Núria Clavero Mateu

THE THANATOMICROBIOME: A NEW DIRECTION IN FORENSIC SCIENCE TO DETERMINE THE POST-MORTEM INTERVAL

Master's Degree in Forensic Genetics, Physics and Chemistry

Dr. Helena Torrell Galceran

Centre for Omic Sciences (Eurecat, Reus)

Dr. Gerard Aragonès Bargalló

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ABSTRACT

For hundreds of years, it has been well known that microbes play an essential role in the decomposition of corpses, but it was not until recent years that researchers gave them the importance they deserved.

The body of a healthy person is made up of organs inhabited by microbes such as the gastrointestinal tract and sterile organs, however, once death has occurred, they are all colonized both by microbes from the gastrointestinal tract and microbes from the environment. The migration from the gastrointestinal tract to the rest of the internal organs follows a specific order and starts earlier at organs closer to the gastrointestinal tract and finishes at more distant organs. Moreover, the microbial communities change with time due that at different stages of the decomposition oxygen availability differs. All these microbes and especially bacteria constitute the thanatomicrobiome and have the potential to determinate the post-mortem interval.

To study the succession of the thanatomicrobiome most researches use animal models but only a few of them human corpses from criminal cases or donations. Microbial communities are mostly analysed with 16S rRNA a genetic marker for bacteria and archaea and metagenomic whole-genome shotgun sequencing. Results are interpreted using alpha and beta diversity metric values and compared to databases.

Results from multiple investigations demonstrate that the most prominent phyla are *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria* which coincide with the GIT microbiome and that with time *Proteobacteria* declines and *Firmicutes* becomes the most abundant phylum. Furthermore, multiple members of the phylum *Firmicutes* such as some species of *Clostridium* and *Lactobacillus* exhibit time signals.

Nevertheless, although the results seem promising the thanatomicrobiome is highly influenced by extrinsic and intrinsic factors and therefore cannot be used as a model to predict the post-mortem interval in the present but probably will be crucial in future forensic investigations.

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1. Introduction

1.1 Microbes in the human body

Multiple studies have revealed that microbes are present on both the internal and external superficies of some organs of the human body such as the skin, gut and lungs and cavities like the mouth, vagina, etc. The term microbiome refers to the population of microorganisms including bacteria, viruses, archaea and protozoan that lives in the human body and its composition and abundance can vary due to a large number of factors. Nevertheless, even though the microbiome includes all these organisms the roles of viruses, archaea, and unicellular eukaryotes are by far less known than the roles of bacteria so the majority of studies about the microbiome focus on the importance of the latest [1,2].

The Human Microbiome Project (HMP) funded by the The US National Institutes of Health (NIH) is a set of projects consisting of an extension of the Human Genome Project responsible for studying the microbiology of the human body. The HMP has generated the largest human metagenomic study to date with the objective of providing resources such as a reference set, data and protocols to be used in future studies [2]. The HMP estimates that there are 100 trillion bacterial cells which means that they outnumber human cells by a ratio of 10:1, highlighting the importance of these organisms in the human body [1,3].

The diversity of microbes of the human body is defined by the number and abundance of the different types of organisms in each organ. Each organ has its population of microbes with a specific function and this is extremely important in order for human beings to stay healthy since the changes that occur in these communities are associated with diseases like inflammatory bowel disease and bacterial vaginosis (Figure 1). Some of these changes are extrinsic such as the temperature, humidity, diet and antibiotics while the others are intrinsic like the health state of the host, sex, etnicity, age and genetics. Although there are a large number of factors that alter microbial populations, in most cases the dynamics of these populations can be predicted, which makes the human microbiome a great source of information about the human body [4].

On the other hand, it is important to note that multiple studies have revealed that even in healthy people there are important differences in the community of microbes that

inhabit each organ and part of that diversity remains inexplicable. Moreover, all these differences mentioned are of great importance considering that the majority of healthy people only share approximately a third of the genes of the metagenome [4,5].

In recent years, thanks to the improvements of sequencing techniques like 16S rRNA gene sequencing and culture techniques (especially anaerobic bacteria), it has been possible to study the human microbiome in greater depth with special attention to the bacterial communities of the gut. These investigations have allowed finding that although most of the microbiome is found in the gut, there are also other parts of the human body that in healthy conditions are inhabited by microbes such as the oral cavity, the skin and reproductive organs such as the vagina (Figure 1) [5].

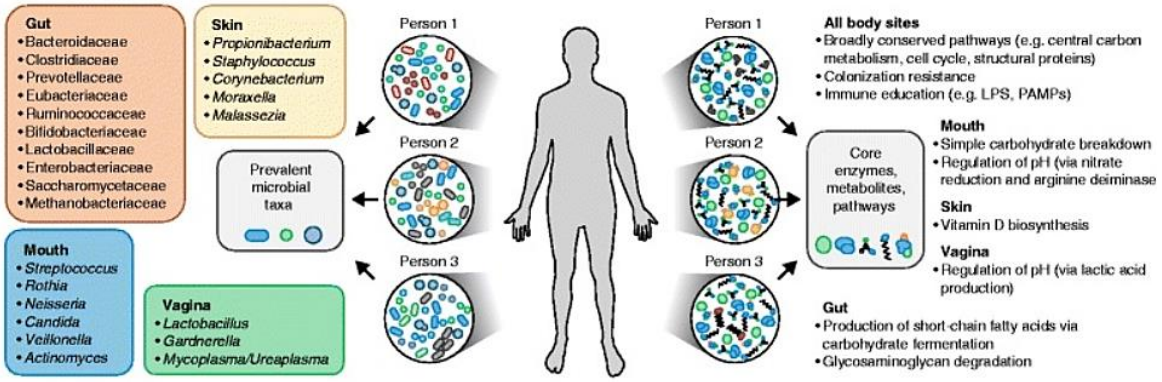

Figure 1. Each body site owns a group of taxa that is expected to be found frequently in that site in healthy people, meaning that the composition of each body site is more similar between the same body site in different individuals than in other places within the same person. The fact that the same parts of the body share populations of similar microbiomes is due to that this allows functionality to be maintained. For example, *Lactobacillus* is a very predominant genus in the vagina and produces lactic acid providing and acidic pH [5].

On the other hand, most places of the body are considered sterile in health conditions such as blood, bones, pleural fluid, peritoneal fluid, pericardial fluid, joint fluid, cerebrospinal fluid and most internal organs like lymph nodes, brain, heart, liver, spleen, kidney, pancreas, etc [6].

To highlight and understand the importance of the microbiome in humans, it is necessary to describe the diversity of microbial communities and the role they play. For this purpose the main focus will be on the gastrointestinal tract (GIT).

1.1.2 The microbiome of the Gastrointestinal tract

The most colonized organ of the body is the GIT which contains approximately 70% of all microbes in the human body mostly due that its environment is rich in molecules that de microbes use as nutrients and its large surface (200 m^2). The GIT microbiota has a

vast number of functions (the fermentation and digestion of carbohydrates and oligosaccharides, the metabolisation of xenobiotics and drugs, antimicrobial protection, etc) and it is crucial to maintain a healthy lifestyle since it contributes to the physiology of the host [1,7]. This means that the different components of the gut microbiota contribute in influencing each aspect of normal development in their own way even though the microbiota as a complete entity displays functional redundancy [1].

As mentioned before, the microbiome can vary due to an extensive number of factors and that obviously doesn't exclude the most colonized organ of the entire body. The microbiome of the GIT of a healthy person is influenced mostly by the diet and ethnicity but sex, age and lifestyle like stress, the consumption of tobacco and alcohol are also important factors to consider (Figure 2) [8].

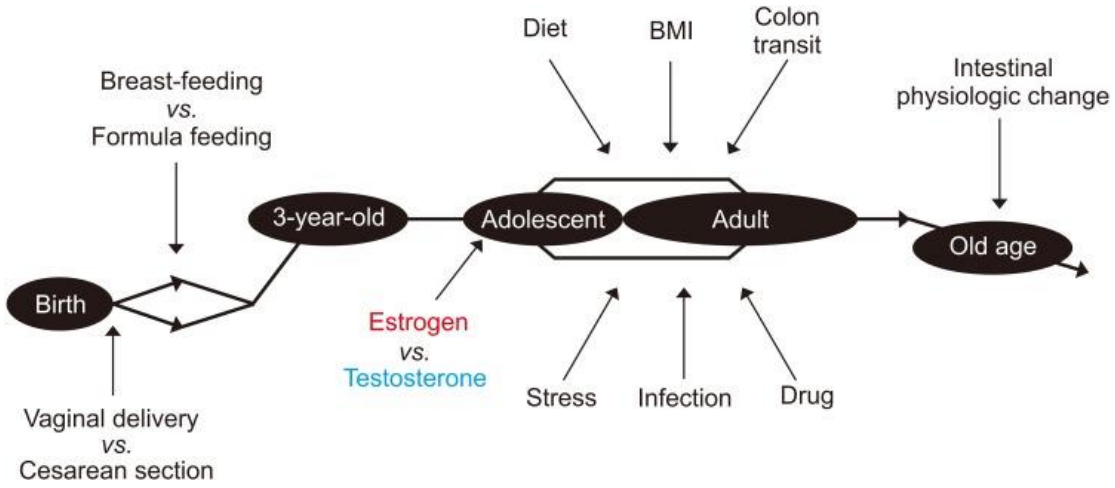

Figure 2. Summary of factors that affect the microbiota during life. The microbiota is in constant change not only by sex and ageing and all the processes associated with it but also by permanent changes like dietary habits and immigration or temporary perturbations like stress. Nevertheless, the microbiota it's necessary to maintain the health of the host and avoid disorders, particularly in early life [8].

The composition of the gut microbiota is suprisingly stable at the phylum level considering all the factors that alter it. Also, even though there are more than 50 phylas described and that the proportions of these groups of bacteria can vary between individuals a healthy human intestinal microbiota is dominated by *Firmicutes* and *Bacteroidetes*, while *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and *Cyanobacteria* are also frequent but in minor proportions (Supplemental information Figure 1.) [1, 5, 9].

A study conducted by Chen et al (2016) analyzed 118 stool samples from healthy people of different sex, ages, demography and lifestyles (BMI, alcohol consumption and

tobacco use) using 16S rRNA and demonstrated that at the phyla level all subjects had a high abundance of *Firmicutes* and *Bacteroidetes* except for an individual that presented an outstanding abundance of *Fusobacteria*. The results also showed an important presence of *Proteobacteria*, *Actinobacteria* and *Fusobacteria* in most samples but with lower abundance (Figure 3) [10].

