A REVIEW OF THE MICROBIOME ASSOCIATED WITH HUMAN DECOMPOSITION

By

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Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported work performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed:

Jack Dowell-Curby
08/12/17

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Part One

Literature Review

A Literature Review of the Microbiome Associated with Human Decomposition

Abstract

The decomposition of human remains involves a complex microbial ecology that few studies have examined in depth. This review investigated the microbiome of human decomposition to further understand their functions within the decomposition process and their potential to increase the accuracy of post-mortem interval (PMI) estimations in forensic applications. The aims of the literature review were to (1) identify the external microbiome responsible for human decomposition, focusing on insect, soil and skin sources, (2) determine the roles of external bacteria in the various stages of human decomposition and (3) to analyse and compare the current contributions of literature in furthering the understanding of the ecological mosaic of decomposition.

The current literature was reviewed and their contributions to necrobiome research was analysed using qualitative and contemporary research techniques. Bacteria were found to play a significant role in each stage of human decomposition with multiple studies demonstrating an observable successive shift in microbial communities through time. This change in community profile was found to be an important biomarker for the estimation of the PMI and potential substitute for entomological techniques currently utilised in forensic investigations. High interpersonal variation between decomposition events, in addition to narrow geographic specificity, represented limitations in the studies which may be remedied by increasing sample size while focusing on different geographic regions and environmental conditions.

Keywords: decomposition, necrobiome, bacteria, post-mortem interval, succession

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List of Abbreviations

ADD	Accumulated degree days
ARISA	Automated ribosomal intergenic spacer analysis
CDI	Cadaver decomposition island
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
GIT	Gastrointestinal tract
NGS	Next generation sequencing
PMI	Post mortem interval
rRNA	Ribosomal ribonucleic acid
T-RFLP	Terminal restriction fragment length polymorphism

Chapter 1 – Introduction

Human decomposition is a continuous process that is divided into multiple stages based on observable changes: commonly known as the fresh, bloat, decay, post-decay and skeletal stages.[1] Each stage attracts different macro and micro species of fauna that introduces numerous varieties of bacteria, both internally (enteric bacteria) and externally (from skin, soil and insects) to the cadaver, known as the necrobiome.[2]

Specific types of bacteria have been known to colonise the cadaver at different intervals using Next Generation Sequencing (NGS), some methods proving more successful than others, such as pyrosequencing,[3] a deoxyribonucleic acid (DNA) sequencing technology that obtains sequences for unculturable microbial taxa.[4] Studies[5,6] have observed bacterial communities providing the successive groundwork for further colonisation until their food source was entirely depleted, with a temporal trend observed within microbial activity. This realisation has implications in forensic investigations as the bacteria present could determine the post-mortem interval (PMI) of the deceased, or the time since death.[7]

The PMI is often estimated using entomological evidence but the prevalence of multiple factors that can interfere with the colonisation process such as weather, location, and clothing, can reduce the accuracy of this singular method. The accuracy of PMI estimations can be increased by combining entomological evidence with quantifiable changes in microbial succession and factors that affect decomposition rates, such as temperature and biological variations, thereby aiding in understanding the role that epinecrotic communities play in human decomposition.[8]

The necrobiome of human decomposition remains a fragmented field of research with little performed in terms of data organisation and classification. A select few[5,9,10] have focused on the topic in geographically specific environments, thereby limiting the potential use of their data in other settings. In contrast, other studies[3,11] have yielded broad results due to the variable, and often uncontrollable, nature of human decomposition research and even less have focused on reviewing and comparing the existing literature as a whole.[8,12]

1.1 Aims

This body of work focused on reviewing the literature associated with the microbiome of human decomposition to further understand their functions within the process and their forensic implications regarding their potential to increase the accuracy of PMI estimations using NGS. The aims of the literature review were to identify the external microbiome responsible for human decomposition, focusing on insect, soil and skin sources, determine the roles of external bacteria in the various stages of human decomposition and to analyse and compare the current contributions of literature in furthering the understanding of the ecological mosaic of decomposition.

In addition, it was hypothesised that, by investigating literature based on the microbiome of human decomposition at each stage, a noticeable shift in bacterial community structure through time would be observed which would increase the accuracy of PMI estimations.

1.2 Methodology

The review was conducted using online and offline resources, focusing on literature that discussed and studied the microbiome of human decomposition. Resources that were examined included journal articles, conference papers, theses, empirical studies,

government agency reports discovered from databases that included Scopus, Web of Science, ProQuest, Wiley Online, and ResearchGate, in addition to government websites, public search engines such as Google Scholar and textbooks from the Murdoch University Library.

Searches were performed using specific keywords and parameters to maximise the amount of relevant results, and included combinations of the following terms:

- “microbiome” AND “human” AND “decomposition”,
- “bacteria” AND “human” AND “decomposition”,
- “human decomposition” AND “bacteria”,
- “human decomposition” AND “bacteria” AND “role”*, and
- “cadaver” AND “decomposition” AND “bacteria”*.
- “soil”*,
- “skin”,
- “insects”, and
- “next generation sequencing”.

Chapter 2 – Background

Bacteria plays a role in almost every facet of human life and death. A decomposing human body contains a complex microbial ecosystem[13], that evolves with each stage of decomposition, with the ultimate goal of consumption resulting in total disintegration of the human body. The microbial communities are separated into two distinct types based on location; the thanatomicrobiome, or internal bacteria, and the epinecrotic community, or surface bacteria,[8] both of which contribute to the individual stages of decomposition. The human microbiota is an ecological community consisting of bacteria, archaea, viruses and eukaryotic microbes that inhabits the body, both internally and externally.[14]

The stages of decomposition vary in number from one to nine, often due to the subjective nature inherent in observing physical variation over time by different individuals and with different animal models. Goff[1] suggests that there are five distinct stages that have been commonly observed, those classified as fresh, bloat, decay, post-decay and skeletal (as shown in Figure 1).

Figure 1. Five stages of decomposition. (1) fresh, (2) bloat, (3) decay, (4) post-decay & (5) skeletonisation. Adapted and used under licence CC-BY-SA-3.0.[15]

The stages are categorised by observable corporeal changes to the cadaver, with the onset beginning approximately 4 minutes after death in a process of self-digestion called autolysis.[11]

2.1 Autolysis

Decomposition begins with the process of self-digestion characterised by cell death. This is caused by the accumulation of waste, the deprivation of oxygen, the increase of carbon dioxide and the decrease of pH levels, alongside the dissolution of cells through enzymatic activity, resulting in cell rupture and the release of their nutrient-filled contents. This process is accelerated in organs that have high fluid and enzyme contents, such as the liver and the brain. Autolysis is first observed several days into the fresh stage of decomposition and once enough nutrients have been released due to cell rupture, the process of putrefaction begins,[11] which is the disintegration and liquefaction of all proteins, tissues and organs, commonly known as decomposition.[16]

2.2 Stages of decomposition

The five distinct stages of decomposition, as classified by Goff,[1] have been demonstrated to be influenced by microbial activity in addition to natural enzymatic processes. Almost every species of known bacteria (making up only 1% of the total microbial population) plays a role in decomposition,[11] indicating the vast and complex nature of the process.

The first stage of decomposition, known as the fresh stage, lasts for approximately one to two days, during which the process of autolysis takes place.[17] Insect colonisation occurs rapidly after death with the arrival of fly species such as blow flies (Calliphoridae) and flesh flies (Sarcophagidae).[18] It is during the initial stage that livor mortis, rigor mortis, and algor

mortis occurs (also known as the mortis triad).[1] Microbial activity assists in digesting internal organs and surrounding tissues and includes the production of gases by anaerobic bacteria from the gut, marking the onset of the bloat stage.

The second stage of decomposition, known as the bloat stage, continues for approximately two to six days and is when putrefaction becomes apparent. The abdomen becomes visibly distended due to the build-up of gases caused by enzymatic and enteric microbial processes.[19] Maggot activity increases which causes a significant rise in body temperature[1] in addition to physical changes such as skin slippage and marbling, resulting from microbial activity in blood vessels. The rupturing of the skin due to the build-up of pressure, distinctly marks the onset of the decay stage and another shift in the bacterial community.[11]

The third stage of decomposition, known as the decay stage, lasts for five to eleven days, during which the body deflates as gases and fluids are released into the surrounding soil, increasing pH levels and altering endogenous bacterial communities.[20] As the odour increases, the level of necrophage activity rises as they are attracted to the scent. The decay stage is defined by the dominant masses of Diptera larvae which would remove the majority of flesh, leaving skin and cartilage.[1] Putrefaction can be halted by environmental conditions, initiating either saponification or mummification.

The fourth stage, known as the post-decay stage, lasts for ten to twenty-four days and is when the body begins to dry out. Beetle populations (common necrophagous beetles include Carrion beetles, (Silphidae*)* and Dermestid beetles (Dermestidae)[17] become the predominant insect species which are capable of feeding off the dried remnants until only bones and hair remain[11], indicating the skeletal stage.

The fifth stage of decomposition, known as the skeletal stage, is observed by the presence of bone and hair which can remain for an indefinite period, from approximately day twenty-four onwards. Necrophagous beetles ingest the remaining tissues and bacterial abundance decreases, as the communities begin to resemble the levels found in the surrounding environment, pre-decomposition.[21]

2.3 Factors affecting decomposition

There are numerous variables that can affect the decomposition rate of a human body. Those identified by Mann et al[22] includes physiological factors such as body size and weight, and environmental conditions such as rainfall.

The greatest influence of the rate of decay is environmental temperature as this factor determines the amount of insect and bacterial activity. A high temperature can increase biological activity and chemical reactions resulting in faster decomposition.[23] Conversely, refrigeration of the cadaver prior to placement for research can also have an effect on microbial communities as found by Micozzi,[24] where refrigerated carcasses exhibited decreased bacteria-mediated putrefaction, as opposed to fresh cadavers.

In addition to environmental factors, lifestyle and health factors can contribute to the variable rate of decomposition. Some pharmaceuticals contain antimicrobial agents that disrupts bacterial activity, causing decomposition rates to decrease.[25] The health of the individual prior to death and the cause of death can influence decomposition. A study by Hayman determined the cause of death of a cadaver to be due to fulminant septicaemia, a bacterial infection, that resulted in an increased abundance of bacteria post-mortem, thereby accelerating decomposition.[26]

Insect access to the body has been described as a major influencing factor in decomposition, second to temperature.[22] The location of a cadaver, clothing and burial depth can determine the prevalence of entomological activity due the level of accessibility.[27] In indoor location can deter external insects from accessing the body and prolonging decomposition, similarly, a buried body can have the same affect.[28]

2.4 Post mortem interval estimation

The ability to estimate the period of time that has elapsed since death (post-mortem interval - PMI) is significant in forensic investigations as it can result in the identification of the criminal or victim through a process of elimination. Even in non-criminal circumstances, establishing the PMI can have legal relevance such as in insurance and inheritance matters, thus the accuracy of the estimation is essential.[29]

There have been multiple methods of calculating the PMI, including observing the mortis', stomach contents, and temperature, which have resulted in crude and unreliable approximations, primarily due to their inaccuracies. In addition, entomology is influenced by uncontrollable variables such as environmental conditions, geographic location, and other unknown factors.[30] Biochemical methods (those based on pathophysiological changes) have been considered more accurate due to the reduced influence of external conditions.[31]

2.5 Accumulated Degree Days

Importance is placed on environmental temperatures during the study of decomposition events due its influence on bacterial growth, in which any fluctuations outside of the bacteria's optimal range (threshold) can result in either increased or decreased growth rates, thereby influencing the rate of decomposition and the PMI estimation. In the majority of

studies, the measure of the degree of decomposition was calculated using ADD's to reflect the influence temperature has on decomposition rates. The ADD is normally calculated by summing the average hourly temperature for each 24-hour period after death. If the threshold is smaller than the result, the number of degrees over the threshold equals the number of degree days or ADD's.[32]

2.6 Entomology

Entomology is the study of insects and their ability to be used in forensic investigations, particularly to establish a PMI, determine body movements and cause of death.[33] Insects are the commonly used method of determining PMI due to their predictable lifecycle during a decomposition event.[34]

Campobasso, et al[27] identified 10 insect families that belong to the Diptera (flies) and Coleoptera (beetles) groups, and are considered the most prevalent during a human decomposition event. The first insects to colonise the cadaver, 2-3 hours after death, are Calliphoridae, Muscidae and Sarcophagidae, which prefer to lay their eggs (oviposition) in natural orifices or wounds,[35] including the mouth, eyes, nasal cavities, and ears. At the onset of the bloat stage, the insects lay eggs in the genitals and after a period of 6 to 40 hours, the eggs hatch influenced by external temperatures.

