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Successive bacterial colonisation of pork and its implications for forensic investigations

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ABSTRACT

Aims: Bacteria are considered one of the major driving forces of the mammalian decomposition process and have only recently been recognised as forensic tools. At this point, little is known about their potential use as 'post-mortem clocks'. This study aimed to establish the *proof of concept* for using bacterial identification as post-mortem interval (PMI) indicators, using a multi-omics approach.
Methods and results: Pieces of pork were placed in the University's outdoor facility and surface swabs were taken at regular intervals up to 60 days. Terminal restriction fragment length polymorphism (T-RFLP) of the 16S rDNA was used to identify bacterial taxa. It succeeded in detecting two out of three key contributors involved in decomposition and represents the first study to reveal *Vibrionaceae* as abundant on decomposing pork. However, a high fraction of present bacterial taxa could not be identified by T-RFLP. Proteomic analyses were also performed at selected time points, and they partially succeeded in the identification of precise strains, subspecies and species of bacteria that colonized the body after different PMIs.
Conclusion: T-RFLP is incapable of reliably and fully identifying bacterial taxa, whereas proteomics could help in the identification of specific strains of bacteria. Nevertheless, microbial identification by next generation sequencing might be used as PMI clock in future investigations and in conjunction with information provided by forensic entomologists.
Significance and impact of the study: To the best of our knowledge, this work represents the first attempt to find a cheaper and easily accessible, culture-independent alternative to high-throughput techniques to establish a 'microbial clock', in combination with proteomic strategies to address this issue.

1. Introduction

Estimating the post-mortem interval (PMI) is a fundamental step within crime scene investigations. Common methods available to forensic pathologists, such as the assessment of the extent of post-mortem changes including *algor,rigor* and *livor mortis*, do not produce sufficiently accurate data, especially if the individual has been dead for longer than 48 h [1]. Forensic entomologists, drawing their inferences from insect colonisation patterns of cadavers, may also provide information about the time since death. However, errors can range from days to months [2], since the growth rate of maggots can be influenced by various factors such as changes in temperature, geographic location, season, etc. [3].

Mammalian decomposition is a complex interplay between a large number of biotic and abiotic factors. The speed of cadaver decomposition mainly depends on climatic and environmental conditions, the ante-mortem intrinsic properties of an individual, insect and scavenger activity, and on microbial activity [4,5]. Although much research has been done on insect colonisation of cadavers and their potential to aid in forensic investigations [6–9], only little attention has been paid to bacteria, which are considered as one of the major driving forces of the human decomposition process [10].

Microbes facilitate the transformation of an individual into its molecular components, providing a pool of energy and nutrients to organisms living in the surrounding environment [11]. If a

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predictable pattern of bacterial succession associated with cadaver decomposition can be recognised, it could present a valuable and readily accessible tool to complement the analysis of insect colonisation patterns. This study aims to establish whether bacterial composition on decomposing tissue changes over time, and if it does so, in a predictable fashion that allows PMI estimation.

Numerous research groups have attempted to reveal the complexity of the microbiome associated with the decomposition process [12–20]. However, the majority of these studies examined the bacterial flora by means of conventional, culture-dependent methods. Since only a minute fraction of bacterial species is cultivatable [21], species richness was likely to be underestimated. Recent studies conducted by Hyde et al. [4,10], Damann et al. [22], Metcalf et al. [2], Pechal et al. [23] and Can et al. [24] based their findings on next generation sequencing (NGS), a culture-independent approach. Hyde et al. [4,10] examined the human microbiome associated with the bloat and non-bloat stage of the decomposition process, documenting a major shift from aerobic to anaerobic bacteria. However, the study further revealed a great variability between bodies, making it difficult to determine a universal succession pattern. Damann et al. [22] investigated the bacterial microbiome associated with bone. They revealed that bones from partially skeletonised bodies maintained a presence of bacteria associated with the human gut, whereas dry skeletal remains increasingly equalled the community profile present in soil [22]. Can et al. [24] investigated the human microbiome associated with internal organs and blood and whether the absence or presence of particular species could potentially be used as PMI predictor. Their main findings indicated that facultative anaerobic bacteria, such as *Lactobacillus* sp. were predominant in all samples obtained at low PMIs, while obligate anaerobic bacteria, such as *Clostridium* sp. were found in large numbers at longer PMIs [24]. Research groups who conducted their studies on animal models have been more successful in establishing a precursor model for PMI estimations [2,25]. They reported a significant and predictable change in relative abundances and taxon richness of bacterial communities through time. While Pechal et al. [25] provided a model able to estimate a PMI within a few hours after death, Metcalf et al. [2] were able to develop a PMI estimation model applicable up to 34 days.

