Forensic Science International: Genetics 39 (2019) 73–85

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen

Research paper

Metabarcoding to investigate changes in soil microbial communities within forensic burial contexts

Noemi Procopio[a,1], Stefano Ghignone[b], Anna Williams[c], Andrew Chamberlain[d], Antonietta Mello[b,*], Michael Buckley[a,*]

[a] Manchester Institute of Biotechnology, The University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK
[b] Istituto per la Protezione Sostenibile delle Piante, C.N.R., Viale P. A. Mattioli 25, 10125, Torino, Italy
[c] School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK
[d] School of Earth and Environmental Sciences, The University of Manchester, Stopford Building, 99 Oxford Road, Manchester, M13 9PG, UK

ARTICLE INFO

Keywords:
Post-mortem interval
Decomposition
Metabarcoding
16S rRNA
Taphonomy

ABSTRACT

The estimation of the time elapsed since death (post-mortem interval, or PMI) is one of the key themes that forensic scientists have to address frequently. However, the estimation of PMI still suffers from poor accuracy and biases especially when decomposition stages are prolonged, so further improvements in methods for PMI estimation are desirable. Soil microbial communities associated with decomposing bodies have been shown to be good candidates for the estimation of the PMI of exposed bodies. Nevertheless, further research is required to better understand the bacterial succession associated with decomposition of buried carcasses in order to test its reliability and applicability for the estimation of PMI and to better understand the dynamics involved with decomposition within this particular scenario. Therefore we explored the succession of soil microbial communities associated with four decomposing pig carcasses (from one to six months PMI) using a metabarcoding approach. The sequencing of the bacterial 16S rRNA variable region 4 (V4) revealed trends linking particular microbial taxa with specific PMIs, and notably an increase in Proteobacteria, Firmicutes and Bacteroidetes at specific PMIs as well as a decrease in Acidobacteria. Our results, in accordance with previous studies conducted on exposed bodies of different mammalian species (including humans), also showed a general reduction of the taxonomic richness from two months PMI onwards, as well as an incomplete re-establishment of the starting soil microbial conditions after six months PMI. We also found specific mammal-derived taxa, such as *Bacteroides* spp., being still present in the soil after six months PMI. As such, this study serves as a baseline for additional research to allow the characterisation of biomarkers associated with specific PMIs. Due to the similarity between the results presented here and those reported in other types of decomposition studies we believe that the metabarcoding approach has considerable potential in the estimation of the PMI, particularly to clarify cases involving heavily skeletonised bodies or for the investigation of clandestine graves in which the carcass has been moved from its original place of deposition.

1. Introduction

Soil consists of a complicated mixture of mineral materials and decaying organic remains of bacteria, fungi, nematodes, mammals, insects and plants [1], as well as living organisms such as microorganisms, invertebrates and small mammals. Due to its ubiquity, soil has been frequently used in forensic investigations to assess the involvement or not of a suspect based on the important and exclusionary information contained in this complex matrix. Classically, forensic analyses on soil were conducted on its chemical and physical characteristics [2], including particle size [3], soil colour [4], organic content [5], elemental composition [5] and mineral abundance [6]. More recently, metagenomic analyses based on the identification of soil microbial communities have been increasingly used to characterise and discriminate different soils of forensic interest [7–9].

Despite the well-known use of molecular approaches to identify different soil types, the application of these techniques to investigate which microbial communities are associated with specific post-mortem

* Corresponding authors.
E-mail addresses: antonietta.mello@ipsp.cnr.it (A. Mello), m.buckley@manchester.ac.uk (M. Buckley).
[1] Current address: Centre for Forensic Science, Faculty of Health & Life Sciences, Northumbria University Newcastle, Ellison Building, NE1 8ST Newcastle Upon Tyne, UK.

https://doi.org/10.1016/j.fsigen.2018.12.002
Received 4 June 2018; Received in revised form 17 November 2018; Accepted 10 December 2018
Available online 12 December 2018
1872-4973/

intervals (PMIs) of carcasses has been much less explored. Bacteria and fungi share the important role of recycling the organic matter within the soil with their carbon and nitrogen cycles [10,11], and the presence of a decomposing body clearly alters the original balances of species and abundances within the microbial communities. The decomposition rate and pattern of a body on the soil surface or within a grave is a very complex phenomenon that strongly depends on some biotic and abiotic factors [12], and on some intrinsic characteristics of the body (e.g., body size [13–15] or trauma [16,17]). In general, it starts with the autolysis process, in which the accumulation of carbon dioxide in tissues causes the release of enzymes leading to cell death [18,19]. Subsequently, the anaerobic intestinal flora starts to break down various macromolecules like proteins, lipids and carbohydrates, inducing the typical changes in colour and bloating of the carcass [20]. During this stage, fluids present in the body start to purge into the soil due to the internal pressure created by expanding gases, and can induce an important shift in the microbial community present within the gravesoil due to the changes in their original environments and due to the presence of newly released carbon, nitrogen and phosphorus compounds [21]. Changes in the availability of these nutrients within soil may induce the growth of some microbial species and inhibit the presence of other species, directly or indirectly [19]. For this reason, the presence and abundance of microbial species existing in the burial environment may vary sequentially during specific decomposition stages, and their identification may be useful when linked to known PMIs of the carcass. The important factors are likely to be (a) the provision of a new food source for existing soil biota, (b) the introduction of a source of new microbiota from the cadaver itself, (c) the attraction of invertebrates to the cadaver which will contribute with further microbiota and (d) the cellular DNA and proteins derived from the decomposition of the cadaver and from the colonising invertebrates.

