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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

enzyme buffer (Promega, Germany), 0.2 μl BSA (Promega, Germany) and nuclease-free water (Qiagen, UK). Samples were incubated for 16 h at 37 °C followed by 20 min heat inactivation at 60 °C.

2.3. Capillary electrophoresis

Capillary electrophoresis (CE) was performed on ABI 3130 Genetic Analyzer with POP-4™ polymer and a 36 cm capillary. 1 μl digested PCR product plus 10 μl HiDi-Formamide and 0.25 μl GeneScan™ LIZ600® dye size standard (all Applied Biosystems, USA) were denatured at 95 °C for 3 min and immediately placed on ice. Injection was performed at 3 kV for 30 s and fragments were separated at 15 kV at 60 °C for 2250 s.

2.4. DNA data analysis

The GeneMapperID v3.2 (Applied Biosystems, USA) and the open access tool box *Tools for T-RFLP data analysis* [34] were used to process generated CE profiles. Only peaks between 50 and 900 bp showing higher intensities than 100 relative fluorescence units (RFU) were included in the analysis; only peaks that were present in at least two of the three replicates and those showing a relative abundance of at least 1% were considered within the analysis. Fragments were binned in 1 bp bins so that peaks with a size difference of less than 1 bp were considered as the same peak.

Bacterial species or families were assigned from consensus profiles by the openly accessible *Microbial Community Analysis* (*MiCa*) *T-RFLP (PAT+)* tool [35]. The Ribosomal Database Project database was used as reference data and only good quality database entries of longer than 1200 bp were considered, resulting in approximately 700,000 entries in total.

2.5. Protein extraction

1 ml of 6 M guanidine hydrochloride (GuHCl) and 100 mM Tris (Sigma–Aldrich, UK) at pH 7.4 at 4 °C were added to each FLOQSwab™ (Copan Diagnostics Inc., USA); after 1 h, 500 μl were separated from the rest and frozen for future analyses, whereas the remaining 500 μl were left at 4 °C for 18 h and further processed. Samples were then ultrafiltered using 10 kDa molecular weight cut-off (MWCO) ultrafiltration units (Vivaspin, UK), then the buffer was exchanged into 50 mM ammonium bicarbonate (ABC; Fluka, UK) and filtered samples were collected in 250 μl of 50 mM ABC. Samples were then reduced using 10.5 μl of 5 mM dithiothreitol (DTT; Sigma–Aldrich, UK) for 40 min at room temperature, alkylated with 42 μl of 15 mM iodoacetamide (IAM; Sigma–Aldrich, UK) in the dark for 45 min and then quenched with the same volume of 5 mM DTT. The digestion step was performed adding 2 μl of sequencing grade trypsin (Promega, UK) (prepared following manufacturer's protocols) and incubating the samples at 37 °C overnight. Digestion was stopped by adding 1% trifluoroacetic acid (TFA; Sigma–Aldrich, UK) resulting in a final TFA concentration of 0.1%. Samples were desalted, purified and concentrated using C18 reversed-phase Zip-Tips C18 (Agilent Technologies, UK) following manufacturer's protocols. Peptides were finally eluted in 100 μl of 50% acetonitrile (ACN)/0.1% TFA, and left to dry under a fume hood at room temperature until completely dry. Samples were then re-suspended in 0.1% formic acid (FA; Fluka, UK)/5% ACN (Fisher Scientific, UK) and subsequently analyzed with a Bruker Ultraflex II MALDI mass spectrometer (MS) or by LC–MS/MS using an UltiMate 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA, USA) coupled with an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer.

2.6. MALDI-TOF mass spectrometry

Purified samples were spotted onto a Bruker target plate (1 μl of sample mixed with 1 μl of a-cyano-4-hydroxycinnamicacid matrix solution) and left dry. Then, mass spectra were collected using the Bruker Ultraflex II MALDI mass spectrometer shooting 5000 laser shots per spot, with a range of m/z 700–3700. The calibration was performed using five calibrant peptides of 0.9–3.7 kDA range (des-Arg^1-bradykinin, angiotensin I, Glu^1-fibrinopeptide, ACTH 1–17 clip, ACTH 18–39 clip and ACTH 7–38 clip). Mass peptide fingerprints were then analysed using mMass software (v5.5.0).

