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Review

"From womb to tomb; we're bound to others": microbiome in forensic science

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Abstract

Microbiome is a new field of interest in clinical medicine with high potential in forensic medicine. It could be used in several applications, such as post-mortem interval (PMI) estimation, personal identification, differential diagnosis of cause of death and toxicology.

Regarding PMI, during the decomposition of a corpse, the passage of time involves changing in microbial population both outside and inside the corpse but also in surrounding soil (cadaver decomposition island). These variations could be hypothetically used as PMI indicators (microbial clock), even thanks to the development of machine learning approach.

Another potential use of skin and saliva microbiome is personal identification thanks to its inter-individual variability and tendency to remain unvarying over time. It may also be helpful to link a person to a specific object that has been touched (microbial fingerprint).

Furthermore, we could infer some information about health state of human subjects, comparing post-mortem and ante-mortem microbiome, but this field of research is quite new and needs further studies.

Moreover, we have to consider the influence of microbiome metabolism in post-mortem toxicological evaluation; microbes could alter substances concentrations – for example of ethanol, gamma-hydroxybutyric acid (GHB) and nitrobenzodiazepines – due to enzymatic degradation and individual microbial metabolism.

Finally, integration of microbiome and human being's transcriptomic analysis may be helpful to depict their complex interactions even after death.

Keywords

Microbiome, forensic science, PMI, personal identification, health state, toxicology.

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Introduction

A new field of interest in microbiology with high potential in many disciplines, including forensic medicine, is the microbiome. This term refers to the set of microorganisms, their genomes, and environmental interactions that they establish in an ecosystem [1]. Paying specific attention to forensic science, the term "forensic microbiology" indicates the great potential use of microbiome in several different applications, for example, post-mortem interval (PMI) estimation, differential diagnosis of the cause of death, personal identification, sexual assault, etc. From this perspective, Javan and Finley [2] introduced the term "thanatomicrobiome", characterized by the succession of microbial communities after death in a body.

The purpose of this review is to highlight some of the possible uses of the microbiome in all the fields mentioned above.

Post-mortem interval (PMI) estimation

In the phases of decomposition of a corpse, an active role of bacterial communities in their temporal succession well marked in time (so-called "microbial clock") [3] has been described, which forms the basis for establishing the time of death, especially in peculiar conditions such as late stages of decomposition. These shifts in microbial populations are due to changes in environmental factors, such as the presence/absence of oxygen, temperature, humidity and pH and a variation in homeostasis within bacterial communities themselves [4, 5]. There are biological interactions which don't produce disequilibrium in bacterial communities, such as neutralism, mutualism, and commensalism; while others – such as amensalism, competition, predator-prey – may throw the equilibrium off balance. To examine in detail these interactions, "neutralism" takes place when bacterial communities don't influence each other; "mutualism" refers to a collaborative relationship and reciprocal benefits; "commensalism" is fulfilled when a microbial population takes advantage of another and doesn't influence it in any way; "amensalism" occurs when a bacterial community is damaged by the presence of another, which on the contrary is not perturbed; "competition" indicates an inverse relationship among two or more species for nutritive factors and ecological niche; lastly the interaction "predator-prey" is established when a microbial population uses all the resources of another to obtain supplies for its survival [6].

Pechal et al. [7] showed how the passage of time involves changing in microbial population, not only in the number of bacteria that tends to increase, but also in their taxonomic diversity which – in contrast – shows a decrease, due to a greater homogenization among taxa. The study identified in all sample sites (eyes, nose, ears, mouth, and rectum) a PMI threshold of about 48 hours for the appearance of this modification, except for the rectum. The best prediction with the lowest error rate (± 2-5 days) is reached 20-25 days post mortem.

A similar study confirms bimodal distribution of microbiome abundance and biodiversity as time goes on, describing that the shift occurs about halfway through the bloat stage (4-7 days after death), time slightly successive compared to Pechal et al. However, the limits of this study are the scarcity of sampling (n = 3) and the single sampling site (caecum) [8]. The presence of temporal variation has also been demonstrated in animal models (rats), underlining that mouth and rectum microbiome is differentiated in early PMIs and tends to homogenize in late ones [9], reflecting the natural difference that exists in a living body.

It is essential to highlight that post-mortem microbial variations exist not just in the body and on it, but also in surrounding soil ("cadaver decomposition island") [10]. Particularly, the occurrence of a breakpoint has been shown 6-7 days after death, corresponding to the beginning of the colliquative stage and releasing of ammonia in soil. This breakpoint is characterized by an increase of *Firmicutes* and a reduction of *Proteobacteria* and soil native phila [10]. Microbial succession in soil ends about 420 days after death, proving the prolonged influence of a decomposing body in the surrounding environment [11].

If so, these data could be hypothetically used as PMI indicators through a sampling of different body location and surrounding soil.