Several attempts have already been made to evaluate the changes in the microbial communities associated with decomposing bodies with different DNA-based strategies. For example, Parkinson et al. [19] performed two separate studies in which they investigated changes in the microbial community associated with three human cadavers left exposed on the ground surface for different time periods using the profiling method of T-RFLP analysis of 16S rRNA bacterial genes and of the ITS region on fungal rRNA. Their results showed that a succession of different bacteria and fungi were associated with different decomposition stages, but they were not able to identify individual biomarker species associated with specific decomposition stages due to the limits of the technique used for the study [19].

Alternative methodologies involving the analyses of bacterial or fungal communities through the use of denaturing gradient gel electrophoresis (DGGE) analyses continued to be used even recently, at least when these studies were applied to forensic sciences. Bergmann and Thompson [22] used an alternative RNA-based approach to evaluate changes in the soil bacterial communities at different depths, after a pig leg was buried in sandy soil. They showed, in accordance with results previously reported in the literature, that the richness of the microbial taxa increased from 3 to 28 days and then decreased from 28 to 77 days after the burial, although the limits of the technique used did not provide any helpful indication about taxa associated with specific PMIs [22]. Olakanye et al. [23] used the same experimental design as Bergmann and Thompson [22] but their study lasted 14 weeks and was focused on DNA instead of RNA. At selected time points, soil at different depths was collected by coring and the 16S rRNA gene from soil bacteria was analysed through PCR-DGGE. They showed an increase in the species diversity within soil collected from the burial site compared with a control soil between 10 and 71 days; however, the intrinsic limits of the technique chosen for the study did not allow the identification of specific biomarkers useful for the estimation of the time elapsed from the beginning of the burial period [23]. Chimutsa et al. [24] focused their study exclusively on fungal community variations to look for cadaver decomposition indicators, using the same experimental design previously adopted by Bergmann and Thompson [22] and Olakanye et al. [23]. In contrast with what they found for bacteria, they did not observe any statistically significant variation in eukaryotic communities comparing the soil collected close to the carcass and the control using the 18S rRNA gene analysis through PCR-DGGE, opening questions about the reliability of this approach when only fungi are analysed [24].

To overcome these limits, new studies using high-throughput DNA sequencing approaches have investigated the microbial community associated with decomposition. Hyde et al. [25] collected bacterial swab samples from different internal and external sample sites on two decomposing human cadavers placed outdoors and analysed the samples using 454 pyrosequencing. They noticed changes in the bacterial community associated with specific PMIs, finding bacteria related to the presence of flies during the bloating and purging phase and bacteria related to soil after the skeletonization phase. Pechal et al. [12] investigated the “necrobiome” succession on the skin of exposed carcasses of freshly killed swine during five days from their initial exposure. Their bacterial 16S rRNA showed that specific families of bacteria were more abundant after specific PMIs, in addition to the observation of an overall decrease of the taxon richness over the course of the decomposition period. Furthermore, they proposed a model to estimate the time of death, though noting that the model required independent validation [12]. Cobaugh et al. [26] explored the bacterial succession beneath four exposed human cadavers during successive decomposition stages, and found that particular phyla were present at specific decay phases up to 198 days post-mortem. Metcalf et al. [27] showed in a study of exposed decomposing mouse bodies that the combination of both prokaryote and eukaryotes datasets collected on skin and on gravesoil for 48 days resulted in an accurate estimation of the PMI. In particular, they found specific taxa that may be diagnostic for potential forensic applications, and more data may improve this knowledge to allow a future application of this method (the “microbial clock” method) to the criminal justice system [27]. In a subsequent paper, Metcalf et al. [28] advanced upon their previous study by studying the bacterial and fungal community assemblies associated with both exposed mouse and human decomposition, also comparing different types of soils. They identified specific networks of decomposers at selected PMIs, and they also highlighted the fact that the three different soil habitats (grass prairie, subalpine forest, and desert environments) did not change the outcome of the results, opening the way to new opportunities in the application of these studies to forensic contexts [28].

Although a limited number of studies have been conducted on this topic, it has been shown that the analysis of the microbial succession within gravesoils may become a useful technique to characterise the decomposition stage and/or the PMI of carcasses even if the body has been removed from its original place of burial. However, significant limitations are still present within this field, in particular for subsurface cadaveric decomposition, as the majority of these studies have been done on above-ground decomposition [11]. For this reason there is the need for new research on this topic to investigate changes in the microbial populations when whole carcasses are buried in the ground instead of being exposed on the surface to the action of invertebrate and vertebrate scavengers. Finley et al. [29] investigated the microbial succession in gravesoil associated with exposed or buried human corpses, but the buried carcasses were only four (in comparison with fourteen bodies left on the surface to decompose) and the time intervals between soil sampling were relatively long (8, 96, 214 and 303 days), leaving gaps in the characterisation of microbial successions during the so-called “active decomposition stage” (the putrefaction/liquefaction stage occurring in general during the first three months post-mortem).

Here we explored the microbial succession of the bacterial community within gravesoil after the burial of whole pig cadavers approximately 40 cm in depth in outdoor settings for six months, using metabarcoding DNA sequencing approaches targeting a specific gene region (16S rRNA) to overcome some of the limits highlighted above.