2.7. LC-Orbitrap Elite mass spectrometry

Peptides were separated on an ethylene bridged hybrid (BEH) C18 analytical column (75 mm × 250 μm i.d., 1.7 μM; Waters) using a gradient from 92% buffer A (0.1% FA in water) and 8% buffer B (0.1% FA in ACN) to 33% B in 44 min at a flow rate of 300 nl min^{-1}. Peptides were then automatically selected for fragmentation by data-dependent analysis; six MS/MS scans (Velos ion trap, product ion scans, rapid scan rate, Centroid data; scan event: 500 count minimum signal threshold, top 6) were acquired per cycle, dynamic exclusion was employed, and one repeat scan (i.e., two MS/MS scans total) was acquired in a 30 s repeat duration with that precursor being excluded for the subsequent 30 s (activation: collision-induced dissociation (CID), 2+ default charge state, 2 m/z isolation width, 35 eV normalized collision energy, 0.25 Activation Q, 10.0 ms activation time).

2.8. Proteomic data analysis

Spectra obtained via LC–MS/MS were searched as .mgf files against the Swiss-Prot database (540,052 entries) using the Mascot search engine (version 2.4.1; Matrix Science, London, UK). Each search included the fixed carbamidomethyl modification of cysteine (+57.02 Da) and the variable modifications for deamidation (+0.98 Da) of asparagine/glutamine and oxidation (+15.99 Da) of lysine, proline, and methionine residues, to account for PTMs and diagenetic alterations; the oxidation of lysine and proline is equivalent to hydroxylation. Enzyme specificity was set to trypsin with up to 2 missed cleavages allowed; mass tolerances were set at 5 ppm for the precursor ions and 0.5 Da for the fragment ions, with all spectra considered as having either 2+ or 3+ precursors.

Progenesis software (version 2.0; Nonlinear Dynamics, Newcastle, UK) was used to get the relative quantitation of the proteins in each sample, to enable comparisons between different experimental conditions to highlight differences and to finally express changes in data. To remove unreliable data, proteins with only one or no unique peptides were excluded from the study as well as proteins with a confidence score smaller than 41 (the score identified by Mascot as reflecting extensive homology).

To ensure that the in-depth proteomic analysis was able to identify subspecies- or strain-specific bacteria, we performed specific searches in the UniProt database for all the identified bacterial proteins, expanding the search to also different subspecies or strains. Then, the amino acidic sequences of the selected protein from the various subspecies and/or strains were aligned with BioEdit (version 7.2.6, Ibis biosciences, Carlsbad, Ca), and each one of the peptides identified with the LC-MS/MS analysis was searched against each sequence, to verify if a specific peptide was detectable only in a specific sequence, giving a subsequent subspecies- or strain-specific identification. Phylogenetic analyses were carried out on selected sequences online with phylogeny.fr, which uses a MUSCLE alignment and analysis by PhyML (with aLRT) and the tree created in TreeDyn.

3. Results

The study was conducted over a period of 60 days from April to June 2015. While the ambient temperature remained relatively stable throughout the entire sampling period (average 11.8 °C ± 2.7 °C SD), the temperature inside the boxes was subject to great fluctuation (Fig. 1). In particular, maximum temperatures fluctuated with the presence of sunlight.

Although approximately 900°-days accumulated after two months of exposure, samples appeared to dry out rather than decompose, perhaps due to the exclusion of insects and scavengers or lack of intestinal bacteria. All pieces of pork had significantly reduced weights at the end of the study, probably due to the loss of water (data not shown).