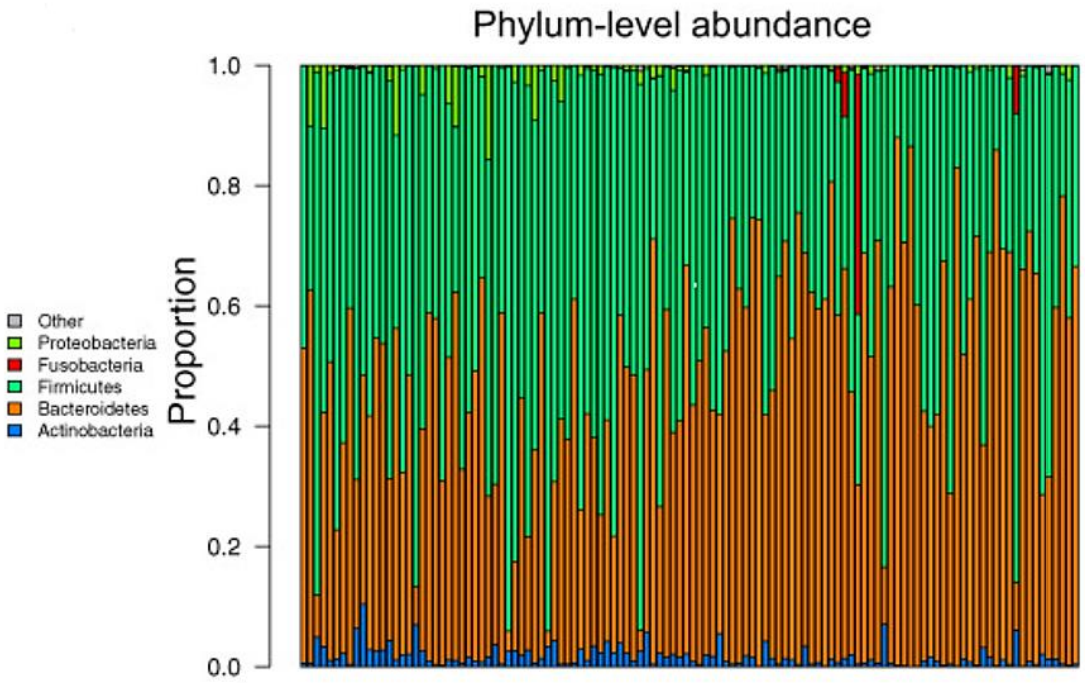

Figure 3. Relative abundance of the most common bacteria of the gut at the phylum level. 116 stool samples were obtained from healthy individuals selected from the Mayo Clinic Biobank. All the individuals that participated had to take a survey to assure the exclusion criteria about bowel symptoms, antibiotic and supplement use, alcohol intake, weight and cancer history. The individuals chosen were classified by age (20–29, 30–39, 40–49, 50–59, 60–69, and 70–79 year groups), sex (10 males and 10 females in each age group), race (white or non-white), BMI (<30 kg/m2 or ≥30 kg/m2), smoking status (current or former/never), and alcohol use (yes or no) and reflect the underlying population in the Upper Midwestern states. The figure illustrates the relative abundance of all the subjects at the phylum level and shows that in most cases *Firmicutes* and *Bacteroidetes* represent 90% of the microbiota [10].

However, when the groups are examined at the family, genera and species levels the differences are obviously more significative which suggests that there's a tendency to maintain certain groups of microbes at the phyla level because of their functionality but not in lower classifications. The number of bacterial species present in the human gut varies widely between different studies, but it is a general consensus that it contains around 500 to 1,000 species, although recent studies suggest that the collective human gut microbiota has more than 35,000 bacterial species [1, 5 ,9].

The results obtained by Chen et (2016) al supports the increase of diversity at the family level and show that even if the most abundant families were *Bacteroidacea (Bacteroidetes)* and *Ruminococcaceae* and *Lachnospiraceae (Firmicutes)* other samples

also had a considerable abundance of *Porphyromonadaceae* and *Prevotellaceae (Bacteroidetes), Enterobacteriaceae (Proteobacteria)* and *Clostridiaceae (Firmicutes)* among others (Figure 4) [10].

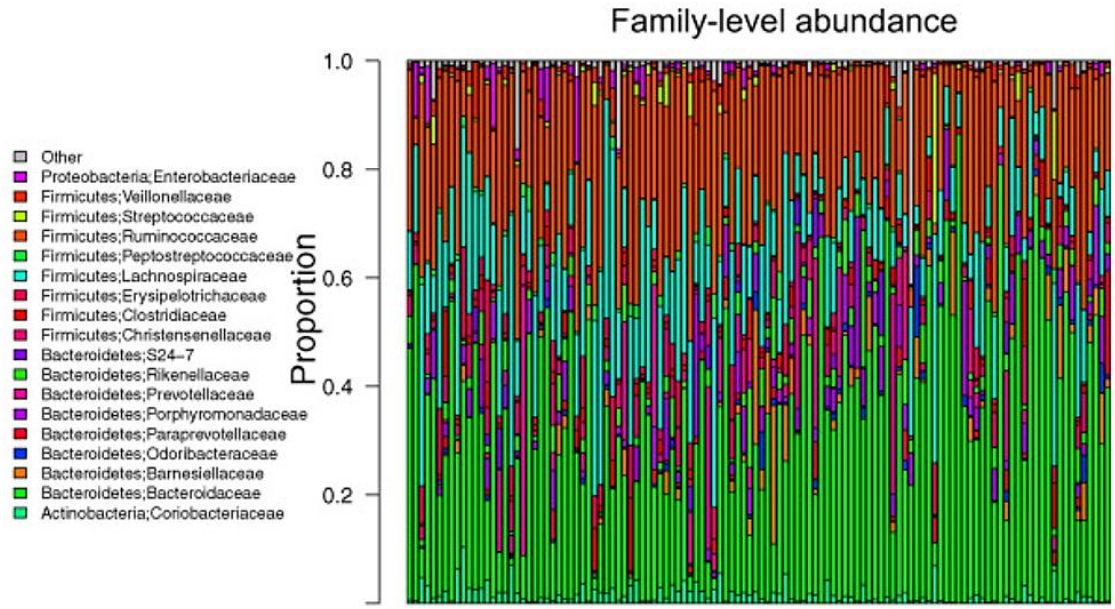

Figure 4. Relative abundance of the most common bacteria of the gut at the family level. The results were obtained from the same samples used by Chen et al, but at a more complex taxonomic level. Thus, from each sample, the abundance of families that existed in the previous phylums can be observed at the same conditions [10].

Chen et al (2016) also demonstrated the effects of BMI, demography, sex, age and alcohol use in the diversity of the gut microbiome which shows how there are a lot of factors that can change the microbe populations so it's crucial to bear them in mind. Of all these factors, the most significant differences were seen in BMI and demography.

In the case of BMI, subjects with higher BMI (> 33) presented less diversity compared to subjects with a lower and healthy BMI ($22 < x > 32$). On the other hand, white subjects generally exhibited a greater bacterial richness in contrast to non-white (Figure 5) [10].

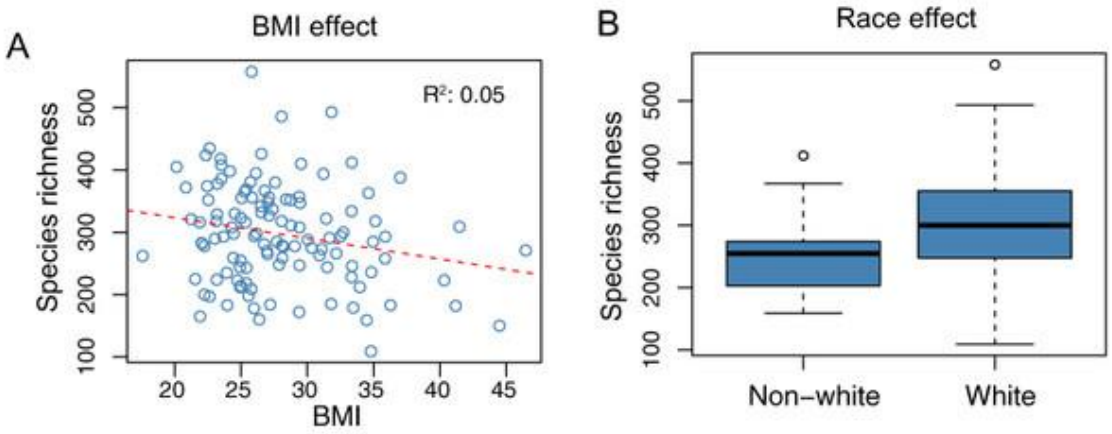

Figure 5 Figure A. Increasing of the BMI is associated with decreased bacterial richness. Figure B. White subjects presented and increase of bacterial richness. The three horizontal lines of the box represent the first, second (median), and third quartiles, respectively, with the whisk extending to the 1.5 interquartile range (IQR) [10].

Moreover, even if the most important microbes are bacteria, The GIT also contains methanogenic archaea (mainly *Methanobrevibacter smithii*), eukarya (for the most part yeast), and an extensive and very under-studied virome (primarily of bacteriophages) [5,9].

1.2 What is the thanatomirobiome?

To date, the research on the human microbiome has been focused on commensal microbes and pathogens that contribute to health outcomes, but currently a large number of articles have been published in which the microbiome is studied for forensic purposes. In this case, we speak about thanatomicrobiome when referring to the microbiome inside the organs and epinecrotic communities when talking about the microbiome on the surfaces of decaying remains (Figure 6) [12].

The word thanatomicrobiome originates from Thanatos who is the god of death in Greek culture and as is mentioned above is defined as the study of microbes that colonize the interior of organs after death. Furthermore, this term is relatively new, but recent articles have highlighted its importance in estimating the time of death of a decomposing body based on the changes that take place in the microbial communities. This is due to the fact that when the host dies, different sequential stages occur that change the conditions of the environment, making certain microbes reproduce without inhibition and the quiescence and death of others. This happens both in internal organs that are already colonized by microbes when the host is alive (such as lungs and gut),

and internal sites that are sterile (such as blood, liver, spleen, heart and brain) so when the host dies both are colonized by microbes following a specific order [6,13, 14, 15].

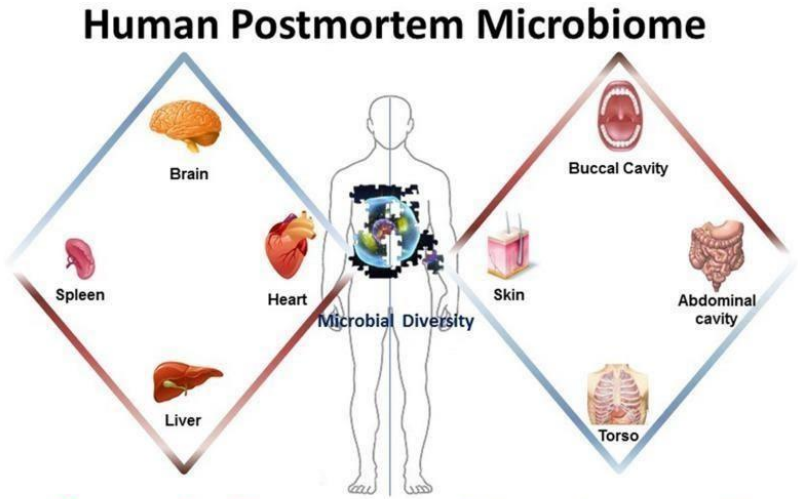

Figure 6. The human postmortem microbiome consists of the thanatomicrobiome that it's the microbiome of the internal organs of the cadavers like the brain, heart, spleen and liver and the epinecrotic microbial communities which are the microbiome of the surfaces of decaying remains and includes the skin, buccal cavity, torso and abdominal cavity [12].

Furthermore, when talking about the thanatomicrobiome, it should also be taken into account that, as in the microbiome, there are intrinsic and extrinsic factors that affect the dynamics of the descomposition and therefore the microbial communities. The slight alteration of any of these factors makes each decomposition unique, something that must be taken into account when calculating the post-mortem interval (PMI) or obtaining other data, since it can produce different and even potentially abnormal results [13, 14, 15].

For the study of the thanatomicrobiome, the Postmortem Human Microbiome Project (HPMP) was created, which is a little-known and still in construction project analogous to HMP in order to characterize the microbes that are part of the thanatomicrobiome, but also the epinecrotic communities, the necrobiome and the soil microbial communities associated with decomposition. In addition, other functions of HPMP are to establish a universal standard so that all laboratories can compare the results thanks to a unification plan that includes sampling, processing, sequencing and bioinformatics analysis and, ultimately, a database [16].