The development of larvae commonly occurs between 3-10 days and pupae develops 6-18 days after oviposition, before taking their adult form.[36] The continuation of fly activity is observed until the post-decay stage of decomposition. Beetles (Silphidae, Staphylinidae and Histeridae) would commonly begin their colonisation at the bloated stage and have a similar life cycle as flies (as shown in Figure 2). The final stages of decomposition would see an influx of beetle families such as Dermestidae, Cleridae and Scarabaeidae.[27]

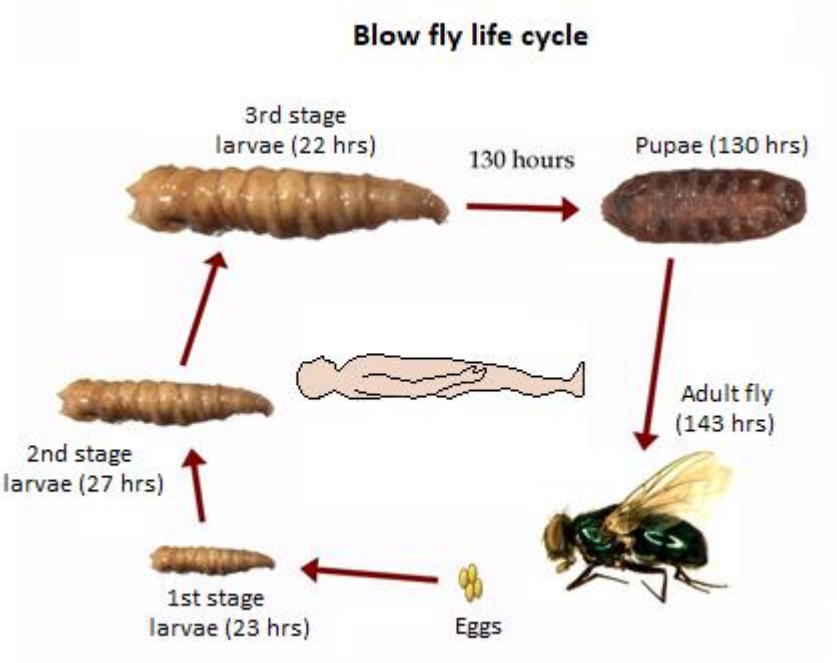

Figure 2. The life cycle of a blow fly. At 21° C, each stage in a blow fly's life cycle takes a known amount of time to complete during human decomposition. Adapted and licenced under Public Domain.[37]

Each insect is adapted to take advantage of the different stages of decomposition which can result in predictable community structure changes due to resource staging. It is this pattern of data from each stage that can be used to estimate the PMI.[9] As with entomological evidence, similar temporal trends have been observed among microbial communities[5] using NGS. Combining the methods has demonstrated an increase in the accuracy of PMI estimations resulting in greater forensic implications, as opposed to the utilisation of a singular method.[8]

2.7 Sources of bacteria

Different types of bacteria have been identified at each stage of the decomposition process using NGS and have been shown to influence the rates of decomposition, in addition to being utilised as a method of PMI estimation.[7] External sources of bacteria that can influence the necrobiome, and therefore the process of decomposition, includes insects, soil and skin, all

of which have their own unique microbial taxa. In addition, internal sources include the natural gut microbiota.

2.7.1 Insects

Entomological evidence can be influenced by bacteria present on the cadaver as it has been demonstrated that an association exists between insect species such as the blow fly (*Lucilia sericata*), and the quorum sensing mechanism that some bacteria exhibit, in addition to volatile compounds produced by the bacteria.[38]

Quorum sensing in bacteria is the creation and release of chemicals called autoinducers that increase in cell concentration, regulating physiological activities including virulence, motility and symbiosis. This communicative ability coordinates the behaviour of the bacterial community in response to its environment and population.[39] Any alteration in the bacterial and insect community may affect the decomposition process of the cadaver, which could lead to mummification or saponification (adipocere formation) instead of decomposition.[9] This may have a negative effect on the accuracy of PMI estimations.

In order to protect themselves from infection, insect larvae such as maggots, have been known to excrete antimicrobial compounds,[40] which may have an impact on the microbial community during decomposition.[41]

Parkinson[12] observed a decrease in bacterial diversity acting concurrently with peaks in maggot mass during decomposition events. Additionally, an increase in chitin levels can be attributed to the presence of maggots. Chitin is a polymer that is a constituent of insect exoskeletons including maggot skin (cuticle), which becomes abundant as maggots shed their skin three times before creating a pupal casing. The chitin polymer can become a food

source for organisms such as the Actinomycetes which are able to degrade chitin using chitinolytic enzymes.[42] Parkinson[12] observed the presence of Actinomycetes in soil.

2.7.2 Soil

Likewise, a shift in the soil bacterial community structure could change the rate of the decomposition process, according to McGuire.[43] Soil evidence can play a significant role in forensic investigations as the properties of soil are diverse and individualising.[44] Only a small number of soil bacteria have been identified through research due its unculturable nature using traditional mediums and the inability to replicate complex soil environments in the laboratory.[45]

Soil influences decomposition by facilitating the involvement of the most abundant microbial group; bacteria,[46] with those present on the cadaver, particularly during the aerobic stages of active decay.[12] The relationship between soil bacteria and cadaver decomposition has been examined in several studies,[43,47,48] primarily focusing on the effects that decomposition has on the localised environment, however, bacterial species have been observed to be associated with the process, specifically those within the cadaver decomposition island (CDI).

The CDI is a specialised and highly fertile habitat surrounding the cadaver, resulting from its decomposition. It is within the CDI that an increase in microbial abundance and activity can be observed in soil communities.[49] During the decay stage, the human body leaches amounts of Nitrogen (32 g per kg), Potassium (4 g per kg), Magnesium (1 g per kg) and Phosphorus (10 g per kg) into the surrounding environment.[50] These nutrients influence the composition and growth of soil bacterial communities and can change the environmental microflora profile for an indefinite period after death.[25] The alteration of

soil microbial communities may therefore, have an influence on the rates of decomposition.

Utilising the bacterial profile in soil, a PMI can be estimated at certain stages of decomposition, particularly the decay and post-decay stages as expulsion of bodily fluids can leach into the surrounding soil and alter the existing biotic structure and interactions.

In addition to bacterial communities, a key factor that affects soil bacterial composition is the pH level of soil surrounding the cadaver. Figure 3 demonstrated a rapid increase in pH of the soil which may result in a decrease in bacterial abundance during the final stages of decomposition due to sub-optimal acidity.

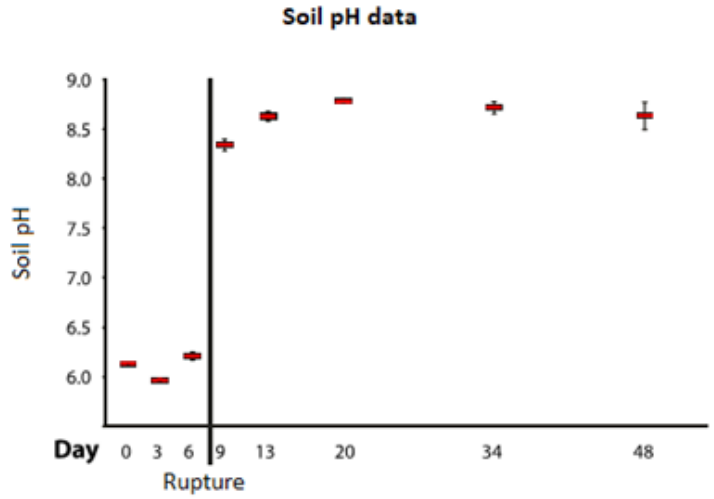

Figure 3. Soil pH levels during decomposition. Relationship between pH and time in soil during the decomposition of mouse cadavers. The rupture event is marked as the obvious shift in stage from bloat to active decay. Adapted and used under licence CC-BY-3.0.[6]

Metcalf noted the observable increase in pH levels after the rupture event had occurred following the bloat stage, as shown in Figure 3. Acidity levels affect bacterial composition in soil as each species has a different pH tolerance, however bacteria are generally neutrophiles that experience optimal growth at neutral pH levels (~7).[51]

2.7.3 Skin

The microbial community structure on skin can display an observable pattern of change in response to different stages of decomposition.[6] As with soil microbiota, the bacterial diversity on skin is complex, however, it is dependent on their location on the body and differs from host to host, often resulting in non-comparable and difficult studies.[52]

The skin is the largest organ on the human body and is comprised of approximately 1.8 m^2 of diverse microbial communities that are individualised depending on host factors such as sex, age, and body site,[52] as shown in Figure 4 using live healthy volunteers.

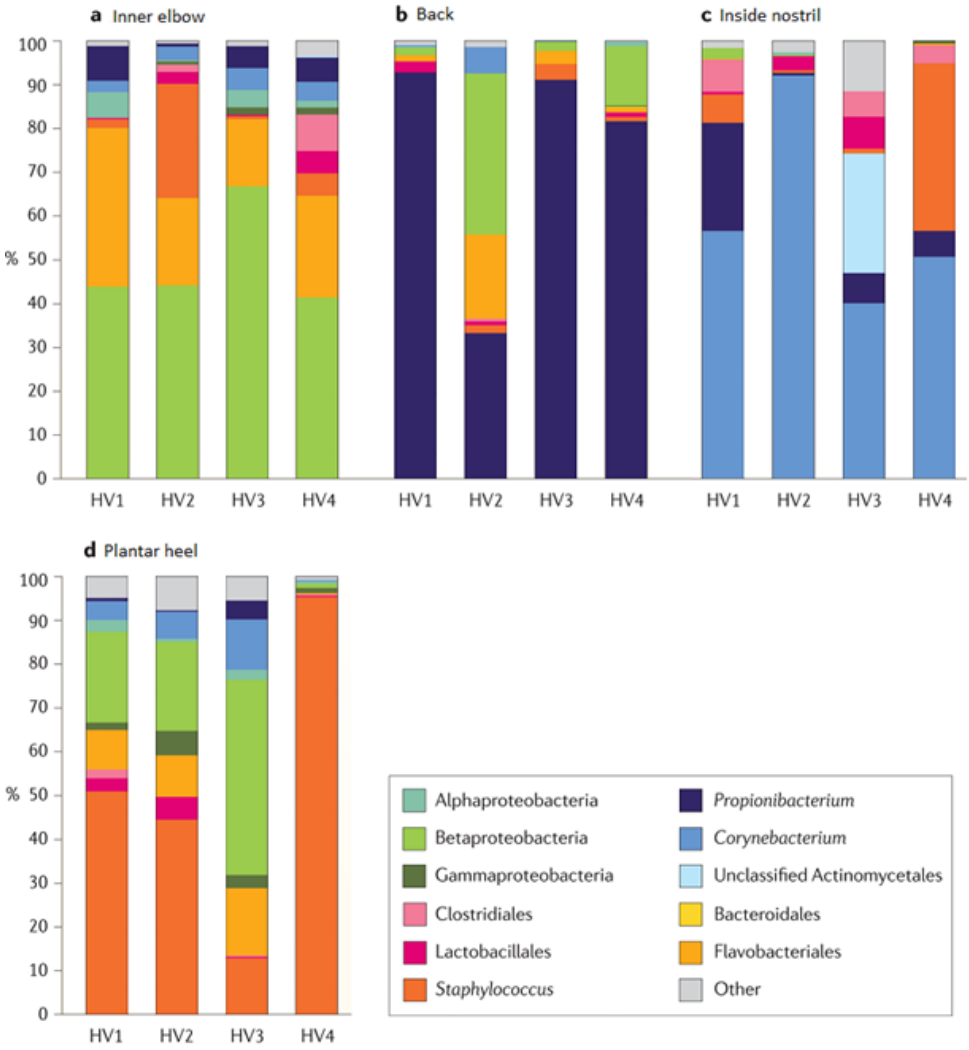

Figure 4. Interpersonal variation of microbial distribution. The microbial distribution of four healthy volunteers (HV1 – HV4) on four sample sites; inner elbow, back, nostril and bottom of the heel. (Reprinted and adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, © 2011).[52]

Observing if there is a change in bacterial population after death was the focus of studies[30] surrounding the use of skin microbiomes in determining a PMI, resulting in a predictor model capable of predicting the PMI within a specific range of accumulated degree days (ADD) as defined by Michaud et al,[53] improving upon the current entomological method and thereby showing that there is a functional relationship between skin bacterial diversity and decomposition rates.[30] Johnson et al[30] suggested that skin microbes are highly influenced by the decomposition process, including the effects of insect colonisation and tissue chemical changes. Using the ear and nasal cavities as sampling sites proved advantageous as it offered a non-invasive method with little chance of evidential disturbance in forensic investigations. It was found that combining microbial data for multiple sampling sites drastically improved PMI predictor results using only skin communities, ultimately establishing that the skin microbiome holds forensic value.

2.7.4 GI tract

The anaerobic microflora found naturally in the gastrointestinal (GI) tract increases in abundance immediately after death due to autolysis,[54] and remains inside the cadaver for approximately five days. The gut bacteria create gases that build-up, causing a rupture event that sees the bacteria purged from the cadaver and released into the surrounding environment. The community profile shifts at this point, however, the change does not last long (usually for 2-3 days) as the bacteria are unable to survive in an aerobic environment.[12]

The ammonifying organisms found naturally in the gut causes the breakdown of muscle tissue during the bloat stage. The pH of the surrounding soil or sand is known to increase when they are released from the cadaver due to an abundance of ammonium. This nutrient

influx had also been recorded during the decay stage and is utilised by ammonia oxidisers such as bacteria from the *Nitrosomonas* genus which converts ammonium to nitrate.[55]

The breakdown of fatty tissue in an anaerobic environment is facilitated by the microbial action of the Clostridiales which causes the hydrolysis and hydrogenation of fatty acids from lipids.[56]

2.8 Next generation sequencing

The ability to identify the bacteria of decomposition has depended on the accuracy of current sequencing technologies, categorised as next generation sequencing or high-throughput sequencing.[57] The most commonly used NGS method is based on pyrosequencing where DNA fragments are sequenced by synthesis using chemiluminescent enzymatic reactions.[58] They include Roche 454, however, updated platforms such as Illumina MiSeq and PacBio have largely replaced this method as a way of assessing genetic diversity.[59]

These NGS platforms employ different procedures but their strategies remain similar. Adaptor sequences are ligated to the ends of fragmented DNA molecules which are amplified to create groups of amplicons. The DNA molecules are sequenced by extending primers to create strands complimentary to the template. Following the end of nucleotide addition, the array is imaged to acquire sequencing data. Sanger sequencing also initiates the process by fragmenting DNA molecules, however, Sanger requires additional cloning and amplification in vivo which is expensive and time-consuming.[60]

NGS is the preferred method for use in microbiome research to identify and study the relationships of pathogenic bacteria and their health effects in medical fields, with little

focus given to the human microbiome post-mortem.[8] The potential to provide forensic data, such as the PMI, can have significance in death investigations. This body of work will focus on reviewing the literature associated with the external microbiome of human decomposition to further understand their functions within the process and their forensic implications regarding their potential to increase the accuracy of PMI estimations through NGS.

Chapter 3 – Decomposition

The continuous process of decomposition, from the initial point of death to skeletonisation, is a continuum divided into multiple stages depending on observable physical changes.[1] The number of individual stages have fluctuated between authors; from one to nine, depending on geographic location.[61] Regardless of the number of stages, a correlation between identified taxa and observable stages in each study does not appear to exist. For example, Table 2 shows that 172 bacterial species were recorded within one stage of decomposition among lizards in a study by Cornaby.[62] In contrast, 133 species were recorded by Early and Goff[63] within five stages of decomposition among cats. This irregularity may be contributed to the variety in taxonomic preferences and sampling methods between studies.