Together, these data allowed us to investigate the changes that take place within a "closed environment", such as the burial grave, when a decomposing body disturbs the normal environmental microbial community. We monitored changes associated with different PMIs (and decomposition stages), with the ultimate aim of identifying specific microbial taxa that may be associated with specific stages of decomposition.

2. Materials and methods

2.1. Experimental design

Experimental forensic burials of four juvenile pigs (*Sus scrofa*) were undertaken at the HuddersFIELD outdoor taphonomy facility (University of Huddersfield, U.K.) between May and November 2016. Soil physicochemical and biological characteristics on a control sample taken at approximately 40 cm in depth prior to the burials (performed by the CNR - Institute for the Study of the Ecosystems, Pisa, Italy) resulted in electrical conductivity (0.53 dS m^{-1}), total N (0.448%), total organic C (6.29%), total P (1.95 g/Kg), available P (62.93 mg/kg), NO3- (69.84 mgNO3-/kg), NH3 (1.93 mgNH3/kg), dehydrogenase activity (0.96 μgINTF/gss*h), β-glucosidase activity (34 μmolMUB/g*h), phosphatases activity (31 μmolMUB/g*h), arylsulfatase activity (314 μmolMUB/g*h), protease activity (15 μmolAMC/g*h). Details of the experiment and of the carcasses were reported in Procopio et al. [30] and are briefly summarised here. The experiment was conducted following the guidelines of the U.K. Department for Environment, Food and Rural Affairs (DEFRA). The pigs, which were of similar age (3–5 weeks old) and weight (4.5–11 kg), died naturally from unknown causes, were frozen immediately after death at −20 °C and were kept frozen until the beginning of the experiment. Due to the nature of the experiment and for ethical and legal reasons, it was difficult to collect animals similar in breeds, size, sex, cause of death, nourishment given *etc.*, but we did make an effort to limit the variability between the animals that is known to exist between carcasses of different sizes [32] as much as possible. Despite being aware that the intestinal biota may vary in relation with different types of diet [31], we could not have control on this specific variable (since the nature of the work excluded the slaughtering of pigs with controlled diets). The experiment time was set to zero when the cadavers had been de-frosted and placed within soil graves. The graves were excavated to approximately 40 cm depth; pigs were placed into metal cages to protect them from the activity of scavengers and then buried at the base of each grave (Supporting Fig. S1). The graves were then completely covered with the grave infill to maintain as much as possible the pre-existing soil conditions, and the various pigs were left to naturally decompose for various PMIs (respectively, one, two, four and six months). One grave was excavated per time point according to the experimental design (P1 after one, P2 after two, P3 after four and P4 after six months PMI). Biological samples were collected simultaneously with soil samples for proteomic analyses that were performed in a separate study (see [30] for bone proteomic analyses performed on these carcasses). Soil samples in contact with the superior part of the carcass were collected from three separate locations, aiming for recovery from within a few centimetres of the remains above the fore-limb area, the abdominal area and the hind-limb area of the body (see Fig. 1 for further details of the sampling locations). These soil samples were then pooled together (i.e., one bag per grave) and three technical replicates per grave, collected from the pooled bag, were used for the metabarcoding analyses. It should be noted that during each of the excavation and sampling events the weather conditions were always favourable, and for this reason soil sampling was not affected by rainfall or by particular leaching effects. Despite the favourable weather conditions during the excavations, it should be noted that rainfall increased from the beginning of the experiment (May 2016) to the end of it (November 2016), as showed in Procopio et al. [30], and this overall affected the humidity condition of the soil

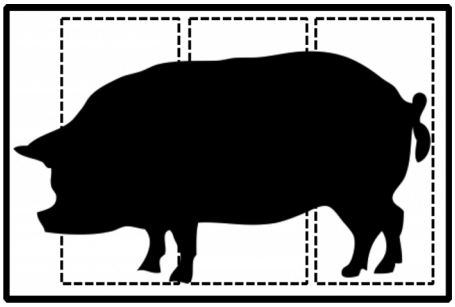

Fig. 1. Illustration of the pig buried in the grave (outer rectangle); dashed rectangles represent the areas sampled in contact with the pig surface (namely, the "fore- limb area" (A), the "abdominal area" (B) and the "hind-limb area" (C)).

recovered at the last time point. Control soil samples (taken at similar soil depths but without a carcass being present) were collected at locations two and six meters away from the graves. The control samples were obtained concurrently with the last sampling performed in November (six months PMI), to provide a representative record of the microbial activity within the field used for the experiment without microbial contamination from the graves. We are confident that these control samples were taken at a sufficient distance from the graves to exclude any significant influence of carcass-derived bacteria, according to the findings of Singh et al. [33]. We assumed that the observable differences within the soil microbial community associated with the close proximity of a decomposing body would be larger and more significant than those caused by variations in local environmental conditions (linked with seasonality, temperature and rainfall), as has been shown by Metcalf et al. [28] for different soil habitats, and for this reason our control samples were collected at different distances from the graves but at a single time point. Soil samples were then placed within individual plastic bags and were frozen immediately at −20 °C until the analyses. Overall, we performed metabarcoding analyses on 18 samples (three samples per grave plus six controls). Soil pHs from each sample were obtained by mixing the soil sample with sterile deionised water in a ratio of 1:2.5 (w/v) and measuring the pH of the supernatant water with a pH probe (Mettler Toledo, Cole-Parmer, U.K.) (Table 1). Average daily rainfalls and temperatures have been taken from the local weather station and were reported in Procopio et al. [30] as well as soil temperatures that were measured using data loggers (Tinytag Plus 2, Gemini Data Loggers, U.K.) [30].