3.1. Bacterial DNA

In order to identify abundant bacterial taxa and to assess the change in bacterial community composition, surface swabs were taken at regular intervals. After the DNA had been extracted, 16S rRNA genes were amplified; the forward primer was fluorescently labelled. The marker was subsequently digested by the restriction endonucleases *MspI*, *RsaI* and *HhaI*. Each digest was carried out singly and every restriction enzyme generated unique patterns.

As a measure of bacterial community composition, the relative abundance of bacterial phyla and families was determined. The relative abundance was finally determined by summing up the height of all present peaks and relating it to the height of the consensus peak referring to one bacterial taxa. The fingerprints generated from three replicate pieces of pork were combined to create consensus profiles. Over a period of 60 days, 18 samples were taken to perform DNA extraction. All the samples taken for the DNA extraction yielded sufficient bacterial DNA levels for the study, apart from samples taken at 20 and 25 days, that were excluded from the analysis. The most abundant bacterial taxa throughout the entire sampling period were Proteobacteria, especially Gammaproteobacteria. Betaproteobacteria were mainly present in samples taken during the first few days. In contrast, Alphaproteobacteria were mainly abundant from 15 days onwards, which corresponds to previous findings [2]. Similarly, Cyanobacteria appeared within later stages of the study, which is in accordance with previous studies [4]. Firmicutes were present within the first two days as well as within the last three samples taken at 40, 50 and 60 days. However, up to 50% of the abundant bacterial phyla could not be identified by means of T-RFLP (Fig. 2). This trend is continued in the relative abundance of bacterial families (Fig. 2); however, it should be noted that by 30 days, up to 75% of the bacterial families present were unknown.

In general, a decreasing trend in bacterial diversity was evident during the first sampling time points, where 11 distinct bacterial families present at time point zero (not considering uncultured and unknown taxa) dropped to three within 24 h after exposure. *Vibrionaceae* (Gammaproteobacteria) were present in large amounts at almost all time points whereas their abundance seemed to increase up to five days followed by a decreasing trend (Fig. 3).

Enterobacteriaceae (Gammaproteobacteria) were highly abundant from 24 h onwards and their presence remained relatively stable within the period of 24 h–60 days. *Comamonadaceae* (Betaproteobacteria) and *Clostridiaceae* (Firmicutes) were only present in samples taken up to 24 h, which is partially contradictory to previous findings. Pechal et al. [23] identified a large