Table 1. Decomposition studies summary. A list of studies focusing on bacterial taxa within decomposing animal and human models showing number of stages and observed microorganisms. Reprinted and adapted by permission of Springer: Experimental and Applied Acarology, © 2009.[1]

Authors	Location	Model	Stages	Bacterial Taxa
Braack[64]	Africa	Impala	4	227 species
Bornemissza[65]	Australia	Guinea Pigs	5	45 groups
Cornaby[62]	Costa Rica	Lizards	1	172 species
Davis and Goff[66]	Hawaii, USA	Pigs	5	85 species
Early and Goff[63]	Hawaii, USA	Cats	5	133 species
Payne[67]	South Carolina, USA	Pigs	6	522 species
Rodriguez and Bass[68]	Tennessee, USA	Humans	4	10 Families
Shean et al[69]	Washington, USA	Pigs	-	48 species

The Megyesi[70] visual body score is a complex method commonly used among studies,[6]

which links each stage to a timeframe based on visual markers such as discolouration, bloat and purging for active decay; and sagging flesh, sinking flesh, caving flesh and mummification for advanced decay (as shown in Figure 5). This review focused on the simplified stages classified by Goff[1] as fresh, bloat, decay, post-decay and skeletal.

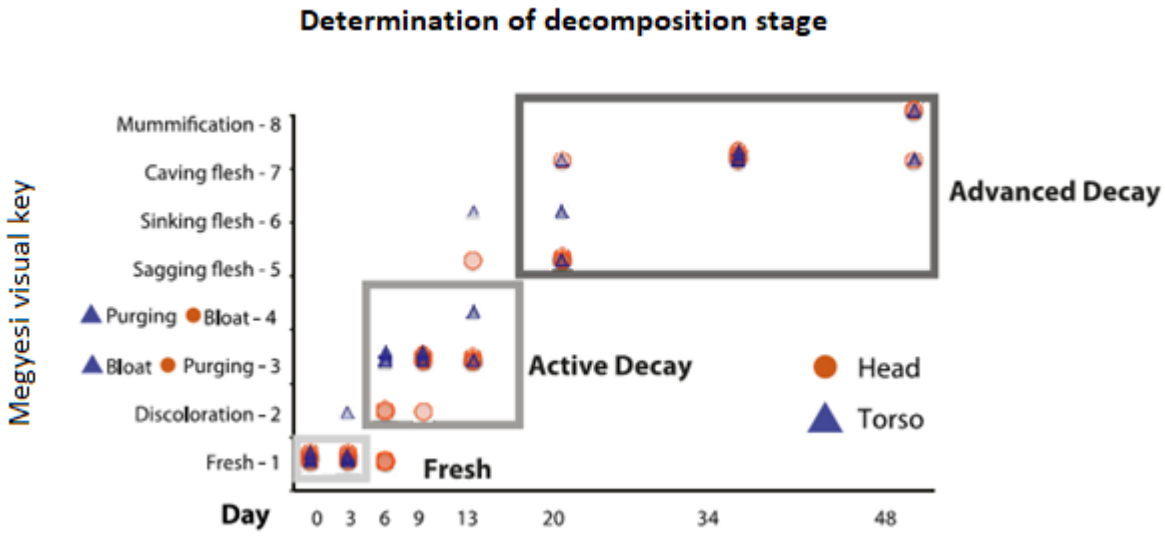

Figure 5. Megyesi visual scoring system for decomposition.[70] Method utilised by Metcalf to determine the stages of decomposition in mouse models. Adapted and used under licence CC-BY-3.0.[6]

3.1 Early post-mortem changes

There are a number of physical changes that occurs soon after death, prior to observable decompositional changes, which are often used to establish the PMI. Some of the changes are influenced by bacterial activity and are important indicators of specific stages of decomposition. They include livor mortis, rigor mortis, algor mortis, discolouration, skin slippage, marbling, mummification, saponification and putrefaction.[1]

3.1.1 Livor mortis

Livor mortis, also called lividity, is characterised by the discolouration of the lowest areas of the body due to pooling of the blood by gravity, and is typically reached between 1-4 hours after death. As the heart ceases to pump blood around the body, the remaining liquid settles

and causes blanching if pressure is applied. Between 9-12 hours after death, the pooled blood pattern becomes fixed.[71] The presence and degree of livor mortis can be used to establish an approximate PMI, however, this method is considered inaccurate as a result of variations in the colour, and distribution of lividity in different cadavers, therefore, this method is primarily used to determine if the body has been moved after death.[72]

3.1.2 Rigor mortis

Rigor mortis is characterised by a stiffening of the body's limbs caused by ATP depletion in the muscle tissues.[73] Rigor is typically first noticed 2-6 hours after death with the smaller extremities affected. The process continues over a 12 hour period in which the larger limbs become stiff and usually lasts for 24-84 hours,[54] or essentially, until decomposition breaks down the tissues, achieving limb flaccidity. These periods are highly variable depending on climate, as hot temperatures are known to accelerate while cool conditions are known to delay this process.[74]

The absence of rigor mortis can be used to determine an approximate PMI which can be less than 2-3 hours since death or more than 72-84 hours since death, assisted if signs of early-stage decomposition are obvious. The presence of rigor mortis can indicate a large time period between 2-84 hours after death, however this estimate can be narrowed down by observing which limbs are affected (smaller muscles, such as eyelids and the jaw, are affected first and continues until larger limbs, such as arms and legs, become affected at a later period).[31]

3.1.3 Algor mortis

Algor mortis is characterised by post-mortem cooling until the body becomes acclimatised to the ambient temperature which is usually reached between 18-20 hours after death.[1] The

process is influenced highly by the presence and/or layering of clothing,[75] which can give false estimates of the time since death.

The temperature of the body after death determines the rate of putrefaction, and therefore, is an important factor that influences bacterial activity. A trend has been identified which states that putrefaction is accelerated if body temperature decreases at a slower rate.[76] The human body can therefore be considered as a type of culture medium for bacteria, with the application of similar principles applying as those in microbiology laboratories to facilitate the growth of microorganisms after death.[77] The ideal temperature required for putrefaction is between 21°C and 38°C; putrefactive bacteria, such as *Clostridia*, require a temperature of 37°C for optimal growth.[78]

Additionally, an increase in ambient temperature can intensify the odour of a decomposing body, thereby drawing extra insect and necrophagous fauna activity. This activity can result in skin penetrations through bites, further enabling the infiltration of exogenous aerobic bacteria, accelerating the rate of decay.[76]

Calculating the PMI from algor mortis commonly involves taking the rectal temperature of the cadaver and subtracting the reading from the normal body temperature of 37°C, indicating overall temperature loss. A result of approximately 9.36°C in temperature loss indicates that the body has been deceased for 12 hours under normal conditions. If the result is less than 9.36°C, than the temperature loss must be divided by 0.78°C, which is the rate of temperature loss per hour prior to the first 12 hours, however, if the result is more than 9.36°C, the answer is subtracted from this number, divided by 0.39°C, and combined with 12.[79]

There are many factors than can affect temperature loss and time since death calculations such as advanced age, absence of clothing, cold, shady or marine environments, all of which can decrease the PMI estimate due an increased rate of heat loss. Factors such as pre-existing fever or illness, direct sunlight, clothing, obesity, enclosed rooms and warm or humid environments can increase the PMI estimate due to a decreased rate of heat loss.[80]

3.2 Fresh stage

The first stage of decomposition transpires in parallel with internal microbial activity and does not become visibly apparent for approximately 3 days after death with the presence of skin slippage, sloughing and fluid-filled blisters indicating the first observable signs of autolysis.[17] It is during the initial stage that the mortis triad occurs (livor mortis, rigor mortis, and algor mortis).[1] Insect colonisation begins minutes after death with the arrival of various fly families such as blow flies (Calliphoridae) and flesh flies (Sarcophagidae)[18] that facilitates the infiltration and dispersal of bacteria, starting at exposed bodily areas and orifices.[2] The fresh stage represents the initial shift in bacterial community structure, in conjunction with an increase in activity as a response to physical disturbance.[19]

3.3 Bloat stage

The second stage of decomposition is characterised by observable signs of putrefaction including visible distention of the abdomen and soft tissues, particularly the face and scrotum. This is due to the build-up of gases such as hydrogen sulfide and carbon dioxide within the body cavities, as a result of enzymatic and enteric microbial processes.[19] The cadaverine and putrescine by-products of this activity causes the generation of a putrid odour, which attracts necrophagous organisms and fauna.[9]

Maggot activity, combined with putrefaction, causes an increase in internal body temperature, significantly higher than ambient temperature (>50°C), to become a separate habitat independent from the external environment.[1] The skin undergoes a visible change in colour with a marbled appearance caused by bacteria moving through the blood vessels, in addition to the expulsion of fluids from orifices, particularly the nose and mouth.[81] The build-up of pressure may cause skin tearing and rupturing which marks the onset of the decay stage and a shift from aerobic to anaerobic bacterial conditions.[82,83]

A secondary effect of the bloat stage, often confused with rigor mortis, is known as putrefactive rigor. This phenomenon is characterised by the erection of limbs in which they appear to stiffen and rise due to the build-up of subcutaneous gases.[73] Confusion with rigor mortis may cause inaccuracies when establishing the PMI and conclusions regarding positioning of the body.[84]

3.4 Decay stage

The decay stage is characterised by the deflation of the body and the purging of gases and fluids into the surrounding environment, causing pH levels to rise and altering endogenous bacterial communities.[20] Masses of Diptera larvae are present at this stage, which would remove the majority of flesh, leaving skin and cartilage.[1] The mass ovipositing behaviour of blow flies can partially be attributed to a phenomenon called interkingdom signalling.[38]

Interkingdom signalling is a form of communication between different species, particularly bacteria and their hosts. Bacteria on the cadaver, such as *Proteus mirabilis*, attracts blow flies, such as *Lucilia sericata,* by releasing odorous compounds including putrescine,[85] ammonia, phenol, sodium hydroxide, and potassium hydroxide,[86] thereby demonstrating a symbiotic relationship between the two species and furthering the decomposition process.

This relationship can also be one of dependence, where the absence of bacteria can cause the failure of development in immature species of flies.[87]

Putrefaction is dependent on environmental conditions, extreme changes in temperature and moisture can lead to saponification or mummification. Saponification is characterised by the formation of adipocere; a waxy, fatty substance, caused by the hydrogenation and hydrolysis of adipose tissue. It is typically observed on cadavers with higher fat contents, that have been subjected to moist, humid environments and holds forensic value as it preserves the body, offering easier identification and determination of cause of death.[88]

Bacteria can accelerate the formation of adipocere on the body, specifically by anaerobic species in the Clostridium genus.[89] Pinheiro[88] specifically identified several microorganisms responsible for producing the fatty-acids necessary for adipocere formation, those of which include *Pseudomonas*, *Staphylococcus aureus*, and *Clostridium perfringens.* Pfeiffer[90] associated the maintenance of adipocere with Gram-negative bacteria and attributed the substances decay to a change in environment, causing a shift in bacterial community to Gram-positive species.

Mummification, on the other hand, is characterised by the dehydration of tissues, resulting in brown, brittle skin resistant to bacterial decay. It is often observed in cadavers that have been subjected to warm, arid and well-ventilated environments.[91] Mummification is a natural method of preservation, therefore, it holds similar forensic value as saponification.

3.5 Post-decay stage

The post-decay stage is characterised by the dehydration of the body, leaving bone, skin and cartilage which are subsequently removed by beetles, commonly from the Dermestidae family,[17] of which become the dominant insect species[27] due to their ability to feed on tough

remains.[92] A distinctly cheese-like odour becomes apparent due to butyric acid fermentation. Bacteria in the surrounding soil, such as those in the Pseudomonas genus, are known to increase in diversity and abundance during this stage due to their ability to thrive on the low nutrient levels presented by the dried remains.[83]

3.6 Skeletal stage

The skeletal stage is characterised by the presence of bone and hair, with a highly variable rate of degradation, depending on factors such as environmental conditions and predation.[93] Goff[1] observed that there were no necrophagous fauna-dependent taxa at this stage, however, there were soil-dwelling taxa, such as Collembola and mites, that could be used to estimate a PMI. In regard to bacterial communities, it was observed that their profiles differed depending on the stage of skeletonisation. Partially skeletonised remains were shown to contain bacteria that resembled those present in the gut and dry remains resembled the bacterial community in soil.[94]

Microbial activity has been demonstrated to influence each stage of decomposition with almost every species of known bacteria (making up only 1% of the total microbial population) playing a role,[11] indicating the vast and complex nature of the process. In addition, the relationship between host and microorganism reflects its function and timing within the epinecrotic community.

Chapter 4 – Epinecrotic Community

Decomposing remains contain a community of organisms that reside on surface regions including skin and mucous membranes of the oral cavity, with prokaryotes, protists and fungi making up the majority of the population.[10] External sources capable of contributing to the microbiome of a cadaver includes the presence of insects and soil. These factors have been focused on in studies of the epinecrotic community in an attempt to further understand the ecological mosaic of decomposition.

4.1 Insect Bacteria

There is an observable symbiotic association between insects and bacteria that is mutually beneficial[95], therefore, the presence of certain insects may determine the existence of their associated bacteria. This has been demonstrated in studies regarding the microbiome of decomposing human remains. A study published in 2015 by Hyde et al[3] found that Proteobacteria such as those in the *Wohlfahrtiimonas* and *Ignatzschineria* genus were a common presence during the bloat and purge stages of decomposition, likewise, the same bacteria commonly detected by Bucheli and Lynne[9] were considered to be associated with flies.

