sugerem que os dados microbiológicos baseados em culturômica, combinados com marcadores bioquímicos, fornecem um suporte multiparamétrico robusto para a estimativa do PMI. Particularmente, a colonização por *E. faecalis* no cérebro e a dinâmica do lactato, ácido úrico, ureia e ferro em diferentes órgãos, emergiram como indicadores confiáveis da progressão *postmortem*. No entanto, é necessária uma validação adicional em condições forenses complexas, incluindo em humanos.

Palavras-chave: Intervalo *postmortem*; Tanatomiologia; Tanatoquímica; *Enterococcus faecalis*; *Escherichia coli*; Biomarcadores.

Abstract:

The determination of the *postmortem interval* (PMI) is fundamental to forensic investigations but remains challenging due to numerous influencing factors, including cause of death and environmental conditions. Traditional indicators such as *Algor Mortis*, *Rigor Mortis*, and *Livor Mortis* are useful early on but often unreliable beyond initial stages. Complementary approaches from entomology, botany, and microbiology show promise but vary in precision. Recent advances in high-throughput sequencing have underscored the potential of *postmortem* microbiology. However, while sequencing has dominated the field recently, culture-based approaches, critical for accurate species-level resolution, as well as biochemistry, remain underexplored in PMI research.

This study aimed to perform a combined microbial and biochemical analysis of different organs (lungs, heart, kidneys, liver, and brain) at six PMI (0, 12, 24, 48, 72, and 96 hours) in male C57BL/6J specific pathogen-free mice (n=3 per timepoint). For microbial analysis, the total bacterial load and the distribution of *Escherichia coli* and *Enterococcus faecalis* were quantified. Feces and organs were homogenized in buffered peptone water and cultured on non-selective (Blood Agar) and selective (MacConkey for *E. coli*, and Slanetz-Bartley for *E. faecalis*) media. Colony-forming units (CFU) per gram of tissue (total and species-specific bacterial loads) were determined after aerobic incubation. For biochemical analysis, tissue homogenates in phosphate buffer (pH 7.4) were processed using the Accent MC240 autoanalyzer (Cormay®). Selected biochemical markers included urea, lactate, uric acid, glucose, total proteins, magnesium, ethanol, and iron. To develop a mathematical model for PMI estimation, markers were normalized to magnesium, and those showing a clear and consistent increasing or decreasing trend, with a determination coefficient (R^2) greater than 0.900, were selected.

Postmortem microbiome dynamics were strongly tissue- and time-dependent, with total aerobic bacterial loads peaking at 72 hours in most organs, except the brain, which peaked at 96 hours, likely due to its anatomical protection. *E. coli* was absent in the liver, heart, and brain. *E. faecalis* exhibited consistent colonization patterns, particularly in the kidneys, lungs, and brain, with initial invasion at 24 hours, peaking between 72 and 96 hours. These findings underscore the value of culture-based methods in detecting taxa relevant to PMI estimation.

Biochemical markers showed distinct tissue- and time-dependent trends. Lactate increased early in most organs, peaking at 24-48 hours PMI, with a sustained increase in the brain, likely consequent to anaerobic metabolism. Uric acid levels also increased

progressively, especially in the kidney, heart, and brain, likely reflecting purine degradation and autolysis. Urea levels exhibited a marked increase at later PMI. Iron increased steadily in the kidney and heart, peaking earlier in the liver. Among the different biochemical markers, lactate was the most informative at early PMI stages, while uric acid and urea correlated strongly with later intervals.

These findings suggest that culture-based microbiological data and/or biochemical markers can provide a robust multiparametric support tool for PMI estimation. Particularly, *E. faecalis* colonization in the brain and the dynamics of lactate, uric acid, urea, and iron in different organs emerged as reliable indicators of *postmortem* progression. Nevertheless, further validation in complex forensic conditions, including studies in humans, is necessary.

Keywords: *Postmortem* interval; ThanatOMICROBIOLOGY; Thanatochemistry; *Enterococcus faecalis*; *Escherichia coli*; Biomarkers.

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List of abbreviations and acronyms

ADDs – Accumulated degree days

ADP – Adenosine diphosphate

AIF – Apoptosis-inducing factor

ALT – Alanine transaminase

AMP – Adenosine monophosphate

AST – Aspartate transaminase

ATP – Adenosine triphosphate

BHI – Brain Heart Infusion

BPW – Buffered Peptone Water

Ca²⁺ – Calcium cation

CBA – Columbia Blood Agar

CFU – Colony Forming Units

CK – Creatine kinase

Cl⁻ – Chloride anion

CNECV – National Ethics Council for Life Sciences

CRP – C-reactive protein

CSF – Cerebrospinal fluid

DNA – Deoxyribonucleic acid

E. coli – *Escherichia coli*

E. faecalis – *Enterococcus faecalis*

HAF – Hypoxia-associated factor

HIF2 α – Hypoxia-inducible factor 2 α

HMGB1 – High mobility group box-1

Hx – Hypoxanthine

ICP-OES – Inductively Coupled Plasma Optical Emission Spectrometry

ID – Identity

HxR – Inosine

i3S – Institute of Research and Innovation in Health

IgM – Immunoglobulin M

IMP – Inosine monophosphate

IOP – Intraocular pressure

K⁺ – Potassium cation

lncRNAs – Long non-coding ribonucleic acids

M. osloensis – *Moraxella osloensis*

meso-DAP – Meso-diaminopimelate

MAC – MacConkey Agar

minPMI – Minimum *postmortem* interval

mRNA – Messenger ribonucleic acid

MALDI-TOF MS – Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry

Na⁺ – Sodium cation

NADH – Nicotinamide adenine dinucleotide reduced form

NGS – Next-generation sequencing

ORBEA-IUCS – Organization responsible for animal welfare at the University Institute of Health Sciences

ORP – Oxidation-reduction potential

PCR – Polymerase chain reaction

PMCT – *Postmortem* computed tomography

PMI – *Postmortem* interval

SD – Standard deviation

SLB – Slanetz & Bartley Agar

SPF – Specific pathogen-free

TSB – Tryptone soy broth

TTC – Triphenyltetrazolium chloride

VOCs – Volatile organic compounds

I. Introduction

Forensic sciences encompass a wide range of specialized disciplines, including forensic medicine, entomology, microbiology, toxicology, anthropology, geology, and ballistics, among others (1). Each field follows rigorous, standardized procedures for the collection, preservation, and interpretation of evidence, and their integration is indispensable for unravelling complex cases and supporting judicial processes (2, 3).

The *postmortem* interval (PMI), the time elapsed since death is a core responsibility in forensic medicine, yet it remains scientifically and procedurally challenging (4). Contrary to some representations, classical PMI estimation methods, including the assessment of *algor mortis*, *rigor mortis*, and *livor mortis*, are neither simple nor readily accessible to all. The accurate application and interpretation of these techniques require highly specialized expertise in forensic pathology, as well as a thorough understanding of the multitude of variables affecting *postmortem* changes (4-7).

Contrary to the perception of being simple or easily accessible, classical PMI estimation methods, such as the evaluation of *algor mortis*, *rigor mortis*, *livor mortis*, and other transformative *postmortem* processes, are complex, highly specialized, and legally restricted. Accurate interpretation requires extensive postgraduate training in forensic pathology or legal medicine, often encompassing a decade or more of academic study and supervised practice. In many jurisdictions, only licensed medical doctors with such specialization are legally authorized to perform autopsies and apply ancillary PMI estimation methods, such as histology, entomology, radiology, or biochemical analysis. Furthermore, the routine implementation of these ancillary investigations is generally limited to accredited forensic institutions with the necessary infrastructure and financial resources, restricting their availability outside major medicolegal centers (8, 9).

The decomposition of a human cadaver is a complex process driven by enzymatic autolysis, microbial proliferation, and environmental factors. Microorganisms, including bacteria, fungi, and protozoa, interact with conditions such as temperature, humidity, pH, and oxygen availability to shape the pace and nature of decay (5, 10, 11). Temperature and humidity are major modulators, while pH and oxygen influence both microbial dynamics and *postmortem* chemical transformations (11).

Although many PMI estimation methods have been proposed, most remain impractical or highly environmentally sensitive, limiting their routine forensic application (12). Complementary disciplines such as microbiology, radiology, omics and botany have

therefore gained importance (4, 13). In particular, the human microbiome follows predictable *postmortem* succession patterns, serving as a potential biological clock for PMI estimation (14). In parallel, thanatochemistry—the study of *postmortem* biochemical changes—offers measurable indicators such as electrolyte shifts, protein degradation, and metabolite accumulation (4, 15-18). Combining microbial and biochemical approaches, supported by computational modeling and machine learning, provides a more robust and evidence-based framework for PMI estimation (14, 19).

This introduction critically examines both classical and emerging PMI estimation methods, with a particular focus on the thanatomicrobiome and thanatochemistry, whose integration promises greater accuracy and reproducibility in forensic practice.

I.1. Methods to estimate the *postmortem* interval

I.1.1. Classical *versus* modern methods for PMI estimation

Classical methods were within us for almost a century. Classical PMI estimation relies on observable transformative processes (thanatomorphological phenomena) such as the progression of *algor mortis*, *livor mortis*, and *rigor mortis*, as well as putrefactive and decomposition changes. The onset and sequence of these processes are not only highly context-dependent, particularly influenced by environmental factors such as temperature and humidity, but also demand expert differentiation from antemortem and perimortem changes. These approaches are rooted in well-documented phenomena and can be applied without the need for advanced laboratory equipment. Simplicity, accessibility, and cost-effectiveness, since they do not require sophisticated tools, make them applicable in resource-limited settings. Although they have been studied and utilized for decades, they have proved to be highly ineffective. The reason why these methods were advocated for more than a century is the proof of concept that forensic autopsies were very resistant to the entrance of science and more accurate methods. In other words, in several countries, PMI estimation follows almost the same procedures as the beginning of the 20th century. While ancillary methods, including histology, entomology, and advanced biochemical analyses, play a central role in advancing the precision of PMI estimation, their implementation is not routine. These techniques require not only additional subspecialist expertise but also access to dedicated laboratory resources and significant financial investment. Moreover, due to strict legal and ethical guidelines

governing the handling of human remains, such investigations are typically reserved for authorized forensic practitioners within institutional settings (9, 20).

Over the last few years, modern biochemical and microbiological methods have emerged within research units as key tools for PMI estimation, though also with advantages and limitations. Modern approaches leverage advances in molecular biology, biochemistry, and microbiology, offering more accurate and reproducible PMI estimations. These include, for instance, the analysis of *postmortem* biochemical changes (*e.g.*, degradation of metabolites or proteins) and shifts in microbial communities (thanatobiome). By employing objective measurements, the subjectivity of the forensic expert is reduced. For example, proteomic analyses can identify protein degradation markers that correlate strongly with time since death. Despite modern methods may require high financial demands as it occurs with next-generation sequencing or mass spectrometry techniques, trained personnel have proven to be the major obstacle. Although for toxicology and genetics we have been developing experts in these fields, estimation of PMI will require proper education of the pathologist to incorporate these techniques in the casework routine. In the following sections, modern methods are presented (9, 21, 22).

1.1.2. Methods based on early *postmortem* changes

Over the years, various methods have been developed to estimate the PMI (Figure 1) (23), all having advantages and disadvantages (Table 1). Early *postmortem* PMI estimation typically relies on *algor mortis*, *livor mortis*, and *rigor mortis*.

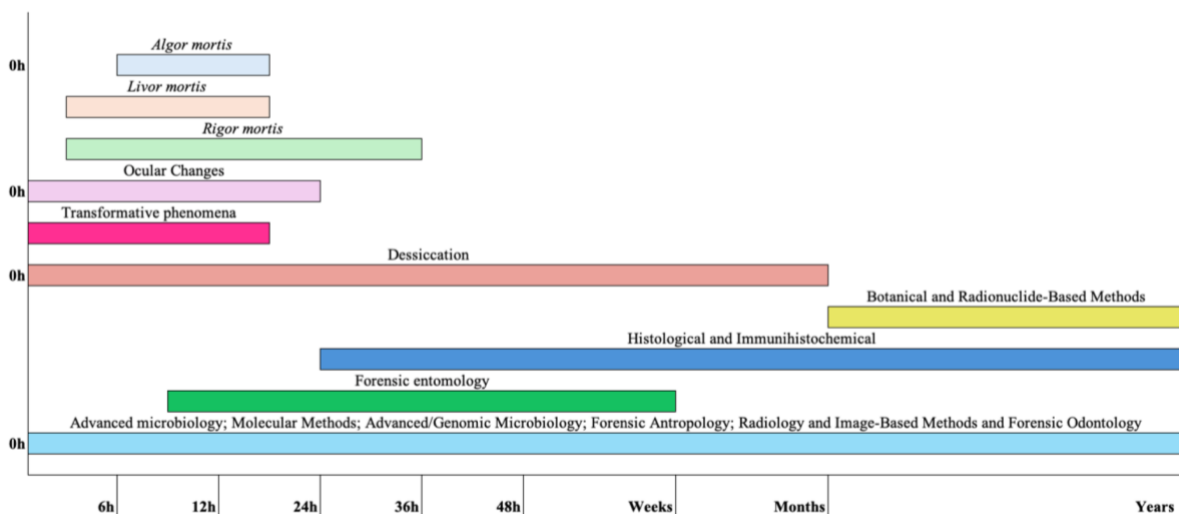


Figure 1. Schematic representation of key methods for estimating PMI and the approximate timeframes during which they can be applied.

These timelines are only indicative, as decomposition is strongly influenced by multiple environmental and biological factors. For instance, transformative processes may occur within a few hours under hot and humid conditions, forensic entomology can provide estimates ranging from hours to weeks (excluding archaeoentomology), and radionuclide dating may still involve errors of several decades.

1.1.2.1. *Algor mortis*

Algor mortis is the *postmortem* cooling of the cadaver, reflecting the cessation of thermoregulation after death. Heat is lost via conduction, convection, radiation, and evaporation (13), with the cooling rate influenced by environmental conditions, body mass, clothing, and position (24, 25).

While frequently cited in textbooks, accurate PMI estimation via *algor mortis* is complex and legally restricted to qualified forensic specialists. Interpretation requires measuring internal temperature (usually rectal or hepatic) and applying models such as the Henssge nomogram, which integrates body weight, clothing, and ambient temperature (26). Moreover, sudden changes in ambient temperature, such as *postmortem* refrigeration, can introduce significant error, and the method is not applicable in advanced decomposition stages (27, 28). Recent developments include noninvasive thermometry and advanced numerical modeling, such as thermodynamic finite-difference algorithms and surrogate model-based parameter optimization, which allow individualized PMI estimation and can accommodate complex scenarios like variable ambient conditions and non-standard body postures (29). These approaches have demonstrated improved accuracy over traditional nomogram methods, with errors reduced to less than one hour in controlled studies (30).

1.1.2.2. *Rigor mortis*

Rigor mortis, the *postmortem* stiffening of muscles, arises from adenosine-5'-triphosphate (ATP) depletion and actin-myosin binding until proteolytic enzymes degrade muscle fibres (13, 24). It typically begins ~2 hours after death, peaks at 6–8 hours, persists for 12–24 hours, and gradually resolves by ~36 hours (13, 31, 32). Although *rigor mortis* can provide indicative PMI estimates, its onset and duration are strongly influenced by

temperature, metabolic state, and *perimortem* activity. Reliable interpretation requires formal training in forensic pathology, and in practice, it is used in conjunction with *algor* and *livor mortis* assessments by certified professionals (24, 25, 33, 34). Interpretation of *rigor mortis* is limited by its sensitivity to environmental and individual factors. Higher ambient temperatures accelerate the onset and resolution, while lower temperatures prolong the process. Muscle fiber type also influences timing, with red muscle developing rigor earlier than white muscle (35, 36). Physical exertion or convulsions before death can hasten ATP depletion and precipitate an earlier onset (37). These confounding variables mean that *rigor mortis* alone cannot provide precise PMI estimates and must be integrated with other *postmortem* findings, such as *algor mortis* and *livor mortis* (8).

1.1.2.3. *Livor mortis*

Livor mortis, or *postmortem* hypostasis, is the purplish-blue discoloration of dependent skin caused by gravitational blood pooling in vessels and capillaries (26). It typically appears within 30 minutes to 2 hours *postmortem*, becomes pronounced and coalescent by 6–12 hours, and eventually fixes as hemolysis and hemoglobin diffusion occur (13, 14, 25, 38). Forensic significance lies in pattern recognition and blanching response, which can indicate time since death and movement of the cadaver (39). Objective methods such as spectrophotometric analysis have improved reliability, but interpretation still requires specialist training to account for confounding variables and to apply mathematical models for PMI estimation (40). Legal restrictions mandate that only qualified forensic practitioners, typically forensic pathologists, may perform and interpret these analyses, as misinterpretation can have significant judicial consequences (25, 26, 39).

1.1.2.4. Ocular changes

Ocular *postmortem* changes, such as corneal opacity, variations in pupil diameter, blood vessel fragmentation, retinal discoloration, and intraocular pressure decline, evolve in a time-dependent manner and can assist in PMI estimation. Among these, corneal opacity and intraocular pressure are the most significant indicators: opacity typically becomes noticeable after ~8 hours, and intraocular pressure decreases markedly within the first 12 hours *postmortem* (10, 41, 42). Additional findings include corneal thickening, transient pupil dilation and fixation, sometimes followed by constriction during *rigor mortis*, and the

“cattle-truck” fragmentation of retinal vessels. Modern tools, such as optical coherence tomography, can visualize *postmortem* changes in retinal layers and reflectivity (33, 34). Interpretation of ocular changes is complex and environment-dependent, requiring consideration of temperature, humidity, and individual physiological variables. Consequently, PMI estimation using ocular signs is legally restricted to qualified forensic pathologists or legal medicine specialists, and often demands access to specialized ophthalmologic or forensic imaging facilities.

The vitreous humor is a particularly valuable matrix for *postmortem* biochemistry (10, 41). Located between the lens and retina, it is relatively protected from bacterial contamination and environmental factors, providing a more stable composition than blood or cerebrospinal fluid. Its predictable biochemical changes (see Section 1.2.2) make it a preferred sample for accredited forensic laboratories, though its collection and analysis remain limited to properly equipped biochemistry facilities. Moreover, collection requires a refined technique to avoid blood contamination, which may compromise the interpretation of results. Cloudy or blood-stained samples should be discarded, as they may lead to erroneous results (43).

1.1.2.5. Histological and immunohistochemical methods

Histological and immunohistochemical analyses provide valuable insights for estimating the PMI by detecting progressive tissue decay and time-dependent protein changes (44). Histological studies document structural alterations such as vacuolation of oral mucosa within ~4 hours and dermal fragmentation by ~24 hours *postmortem* (45). Immunohistochemistry (IHC) allows the detection of specific antigens, including glucagon in pancreatic tissue for up to 6 days or collagen fibers via specialized staining (46). Other markers, such as insulin, thyroglobulin, and calcitonin, can support approximate PMI estimation in selected cases (26).

Recent advances, such as multi-marker panels and proteomic profiling, have improved early PMI estimation, particularly within the first 24 hours (44). However, environmental conditions, inter-individual variability, and tissue integrity significantly affect reliability, and many protein markers are useful only within narrow *postmortem* windows. Consequently, histology and IHC are considered supplementary tools rather than standalone methods for PMI determination (10, 41).

Importantly, the application of these microscopic and molecular techniques is legally restricted to accredited forensic laboratories and requires highly trained professionals, typically forensic anatomopathologists. Routine implementation also depends on specialized resources and infrastructure, limiting their availability outside major medicolegal or academic centers. Indeed, microscopic and molecular analyses, such as collagen degradation assessment, osteocyte quantification, and immunohistochemical staining, are highly sensitive to pre-analytical variables, environmental exposure, and taphonomic processes, which can confound results if not properly controlled and interpreted by subspecialists (47).

I.1.2.6. Desiccation

Postmortem desiccation produces progressive color and texture changes in mucous membranes and delicate skin surfaces, advancing most rapidly in moist areas like the eyes, lips, and genitalia (41). A classic sign is "tache noir", a black discoloration along the scleral equator caused by corneal drying. As tissues dehydrate, the skin and mucous membranes become dry and leathery, and in arid environments, this process may culminate in natural mummification within 2–4 weeks (48).

Although desiccation patterns provide supportive information for PMI estimation, their interpretation is highly context-dependent, influenced by environmental conditions, body position, and exposure. Accurate forensic assessment of these changes is legally restricted to qualified specialists, typically forensic pathologists, and should be performed within accredited facilities as part of an integrated, multi-method evaluation. This context dependency and need for expertise are consistent with the broader challenges in PMI estimation in forensic anthropology, where visual and morphological skeletal assessments, taphonomic analysis, and contextual/archaeological indicators all require integration with environmental and case-specific data to improve reliability (49).

I.1.3. Methods based on late *postmortem* changes

In later *postmortem* periods, classical methods become more challenging to apply, making techniques like forensic entomology and molecular testing more relevant (50).

I.1.3.1. Forensic entomology

Forensic entomology estimates the minimum *postmortem* interval (minPMI) by analyzing the arrival, colonization, and development of insects on human cadavers (51, 52). Decomposing tissues provide both nutritional resources and oviposition sites, supporting the life cycles of various insect species. Early colonizing flies (Order Diptera) are often the first to arrive, and estimating the age of their oldest immature stages, combined with the likely time of oviposition, allows entomologists to determine how long the remains have been exposed (52, 53). Since insect development is temperature-dependent, accurate estimation also requires meteorological data for the relevant period.

Forensic entomology can inform criminal investigations by supporting PMI estimation, and in some cases, the location or manner of death (52, 53). The applicability of entomological evidence spans from hours to several weeks *postmortem*, depending on species succession, environmental access, and whether archaeoentomological timelines are excluded. Despite its utility, entomological analysis is highly specialized and requires formal training and access to accredited laboratories capable of species identification and developmental staging. These analyses are therefore legally restricted to qualified forensic professionals, and their results are most reliable when integrated with other *postmortem* indicators such as *algor mortis*, *rigor mortis*, *livor mortis*, and biochemical findings (14).

I.1.3.2. Transformative processes induced by autolysis, putrefaction, and decomposition

The physical and chemical transformations of a human cadaver, such as skin discoloration, bloating, fluid release, and tissue breakdown, form the basis of classical PMI estimation (54). Human decomposition proceeds through distinct phases—fresh, bloating, decay, and drying—each marked by specific thanatomorphological phenomena and chemical changes (4, 10). Decomposition begins immediately with autolysis, the enzymatic self-digestion of tissues, which disrupts cellular compartments, alters ionic balances ($\uparrow K^+$, $\downarrow Na^+/Cl^-$), and lowers pH. Organs rich in enzymes, such as the pancreas, gastric mucosa, and adrenal medulla, degrade first. Within 24–72 hours, putrefaction predominates: endogenous bacteria (notably *Clostridia* and *Proteus* spp.) spread through tissues, producing gases (H_2S , NH_3), bloating, discoloration, fluid leakage, and venous marbling. Amino acid, lipid, and

carbohydrate catabolism generate biogenic amines, organic acids, and volatile compounds responsible for the characteristic odor of decomposition (26, 55, 56).

Environmental and intrinsic factors, including temperature, humidity, oxygen availability, and body storage conditions, heavily influence decomposition rates. Plastic wrapping, water submersion, or sealed environments can significantly delay putrefaction, while arid conditions promote desiccation and eventual natural mummification. The dry phase culminates in skeletonization, whose timing varies widely from weeks to months depending on conditions (57).