Nevertheless, these research groups relied on high-throughput techniques, either 454 Pyrosequencing or Illumina [2,25]. However, at the moment, NGS technology is expensive and the equipment is rarely available to forensic institutes. For this reason, this study aimed to research the ability of using terminal restriction fragment length polymorphism (T-RFLP) to reliably identify bacterial taxa from decomposing pork. In order to establish a reliable PMI estimation model, which also applies to humans, it is essential to understand the relative importance and interplay of ecological factors influencing the decomposition process. It is necessary to strip the empirical data back to the baseline, and control as many variables as possible. T-RFLP relies on the same laboratory equipment as human DNA-profiling, which implies it could be easily implemented into forensic laboratories. This is an attempt to find a cheaper and more easily accessible, culture-independent alternative to NGS.

We further explored the possibility to apply other 'cheap' non-DNA based methods to this study, to overcome the possible limitations that the T-RLFP methodology could lead to. In particular, it has already been shown that mass spectrometry performed on proteins or on peptides is a powerful tool to typify microorganisms [26], and some of its features like its sensitivity, its rapidity and its broad spectrum in characterising organisms make it an intriguing option to address the aims of this work in an easily accessible way. Based on the paradigm proposed by Anhalt and Fenselau [27], each individual organism is characterised by a characteristic mass spectral "fingerprint", that can be used to perform taxonomic distinctions based on these assumptions. In particular, bottom-up proteomic approaches have already been used to rapidly identify bacteria, both using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry [28] and both using liquid chromatography–tandem mass spectrometry (LC–MS/MS) approaches [29]. For these reasons, we also applied both these strategies to this study, to evaluate limits and potentials of these techniques and to compare them with the results obtained using the most common DNA-based approach.

2. Materials and methods

Three replicate pieces of pork loin were each cut in two (0.25–0.385 kg) for DNA and protein analysis and kept in sterile plastic boxes, which allowed airflow, but no insect colonisation, were placed in the outdoor decomposition facility at the University of Huddersfield. Sterile wet cotton swabs (FLOQSwab™ Copan Diagnostics Inc., USA), were used to sample bacterial DNA and protein at regular intervals ranging from hours to 60 days after exposure, never sampling an area of pork twice. Samples were stored at −20 °C until processed further.

Weather data was derived from the weather station located in the same facility at the University of Huddersfield. The local temperature inside the sampling boxes was measured hourly by RC-5 USB Temperature Data Loggers (Elitech, China). Temperature data was converted into accumulated degree-days (ADD) according to the method of Micozzi [30] using a base temperature of 4 °C and a maximum of 47 °C.

2.1. DNA extraction and amplification

DNA extractions were carried out based on the Bacterial DNA Isolation CTAB Protocol by William et al. [31]. Once DNA was dissolved in 50 μl nuclease-free water (Qiagen, UK), DNA yield was determined by the NanoDrop 2000 UV–vis Spectrophotometer at 260/280 nm (Thermo Scientific, USA).

Amplification of the bacterial 16S rDNA marker was performed using two different primer sets in order to consider possible preferential amplification. The eubacterial 5′-FAM labelled forward primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) were singly combined with the 1392R (5′-ACG GGC GGT GTG TAC A-3′) reverse primer (Eurofins, Germany). PCR reactions were set up following the manufacturer's protocol for GoTaq DNA polymerase (Promega, Germany) with approximately 70 ng template DNA and 0.6 μmol l^{-1} forward and reverse primer. Samples underwent the following temperature programme: 2 min at 95 °C, 28 cycles of 45 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C. Final extension was carried out at 72 °C for 7 min.

2.2. T-RFLP

Generated PCR products were digested by *MspI*, *RsaI* and *HhaI* (Promega, Germany). The selection of *MspI* and *HhaI* was based on previous studies conducted by Engebretson and Moyer [32] and Quaak and Kuiper [33]. These restriction enzymes were found to resolve bacterial populations best by generating fragments showing great length polymorphism. However, the use of only two restriction enzymes possibly results in the generation of peaks of multiple bacterial species. In order to limit the number of possible species that could be assigned to a single peak, PCR products were additionally digested by *RsaI*. All reactions were carried out as single digests. 20 μl reaction mixtures contained 8 μl PCR product, 10 μl restriction enzyme, 2 μl 10X restriction