Parkinson's study identified bacterial orders in samples that were primarily associated with insect colonisation, indicating that the bacterial community may have been influenced by the insects themselves. The introduction of bacterial orders associated with insect larvae, such as Xanthomonadales, *Myroides* and *Providencia,* were seen in samples retrieved from carcasses that had exhibited insect activity and were absent from those without insect colonisation.[12]

4.1.1 Insect microbiome

The relationship between bacteria and insect host has been regarded as symbiotic, with significant differences observed in the relative abundance of anaerobic bacteria, dependant on the host's diet, phylogeny (the evolutionary associations among biological species) and developmental stage. It was found that the diversity of bacteria in the gut communities of carnivorous insects were lower than those found in omnivorous insects, with the predominant phyla being Proteobacteria and Firmicutes.[96] This was supported in a study by Colman et al[97] where it was also found that Proteobacteria and Firmicutes were the most prevalent phyla.

In a study by Yun et al,[96] it was found that some insect gut populations were dominated by Bacteroidetes, Actinobacteria, and Tenericutes, in addition to Proteobacteria and Firmicutes. They also established a point of difference between mammalian and insect bacterial gut communities, in which the diversity was greater within the insect microbiome than in mammals. Furthermore, the insect microbiome changed depending on its life cycle,[98] insect order,[99] bacterial metabolism,[100] and insect gut pH levels.[101] These variations in insect gut conditions may cause the differences in host-specific insect microbiota.[96]

The observed dominant bacterial phyla in insect species may be associated with those found during human decomposition due to the correlation between insect prevalence and decomposition stage, particularly necrophagous fly species and their larvae.

4.1.2 *Ignatzschineria*

Ignatzschineria are Proteobacteria from the Xanthomonadaceae family and have been associated with a species of parasitic fly,[102] *Wohlfahrtia magnifica*, a flesh fly belonging to the Sarcophagidae family. The adult *W. magnifica* fly is a necrophagous insect whose larvae

ingests decaying tissue and plays an important role in decomposition and forensic entomology.[103]

Ignatzschineria genera have been isolated from the first and second larval stages of the *W. magnifica* fly. The non-motile, aerobic species is observed as Gram-negative and rod-shaped.[104] Hyde demonstrated that *Ignatzschineria* was the most abundant genus at all body sites during the decay stage of decomposition and decreased in numbers over time. This fluctuation occurred in parallel with insect activity during the same stages of decomposition; an increase in activity during the decay stage and a decrease in activity during the post-decay stage.

4.1.3 *Wohlfahrtiimonas*

Wohlfahrtiimonas genera are Proteobacteria from the same Xanthomonadaceae family and has been isolated from the third larval stage of the *W. magnifica* fly. The genus exhibits similar morphology as its close lineal relatives *Ignatzschineria;* in which they are classified as Gram-negative, non-motile, aerobic rods.[102] *Wohlfahrtiimonas* were found to be abundant at the same stages of decomposition and insect activity as those from the *Ignatzschineria* genus.

The predictive prevalence and relative abundance of Xanthomonadaceae related bacteria associated with necrophagous fly species, such as the flesh fly, indicates that they are major contributors to the decomposition process. This was consistent with the findings by Metcalf et al[6] who demonstrated an observable increase in Xanthomonadaceae bacteria on mouse skin over time. Similarly, Pechal et al[10] noted the presence of the Xanthomonadaceae family on pig corpses. This confirms the relevance of these studies by suggesting that the presence

of Xanthomonadaceae bacteria, and therefore flesh fly species, are not dependent on host type.

4.1.4 *Pseudomonas*

Pseudomonas genera, a group of Proteobacteria, were found to be the first to colonise at the fresh stage[6] and were observed by Hyde[5] to be in high abundance during the post-decay stage. Due to its diverse ecology and morphology, *Pseudomonas* spp. can inhabit multiple sources including soil as saprophytes and pathogens in humans and insects. The genus plays an important role in decomposition as they can solubilise the normally insoluble phosphorus, a natural compound (among carbon and nitrogen) that is produced during decomposition.[105]

4.1.5 *Proteus mirabilias*

The enterobacteria *Proteus mirabilias* has been linked to interkingdom signalling between insects and bacteria by Ma et al,[86] further facilitating decomposition. *P. mirabilis* is a Gram-negative, rod-shaped, anaerobic bacterium that is motile and originates from soil, however, the bacteria are commonly distributed via insect activity.

In a study conducted by Ma,[86] *P. mirabilis* was obtained from *Lucilia sericata*, a species of necrophagous blow fly from the Calliphoridae family that dominates the ecological ecosystem during early decomposition.[106] It was determined that the swarming behaviour and emitted compounds from *P. mirabilis*, were a form of interspecies communication to attract more flies and further increase the abundance of the bacteria.

4.1.6 *Clostridium*

Clostridium, from the phylum Firmicutes, are an environmentally diverse anaerobic, Gram-positive, rod-shaped genus. Hyde et al[3] noted that the relative abundance of *Clostridium*

was significant due to its association with insects. The role of *Clostridium* genera in the facilitation of decomposition is significant as the bacteria breaks down lipids and carbohydrates in human tissues.[21] It was suggested by Hyde et al[5] that the *Clostridium* lipases encourage fat hydrolysis and carbohydrates are transformed into acids and alcohols by enzymes.

Hyde et al[5] found that unclassified *Clostridiaceae* genera made up 55% of the bacterial community before purge and 64% during the post-decay stage. Bucheli[9] observed a pattern between seasonal changes, insect fluctuations and bacterial abundance, in which fly-related bacteria was absent when insect activity was reduced due to lower environmental temperatures, thereby reducing the rate of decomposition. This correlation demonstrates the significance of bacteria in the decomposition process and their entomological relevance.

Pechal found that Firmicutes were the most abundant phylum, in addition to Proteobacteria, during the decomposition of pig carcasses. The prevalence of the *Clostridiaceae* genera can be seen in Figure 6.

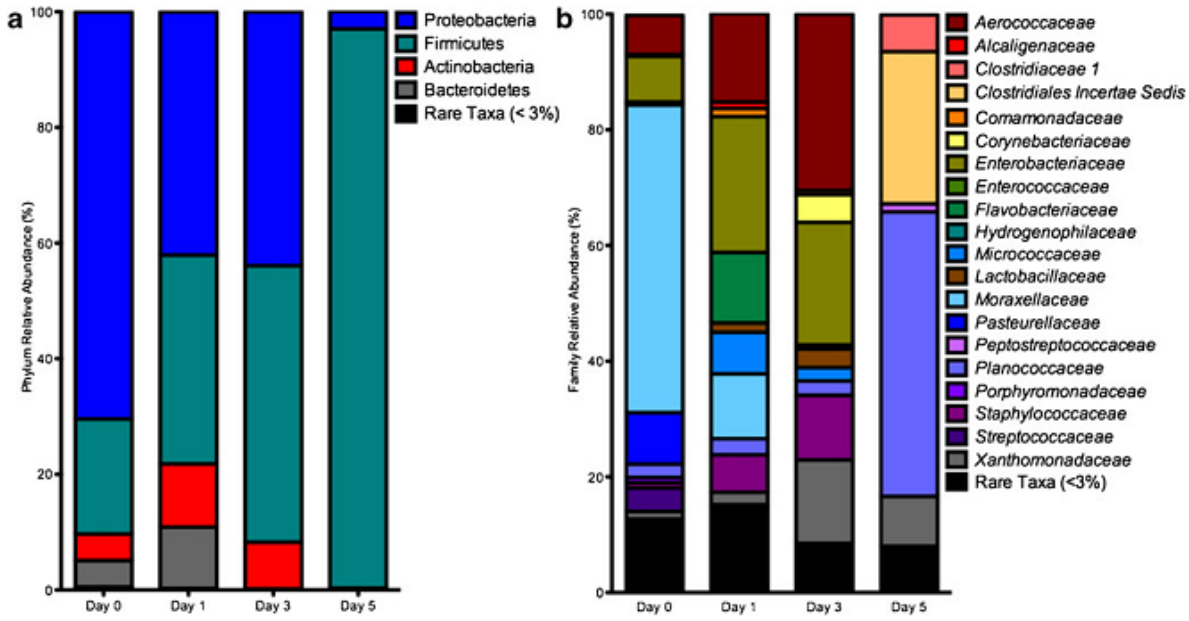

Figure 6. Bacterial community abundance in pig models. The relative abundance of bacterial communities where (A) phylum abundance levels and (B) family abundance levels. Reprinted and adapted by permission of Springer: International Journal of Legal Medicine.[10]

4.2 Soil Bacteria

The microbial soil population is dominated by bacteria with Harris[107] estimating that, in each gram of soil, there may exist 10^9 bacterial cells. The complexity of the soil environment results in up to 99% of the bacterial cells being unculturable, particularly through traditional methods.[45] The following bacterial species were considered the most prevalent between studies of soil microbes in relation to decomposition.

4.2.1 *Acinetobacter*

Acinetobacter genera, a Gammaproteobacteria from the family Moraxellaceae, are classified as Gram-negative, aerobic and non-motile coccobacilli.[108] *Acinetobacter* play an important role in decomposition as they facilitate the breakdown of organic compounds.[7]

In the study by Bucheli,[9] it was found that soil associated bacteria such as *Acinetobacter*, were among the most abundant species during the skeletonisation of cadavers. Likewise,

Hyde[3] observed that *Acinetobacter* were the most prevalent before purge and during the dry stage of decomposition. This was attributed to contact with soil as these bacteria are commonly found in soil as they can survive in dry substrates better than other bacteria.[109] Metcalf[7] observed significant numbers of Gammaproteobacteria in soil samples surrounding mouse corpses. Similarly, pig corpses in Pechal's[10] study exhibited an abundance of Moraxellaceae. This interspecies trend may signify a correlation between the Moraxellaceae family and the decomposition process.

Proteobacteria have been observed as the most abundant phyla among studies, particularly from Damann et al,[94] in which they dominated in all samples and demonstrated their omnipresence in soils, as shown in Figure 7. Additionally, Damann discovered that dry remains contained the highest levels of Actinobacteria compared to other stages of decomposition, demonstrating a community profile shift from one that resembles the human gut (Firmicutes and Bacteroidetes) to one that resembles non-cadaver soil (Actinobacteria and Acidobacteria).

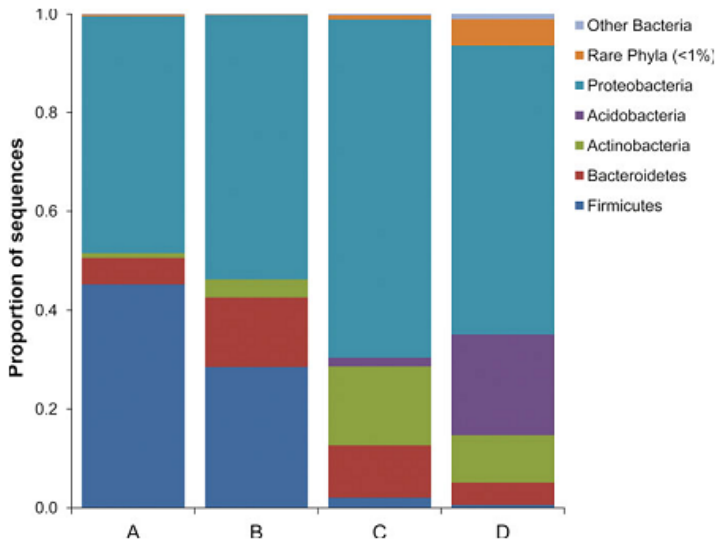

Figure 7. Relative abundance of bacterial phyla in bone and soil. The abundance of bacteria in (A) partially skeletonised, (B) skeletonised, (C) dry remains and (D) soil. Reprinted and adapted by permission of John Wiley and Sons: Journal of Forensic Sciences, © 2015.[94]

4.2.2 Chromatiaceae

Chromatiaceae, known as purple sulfur bacteria,[110] are a family of phototrophic, obligate anaerobes.[111] Metcalf[7] stated that Chromatiaceae, from the Proteobacteria phylum, could be observed during advanced decay. No other study had mentioned its prevalence which was considered interesting due to the significant role of the bacterium, as explained by Metcalf, that Chromatiaceae catalyses the denitrification and nitrate reduction processes during decomposition.

4.2.3 *Myroides*

The *Myroides* genera from the family Flavobacteriaceae are non-motile, Gram-negative, aerobic rods,[112] classified as environmental bacteria found in water and soil habitats.[113] Metcalf[7] observed the presence of *Myroides*, from the Bacteroidetes phylum, during active decay stage.

A soil microbial study by Cobaugh[114] used Bacteroidetes as a representative population and found that bacterial activity and biomass increased in soils beneath cadavers during the bloat stage. It was found that microbial diversity in CDI soils did not significantly change until the later stages of decay where an increase was observed. A shift in community structure was noted in cadavers between the bloat and decay stages, consistent with the leaching of decomposition fluids into the soil, however, several outliers for one cadaver (shown as diamonds in Figure 8) existed indicating the presence of inter-individual variability.[114]

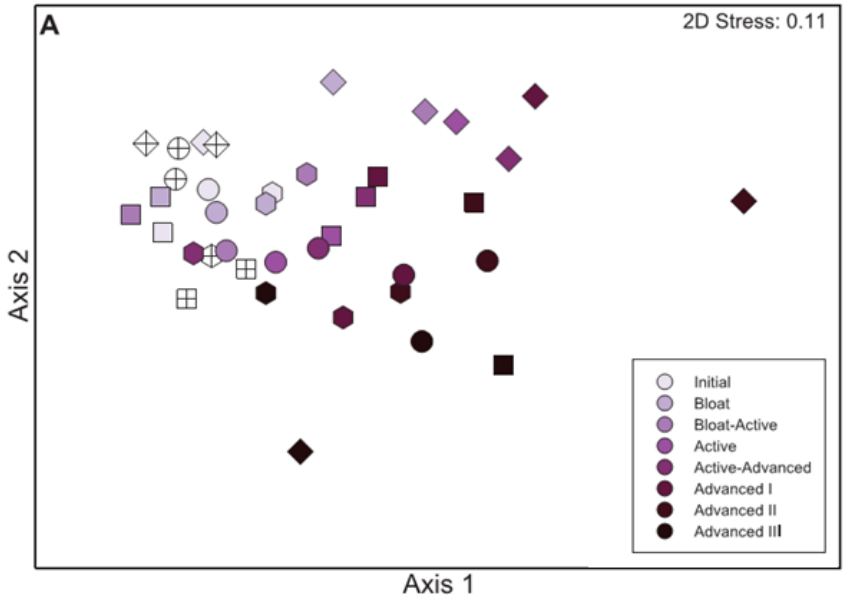

Figure 8. Soil bacterial community structure beneath decomposing cadavers. Relative bacterial abundance using NMDS ordination of Bray Curtis dissimilarities. Each shape indicates the different cadavers and crossed shapes indicates the control (no cadaver) sample. Adapted and used under licence CC-BY-4.0.[114]

4.3 Skin Bacteria

Human skin comprises the second largest abundance of microflora (the largest being the gastrointestinal tract - GIT) due to its size, with both temporary and permanent organisms,[115] however the skin microbial community varies between individuals. Microflora observed on the skin of a healthy, living person can include *Brevibacteria*, *Corynebacterium*, *Micrococcus*, *Propionibacteria* and *Staphylococcus,*[116] in addition, a survey by Gao[115] found that the phyla Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes were also present on skin.