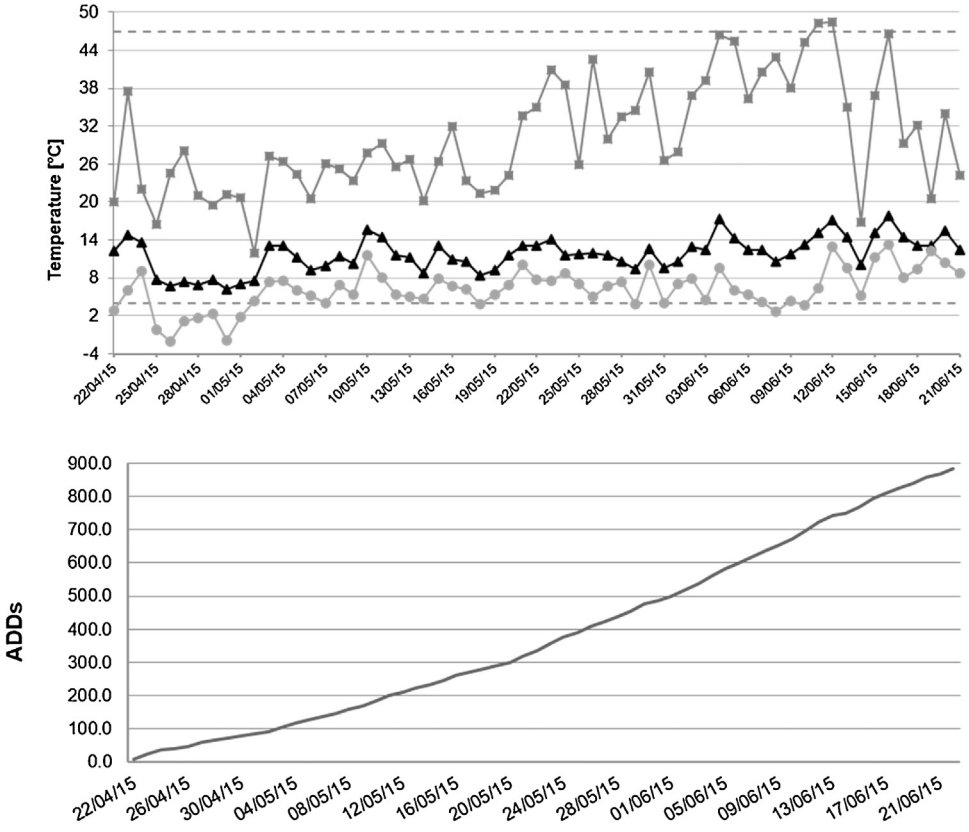

Fig. 1. Temperature data generated by the University's weather station and by temperature data loggers placed into sampling boxes. Weather data was recorded throughout the entire sampling period of 60 days. (▲) Average ambient temperature, (●) minimum and (■) maximum temperatures inside sampling boxes. Dashed lines at 4 °C and 47 °C represent thresholds on which ADD calculations were based. Linear accumulation of heat units up to 60 days after exposure. ADDs were calculated on a base of 4 °C as follows: (maximum temperature boxes + minimum temperature boxes)/2 − base temperature.