Although these transformative processes provide critical forensic information, their interpretation is complex and context-dependent, requiring specialized training in forensic pathology or legal medicine. Accurate PMI estimation from decomposition changes is legally restricted in most jurisdictions to accredited professionals and is most reliable when integrated with other *postmortem* indicators, including entomology, biochemistry, and thanatobiome analyses. Indeed, the medical literature consistently emphasizes that decomposition rates and patterns are influenced by a multitude of biological and environmental factors, including temperature, humidity, burial conditions, and individual characteristics, making accurate assessment highly challenging and necessitating expert knowledge for reliable interpretation. Moreover, these assessments have significant medicolegal implications and require adherence to strict protocols and ethical guidelines (8, 58).

1.1.3.2.1. Accumulated degree days (adds) method

Megyesi *et al.* (59) proposed estimating the PMI of decomposed human remains using accumulated degree days (ADDs), which account for the cumulative effect of ambient temperature on soft tissue decomposition. Recognizing that decomposition is a gradual, region-specific process, the method scores observable changes in three cadaver regions: the head and neck (including cervical vertebrae), trunk (thorax, pectoral girdle, abdomen, and pelvis), and limbs (hands and feet). By incorporating temperature-dependent progression rather than relying solely on elapsed time, this approach offers more refined PMI estimates. However, the applicability of ADD-based models is highly context-dependent, as decomposition rates vary with geography, climate, and environmental conditions (60). Forensic interpretation of ADD scoring therefore requires specialized training and is legally restricted to qualified practitioners in accredited settings, often in combination with other

postmortem indicators. Indeed, specialized forensic training is essential to correctly score decomposition, select appropriate models, and interpret confounding variables, as improper application can lead to substantial errors, especially when models are used outside their validated geographic or environmental context and it is needed strict adherence to protocols, chain of custody, and ethical standards (61, 62).

1.1.3.3. Molecular methods

Molecular approaches for PMI estimation rely on the analysis of nucleic acid integrity and degradation, including deoxyribonucleic acid (DNA), messenger ribonucleic acid (mRNA), and long non-coding RNA (lncRNA) (10, 41). Progressive nucleic acid breakdown occurs *postmortem* due to enzymatic activity, microbial invasion, and environmental conditions, with temperature and humidity strongly influencing degradation rates (56). Several experimental studies illustrate the potential of these methods. For example, Peng *et al.* (55) developed mathematical models based on the *postmortem* expression of hypoxia- and apoptosis-related mRNA markers, such as HAF, AIF, and HIF-2 α . Similarly, lncRNAs in brain tissue have shown promise as early PMI markers in animal studies (63), while DNA degradation patterns in dental pulp are being explored with next-generation sequencing (NGS) (64).

Emerging strategies aim to enhance reliability. These include combining RNA and DNA analysis to develop more reliable models, using ADDs instead of calendar days to better account for temperature variations, and investigating the potential of stable RNA types, such as certain lncRNAs, for more precise PMI determination (63, 64). While molecular methods are promising and highly innovative, they remain experimental, tissue-specific, and resource-intensive, and their forensic application is currently limited to accredited laboratories with specialized expertise. Indeed, the tissue specificity arises because molecular degradation rates vary widely between tissues (*e.g.*, muscle, liver, brain, and dental pulp), and are influenced by environmental factors, agonal events, and cause of death, making universal models unreliable and necessitating careful selection and interpretation of the biological matrix. Moreover, most studies focus on short PMIs and animal models, with limited human data and lack of standardized protocols, further complicating clinical translation (9, 65).

I.1.3.4. Botanical evidence

Botanical evidence can provide contextual information about the environment surrounding human remains, although its application in PMI estimation is limited (10, 66). Plant succession patterns, such as grasses colonizing disturbed soil before shrubs and eventually trees, may indicate time since burial site disturbance, while seasonal cues such as the state of leaves, flowers, or fruits can offer approximate time-of-year indicators (66). *In situ* plant growth analysis can help estimate the minimum time a cadaver has been at a location, but maximum PMI cannot be determined reliably. Plant species may also provide geographical context, helping to identify whether remains were relocated. Modern methods such as DNA barcoding and microscopic algal analysis can support species identification and link remains to nearby environments, including aquatic sites (67). The forensic utility of botanical evidence is highly dependent on expert collection, documentation, and preservation, while remaining sensitive to climate, species variability, and environmental conditions. As a result, forensic botany is underutilized, requiring specialized knowledge and integration with other forensic methods to be effective (68).

I.1.3.5. Forensic radiology

Forensic radiology applies imaging techniques such as X-ray, computed tomography (CT), and magnetic resonance imaging (MRI) to document *postmortem* changes (26). These methods can identify gas accumulation, tissue alterations, and other structural, or internal changes that occur after death. Interestingly, *postmortem* imaging can sometimes yield superior image quality due to the absence of motion artifacts. However, gas formation and low cadaver temperature may also affect image quality (69).

Recent research has investigated *postmortem* CT (PMCT) combined with machine learning models for PMI estimation. In an experimental rabbit model, PMCT data from multiple tissues, including the brain, eyes, myocardium, liver, kidneys, and muscles, were successfully used to train a stacking model for PMI prediction (70). Despite its promise, forensic radiology for PMI estimation is highly specialized, requiring advanced imaging infrastructure and expert interpretation, and remains restricted to accredited forensic centers. *Postmortem* imaging modalities—such as *postmortem* CT (PMCT) and MRI—demand not only technical proficiency in radiological acquisition and processing, but also deep understanding of *postmortem* anatomical and physiological changes, which differ

substantially from clinical imaging findings. Moreover, dedicated *postmortem* imaging suites, specialized scanners, and advanced post-processing software are required.

I.1.3.6. Radionuclide-based methods

Radionuclide-based methods, including the analysis of isotopes such as ^{210}Pb , ^{210}Po , and ^{14}C , are particularly useful for aging skeletonized remains (10). These methods, such as radiocarbon (^{14}C) dating, are primarily applicable for relatively old remains (several years to decades *postmortem*) and are currently limited by both their precision (often $\pm$ several years or decades) and the requirement for preserved soft tissues. Their practical applicability for more acute forensic timeframes is, at present, minima (26, 71, 72).

I.1.3.7. Forensic odontology

Forensic odontology primarily supports identification and age estimation through the examination of dental tissues, particularly dental pulp, and can also contribute to PMI estimation under certain conditions (10, 73). Teeth provide natural protection from environmental factors, preserving tissues for early and late *postmortem* analysis.

Techniques include morphological and histopathological assessments of mineral density and microscopic alterations; molecular analyses of DNA and RNA, including by PCR and NGS. Recent advancements demonstrate the potential of NGS for PMI estimation, especially beyond 7–10 days *postmortem*, with specific DNA degradation patterns and mutations in dental pulp correlating with PMI intervals (64). Despite this promise, forensic odontology for PMI estimation is highly specialized, requiring accredited laboratories, expert interpretation, and further validation studies to improve accuracy and standardization (74).

I.1.3.8. Forensic anthropology

Forensic anthropology applies the study of skeletal remains to medicolegal investigations, supporting identification, age and sex estimation, ancestry assessment, trauma analysis, and, in some cases, PMI estimation (10). Anthropological evaluations are particularly relevant in late PMI scenarios, where soft tissues have largely decomposed or skeletonization has occurred.

PMI estimation in anthropology is typically based on (58):

1. Visual and morphological assessment of skeletal changes, including bone weathering, cortical exfoliation, and surface cracking;
2. Taphonomic analysis, examining how environmental factors (*e.g.*, soil composition, moisture, pH, temperature, and vegetation) have affected bone preservation;
3. Contextual and archaeological indicators, such as soil staining, associated artifacts, and stratigraphy, which provide insight into deposition time.

Age-related skeletal changes can help estimate age at death, which is often necessary to contextualize PMI. However, precision is limited, and late PMIs usually require broad time ranges due to variability in environmental exposure, burial conditions, and scavenger activity (75). Advanced methods, including histological bone analysis, stable isotope studies, and radiocarbon or luminescence dating, can complement traditional assessments but are resource-intensive and typically restricted to specialized laboratories (76).

It is important to note that no single method provides a precise and secure determination of time since death (Table 1), especially for late PMIs. The most effective approach often involves combining multiple techniques and considering various factors that influence the decomposition rate.

Table 1: Overview of different methods used to estimate the *postmortem* interval, highlighting their key advantages and disadvantages.

Methods	Advantages	Disadvantages	References
<i>Algor mortis</i>	Simple and low-cost; useful in the first 24 hours.	Cadaver temperature can be influenced by many factors (<i>e.g.</i> , ambient temperature, clothing, and cadaver size).	(13)
<i>Rigor mortis</i>	Observable and quite predictable sequence. Offers the longest <i>postmortem</i> interval estimates.	Influenced by various factors, including ambient temperature and the individual's metabolic state at the time of death.	(24, 25, 77)
<i>Livor mortis</i>	The progression of <i>livor mortis</i> occurs in distinct stages. Helpful for early PMI.	Only useful during the first few hours after death (becomes fixed around 8–12 hours).	(14)

	Environmental factors (<i>e.g.</i> , temperature) can affect its progression, making it an imprecise indicator of time of death.		
Ocular changes	Offers measurable changes (<i>e.g.</i> , IOP, opacity) and a stable matrix for estimating the <i>postmortem</i> interval due to its resistance to contamination and environmental factors.	Requires careful interpretation due to environmental and individual variability.	(10, 33, 41)
Histological and immunohistochemical	Changes in specific proteins/tissues provide valuable information. Useful in early PMIs.	Antigens such as insulin, thyroglobulin, or calcitonin allow only a rough PMI estimation, as many markers are effective only within limited timeframes, and environmental factors and individual variability can influence results. Requires specialized techniques and expertise.	(6, 24, 38, 45, 48)

Desiccation	Visible progression.	Influenced by climate.	(41, 48)
	Helpful in advanced Decomposition.	Not suitable for precise PMI.	
Forensic entomology	Useful in intermediate periods (days to weeks) using early colonizers.	Dependent on timely insect access to the remains.	(14)
	Well-documented insect succession.	May be compromised by delays in oviposition.	
Transformative processes	Gross observation is possible in the field.	High variability.	(4, 10, 26, 54)
	Useful in late stages.	Environmental and individual differences reduce precision.	
Accumulated degree days (ADDs) method	Improves PMI estimates by integrating temperature into decomposition, allowing regional adaptation.	Its applicability may vary across different geographical regions and environmental conditions.	(60)

Molecular methods	High specificity.	RNA/DNA degrades quickly.	(56)
	Potential for tissue-specific models.	Sensitive to environmental factors.	
Botanical evidence	Uses seasonal plant indicators, growth analysis, and species identification to assess time of death and cadaver relocation.	Effectiveness depends on proper collection and preservation.	(10, 66-68)
	Useful in buried bodies and outdoor settings.	Growth rates are influenced by climate and species variation. Requires ecological expertise.	
Forensic radiology	Visualizes internal changes like gas accumulation.	Requires imaging technology.	(69)
	Supports decomposition scoring.	Factors like gas formation and low cadaver temperature can affect image quality.	
Radionuclide-based methods	Radiocarbon in soft tissues reflects atmospheric levels.	Requires preserved soft tissues.	(71, 72)
		Costly and complex.	

	Estimates the year of death within ~3 years.	Limited use for older remains. Results can be affected by environmental contamination.	
Forensic odontology	Teeth preserve well beyond 7–10 days. Dental exams help estimate age and identity.	Need for standardization, improved accuracy, and larger validation studies. Results can be slow.	(73, 74)
Forensic anthropology	Effective in skeletonized remains. Helps to estimate age, ID, gender, and PMI.	Estimates age at death visually. Large error range for PMI. Not suitable for early PMI.	(10, 75)
Biochemical Markers	Predictable changes in markers like K ⁺ , Hx, and glucose. Stability of fluids (<i>e.g.</i> , vitreous humor).	Accuracy declines over time. Affected by temperature and health status.	(78)

Proteomics	Protein degradation follows time-dependent trends.	Requires expensive equipment (<i>e.g.</i> , mass spectrometry).	(79-81)
	Robust in degraded samples.	Trained personnel needed.	
Volatile Organic Compounds	Decomposition-specific chemical fingerprints.	Requires gas analysis tools.	(82-84)
	Useful for PMI fingerprinting.	Affected by storage/environment.	
Metagenomics	Predictable microbial succession.	Influenced by external factors (humidity, oxygen).	(10, 85)
	Useful in late PMI.	Human cadaver studies are limited.	
	Supports “microbial clock” models.		
Metatranscriptomics	Reflects gene expression.	RNA instability.	(26, 86, 87)
	Time-sensitive Markers.	Requires advanced laboratory techniques.	

	Integrates metabolic fingerprints.	Complex interpretation.	
Metabolomics	Highly sensitive.	Influenced by multiple internal/external variables.	(85, 86, 88)

Abbreviations: DNA, Deoxyribonucleic acid; Hx, Hypoxanthine; ID, identity; IOP, Intraocular pressure; K⁺, Potassium cation; PMI, *postmortem* interval; RNA, Ribonucleic acid.

I.2. Unveiling microbial and biochemical markers for PMI estimation

I.2.1. Thanatomiobiome analysis: an emerging tool for PMI estimation

Before the emergence of the microbiome era in forensic science, forensic investigations primarily relied on traditional physical evidence, such as footprints, tool marks, fingerprints, and blood spatter. These methods were rooted in Locard's Exchange Principle, which postulates that physical evidence is inevitably left at nearly every crime scene. In the late 19th century, microbes were first employed as forensic evidence, but their use was narrowly focused on assessing the pathogenicity and lethality of microorganisms (89). The field was constrained by the technological limitations of the time and a limited understanding of microbial communities, which restricted its broader application in forensic investigations. However, the advent of NGS and other advanced molecular techniques has significantly expanded the role of microbiology in forensic science, opening new avenues for investigation and analysis (10).

The study of *postmortem* microbial communities, known as the “thanatomiobiome”, introduced in 2014 by Can *et al.* (90), has emerged as a crucial area in forensic sciences, providing valuable tools for estimating the PMI (4). After death, the breakdown of physical and immunological defenses allows microorganisms, predominantly from the gastrointestinal tract, to colonize less accessible areas. Bacteria such as those from the Bacillota (*e.g.*, *Clostridium*, *Bacillus*, and *Peptoniphilus*) and Pseudomonadota phyla are prominent predictors of PMI, contributing to decomposition by breaking down complex tissues through enzymatic activity (91). These microbial shifts, collectively referred to as the "microbial clock", follow predictable patterns and serve as temporal indicators during decomposition (6). Microbial markers provide a unique advantage in PMI estimation, especially in later *postmortem* periods when traditional methods such as *rigor mortis* or *livor mortis* are no longer applicable.

The necrobiome, including internal (endonecrotic) and external (epinecrotic) microbial communities, is integral to understanding decomposition. Internal communities, or the thanatomiobiome, are found in the blood, cadaver fluids, and internal organs, while external epinecrotic communities inhabit surfaces like the skin and orifices (92). Although easier to

sample, epinecrotic communities are more susceptible to environmental and biological factors, which can affect their reliability in forensic applications (10).

Research using advanced techniques such as 16S rRNA sequencing and shotgun metagenomics has highlighted the utility of microbial markers in PMI estimation. Predictable microbial shifts occur in internal organs, while the soil surrounding a cadaver undergoes changes influenced by leachates. The combination of microbial data from multiple sites, such as skin, caecum, and soil, with computational models has significantly improved PMI accuracy. For example, microbial taxa such as *Clostridium* and *Rhizobiales* have been identified as strong predictors of PMI, particularly during the active decay phase (93-96). Despite these advancements, challenges remain. Variability in microbial composition due to lifestyle, diet, environmental conditions, and sampling methods can lead to inconsistencies in findings. Environmental factors like humidity, oxygen, and temperature also influence microbial dynamics but are often underexplored in forensic models (10). While the influence of these variables on microbial community changes is not fully understood, different studies suggest that bacterial succession could help estimate PMI, as certain groups (mainly from Gammaproteobacteria, Lactobacillaceae, and Clostridiaceae) are strongly linked to decomposition (97). Specific bacterial taxa play key roles in decomposition, and some, like those from Pseudomonadota and Bacillota, have been proposed as PMI biomarkers (10). Javan *et al.* (98) highlighted species from the Bacillota phylum (*Clostridium*, *Bacillus*, *Peptoniphilus*, *Blautia*, *Lactobacillus*) as potential biomarkers. Nevertheless, contamination during sample collection and limited access to human cadavers remain significant challenges. Moreover, conflicting results across studies continue to hinder the identification of reliable biomarkers, due to variations in experimental models, sampled cadaver sites, microbial communities, and decomposition conditions. Additionally, most PMI studies rely on animal models, complicating the translation of results to human decomposition scenarios (10).

The incredible advancements in microbiome analysis, including high-throughput sequencing and machine learning, such as Random Forest regression models, have enabled the rapid identification of microbial communities even in degraded samples (6). These tools have expanded the potential of microbial markers, with the microbial clock showing particular accuracy in the first 48 hours after death (10). Moreover, microbial signatures persist in the soil long after decomposition, helping in locating clandestine graves. To further refine PMI estimation, future efforts should focus on standardizing protocols, expanding datasets with human cadavers from diverse environments, and incorporating environmental

variables into predictive models. By addressing these challenges, the integration of microbial and computational approaches holds great promise for advancing forensic investigations.

1.2.2. Thanatochemistry and biochemical markers

Thanatochemistry, commonly referred to as "*postmortem* chemistry," investigates the chemical changes that occur in various cadaver fluids and tissues after death, with a focus on decomposition and biochemical transformations, while accounting for various influencing factors (4, 78). This field plays a critical role in understanding *postmortem* biochemical shifts, offering valuable insights for estimating the PMI (99). By analyzing a range of biochemical markers, thanatochemistry provides a more robust and potentially reliable approach to PMI estimation, helping to address the limitations of traditional methods (15-18). However, despite its promise, this approach may still lack precision in certain contexts (100).

Death initiates profound biochemical changes across all cadaver tissues, driven by the cessation of oxygen circulation, disruption of enzymatic reactions, cellular breakdown, and the halt in anabolic metabolite production (100). Various biochemical markers, including Na^+ , K^+ , Ca^{2+} , Cl^- , urea, creatinine, and glucose, are present in different biological fluids and play a critical role in estimating PMI (101). Among these fluids, vitreous humor and synovial fluid are particularly significant due to their protection from external influences, such as temperature fluctuations and the effects of chemical and microbial agents, as they are encapsulated within specific anatomical structures (99).

Vitreous humor analysis has emerged as a valuable tool in thanatochemistry due to its protected anatomical location and slower autolytic changes compared to other biological matrices (102). Several biochemical markers in vitreous humor exhibit predictable trends *postmortem*, helping in PMI estimation. K^+ concentration is one of the most extensively studied parameters for PMI estimation, as K^+ levels rise linearly after death due to cell membrane breakdown and diffusion from surrounding tissues. This predictable increase has enabled the development of formulas to estimate the PMI. However, the accuracy of the method decreases with extended PMIs, particularly beyond a few days, and can be influenced by factors such as temperature and individual baseline variations. In addition to K^+ , other biochemical markers in vitreous humor, such as Na^+ , Cl^- , glucose, and hypoxanthine (Hx), also display predictable *postmortem* trends. Hx, in particular, rises significantly after death and serves as a complementary indicator, while Na^+ , Cl^- , and glucose levels gradually decrease (78). Recent advancements have enhanced PMI prediction by integrating multi-

parameter approaches, such as combining K^+ and Hx measurements, and by analyzing other electrolytes like Ca^{2+} . Advanced techniques, including spectroscopy, chemometrics, thermogravimetry, and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), have further refined data interpretation and improved model accuracy (103, 104). Machine learning applications are also being explored to optimize these methods, providing more robust and precise PMI estimation tools (102).

Synovial fluid becomes especially valuable in PMI estimation when vitreous humor is unavailable, as the progression of its K^+ concentration progression closely mirrors that of vitreous humor (105). Tumram *et al.* (106) demonstrated that increasing K^+ and decreasing glucose levels in synovial fluid are at least as accurate, if not more accurate, than those in vitreous humor for determining the PMI. More recently, K^+ concentration in synovial fluid was proposed as the most promising biomarker to estimate PMI (99). Furthermore, a study by Srettabunjong *et al.* (107) revealed significant correlations between the concentrations of Na^+ , K^+ , glucose, lactate, urea, uric acid, and creatinine in both fluids (Table 2), although Cl^- and Mg^{2+} concentrations did not show the same level of correlation.

Blood analysis can also provide valuable insights for estimating the PMI, as several biochemical parameters in blood undergo significant changes after death. Notably, total and direct bilirubin, urea (breakdown of amino acids and pyrimidine bases, with nitrogen being converted to urea primarily in the liver), uric acid, transferrin, and immunoglobulin M (IgM), along with enzymes such as creatine kinase (CK) and aspartate transaminase (AST), show linear correlations with PMI (Table 2). Additionally, electrolytes such as Ca^{2+} and iron also exhibit notable *postmortem* changes (16). Blood glucose concentrations decrease rapidly after death, while lactate levels increase, often reaching up to 60 times their *antemortem* values. This dramatic increase is attributed to the metabolic shift from aerobic to anaerobic pathways following death (16). Creatinine, a byproduct of phosphocreatine degradation, is also released into the bloodstream *postmortem*. Research indicates that creatinine concentrations rise sharply within the first 24 hours after death, after which they stabilize. pH levels decrease slightly during the first five days and then continuously increase thereafter. High mobility group box-1 (HMGB1) has been proposed as a potential protein marker released upon necrosis as it shows a time-dependent increase, peaking at day 3 at 14°C before decreasing and plateauing, while at 24°C, it peaks at day 2 (108). The oxidation-reduction potential (ORP), has a strong positive correlation with PMI across different temperatures (109). Myoglobin levels in blood, but also in liver and parotid glands, have been shown to correlate significantly with PMI: as the PMI increases, myoglobin concentration also rises, making it

a reliable biochemical marker for estimating the PMI (110, 111). These biochemical alterations in blood can be used to create mathematical models to estimate PMI more accurately. However, environmental factors and individual variations can influence these changes. Thus, combining multiple biomarkers often yields the most reliable results. C-reactive protein (CRP) is a significant biochemical marker widely studied in forensic and clinical settings for its role in determining the cause of death and estimating survival time. *Postmortem* CRP analysis reveals distinct patterns that can provide valuable insights into the physiological state preceding death. Generally, CRP levels decrease by approximately 35% *postmortem* but remain relatively stable in blood for up to six days (112). Elevated *postmortem* CRP levels are often indicative of an inflammatory process that was active prior to death. CRP levels also correlate with survival time, offering predictive value in various scenarios. In non-small cell lung cancer patients, higher CRP concentrations are associated with shorter survival times (median survival of 5.3 months compared to 18.5 months for patients with lower CRP levels) (113). Additionally, in acute deaths, CRP levels are generally lower in individuals who died immediately or within six hours of the triggering event (114). These findings underscore the potential of CRP as a reliable marker for estimating survival time and enhancing forensic investigations (115).