2. Objectives

To acknowledge the importance and role of microbes in the human body during life and death and the most abundant phyla that inhabit the gastrointestinal tract.

To analyse the microbial communities and chronological changes that occur in the thanatomicrobiome of the most studied organs to have an approximation of the post-mortem interval.

To review the most widely used mass sequencing techniques when studying the microbiome and thanatomicrobiome and their advantages and limitations.

To study the intrinsic and extrinsic factors that affect the thanatomicrobiome and the consequences of their alteration.

To discuss the future directions of thanatomicrobiome studies to calculate the post-mortem interval but also other crucial data in forensic investigations and the advances on the techniques that allow its study.

3. Bacteria during descomposition

The decomposition is a dynamic process that is greatly affected by the metabolism of microbes, however, little is known about the bacterial basis of human decomposition. This process is also affected by intrinsic factors that depend on the individual and extrinsic factors such as the environment, availability to insects, the clothing and others. All this causes that each decomposition processes to be practically unique, complicating the obtainment of accurate and reliable results. Decomposition can be described with 3 stages (autolysis, putrefaction and diagenesis) but some literature divide it with 5 stages (fresh that it's the same as autolysis, bloat, active decay and advanced decay that are all included in putrefaction and dry that substitutes diagenesis) but sometimes active and advanced decay can be both included in one stage named decay (Figure 7).

It is important to highlight that the different stages overlap with each other because the decomposition is a continuous process rather than a series of differentiated steps, also these stages can't be referred to as a especific frame time since their duration is affected by the factors mentioned before [17].

3.1 Fresh or autolysis

Human decomposition begins around 3-7 minutes after death has occurred and the first stage is called fresh or autolysis that lasts about 1 or 2 days and is dominated by a process called autolysis or self-digestion and physical changes such as *pallor mortis, livor mortis, algor mortis,* and *rigor mortis*.

Pallor mortis occurs only about 15-20 minutes after death and involves a physical change of colour of the skin due to the lack of capillary circulation.

Livor mortis starts about 1-2 hours after death, is well-formed around 3 to 4 hours and settles 6-10 hours later and it's the reddish-purple colouration of the skin caused by the formation of blood pools in the blood vessels as a result of gravity.

Algor mortis describes the constant loss of temperature that the corpse sufferers by the cessation of circulation and failure of the mechanisms that produce heat, in order to reach thermal equilibrium with the environment in which it is found and it normally lasts about 24 hours.

Rigor mortis is defined as the stiffness of death and describes the changes of flexibility that happens in the body due to the binding of actin and myosin filaments and the gelling of the cytoplasm. *Rigor mortis* starts 3-7 hours after death, peaks 12 hours later and dissipates in 24-48 hours as individual cells decay and myosin heads detach from actin filaments [17, 18].

After death, the respiration ceases and the heart no longer pumps blood through the body so cells are deprived of oxygen. Also, the blood becomes rich in carbon dioxide which forms carbonic acid, the pH decreases and the environment becomes acidic, and cellular by-products accumulate and act as a poison for the cells. The loss of the remaining oxygen is caused by the metabolisms of human cells and aerobic microbes mainly from the gut and lungs which changes the environment creating the ideal conditions for the proliferation of anaerobic microbes. At the same time, cells are lysed by uncontrolled lysosomal enzymes such as lipases, proteases and amylases that eventually causes them to release their contents that it's a fluid abundant in nutrients and gases. All of this causes the break down of the tissues but also the immuno system stops functioning, so bacteria mostly found in the GIT of the host begin to grow and spread to other organs.

Obviously, autolysis is faster in organs that have higher content in enzymes like the liver, but also in those that are high in water like the brain, but it ends up affecting all the cells of the body equally. Autolysis generally does not become visually apparent until a few days and is initially observed by the appearance of fluid-filled blisters and slipping of large sheets of the skin [17, 19, 20].

Moreover, some insects start to be attracted to the body towards the end of the fresh stage but some species of flies detect the corpse only after a few minutes after death. Flies are the first insects to interact with the body during decomposition and start their life cycle consisting in oviposition, hatching of eggs, feeding activity of larvae or maggots and the transformation of maggots to adult flies that normally lasts through the bloated and decay stages. Other insects present are ants that feed both of flesh, eggs and larvae [19].

3.2 Putrefaction

In this stage, the internal organs decompose but it happens following and order, It usually starts in the abdomen and then slowly affects other parts, including the heart, brain, and finally the uterus or prostate [17].

3.2.1 Bloat

Putrefaction is marked by the beginning of the bloat stage where the soft tissues of the body are destroyed by the actions of microorganisms like bacteria, fungi and protozoa resulting in the catabolism of tissue into gases, liquids and simple molecules. The first visible sign of putrefaction is a greenish discolouration of the skin due to the formation of sulfhaemoglobin by anaerobic bacteria in settled blood.

The main process in this step is the anaerobic fermentation by bacteria from the gut and other parts of the body that release by-products rich in volatile fatty acids like butyric and propionic acids and progresses with the accumulation of gases (hydrogen sulfide, carbon dioxide, methane, ammonia, sulfur dioxide and hydrogen) that causes expansion of the abdomen, the tongue and eyes to protrude and fluid to gush out of orifices like the nose, mouth and rectum. Sometimes, the gas and fluid that accumulates in the intestines can purge from the rectum and be strong enough to rip apart the skin provoking additional post-mortem injuries. Furthermore, the bloat stage is characteristic for the strong odour that the cadaver emanates due the liberation of sulfur-containing gases and amonia. Also, in the course of bloat flies continue to arrive to lay their eggs while ants keep feeding on them and other insects such as beetles start colonizing the corpse. This process lasts about 4 days and soon after the purging of gases is finished, active decay begins [18, 19, 20].

3.2.2 Active decay

The third stage is active decay also named purge and usually starts the fifth day and finishes the eleventh. Active decay stands out because the abdomen deflates and the greenish pigmentation turns yellow-brown-blackish with time. In this stage, black putrefaction occurs since there is a continuous tissue breakdown due to microbial and maggot activity and normally is first apparent in the head because fly larvae tend to colonize orificies like the nose accelerating the degradation. During active decay, the environment changes from anaerobic to aerobic since the pressure of the liquids and gases ruptured the skin allowing oxygen to enter the body again and providing more surface for the insects. So, the anaerobic bacteria remaining and aerobic bacteria further break break down proteins and fat liberating phenolic compounds and glycerols including indole, 3-methylindole (skatole), putrescine, cadaverine and various fatty acids. This stage is also known for the loss of electrolytes, weight loss from the prominent activity of insects and fluid purge and depending on the situation carnivores can also contribute to the decomposition of the body [19, 20, 21].

3.2.3 Advanced decay

Advanced decay is known for the mass migration of fly larvae away from the corpse due to the lack of nutrients. During advanced decay, the remains include portions of organs, bone, tendons, ligaments and some muscle and skin though there still can be some remaining flesh in the abdominal cavity and mould and microbes can mostly be seen in dry surfaces such as the skin, bone, clothing, soil and others. Moreover, even if most of the nutrients have already been consumed there are insects that can still be attracted to the body like adult beetles.

Sometimes, depending on the conditions in which the corpse is found, putrefaction can be replaced by other processes such as adipocere or saponification and mummification. Saponification results from the bacterial anaerobic hydrolysis of body fat and usually occurs in humid environments resulting in the hydrolysis of the triglycerides of the adipose tissue. Mummification typically occurs in warm, dry and arid conditions like a desert and substitutes putrefaction with the dissection of remaining tissue (normally skin) giving it a leather-like texture that clings to the bone [19, 20].

3.3 Dry or diagenesis

Diagenesis is a natural process that usually starts 24 days after death and in this stage only bone, cartilage and some skin are present therefore most insects have already left but in some cases centipedes and snails can be seen. Also, there is a cessation of activity of the individual's own microbes. During diagenesis, the amount of organic (collagen) and inorganic (hydroxyapatite, calcium, magnesium) compounds of the bone are altered as a result of the exposure to environmental conditions like moisture. This is achieved by the exchange of natural bone constituents, deposition in voids, adsorption onto the bone surface and leaching from the bone. It's important to keep in mind that putrid remains may still have a layer of adipocere and dry remains can have mummified tissues (some exposed bones may be sun-bleached if they are exposed) and become skeletalized [19, 20].

Figure 7. The 5 stages of decomposition in an animal model, in this case a pig. 1) Fresh or autolysis. 2) Bloat. 3) Activate decay. 4) Advanced decay. 5) Dry or diagenesis [19].

4. Materials and methods used in thanatomicrobiome studies

Most postmortem microbiological studies use animal models such as pigs and juvenile rodents like rats, because their decomposition process is similar from the one that happens to humans. In addition, the use of animal models allows greater control of the PMI and replicas but, sometimes human corpses are also used to characterize the thanatomicrobiome and in this case, most of these bodies are obtained from donations or from autopsies in criminal cases [12, 22].

However, the techniques and methods used in live animals and humans to describe the antemortem microbiome and the thanatomicrobiome and thus determine the PMI from bodies in criminal cases are practically the same [2, 12].

4.1 Sample collection

Currently, there is no protocol that indicates how the sample should be obtained but in most cases, the samples are collected by cutting the organ to be studied with a sterile scalpel or by scratching it vigorously with a sterile cotton tip [13].

4.2 Microbial cultivation

The composition of the microbiome and thanatomicrobiome can be investigated using culture-dependent or independent methods [14].

The culture dependent method is based on isolating or cultivating live microbes from environmental samples in order to characterize them with high taxonomic resolution. The main disadvantage is that more than 50% of microbes associated with humans have been estimated to be "unculturable". In this context, “unculturable” means that current culture techniques that laboratories use are unable to grow a given microbe so "unculturable" does not mean that they cannot be cultivated. On the other hand, although cultivation techniques have now been improved, which has allowed cultivating microbes that were previously considered "unculturable", it must be taken into account that microbes have a different capacity to grow depending on growth conditions so, it is difficult to deduce the abundance of microbes that will grow in the cultures [14, 23].

Moreover, with this method, only alive bacteria can be detected and there is a high risk of obtaining a false negatives. Nevertheless, bacterial culturing is able to detect most bacteria that are considered clinically important in human microbial studies [14, 24].

The independent culture method, unlike the previous method, consists of extracting and sequencing the genetic material directly from the sample, which allows the results to be more representative but also helps the investigators to obtain a more complete and higher quality profile with the comprehension of the interactions between the communities [14]. This is possible because independent culture methods are able to analyze both alive and dead bacteria but also fragmented bacteria DNA [24]. On the other hand, there is a loss of taxonomic ressolution [14, 24].

Common approaches of this method include metagenomic whole genome sequencing (mWGS) and marker gene sequencings like the 16S RNA gene for bacteria and archaea and internal transcribed spacer (ITS) for fungi [14].

4.3 Analysis of the communities

To examine microbial communities, genetic analysis is combined with metagenomics as a complementary method. Metagenomics is the direct sequencing of the complete microbial communities which allows the analysis of the whole genomic content hence obtaining information about the complexity of the microbial communities. Moreover, metagenomics enables to study both culturable and unculturable bacteria that can be found in each community, since it directly analyses the environmental sample without the need for cultures [25]. To extract the DNA most researchers use widely employed protocols and/or commercially available kits specific to the sample type and the same happens with sequencing with Illumina MiSeq and 454 pyrosequencing platforms being the most used. In addition, The HMP indicates and explains the optimal kits, precautions against contamination, the instruments, databases and most indicated software to obtain optimal results. Most studies follow the recommendations of the HMP but there are articles that suggest other methods that provide a higher microbial diversity [2].