4.3.1 Site location

A study by Hyde et al[5] focused on bacteria during the bloat stage, which found that there was a relationship between body sample site location and biome richness. It was shown in Figure 11, the observable differences between location and bacterial abundance, with the concentration of bacteria increasing while moving from the mouth, stomach and small

intestine (upper GIT) to the colon and rectum (lower GIT). The study included the mucosal surfaces of the oral cavity as a part of the collective skin habitat.

The results in Figure 9 may show a limitation to Hyde's study due to the pyrosequencing method chosen (Roche 454), whereby the sample depth of 1600 may not have been sufficient to determine an accurate assessment of species diversity. This can be observed by the continued increase of the fecal line at 1600 sequences and could be remedied by using Illumina which offers a deeper sampling depth.[117]

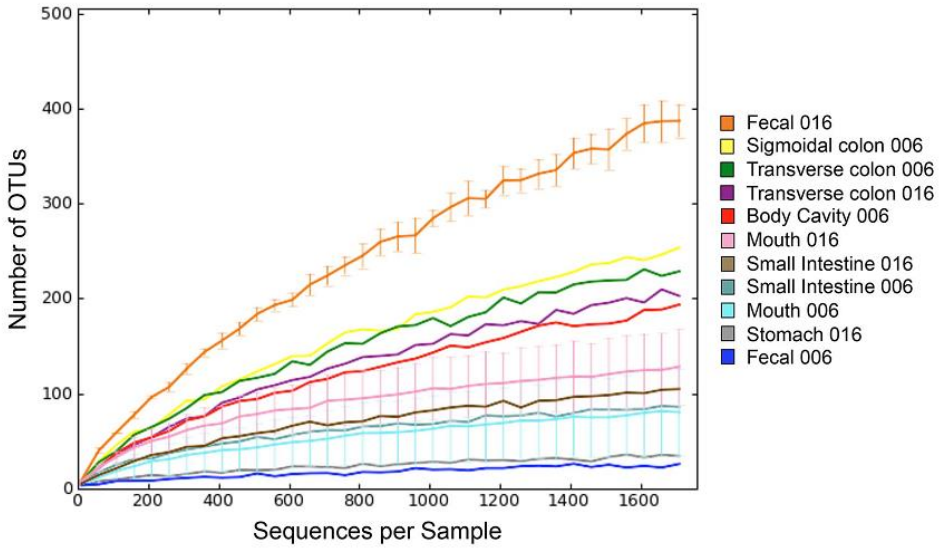

Figure 9. Variation of microbiome concentration according to location. A rarefaction curve plotting the microbiome richness (OTUs) over average samples sequenced and relating to body sample site. Adapted and used under licence CC-BY-4.0.[5]

Hyde et al[5] continued to analyse the bacterial community by observing the sample diversity (beta diversity) across all sites and confirmed that an obvious difference existed in communities depending on location. Other reports have confirmed this point of difference between GIT and oral cavity microbiomes.[118,119] It was noted that a possible source of the discovered communities could be attributed to bacterial migration from the environment (i.e. soil) or external areas (i.e. skin) as decomposition progresses, however, no formal conclusions could be drawn as no external samples were collected.

Furthermore, Hyde et al[5] analysed the phyla in each sample to determine their relative abundance, as shown in Figure 10. It was found that the composition of the community varied across body sites with collection methodology (swab and scrape) being a significant factor. The most common phylum was reported as being Firmicutes, followed by Proteobacteria and Actinobacteria.

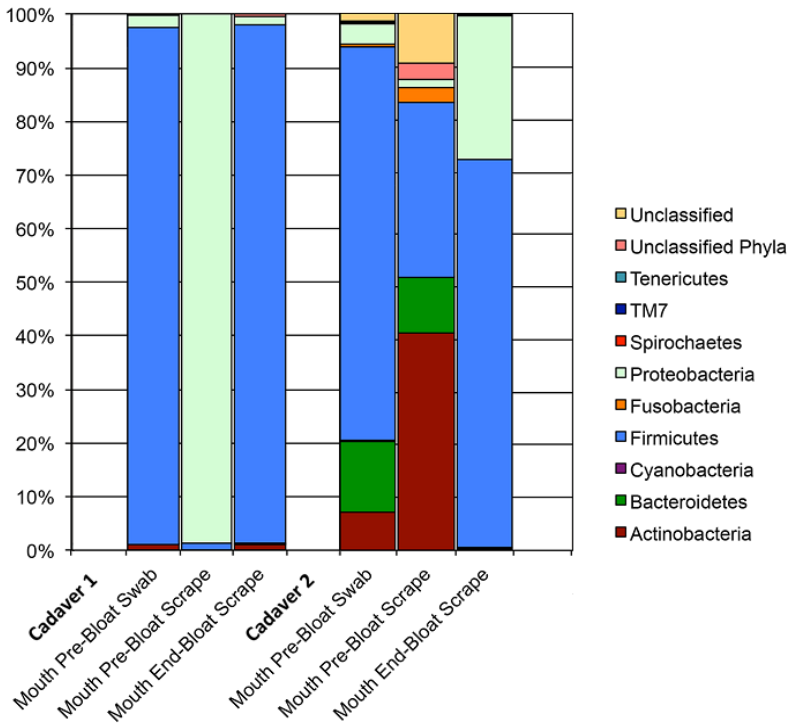

Figure 10. Phyla abundance between skin sites. Two cadavers were sampled using two methods; swab and scrape, with the relative abundance of each phyla recorded. Adapted and used under licence CC-BY-4.0.[5]

Similarly, Metcalf[6] observed the dominance of enteric aerobic bacteria, specifically Rhizobiales from the Proteobacteria phylum on skin samples of mice following the rupture event. This increase in aerobic taxa was attributed to the exposure of the cadaver to oxygen.

It was found that oral cavity samples for one cadaver at the fresh stage, closely resembled the bacteria present in healthy individuals, with *Streptococcus*, *Prevotella*, and *Veillonella* detected. Interestingly, variations of abundance and diversity in taxa were observed between the left and right sides of the cadavers head in Hyde's[5] study, of which an

explanation was attributed to the passive head movements during decomposition that may have changed the microhabitat of the sites.[3]

Overall, Hyde's[5] study attempted to catalogue the bacteria present at different stages of decomposition in an exploratory effort, and highlighted the number of microbes yet to be classified. They were not able to determine the existence of a shift in community structure through time due to their limited resources (consisting of two cadavers), however, they were able to identify key areas of investigation for future studies.

4.3.2 Pseudomonadaceae

In addition to the bacteria discovered by Hyde, Pseudomonadaceae, were found to be a significant member of the mouse skin microbial community by Metcalf.[6] Pseudomonadaceae are a diverse family of gram-negative, rod-shaped cells that inhabit various environments, from soil and water to animal and human hosts. They can be aerobic or anaerobic, motile or non-motile.[120] The study by Damann,[94] displayed the dominating abundance of Pseudomonadaceae in partially and wholly skeletonised remains, as shown in Figure 11.

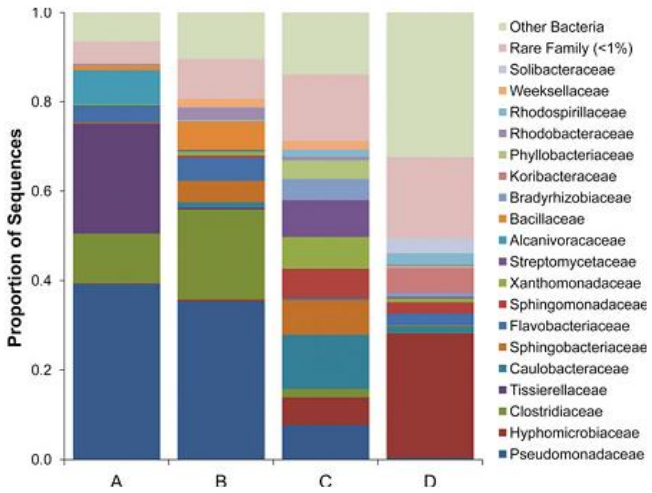

Figure 11. Relative abundance of bacterial families from bone and soil. Bacterial abundance in (A) partially skeletonised, (B) skeletonised, (C) dry remains and (D) soil (adapted). Reprinted and adapted by permission of John Wiley and Sons: Journal of Forensic Sciences, © 2015.[94]

4.3.3 Rhizobiales

Rhizobiales are an order of gram-negative Proteobacteria that are known for their nitrogen-fixing abilities. Metcalf[6] found that Rhizobiales, particularly from the Phyllobacteriaceae, Brucellaceae and Hyphomicrobiaceae families (e.g. *Pseudochrobactrum)* were the most abundant taxa following the rupture event, which marked the shift from aerobic to anaerobic conditions. Due to its prevalence at all sites, it was determined that Rhizobiales held the most predictive power for establishing a PMI.

4.3.4 Actinobacteria

A study by Johnson[30] determined that the bacterial behaviour of organisms within the phyla Actinobacteria, were considered the most valuable in their model and were powerful indicators, particularly of PMI. This was due to their abundance and predictive behaviour.

Actinobacteria are one of the largest phyla of gram-positive bacteria with a diverse range of morphologies and habitats. The Bifidobacteriaceae family within the Actinobacteria phylum, can be found in oral cavities due to its ability to adhere to mucus and the GIT. They are non-motile, anaerobic bacteria. A trend was discovered in a study by Knight et al,[121] in which the diversity of post-mortem skin microbiota showed progressive changes over time between bacteria and decomposing skin. This demonstrable successive behaviour assists in substantiating the value of the bacteria in time models for PMI.

4.4 Discussion

There were a number of studies conducted to examine the microbiome at various stages of human decomposition. An observable trend between time and bacterial successional shift was concluded by many, however the overwhelming majority found that there were many

microbes yet to be classified. Such is the case in Hyde et al's[3,5] studies which attempted to catalogue the bacteria present at different stages of decomposition in an exploratory effort. Due to the number of unidentified bacteria and resources needed to perform analysis on such a large scale, they were unable to determine the existence of a shift in community structure, however, they were able to identify key areas of investigation for future studies.

Evans[82] and Carter[23] were able to catalogue various organisms that were significant during the bloat stage of decomposition, alongside Vass[11] and Janaway[21] whom were able to identify a shift from aerobic to anaerobic bacteria and stated that autolysis initiated the breakdown of tissues in addition to bacterial activity. Due to the complex and diverse nature of decomposition, Vass was overwhelmed by the abundance of isolated organisms during their attempt to characterise them. Pechal et al[10] stated the unculturable nature of up to 99% of bacterial species using traditional methods and called for this gap in knowledge to be addressed through the continued use of NGS techniques, such as pyrosequencing, to further structure the microbial community.

Most studies identified a difference in bacterial abundance and diversity depending on site location with Firmicutes dominating mouth sites and Rhizobiales on skin samples, while the GI tract exhibited greater variation and concentration of bacterial communities. This was attributed to the presence of natural microflora before death. In addition, the influence of the insect microbiome was demonstrated with bacterial populations closely resembling those found in the gut of necrophagous insects present during decomposition.

Damann et al[94] focused on the use of bacterial succession (family level taxonomic resolution) in late stage decomposition to establish the PMI in skeletonised remains. It was discovered that remains in the skeletal stage contained the highest levels of Bacteroidetes than in any

other stage, and likewise with Firmicutes in partially skeletonised remains and Actinobacteria in dry remains. This level of succession demonstrates the ongoing progressive nature of bacteria in the late stages of decomposition in which the community profile shifts from one that resembles the human gut (Firmicutes and Bacteroidetes) to one that resembles non-cadaver soil (Actinobacteria and Acidobacteria).

Damann's study focused more on identifying bacterial community abundance at the different late stages of decomposition and highlighting the potential use of their data in establishing a PMI, however the data was not directly applied, therefore future research could be aimed at using this data for PMI estimation in skeletal remains. Overall, the difference in bacterial community variance between the late stages of decomposition showed a great potential for utilising temporal bacterial succession data for PMI estimation in skeletal remains.

Parkinson[12] observed the relationship between insect colonisation and decomposition by noticing a change in community structure that correlated with the appearance of insect larvae on colonised cadavers. Additionally, the purge of fluids following the bloat stage was attributed to an increase in microbial abundance and shift in community profile to one that resembled GI tract bacteria. These findings are consistent with those from other studies[3,10,94] suggesting that a strong relationship exists between bacterial community shifts and stages of decomposition over time.

Chapter 5 – Research methods

Studies associated with the human thanatomicrobiome have focused on identifying and classifying the microbiome of human decomposition, in addition to discovering a shift in the epinecrotic bacterial community over time, utilising NGS to determine abundance.

The studies have commonly utilised Roche 454 pyrosequencing to identify taxa, however, their research models and sampling methods have varied greatly. Pigs were commonly used as models for human decomposition as they have similar physiological and anatomical features[122] and are believed to go through comparable decomposition processes.[61] In most studies, the 16S rRNA gene was amplified, however, different regions were targeted. Likewise, similar sampling protocols were used throughout the range of studies, involving non-invasive swabbing or scraping. A strong point of difference in each study was the geographical location, predominantly in varying States across North America.