Cerebrospinal fluid (CSF) undergoes various biochemical and cellular changes after death, offering valuable insights for estimating the PMI and understanding the circumstances of death. Electrolyte levels in CSF shift noticeably *postmortem* (Table 2). While Na and chloride concentrations decrease, K⁺ levels rise significantly, showing an average increase of 1.21 meq/h up to 25 hours *postmortem*. Calcium levels remain relatively stable, and magnesium may show a slight increase, although no strong correlation with PMI has been established. In contrast, phosphate levels increase markedly due to *postmortem* esterase activity (116-118). Other biochemical markers in CSF, such as glucose and protein concentrations, change predictably after death. Glucose levels decline rapidly, while total protein levels increase, reflecting changes in protein profiles over time (119). Overall, the Na⁺-K⁺ ratio in CSF appears to be a particularly promising parameter for more accurate PMI estimation compared to individual ion values (117).

Enzymatic activities, including AST and alanine transaminase (ALT), rise *postmortem*, alongside a pronounced exponential increase in Hx during the early *postmortem* period (Table 2) (117, 120). Cellular changes in CSF also occur. Red blood cell counts increase as PMI progresses, while blood cells (pleocytosis) become more common, particularly in acute infections before death (120). While changes in CSF can offer valuable insights for PMI

estimation, their reliability is influenced by numerous factors, including environmental conditions, injuries, individual characteristics, and the cause and timing of death. These variables can limit the accuracy of CSF-based methods, particularly for extended PMIs (23).

Muscle and organ tissues undergo significant biochemical and structural changes after death, also offering valuable insights for estimating the PMI (121). Among the key biochemical markers, ATP and its degradation products provide a reliable framework for PMI estimation (Table 2). ATP levels in tissues decrease rapidly after death, following a predictable breakdown pattern: ATP → adenosine-5'-diphosphate (ADP) → adenosine-5'-monophosphate (AMP) → inosine-5'-monophosphate (IMP) → inosine (HxR) → Hx. This process occurs across multiple organs, including the brain, spleen, and kidney, and is temperature-dependent, with faster degradation at higher temperatures (122). Enzymatic activity also changes *postmortem* and varies by tissue type. In dermal connective tissues near wound edges, alkaline phosphatase, acid phosphatase, and esterase activities decrease within two hours after death, while esterase activity in the epidermis and hair follicles may increase during the same period (123). These enzymatic changes, however, are influenced by environmental factors, which can complicate their use in PMI estimation (124).

Skeletal muscle tissue undergoes notable biochemical and structural alterations after death. While passive mechanical properties of muscle bundles and collagen content remain stable for up to seven days, the degradation of titin, a protein essential for muscle elasticity, decreases up to 80% over this period. Myosin heavy chain composition also remains unchanged within this timeframe (121). Histological examinations reveal no significant changes in muscle tissue at three hours *postmortem*, but focal areas of autolysis appear by 24 hours, with progressive autolysis occurring in subsequent days (125).

Volatile Organic Compounds (VOCs) have emerged as valuable tools for estimating the PMI by analyzing the chemical changes that occur during decomposition. Among these compounds, putrescine and cadaverine stand out as particularly significant biomarkers. Different studies have explored their potential to provide reliable PMI estimates. Zhiyuan *et al.* (84) analyzed VOCs in rat muscle samples collected at different PMIs, revealing a clear pattern of progression during decomposition. A study using rat muscle samples identified a three-stage decomposition model based on the number and total peak area of VOCs detected. In the first stage, spanning 1 day *postmortem*, no VOCs were observed. The second stage, covering days 2 to 5, showed a rapid increase in both VOC species and their total peak areas. During the final stage, from days 6 to 10, the increase in VOCs continued but at a slower rate (84).

Another study in brain tissue showed significant correlations of both putrescine and cadaverine with PMI (82). Notably, putrescine proved to be a more accurate biomarker, with 99.5% of its variability linked to the timing of sampling, compared to 75.2% for cadaverine. This underscores the potential of these polyamines as precise indicators of decomposition stages. Using an electronic nose, Mazzatenta *et al.* (83) recorded volatile metabolites emitted from human tissues and isolated muscle cells at various intervals (0, 24, 48, and 72 hours *postmortem*). This approach enabled the creation of PMI volatomics fingerprints, highlighting critical decomposition points at 24 hours for whole tissues and at 48 hours for isolated cells.

All the biochemical changes aforementioned occur at different rates and can be influenced by various factors such as ambient temperature, cadaver composition, and the cause of death. Therefore, a combination of multiple biochemical markers is often used for more accurate PMI estimation. Ongoing research continues to identify and validate new biochemical markers for improved PMI determination (83).

Table 2: Evidence of *postmortem* fluctuations of biochemical markers.

	Biochemical Markers	Levels after death	References
Glucose	Vitreous humor	↓ gradually	(78)
	Synovial fluid	↓	(106)
	Blood	↓ quickly	(16)
	Cerebrospinal fluid	↓ quickly	(119)
Potassium	Vitreous humor	↑ linearly	(78)
	Synovial fluid	↑ linearly	(106)
	Cerebrospinal fluid	↑ significantly	(116-118)

Sodium	Vitreous humor	↓ gradually	(78)
	Cerebrospinal fluid	↓	(116-118)
Chlorine	Vitreous humor	↓ gradually	(78)
Lactate	Blood	↑ drastically	(16)
Creatinine	Synovial fluid	↑	(126)
	Blood	↑ sharply in the first 24 hours and then stabilizes	(108)
pH	Blood	↓ slightly during the first 5 days and then ↑ continuously	(108)
Myoglobin	Blood	↑ in the blood, liver, and parotid glands	(110, 111)

C-reactive protein	Blood	↓ approximately 35%, but remain relatively stable for up to 6 days	(112)
Chloride	Synovial fluid	↓	(106)
	Cerebrospinal fluid	↓	(116-118)
Calcium	Blood	↓ significantly and linearly with the putrefaction time	(16)
	Cerebrospinal fluid	Relatively stable	(116-118)
Phosphate	Cerebrospinal fluid	↑ sharply	(116-118)
Total protein	Cerebrospinal fluid	↑	(119)
Enzymatic activity	Cerebrospinal fluid	↑	(117, 120)

Hypoxanthine	Vitreous humor	↑ significantly	(78)
	Cerebrospinal fluid	Exponential ↑ observed during the early <i>postmortem</i> period	(117, 120)
Urea	Synovial fluid	↑	(126)
	Blood	↑	(16)
Uric Acid	Synovial fluid	↓	(126)
	Blood	↓ linearly	(16)
Total and Direct Bilirubin	Blood	↓ significantly and linearly with the putrefaction time	(16)
Transferrin	Blood	↓ significantly and linearly with the putrefaction time	(16)

Immunoglobulin M	Blood	↓	(16)
Creatine kinase	Blood	↑	(16)
Iron	Blood	↑	(16)
Adenosine Triphosphate	Muscle and organ tissues	↓ quickly in the brain, spleen, and kidney	(122)
Alkaline phosphatase	Muscle and organ tissues	↓ over the next 2 hours in the dermal connective tissues	(123)
Acid phosphatase	Muscle and organ tissues	↓ over the next 2 hours in the dermal connective tissues	(123)

Esterase enzymes	Muscle and organ tissues	↓ over the next 2 hours in the dermal connective tissues ↑ in hair follicles and epidermis	(123)
Titin	Muscle and organ tissues	↓ quickly	(121)
Putrescine and cadaverine	Volatile Organic Compounds	Rapid ↑ 2 to 5 days in the muscle Continuous ↑ from 6 to 10 days, but at a slower pace ↑ in brain tissue	(82, 84)
High mobility group box-1	Blood	Time-dependent ↑, peaking at day 3 at 14°C before ↓ and plateauing; at 24°C, it peaks at day 2	(108)

Magnesium

Blood

may show a slight ↑

(116-118)

I.3. Integrating biochemical and microbial signatures for enhanced PMI estimation

The integration of biochemical and microbial signatures of death presents a promising avenue for enhancing the accuracy of PMI estimation in forensic investigations. Traditional methods often yield significant margins of error, particularly beyond 48 hours *postmortem*. Recent advancements in metabolomics, microbial analysis, and proteomics offer innovative approaches to refine PMI predictions. The integration of multiple approaches could yield the most reliable PMI estimations, balancing the strengths and weaknesses of each method.

Proteomics, the study of all expressed proteins and their changes under physiological and environmental conditions, is a versatile technique with numerous forensic applications due to its molecular-level insights (127). While DNA is often associated with biological evidence in forensic investigations, proteins are more robust and can endure in degraded samples, making them a valuable alternative or complement when DNA is damaged or unavailable (79). Protein degradation into smaller peptides, and ultimately, amino acids, is catalyzed by proteolytic enzymes (proteases), especially by the calpains, which are recognized to be responsible for a large part of proteolytic activity, as this enzyme class is ATP-independent in its activation (26). Recent evidence shows that *postmortem* degradation of certain proteins occurs in a predictable manner, making protein analysis a promising tool for PMI estimation (80, 81). Researchers propose examining the progression of protein degradation to correlate specific alterations with PMI ranges. This approach involves identifying suitable marker proteins, analyzing *postmortem* changes, and accounting for influencing factors. Degradation profiles can provide a relative timeline of events or, ideally, back-calculate to the time of death based on time-dependent curves. Proteomics directly detects biomolecular sequences, making it well-suited for analyzing poorly characterized evidence frequently encountered in forensic cases. For example, *postmortem* changes in bone protein profiles *postmortem* can provide insights into decomposition patterns, utilizing techniques like mass spectroscopy to link protein alterations to specific PMIs (128).

During decomposition, the evolving necrobiome produces various metabolites that can be biochemically detected. For instance, the *postmortem* increase in formic acid is likely due to bacterial putrefaction rather than anaerobic metabolism (100). Tracking changes in microbial communities alongside metabolite concentrations could yield more accurate PMI estimates. Blood pH decreases rapidly *postmortem*, influencing microbial growth and succession patterns. Exploring the interplay between pH changes and microbial shifts can

provide a more comprehensive framework for estimating the PMI (100, 129). Microbial colonization occurs at organ-specific rates after death. For example, bacterial presence is detectable in the liver after one day, while 50% of pericardial fluid samples remain sterile even after five days (10, 129). *Postmortem* anaerobic metabolism leads to the accumulation of lactic acid and nicotinamide adenine dinucleotide reduced form (NADH), altering the biochemical environment and influencing microbial community composition (100).

Metagenomics, metatranscriptomics, and metabolomics offer powerful approaches for analyzing microbial and biochemical activities during decomposition, providing valuable insights for PMI estimation. Metagenomics, the study of microbial community genomes, serves as the foundational step in microbiome research. Its primary goal is to infer the taxonomic profile of a microbial community. While whole-metagenome sequencing offers limited insights into functional profiles, metatranscriptomics—sequencing the community's entire transcriptome—provides a deeper understanding by identifying genes actively expressed under specific conditions. While metagenomics answers "what is the microbial community composition?", metatranscriptomics explores "what genes are expressed together?", and metabolomics focuses on "what byproducts are produced?" (85). Metatranscriptomics can be associated with a thanatobiological approach for PMI estimation, which corresponds to a comprehensive gene expression analysis in the field of thanatobiology (26). This approach has shown marked changes in the expression of specific genes after death, and this change is independent of *postmortem* RNA degradation, enabling the identification of potential thanatotranscriptome biomarkers (86). Metabolomics offers a comprehensive view of biochemical changes during decomposition, with metabolite analysis revealing potential biomarkers for PMI estimation (87). These changes can serve as biomarkers or "fingerprints" that can be complex to interpret due to variability influenced by external and internal factors. In this context, machine learning models have been applied to metabolomic data, uncovering hidden correlations and improving PMI prediction accuracy compared to traditional methods (88).

Nevertheless, scarce studies have simultaneously assessed microbial changes along with biochemical analysis. Two remarkable studies by Liu *et al* (130, 131) combined microbial community characterization, microbiome sequencing from different organs, and machine learning algorithms, while examining differences in the metabolism among different organs. In the first study, dating from 2020 (130), the authors described significant differences in the prevalence of particular bacterial species between the death point and advanced decay stages, suggesting *Enterococcus faecalis*, *Anaerosalibacter bizertensis*, and *Lactobacillus reuteri* as

promising biomarkers in the decomposition process (130). Simultaneously, they reported that following a decrease after cadaver rupture on day 3, there was a marked increase in genes associated with carbohydrate and amino acid metabolism on days 7 and 15. In the other study, dating from 2023 (131), the authors demonstrated that during the first day of decomposition, genera such as *Agrobacterium*, *Prevotella*, *Bacillus*, and *Turicibacter* were identified as time-relevant in internal organs. Pathways linked to lipid, amino acid, carbohydrate, and terpenoid metabolism showed significant enrichment at 8 hours compared to 0.5 and 4 hours. These changes in microbiome composition and metabolic pathways were not only time-dependent but also organ-specific. Some amino acid metabolism pathways, such as those for glycine, serine, threonine, histidine, alanine, aspartate, glutamate, and lysine, were activated because of some bacterial behavior. For example, the lysine biosynthetic pathway is closely associated with bacterial activity, and two products of this pathway, lysine and meso-diaminopimelate (m-DAP), are directly involved in bacterial cell wall synthesis. After 12 hours of decomposition, the pyruvate metabolism pathway was enriched in the kidney compared with the other organs. Lactic acid bacteria can produce pyruvate and lactate from carbohydrates, organic acids, and amino acids. These findings underscore the intricate relationship between microbial activity and metabolic pathways, highlighting their direct impact on biomarker fluctuations during *postmortem* decomposition. Another study by Hu *et al.* (132) integrated microbial data with biochemical markers through a multimodal approach to improve the accuracy of PMI estimation. They highlighted the dynamic changes in microbial communities during decomposition, such as the shift from anaerobic to aerobic bacteria, and their potential as reliable PMI indicators. Additionally, biochemical markers like metabolites and VOCs offer complementary insights into the decomposition process. The authors advocated for a multimodal approach combining microbial and biochemical data, which provided more accurate and robust PMI estimation models when paired with machine learning. This integration represents a significant advancement in forensic sciences, offering objective tools for later-stage decomposition analysis. Zapico *et al.* (4) discussed new methodologies for PMI estimation by analyzing changes in human tissues and microbial communities, exploring the fields of thanatobiology and thanatomicrobiome.

Although studies integrating different research areas remain limited, the intricate interplay between metabolites, proteins, and microbes is undeniable. Protein degradation after death is systematically driven by microbial and enzymatic activity, which begins as metabolic processes cease (133). Addressing these dynamic changes over time through multi-omics integration may provide a more comprehensive understanding of *postmortem*

processes. By leveraging the combined strengths of metagenomics, metatranscriptomics, and metabolomics, researchers can uncover a detailed picture of the intricate relationship between microbial activity and biochemical changes during decomposition, enabling the development of more accurate and robust PMI estimation methods.

2. Objectives

The decomposition of the human body is a multifaceted process involving both biological and chemical changes that are instrumental in forensic analysis. The methods used to characterize *postmortem* changes can be broadly categorized into six main types: physical, physicochemical, biochemical, microbiological, entomological, and botanical (38). A growing body of research underscores the significant potential of utilizing *postmortem* microbial succession and biochemical transformations as forensic tools for accurate PMI estimation.

Most studies focus on extensive bacterial sequencing of *postmortem* samples, but culture-based experiments for higher taxonomic resolution of bacteria are still limited. The combination of microbial and biochemical/metabolic analyses is also underexplored (131).

Based on preliminary results of the research team, the main goal of this project was to perform a combined microbial and biochemical analysis of different tissues at different *postmortem* time points under natural conditions. Thus, using specific-pathogen-free (SPF) mice, to elucidate the dynamic interplay between microbial activity and biochemical changes during decomposition, the following specific objectives were defined:

- To analyze the total bacterial load and map two bacterial species *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*), both part of the intestinal microbiota, in different tissues (lungs, heart, kidneys, liver, and brain), and assess their evolution over different *postmortem* time points;
- To evaluate the dynamics of different biochemical parameters, including ethanol, glucose, lactate, magnesium, uric acid, urea, iron, and total proteins in the supernatant of homogenized tissue samples over different *postmortem* time points.

3. Materials and methods

3.1. Ethics statement

Animal experimentation was carried out in compliance with the European Guidelines for Animal Welfare (2010/63/EU), transposed into Portuguese legislation through Law 113/7 of August 2013, and adhered to the National Ethics Council for Life Sciences (CNECV) recommendations. All animal procedures were approved by the Organization Responsible for Animal Welfare at the University Institute of Health Sciences (ORBEA-IUCS), under reference ORBEA/IUCS/CESPU-001/2023.

3.2. Animal model, sacrifice and time points

Male C57BL/6J SPF and immunocompetent mice, aged 6–7 weeks, were purchased from the Institute of Research and Innovation in Health (i3S) and group-housed in the Animal Facility of the University Institute of Health Sciences under standard conditions, including a 12-hour light/dark cycle, a temperature of 21.0 ± 1.5 °C, and a relative humidity of $50.0 \pm 7.0\%$, with *ad libitum* access to food and water. The animals were acclimatized under these conditions for 20 days before the experiments began.

A single experiment was conducted. At the start of the study (9–10 weeks of age), the mice weighed between 22.3 g and 27.8 g. They were anesthetized in an isoflurane chamber, followed by euthanasia by cervical dislocation (Figure 2), and then left at a room temperature of 21 °C for the desired PMI. A total of 18 mice were used, with three animals assigned to each of six different PMI: 0, 12, 24, 48, 72, and 96 hours. At each PMI, various organs were collected, including the brain, heart, lungs, liver, and kidneys (Figure 3).

All surgical instruments were sterilized by immersion in 70% ethanol and allowed to dry at room temperature before organ collection. The organs were harvested on a clean and disinfected dissection board.

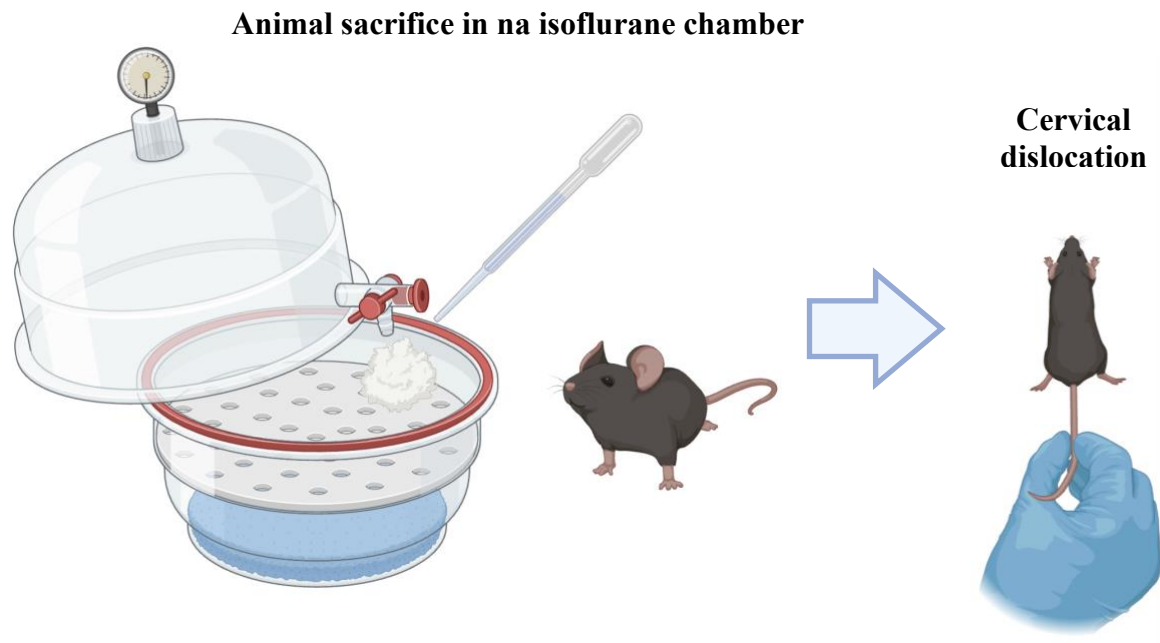


Figure 2. Illustration of the procedure used for animal sacrifice in an isoflurane chamber followed by cervical dislocation.

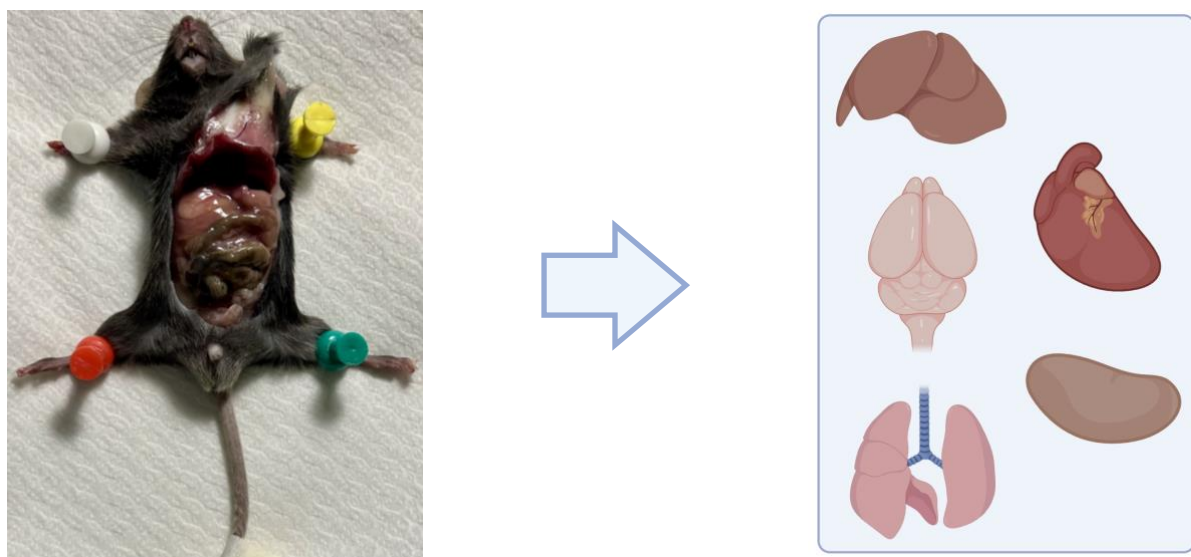


Figure 3. Procedure used for organ collection.

3.3. Sample collection

Initially, fecal pellets from the boxes where the mice were housed were collected to confirm colonization by *E. faecalis* and *E. coli*.

To ensure the best possible collection of organs, the mouse carcass was placed in the dorsal decubitus position. An incision was made along the midline to open the abdominal cavity, extending cranially to the thorax and caudally to the pubis, with the abdominal walls retracted laterally. Once the relevant organs (liver, kidneys, lungs, and heart) were exposed, they were carefully harvested using sterile forceps and scissors to avoid cross-contamination. Each organ was immediately weighed after harvesting and quickly frozen in liquid nitrogen in separate 15 ml tubes. Small scissors were used to collect the brain. The lower blade was inserted into the opening where the skull meets the spinal canal, and a straight cut was made upwards along the midline of the skull. With tweezers, the two halves of the skull were separated to expose the brain, which was then carefully extracted. All organs were stored at -80 °C until further microbiological and biochemical analyses.

3.4. Culture media used

The culture media were prepared according to the manufacturers' instructions.