4.5.1 16S rRNA sequencing

Most studies about microbial communities use sequencing technics that target specific genetic markers like the 16S rRNA in the case of bacteria and archaea (especially with the culture-independent method) and ITS for fungi [14].

After DNA isolation, the DNA is selectively amplified using primers targeting regions of the 16S rRNA, this gene codes for one of the components of the minor subunit (30S) of the ribosome in bacteria and archaea and it’s vital for their survival.

The prokaryotic 16S rRNA gene contains nine hypervariable regions (V1-V9) that are flanked by conserved stretches that allow the PCR to amplify the target regions using universal primers (Figure 8). These hypervariable regions are the ones that allow distinguishing the different types of bacteria and archaea because they show great diversity. However, hypervariable regions exhibit different degrees of diversity between them and no region is able to distinguish all bacteria and archaea, this is why usually a

group of regions is analysed instead of a single region, the most common ones being V1-V3, V3 -V5 and V4-V5. Moreover, recent studies have discovered that the fourth hypervariable region (V4) has the highest discriminatory power and sensitivity to differentiate microbes and that the combination of V2, V3 and V4 gives the best accuracy for taxonomic determination [26, 27].

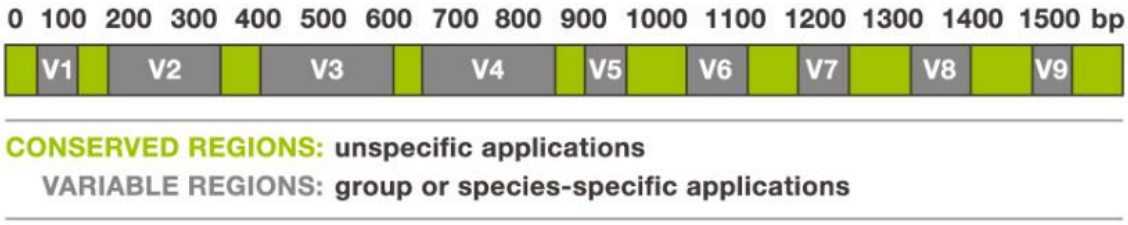

Figure 8. Structure of the 16S rRNA gene. The 16S rRNA gene is approximately 1550 bp in length and contains 9 hypervariable regions flanked by conserved regions in all bacteria and achaea that are targeted by the primers for PCR amplification so all the 16S rRNA genes in the sample are amplified [14, 28].

The main reasons why this marker is used is because it is distributed across all bacteria and archaea lineages and since it is responsible for an essential process in the cell, the function of the gene has not changed, which suggests that the variations that have occurred are random so organisms still can be differentiated according to their evolution, the gene is long enough that the information can be processed through computers and the technique is relatively cheap (costs as little as 5$ for a partial sequence or 25$ for the full-length sequence) and fast [14, 28].

However, the main drawback of this technique is that it has a limitation in the differentiation of sequences at the species level, so it cannot guarantee that two strains are not of the same species. For this reason, this technique is mainly used to distinguish organisms at the phylum, family and genus level that, although this doesn't bring specific information as the species level, provides more reliable results. One of the reasons why this technique is not optimal at the species level is the generation of chimeras and the intrinsic error rate associated with PCR resulting in sequences that can be identified as new species. It should also be borne in mind that some 16S rRNA genes can be amplified with different efficiency due to the affinity of the primers and the GC content and that the allocation of taxonomy is dependent on the integrity of the databases used as reference [14, 28].

4.5.2 Metagenomic whole-genome shotgun sequencing (mWGS)

Metagenomic whole-genome shotgun sequencing (mWGS) unlike marker gene sequencing techniques sequences directly all DNA material extracted from a sample providing a better insight of the biodiversity and functionality of a community with a higher taxonomic resolution. In addition, mWGS data has been demonstrated to distinguish microbial communities at the species level and to strictly monitor transmissions and evolutions at the strain level compared to 16S rRNA. It should also be mentioned that the mWGS was developed as an alternative to avoid all the errors that happen in the gene marker sequencing techniques like 16S rRNA, especially in the case of unculturable bacteria [14, 29].

On the other hand, the main disadvantage of mWGS is the complexity and difficult interpretation of the obtained data associated with the nature of the technique. Fortunately, new tools and data resources have been specifically developed to bypass these complications allowing researchers to determine which microbes are present in the community and their functionality [29]. Furthermore, sometimes is not possible to sequence the entire genome of every single organism so, the most abundant organisms end up having the more complete genomes due to being more represented in the sequences and the less abundant bacteria have much more incomplete genomes. [30]

As mentioned before when using mWGS all the DNA from the sample is extracted and instead of targeting a specific locus for amplification, all DNA is cut into small fragments that will be independently sequenced multiple times. The resulting sequences (reads) are aligned together with computer programs since they overlap at the end with each other to create a consensus sequence which is representative of the original DNA sequences in the bacterial genome. Finally the consensus sequences are introduced in databases (Figure 9). [29, 30]

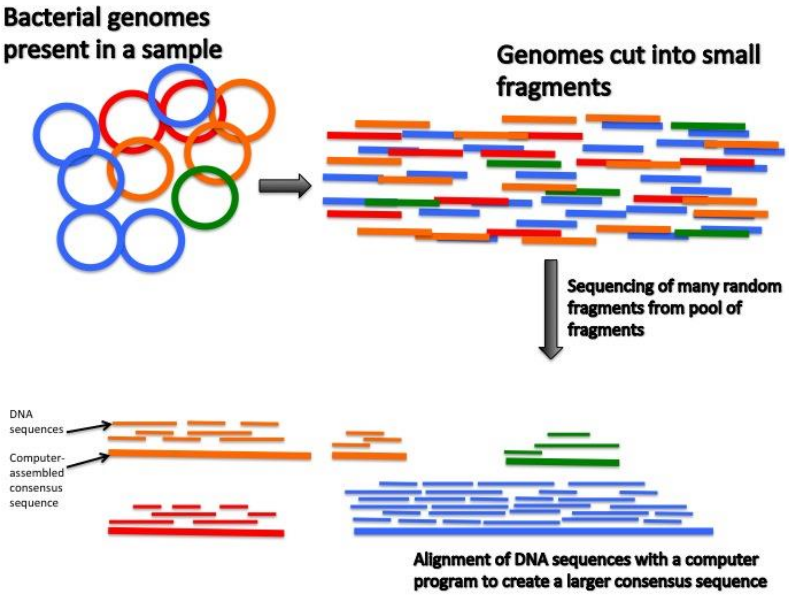

Figure 9. After the sample is collected all the genomes are cut into smaller fragments in order to be sequenced. Then the fragments are sequenced multiple times to assure that most fragments are sequenced. After sequencing, the reads are aligned in order to create a longer sequence with computer programs called a consensus sequence which is finally introduced in databases. In some cases, consensus sequences missing from databases define new species that can be annotated and incorporated into databases [30].

4.4 Bioinformatic Analysis

After sequencing, all the data obtained is processed and analysed using a standard microbial diversity analysis pipeline consisting of two major stages, the denoising and chimera detection stage and the microbial diversity analysis stage. During the first stage, denoising removes short and singleton sequences, and noisy reads while chimera detection eliminates chimeric sequences. Finally, the remaining sequences are corrected base-by-base to remove noise from within each sequence [13, 28, 31].

In the case of 16S rRNA, to obtain the relative taxonomic abundance and the phylogenetic classification each sample runs through an analysis pipeline to cluster the reads into Operational Taxonomic Units (OTUs) (in most cases all reads that have a similarity of 97% cluster into one OTU) and then each OTU is globally compared to a high-quality database like Silva or GreenGenes to determine the taxonomic classifications (phylum, genus, family being the most common) (Figure 10) [2, 13, 31].

Finally, the results can be interpreted using alpha or beta diversity metric values. Alpha diversity is the diversity of microbes in one sample and measures the richness using the number of species or OTUs or like in most cases the relative abundance which can be illustrated with an octave plot. To obtain these results the most used indices are Shannnon diversity index for richness and Shannon evenness index for abundance. On the other hand, beta diversity compares two samples by calculating a distance. For example, Bray-Curtis dissimilarity and Jaccard distances calculate a number that ranges

from 0 to 1 which indicates the similarity or difference between two samples and is represented in a distance matrix (increasing values indicate lower similarity and increasing distance). Nonetheless, the most used algorithm is UniFrac that is based on the fraction of branch length that is shared between two samples or unique to one or the other sample and can be unweighted (qualitative) or weighed (quantitative). Samples can be clustered to bring similar samples together, producing a tree or represented with principal coordinates analysis (PCoA) [2, 13, 28, 31].

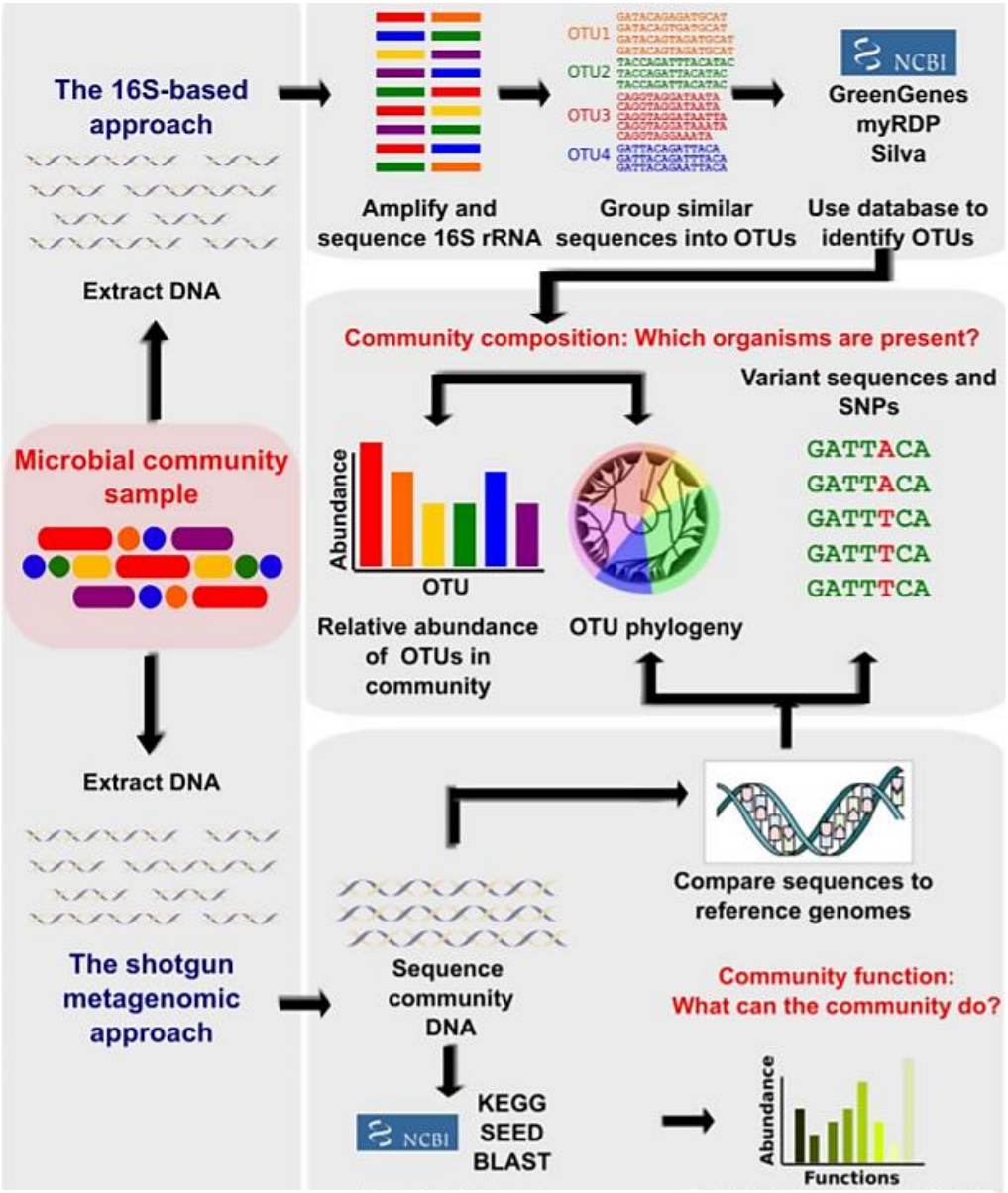

Figure 10. Summary of the most common bioinformatics analysis to study results obtained through 16S rRNA and mWGS [30].