2.2. Chemicals and materials

Primers and agarose gel were purchased from Sigma-Aldrich (U.K.), Platinum Hot Start PCR Master Mix 2x was purchased from Thermo Fisher Scientific (U.K.), Wizard® SV Gel and PCR Clean-UpSystem was purchased from Promega (U.K.), FastDNA® SPIN Kit for Soil was purchased from MP Biomedicals (Europe). All PCRs were performed using a T3000 thermal cycler (Biometra GmbH, Göttingen, Germany).

2.3. DNA extraction, amplification and sequencing

Genomic DNA was extracted from 500 mg of soil sample using the FastDNA® SPIN Kit for Soil, following the guidelines provided by the manufacturer and Eubacterial communities were specifically targeted amplifying the 16S rRNA locus [34]. Forward 515FB (GTGYCAGCMGCCGCGGTAA) and reverse 806RB (GGACTACNVGGGTWTCTAAT) primers ([35,36] respectively) were used in combination with overhangs following the guide for 16S metagenomic sequencing library preparation, suggested for the Illumina MiSeq system. Degeneracies were allowed in the primers to decrease eventual biases and to increase the ability to detect organisms characterised by slight modifications in

Table 1
Samples used in the experiment with information about sampling location, depth, PMI of the corpses and pH.

Samples	C1	C2	P1	P2	P3	P4
Collection site	2 m from graves, 40 cm depth	6 m from graves, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth
PMI of the carcasses	–	–	1 month	2 months	4 months	6 months
Body size (kg)	–	–	4.5	6.1	10.8	11.0
Approximate age (weeks)	–	–	3-4	3-4	4-5	4-5
pH	7.3	7.2	7.7	8.1	7.6	7.2

their binding site's sequences. PCR negative controls were run in each analysis to perform a quality check of the amplifications and in all cases these controls gave negative results. PCR reaction mixtures were set up as follows: 12.5 μL master mix (Platinum Hot Start PCR Master Mix 2x), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM) and 0.5–1.5 μL template DNA in a final reaction volume of 25 μL. The thermocycler conditions were set up as follows: denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 40 s and extension at 68 °C for 30 s; final extension at 68 °C for 10 min and maintenance of the samples at 4 °C. The PCR products obtained were checked on 1.5% (w/v) agarose gels, purified (Wizard® SV Gel and PCR Clean-UpSystem), quantified with Qubit (Qubit Fluorometric Quantitation, Thermo Fisher Scientific, U.K.) and sent to IGA Technology Services (Udine, Italy) for paired-end sequencing using the Illumina MiSeq technology (2 × 250 bp).

2.4. DNA data analysis

Paired-end reads from each library were merged using Pear v.0.9.2 [37], with the quality score threshold for trimming the low-quality part of a read set at 28, the minimum length of reads after the trimming process set at 200 bp and the minimum possible length of the assembled sequences set at 200 bp. Unix bash commands were used to select assembled sequences beginning with the recognisable forward primer (no mismatch allowed), to trim initial and terminal 19 and 20 bases (corresponding to forward and reverse primers respectively) and to assign a sample specific progressive count to each fragment. Assembled sequences from each library were clustered into Operational Taxonomic Units (OTUs) using a closed reference-based clustering strategy with VSEARCH v2.3.4 (https://github.com/torognes/vsearch [38]) at 97% similarity; only clusters encompassing at least 2 sequences were initially retained. The GreenGenes v.13_8 database [39] was used as a reference for OTU picking and taxonomy assignment. Since not all the entries in the GreenGenes database are defined for the seven conventional taxonomic levels, the missing levels have been filled using the epithet "Unknown" associated with the last available taxonomic level. VSEARCH output was converted into biom format (Biological Observation Matrix format) with biom v2.1.5 program [40] for subsequent statistical analyses.

2.5. Statistical analyses

Numerical ecology statistical analyses were performed within the computing environment R [41] (https://www.R-project.org/).

Soil controls were collected from two different locations to better describe the variability of the basal microbiome. DNA from three technical replicates was extracted from each of the two control locations (replicate #1, #2 and #3). During the data analysis, the two control samples collected from the distinct locations were unified arbitrarily into a single control sample, summing up the reads of each of the three replicates (the first two replicates of #1 from both soils were unified together to obtain a single "replicate #1" and so for the other two replicates). In other words, we did [replicate 1 + replicate 1]$_{C1,C2}$ + [replicate 2 + replicate 2]$_{C1,C2}$ + [replicate 3 + replicate 3]$_{C1,C2}$ to obtain our three replicates for control samples. From the four time point samples, overall we extracted 12 DNA samples (three DNA samples extracted from the three technical replicates per grave) that were further analysed together with the arbitrarily unified three control samples.

A number of filtering steps were applied to gather the final OTU dataset: OTUs labelled as "mitochondria" (recognised as contaminant in GreenGenes, but not removed from the database) were removed; OTUs with fewer than 50 reads were filtered out as well as samples with fewer than 20 reads and OTUs showing a Coefficient of Variation greater than 3.0 were also removed.