- To isolate and enumerate *Enterococcus* species relative to other genera, Slanetz & Bartley Agar (SLB) medium supplemented with triphenyltetrazolium chloride (TTC) was used. This highly selective medium contains sodium azide, which inhibits the growth of Gram-negative bacteria. Triphenyltetrazolium chloride is reduced to insoluble formazan within bacterial cells, producing colonies that range in color from light pink to dark red. The agar plates were incubated at 37 °C for 48 hours to allow bacterial growth and color development. *E. faecalis*, the target species in this study, forms dark wine-colored colonies, whereas *E. faecium* typically produces pink colonies.
- MacConkey Agar (MAC) is a specialized medium that selectively supports Gram-negative bacteria and differentiates them based on lactose metabolism. Lactose fermenters, such as *E. coli*, form pink colonies.
- Columbia Blood Agar (CBA), specifically Columbia Agar with 5% sheep's blood, is a nutrient-rich microbiological medium used to cultivate and enumerate a wide range of microorganisms. It contains agar, essential nutrients, and 5% sheep's blood, supporting bacterial growth while enabling differentiation based on hemolytic activity. Bacteria exhibit distinct patterns of red blood cell degradation, including alpha-hemolysis, beta-hemolysis, and gamma-hemolysis. Additionally, this medium

is proper for bacterial isolation to obtain fresh cultures. It was used in this study to allow the growth of several bacterial species to enable the total bacterial load count.

- Brain Heart Infusion (BHI) Agar, a non-selective nutrient-rich medium, supports the growth of a wide range of microorganisms, including bacteria and fungi. In this study, it was used to cultivate bacteria and generate new colonies, ensuring the availability of fresh pure cultures whenever needed.

MAC, CBA, and BHI Agar media were autoclaved, cooled, and poured into Petri dishes under aseptic conditions. The SLB medium was not autoclaved, as triphenyltetrazolium chloride is heat-sensitive.

- Buffered Peptone Water (BPW) is a versatile pre-enrichment medium to recover bacteria with sublethal damage. Its neutral pH and nutrient-rich composition facilitate bacterial repair and growth before isolation on selective media.
- Tryptone Soya Broth (TSB) medium with 15% glycerol was sterilized by autoclaving. Glycerol is an additional carbon source and a cryoprotectant for long-term storage. This medium is widely used for bacterial cultivation, maintenance, and preservation.

3.5. Culturomic analysis: Total aerobic bacterial load and counts of *Escherichia coli* and *Enterococcus faecalis*

Fecal samples were resuspended and homogenized in BPW at a 1:10 ratio (Figure 4). Serial 1:10 dilutions were then prepared by adding 100 µL of the solution to 900 µL of sterile saline. Next, 100 µL of each dilution were inoculated onto selective culture media: SLB for *Enterococcus* spp. and MAC medium for *Enterobacteriaceae*, enabling the quantitative detection of *E. faecalis* and *E. coli*, respectively (134). For total bacterial count, 100 µL of each sample were plated onto blood agar and incubated under aerobic conditions at 37 °C for 24-48 hours.

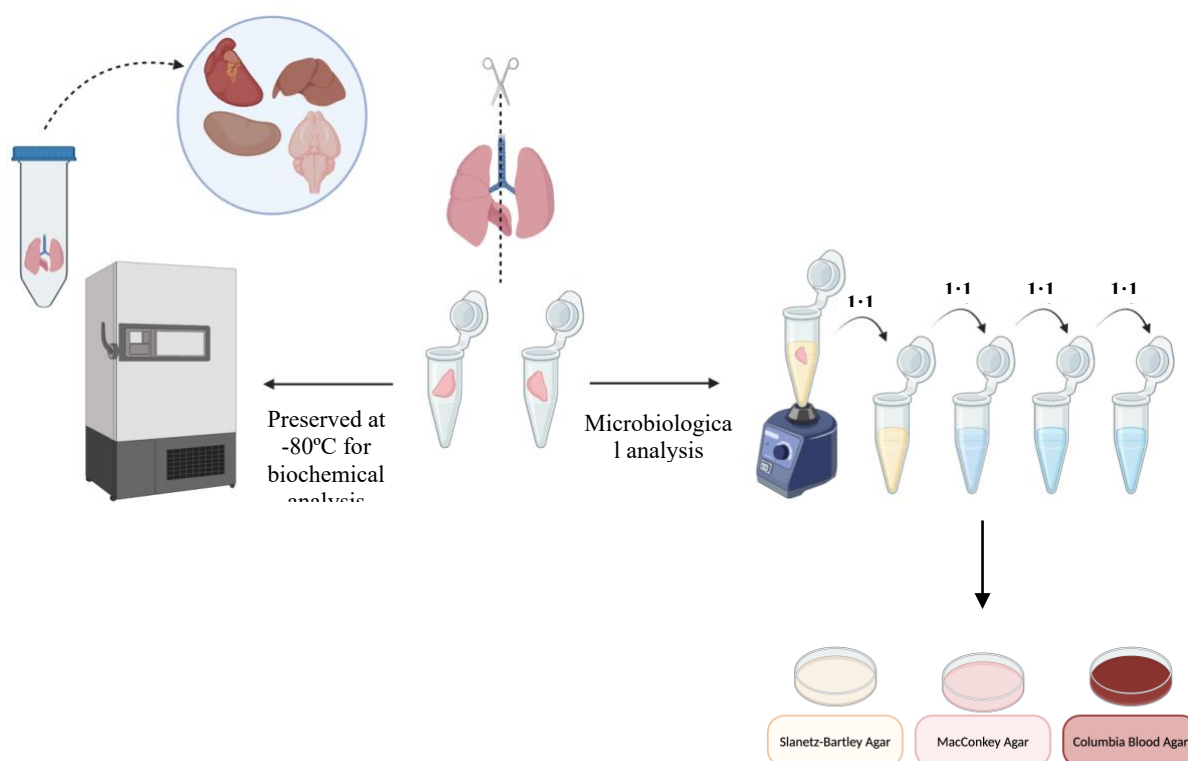


Figure 4. Illustration of the sample processing protocol for microbiological quantifications in fecal samples. Processed samples were inoculated in different media, including MacConkey Agar (MAC), Slanetz & Bartley Agar (SLB), and Columbia Blood Agar (CBA).

The collected organs were divided into two portions - one for microbiological analyses (Figure 5) and the other for biochemical analyses.

For microbiological analyses of the different organs, BPW was added at a 1:10 ratio, and the organs were homogenized by vortexing, followed by mechanical disruption using a manual pellet pestle homogenizer. Subsequently, 100 μ L of each dilution were inoculated onto SLB, MAC and CBA media, and incubated under aerobic conditions for 24-48 hours.

The total bacterial count and the count of the two bacterial species of interest were expressed in Colony Forming Units (CFU) per gram of tissue (CFU/g), considering the initial quantity of tissue used (grams). In a few cases, the CFU number was estimated based on plates with fewer than 30 colonies in the absence of growth in other dilutions, acknowledging the limitations in statistical reliability as per ISO 7218 guidelines (135).

Enterococcus faecalis ATCC 29212 and *Escherichia coli* ATCC 25922 (American Type Culture Collection) were used as control strains. Colonies with typical and distinct morphologies were selected for later identification for each condition. These colonies were

purified on BHI agar and preserved at -80 °C in TSB medium with 15% glycerol. Each frozen isolate was given a code number for subsequent studies.

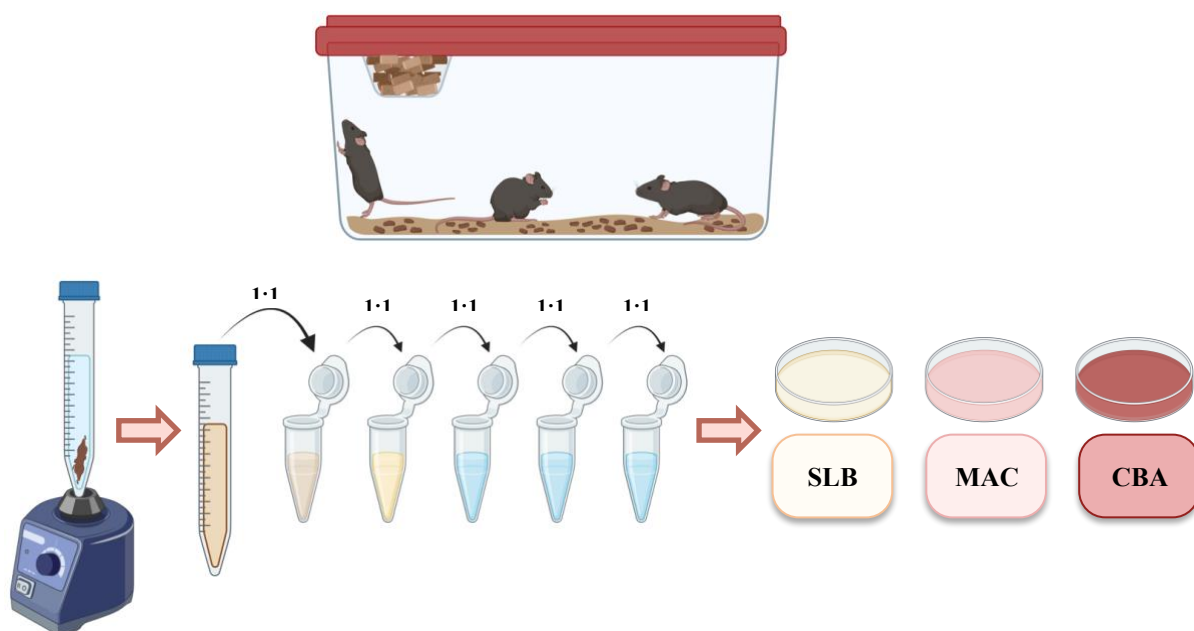


Figure 5. Diagram of the sample processing protocol for microbiological quantification of collected organ samples.

3.6. Bacterial species identification

Bacterial identification was carried out using the reference spectroscopy method with a Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) device (Figure 6). This essential diagnostic tool generates protein mass spectra for identification. Protein samples are ionized by mixing with a matrix solution, producing mass spectra within defined molecular weight ranges (136). Each distinct mass spectrum is a unique microbial fingerprint, enabling precise species-level identification for most bacterial strains.

The device employs a laser to fragment the sample into its constituent particles and molecules, measuring the time they travel through a defined space before reaching the detector. The resulting mass signals are then compared against bacterial strain libraries or public proteomics/genomics databases (136). Fresh pure bacterial cultures grown on solid media (Brain Heart Infusion agar) were used for analysis.

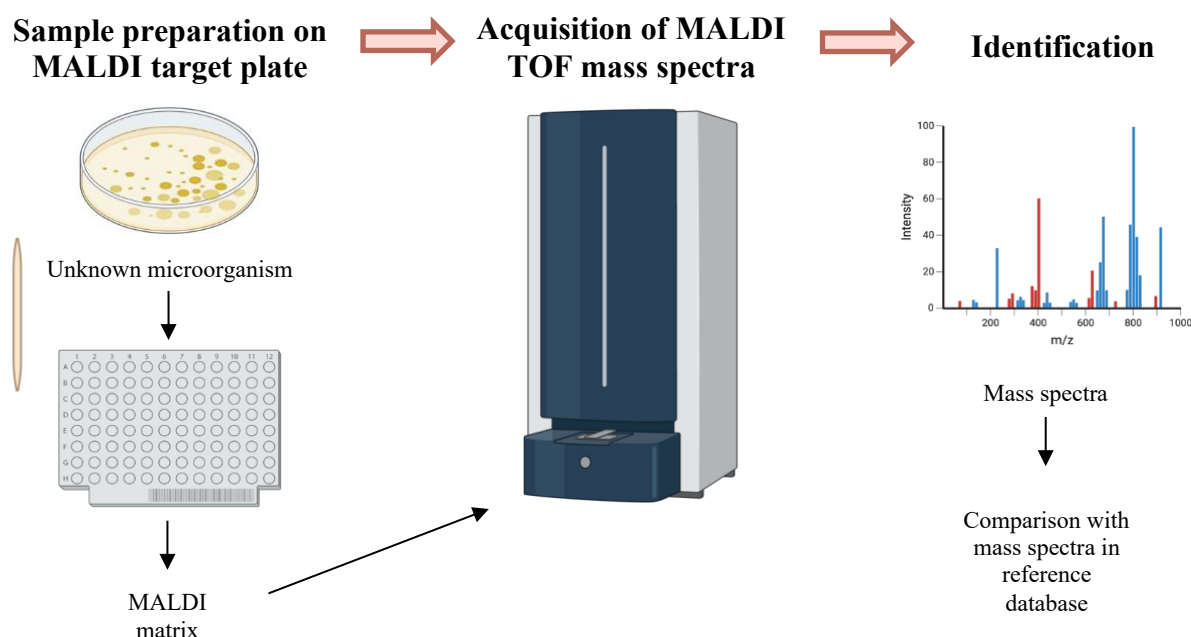


Figure 6. Schematic of the MALDI-TOF MS procedure for bacterial identification [adapted from (136)].

3.7. Biochemical analysis of microbial metabolism parameters and data analysis

3.7.1. Sample preparation

A 50 mM phosphate buffer (pH 7.4) was used for sample preparation. The buffer solution was prepared by dissolving 2.59 g of KH_2PO_4 and 5.52 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in approximately 900 mL of distilled water (dH_2O). As needed, the pH was adjusted to 7.4 using small volumes of 1 M HCl or 1 M NaOH. The final volume was brought to 1 L with dH_2O , and the solution was stored at 4 °C until use.

Tissue samples (~0.1 g) were homogenized in 1.5 mL tubes at a 1:4 ratio (w/v) in ice-cold 50 mM phosphate buffer (pH 7.4) using a manual pellet pestle homogenizer. Homogenization was performed on ice to prevent enzymatic degradation. The resulting homogenates were centrifuged at 13,000 rpm for 10 minutes at 4 °C in a refrigerated Eppendorf 5810 R centrifuge (Eppendorf, Hamburg, Germany). After centrifugation, the supernatants were carefully transferred to sterile 1.5 mL tubes and stored at -80 °C until biochemical analysis.

3.7.2. Automated biochemical analysis

Biochemical analyses were performed using the Accent MC240 automatic autoanalyzer (Cormay®, Łomianki, Poland), a benchtop device designed for the quantitative determination of analytes in biological samples such as serum, plasma, and urine, in accordance with the manufacturer's instructions, as previously described (137). The system operates based on spectrophotometric methods, utilizing specific enzymatic or colorimetric reactions for each parameter analyzed.

The Accent MC240 is widely employed in both clinical and research laboratories due to its efficiency, flexibility, and analytical robustness, making it suitable for routine diagnostics as well as scientific investigations. Calibration was performed using a multipoint curve with two appropriate calibrators per parameter, following the manufacturer's protocols. Results were validated through internal quality controls. All reagents used with the analyzer were supplied by Cormay®. To ensure analytical accuracy and prevent cross-contamination, the ACCENT Washing Solution (Cormay®) was used to clean both the sample and reagent probes. This cleaning procedure was carried out automatically, ensuring the removal of residual substances and minimizing carry-over effects.

The biochemical parameters analyzed included ethanol, glucose, lactate, magnesium, uric acid, urea, iron, and total proteins.

3.8. Statistical Data Analyses

Statistical analysis was performed using GraphPad Prism software, version 10.1.1 for macOS (GraphPad Software, Inc., San Diego, CA, USA). The values in the figures correspond to the mean $\pm$ standard deviation (SD) of the data obtained experimentally, calculated based on the number of PMI indicated in the respective captions. The normality of the data was assessed using the Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Statistical comparisons among groups were performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons.

Specifically, to generate a single formula for PMI calculation, the results of biochemical parameters were normalized to magnesium. Determination coefficient analysis was then used to identify parameters with determination values (R^2) greater than 0.900, which were subsequently used in the formula calculation. The mathematical models were developed

based on 96-hour kinetics, using regression analysis performed with the Microsoft Excel 2020 supplement for macOS (16).

4. Results and discussion

4.1. Culturomics for characterization of detectable *postmortem* bacterial communities

In contrast to DNA-based methods that detect both live and dead microbes, culturomics focuses on isolating and identifying viable bacterial species, providing a functional perspective on the dynamic *postmortem* microbiota. Prior to the collection of each organ, a photograph was taken of the corresponding mouse at each experimental time point, allowing for a more detailed analysis and documentation of the initial anatomical changes. Overall, a reduction in blood content within the organs was observed, resulting in a paler appearance, particularly notable in the liver (Figure 7). In the intestines, the presence of air bubbles became apparent around 24 hours *postmortem* and was more pronounced after 48 hours. The brain began to exhibit a gelatinous consistency approximately 24 hours after death.

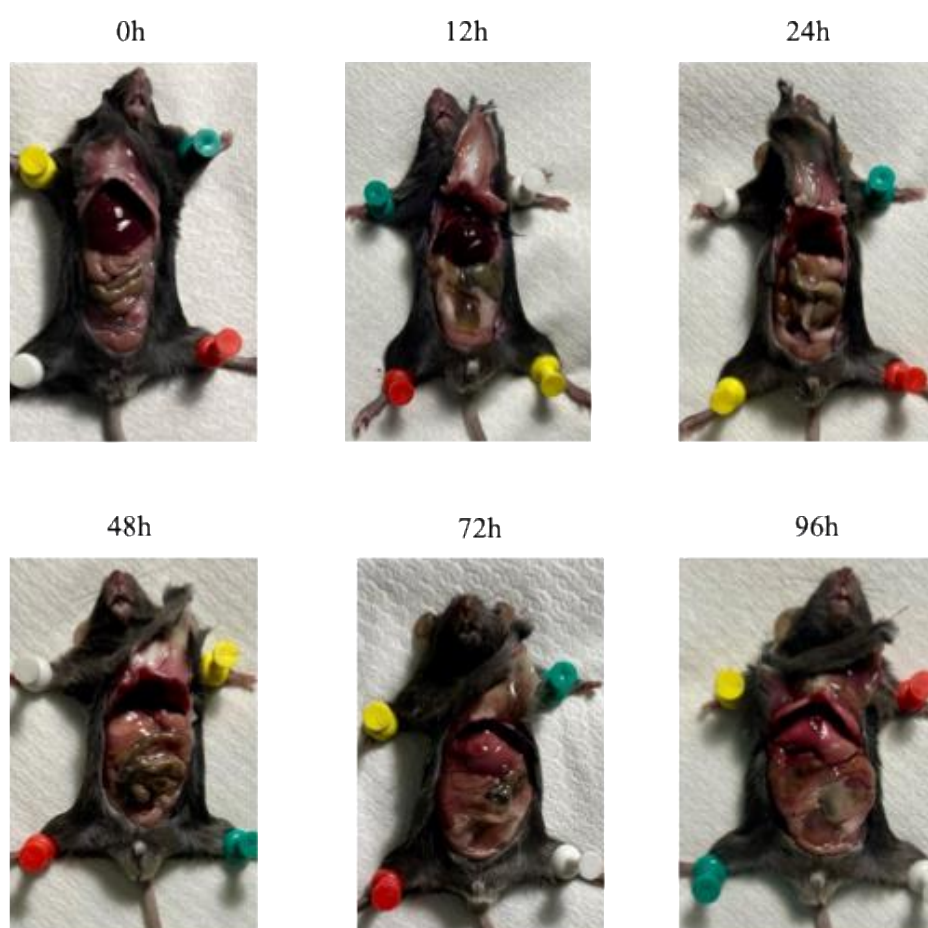


Figure 7. Photographic documentation of anatomical changes observed in dead animals after different PMIs (0, 12, 24, 48, 72, and 96 hours).

Additionally, an initial assessment of the fecal microbiota was carried out to identify potential interindividual variations that could influence experimental outcomes, which is an approach recommended in studies involving murine models (138). Fecal samples were cultured on selective media specific for the detection of *E. faecalis* (SLB) and *E. coli* (MAC), confirming the presence of both bacterial species. These findings align with the known commensal presence of *E. faecalis* and *E. coli* in the intestinal microbiota of both rodents and humans (139).

As part of the microbiological culture analysis, a total of 90 samples were collected from 18 animals, distributed across six time points over a 96-hour decomposition period. *Postmortem* changes in the microbial composition of various organs were assessed through visual inspection and quantified as CFU per gram of tissue in the brain, heart, lungs, kidneys, and liver.

Considering only the CBA medium (n = 90), which supports the growth of a broad range of bacterial species, including fastidious microorganisms, bacterial growth was detected in 31 samples (34%), while 59 samples showed no microbial development. Among the positive samples, most were classified as monocultures (20/31; 65%), whereas a smaller proportion were identified as mixed cultures (11/31; 35%). Both types of cultures, monocultures and mixed cultures, were observed across all organs analyzed (Figure 8).

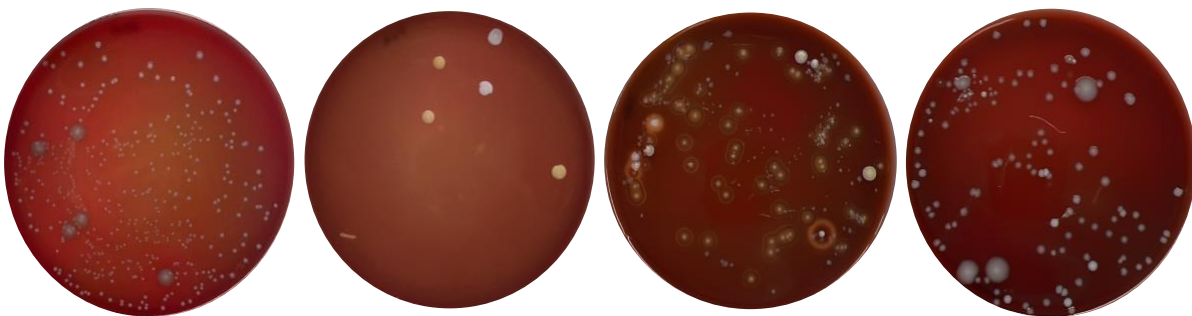


Figure 8. Representative Columbia Blood Agar plates illustrating distinct bacterial growth patterns of mixed cultures.

Regarding the cultures obtained on MAC (n = 90), a medium specifically formulated for the growth of Gram-negative bacteria, 84 samples showed no bacterial development, while 6 exhibited microbial growth. The majority of positive samples were identified as *E. coli* monocultures (66,7%) (Figure 9).

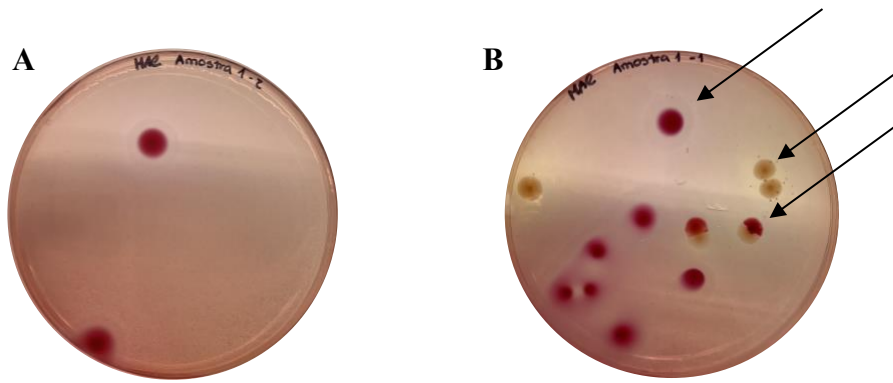


Figure 9. Representative MacConkey agar plates illustrating distinct bacterial growth patterns. (A) Typical growth of *E. coli* monocultures. (B) Typical growth of mixed cultures.