On the other hand, in the case of mWGS the consensus sequences can be directly introduced in functional databases like KEGG or SEED which allows to describe the community as relative abundances or PCA of its genes and pathways or compared to reference genomes to be analysed using OTUs like 16S rRNA. Finally, sometimes sequences can be studied through the observation of single nucleotide polymorphisms which is more expensive but provides better taxonomic resolution. (Figure 10) [29, 30].

5. Progression of the thanatomicrobiome

Recently in the past few years, plentiful articles have been published about the thanatomicrobiome and all of them conclude that the diversity of microbes in the human thanatomicrobiome varies by organ and mostly changes as a function of time and temperature. To understand how the thanatomicrobiome changes during time it's important to know that in health most internal organs such as the heart, brain, liver and spleen are considered sterile so the composition of their thanatomicrobiome comes principally from horizontal migration of the microbes of the host's digestive tract and the environment in contact with the corpse. Nevertheless, the microbial contribution from the soil and insects like flies can be easily differentiated from the gut even if at the phylum level they are similar.

Horizontal migration from the GIT to other body sites it's possible because after death all the mechanisms that suppress microbial growth no longer function which includes the innate and adaptative responses and tissue barriers but also because the temperature changes altering the environment. This causes that 24 hours after death microbial cells overcome the protective systems of the body and migrate to the internal organs [32].

The migration of microbes from the GIT is the principal factor that decides in which order internal organs are colonized. During the end of bloat and start off decay, the internal organs closer the GIT like the liver, pancreas and spleen are invaded by microbes next goes the heart and lungs and following with the brain, kidneys, bladder and at the very last the prostate or uterus (Figure 11). Even so, the characteristics of the organ itself also influence the rate of decomposition [32].

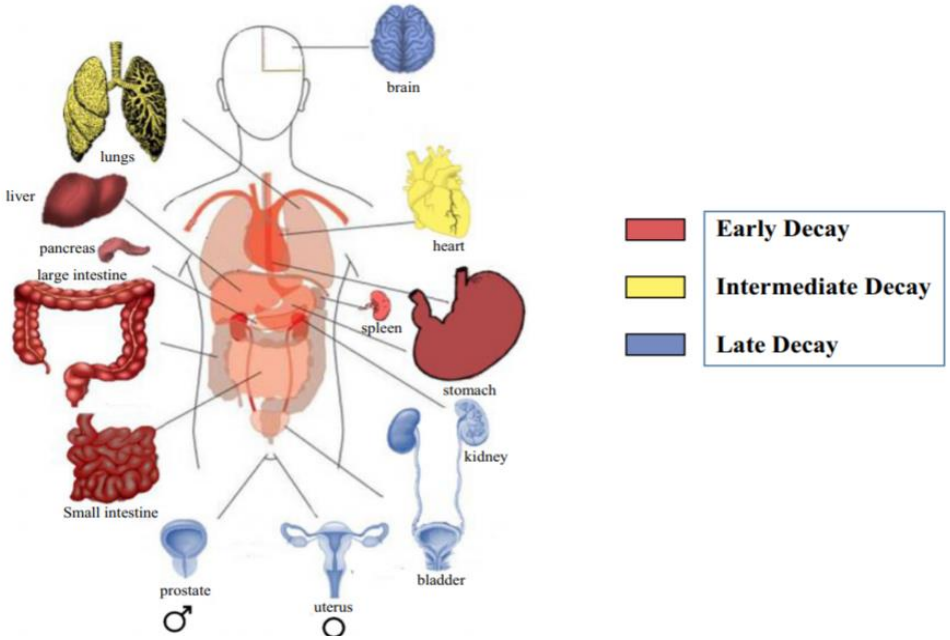

Figure 11. Internal organs closer to the GIT are the ones that are colonized first by microbes due to their proximity like the liver, pancreas and spleen. The next internal organs that are invaded are the heart and lungs preceding the brain, kidneys, bladder and at the very end the reproductive organs [32].

The migration of GIT microbes has been proven by several investigations mainly using animal models. It has been demonstrated that removing protective barriers, such as the epithelial mucosa of live mice, as it occurs 24 hours after death, facilitates the colonization of gut microbes to other organs. Other studies, using luminal gut contents of sacrificed mice, show that gut microbes can colonize organs such as the liver, cardiac blood, and kidneys even 5 minutes after death. Finally, bacterial translocation studies have also been performed using whole-body fluorescent imaging techniques and culture-based techniques in mouse models to track the invasion of particular gut bacteria.

Table 1. Order of degradation of the organs starting with those with an early decay such as the stomach and larynx and ending with late decay organs as the reproductive organs [32**]**.

	Body sites
Early decay	Larynx and trachea
	Stomach and intestines
	Spleen
	Mesentery
	Liver
	Pancreas
	Adrenal tissues
	Pregnant uterus
Intermediate decay	Heart
	Lungs
	Kidneys
	Oesophagus and diaphragm
	Blood vessels
Late decay	Brain
	Urinary Bladder
	Prostate and nulligravid uterus
	Skin, muscles and tendons
	Bones

Moreover, to determine the order in which the migration of microbes from the GIT to the rest of the internal organs occurs (Tabe 1) and, therefore, the development of the decomposition

process, several studies analyze the rate of decay of the DNA from the cells of each organ with flow cytometry. The results of this method show that there is a dependent relationship between DNA degradation and the proximity of the organs to the gut and time which mean that organs closer to the GIT have a faster DNA degradation compared to those who are in far distances.

Nevertheless, it's important to say that the cause of death can alter the order in which the thanatomicrobiome migrates meaning that the microbes don't always follow the predicted sequence described in this review [32].

In terms of body sites, brain, heart, liver, spleen and reproductive organs have the most stable community composition over time so most studies about the thanatomicrobiome focus on them, especially the liver [14].

5.1 Spleen

The spleen is the largest ductless gland and the biggest single lymphoid organ in the human body and as the most vascular organ so it decomposes easily but it can be slower if it is in a low blood level which provokes large knowledge gaps concerning the mechanisms of its decay. Nevertheless, recent studies have revealed that the degradation rate is low up to 5 days after death but it gets much faster after an extended period of time. Moreover, it has been demonstrated that the spleen shows a very diverse thanatomicrobiome compared to other internal organs [32, 33].

5.2 Liver

The liver is the internal organ that has the most abundant and diverse thanatomicrobiome and is considered predominantly free of microbes for the 5 first days after death. The reasons behind its wide diversity include the fact that it is the biggest gland in the human body, it has an important role in all metabolic processes, its location close to other abdominal organs full of microbes like the GIT and its fast autolysis due to pancreatic enzymes, stomach acids and gallbladder fluids that accelerate its putrefaction. Moreover, it has been demonstrated that the liver can be used as a control for postmortem contamination meaning that if monomicrobial growth is seen it is considered a true positive [14, 32].

5.3 Heart

The heart is relatively resistant to decomposition compared to other internal organs. Cardiac tissues can persist up to 4 days after death, but it is also possible to detect coronary arteries even months later. In addition, cardiac cells support differential rates of autolysis depending on their location, which means that cardiac cells on external surfaces break down faster than those found in deeper parts of the organ (it is considered that there is a delay of 2 days between the subendocardic and subepicardic autolysis) [32].

5.5 Brain

Even though little is know about the post-mortem brain it has been demonstrated that brain cells are the first type of cells to decay and their death normally occurs 3 to 7 minutes after death. However, some studies have reported that sufficient DNA can be collected in brain samples after 3 weeks for a DNA-fingerprint. The main reason why it's still possible to obtain enough DNA after all this time compared to other organs is due to low concentrations of digestive enzyme especially nucleases [32, 33].

5. Reproductive organs

The prostate and nulligravid uterus are the last organs to decompose (but the gravid uterus goes through putrefaction earlier) due to the anatomy of both organs, their active immune systems and protective accessory organs (the vagina and penis, respectively).

In the case of the uterus, there is growing evidence of the existence of a microbiome in the gravid uterus compared to the non-gravid uterus that it's considered sterile and with slower rates and decay and less enzymatic activity. Also, it's been demonstrated that putrefaction takes place faster in organs that are made of less muscle and fibrous tissues and that circulate a relatively large amount of blood. This also explains why the nulligravid uterus decomposes slower compared to the gravid uterus since the later becomes highly vascularized, less fibromuscular and undergoes through an increase in blood flow during pregnancy.

In the other hand, the prostate is an organ rich of fibromuscular tissue and it is inside portion is made up of smooth muscle that is surrounded by collagen. Collagen creates a

protective shell with high resistance to decomposition so only a handful number of microbes with specific the enzymes can break it down [32].

6. Determination of the PMI

The PMI describes the time that has passed since someone has died and its estimation is crucial during criminal and historical investigations. Knowing the PMI can help investigators eliminate suspects if they have a valid alibi for when the death occurred allowing investigators to focus on other clues, support the integrity of complementary physical evidence, determine the exact cause of death or if the scene was set by the criminal, solve historical cases, etc [34].

Currently, the thanatomicrobiome is not the first method taken into account to estimate the PMI. In the case of early PMI, physical (Livor Mortis, Algor Mortis and Rigor Mortis), biochemical (vitreous humour, synovial fluid, pericardial fluid, urine, and cerebrospinal fluid) and histo-morphological changes (analysis of blood cells and skin structure) are normally the most used methods.

On the other hand, entomology, mRNA and DNA degeneration, inorganic consitutents of the bone and even proteins are typically used to determine the late PMI [17].

As discussed in the preceding section, the GIT microbiome invades the internal organs following a particular order but the microbes that migrate are not always the same, because during descomposition the microbial communities shift to adapt with the environmental changes of each stage.

In general, during decomposition, important changes in the environment occur influenced by oxygen availability that affect the succession of microbial communities. During the beginning of the bloat stage, the conditions go from being aerobic to anaerobic due to the metabolism of the remaining cells and microbes of the body, but once the skin ruptures at the beginning of decay, oxygen is reintroduced into the previously hypoxic body causing an increase of aerobic bacteria and preventing the growth of anaerobic bacteria. These oxygen switches are the ones that cause the quiescence of certain microbes and the domination of others so as decomposition progresses, microbial diversity decreases but richness increases. Nevertheless, overall the most prominent phyla are *Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria* which coincide with the GIT microbiome. [15, 32]. Furthermore,

Proteobacteria is the most abundant phylum in the early stages of decomposition, while *Firmicutes* dominates in the late stages [37].