In order to standardize sampling efforts and to allow for comparisons of samples with non-uniform coverage, the OTU table has been normalized by subsampling at even sequencing depth from each sample (38,684 sequences per sample) by means of the *rarefy_even_depth* function in the R package PHYLOSEQ v.1.22.3 [42]. The rarefaction effects on an OTU table are commonly rendered by means of the rarefaction curves, in order to graphically estimate species richness. Raw species richness counts can only be compared when the species richness has reached a clear asymptote. All the species present in a sample are well described when the curve ascribed to each sample reach its plateau. Rarefaction curves have been rendered by means of the function *ggrare*, provided by the richness.R script from the phyloseq extension package by Mahendra Mariadassou (https://github.com/mahendra-mariadassou/phyloseq-extended).

All the taxon abundances have been calculated and graphically plotted with the aid of the R package PHYLOSEQ [42].

Biodiversity analyses were carried out by comparing the richness and evenness of microbial communities of the PMIs. Richness is merely a measure of the number of species in each sample, without taking into account the relative abundance of each species. Evenness, in contrast, takes into account both the number of species, and the relative abundance of each species. Within-sample (Alpha) diversity was assessed by six estimators: "observed number of species", "Chao1″, "abundance-based coverage estimators (ACE)", "Shannon", "Simpson", "Fisher". The Alpha diversity indices were calculated and plotted by means of the functions *estimate_richness* and *plot_richness* implemented in the R package PHYLOSEQ [42].

In order to test whether communities were statistically different from each other (Beta diversity), a multivariate homogeneity of group dispersions among the different Control and PMI groups was assessed before proceeding further with the analyses by means of the *betadisper* and *permutest* (with 999 permutations) functions in the R package VEGAN v.2.5.2 [43]. The differences in the composition of bacterial communities in PMIs were rendered by means of a nonmetric multidimensional scaling ordination (NMDS), to visualise Bray-Curtis distances, using the functions *vegdist* and *metaMDS* in the R package VEGAN [43].

The permutational multivariate analysis of variance (PERMANOVA, 999 permutations), as implemented in the *adonis* function of the VEGAN package of R [43], were applied to access whether communities were statistically different from each other.

Indicator species analysis (a classification-based method to measure associations between species and groups of sites [44]) was carried out using the *multipatt* function in the INDICSPECIES v.1.7.6 R package, with

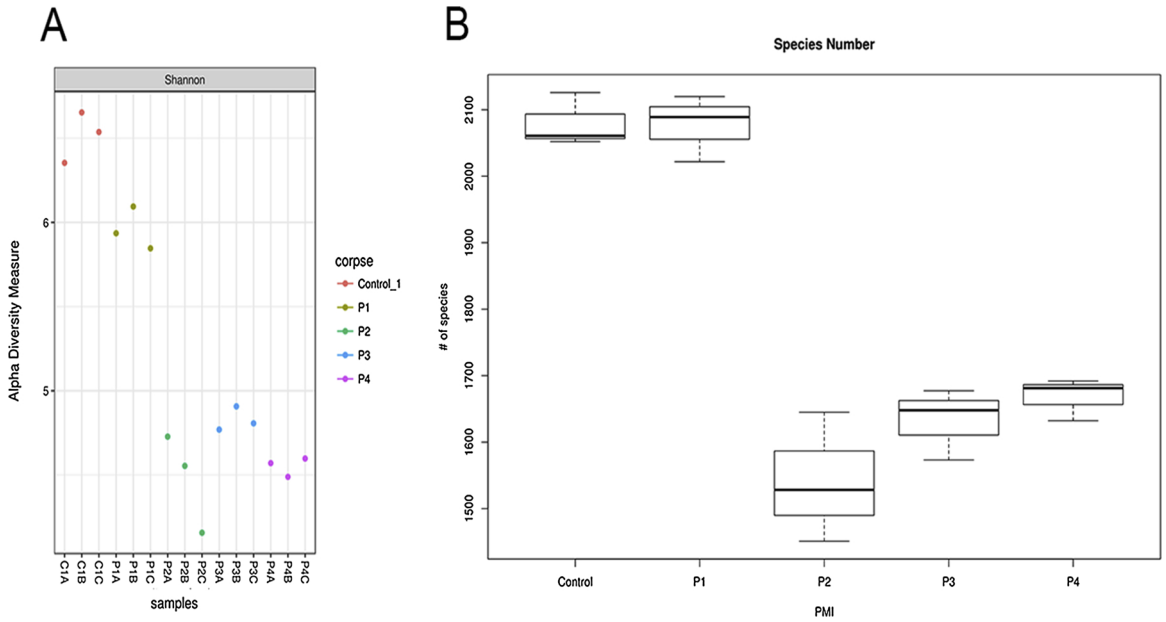

Fig. 2. A) Shannon alpha diversity measure of the samples collected after one (P1), two (P2), four (P3) and six (P4) months post-mortem and B) box plot of the microbial richness of soil samples showing the raw number of species per sample, with standard deviations represented with vertical error bars and medians represented with a bold line in each box.

999 permutations [45] in order to assess whether OTUs (and, if so, which ones) were significantly associated with a particular PMI.

To explore the shifts in the basal communities at different decomposition stages we evaluated which families showed statistically significant differences in abundance at different PMIs applying the Student's *t*-test with Benjamini and Hochberg False Discovery Rate (FDR) correction [46] with the T.TEST and P.ADJUST functions in R. This generated both *p* values and adjusted *p* values (*q* values), that were used to evaluate the statistical significance of the results reported in the work. All the plots have been obtained by R base or packages' accessory functions.