The SLB medium ($n = 90$), in which 64 samples showed no growth and 26 exhibited bacterial development, demonstrated the highest degree of specificity. About half cases showing bacterial growth corresponded to monocultures (14/26; 54%), exclusively linked to the isolation of *E. faecalis* (Figure 10).



Figure 10. Representative Slanetz & Bartley agar plates illustrating the typical growth of *E. faecalis* monocultures.

The bacterial growth observed across different culture media can theoretically be attributed to several factors, including genuine infection, agonal dissemination (a topic that remains controversial), *postmortem* transmigration, and contamination. It is widely accepted that the presence of a pure culture of a pathogen indicates a true infection and can be considered a valid result. Conversely, detecting mixed cultures composed of non-pathogenic microorganisms is generally interpreted as resulting from bacterial migration occurring after death (140).

4.2. Organ-specific dynamics of aerobic bacterial load and target species fluctuations during *postmortem* decomposition

Postmortem decomposition is a dynamic and progressive process marked by distinct microbial shifts that vary according to the organ and the PMI. During this process, a general increase in aerobic bacterial load was observed across the organs analyzed, reflecting ongoing microbial invasion and proliferation. The analysis of the total bacterial load (CFU/g) in the different organs across time was performed by a kinetic survey of *postmortem* changes in the main bacterial species that can grow aerobically by routine culture media (Figures 11-15).

Concurrently, the identification of target species cultured on selective media provided deeper insight into organ-specific microbial dynamics (see below). With a single exception (see below), we could not identify significant differences in bacterial counts between the different time points for each organ analyzed, probably due to the variability in results across the three assays and the limited number of animals used.

4.2.1. Kidneys

In total, 54 microbiological analyses were conducted on renal samples, corresponding to three distinct culture media evaluated across six *postmortem* time points (18 analyses per medium), as shown in Table 3. In the CBA medium, monocultures and mixed cultures were observed with comparable frequency. Similarly, in the selective media for *E. coli*, growth, when present, occurred exclusively as monocultures, reinforcing the hypothesis of selective, non-random colonization by this species and arguing against external contamination.

Analysis of kidney cultures revealed a mean total bacterial load ranging from a minimum of 4.0×10^3 CFU/g at 24 hours to a maximum of 2.5×10^6 CFU/g at 72 hours *postmortem*. The temporal progression of bacterial load exhibited a pattern consistent with bacterial transmigration, characterized by progressive growth up to 72 hours, followed by stabilization at 96 hours. Bacterial positivity followed an undulating pattern, with a marked peak at 72 hours, preceded by a transient decrease in load between 24 and 48 hours (Table 3, Figure 11.A), mirroring the temporal fluctuations described by Burcham *et al.* (141) who reported progressive and oscillatory colonization of previously sterile organs by commensal bacteria.

Table 3. Microbiological results of kidney samples at different *postmortem* time points.

Time points	Kidneys	Weight (g)	CFU/g CBA	CFU/g SLB	CFU/g MAC
0	1A	0.2273	0	0	0
	1B	0.1752	0	0	0
	1C	0.189	0	0	0
12	2A	0.2254	0	0	0
	2B	0.1893	0	0	0
	2C	0.2049	0	0	0
24	3A	0.1367	1.4×10^4 (P)	1.4×10^4 (P)	7.0×10^3 (M)
	3B	0.1985	4.0×10^5 (M)	5.0×10^5 (M)	0
	3C	0.1655	4.0×10^3 (M)	0	0
48	4A	0.1672	0	0	0
	4B	0.2102	0	0	0
	4C	0.1712	1.7×10^4 (M)	1.2×10^4 (P)	
72	5A	0.1787	2.5×10^6 (M)	3.2×10^6 (M)	5.0×10^3 (M)
	5B	0.1433	8.0×10^3 (P)	4.0×10^3 (P)	0
	5C	0.1628	8.0×10^3 (P)	5.0×10^3 (M)	0
96	6A	0.119	0	0	0
	6B	0.1759	0	0	0
	6C	0.1721	1.8×10^4 (P)	8.0×10^3 (M)	0

Abbreviations: CBA (Columbia Blood Agar medium); CFU/g (colony-forming units per gram of tissue); MAC (MacConkey Agar medium); SLB (Slanetz & Bartley Agar medium). (M) indicates monoculture growth; (P) indicates polyculture growth.

Specific analysis of *E. faecalis* (Table 3, Figure 11.B) revealed colonization beginning at 24 hours *postmortem*, followed by qualitative fluctuations and a peak at 72 hours. In contrast, *E. coli* (Table 3, Figure 11.C) displayed a more sporadic presence and only for one experiment, with detection restricted to 24 and 72 hours *postmortem*. This intermittent pattern, marked by delayed peaks, may be associated with local biochemical changes since it has been proposed that the kidneys may sense changes in the levels of metabolites derived

from the gastrointestinal microbiome and selectively favor the proliferation of certain bacterial species (142).

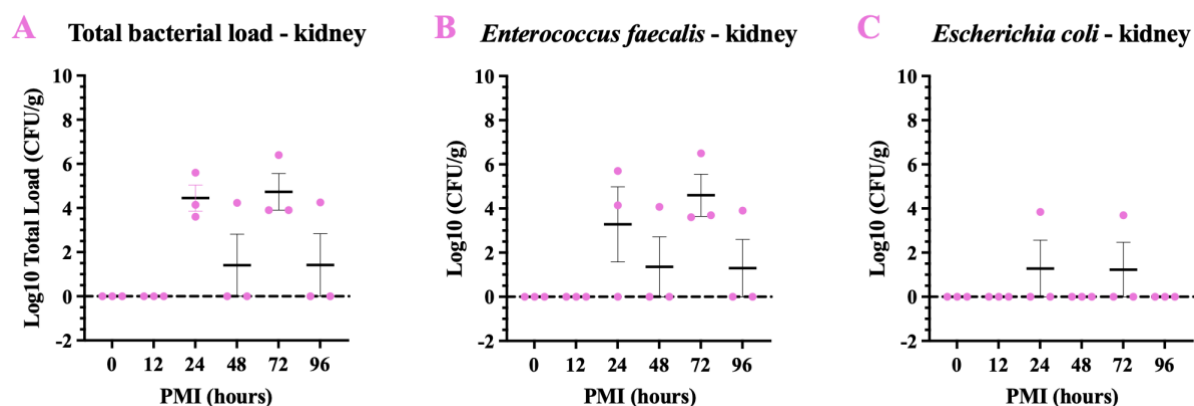


Figure 11. Kinetic cultural analysis of the *postmortem* kidney microbiota.

Total bacterial load (A), *E. faecalis* load (B), and *E. coli* load (C) were quantified as colony-forming units (CFU) per gram of kidney tissue using selective culture media: Columbia Blood Agar, Slanetz & Bartley Agar, and MacConkey Agar, respectively. Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons.

In all cases, bacterial growth was only detected after 24 hours. Liu *et al.* (131) demonstrated that, after 12 hours of decomposition, the kidneys show a specific enrichment in the pyruvate metabolic pathway, potentially acting as a metabolic driver for bacterial growth from this timepoint onward and distinguishing this organ in terms of susceptibility to colonization. More studies focusing on *E. faecalis*, in particular, may be useful for better exploring the potential of this species as a potential biomarker of kidney invasion after the 24-hour mark.

4.2.2. Liver

A total of 54 microbiological analyses were performed on liver samples from three different culture media evaluated at six *postmortem* time points (18 analyses per medium), as shown in Table 4. In CBA medium, growth was predominantly observed as mixed cultures. Similarly, on selective media for *E. faecalis*, growth, when present, occurred with both mono- and polycultures. In all cases, no *E. coli* growth was detected on MAC medium.

Table 4. Microbiological results of liver samples at different *postmortem* time points.

Time points	Liver	Weight (g)	CFU/g CBA	CFU/g SLB	CFU/g MAC
0	1A	0.4719	0	0	0
	1B	0.8739	3.4×10^4 (P)	2.8×10^4 (M)	0
	1C	0.5148	0	0	0
12	2A	0.6887	4.0×10^3 (M)	0	0
	2B	0.5604	0	0	0
	2C	0.4464	0	0	0
24	3A	0.6375	0	0	0
	3B	0.7614	0	0	0
	3C	0.7052	0	0	0
48	4A	0.6556	9.0×10^3 (P)	4.0×10^3 (P)	0
	4B	0.5394	0	0	0
	4C	0.6039	0	0	0
72	5A	0.5485	0	0	0
	5B	0.4024	4.0×10^3 (P)	0	0
	5C	0.4616	0	0	0
96	6A	0.3255	0	0	0
	6B	0.4028	0	0	0
	6C	0.3865	0	0	0

Abbreviations: CBA (Columbia Blood Agar medium); CFU/g (colony-forming units per gram of tissue); MAC (MacConkey Agar medium); SLB (Slanetz & Bartley Agar medium). (M) indicates monoculture growth; (P) indicates polyculture growth.

The analysis of liver samples showed an average load ranging from a maximum of 3.4×10^4 CFU/g at 0 hours to a lower values at subsequent time points, reaching no growth at 24 hours. From that point onward, there was a renewed increase up to 9×10^3 CFU/g at 48 hours, followed by a slight reduction but consistent peak at 72 hours (4×10^3 CFU/g). The absence of detectable growth at 96 hours suggests stabilization or a decline in bacterial activity during this final phase (Table 4, Figure 12.A).

This undulating profile, marked by an initial reduction in bacterial load followed by fluctuations, aligns with the phenomena described by Heimesaat *et al.* (143), who also described a maximum peak at 72 hours but did not test longer time points. The detection of a substantial bacterial load at the 0-hour time point, despite variability across experiments, may reflect peri-agonal contamination or rapid bacterial translocation occurring immediately after death. However, comparison with similar studies using murine models remains difficult, as

none included a 0-hour time point measurement or quantified bacteria through culture-based methods as applied in the present work (131, 141, 143).

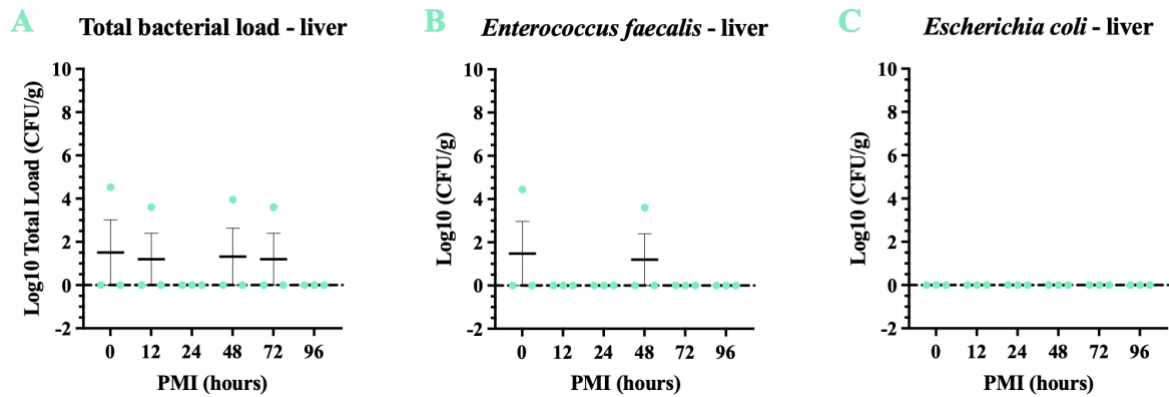


Figure 12. Kinetic cultural analysis of the *postmortem* hepatic microbiota.

Total bacterial load (A), *E. faecalis* load (B), and *E. coli* load (C) were quantified as colony-forming units (CFU) per gram of liver tissue using selective culture media: Columbia Blood Agar, Slanetz & Bartley Agar, and MacConkey Agar, respectively. Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons.

The detection of *E. faecalis* in liver tissue demonstrated a pattern of intermittent colonization throughout the *postmortem* period. This species was identified at 0 hours with an average load of 2.8×10^4 CFU/g, which declined to no growth from 12 to 24 hours, but the species reemerged at 48 hours (4×10^3 CFU/g). The absence of growth at certain time points may indicate the cultural limit of detection or fluctuating ability of *E. faecalis* to colonize hepatic tissue, possibly influenced by local viability factors or microbial competition (Table 4, Figure 12.B). This oscillatory pattern mirrors the dynamics reported by Burcham *et al.* (141), in which *postmortem* colonization of internal organs by commensal species follows a nonlinear trajectory shaped by the breakdown of anatomical barriers and the shifting composition of the intestinal microbiota. The early detection of *E. faecalis* at 0 hours may be attributable to peri-agonal contamination or rapid translocation, while its reappearance at later time points supports the hypothesis of intermittent migration from the intestine, as previously proposed by Heimesaat *et al.* (143). Also, Campobasso *et al.* (144) have described *Enterococcus* spp. as the dominant genus in liver samples of human cadavers, starting from the first samples, which were only collected after ~ 72 hours *postmortem*, and it resisted at

least until 168 hours *postmortem*. More studies, including a larger number of samples, are necessary to better assess the behavior of *E. faecalis* over time.

Selective cultures using MAC medium did not detect *E. coli* in liver samples across the PMIs examined (0–96 hours), as no bacterial growth was observed (Table 4, Figure 12.C). This absence may reflect either an inability of *E. coli* to colonize hepatic tissue within the examined timeframe or methodological limitations, such as insufficient sensitivity of the culture medium or compromised bacterial viability in liver samples. These findings contrast with patterns observed in other organs and with previous studies suggesting *postmortem* translocation of enteric bacteria like *E. coli* to internal tissues (143). Nevertheless, as Burcham *et al.* (141) noted, *postmortem* colonization can follow temporally and organ-specific trajectories, and it is plausible that *E. coli* colonizes the liver at later time points, or that its detection requires more sensitive, molecular-based techniques.

4.2.3. Lungs

A total of 54 microbiological analyses were performed on lung samples from three different culture media evaluated at six *postmortem* time points (18 analyses per medium), as shown in Table 5. In CBA medium, monoculture growth was predominant. Similarly, in selective media for *E. coli* and *E. faecalis*, growth, when present, was predominantly as monocultures, reinforcing the hypothesis of selective, non-random colonization by these species and contradicting external contamination.

Analysis of lung sample cultures revealed a mean total bacterial load ranging from a minimum of 4.0×10^3 CFU/g at 0 hours to a maximum of 2.28×10^8 CFU/g at 72 hours *postmortem*. The temporal evolution of the bacterial load followed a pattern consistent with *postmortem* bacterial transmigration, characterized by a progressive increase up to 72 hours, followed by stabilization at 96 hours (Table 5, Figure 13.A). There are currently no studies available that allow for a direct comparison with our findings regarding lungs. Bacterial growth was already detected at the 0-hour time point, although a more robust result was obtained for 12 hours (>30 colonies). *Postmortem* bacterial growth in the lungs can begin as early as 1 hour after death (141), challenging the notion that lungs are completely sterile. Moreover, lung microbiota is dynamic and highly variable even in live animals.

Table 5. Microbiological results of lung samples at different *postmortem* time points.

Time points	Lungs	Weight (g)	CFU/g CBA	CFU/g SLB	CFU/g MAC
0	1A	0.0357	5.0×10 ³ (P)	0	0
	1B	0.0615	0	0	0
	1C	0.1243	0	0	0
12	2A	0.1026	0	0	0
	2B	0.0872	6.0×10 ³ (M)	0	0
	2C	0.1185	3.0×10 ⁵ (M)	0	0
24	3A	0.0603	1.37×10 ⁵ (M)	1.37×10 ⁵ (P)	9.0×10 ³ (P)
	3B	0.1048	1.25×10 ⁵ (P)	8.9×10 ⁴ (P)	0
	3C	0.1028	6.0×10 ³ (M)	0	0
48	4A	0.113	1.14×10 ⁵ (P)	6.1×10 ⁴ (P)	0
	4B	0.1196	1.3×10 ⁷ (M)	3.0×10 ⁶ (M)	9.0×10 ⁴ (M)
	4C	0.0825	7.4×10 ⁷ (M)	7.6×10 ⁷ (M)	0
72	5A	0.0346	2.28×10 ⁸ (P)	4.0×10 ⁷ (M)	8.0×10 ⁶ (M)
	5B	0.0656	0	1.0×10 ⁶ (M)	0
	5C	0.0701	3.0×10 ⁶ (M)	1.1×10 ⁷ (P)	0
96	6A	0.1086	4.0×10 ³ (M)	7.0×10 ³ (M)	6.0×10 ³ (M)
	6B	0.0647	0	0	0
	6C	0.0832	0	0	0

Abbreviations: CBA (Columbia Blood Agar medium); CFU/g (colony-forming units per gram of tissue); MAC (MacConkey Agar medium); SLB (Slanetz & Bartley Agar medium). (M) indicates monoculture growth; (P) indicates polyculture growth.

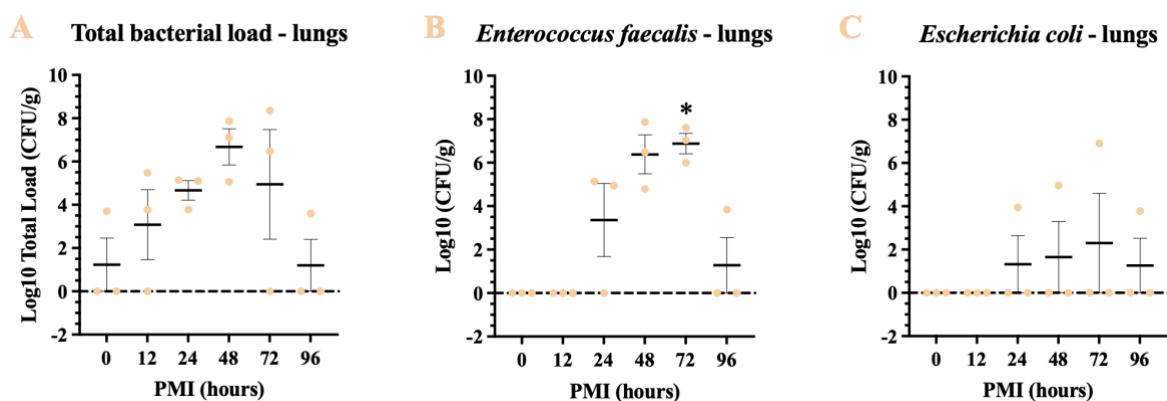


Figure 13. Kinetic cultural analysis of the *postmortem* lung microbiota.

Total bacterial load (A), *E. faecalis* load (B), and *E. coli* load (C) were quantified as colony-forming units (CFU) per gram of lung tissue using selective culture media: blood agar, Slanetz & Bartley agar, and MacConkey agar, respectively. Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$ versus 0-hour time point.

E. faecalis exhibited detectable colonization at 24 hours, reaching a peak between 48 and 72 hours (Table 5, Figure 13.B), with consistent results across all three experiments during this time frame. These results included the single statistically significant result at 72 hours with three highly consistent values. They also support the hypothesis of intestinal bacterial migration to the lungs after death, likely via hematogenous spread or bacterial translocation mechanisms, as proposed by Heimesaat *et al.* (143). Further studies are essential to determine whether our results are reproducible and *E. faecalis* can be a promising biomarker of lung invasion after death, namely by testing longer *postmortem* time points after 96 hours.

E. coli was detected in lung tissue at increasing concentrations over time, with minimal presence at 24 hours and a peak at 72 hours (Table 5, Figure 13.C), suggesting colonization of enteric origin. However, these *E. coli* counts were more inconsistent, being detected at 24, 48, 72, and 96 hours only in one of the three experiments, precluding reliable conclusions.

Historically, the lungs of healthy individuals were regarded as sterile organs. However, recent studies, including that of Dickson *et al.* (145), have demonstrated the presence of a distinct pulmonary microbiota, albeit composed of low-biomass bacterial communities. This represents a paradigm shift, emphasizing the role of the lung microbiota in maintaining homeostasis and regulating local immune responses. Moreover, the composition of the lung microbiota exhibits high interindividual variability, even among genetically identical animals such as C57BL/6J SPF mice. This variability has been attributed primarily to environmental factors, such as housing conditions, transport, and vendor origin, rather than genetic background. The ecological plasticity of the lung microbiota becomes especially apparent when animals from different cages are co-housed, leading to a convergence of their microbial communities. This phenomenon underscores the significant influence of environmental conditions, even in SPF facilities. In the *postmortem* context, such environmental variability may further amplify the diversity of bacterial profiles observed, representing an important factor to consider when interpreting microbial data from lung samples.

4.2.4. Heart

A total of 54 microbiological analyses were conducted on heart samples using three different culture media, evaluated across six *postmortem* time points (18 analyses per medium), as shown in Table 6. In the CBA medium, only monoculture growth was detected, while the selective SLB medium showed a predominance of mixed cultures. No growth occurred in the selective medium for *E. coli*.

Table 6. Microbiological results of heart samples at different *postmortem* time points.

Time points	Heart	Weight (g)	CFU/g CBA	CFU/g SLB	CFU/g MAC
0	1A	0.1173	0	0	0
	1B	0.0885	0	1.0×10 ⁴ (P)	0
	1C	0.0737	0	0	0
12	2A	0.0794	0	0	0
	2B	0.0352	0	0	0
	2C	0.07	0	0	0
24	3A	0.0699	0	0	0
	3B	0.0718	4.3×10 ⁴ (M)	5.4×10 ⁴ (P)	0
	3C	0.0565	0	0	0
48	4A	0.0718	0	0	0
	4B	0.0661	4.2×10 ⁴ (M)	8.1×10 ⁴ (P)	0
	4C	0.0606	0	0	0
72	5A	0.0658	3.9×10 ⁴ (M)	7.4×10 ⁴ (M)	0
	5B	0.0543	0	0	0
	5C	0.066	4.9×10 ⁴ (M)	8.9×10 ⁴ (P)	0
96	6A	0.0448	0	0	0
	6B	0.0659	0	0	0
	6C	0.0453	0	0	0

Abbreviations: CBA (Columbia Blood Agar medium); CFU/g (colony-forming units per gram of tissue); MAC (MacConkey Agar medium); SLB (Slanetz & Bartley Agar medium). (M) indicates monoculture growth; (P) indicates polyculture growth.

The microbiological analysis of *postmortem* cardiac tissue revealed distinct trends in the quantification of total bacterial and *E. faecalis* loads, as a function of the PMI.

The total bacterial load in the heart (Table 6, Figure 14.A) followed a general pattern of increase throughout the PMI, recording 4.3×10⁴ CFU/g at 24 hours and remaining

relatively stable until peaking at 72 hours with 4.9×10^4 CFU/g. However, variability across the three experiments prevents drawing definitive conclusions.

As illustrated in Table 6 and Figure 14.B, the count of *E. faecalis* showed detectable bacterial presence as early as 0 hours *postmortem*. Following this initial observation, a slight but gradual increase was recorded from 24 hours onward, peaking around 72 hours PMI. These results suggest a progressive colonization of cardiac tissue by *E. faecalis* during more advanced *postmortem* stages, but the lack of reproducibility between the three experiments was notable.