As tissues break down during autolysis and as oxygen declines, the microbes from the GIT have a free path to migrate to other organs and before the environment becomes anaerobic, facultative aerobic species *Staphylococcus (Firmicutes)* is the first one to migrate from the small intestine. Moreover, in early PMI's (under 50 hours) aerobic and anaerobic facultative such as the genera *Lactobacillus, Veillonella (Firmicutes), Prevotella (Bacteroides), Streptococcus (Firmicutes)* and *Gemella (Firmicutes)* are abundant [14, 38].

As time passes by and due to the release of oxidative gases during autolysis there is an ongoing decline of anaerobic taxa such as *Bacteroidetes* and *Lactobacillus (Firmicutes)* and a higher abundance of *Enterobacteriacae (Proteobacteria)* [14].

However, the relative abundances of anaerobic gut bacteria such as *Lactobacillaceae, Bacteroidaceae (Proteobacteria)* and *Clostridia (Firmicutes)* increase at the bloat stage [37].

Later, with the reintroduction of oxygen in the hypoxic bodies, there is another switch and the environment changes from anaerobic to aerobic causing an increase in aerobic bacteria but promoting the quienescense of anaerobic bacteria [15, 32].

This causes an increase of aerobic bacteria and facultative anaerobic bacteria like some members of the *Enterobacteriaceae* family *(Proteobacteria)* such as *Enterobacter* and species of *Escherichia* like *E. coli* and *E. albertii* (these *Escherichia* species are especially noticeable at PMIs longer than 240 hours) and finally anaerobic bacteria such as the class *Clostridia (Firmicutes)* that also include facultative anaerobic species and the genus *Bacteroides (Bacteroidetes)* [15, 37, 38].

To summarize, most investigations highlight the potential of some members of the phylum *Firmicutes* like *Lactobacillus* and *Clostridia* as the best biomarkers since they have shown to exhibit time signal. In the first case, *Lactobacillus* has been demonstrated to be more abundant in cadavers with short PMIs [13, 14]. On the other hand, although at first it was believed that *Clostridia* was only abundant in late PMI's, recent studies have revealed their ubiquitous nature and sometimes they can even be the most abundant prokaryotes in early PMI's like 4 hours after death [13, 31].

Javan et al (2017) also evidenced in on of their latest studies in 2017 that the facultative anaerobic species *Clostridium sp.* are capable of translocating to surrounding tissues within 5–48 h after death at 25◦C. Nevertheless, even though the presence of *Clostridium sp*. was continuous, other species of *Clostridia* class change with time and some were more prevalent in early PMI's while others at later PMI's [31].

For example, Javan et al also discovered that *C. perfringens*, has the fastest generation time of approximately 7.4 min at optimal temperatures (37–45◦C) [31]. Other species present in early PMIs include *C. sordelii, C. difficile, C. barlettii, C. bifermentans y C. limosum* while *C. haemolyticum, C. botulinum y C. novyi* develop in PMIs superior to 240h [38].

This year Liu et al (2020) published a new article with the aim to develop an accurate model to estimate the PMI. This study shows how at the phylum level, *Proteobacteria* has a higher abundance in early PMIs but later their abundance declines as *Firmicutes* dominates the organs. Also, the presence of *Bacteroidetes* declines drastically after the two firsts days in the brain and heart probably due to the release of gases during autolysis but it's slower in the cecum. Moreover, the cecum which seems to behave differently than the other organs also presents a decrease in abundance of *Actinobacteria* (Figure 12) [37].

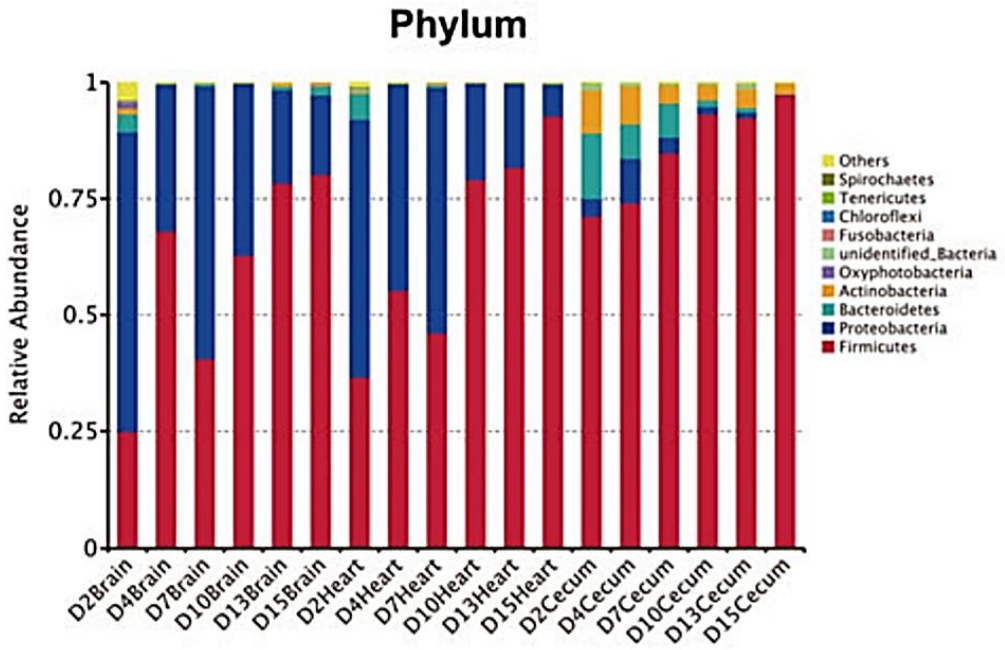

Figure 12. Relative abundance at the phylum level. Lui et al (2020) analyzed 176 internal organs (heart, brain and cecum) of 80 rodent cadavers during the firsts 15 days of decomposition using 16S rRNA. The samples were named taking into account the organ and the PMI in days [38].

Liu et al (2020) also analyzed the succession of the thanatomicrobiome at the family level. In this case, there is a significative abundance of *Staphylococoaceae* and *Lactobacillae* at the earliest brain and heart samples respectively and a tendency of a decline of the abundance of *Enterobacteriacea* in all organs even though the abundance rises the seventh day for the brain and heart meanwhile for the cecum this happens later. Furthermore, *Clostridiales* are seen in all samples at all PMIs except at the first two days after death at the brain and the abundance of this family mostly increases with time but it can change due to the oxygen availability. Another observation is how *Erysipelotrichaceae (Firmicutes)* which is only found in the cecum in high abundances suffers an important drop of abundance in samples collected in the seventh and tenth day. This could be caused by the loss of oxygen availability during bloat since this family is made of aerobes and facultative anaerobes. (Figure 13) [37].

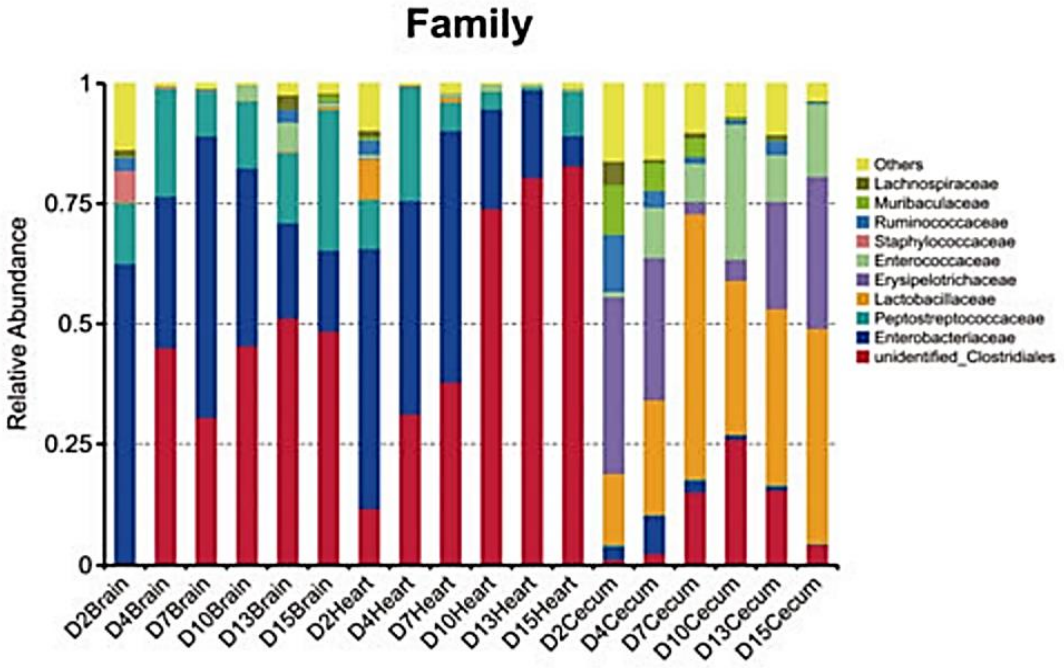

Figure 13. Relative abundance at the family level. Lui et al (2020) analyzed 176 internal organs (heart, brain and cecum) of 80 rodent cadavers during the firsts 15 days of decomposition using 16S rRNA. The samples were named taking into count the organ and the PMI in days [38].

Furthermore, beta diversity exhibits that samples from the brain and heart have similar microbial communities at different time points because the microbes from the GIT migrate first to the heart and then to the brain so the samples cluster together at different time points (Figure 14) [38].

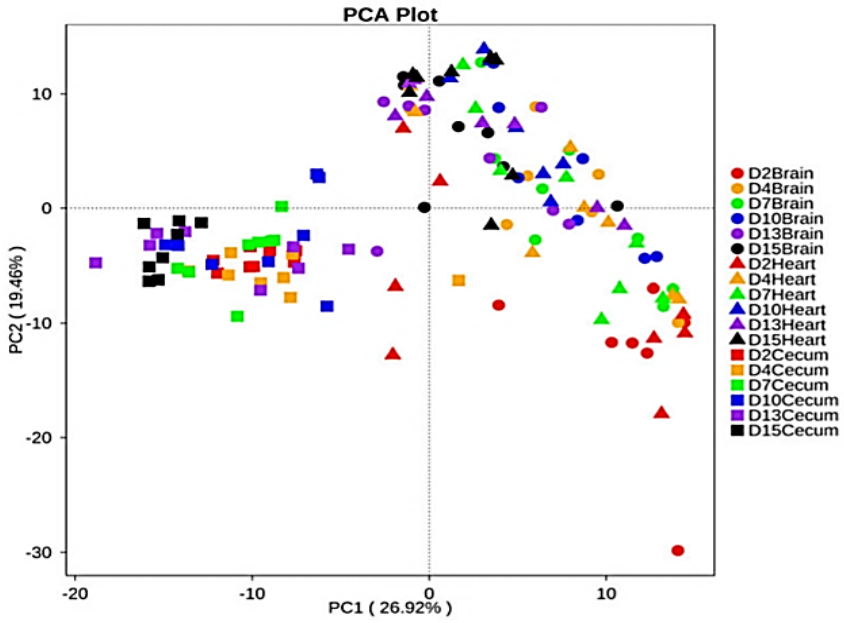

Figure 14. PCA plot of metabolic pathways expression. The PCA plot shows all the samples distributed depending on their relative abundance of certain metabolic pathways. To define the correlation between postmortem microbiota and metabolic changes within the decomposition all the sequences from each sample were introduced in the KEGG database and then distributed and clustered according to the relative abundance expression level of KEGG pathways. Samples from the brain and heart are clustered together at different time points due to the similarity of their microbial communities while samples from the cecum have a high significative difference compared to brain and heart samples. Samples from the same organ and similar PMIs also cluster together in most cases especially the samples from the cecum [37].