5.1 Sampling and methodology

Damann et al[94] focused on bacterial communities sampled from human bones and compared with those in soil. DNA was extracted from a single rib collected from 12 decomposing human cadavers on a ground surface, in addition to 3 soil samples in Tennessee, USA. The V3 region of the 16S rRNA gene was targeted for amplification. Family and phylum results were then plotted in relative abundance charts.

In Pechal et al,[10] three pig carcasses were sampled in Ohio, USA, at 0, 1, 3, and 5 day intervals from oral cavities and skin. DNA was extracted and analysed by Roche 454 pyrosequencing. The V1 to V3 regions of the 16S rRNA gene were amplified and taxon abundance was determined for each sample at family and phylum levels.

In Hyde et al,[5] two human cadavers were sampled in Texas, USA, at time of placement and conclusion from mouth and rectal sites. Destructive internal samples were taken during dissection at the concluding period. Similarly, the 16S rRNA gene was amplified and 454 pyrosequencing was performed. Similarly, Bucheli et al[9] conducted a long-term study on human cadavers in Texas, collecting approximately 20,000 swabs over a three year period. In contrast, Metcalf et al[6] sampled mouse corpses on soil and amplified the 16S and 18S rRNA genes.

Similar studies were conducted by Lang et al[123] and Dickson et al,[124] with an emphasis on pig carcasses in marine habitats. Bacterial communities were examined in Pennsylvania, USA and Dunedin, New Zealand respectively. Dickson utilised capillary electrophoresis to sequence while Lang employed the ARISA (automated ribosomal intergenic spacer analysis) method, focusing on the 16S-23S bacterial gene. The ARISA approach differs from sequencing in that it creates community profiles that estimates the bacterial diversity and composition, rather than identifying the organisms.[125]

The sampling method remained constant across most studies, however, Hyde[5] found that the bacterial community composition varied depending on collection methodology (swab or scrape) and was classified as a significant factor that may influence the overall results.

5.2 Next generation sequencing

Identify unculturable microbial taxa[4] associated with decomposition has depended on the accuracy of NGS technologies. The most commonly used method to sequence includes Roche 454, a technique based on pyrosequencing where DNA fragments are sequenced by synthesis using chemiluminescent enzymatic reactions.[58] This method is utilised for its large sampling depth and the ability to detect uncommon microbial taxa that exists in low

numbers.[126] The genes that can be targeted for amplification are the 16S rRNA gene, regions V1 to V5. This gene is used as it is ubiquitous in bacteria and has a slow rate of evolutionary change.[127]

Gene sequencing using the 16S rRNA gene has proven to be a suitable method for identifying bacterial species, however, there have been multiple genus from the Enterobacteriaceae family that had been falsely identified, such as *Escherichia* and *Shigella,* due to their similarities.[128] Sequencing is particular useful when organisms are difficult to identify using standard culture methods, however, 1 to 14% of the population remain unidentified after testing.[127] In addition, the ability to differentiate between bacteria can be impaired when the V1 variable region of the 16S gene is often discarded during the purification process due to the presence of residual dyes.[128]

In addition to NGS, the change in bacterial communities during the decomposition process was explored by Parkinson,[12] using molecular methods; T-RFLP (terminal restriction fragment length polymorphism) and DGGE (denaturing gradient gel electrophoresis). These tools are commonly utilised in ecological studies to observe and typify changes in microbial communities. The results of Parkinson's study using the T-RFLP and DGGE methods, indicated conflicting results on bacterial diversity among samples and was attributed to common problems with the approaches such as inter-gel variation with DGGE.[129]

The majority of studies utilised Roche 454 pyrosequencing which has been considered a reliable technology for the quantitative assessment of genetic diversity,[59] however, updated sequencing technology could yield better results. Current systems, such as Illumina MiSeq, can produce longer contigs (DNA segments that overlap to represent a consensus region of DNA)[130] with greater accuracy and at a lesser expense than 454.[131] The PacBio system has

been preferred when species identification is known in a sample[132] and is therefore best used in bacterial abundance studies, in addition to providing longer read lengths.[133]

5.3 Discussion

Although there are many commonalities among the human thanatomicrobiome studies, there exist just as many variances in methodology that can cause inconsistencies in data, particularly among research models. Geographic location should be focused on more to highlight the many differences in climates and environments throughout the world and establish if patterns exists between each region. Studies have been focused in the USA, mainly due to their resources and ability to conduct research on human cadavers that would otherwise be problematic in other countries. Additionally, the NGS technologies used by the studies can become quickly outdated and a shift to current systems can ensure more accurate data.

Chapter 6 – Forensic applications

Identifying the bacteria involved in decomposition, the roles they play and their successional changes, helps to understand the overall process of decomposition, however, bacteria offers additional insights into details that have forensic significance, such as establishing the PMI.[9]

6.1 PMI

The amount of time that has passed since death is one of the most challenging types of physical evidence to establish, however it is important in forensic investigations as it can result in the identification of the victim or possible suspects.[6] There have been multiple methods of calculating the PMI, including observing the mortis triad, stomach contents, and temperature which have resulted in imprecise approximations. Biochemical methods (those based on pathophysiological changes) have been considered more accurate due to the reduced influence of external conditions, such as temperature and predation.[31] Insects have been considered as significant biomarkers to use for PMI estimation due to their successional community shifts.[67]

6.3 Successional shifts

A demonstrated shift in abundance could be observed as the stage of decomposition shifted from partially skeletonised to dry remains. By observing bone decay, it was found that initial bacterial communities resembled those found in the human gut, while each successive stage resulted in a decrease in microbial abundance and overall profile until the community resembled those found in non-cadaver soil.[94]

Additionally, Pechal et al[10] noted that a clear bacterial community shift existed from as early as day one of decomposition, and established how this community structure could be used to distinguish time of decomposition using simple and complex modelling systems (from relative abundance modelling to general algebraic and generalised linear modelling). Using their approach, it was determined that the shift in community structure could be utilised when there is a lack of entomological evidence that would ordinarily be used to establish the PMI.

6.3.1 Successional study review 1

Metcalf et al[6] observed that the rupture point of decomposition (following bloat) marks a major shift in bacterial community structure as the presence of anaerobic, gut-microbes found during the initial stages, changes to non-enteric aerobic species (from soil and skin). It was observed that the abundance of endogenous anaerobes, such as Firmicutes and Bacteroidetes, decreased considerably due to the rupture event, allowing aerobes such as *Rhizobiales* to dominate, as shown in Figure 12.

These taxa shifts were common in the advanced decay stage of soil samples, after approximately 20 days, which were significantly different to the zero-day soil microbe populations and the no-cadaver control samples, as demonstrated in Figure 13. It was noted that the control samples (non-cadaver) did not change considerably over time, connecting the soil microbial community shift to the presence of a decomposition event.

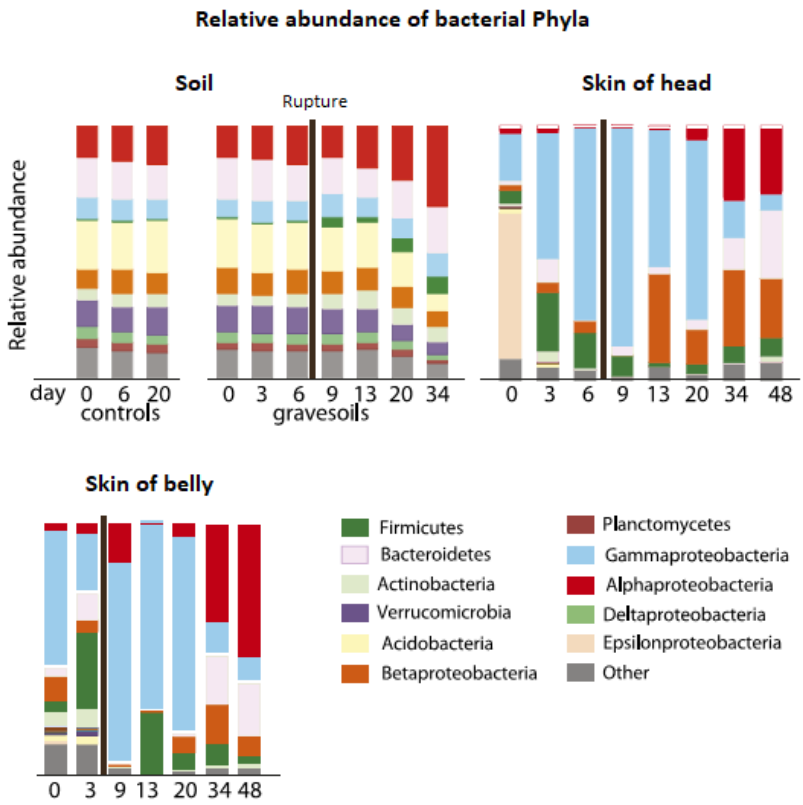

Figure 12. Bacterial phyla abundance in mouse carcases. The relative abundance of phyla in soil and skit sites where control samples contained no-cadaver soil. Adapted and used under licence CC-BY-3.0.[6]

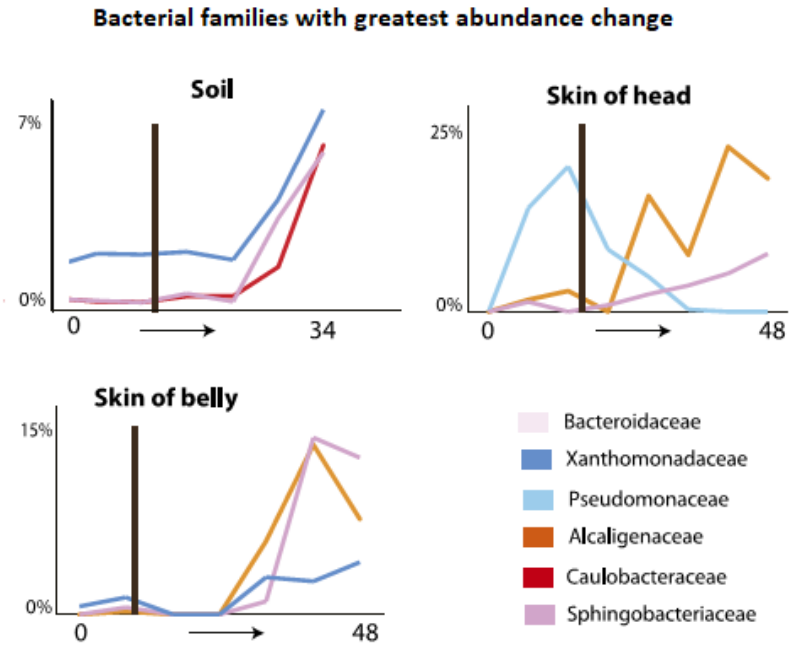

Figure 13. Change in abundance of microbial families on mice corpses. Trajectories of bacterial families that exhibited the largest change in abundance over time. Adapted and used under licence CC-BY-3.0.[6]

Microbial communities associated with the decomposition process of mice, varied greatly with time in contrast to starting communities on soil and skin sites, where an increase in abundance of several families were observed during advanced decay (such as Bacteroidetes and Alphaproteobacteria). Metcalf et al[6] found that this was consistent with previous studies suggesting that skin microbial communities are reflected from the surrounding environment.[134]

The rupture event has been classified as the most visually obvious marker of the shift from bloat to active decay stage,[83] therefore, the ability to determine whether a cadaver's bacterial community has shifted beyond the rupture event with the presence of dominating aerobic species, can help establish timeframe more accurately.

Metcalf et al[6] was able to ascertain the presence of consistent shifts in the abundance of the microbial community during specific stages of decomposition, suggesting that bacterial succession may be utilised to estimate the PMI. Similarly, Hauther et al[135] determined that abundances of Bacteroides and Lactobacillus could be used as PMI indicators. Additionally, it was found that temporal shifts in the bacterial communities of the skin (head) permitted an estimation of the PMI to within 3 days (+/- 2 days).

Interestingly, it was discovered that the major shift from anaerobic to aerobic bacteria during the rupture event, was inconsistent between cadavers, and therefore, was not considered an accurate marker of timing and offered less useful data to estimate the PMI. Furthermore, it was found that the most significantly predictive taxa belonged to the *Rhizobiales* order at all sample sites.

6.3.2 Successional study review 2

Parkinson's[12] study focused on exploring the change in bacterial communities, in which successional shifts were evident in the results with plots indicating deliberate successive changes rather than random. Specifically, it was found that bacterial communities experienced major early changes in composition at the commencement of active decay, between 7 and 9 days (114 and 148 ADD's) into decomposition, which correlated with initial insect colonisation. The observable change in bacterial composition could be attributed to the detection of skin bacteria during the fresh/bloat stage which shifted once rupture occurred and gut microflora was released.

A second shift occurred at day 16 to 18 (259 to 283 ADD's) which was related to the onset of insect larvae migration and when the residing substrate began to dry (soil and sand). A third shift occurred at day 50 and 115 (758 and 1440 ADD's) which was associated with skeletonisation in insect-colonised cadavers, or mummification in non-insect-colonised cadavers. The difference between colonised and non-colonised cadavers suggested that, prior to the hatching of insect larvae during the fresh/bloat stage, the cadavers possessed similar bacterial communities, however, once tissue decomposition began due to larval activity, the composition of the community diverged and persisted throughout the process. These results indicate the influence insect-colonised cadavers have on bacterial composition, and therefore, PMI estimation.

Parkinson's[12] study was performed on various substrates (sand and soil) to compare their influence on bacterial composition, however, patterns were similar on both types suggesting that the shifts were facilitated by body-hosted bacteria instead of an organism's environmental response to the increase in nutrients. In the same study, significant amounts

of decompositional variation was encountered with the human cadavers, which was attributed to inter-specimen variability among factors such as age, sex and physiological characteristics. One cadaver exhibited decelerated decomposition which could have been caused by anti-microbial pharmaceuticals consumed prior to death, disturbing the body's natural microflora. This was corroborated by a proliferation of fungal species due to the absence of a strong microbial community.[136]

The identified bacterial orders were collated in the study by Parkinson,[12] and it was found that common bacterial orders existed between samples (pig and human cadavers) and over all decomposition stages. Several trends emerged from the study, including the presence of Xanthomonadales in relation to insect colonisation, the detection of Sphingobacteriales only beneath pig models, and the presence of Myxococcales and Rhodospirillales associated only with human cadavers. Bacteria common to all samples included those from the Pseudomonadales, Flavobacteriales and Clostridiales orders. It was important to note that there were no time-specific bacteria identified.