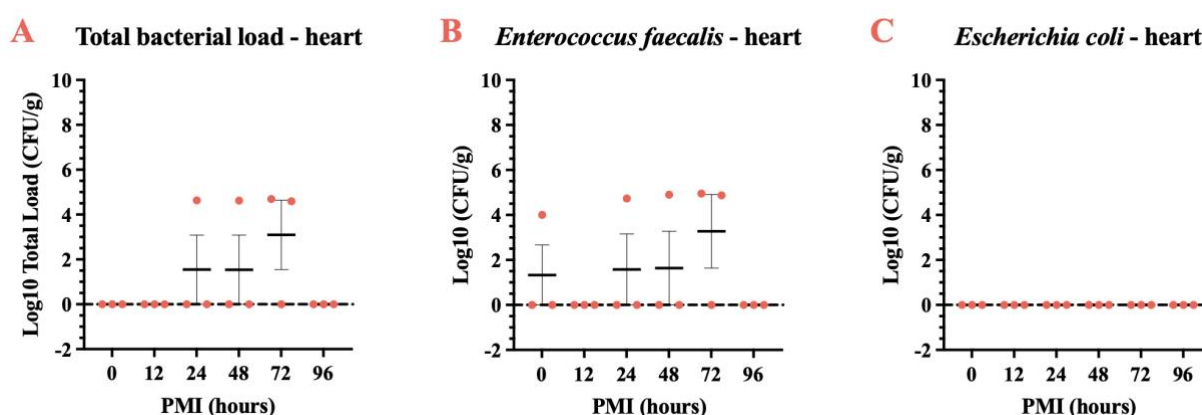


Figure 14. Kinetic cultural analysis of *postmortem* cardiac microbiota.

Total bacterial load (A), *E. faecalis* load (B), and *E. coli* load (C) were quantified as colony-forming units (CFU) per gram of heart tissue using selective culture media: blood agar, Slanetz & Bartley agar, and MacConkey agar, respectively. Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons.

In contrast, *E. coli* (Table 6, Figure 14.C) displayed a markedly different pattern. Microbiological analysis revealed a general absence of bacterial growth in cardiac tissue across all examined PMI (0–96 hours). The consistent absence of *E. coli* in cardiac tissue up to 96 hours *postmortem* supports the notion that the heart is relatively protected during early decomposition, as previously reported (94, 146). This aligns with the stepwise progression of *postmortem* bacterial translocation, which typically begins in organs near the gut (*e.g.*, liver or spleen) before reaching more distant sites like the heart or brain (147). Under the environmental and laboratory conditions of this study, MAC medium-based microbiological analysis suggests that the heart is not a suitable tissue for early PMI estimation based on enteric bacteria.

4.2.5. Brain

A total of 54 microbiological analyses were performed on cerebral samples from three different culture media evaluated at six *postmortem* time points (18 analyses per medium), as shown in Table 7. In the CBA medium, only monoculture growth was observed. Similarly, in selective media, *E. faecalis* growth occurred exclusively in monocultures, whereas growth was not observed for *E. coli*. Notably, bacterial growth in the SLB medium closely mirrored that observed in the CBA medium (Table 7).

Table 7. Microbiological results of brain samples at different *postmortem* time points.

Time points	Brain	Weight (g)	CFU/g CBA	CFU/g SLB	CFU/g MAC
0	1A	0.2083	0	0	0
	1B	0.15	0	0	0
	1C	0.1502	0	0	0
12	2A	0.15	0	0	0
	2B	0.1875	0	0	0
	2C	0.1238	0	0	0
24	3A	0.0991	0	0	0
	3B	0.0766	0	0	0
	3C	0.1419	0	0	0
48	4A	0.0929	0	0	0
	4B	0.0696	0	0	0
	4C	0.1455	0	0	0
72	5A	0.1299	1.4×10 ⁴ (M)	1.3×10 ⁴ (M)	0
	5B	0.1475	0	0	0
	5C	0.1132	6.0×10 ³ (M)	4.0×10 ³ (M)	0
96	6A	0.171	0	0	0
	6B	0.1481	0	0	0
	6C	0.1593	8.0×10 ⁸ (M)	5.0×10 ⁸ (M)	0

Abbreviations: CBA (Columbia Blood Agar); CFU/g (colony-forming units per gram of tissue); MAC (MacConkey Agar medium); SLB (Slanetz & Bartley Agar medium). (M) indicates monoculture growth; (P) indicates polyculture growth.

Bacterial colonization of the brain during *postmortem* decomposition has been shown to be both delayed and gradual, consistent with the relative anatomical and immunological protection of the brain, conferred primarily by the blood–brain barrier. The total bacterial load remained undetectable until 72 hours. From this time point onward, a progressive

increase was observed, reaching 1.4×10^4 CFU/g at 72 hours and peaking sharply at 96 hours, with a maximum of 8×10^8 CFU/g (Table 7, Figure 15.A).

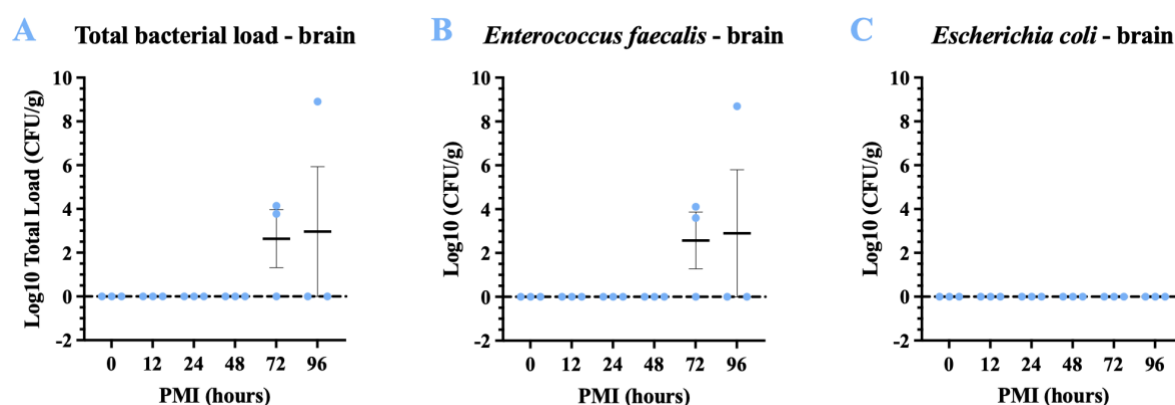


Figure 15. Kinetic cultural analysis of *postmortem* brain microbiota.

Total bacterial load (A), *E. faecalis* (B), and *E. coli* (C) were quantified as colony-forming units (CFU) per gram of brain tissue using selective culture media: Columbia Blood Agar, Slanetz & Bartley Agar, and MacConkey Agar, respectively. Data are presented as mean ± SD. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons.

This growth pattern is indicative of bacterial transmigration, suggesting that, despite the blood–brain barrier's protective role during life, the brain gradually becomes susceptible to microbial colonization as the structural integrity of these barriers deteriorates after death. The initial phase of relative stability may reflect a colonization latency period, during which bacterial proliferation remains limited by residual structural integrity and a scarcity of nutrients in the cerebral microenvironment. This dynamic is consistent with observations by Heimesaat *et al.* (143), who highlighted the delayed invasion of the brain compared to other more exposed or vascularized organs. The data support the hypothesis that *postmortem* bacterial migration is not confined to directly exposed tissues, but may also extend to the central nervous system. This finding is particularly relevant for understanding decomposition processes and interpreting microbiological evidence in both forensic and experimental contexts. Liu *et al.* (131) reported bacterial growth in brain samples at 0.5 hours *postmortem* by sequencing, meaning that our culture-based approach may not reach the desirable limits of detection. On the other hand, other studies reported undetectable bacterial growth in murine organs for up to seven days (148) and detection of thanatobiota in human brain

and heart only after seven days *postmortem* (144). It becomes evident that more research on this organ is needed.

Species-specific analysis revealed distinct dynamics between *E. faecalis* and *E. coli*. At no time point did all three assays yield positive results, and only *E. faecalis* was detected between 72 and 96 hours. *E. faecalis* was first detected at 72 hours (Table 7, Figure 15.B), with consistent growth observed up to 96 hours, a pattern consistent with its late detection in other organs, such as the kidneys (141). The recurrent presence of this species in the later stages of decomposition may be attributed to its high environmental resilience and notable adaptability, positioning it as a potential biomarker of late-stage brain colonization.

In contrast, *E. coli* was not detected at any PMI (Table 7, Figure 15.C), suggesting more limited access to brain parenchyma. This restriction may result from its specific nutritional requirements or its reduced capacity to traverse the anatomical barriers of the central nervous system. Liu *et al.* (131) found that brain microbial shifts begin after 8 hours *postmortem*, notably with depletion of metabolic pathways. They suggested that microbial diversity declines more slowly in the brain, likely due to its distance from the gut. Thapa *et al.* (149) showed that gut bacteria can reach the brain under dysbiosis, supporting our hypothesis that *E. faecalis* may translocate *postmortem* and serve as a brain invasion marker around 48 hours. The low detection of *E. coli* may reflect limitations inherent to the culturomics approach employed, which, although effective at identifying viable species, has a higher detection threshold compared to sequencing-based methods (142, 143).

In summary, these findings indicate that bacterial colonization of the brain occurs late and selectively during decomposition. Given the similarity in CFU/g values and time points between total aerobic bacterial counts (CBA medium) and the selective SBA medium targeting *Enterococcus*, *E. faecalis* stands out as the most prevalent and consistently detected species at later PMI. This consistent presence highlights its potential as a valuable biomarker for estimating extended PMI and indicating central nervous system involvement, with more studies being needed.

Altogether, the results from the culturomics assays highlight that *postmortem* microbiome dynamics are strongly tissue- and time-dependent. In all organs examined, total aerobic bacterial load peaked at 72 hours and then declined or reached undetectable levels by 96 hours, except in the brain, where the peak was observed at 96 hours. This delayed colonization likely reflects the brain's anatomical distance from the gut, rendering it one of the last organs to be invaded. Considering all data, the brain-*E. faecalis* association appears particularly promising for future investigations aimed at estimating the PMI.

Building on the recognition that Bacillota and Pseudomonadota dominate the *postmortem* microbiota (130), our study offers key insights into the dynamics of *E. faecalis* and *E. coli*, as representative species of these phyla, with potential as stable biomarkers. Despite known limitations of culturing methods, such as possible false negatives and unculturable species, our approach targeted bacteria that are both relevant to the human microbiota and readily recoverable under standard laboratory conditions. While sequencing can miss species-level resolution, our culture-based strategy enabled accurate identification down to the species level. Overall, our findings support previous work highlighting *Enterococcus*, particularly *E. faecalis*, as one of the most informative bacteria in the decomposition process (130, 143, 150).

4.3. Biochemical changes in *postmortem* tissues

4.3.1. Analysis of Raw Biochemical Parameters

4.3.1.1. Kidneys

Figure 16 illustrates the temporal variation of multiple biochemical markers in kidney tissue over a 96-hour PMI. The analysis revealed distinct patterns for each marker. Ethanol concentrations were relatively high at 0 hours and remained stable until 48 hours, after which a slight decrease was observed (Figure 16A). Glucose levels were initially very low, markedly increasing at later time points (48–96 hours), with substantial variability among samples (Figure 16B). Lactate levels increased sharply between 0 and 24 hours and remained relatively stable from 24 to 96 hours (Figure 16C). Magnesium levels showed minimal variation across all time points (Figure 16D). Uric acid exhibited a continuous and consistent increase over time (Figure 16F). This trend may reflect ongoing purine catabolism and cellular breakdown (100). Urea concentrations remained relatively stable during the first 48 hours and rose significantly at 72 and 96 hours (Figure 16G), likely due to the cessation of renal clearance and progressive tissue autolysis (101). Iron levels increased linearly throughout the PMI (Figure 16H), which may be attributed to hemolysis and degradation of iron-rich structures (151). Total protein concentrations decreased progressively, particularly after 48 hours (Figure 16E), likely as a consequence of tissue degradation (151).

Together, these data reveal temporal dynamics that are marker-specific, with uric acid and iron showing particularly strong time-dependent profiles, suggesting their potential forensic applicability in PMI estimation.

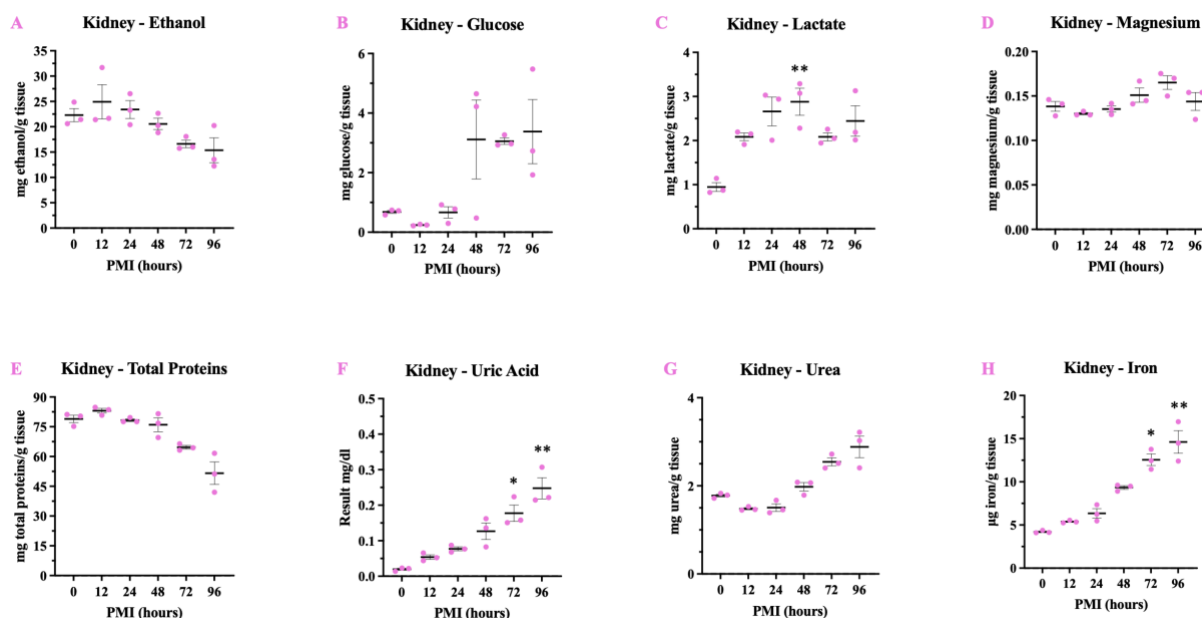


Figure 16. Temporal dynamics of biochemical markers in kidney tissue as a function of PMI. Data are presented as mean $\pm$ standard deviation. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$; ** $p < 0.01$ versus 0-hour time point.

4.3.1.2. Liver

Figure 17 shows the temporal variation of biochemical markers in liver tissue over a 96-hour PMI. Similar to the kidneys, biochemical analysis in the liver revealed distinct patterns for each marker. Ethanol levels were initially high and peaked at 24 hours, followed by a gradual decline (Figure 17A). Glucose showed a biphasic trend, with low values at early time points, a peak at 48 hours *postmortem*, followed by a decrease up to the 96-hour time point (Figure 17B). Lactate levels increased abruptly from 0 to 24 hours *postmortem*, followed by a relatively stable progression up to 96 hours (Figure 17C). On the other hand, magnesium levels remained relatively stable over the entire *postmortem* period studied (Figure 17D). In contrast to these parameters, total protein (Figure 17E) and uric acid (Figure 17F) levels showed a continuous decline from 0 hours until the 96-hour time point. The ongoing decrease in total protein levels likely reflects expected protein degradation over time

(152, 153), while the gradual decline in uric acid suggests hepatic consumption or redistribution (100). Additionally, both urea (Figure 17G) and iron (Figure 17H) levels showed a trend of continuous increase. The over time increase in urea levels likely reflects *postmortem* release from liver cells, and the gradual increase in iron levels is consistent with hemolysis and heme breakdown (154).

Overall, these data indicate marker-specific temporal dynamics, with uric acid, urea, and iron showing nearly linear trends. Additionally, the dynamics of lactate appear particularly interesting at early time points.

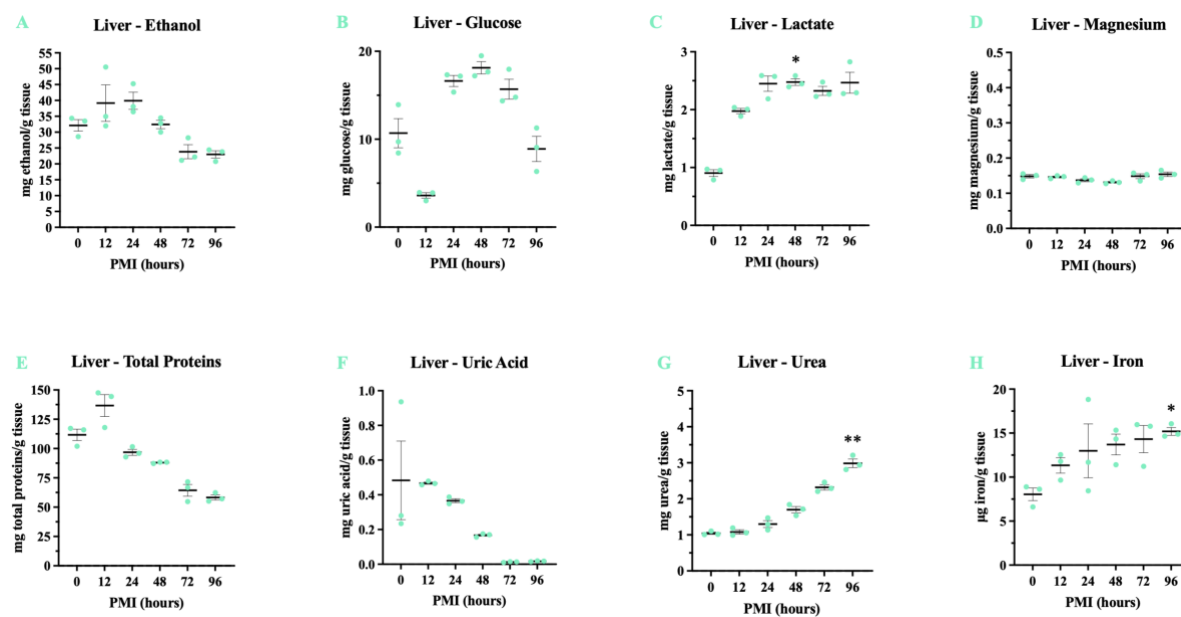


Figure 17. Temporal dynamics of biochemical markers in liver tissue as a function of PMI. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn’s post hoc test for multiple comparisons. * $p<0.05$; ** $p<0.01$ versus 0-hour time point.

4.3.1.3. Lungs

Figure 18 displays the temporal variation of biochemical markers in lung tissue over a 96-hour PMI. Ethanol levels were highly variable among different organs, showing a general decreasing trend until 48 hours, followed by a gradual increase up to 96 hours (Figure 18A). Glucose levels remained low until 48 hours, after which a gradual increase was observed, peaking at the 96-hour time point (Figure 18B). Lactate levels showed a progressive increase, stabilizing between 48 and 96 hours (Figure 18C), likely reflecting anaerobic metabolism

(155). In contrast to liver and kidney tissues, magnesium levels in the lungs showed a gradual increase over time (Figure 18D). Both uric acid (Figure 18F) and iron (Figure 18H) showed similar temporal profiles, with a gradual increase over time, peaking at the 48-hour time point, followed by a slight decrease at later time points. Similar to the liver, urea levels in lung tissue showed a gradual increase over time, reaching maximum levels at 96 hours *postmortem* (Figure 18G). In contrast to other organs, total protein levels in lung tissue gradually increased until the 48-hour time point, followed by a plateau (Figure 18F). This was an unexpected result, as tissue degradation would typically result in a decrease in total protein levels. However, this could reflect microbial activity, which could contribute with additional proteins to the tissue, as a gradual increase in total bacterial load was observed until the 48-hour time point, followed by a plateau.

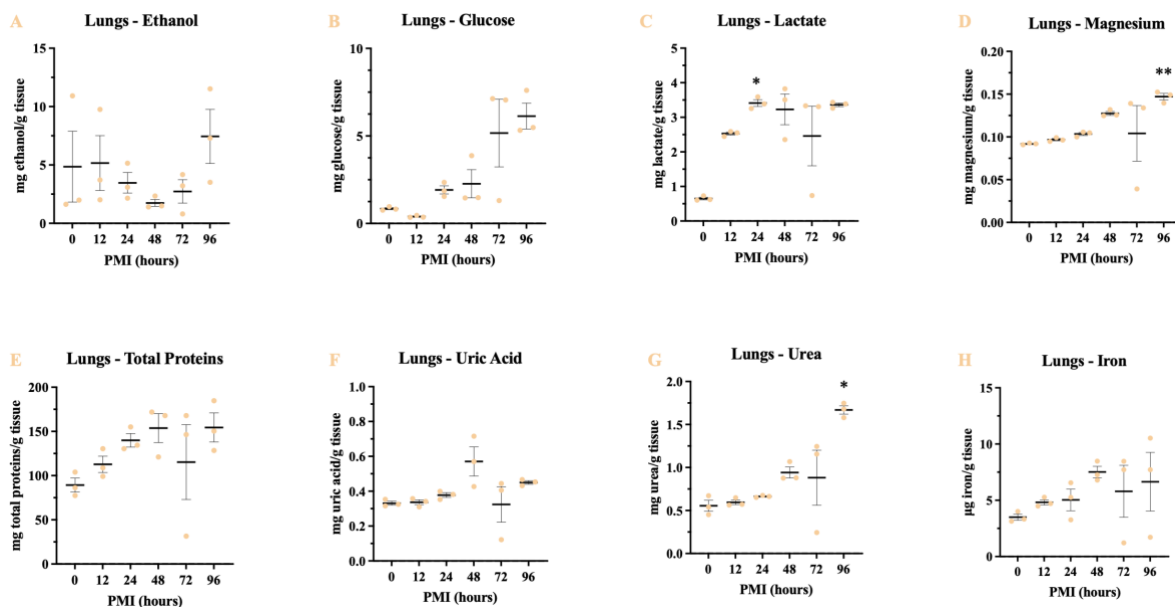


Figure 18. Temporal dynamics of biochemical markers in lung tissue as a function of PMI. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p<0.05$; ** $p<0.01$ versus 0-hour time point.

These results highlight glucose and lactate at early time points, and urea throughout the entire 96-hour period, as potentially informative markers for PMI estimation in lung tissue.

4.3.1.4. Heart

Figure 19 presents the biochemical variations in heart tissue throughout the 96-hour PMI. Although ethanol levels over the PMI were highly variable, they remained low and nearly constant until 72 hours, followed by a late increase at 96 hours (Figure 19A). Similarly, magnesium (Figure 19D) and total protein (Figure 19E) levels in heart tissue remained relatively constant throughout the PMIs studied. In contrast with these parameters, glucose, lactate, uric acid, urea, and iron continuously increased over PMI. Glucose levels showed a progressive increase from 12 hours onward, peaking at 96 hours (Figure 19B). Lactate increased steadily over time, reaching a plateau between 72 and 96 hours (Figure 19C), reflecting sustained anaerobic metabolism (156). The gradual increase of uric acid (Figure 19F) and urea (Figure 19G) levels over time likely reflects continuous purine degradation and progressive tissue breakdown (101). Additionally, the continuous increase observed for iron (Figure 19H) may reflect hemolysis and iron release from cardiac tissue (157).