Moreover, samples from the same organ and similar PMIs also cluster together in most cases especially samples from the cecum. On the other hand, samples from the cecum have a high significative difference compared to brain and heart samples [38].

The optimal internal organ for studying the thanatomicrobiome and therefore obtaining the PMI is the liver and as mentioned before this organ is considered highlty sterile up to 5 days after death [24].

A study conducted by Tumisto et al (2013) analyzed the liver from 33 corpses from criminal cases using bacterial culturing and real-time quantitative polymerase chain reaction (RT-qPCR) shows that with time the amount of GIT bacteria increases in this organ. Both techniques demonstrate that the sterility of the liver remains stable for the first 5 days but after that, it considerably decreases so the liver is one of the most

appropriate organs for microbiological sampling at least before 5 days post-mortem (Figure 15) [24].

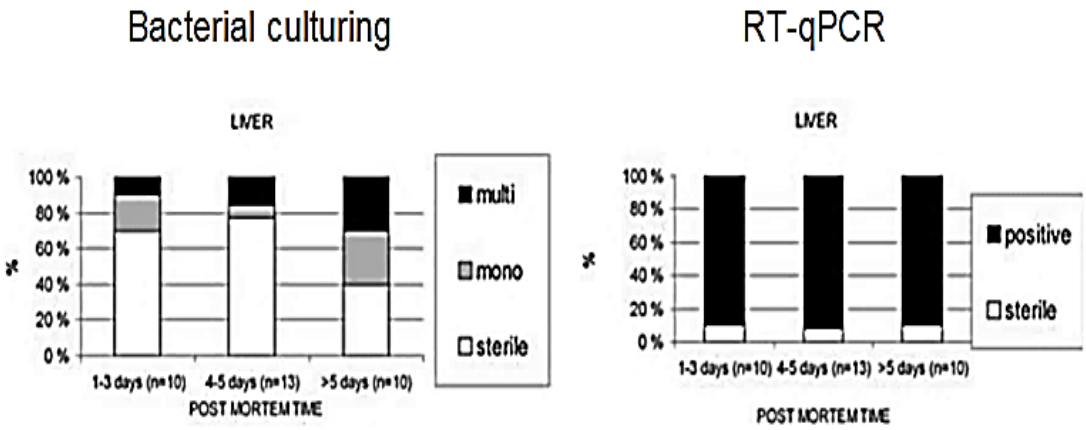

Figure 15. Percentage of bacterial findings in the liver using bacterial culturing and RT-qPCR with specific primers and probes. In culturing, the number of bacterial isolates per sample is represented as sterile (no bacterial growth), mono (single isolates) and multi (more than one isolate). However, in RT-qPCR, sterile means no bacterial DNA present meanwhile positive means there is bacterial DNA [24].

Tumisto et al (2013) documented how *Staphylococcus* that is the first bacteria that transclotates from the gut has a high abundance in low PMIs but decreases fast with time. Their results also show the ubiquitinous nature of *Clostridium sp.* since this species appear in all the samples even if they have different PMIs. On the other hand, *Bacteroides* and *Streptococcus* seem to remain stable at all PMIs while *Bifidobacterium* and *Enterobacter* proliferate with time especially *Bifidobacterium* in PMI's longer than 5 days probably due the loss of oxygen since they are anaerobic (Figure 16) [24, 39].

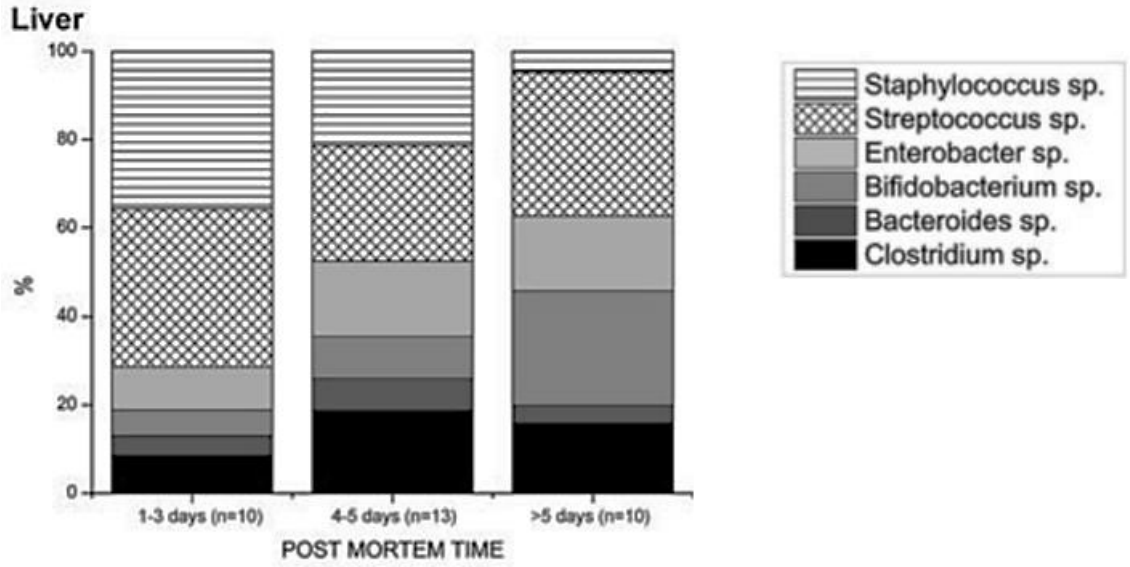

Figure 16. Relative amount (%) of the most common genera found in liver bacterial DNA positive samples in RT-qPCR. In the liver, there were seven samples likely to be true positive (*Clostridium sp., Streptococcus sp., Enterobacter sp., Enterococcus sp., Escherichia sp., Staphylococcus sp., or Streptococcus sp*.) [24].

In addition to the highly active temporal changes at each body site, there is also an exchange of microbes among body sites as well as between the body and the environment. This transmission of microbes to the environment also has the potential to predict the PMI and it has been demonstrated that it is similar to the one that occurs for the thanatomicrobiome [14].

A study conducted by Cobaugh et al (2015) demonstrated that the phylum *Firmicutes* rises in abundance in each step of decomposition because the bacteria in the body are transferred to the soil reducing the abundance of the typical taxa of the soil. The experiment also shows that over time (at late decay) the soil's own microbiome ends up replacing the transferred bacteria and therefore, the abundance of *Firmicutes* declines (Figure 17). [14, 40].

Moreover, it has also been observed that there is a transmission of bacterias from insects like flies to the body especially taxa such as *Ignatzschineria* and *Wohlfahrtiimonas* that comes from flies. In addition, it's important to mention that the microbial communities from the environment can vary significantly depending on the geographical location so the identification of there strains in the thanatomicrobiome can potentially discover if the body was moved which is also crucial in forensic investigations [14].

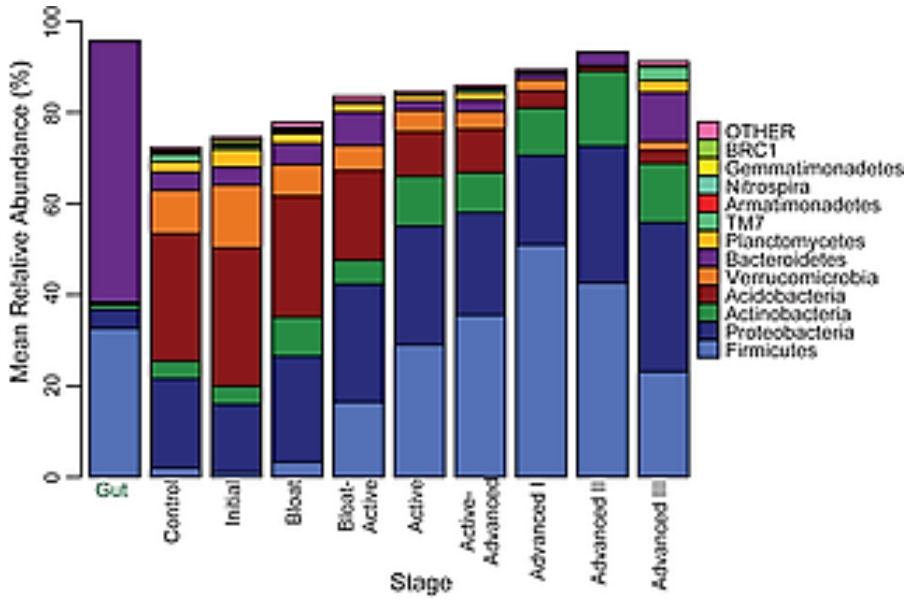

Figure 17. Transference of the gut microbiome to the soil. *Firmicutes* a phylum predominant in the gut but inexistent in soils trasnfers from the gut to the soil so soil samples from under the torax of the body present *Firmicutes*. As time passes *Firmicutes* dominates the soil's taxa peaking at advanced decay 1 [39].

7. Factors that alter the descomposition and the thanatomicrobiome

The development of the thanatomicrobiome depends on exogenous and endogenous factors that alter the progression of the microbial communities and therefore affect the accuracy of the PMI and other information that can be useful in forensic cases (Figure 18) [32].

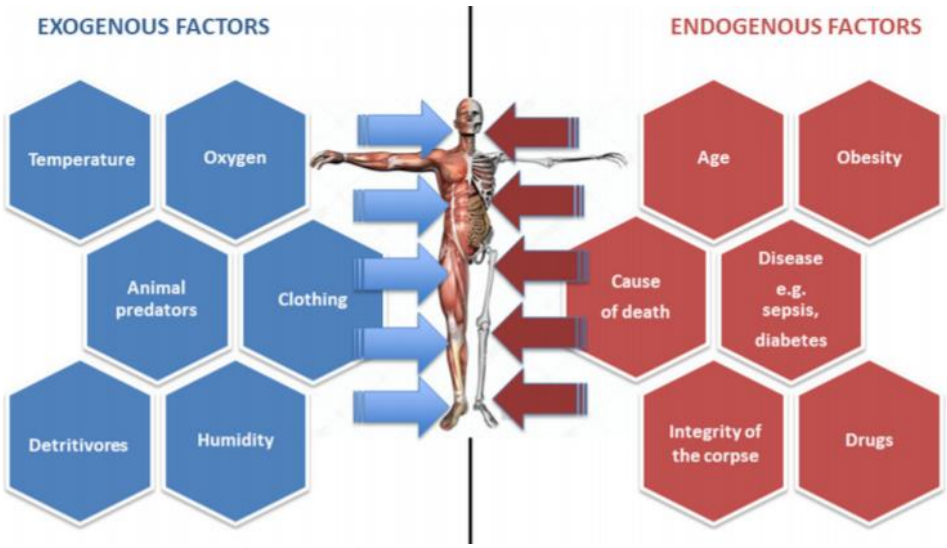

Figure 18. Exogenous and endogenous factors. This factors are the most important variables that alter decomposition making every discomposition unique and therefore alsoparticipate in the succession of microbial communities [32].

7.1 Exogenus or extrinsic factors

Exogenous or extrinsic factors are those that aren't inherent of the individual but of the environment. The most important exogenous factors are the temperature and humidity because high temperatures and humidity greatly accelerate the decomposition rate and the metabolism off microbes and insects. Moreover, oxygen is also a crucial factor and the more oxygen is available the fastest the decomposition. For example, bodies decompose slower in the water and soils with less oxygen content. Other factors that facilitate decomposition are the presence of animal predators such as bears and detritivores like insects. Finally, if the body is clothed the decomposition rate will decay since it will diminish the exposure to the environment and slow down the rate of cooling of the body. Moreover, thick and tight clothing slow decomposition even further [15, 17, 32, 40].