PMI estimation related to microbial changing represents an up-and-coming field for the development of a machine learning approach. A meaningful example has been shown by Belk et al. [12] with the application of Random Forest regression models based on Metcalf et al. studies, one on human bodies [3] and the other on the animal model [13]. Specifically, Belk's study has shown that the most robust prediction model uses soil and skin as sampling location, rRNA16s (indicative of bacteria) as marker and class or phila as taxonomic rank, with an estimated error of 2-3 days in the first 2 weeks after death.

However, numerous hot points have to be considered in study design. One critical point is the environmental influence (both microclimate and fauna) on the timing of passage through various stages of decay and consequently on bacterial communities succession. Discordant opinions exist in literature about the influence of microbial colonization and insects. Some studies demonstrated the irrelevance of presence/absence of insects in microbial succession [9]; while others proved a significant influence [3]. This controversy could be explained by the complexity of interactions between microbiome and insects: there are collaborative mechanisms – in which bacteria release gases (mercaptan) capable of attracting vertebrate and invertebrate animals on the body [14] – and competitive mechanisms, in which microbes produce toxins against eukaryotic organisms and insects produce antimicrobial peptides. In light of the preceding, it is essential to pay attention to environmental variables (moisture, temperature, oxygen, etc.) and entomology for the creation of the regression models. The more parameters are included, the more PMI estimation is accurate [15].

Variability of results depending on site sampling – especially in the early stages of decomposition – and the sampling method (such as swab vs. scrapes) [16] are other limits. Furthermore, data from animal model can't be transferred as a whole to humans, and few studies using human corpses have been developed because of the shortage of donors.

The creation of standardized analytical tools and differentiated protocols depending on clinical situations [17] with a well-established error rate would allow a sufficiently accurate PMI prediction and their application in the forensic field, with a sufficient level of scientific reliability in the court, following the U.S.A. Daubert criteria [14, 18].

Personal identification

Several studies have shown potentiality to identify an individual within a population based on skin microbiome because the microbial communities have a sufficient level of inter-individual variability [19]. Furthermore, the "personal" microbiome tends to remain unvarying over time. The microbiota profile of the skin, therefore, can be helpful to link a person to a specific object that has been touched and the extraction of bacterial DNA could be useful to demonstrate or exclude the presence of an individual in a crime scene when human DNA (detected from blood, semen, saliva, etc.) is not sufficient to determine a genetic profile. This is possible because bacterial DNA is more stable than the human one, due to its circular shape, its preferred location in a nucleoid and the presence of bacterial cell wall. All these data lead to the creation of the so-called "microbial fingerprint" [20]. However, it is remarkable that the skin microbiome of cohabitants can be similar. Ross et al. proved the ability to match cohabiting couples that had lived together for a period ranging from 4 months to 14 years through a machine learning approach in more than 86% of cases. The skin microbiome was evaluated in 17 skin sites with the best matching achieved in foot samples [21].

Not just skin microbiome could be used for personal identification, but also saliva microbiome, that has been studied in term of intra- and inter-individual variability. Costello et al. [22] demonstrated that oral microbiome – compared to skin and gut ones – is less variable within an individual as time goes on in term of bacterial membership. Regarding inter-individual variability, Stahringer et al. [23] showed that salivary microbiome of homozygous twins shows the same degree of diversity identifiable in a couple of random subjects. This feature confirms the wide influence of environmental factors – such as diet, smoke, alcohol use, oral hygiene – on microbiome composition. Antibiotic use has an important role in the changing of the salivary microbiome. After antibiotic therapy, microbiome composition tends to vary, returning to the previous state in about 3 weeks [24]. As a whole, this information could be usefully employed in sexual assault cases, when aggressor's DNA is often mixed with DNA of the victim, barely detectable and insufficient to give a complete genetic profile. In such a situation, the perpetrator of a sexual crime could be recognizable through the study of salivary microbiome detected

on saliva stain possibly recovered by the victim's body [25].

Up to now this application – even if intriguing – presents several limits, especially in study design, like the small sample size. Also, it is recommended to choose the correct analytical tool, such as the metagenome method rather than culturing on common media because the latter recovers only 0.1-1% of total bacteria.

Wherever personal identification is not the principal scope of analysis, microbiome – especially of the skin – can be used for intelligence purposes, providing clues that are potentially relevant for identifying lifestyle, personal habits and even the ethnic group of an individual [18].

Microbiome and health state

An intriguing role provided by the study of the microbiome has been recently proposed by Pechal et al. [7]. The study tries to correlate the post-mortem microbiome and ante-mortem state of health of individuals. They demonstrated that the taxonomic diversity of the first 2 days after death – which still reflects the ante-mortem microbiome populations – and the biological functions investigated by KEGG ortholog (KO) pathways make possible to hypothesize that the microbial composition in the first 48 hours after death is a potential indicator of ante-mortem health conditions. An increase in biodiversity is a sign of a good general state of health; indeed, a high level of microbiome variability – similar to healthy people's one – suggests violent or sudden death. A reduction of variability is related instead to the presence of heart pathologies and other chronic diseases, as a putative role of death. Genus *Rothia* showed an increase in heart disease cases, compatible with a causal role in the development of infective endocarditis. Researchers have also demonstrated that the best sampling site for this purpose is the mouth.