6.3.3 Successional study review 3

A study by Pechal et al[10] used pyrosequencing to identify bacterial communities and their relative abundance in human decomposition events, in order to create a model that estimates physiological time, thereby corresponding with a minimum PMI estimate. Their data illuminated the presence of differing bacterial communities over time as decomposition progressed. Furthermore, they identified the need to distinguish between taxonomic resolution (i.e. family or phylum) to properly discriminate between bacterial communities.

It was determined that the phylum taxa were the best level of resolution to distinguish decomposition time, however, the family level was appropriate for estimating physiological

time, considered a finer measurement that can be used as a surrogate for decomposition time. Additionally, the study found that taxon richness could be influenced by external sources of bacteria from fly competition,[136] resulting in a decrease of bacterial variation over time. This utilisation of cadaver resources (through mating, oviposition and nutrient supply) by insect colonisers may disrupt existing bacterial communities through competition.

Using the bacterial community to estimate the PMI is similar to using blow fly developmental data in forensic entomology, however, microbial data may be more beneficial if insect colonisation has not occurred. Based on their research, Pechal[10] developed a theoretical framework to demonstrate forensic application, as shown in Figure 14. This framework is comparable to the methods used by forensic entomologists where insect samples are collected and compared to insect life cycle stage data and known time ranges of each stage.

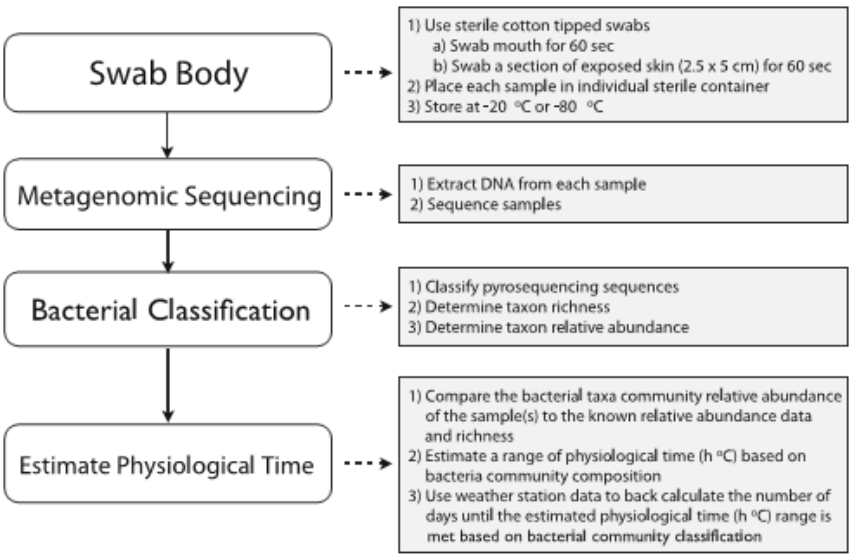

Figure 14. Framework for using bacterial communities for estimating PMI. Reprinted and adapted by permission of Springer: International Journal of Legal Medicine, © 2013.[10]

6.4 Discussion

Bacteria have been the focus of studies regarding PMI estimation in death investigations due to an observable successional change over time, through which the authors have attempted

to catalogue and explain. Metcalf et al[6] observed the presence of shifts in the abundance of the bacterial community during different stages of decomposition and suggested that recent deaths had a higher accuracy of PMI prediction. They suggested that external sources of change (vegetation or entomology) should be focused on during late stage of decomposition, however, Damann et al[94] observed a noticeable shift in bacterial succession during the dry and skeletal stages that had the potential to establish a PMI.

Pechal et al[10] demonstrated the importance of considering necrophagous flies as contributors of bacteria to the human necrobiome and the impact insects have when establishing a PMI, in addition to creating a model and framework for estimating the PMI in a forensic setting. The aforementioned studies provide an overview of bacterial succession research with quantifiable data and demonstrable results, and in combination with smaller studies,[3,94] can have the potential to create an accurate model for PMI estimation associated with all stages of decomposition.

Overall, the studies demonstrate the presence of multiple successional shifts within bacterial communities that are associated with major taphonomic events during human decomposition. The shifts were observed to be common among all samples and cadavers with slight variances in regard to bacterial abundance, depending on taxonomic resolution (with the majority of studies focusing on bacterial families).

Chapter 7 – Conclusion

The human necrobiome has become the focus of several studies attempting to catalogue the identification of bacteria and explain the role the microbial community plays in human decomposition. Distinct profile shifts were observed in the studies of bacterial communities which can be used to establish a post-mortem interval estimation, data which may be

valuable when forensic evidence is deficient: for example, when forensic entomology cannot be utilised due to a lack of insect colonisation. This method, however, contains limitations that must be addressed in future studies.

7.1 Limitations

Necrobiome studies in human models can present difficulties when controlling variables between subjects, such as medical history and pharmaceutical usage before death, and may account for inconsistencies in results. Several studies have already utilised pig models as substitutes, due to their physiological similarities and comparable decomposition processes, in order to decrease the impact of these limitations. Likewise, the high levels of variance between human cadavers in addition to the limited resource size in studies, may not provide data representative of human models, indicating that sample size should be increased to account for this.

There are limitations to pyrosequencing bacterial diversity that limits its use in forensics, such as a lack of known genomic references for comparison, an increased insertion-deletion error rate, and the expense of using NGS technology. These problems could be remedied by using updated technology, such as Illumina and PacBio, which have demonstrated greater sequence sampling depth at a lesser expense than previous generations, such as Roche 454, resulting in a more representative assessment of species diversity.

Establishing an accurate PMI using bacterial communities can be influenced by multiple variables, such as base temperature, that could result in over or under estimations. PMI models should consider an appropriate temperature range, as those used in some studies (0 - 60°C), may not be appropriate for bacteria found outside this range, thereby limiting the accuracy of PMI measurements. Limitations exist in regard to using bacterial communities

as a biomarker for PMI establishment, due to large interpersonal variation, whereby generalisations are used which can decrease the accuracy of the PMI. Further research should be conducted using larger sample sizes to observe a deeper trend among bacterial communities.

7.2 Future research

The relationship between soil bacteria and cadaver decomposition have been examined in studies, primarily focusing on the effects that decomposition has on the localised environment and neglecting the effect that environmental bacteria has on cadaver decomposition rates, which can be focused on in future research. Additionally, future research should examine stable bacterial communities over time and the refinement of PMI models by addressing base temperature ranges. Due to the variable nature of bacterial communities, more data is required to account for this variation related to human remains in different geographic regions and environmental conditions, of which future studies can focus on different biomes and habitats, adding to the pool of knowledge with a goal of increasing the accuracy of PMI estimations using bacterial communities.

Reviewing the studies of bacterial succession during decomposition indicated that the formulated hypothesis may be plausible, whereby, a noticeable shift in bacterial community structure through time can be observed with the ability to establish a PMI, however additional studies are required to address this hypothesis further with forensic application in mind. Further research has the additional potential for providing an insight into other scientific areas, including the discovery of unidentified organisms with capabilities that can be utilised in the medical field by adding to the existing knowledge of the human microbiome. This review focused on comparing the current contributions of literature to further

understand the ecological mosaic of human decomposition and with which future research can be based on.

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Part Two

Manuscript

A Literature Review of the Microbiome Associated with Human Decomposition

Abstract

Human decomposition consists of a complex microbial ecology that few studies have examined in depth. This review investigated the microbiome of human decomposition to further understand their functions within the decomposition process and their potential to increase the accuracy of post-mortem interval (PMI) estimations in forensic applications. The aims of the literature review were to (1) identify the external microbiome responsible for human decomposition, focusing on insect, soil and skin sources, (2) determine the roles of external bacteria in the various stages of human decomposition and (3) to analyse and compare the current contributions of literature in furthering the understanding of the ecological mosaic of decomposition.

The current literature was reviewed and their contributions to necrobiome research was analysed using qualitative and contemporary research techniques. Bacteria were found to play a significant role in each stage of human decomposition with multiple studies demonstrating an observable successive shift in microbial communities through time. This change in community profile was found to be an important biomarker for the estimation of the PMI and potential substitute for entomological techniques currently used in forensic investigations. High interpersonal variation between decomposition events, in addition to narrow geographic specificity, represented limitations in the studies which may be remedied by increasing sample size while focusing on different geographic regions and environmental conditions.

Keywords: *decomposition, necrobiome, bacteria, post-mortem interval, succession*

Table of Contents

1.0 Introduction

Human decomposition is a process divided into stages based on observable physical changes: known as the fresh, bloat, decay, post-decay and skeletal stages.[1] Each stage attracts different species of macro and micro-fauna that introduces additional bacteria, both internally (enteric bacteria) and externally (from skin, soil and insects) to the cadaver, collectively known as the necrobiome.[2]

Specific types of bacteria have been found to colonise the cadaver at different intervals using Next Generation Sequencing (NGS) methods including pyrosequencing,[3] a DNA (deoxyribonucleic acid) sequencing technology that acquires sequences for microbial taxa that are considered unculturable.[4] A temporal trend had been observed among studies[5,6] which had found that each bacterial community provided the foundation for the subsequent colonisation of different microbial species. The implications of this discovery holds significant weight in forensic investigations as the identified bacteria could determine the post-mortem interval (PMI) of the deceased, or the time since death.[7]

Entomological (insect) evidence is commonly utilised to estimate the PMI, however, there are many variables that can interfere with the colonisation process such as weather, location, and clothing, which reduces the accuracy of this method. Changes in microbial succession and factors that affect the rate of decomposition, such as temperature and biological variations, can be combined with entomological methods to increase the accuracy of PMI estimations, which can assist in understanding the role that epinecrotic communities play in human decomposition.[8]

Three necrobiome studies[5,9,10] have been conducted in similar geographic locations, which can limit the use of their data in different environments. Other studies[3,11] have contrastingly

generated broad results due to uncontrollable variables when utilising human models. Even fewer studies[8,12] have focused on reviewing and comparing the existing literature as a whole.

This review aimed to compare the current studies associated with the human necrobiome to further the understanding of its functions and implications in forensic science. The objective involved identifying the external microbiome and their roles in human decomposition, specifically associated with insects, skin and soil. In addition, it was hypothesised that a shift in the structure of bacterial communities would be observed over time which would assist in increasing the accuracy of post-mortem interval estimations.

This review was conducted using online and offline resources, focusing on literature that discussed and studied the microbiome of human decomposition, including journal articles, conference papers, theses, empirical studies, and reports from databases such as Scopus, ResearchGate, and ProQuest. Searches were performed using specific keywords and parameters related to human decomposition and bacteria to maximise the amount of relevant results retrieved.

2.0 Background

The human microbiome is a complex ecosystem[13] that has a large influence on the rate of decomposition, with the objective of total nutrient consumption. The various communities are categorised by body site location, with clear delineations existing between internal (predominantly from the gastrointestinal – GI - tract) and external (existing on the skin)[8] locations and the bacteria that inhabits them. The natural microbial community in healthy, living humans consists of bacteria, archaea, viruses and eukaryotes,[14] with many that are allowed to flourish when host death occurs and decomposition begins.

The different stages of decomposition, as seen in Figure 1, have been shown to be consistent among various human and animal models,[15] with similar bacterial profiles exhibited, particularly in swine models due to their similar physiology.[16] The bacteria have been demonstrated to influence each stage of decomposition, in association with natural enzymatic processes such as those during autolysis in the fresh stage, a method of self-digestion characterised by cell death.[13]

Figure 15. Stages of decomposition. (1) fresh, (2) bloat, (3) decay, (4) post-decay & (5) skeletonisation. Adapted and used under licence CC-BY-SA-3.0[17]

3.0 Decompositional bacteria

Different bacterial species have been discovered during specific stages of decomposition and can be linked to each successive stage and community shift. Most studies have attempted to identify and examine the bacterial abundance at specific stages.

3.1 Fresh stage

The first stage of decomposition, known as the fresh stage, lasts for approximately one to two days, and is where rapid insect colonisation from fly species such as blow flies (Calliphoridae) and flesh flies (Sarcophagidae) can be observed,[18] facilitating bacterial dispersal through physical transference.[2] The fresh stage occurs in parallel with internal microbial activity during

the autolysis process. Anaerobic bacteria found naturally in the GI tract, assists in digesting organs and tissues, creating gaseous by-products that marks the onset of the bloat stage. The initial shift in bacterial community structure can be observed in the fresh stage, coinciding with the increase in activity as a response to physical disturbance.[19]

The *Pseudomonas* genera from the Proteobacteria group, were the first bacterial colonies observed at the fresh stage in the study by Metcalf et al.[6] The role of *Pseudomonas* is to solubilise phosphorus, a natural compound produced during decomposition.[20] *Proteus mirabilias*, a species of enterobacteria, becomes abundant during the fresh stage and was demonstrated by Ma et al[21] to facilitate decomposition using interkingdom signalling, a form of interspecies communication to attract more flies. This bacterium is commonly distributed by *Lucilia sericata*, a species of necrophagous fly from the Calliphoridae family that is prevalent during early decomposition.[22]

The *Wohlfahrtiimonas* and *Ignatzschineria* genera are both Proteobacteria from the Xanthomonadaceae family and have been associated with the parasitic fly[23] *Wohlfahrtia magnifica*, a species of flesh fly from the Sarcophagidae family. These genera have been observed in the early stages of decomposition due to the increase of insect activity soon after death.[6] A study by Hyde[5] demonstrated that *Ignatzschineria* abundance decreased over time, in parallel with insect activity as each stage progressed.