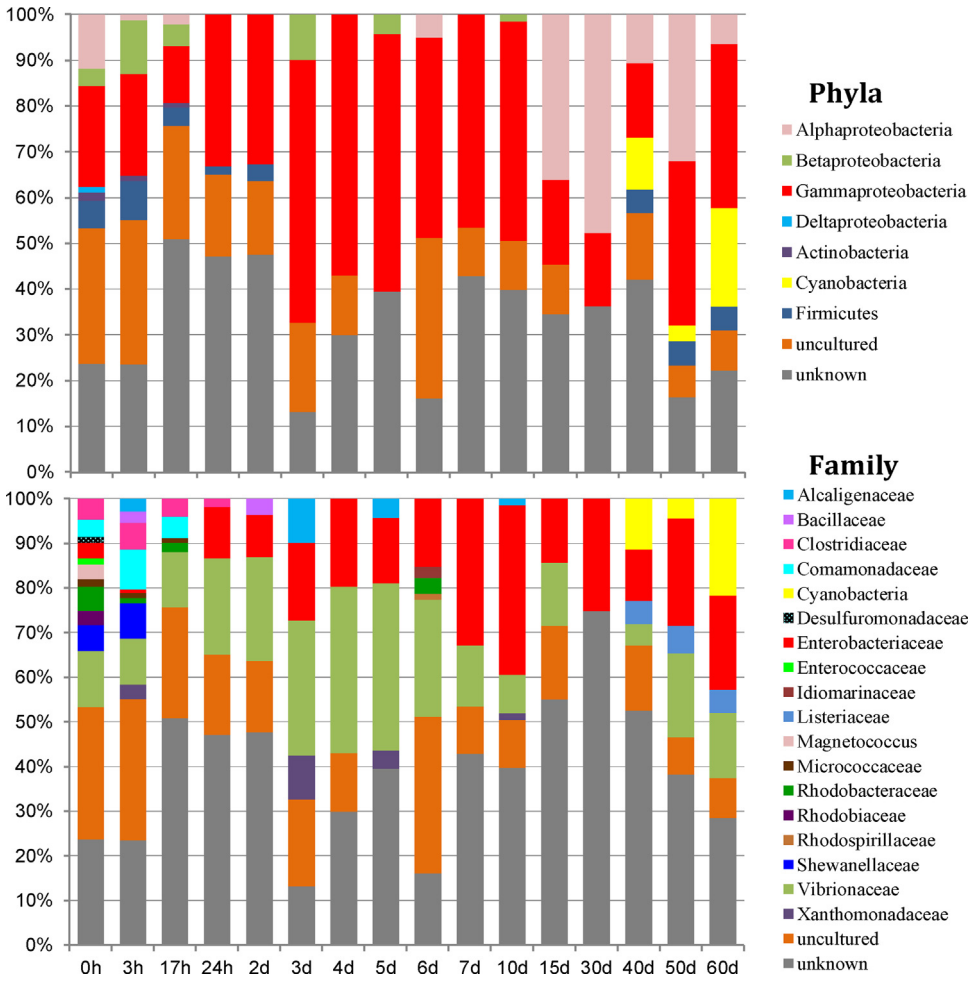

Fig. 2. Relative abundance of bacterial phyla and families throughout the entire sampling period. Samples have been taken from 0 h up to 60 days at regular intervals, which increased as time proceeded. The T-RFLP data generated by both primer sets and by all three restriction enzymes was analysed, averaged and summarised in percentage abundance of bacterial phyla. The T-RFLP data generated by both primer sets and by all restriction enzymes was analysed, averaged and summarised in percentage abundance of bacterial families.

fraction of *Clostridiaceae* in samples taken from swine carcasses five days after death, and only minor levels in samples, which were obtained one day after death. As in Fig. 2, Cyanobacteria only appeared during the last three sampling time points. These likely included families such as *Microcystaceae*, *Leptolynbyaceae* and *Cyanothece*. However, an unambiguous identification could not be achieved. As Cyanobacteria, *Listeriaceae* could also be exclusively identified in samples of 40, 50 and 60 days. It should be noted that 50 and 51 days after exposure, temperatures exceeded the upper threshold of 47 °C (Fig. 1); while Cyanobacteria increased in abundance between 50 and 60 days, the relative abundance of all other bacterial families remained unchanged. This was likely due to the fact that Cyanobacteria are able to grow at extreme temperatures [36]. In contrast, the majority of bacterial species

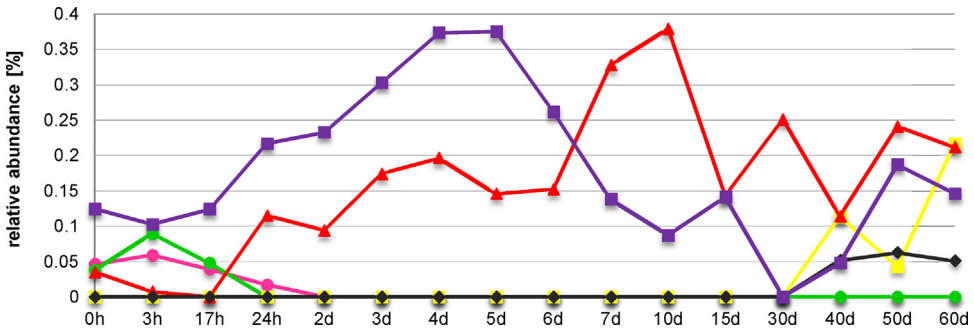

Fig. 3. Relative abundance of selected bacterial families throughout a sampling period of 60 days, obtained by T-RFLP. Some bacterial families were abundant at various PMIs throughout the entire sampling period, including (purple □) *Vibrionaceae* and (red ▲) *Enterobacteriaceae*. Others could only be detected at certain stages of the study. While (green ●) *Comamonadaceae* and (pink ●) *Clostridiaceae* were present in the beginning, (yellow ■) Cyanobacteria and (black ◆) *Listeriaceae* could be identified in samples taken at later stages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

belonging to the *Enterobacteriaceae*, *Vibrionaceae* and *Listeriaceae* families are killed at temperatures above 47 °C [37,38], and therefore, were not able to further increase in abundance.

3.2. Proteomics

In order to test the efficacy of the proteomic approach in the identification of unknown bacterial species, the pork loins were swabbed at six different time points representative of the overall duration of the experiment, as previously described, and proteins were extracted and following analysed with both MALDI-TOF and LC–MS/MS.