This field of research is quite new and totally at the beginning: preliminary results have to be taken carefully, waiting for additional demonstrations.

Other studies confirm the usefulness of post-mortem microbiome analysis in the determination of the cause of death. Several authors showed that blood and cerebrospinal fluid (CSF) cultures which present a single isolate of known pathogen occurred in association with genuine infection; on the contrary, polymicrobial growth detected in the cultures is most likely due to contamination during collection. This applies to all ages, including perinatal period. Especially in sudden unexpected death in infancy (SUDI), post-mortem microbial analysis should be one of the main steps for determination of the cause of death, which always has to be carefully read into clinical and autoptic data [26].

Microbiome and post-mortem toxicology

One of the most relevant challenges in toxicology is the detection of drugs eventually involved in the cause of death, especially when microbes alter their concentrations, due to enzymatic degradation, microbial metabolism, and instability in the cell matrix. A case in point is ethanol and gamma-hydroxybutyric acid (GHB) production by bacterial species.

Several studies investigated the quantification of ethanol produced after death. Boumba et al. [27] proposed mathematical models – mainly based on *in vitro* studies – to infer the amount of ethanol produced by *E. coli* cultures basing on its correlation with other endogenous alcohols. The lapse of time evaluated was from day 1 to day 30. The model based on the quantification of 1-propanol and 1-butanol – the main alcohols produced following ethanol – had a sufficient level of correlation with ethanol. Researchers proved the validity of mathematical models retrospectively in 60 real post-mortem cases, finding that the model constructed by 1-propanol and 1-butanol was successful in 42% of cases. Despite the clear limitations about the study – an exact quantification of alcohol produced by bacteria is still impossible – it is interesting the prospect to find a tool to detect the neo-formation of alcohols.

Regarding GHB, an endogenous post-mortem production has been demonstrated, even if the source is still unclear. One of the possible mechanisms of GHB production after death – in addition to oxidative metabolism – could be bacterial metabolism, specifically of *Pseudomonas spp.* [28]. Researchers attempted to identify a cut-off level to differentiate endogenous production from the external origin of GHB; in this case, choosing the better source for sampling is crucial. Blood indeed is highly affected by post-mortem modifications, and it is therefore not an extremely valid matrix. On the contrary, vitreous humor is a stable matrix, due to its anatomic position, providing a suitable sampling [29]. Further studies are needed both to clarify the production of GHB from *Pseudomonas spp.* and to detect an exact cut-off level for all the suitable matrixes.

Not only ethanol and GHB but also drugs – such as benzodiazepines, antidepressants, antipsychotics – and poisons, such as cyanide, may be affected by bacterial species after death [30].

For example, in the former category, nitrobenzodiazepines seem to be almost completely degraded in their respective 7-amino-metabolites after death in 8 hours at 22°C. Some precautions are needed to avoid this event: preservation of samples with 1% (w/v) sodium fluoride and storage at -20°C [31].

Regarding poisons, the importance of the microbiome in the concentrations of the substances is the activity of *P. aeruginosa* that may lead to the post-mortem formation of cyanide, using glycine as a substrate [32].

As described above, it is necessary to take into account the influence of microbiome metabolism in every toxicology evaluation, to get a valid interpretation of final data, considering cadaver decomposition and specimen contamination.

Conclusions and future perspectives

Establishing a correlation between gene expression in human cells after death and microbial colonization of the body might be of forensic interest. Noble and Pozhitkov carried out a preliminary study on animals, specifically zebrafish and mouse, proving that 1% of the gene transcripts significantly increased from 48 to 96 hours after death [33]. Later Ferreira et al. [34] have shown that after death some human genes are up-regulated while others are down-regulated, proving the existence of an active post-mortem regulation which reaches a peak between 7 and 14 hours after death and settles between 14 and 24 hours. The purpose of up- and down-regulated genes seems to be the reaction to external stress as hypoxia provoked by the absence of blood flow. This kind of stress produces variations of energetic pathways, such as down-regulation of tricarboxylic acid cycle and up-regulation of glycolysis and response to hypoxia-mediated by hypoxia-inducible factor (HIF). Depletion of energy production pathways, concomitant progressive down-regulation of immune response and deterioration of anatomical cellular and tissue integrity, may enhance bacterial growth and promote colonization responsible for putrefaction. This "sequence" could explain the reason why post-mortem and ante-mortem microbiome is about the same in the first hours after death.

Integration of microbiome and human being's transcriptomic analysis may be helpful to depict their complex interactions even after death.

An important point to consider in the development of future researches requesting sampling *in vivo* is the formulation of an appropriate consent to human subjects involved in microbiome studies, able to highlight ethical implications of donating samples potentially able to provide several personal information, such as sexual behavior, health state, etc.

It is clear that, currently, the microbiome is one of the hot topics in the research community, not only in forensic science, due to its wide range of applications in many fields of research. Every study, therefore, has to be designed with solid scientific reasoning, and the scientific method must be applied, to avoid waste of money and technical resources.

Declaration of interest

The Authors declare that there is no conflict of interest.

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