3. Results and discussion

3.1. Decomposition stages at selected PMIs

One month after burial (pig P1), the carcass was in the putrefaction stage of decomposition but was still recognizable, after two months (pig P2) the carcass was in the putrefaction/liquefaction stage of decomposition, with bones still connected by the ligaments. After four months the carcass P3 was partially skeletonised with still some soft tissues present, and after six months (pig P4) the carcass was fully skeletonised. It has to be noted that the sizes of the carcasses might also have played a role in the decomposition rate of the various animals, as the first two pigs (P1 and P2 were smaller than the last two (Table 1). Notably, bodies with a smaller size are characterised by quicker decomposition than bigger ones [14,15] although we note that the latter studies involved much larger (i.e. order of magnitude or greater) differences in body mass between size categories when compared to the twofold differences in body mass amongst the carcasses in our study. Despite body size variation being a potential confounding factor for the estimation of PMI from carcass decomposition, we believe that this had only a minor impact on our study due to the extended time length of the experiment and the focus on soil microorganisms instead of on the decomposition stage of the animal itself. Samples collected after one month were visually different from samples collected after two and four months, which were characterised by the presence of some adipocere (in particular after two months), that was instead missing in the first grave. We believe that these differences were mostly due to the burial conditions (e.g., moisture, oxygen availability, temperature) and not to the body size of the carcasses (fatter bodies originate more adipocere than smaller bodies, however we have noticed more adipocere in one of the smaller pigs used in the study than in bigger ones). The soil in the fourth grave was more muddy and slimy, due to the heavy rainfall that introduced non-percolated water within the grave prior to the time of the sample collection.

3.2. Metabarcoding analysis of soil bacteria

From the 18 soil samples, a total of 5,120,369 sequences were collected after libraries' pre-processing: soil controls yielded 758,986 sequences (ranging from 36,799 to 321,857) and PMI soil samples yielded 4,361,383 sequences (ranging from 51,328 to 2,326,277). Those sequences were clustered with GreenGenes v.13_8, and 3,865,209 out of 5,120,369 (75%) found a match at 97% identity with database entries, providing an initial set of 12,229 OTUs. After filtering, 9 OTUs were recognized as contaminants ("mitochondria"), 9307 OTUs were removed since their cluster size was smaller than 50 reads, and 90 OTUs were removed because of their Coefficient of Variation being greater than 3.0. A final number of 2823 OTUs was obtained (Supporting Fig. S2) showed that the sequence coverage was adequate for the study and that C1 and P1 were similar each other as well as P2 and P3, as will be discussed further in the text.

A preliminary data analysis inclusive of all samples analysed within this work, performed on the sum of the abundances of the rarefied reads, showed overall that the 99.5% of the microorganisms present within the samples belonged to the Eubacteria kingdom (according to GreenGenes classification). For this reason, all subsequent analyses were done on the Eubacteria fraction only. Within this study, Eubacteria overall were characterised by 2809 different OTUs which are elucidated further below.

3.3. Bacterial community changes during progressive stages of decomposition

The first step in the analysis focused on the evaluation of the richness (alpha diversity) of the microbial taxa associated with different PMIs using the Shannon-Wiener index and with boxplots representing

the species distribution among samples, with replicates grouped together, in term of raw species number (Fig. 2). Other indices for alpha diversity such as Chao1, ACE, Simpson's and Fisher's index have been also applied, as recommended by Bandeira et al. [47], in order to better define the communities (Supporting Fig. S3). Taxonomic richness was roughly constant across all diversity metrics showed in Supporting Fig. S3 between control (C1) and one month post-mortem (P1), with ~2100 different species observed on average (Student's *t*-test p value = 0.95) but decreased compared to the control by approximately 30% at two months (P2) (p value = 0.0009) and remained lower than the control at four months (P3) (p value = 0.0003) and at six months (P4) (p value = 0.0002) despite a non-significant increase observed starting from P3 onwards. The Shannon-Wiener diversity indices showed statistically significant differences between C1 and P1 (p value = 0.008), C1 and P2 (p value = 0.0004), C1 and P3 (p value < 0.0001) and C1 and P4 (p value < 0.0001).

A decrease in the richness (variability) of the soil microbial population during body decomposition was also observed by Cobaugh et al. [26] in soils underneath four human carcasses, in agreement with our findings. Finley et al. [29] observed similar results for buried swine carcasses, but the extended time intervals utilised in their study did not allow for a precise comparison with our data. In fact, despite the decrease in species richness that they observed between 0–3 months, they did not collect intermediate data. Furthermore, they demonstrated an increase in taxonomic richness after prolonged PMIs, which is in accordance with our findings. It should also be noted that there are environmental differences between the geographical locations of their experiment and ours (e.g., higher average temperatures and higher humidity at the University of Tennessee ARF than at the HuddersFIELD outdoor taphonomy facility, data from the relevant weather stations are available online respectively at https://www.weather.gov/mrx/tysclimate and at http://www.weatherforce.org.uk/). These climatic differences may have caused the discrepancies observed here, highlighting the need for additional studies with extended datasets and varying environmental conditions. Due to the potential variations observed here, we are confident that the choice of an U.K. facility for this experiment made this study more applicable to forensic cases in the U.K.