The MALDI-TOF analyses did not give the expected outcome, since the spectra were swamped by peaks originated by the pig proteins. In fact, the most abundant peptides visible in the spectra were attributed to pig muscle proteins, like myosin-regulatory light chain, different types of myosin light chains, myomesin-2, titin, fibronectin and tropomyosin (data not shown).

To overcome this limitation, an in-depth sequencing using LC–MS/MS was performed on the same 6 samples to go past the dominant pig proteins and to better resolve also the bacteria species collected on the pork loin. These analyses comprehensively resulted in the identification of 321 different proteins; among these, 43 where attributed to non-mammalian species (13.4% of the totality). It can be assumed that the mammalian proteins recovered were originated from the pig muscles; given the lack of a complete pig proteome database, some proteins were identified as originated from pig and some other from different mammalian species (e.g. bovid, human etc.). However, they have been excluded from the study given the interest in focusing on the microbial proteins instead of on the overall set of recovered proteins.

It was possible to identify that different groups of non-mammalian proteins were more abundant after specific time points, with 17 proteins out of 43 which levels were higher at time 0 and then decreased, and with other smaller groups of proteins with a different behaviour during the time course (e.g. higher levels after 24 h than during the other time points, etc.) (Fig. 4). It should also be noticed that the average relative abundance of the proteins recovered at higher levels at time 0 was at least one order of magnitude bigger than all the other groups showed in Fig. 4, also in agreement with what observed with the DNA analyses.

The analyses done to verify the presence of specific peptides able to give a subspecies- or strain-specific identification gave some positive results, allowing the recognition of specific strains of microorganisms in two cases and the recognition of a specific subspecies in another one. In particular, the peptide VPPVVPKNNDNLK identified uniquely the presence of the *Saccharomyces cerevisiae* strain JAY291 out of five different strains, the two peptides LLLGEGR and RNSPCVIIQGSDEKNDPDGAQIIVK allowed the identification of the *Rhodopseudomonas palustris* strain BisB18 out of three different strains, and the two SPEMIALK and NVSGDLK identified uniquely the *Buchnera aphidicola* subspecies *Baizongia pistaciae* strain Bp out of five different subspecies (Fig. 5). Relative abundances per each time point of the three proteins that allowed the above-mentioned identifications are also reported in Fig. 5, together with the levels of the most abundant protein recovered in the samples, the myosin regulatory light chain 2 (MLRS_PIG). It should be noticed that the scale used to report the data is a base 10 log, to facilitate the visualisation of the data due to the high abundance of the pig protein in comparison with the other proteins in the graph.

4. Discussion

The data indicated that, although up to 75% of the abundant bacterial families could not be identified by means of T-RFLP, it was possible to unambiguously assign a set of detected peaks to specific bacterial phyla and families. Some data was generated considering only two restriction enzymes since using all three would have resulted in no identification. The method itself is reproducible, as samples, which were analysed twice, resulted in identical profiles generated by the Genetic Analyzer. The high fraction of unidentified bacterial taxa might also have been due to the paucity of the reference database used, which was restricted to 700,000 entries.

The high proportion of unidentifiable bacterial taxa present complicated the assessment of the change in bacterial community composition over time. However, results indicated that the bacterial family richness decreased over time. While some taxa, such as *Vibrionaceae* and *Enterobacteriaceae* were present throughout the entire course of the study, at almost all time points, others could exclusively be identified in early or late stages such as *Comamonadaceae* and *Clostridiaceae* or Cyanobacteria and *Listeriaceae*, respectively. The abundance of Cyanobacteria at later PMIs is in accordance with previous studies [4]. Surprisingly, none of these studies identified any species belonging to the *Vibrionaceae* family, which is one of the two most abundant families found here.