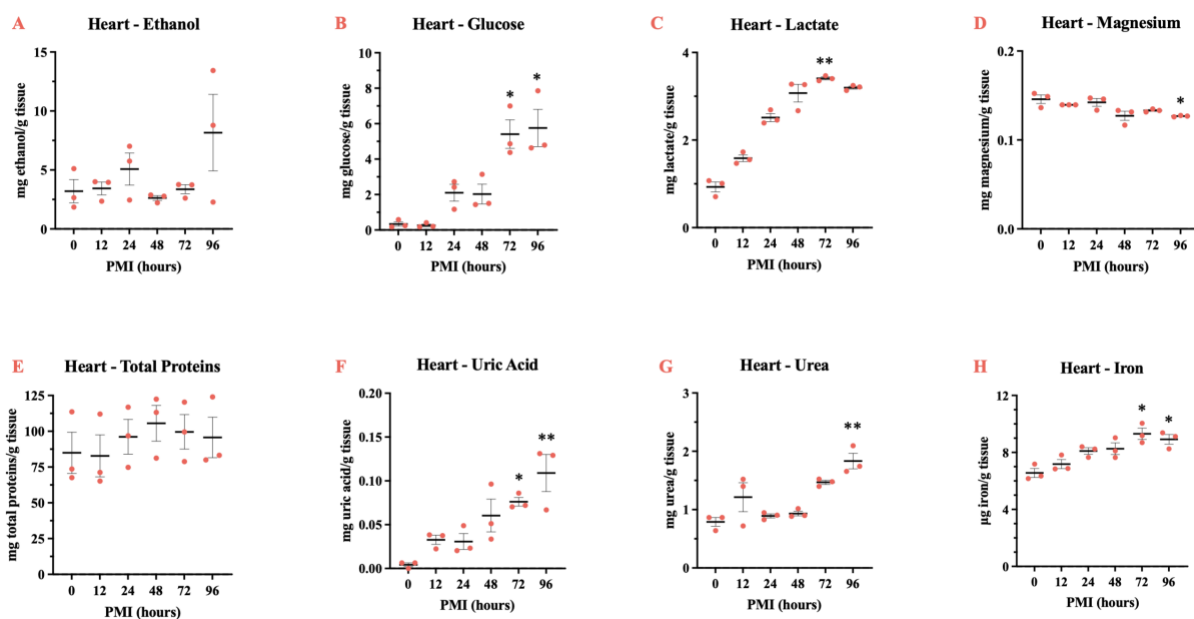


Figure 19. Temporal dynamics of biochemical markers in heart tissue as a function of PMI. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p<0.05$; ** $p<0.01$ versus 0-hour time point.

Lactate, glucose, uric acid, urea, and iron demonstrated marked *postmortem* progression and may serve as valuable markers for PMI estimation in cardiac tissue.

4.3.1.5. Brain

Figure 20 illustrates the evolution of biochemical parameters in brain tissue across the 96-hour PMI. Distinct temporal trends were observed in this tissue for the different biochemical markers analyzed. Ethanol levels in the brain remained relatively stable over the 96-hour period (Figure 20A). Likewise, glucose (Figure 20B) and iron (Figure 20H) levels also showed stable trends over time. In contrast, lactate levels increased steadily until 96 hours (Figure 20C), suggesting progressive anaerobic metabolism (100). Magnesium levels remained relatively stable throughout the investigated PMIs (Figure 20D). Total protein levels were initially high up to 12 hours *postmortem*, followed by a decline until 48 hours, after which they stabilized (Figure 20E). Uric acid was undetectable until 48 hours, after which it increased modestly, peaking at 96 hours (Figure 20F). Stable urea levels were also observed until 24 hours, followed by a continuous increase over time (Figure 20G).

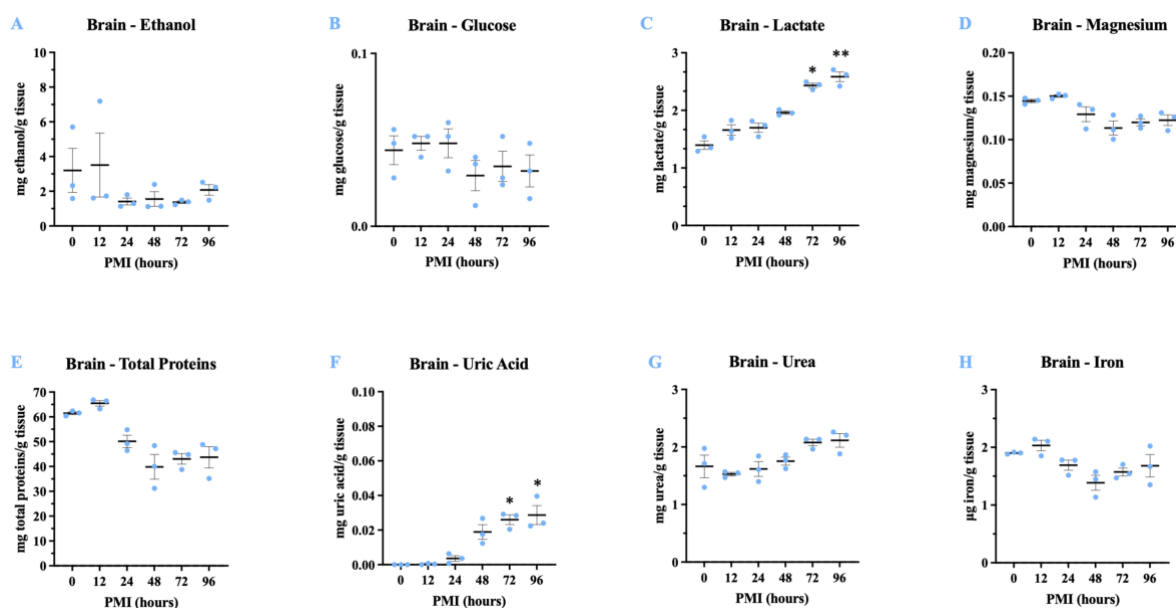


Figure 20. Temporal dynamics of biochemical markers in brain tissue as a function of PMI. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p<0.05$; ** $p<0.01$ versus 0-hour time point.

Overall, although some markers, including lactate, uric acid, and urea, showed a clear and pronounced time-dependent increase, the remaining biochemical markers exhibited more modest fluctuations than in other tissues, likely reflecting the brain's relative biochemical stability *postmortem*.

A temporal analysis of various biochemical markers revealed distinct distribution patterns across tissues throughout the PMI, highlighting both general trends and noteworthy variations.

The ethanol distribution patterns observed in this study align with those reported by Shi *et al.* (158), who examined *postmortem* ethanol dynamics and its metabolites in rabbits. Similar to our findings in the kidneys and liver, their study showed initially elevated levels followed by gradual declines in these organs. The brain exhibited consistently low ethanol levels in both studies, suggesting a reduced susceptibility to redistribution. Despite some organ-specific differences, these data collectively suggest that different tissues follow unique accumulation and elimination profiles during the PMI.

Other studies have assessed glucose dynamics in various rat tissues over a 60-hour PMI. While a decreasing trend for hepatic glucose was reported, renal glucose levels increased over time (159). Consistent with their results for the kidneys, we observed a progressive increase in glucose after the initial PMI, particularly between 24 and 60 hours. More strikingly, we observed a significant increase in glucose levels in the heart and a trend of increasing levels over time in the lungs, both of which are located in the thoracic cavity. The similar glucose pattern observed in the heart and lungs may reflect the diffusion of unbound glucose from adjacent tissues, including liver and muscle, via the vasculature, driven by a concentration gradient (159). This is consistent with the biphasic pattern identified for glucose in the liver, characterized by a peak at 48 hours, followed by a decline.

Previous studies have shown contrasting results regarding brain glucose. While some studies reported increasing levels of glucose in the brain of female balb-c albino mice (159) and canine cerebrospinal fluid (160), other studies found that *postmortem* glucose levels decrease in human cerebrospinal fluid (161, 162). However, our study found stable glucose levels over time, potentially reflecting species-specific metabolic responses and the brain's limited glycolytic activity.

Lactate levels in our study showed a rapid accumulation in the kidneys and liver during the first 24 hours *postmortem*, followed by stabilization up to 96 hours. In the lungs, lactate levels increased progressively and plateaued between 48 and 96 hours. Conversely, the heart and brain exhibited a continuous rise in lactate concentrations throughout the PMI, without early stabilization, suggesting organ-specific metabolic differences. Costa *et al.* (16) found an over time increase in lactate levels in their *postmortem* blood analysis, although it was more pronounced during earlier periods (up to 100 hours *postmortem*). This suggests the existence of a dynamic redistribution of lactate between the blood and tissues over time.

Nevertheless, it underscores the complexity of interpreting *postmortem* lactate and highlights the importance of considering organ variability and distinct metabolic activities when evaluating biomarkers for estimating the PMI.

Magnesium levels remained relatively constant in organs such as the kidneys, liver, heart, and brain. This stability profile aligns with findings by Farmer *et al.* (163), who observed only minor variations in magnesium concentrations in the vitreous humor throughout the PMI, suggesting *postmortem* stability of this electrolyte. In contrast, the lungs gradually increased in magnesium levels over time. In fact, Costa *et al.* (16) found a pronounced increase in magnesium in their *in vitro postmortem* analysis of blood biochemical markers during the first 24 hours. Considering that the lungs have an extensive blood supply, it can be assumed that magnesium from the blood may accumulate more readily in lung tissue after death. This phenomenon is favoured by the extensive and highly permeable capillary network designed for gas exchange characteristic of the lungs (164). Additionally, the lungs are more prone to *postmortem* edema (fluid buildup) (165), which further concentrates ions like magnesium. Other organs may not experience the same degree of fluid accumulation, explaining the organ-specific variations in magnesium dynamics observed in this study.

Analysis of total protein levels revealed organ-specific variations throughout the PMI. Kidneys and liver displayed a marked, progressive decrease in total protein levels throughout the PMI. Characteristically, hepatocytes carry out extensive metabolic, detoxification, and recycling functions, requiring a high abundance of lysosomes (166). Similarly, in the kidneys, especially in proximal tubule cells, lysosomes are essential for degrading reabsorbed proteins and waste materials (167). As these organs have a higher lysosome content than the heart, brain, or lungs, an accelerated and widespread protein degradation is anticipated, consistent with our findings. In contrast, total protein levels in the heart remained relatively constant throughout the PMI. Although just speculative, this may be attributed to both the resistant ultrastructure of myocardial tissue and the limited proteolytic environment in the heart compared to more metabolically active organs. Additionally, a pattern of an initial increase in total protein levels, followed by a stabilization period, was observed for both the brain and lungs. Similar observations were made by Costa *et al.* (16) in *in vitro postmortem* analysis of total proteins in blood. The initial increase in total protein levels may be speculated to result from an accentuated import of blood proteins at the early time points. As the PMI advances, the exhaustion of readily degradable protein pools and the slower degradation of more stable proteins may account for the stable total protein content observed. Nevertheless, these remain speculative hypotheses, requiring further investigation.

In this study, uric acid levels varied distinctly among the organs studied, showing a continuous increase in the kidneys and heart, remained undetectable in the brain for up to 48 hours, and declined in the liver over time. Previous studies in blood have also documented distinct *postmortem* profiles of uric acid. Costa *et al.* (16) observed a linear decrease in uric acid concentration over time *in vitro*. Donaldson and Lamont (100) reported several distinct scenarios: a gradual increase in uric acid levels up to 48 hours in rat blood *in vitro*; a delayed increase only at later time points (from 32 to 96 hours) in rat blood *in situ*; and a stable profile in both *in vivo* pig blood and *in vitro* human blood. These results suggest that the variations in uric acid levels across different organs may reflect intrinsic characteristics of each tissue, such as enzymatic activity, substrate availability (e.g., purines), vascularization, and local antioxidant capacity. Understanding the factors contributing to these organ-specific uric acid profiles may provide key insights into the biochemical processes occurring after death.

Urea levels in this study remained stable in the kidneys for up to 48 hours *postmortem*, followed by a significant increase. However, urea levels gradually increased throughout the PMI in the liver, lungs, heart, and brain, peaking between 72 and 96 hours *postmortem*. A similar pattern of increasing urea levels was reported in *postmortem* blood (16). However, a stable urea profile was shown in vitreous humor and synovial fluid (106). The oxidation of amino acids releases nitrogen, which is primarily converted into urea (168). Thus, an increase in urea levels is expected to result from amino acid oxidation during *postmortem* decomposition. As such, the results of this study may indicate that the slight variations in *postmortem* urea dynamics among the different organs studied reflect tissue-specific metabolic differences and decomposition processes. Additionally, these observations support urea as a reliable marker for PMI estimation, particularly in intermediate to late stages.

Finally, the continuous increase in iron levels in the kidneys, liver, and heart is consistent with the findings of Conte *et al.* (157) and Costa *et al.* (16), who attributed iron release into *postmortem* fluids to hemolysis and tissue autolysis. However, the lungs peaked at 48 hours followed by a slight decrease. This slight decrease at later time points (72 and 96 hours) may be attributed to the variability of the results, as discordant findings were observed in one animal at each time point. Additionally, the brain maintained stable iron levels throughout the period analyzed. Different hypotheses may explain this result. First, the blood-brain barrier may limit the influx or efflux of iron even after death, helping to maintain stable iron levels. Second, the brain's protection inside the cranium may slow *postmortem* cellular breakdown and iron release, resulting in more stable iron levels. Nevertheless, these hypotheses require further investigation.

4.3.2. Generation of a formula for PMI estimation based on biochemical markers

In a subsequent step, we aimed to integrate the different biochemical parameters measured across various organs into a formula to estimate the PMI. For this purpose, the biochemical markers were initially normalized to magnesium concentrations, given its relatively stable *postmortem* profile. The normalization of the biochemical markers to magnesium allows for the effective integration of an additional marker into the formula, enhancing the robustness and interpretability of the PMI estimation model.

From the normalized parameters, specific parameters were selected based on two main criteria: (1) the presence of a clear and consistent increasing trend throughout the PMI, and a determination coefficient (R^2) greater than 0.900. This statistical threshold ensured that the normalized data reflected meaningful biological trends rather than random variation. Overall, this selection highlighted the most promising and interpretable biochemical markers for PMI estimation.

4.3.2.1. Kidneys

Magnesium-normalized uric acid levels in the kidneys showed a steady and progressive increase throughout the 96-hour interval ($R^2 = 0.9828$; Figure 21A). A similarly linear and consistent increase across the PMIs was also observed for iron ($R^2 = 0.9928$; Figure 21B). The remaining normalized biochemical markers for the kidneys showed R^2 -values below 0.900 and were therefore not considered for the generation of the PMI formula (ethanol: $R^2 = 0.8908$; glucose: $R^2 = 0.8899$; lactate: $R^2 = 0.3205$; total proteins: $R^2 = 0.9454$; urea: $R^2 = 0.8746$).

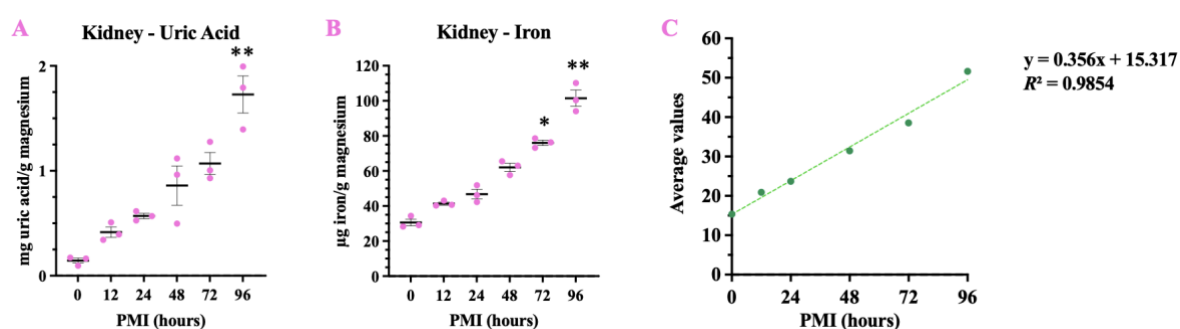


Figure 21. Temporal evolution of selected kidney parameters normalized to magnesium and the combined graph reflecting the average of their values at each time point.

Only parameters with a r value greater than 0.900 are shown. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$; ** < 0.01 versus 0-hour time point.

Based on these two normalized markers (uric acid and iron), a combined graph reflecting the average of the values of both parameters at each time point was generated (Figure 21C). The high coefficient of determination observed for the combined data ($R^2 = 0.9854$) supports the presence of a strong positive linear determination between the PMI and the observed mean values.

The kidneys exhibited one of the strongest determinations with the PMI ($R^2 = 0.9854$), suggesting that the selected biochemical parameters are particularly informative in this organ.

4.3.2.2. Liver

Magnesium-normalized urea concentrations in the liver showed a gradual and continuous increase throughout the PMIs studied ($R^2 = 0.9958$; Figure 22A). The remaining normalized biochemical markers for the liver showed R^2 -values below 0.900 and were therefore not considered for the generation of the PMI formula (ethanol: $R^2 = 0.7564$; glucose; $R^2 = 0.1453$; lactate: $R^2 = 0.5474$; total proteins: $R^2 = 0.9092$; iron: $R^2 = 0.7394$). In particular, normalized uric acid concentrations showed a gradual and continuous decrease, and therefore, it was not considered ($R^2 = 0.9689$).

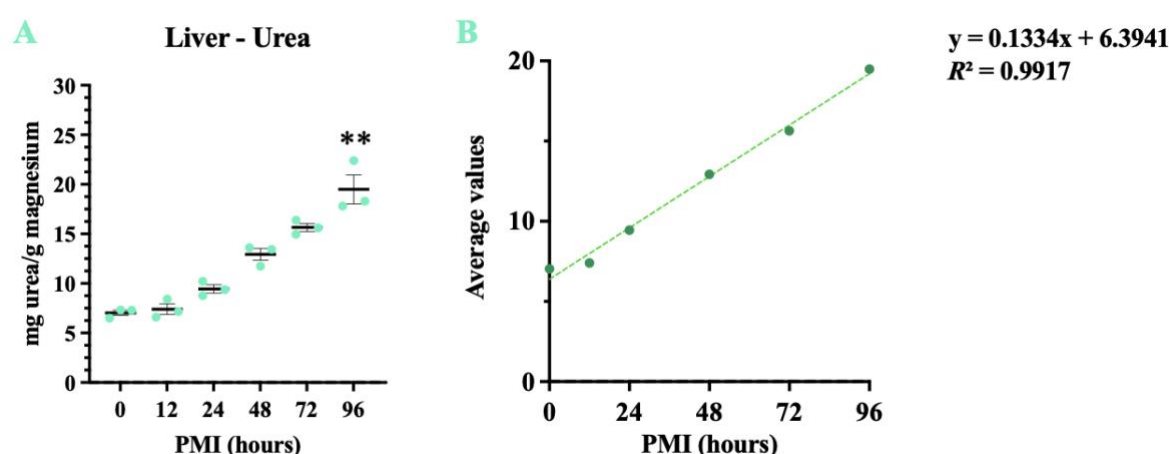


Figure 22. Temporal evolution of magnesium-normalized urea levels in the liver.

Only parameters with an increasing trend and an r value greater than 0.900 are shown. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. ** $p < 0.01$ versus 0-hour time point.

The high coefficient of determination observed for magnesium-normalized urea levels in the liver ($R^2 = 0.9739$) indicates a strong positive linear determination with the PMI (Figure 22B).

4.3.2.3. Lungs

Magnesium-normalized glucose levels in the lungs showed a gradual, but intermittent increase throughout the entire PMIs studied and determination throughout the 96-hour interval ($R^2 = 0.9134$; Figure 23A). Additionally, normalized urea levels showed a steady and progressive increase throughout the 96-hour interval ($R^2 = 0.9205$; Figure 23B). The remaining normalized biochemical markers for the lungs showed R^2 -values below 0.900 and were therefore not considered for the generation of the PMI formula (ethanol: $R^2 = 0.2872$; lactate: $R^2 = 0.2429$; total proteins: $R^2 = 0.2017$; uric acid; $R^2 = 0.3843$; iron: $R^2 = 0.2775$).

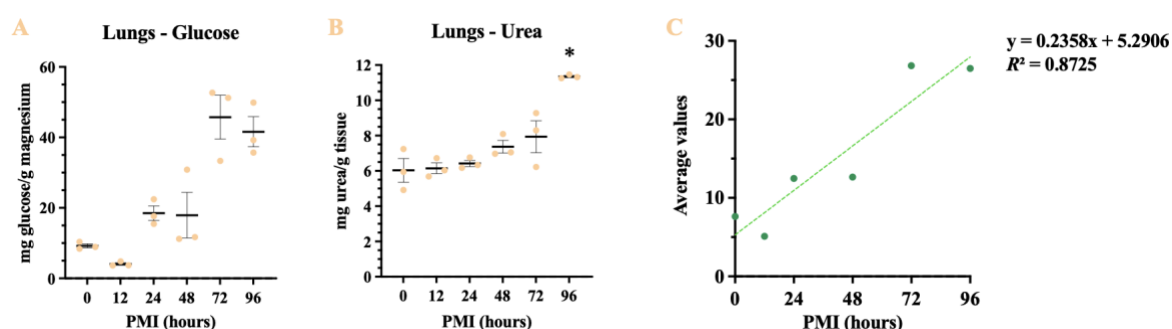


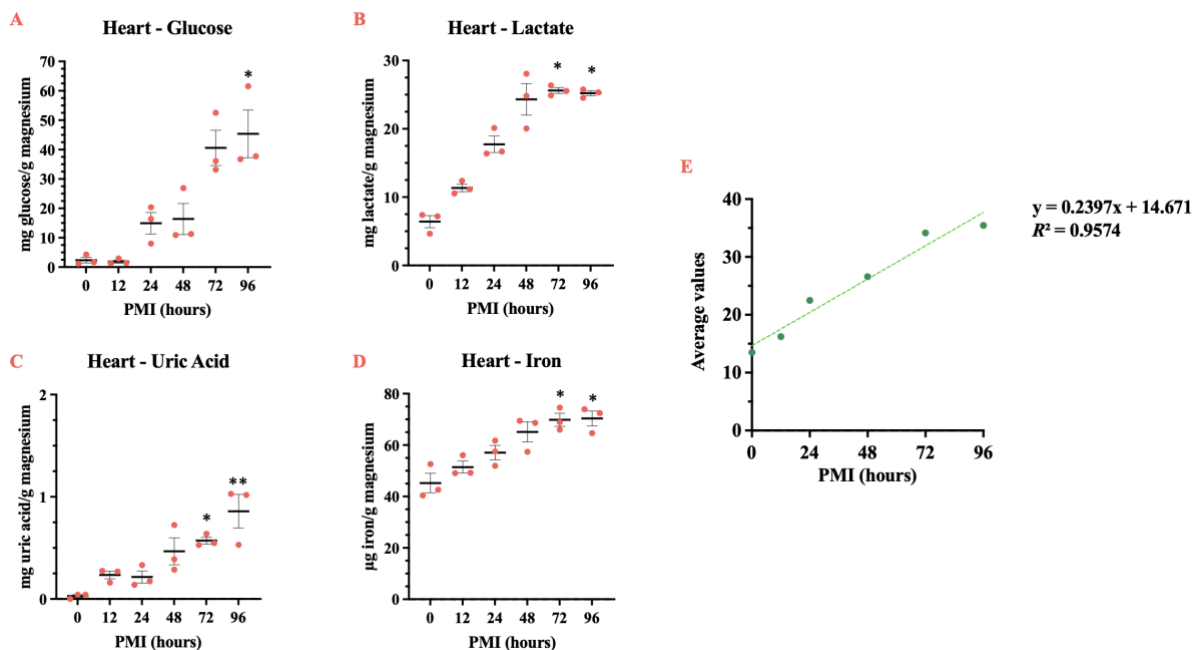
Figure 23: Temporal evolution of selected lung parameters normalized to magnesium and the combined graph reflecting the average of their values at each time point.