Overall, these results suggest that the presence of the body alters the soil endogenous microbiome resulting in a decrease of the species richness particularly after two months from the burial, at a time period in which the bodies were in their active decomposition stage. The timeline of the progress of decomposition of swine carcasses in shallow burials was also elucidated by Belle et al. [48], and their results agree with our findings despite the animals used in their study being older (and heavier) than ours. The availability of a large nutrient resource supplemented by the colonisation of the bacteria from the abdominal cavity altered the original microbial community; the new microorganisms originated by the carcasses appeared to overwhelm the pre-existing community resulting in an overall decrease of the species richness over time. The slight increase in richness observed during the advanced decomposition stages suggests that after a few months the microbial communities appear to start returning toward original values, but more than six months are required to return to their original state. This observation may be particularly useful in the investigation of clandestine movements of buried carcasses from their original place of burial to another one, despite the absence of the characteristic traits of the vegetation that are instead often used as an indicator for these situations in case of exposed bodies (e.g. presence of cadaveric decomposition islands, CDIs [49]).

In order to test whether communities were statistically different from each other, a multivariate homogeneity of group dispersions among the different Control and PMI groups was first assessed by means of the betadisper and permutest (with 9999 permutations) functions in the R package vegan [43] (Fig. 3A) and then assessed by non-metric multidimensional scaling (NMDS) (Fig. 3). The homogeneity of dispersion among groups was supported by a non-significant result in permutest (p value = 0.4732). Subsequently, PERMANOVA provided significant results (R2 = 0.91282 and p = 1e-04), thus indicating that the dissimilarity was influenced by difference in composition between groups. Consequently, we analysed the differences in the taxonomic abundances from different samples to evaluate the beta diversity between different samples (Fig. 3). Samples collected from the same location clustered together, with P2 being the only one in which the three subsamples did not cluster in a compact way, potentially due to the presence of the adipocere in the soil that may have resulted in a less homogeneous sample. Overall, P1 was closer to the control, whereas samples collected after increased decomposition stages were more distant from the control (Fig. 3).

In order to characterise and identify which were the major differences observed between the different groups, we initially focused on the abundances of phyla. Overall, Proteobacteria were the most abundant phylum followed by Bacteroidetes, then by Acidobacteria which were almost as abundant as Actinobacteria, and by Firmicutes (Supporting Fig. S4). Comparing the different PMIs, specific trends were observed at specific decomposition stages (Fig. 4); in particular, Proteobacteria increased in their abundance from P1 to P4 (with a slight decrease in P3) and were the dominant phylum at the first time point analysed here but also after two and six months post-mortem. Bacteroidetes also increased with increasing PMIs, reaching their maximum after four months PMI and overcoming the abundance of Proteobacteria at this specific time point. Finally, Firmicutes abundances increased during the later stages of decomposition. Control soil was dominated by Acidobacteria followed by Proteobacteria. Acidobacteria were the only phylum for which abundance decreased constantly after the introduction of the carcasses in the soil, with decreasing levels from one to four months and with a partial recovery after six months. Some other phyla showed a similar behaviour to this latter example, with decreasing abundances accompanying with increasing PMIs compared with the control soil (Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia).

The observation that Proteobacteria are the most abundant phylum within the majority of the experimental samples analysed here is in accordance with previous studies [12,27,29,50]. Finley et al. [29] found Proteobacteria, Acidobacteria and Actinobacteria to be the most abundant phyla in all samples, similar to what was found here, even though they found lower levels of Bacteroidetes in comparison with our study (Fig. 4). Similar observations for Acidobacteria, Nitrospirae and Verrocumicrobia were obtained by Cobaugh et al. [26], where the relative abundances of these phyla decreased to less than 15% of their abundances in the control samples during later stages of decomposition. The increase of Bacteroidetes has not previously been shown in the literature, despite the fact that here they reached high abundances over the course of our experiment. On the contrary, the increase of Firmicutes was already documented in previous works; Metcalf et al. [27] showed that Firmicutes increased with prolonged PMIs and Pechal et al. [12] found Firmicutes to be the most abundant phylum at prolonged decomposition stages, in contrast with our findings (namely that Proteobacteria dominated the later decomposition stages). However, the results for Firmicutes reported by Pechal et al. [12] could be due to the presence of insects, particularly flies, accessing the carcass. Another study performed by Pechal et al. [51] on swine decomposition, which excluded the exposure of the carcasses to flies, in fact showed that Proteobacteria remained the most abundant phylum after prolonged decomposition stages. Therefore, it is likely that our experimental design excluded access by insects such as flies to our carcasses, and this may explain the reduced abundance of Firmicutes observed here in contrast with the previously mentioned findings obtained on exposed remains.

The pH of the soil associated with the graves increased from 7.2 in the controls to 8.1 after two months PMI and decreased again over the course of the experiment returning to the starting value after six months