7.2 Endogenous or Intrinsic factors

Intrinsic factors are those that depend on the individual. In this case, some of the most decisive factors are the cause of death and the integrity of the corpse. The cause of death can alter the rate of decomposition, for example, people who die due to infections undergo a faster decomposition in comparison to people who die of malnutrition but also it can affect the order of the succession of the thanatomicrobiome. Moreover, people who suffer from certain diseases can have an altered microbiome. On the other hand, the integrity of the corpse can also change the speed of decomposition and the succession of the thanatomicrobiome because putrefaction starts with the rupture of the skin so bodies with open wounds led to faster decomposition especially in the affected area. Another important factor is the fat content in the body since a higher percentage of fat means a higher water content and better retention of temperature and therefore a faster decomposition which makes putrefaction faster in woman and people with more adipose tissues. In addition, drugs like antimicrobials slow down decomposition while cocaine and methamfetamine will make insects decompose the body faster. Finally, younger people decompose faster than older individuals primally because the firsts have higher water content [17, 32, 40].

7.3 Sex related differences

As seen above, most studies about the thanatomicrobiome determinate the PMI using bacteria that can be found both in male and female cadavers but recent studies have shown that there are distinct bacteria that depend on sex that also are influenced by time and therefore have the potential to determinate the PMI.

Javan et al (2016) analysed the brain, liver, heart and spleen from 27 human corpses from criminal cases with postmortem intervals between 3,5-240 hours using 16S rRNA and found out that apart from temperature and time another important factor that influenced the microbial communities was sex. The main differences were that female cadavers had a higher abundance of the aerobe bacteria *Pseudomonas (Proteobacteria)* compared to men while only male presented *Rothia (Actinobacteria)* and higher abundances of *Lactobacillus (Firmicutes)* and *Streptococcus (Firmicutes)* (Figure 19) for reasons that are not yet known [13].

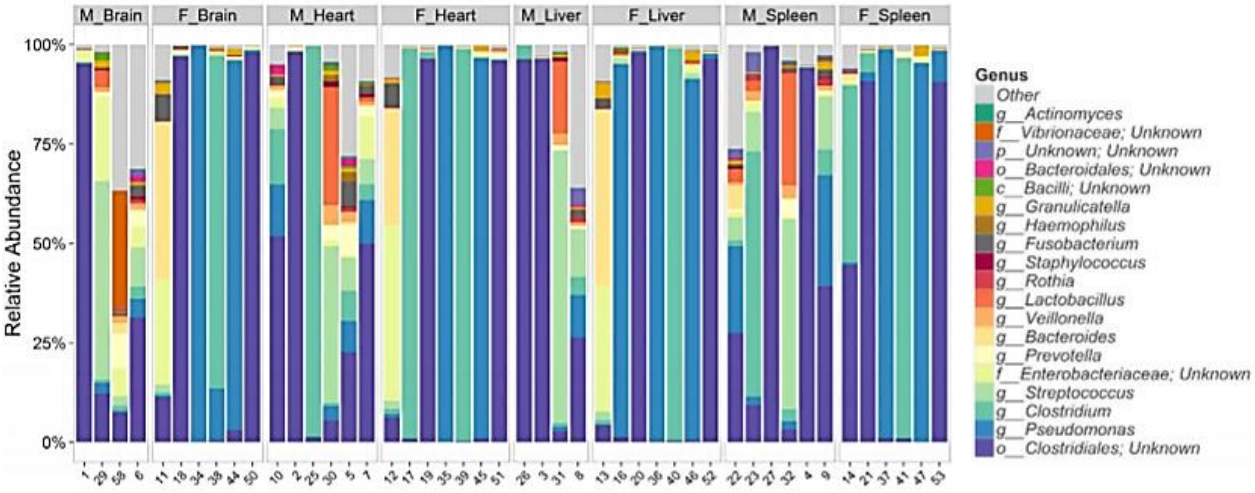

Figure 19. Relative abundance of the most representative genera in samples of brain, heart, liver and spleen from 27 human corpses (12 female and 15 male) with postmortem intervals between 3,5-240 using 16S rRNA. The graph shows that there is no significant difference between organs pertaining to the same sex but there are significant differences between female and male samples. It should be noted that only the samples obtained from women contain *Pseudomonas* and also in high abundances in all organs and that only the samples obtained from men contain *Rothia* [13].

These results were again supported by another study also led by Javan et al in 2017 but this new investigation also showed that apart from *Pseudomonas*, female cadavers also had hight abundances of *Escherichia coli (Proteobacteria)* and *Clostridium septicum (Firmicutes)* [31].

Nevertheless, most literature indicates that until now of all the bacteria mentioned above, *Pseudomonas* and *Streptococcus* has the best potential to determinate the PMI [14].

In women, *Pseudomonas* show two high peaks of abundance before 25 hours after death and another peak at 50 hours. After this, the abundance declines significantly but increases gradually until 250 hours. In the case of men, *Rothia* has a high peak just before 20 hours after the death and after that the abundance drops until it rises again at 40 hours to remain fairly stable. In addition, in the case of men we can also observe *Streptococcus* that presents three very high and wide peaks of abundance, approximately one every twenty hours compared to women who only have one. Another difference is that women have a really an ascendent wide peak of *Clostridia* later in decomposition compared to men that have also hight peaks of this class but earllier which are also seen in women. (Figure 19) [13].

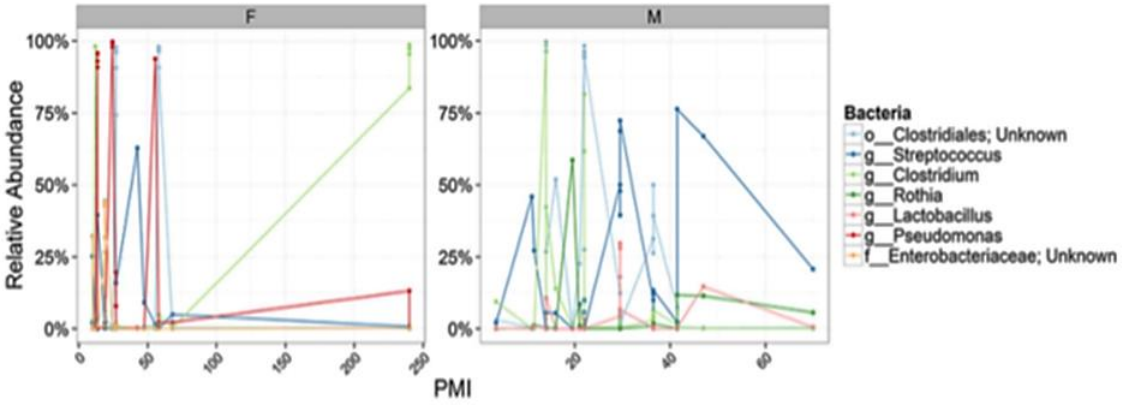

Figure 19. Relative abundance of the most predominant bacteria between females and males samples over time. Although there are differences between both sexes, it can also be observed how other bacteria behave in a similar way. For example, *Streptococcus* presents a relevant peak of abundance between 20 and 40 hours for men and women, although in men it has other peaks but its abundance decreases after approximately 60 hours in both cases. In the case of *Clostridia*, it is abundant in both sexes which both present a couple of high peaks before 8:00 p.m., however, women present a very wide peak later that has only been documented until 250 hours [13].

8. The Future

Presently, the HMPM is working to be the largest catalog of sequencing and bioinformatics data for relevant microorganisms associated with internal organs and cadaveric soil associated with human decomposition, and it is not ruled out that it will play an important role in future criminal cases since this tools and databases will be potentially used by medicolegal and forensic researchers. The HMPM is also improving, validating and standardizing protocols with the objective of attracting collaborators and contributors. Moreover, this project is also arranging a forum to provide information exchange and promote the importance of the thanatomicrobiome [16].

Another important future perspective it's the study of the thanatomicrobiome beyond bacteria. Nowadays, researchers are studying the use of ITS to characterize fungi and the use of archaea to further advance on the understanding of the role of microbes in human putrefaction [12].

9. Conclusions

One of the most studied areas in forensic science is the determination of the PMI given its crucial importance in both criminal and historical investigations. However, the current methods are highly sensitive to the environmental conditions and the characteristics of the individual and, therefore, scientists are looking for other choices

such as the thanatomicrobiome since microbes and especially bacteria are essential in decomposition.

In recent years, it has been discovered that after death the microbes from the GIT migrate to the internal organs following a specific order and that these microbes change depending on the stage of the decomposition principally due to the oxygen availability. These discoveries suggested that microbes change with time in a predictive manner, especially considering that the composition of the GIT is relatively stable among individuals, especially at the phylum level. Of all these microbes, the phylum *Firmicutes* stands out, particularly species of the genuses *Clostridium* and *Lactobacillus* since they exhibit time signal. Other important findings are the exchange of microbes with the soil (primarly the exchange of *Firmicutes*) and insects and some sex specific bacteria like *Pseudomonas* for females *Streptococcus* and *Rothia* for males.

Nonetheless, the thanatomicrobiome is altered by a vast amount of factors so in order to predict PMI is necessary to establish a universal model that includes not only time-related alterations of the microbial abundance but also other sources of variation like the host characteristics and the environment. Unfortunately, the thanatomicrobiome is a complex and understudied topic that has been mostly investigated using animal models so there's still the need for more research as there are still no studies with conclusive results. This is evidenced by the lack of standard protocols that cover sampling, processing, sequencing platforms, databases and prediction models. Moreover, it's also important to bear in mind the limitations of the actual techniques, especially in culturing and mass sequencing that difficult the determination of bacteria principally at the species level.

However, the thanatomicrobiome could be useful in specific circumstances where insects cannot access the body either because it is in a closed room or protected or depending on the weather and season because that can modify the oviposition time. Furthermore, in more advanced stages of decomposition, insects become less useful as multiple generations inhabit the body, which causes the PMI estimate to be more inaccurate with an error of months or may not even possible to be determined. Therefore, in cases like this, using methods that take into consideration information inherent to the body itself may be indispensable. Furthermore, the thanatomicrobiome

has the potential to give other crucial information such as if the body was moved from the crime scene, connect a suspect with a crime and determine the cause of a death.

In conclusion, at present, the thanatomicrobiome is not a consistent method to determine the PMI but all research indicates that microbial communities will probably have an important role in future forensic investigations. Nevertheless, this requires more research and therefore time, funding, more corpse donation, and a standardized protocol especific for organs in a state of decomposition.

10. Abbreviations

GIT = Gastrointestinal tract GIT

HMP = The Human Microbiome Project

HPMP = The Human Postmortem Microbiome Project

ITS = Internal transcribed spacer

mWGS = Metagenomic whole-genome shotgun sequencing

NIH = The US National Institutes of Health

OTU = Operational taxonomic unit

PMI = Interval post mortem

RT-qPCR = Real-time quantitative polymerase chain reaction

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12. Supplemental information

Figure 1. Some examples of taxonomic gut microbiota composition. Approximately 90% of the gut microbiota consists of bacteria from the phylum *Firmicutes* and *Bacteroidetes*. The phylum *Firmicutes* is the most abundant and consists of genera such as *Lactobacillus, Bacillus, Clostridium, Enterococcus,* and *Ruminicoccus* with *Clostridium* representing 95% of this phylum. This illustration doesn't exemplify all bacteria that can be commonly found in the gut like some species such as *Streptococcus* from the phylum *Firmicutes* and *Enterobacter* and *Pseudomonas* from the phylum *Proteobacteria* [11].

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