Only parameters with an R^2 value greater than 0.900 are shown. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$ versus 0-hour time point.

The combined graph reflecting the average of the normalized values of glucose and urea at each time point revealed a lower determination value throughout the PMI than other organs ($R^2 = 0.8725$; Figure 23C). This is likely the reflex of the gradual, but intermittent, increase in glucose values over time.

4.3.2.4. Heart

Magnesium-normalized levels of glucose, lactate, uric acid, and iron in the heart showed a steady and progressive increase over the 96-hour interval (glucose: $R^2 = 0.9670$; lactate: $R^2 = 0.9003$; uric acid: $R^2 = 0.9838$; iron: $R^2 = 0.9529$; Figure 24A-D). This suggests that the heart is an organ with significant predictive value for PMI estimation. In contrast, the remaining normalized biochemical markers for the heart showed R^2 -values below 0.900 and



were therefore not included in the generation of the PMI formula (ethanol: $R^2 = 0.6742$; total proteins: $R^2 = 0.7403$; urea: $R^2 = 0.8891$).

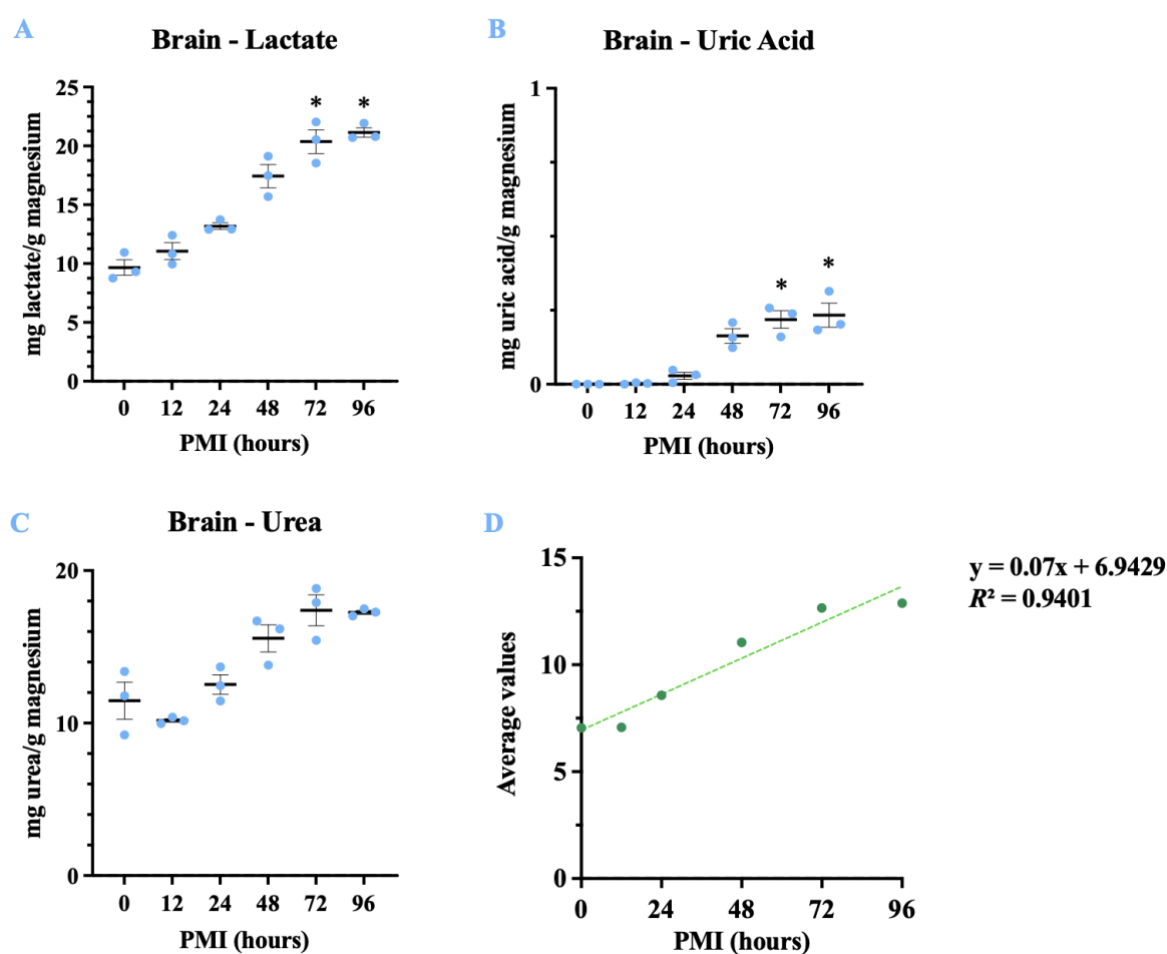
Figure 24. Temporal evolution of selected heart parameters normalized to magnesium and the combined graph reflecting the average of their values at each time point.

Only parameters with an R^2 value greater than 0.900 are shown. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$; ** $p < 0.01$ versus 0-hour time point.

Based on the four normalized markers selected (glucose, lactate, uric acid, and iron), a combined graph reflecting the average of the four parameters at each time point was generated (Figure 24E). The elevated coefficient of determination for the combined data set ($R^2 = 0.9574$) indicates a strong and expressive linear relationship between the PMI and the corresponding average values.

4.3.2.5. Brain

Magnesium-normalized lactate levels in the brain continuously and markedly increased throughout the 96-hour *postmortem* period, demonstrating a clear positive determination with PMI ($R^2 = 0.9805$; Figure 25A). Normalized levels of uric acid remained relatively stable up to 24 hours, followed by a progressive rise until 72 hours, with a tendency to stabilize thereafter. However, it positively correlated with PMI ($R^2 = 0.9642$; Figure 25B). Additionally, normalized urea levels remained relatively stable until 24 hours, gradually increasing thereafter ($R^2 = 0.9358$; Figure 25C). The remaining normalized biochemical



markers for the brain showed R^2 -values below 0.900 and were therefore not considered for the generation of the PMI formula (ethanol: $R^2 = 0.4782$; glucose; $R^2 = 0.6132$; total proteins: $R^2 = 0.8452$; iron: $R^2 = 0.1068$).

Figure 25. Temporal evolution of selected brain parameters normalized to magnesium and the combined graph reflecting the average of their values at each time point.

Only parameters with a R^2 value greater than 0.900 are shown. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple comparisons. * $p < 0.05$ versus 0-hour time point.

Based on the three normalized markers selected for the brain (lactate, uric acid and urea), a combined graph reflecting the average of the three parameters at each time point was generated (Figure 25D). The elevated coefficient of determination for the combined data set ($R^2 = 0.9401$) indicates a strong and expressive linear relationship between the PMI and the corresponding average values.

Taking into account all parameters with determination coefficient values greater than 0.900, selected from the different organs mentioned above (normalized values for uric acid and iron in the kidneys, urea in the liver, glucose and urea in the lungs, glucose, lactate, uric acid and iron in the heart, and lactate, uric acid and urea in the brain - 12 parameters), a combined graph reflecting the average of all these parameters at each time point was generated (Figure 26), and a global formula integrating these markers for PMI estimation was developed (Figure 27 - equation A). Parameters with magnesium-normalized values that showed r values lower than 0.900 were excluded from the model.

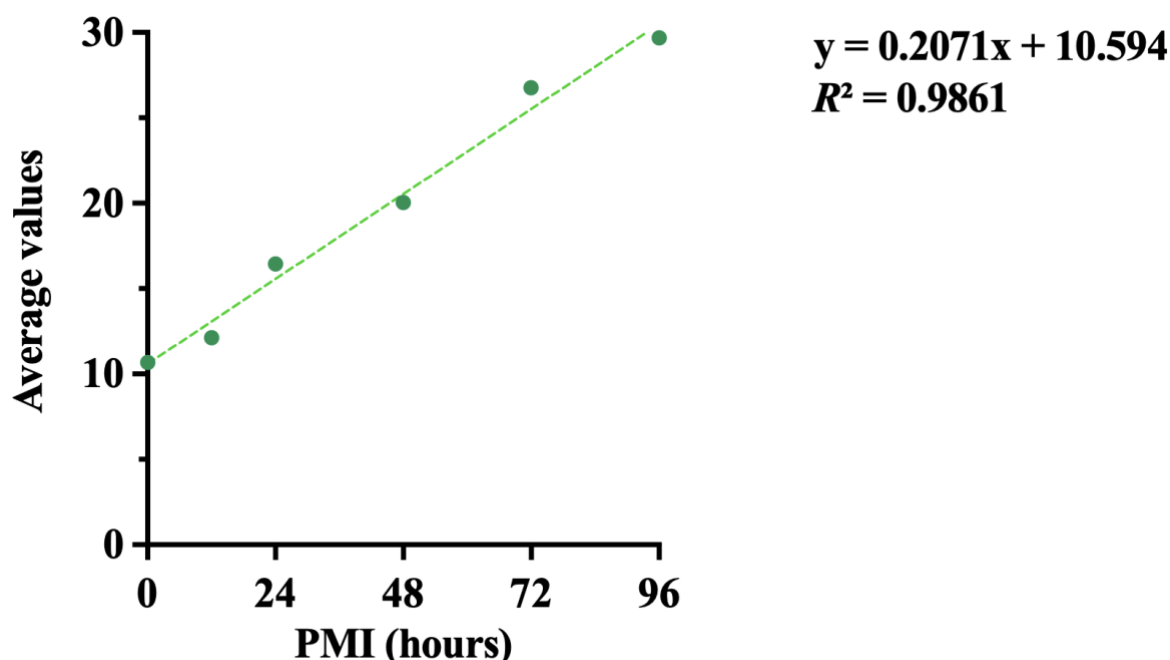


Figure 26. Mean graphical concentrations of the magnesium-normalized biochemical parameters used to generate a global formula to estimate the PMI. Normalized parameters

include uric acid and iron in the kidneys, urea in the liver, glucose and urea in the lungs, glucose, lactate, uric acid, and iron in the heart, and lactate, uric acid and urea in the brain.

The goal of the linear regression analysis was to determine the best estimates for the slope and intercept by minimizing the residual error between the experimental values of y and the predicted values based on the regression line. This analysis relies on three fundamental assumptions: (1) deviations between the observed data and the regression line are due to random errors affecting the y – *values*; (2) these errors are normally distributed; and (3) the errors in y are independent of the values of x .

Based on these calculations, a regression equation was defined to estimate the PMI, accounting for parameters with positive slopes (Figure 27, equation A). Based on this equation, the mean signal of the 12 parameters ($\bar{Y}x$) calculated can be used to determine the PMI.

The standard deviation associated with the estimated value of x (S_x) is calculated using the formula presented in Figure 27 (equation B), where K is the number of replicate samples (in this case, $K = 12$ parameters, analysed in triplicate = 36), n is the number of time points measured ($n = 6$), $\bar{y}$ is the average signal of the 12 parameters for each time point, and $\sum xx$ represents the sum of squared deviations $(x - \bar{x})^2$. Once S_x is known, a confidence interval for the PMI can be calculated using the appropriate t – *value* for the desired confidence level ($p < 0.05$) and $n - 2$ degrees of freedom.

A	$\text{PMI} = \frac{(10.04 - \bar{Y}x)}{0.19} \pm 2.78 \times \left[4.89 \times \sqrt{0.19 + \frac{(\bar{Y}x + 17.95)^2}{242.39}} \right]$
B	$S_x = \frac{S_y}{m} \sqrt{\frac{1}{K} + \frac{1}{n} + \frac{(\bar{Y}x - \bar{y})^2}{m^2 \sum xx}}$

Figure 27: Global model for PMI estimation. Equation A allows the calculation of the PMI, the mean value of the following magnesium-normalized parameters: uric acid and iron in the

kidneys, urea in the liver, glucose and urea in the lungs, glucose, lactate, uric acid and iron in the heart, and lactate, uric acid and urea in the brain. Equation B estimates the error.

5. Conclusions and Future Perspectives

The dynamics of the *postmortem* microbiome were strongly dependent on tissue type and time elapsed since death. Total aerobic bacterial load peaked at 72 hours in most organs, except for the brain, which peaked at 96 hours *postmortem*, likely due to its anatomical protection. *E. coli* was absent from the liver, heart, and brain, whereas *E. faecalis* consistently colonized the kidneys, lungs, and brain, initiating invasion as early as 24 hours and reaching peak levels between 72 and 96 hours. These findings underscore the value of culture-based methods for detecting viable bacteria relevant to estimating the PMI.

The biochemical markers analyzed exhibited organ-specific and temporal trends. Lactate levels rose early in nearly all organs, peaking between 24 and 48 hours, with a sustained increase observed in the brain, likely reflecting anaerobic metabolism after death. Uric acid progressively increased, primarily in the kidneys, heart, and brain, likely linked to purine degradation and autolysis. Urea levels rose sharply during the late stages of the PMI, while iron increased steadily in the kidneys and heart, with an earlier peak observed in the liver. Among these markers, lactate was the most informative for early PMI estimation, whereas uric acid and urea showed stronger correlations with later *postmortem* stages.

Integrating culture-based microbiological data and biochemical markers offers robust, multiparametric support for PMI estimation. Notably, late brain colonization by *E. faecalis* and the temporal patterns of lactate, uric acid, urea, and iron in various organs emerge as reliable indicators of *postmortem* progression. However, further validation in more complex forensic scenarios, particularly involving human cases, is necessary to fully establish the potential of this multidisciplinary approach in forensic practice.

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7. Appendix

7.1. Poster communication at the IV TOXRUN International Congress, 2025

Bacterial Load and Dynamics:

Exploring Their Potential as Biomarkers for *postmortem* Interval Estimation

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Background

Establishing the *postmortem* interval (PMI), the time since death, is vital in forensic investigations but remains challenging due to multiple factors like the cause of death, body location, and environmental conditions during decomposition [1]. Traditional PMI estimation methods include *Algor Mortis*, *Rigor Mortis*, and *Livor Mortis*, along with complementary approaches from entomology, botany, and microbiology, though all carry some degree of inaccuracy [2]. Microbiology and *postmortem* biochemistry or "thanatochemistry" are gaining prominence in PMI determination, as microbial succession and biochemical analysis offer valuable insights into the time since death, despite certain limitations into PMI estimation [3].

Objective

Objective: This study aimed to explore the potential of using bacterial translocation dynamics for PMI estimation by quantifying total bacterial load and tracking the dissemination of two gut microbiota species, *Escherichia coli* and *Enterococcus faecalis*, across various organs (lungs, heart, kidneys, liver, and brain) at defined PMIs under controlled, pathogen-free conditions.

Methods

Male C57BL/6J SPF mice were analyzed at six *postmortem* timepoints (0, 12, 24, 48, 72, and 96 h). Feces and organs (n=3 animals/assay) were collected, thoroughly resuspended in buffered peptone water, and then seeded onto non-selective (Blood agar) and selective (MacConkey-MC for *Escherichia coli* and Slanetz-Bartley-SB for *Enterococcus faecalis*) media. After routine aerobic incubation, colony-forming units (CFU) were quantified per gram of tissue to assess total and species-specific bacterial loads.

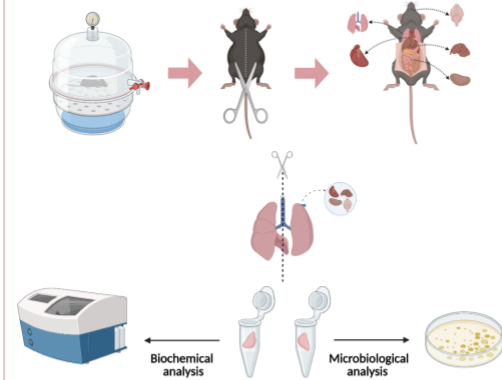


Figure 1. Schematic representation of animal sacrifice using an isoflurane chamber followed by cervical dislocation and sample collection. Tissues were preserved at -80 °C for biochemical and microbiological analysis.



Figure 2. Workflow for microbiological quantification in the collected organ samples, including homogenization, dilutions, on Columbia Blood Agar, Slanetz Bartley Agar and MacConkey Agar media.

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Results and Discussion

➤ The total bacterial load increased at later *postmortem* time points.

➤ **Lungs:**

- Showed an undulating pattern of bacterial presence throughout decomposition.
- Bacterial load peaked at 72 h *postmortem*, followed by a decline.

➤ **Liver and Heart:**

- Both organs, being highly vascularized, showed detectable bacterial loads at the initial time point (0 h).

➤ **Kidneys:**

- Initially had low or undetectable bacterial loads due to their sterility.
- An undulating pattern of bacterial presence developed over time.
- A pronounced peak in bacterial load occurred at 72 h, after a temporary decline between 24 and 48 h.

➤ **Brain:**

- No bacterial load was initially detectable.
- An increase in bacterial load was observed between 72 and 96 h *postmortem*.

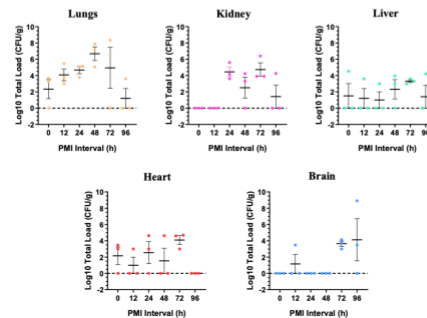


Figure 3. Aerobic bacterial load (Log₁₀ CFU/g) for lungs, kidney, liver, heart and brain across different PMI.

➤ *Enterococcus faecalis* findings (according growth in SB agar):

- Bacterial growth detected only at later time points.
- Showed the most promise as a potential biomarker for lung invasion, with additional relevance for kidney, liver, and possibly brain invasion at later time points.

➤ *Escherichia coli* findings (according growth in MC agar):

- Organ-dependent growth.
- Bacterial growth was only detectable after 24 h.
- Not detected in liver samples in this medium and was almost undetectable in the heart, suggesting its potential as a specific biomarker for lung invasion.

➤ Biochemical analyses are ongoing to support the microbiological results.

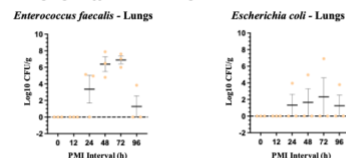


Figure 4. Numbers of *Enterococcus faecalis* and *Escherichia coli* determined by detection of colony forming units (CFU) per gram/organ on appropriate culture media on Slanetz-Bartley and MacConkey agar, respectively.

Conclusions

This quantitative, culture-based study indicates that *Enterococcus faecalis* shows potential as a biomarker for the invasion of the kidneys, liver, lungs, and possibly the brain at later *postmortem* stages. Conversely, *Escherichia coli* was virtually absent in liver samples cultured in MC medium and nearly undetectable in the heart, suggesting its potential as a specific biomarker for lung invasion. These findings highlight the impact of the PMI on the growth dynamics of these bacteria and underscore their potential forensic value as traceable biological markers. Nonetheless, further research is required to validate and expand upon these findings in more complex scenarios.

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7.2. Publication of congress abstract in *Scientific Letters*

PC25 Bacterial load and dynamics: exploring their potential as biomarkers for postmortem interval estimation

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ABSTRACT

Background: Establishing the *postmortem* interval (PMI), the time since death, is vital in forensic investigations but remains challenging due to multiple factors like the cause of death, body location, and environmental conditions [1]. Traditional PMI estimation methods include *Algor Mortis*, *Rigor Mortis*, and *Livor Mortis*, along with complementary approaches from entomology, botany, and microbiology, though all carry some degree of inaccuracy [2]. Recently, microbiology and *postmortem* biochemistry (thanatochemistry) have gained attention, as microbial succession and biochemical changes offer promising, though still limited, insights into PMI estimation [3]. **Objective:** This study aimed to quantify the total bacterial load and map two bacterial species, *Escherichia coli* (Ec) and *Enterococcus faecalis* (Efs), two gut microbiota species, across various organs (lungs, heart, kidneys, liver, and brain) at varying PMIs under controlled, pathogen-free conditions. **Methods:** Male C57BL/6J SPF mice were analyzed at six *postmortem* timepoints (0, 12, 24, 48, 72, and 96h). Feces and organs (n=3 animals/assay) were collected, thoroughly resuspended in buffered peptone water, and then seeded onto non-selective (Blood agar) and selective (MacConkey-MC for Ec and Slanetz-Bartley-SB for Efs) media. After routine aerobic incubation, colony-forming units (CFU) were quantified per gram of tissue to assess total and species-specific bacterial loads. **Results:** The total bacterial load increases at later *postmortem* timepoints. The heart and lungs (highly vascularized organs) showed a detectable bacterial load at the initial timepoint (0 h). Organs typically considered more sterile, such as the kidneys and brain, showed low or undetectable bacterial loads initially, although with a gradual increase over time. In SB, used for the quantitative detection of Efs, bacterial growth emerged at later timepoints. On MC, selective for Enterobacteriaceae, and used for the detection of Ec, bacterial growth was organ-dependent and detectable only after 24 h. Notably, *E. coli* was absent from liver samples on this medium. Biochemical analyses are ongoing to complement microbiological analysis. **Conclusions:** This culture-based quantitative study shows how PMI impacts the growth and dynamics of *E. faecalis* and *E. coli*, suggesting their potential as traceable biomarkers for PMI in forensic contexts. Nonetheless, further studies are required to validate and extend these findings to more complex scenarios.

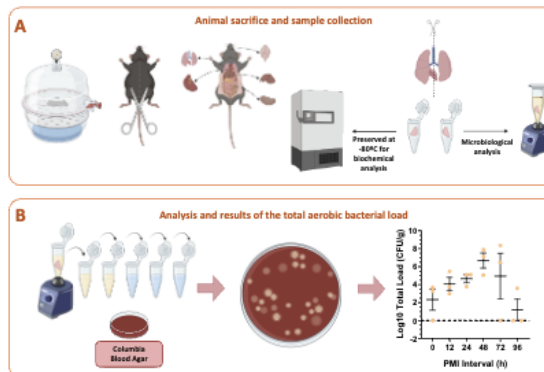


Figure 1. (A) Schematic representation of animal sacrifice using an isoflurane chamber followed by cervical dislocation and sample collection. Tissues were preserved at -80 °C for biochemical and microbiological analysis. (B) Workflow for microbiological quantification of collected organ samples, including homogenization, culture on Columbia Blood Agar, and total bacterial load assessment. The graph on the right shows total aerobic bacterial load (Log10 CFU/g) for lungs across different *postmortem* intervals.

Keywords: *postmortem* interval; thanatomicrobiome; bacterial translocation

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PC26 Exploring the potential of dichloroacetate in targeting oral cancer metabolism

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ABSTRACT

Background: Oral cancer represents a significant global health burden, and its incidence is steadily rising [1]. A hallmark of cancer is metabolic reprogramming - most notably, a shift toward aerobic glycolysis, commonly known as the Warburg effect. This altered metabolic phenotype supports rapid proliferation, enhances survival under hypoxic conditions, and contributes to