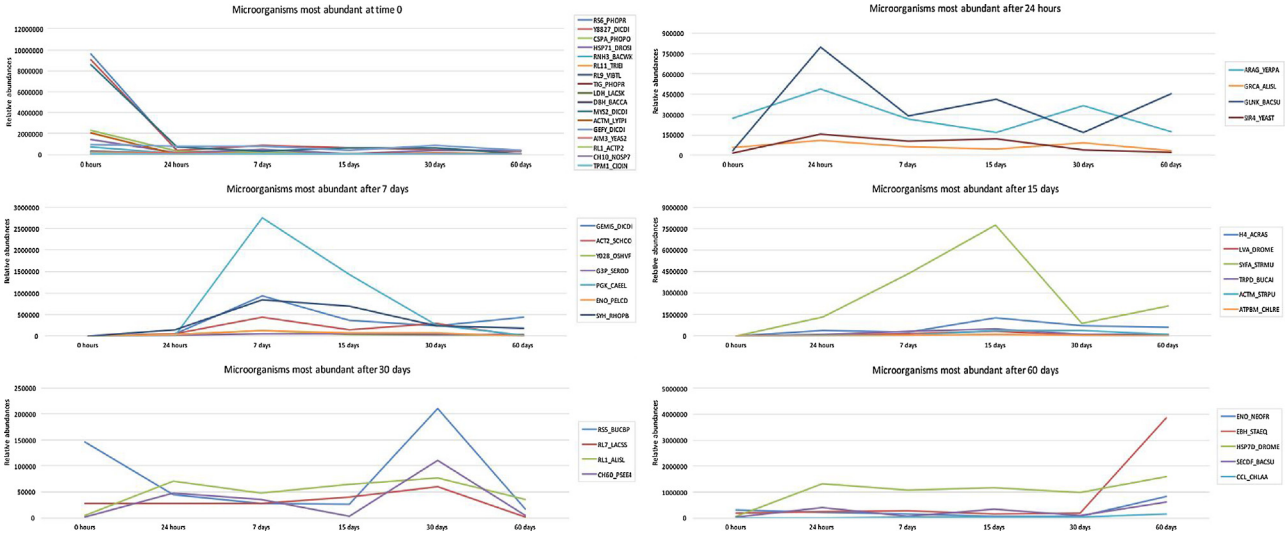

Fig. 4. Relative abundances of selected bacterial proteins throughout a sampling period of 60 days. Each box shows bacterial proteins with a similar trend over the time. Each colour in each single box represents a different protein, and for each of them, the entry name on Uniprot is reported.

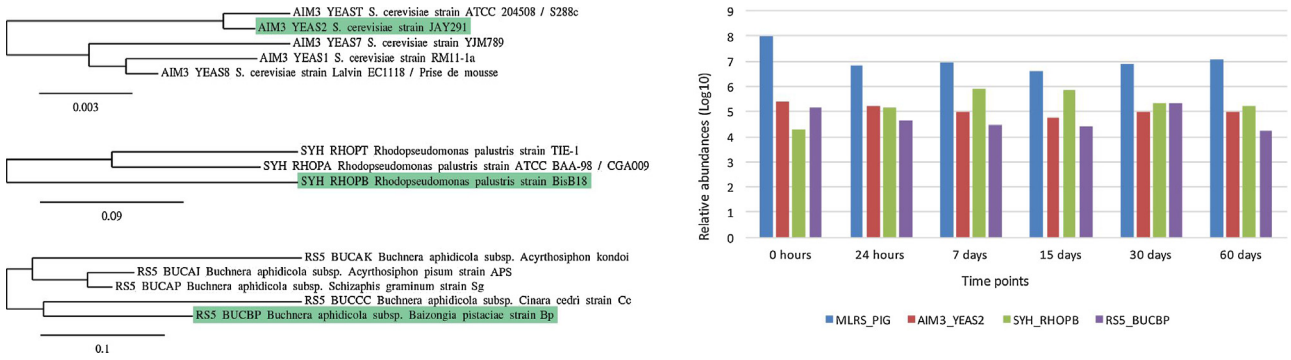

Fig. 5. Phylogenetic (left) and relative abundance analysis (right) of the three proteins identified to the sub-species or strain level in all samples; the latter is also in comparison to the myosin regulatory light chain-2 protein as the most abundant pig protein recovered in each sample.