3.2 Bloat stage

Putrefaction and visible bodily distention is characteristic of the second stage of decomposition, due to an accumulation of gases (including hydrogen sulfide and carbon dioxide) as a result of enteric microbial and enzymatic activity.[19] A marbled appearance and change of colour can often be observed, due to the movement of bacteria through blood

vessels.[24] The presence of oxygen causes aerobic bacteria in the gastrointestinal tract to thrive during the initial stages of decomposition, however, the increase in pressure as a result of the microbial fermentation, causes the skin to tear and rupture, purging the bacteria from the body and transforming the environment into anaerobic conditions.[25,26]

Wohlfahrtiimonas and *Ignatzschineria* genera are commonly found during the bloat and purge events.[3] *Pseudomonas* were observed during the aerobic phase of the bloat stage, however, following the rupture event and change to anaerobic conditions, the abundance of *Pseudomonas* were replaced with Firmicutes (specifically *Peptoniphilus* and *Clostridium*),[5] demonstrating the community shift. Bacteroidetes populations, such as *Myroides*, were found to increase in abundance in soil communities surrounding the body and were an indication of another shift due to the leaching of fluids into the soil from the purge event.[7,26]

3.3 Decay stage

The body deflates when liquids and gases are purged into the environment during the decay stage, modifying the external microbiome on skin and soil surfaces.[27] Interkingdom signalling is exhibited at the decay stage[28] and can result in an over-abundance of Diptera larvae (maggots) which feed on soft decaying tissue, leaving only skin and cartilage in preparation for the post-decay stage.[1] The presence and signalling behaviour of bacteria can further influence the development of fly larvae into adults, demonstrating the hosts' dependence on the relationship.[29] During this stage, the putrefaction process can be altered to exhibit saponification or mummification, depending on environmental conditions such as temperature and humidity. Saponification is the formation of a fatty, waxy substance called adipocere and occurs in moist, humid conditions,[30] while mummification is the dehydration of tissues due to arid, ventilated environments, leading to brown and brittle skin that is resistant

to bacterial decay.[31] Both processes can preserve the body, thereby offering significant forensic value.

Saponification can be accelerated by bacterial activity, particularly from anaerobic species in the Clostridium genus.[32] Microorganisms such as *Pseudomonas*, *Staphylococcus aureus*, and *Clostridium perfringens,* have been identified as being responsible for the production of fatty-acids required for adipocere formation.[30] Pfeiffer[33] identified a bacterial community shift from Gram-negative to Gram-positive species during the saponification process.

3.4 Post-decay stage

The body becomes dehydrated during the post-decay stage where the dominant insect species, Dermestid beetles, remove the remaining skin and cartilage.[34] Bacterial communities in the surrounding soil, such as Pseudomonas, increase in abundance at this stage due to their abilities to feed off low-nutrient sources presented by the dried remains.[35] *Ignatzschineria* abundance was shown to decrease by Hyde[5] at this stage, in parallel with a decrease in insect activity. It was found that the *Clostridiaceae* genera made up 64% of the bacterial community during post-decay.[5]

3.5 Skeletal stage

Hair and bone are the final physiological vestiges of the skeletal stage and can remain for a highly variable period of time depending on environmental conditions and predation.[36] Damann et al[37] found that each stage of skeletonisation influenced the bacterial profile, where partially skeletonised remains contained bacteria resembling the gut at earlier stages (Firmicutes and Bacteroidetes), while dry remains resembled the soil community, such as *Acinetobacter* found by Bucheli et al[9] and Hyde et al.[3] The similar soil profile was attributed to greater contact with soil surfaces at the dry stage and the bacteria's ability to survive on dry

remains.[38] Damann et al[37] also observed an abundance of Pseudomonadaceae in partially and wholly skeletonised remains, in addition to the highest abundance of Bacteroidetes than any other stage.

Due to the varying nature and focus of each study into the necrobiome of human decomposition, a comparison of relative abundance was produced to highlight the differences and similarities between phyla abundance in three studies, those by Damann et al,[37] Hyde et al,[5] and Pechal et al,[10] as shown in Figure 2. The three particular studies were chosen due to their focus on phyla in addition to their varying location sample site, which demonstrates the variances attributed to location among bacterial populations. It was found that Studies 1 and 3 displayed the highest abundance of Firmicutes, while Study 2 had the highest levels of Proteobacteria. The Actinobacteria and Bacteroidetes phyla represented the smallest abundance in all studies.

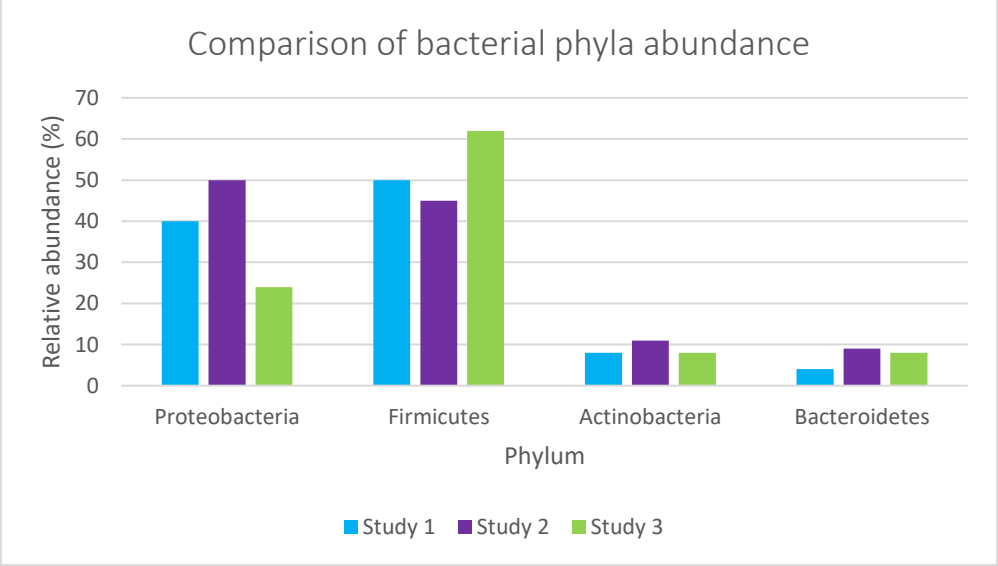

Figure 16. Bacterial abundance comparison. Studies (1) Pechal et al,[10] (2) Damann et al,[37] & (3) Hyde et al.[5] Study 1 focused on gut and internal locations, study 2 focused on soil locations and study 3 focused on skin locations.

Each stage of decomposition was shown by multiple studies to be influenced by specific bacterial organisms, with different levels of diversity and abundance within the communities.

These observations can have significance in forensic contexts where the establishment of a post-mortem interval (PMI) is required by observing temporal-based successional shifts in relation to bacterial identification.[9]

4.0 Successional shifts

Establishing a PMI, or the amount of time that has passed since death, can be integral to a forensic investigation when ascertaining timelines that can often result in the identification of the victim or suspects.[6] Past and current methods of establishing the PMI have focused on inexact markers of time that give rise to crude approximations, such as observing the mortis triad (livor, rigor and algor mortis), stomach contents and body temperature. Biochemical methods that focus on pathophysiological changes are influenced less by external variables such as environment and predation, and are therefore considered more accurate markers.[39] Entomological evidence is currently utilised to calculate the time since death due to similar successional shifts between necrophagous insect species.[40]

A study by Pechal et al[10] found the earliest signs of an obvious bacterial community shift from day one of decomposition, and determined through various modelling systems, how these shifts could determine time since death. Their approach utilised the abundance and diversity of bacteria within the decomposition process to potentially establish a PMI when entomological evidence was lacking.

The rupture event following the bloat stage, was marked as a major shifting point in the bacterial community, according to Metcalf et al,[6] where anaerobic gut microbes (such as Firmicutes and Bacteroidetes) gave way to aerobic species commonly associated with skin and soil (such as Rhizobiales), as shown in Figure 3.

Several separate bacterial families were observed during advanced decay that differed greatly from the initial community profile and was consistent with their previous studies, which Metcalf et al suggested was confirmation that skin microbial communities were a reflection of the surrounding environment.[41] As a result of the obvious successional shifts over time, an accurate PMI could potentially be established to within 3 days (+/- 2 days), according to Hauther et al,[42] by observing the abundance of Bacteroides and Lactobacillus as indicators. In contrast, Hauther et al found multiple community discrepancies between cadavers during the rupture event, resulting in decreased reliability as a PMI marker. Furthermore, they established that the most dominant predictive taxa across all sites, belonged to the Rhizobiales order.[42]

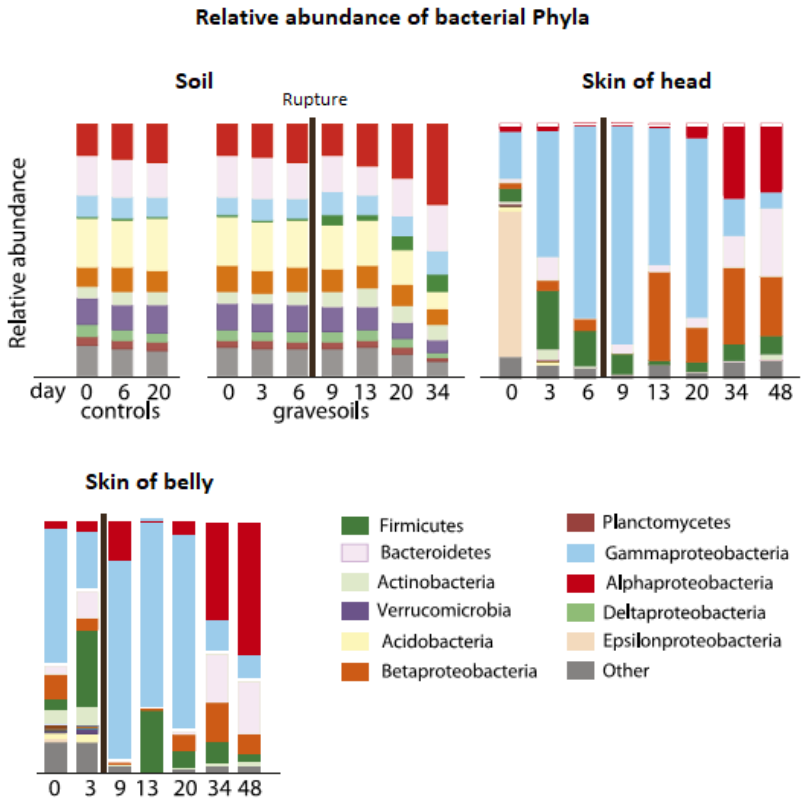

Figure 17.Bacterial phyla abundance in mouse carcases. The relative abundance of phyla in soil and skin sites where control samples contained no-cadaver soil. Adapted and used under licence CC-BY-3.0.[6]

A study by Parkinson[12] found that each shift was deliberate rather than random, with initial community changes commencing 7 to 9 days into decomposition, and was connected with

insect colonisation. This shift was attributed to the detection of skin bacteria observed during the fresh and bloat stage which changed during the transpiration of the rupture event, a similar observation noted by Metcalf et al. Parkinson observed a second shift at days 16 to 18 which was attributed to the migration of insect larvae and the desiccation of the body and surrounding soil. A third shift was apparent at days 50 to 115 which was associated with skeletonisation and mummification, depending on the presence or absence of insect activity, indicating the influence insect colonies have on the decomposition process.[12]

Establishing the PMI using bacterial data as a substitute for entomological data is possible, according to Pechal et al,[10] whom developed a theoretical framework for forensic application based on their research. The framework detailed the process starting from sample collection via body swabs, extraction and sequencing of DNA from the samples, classifying the bacteria according to taxon richness and abundance and estimating physiological time by comparing the composition of the bacterial community and using weather data to calculate the number of days based on bacterial classification.

5.0 Discussion

Determining the utility of successional shifts within bacterial communities has been the focus of several studies, in order to examine its worth as a forensic tool to establish PMI. Recent deaths were found to provide more accurate PMI data, as determined by Metcalf et al,[6] and suggested that the later stages of decomposition should focus on external variables, such as vegetation or insects. In contrast, Damann et al[37] found that an observable successional shift did occur during the skeletal stage that could be used to establish a PMI.

The contribution of necrophagous flies to the decomposition process was demonstrated by Pechal et al,[10] with each study demonstrating overall, the observation of bacterial successional

shifts within communities associated with taphonomic events. This trend was commonly seen among all animal and human models, with some variation in abundance as this depended on taxonomic resolution, where the majority of studies focused on bacterial families.

6.0 Conclusion

The role that the microbial community plays in human decomposition has been studied in an attempt to identify and explain the natural process and associate it with possible forensic application by utilising the bacterial community shifts to establish a PMI. This may prove beneficial if other methods are inconclusive, for example; due to a lack of entomological evidence, however, limitation exist that future studies should address.

6.1 Limitations

There have been several external variables that are difficult to control in necrobiome studies using human models, which may account for data discrepancies, such as medical history, diet and pharmaceutical usage prior to death. Due to these difficulties, several studies had substituted human cadavers for animal models, including pigs and mice, due to their physiological similarities. In addition, sample size can present a problem in studies using human models as there are difficulties with acquisition and legal requirements pertaining to human studies that may result in non-representative data.

An additional factor that can influence the accuracy of PMI estimations include interpersonal variation, which have been found to provide under or over-estimates as a result of inconsistent data between individual human cadavers. Overall, a larger sample size could remedy the majority of issues present in human-related studies.

6.2 Future studies

The majority of studies have examined the effects of decomposition on the surrounding environment, however, the environmental impact on the cadaver could be focused on in future research to ascertain the effects on decomposition rates and insect activity. Additionally, stable bacterial communities could be examined over time to refine and increase the accuracy of PMI models and discover further trends. Future research could also focus on decomposition studies in different ecological biomes and habitats. Further research has the potential for providing an insight into other scientific areas, including the discovery of unidentified organisms capable of being utilised in other fields, and thereby adding to the existing knowledge of the human microbiome

Reviewing the studies of bacterial succession during decomposition indicated that the formulated hypothesis may be plausible, whereby, a noticeable shift in bacterial community structure through time can be observed with the ability to establish a PMI, however additional studies are required to address this hypothesis further with forensic application in mind. This review focused on comparing the current contributions of literature to further understand the ecological mosaic of human decomposition and with which future research can be based on.

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