Vibrionacaeae is a family of gram-negative, rod-shaped, facultatively anaerobic motile bacteria found in fresh or sea water and, occasionally, in fish and humans. *Campylobacter* and *Helicobacter*, responsible for gastroenteritis in humans, are members of this family.

Previous studies demonstrated the presence of *Clostridiaceae*, *Moraxellaceae* and *Xanthomonadaceae* at some point during decomposition [2,4,25], indicating that these might be key contributors to the decomposition process. The abundance of *Clostridiaceae* and *Xanthomonadaceae* could be confirmed within this study, too. However, no species belonging to the harmless, parasitic *Moraxellaceae* family could be identified within this study.

Although the proteomic analyses were only performed on a limited number of samples, it was shown that the MALDI-TOF results were not appropriate to identify bacteria-specific peaks due to the high peptide signals originating from the muscles proteins. However, it was possible to identify a variety of microorganisms, sometimes also at a very low taxonomic level (subspecies or strain specificity) using the in-depth protein sequencing. The main limitation in this case was due to the complexity and uncertain limitations of database entries within Swiss-Prot, an issue overcome by the standard MALDI-based approach (e.g. [39,40]) of plating/growing bacterial colonies which works in such high-throughput that it has developed vast database entries, but without attempting to understand the origins of the peptides present within their fingerprints.

This study tried to reduce all external factors such as insect colonisation, microbial composition of soil, local and seasonal variations, which could alter the bacterial population present on cadaveric material as it decomposes. Insects secrete substances, which have an antimicrobial effect or initiate immune responses destroying existing bacterial communities [41]. Carter et al. [42] suggested that the microbial activity is highly dependent on seasonal variations. Ambient temperatures and solar irradiance have a significant impact on the decomposition process and therefore, on bacterial community compositions [43]. The study also tried to eliminate potential variations in bacterial populations caused by factors intrinsic to the deceased individual, by using uniform porcine loin meat pieces. Muscle tissue was used to study the decomposition microbiome, as a preliminary *proof of concept* study. This research should serve as a springboard for further research aimed at the development of an alternative method for PMI estimations. Bacterial communities inhabiting the gastrointestinal tract are highly dependent on diet, local variations and lifestyle of the individual [44]. Future research will inevitably have to consider this complex factor. Additionally, Stokes et al. [45] investigated how far animal models of muscle tissue loss are able to accurately model human decomposition dynamics, but with pork, beef and lamb, they found no single analogue to be a precise approximation modelling human decomposition dynamics. However, Pittner et al. [46,47] have recently revealed promising results by looking at the protein changes using Western Blotting and casein zymography. To conclude, the human decomposition process presents a complex interplay between all these variables, which inevitably need to be considered in order to establish an alternative model to determine the time since death. This will be the most challenging task in future research.

5. Conclusions

This study aimed to establish the *proof of concept* that it is possible to identify which bacterial taxa are present on cadaveric material as it decomposes using T-RFLP and MALDI-TOF analyses. It succeeded in identifying two out of three previously defined key contributors involved in the decomposition process [4] and represents the first study to reveal *Vibrionaceae* as present on rotting pork at various PMIs. However, currently, this model is not yet predictive and precise enough to be used for PMI estimations. Considering the high fraction of bacteria, which could not be identified by means of T-RFLP, NGS might lead to the generation of more complete and reliable results, and thus, be better used in future death microbiome research. If it succeeds in exploring the human decomposition microbiome and identifying bacterial taxa specific to certain PMIs, the 'microbial clock' may be used as an additional measure to estimate the time since death. Nevertheless, it should be seen as complementary to conventional means rather than a stand-alone model. Combining this model with different expertise such as pathology and entomology and will allow more reliable PMI estimates in future.

Conflict of interest

No conflict of interest declared.

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