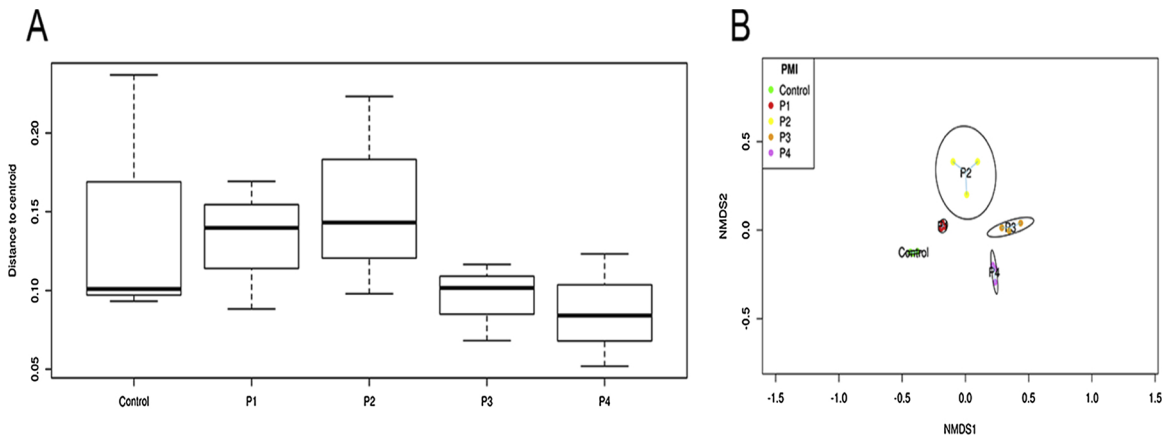

Fig. 3. NMDS plot showing an increase in beta diversity between the control soil and the gravesoil, with increasing variability after prolonged PMIs compared with shorter ones represented by expanded ellipses for P2, P3 and P4 (P1 = 1 month, P2 = 2 months, P3 = 4 months, P4 = 6 months). Standard deviation is represented by the dimension of the ellipses.

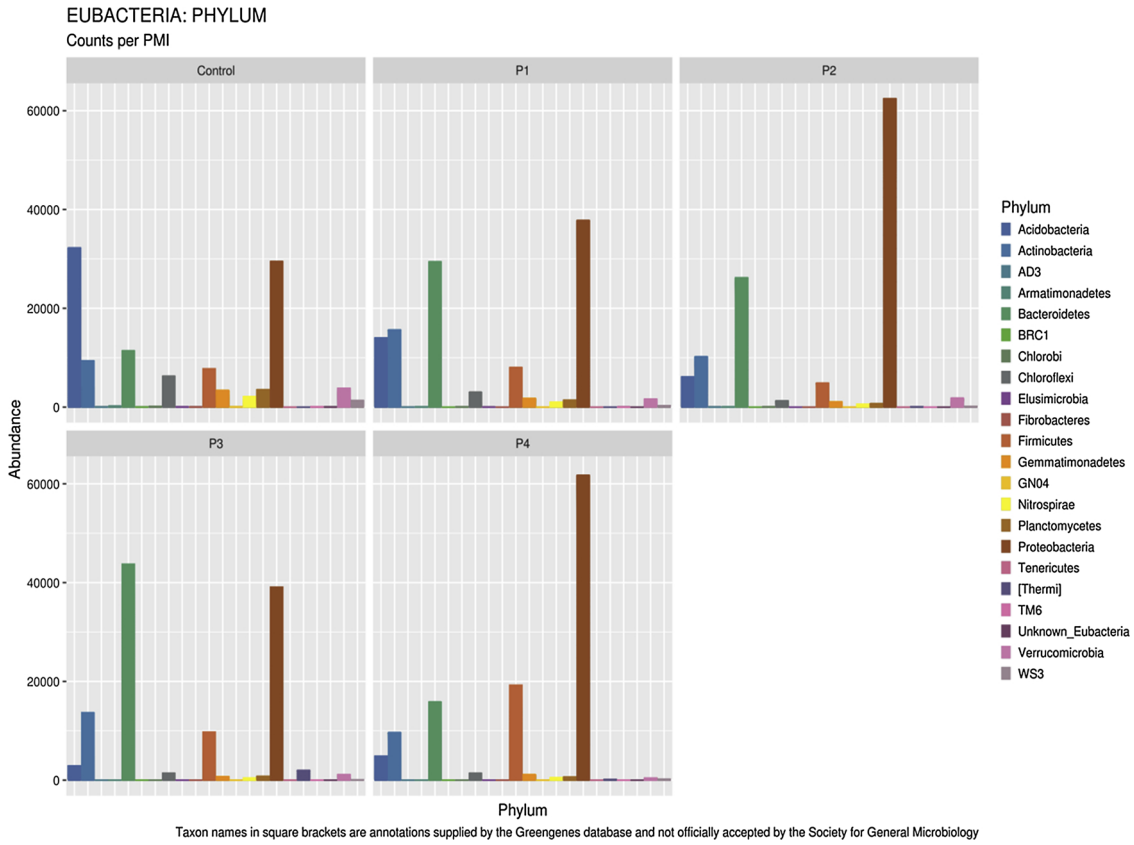

Fig. 4. Bar chart with abundances of taxa at a phylum level associated with control soil and with the experimental samples.

(Table 1). This finding is in contrast to the results of Cobaugh et al. [26] but is supported by other studies [23,28,52,53]. The presence of a linkage between the alkalinity of the soil and the formation of adipocere proposed by Forbes [54] and by Olakanye et al. [23] found support in this study, where the most alkaline soil contained the greatest amount of adipocere. The increase in gravesoil pH is associated with the active decay of the carcass, and in particular with the rupture of the skin and the release of ammonia-rich nutrients within the soil. Data obtained here suggest that the rupture of the corpses started approximately after one month from the deposition of the carcasses but the release of the nutrients reached its maximum only after two months, supporting also our findings about the taxonomic richness of the samples previously discussed. This finding correlates well with other studies on Acidobacteria associated with decomposing carcasses [27,55]. Acidobacteria is a phylum of bacteria characterised by different subgroups adapted to survive in various pH conditions [56], with some groups preferring basic pH and others preferring acidic conditions. However, the groups which here showed the most significant changes between control and gravesoil were groups III and V [57] (families iii1-15 and RB41 respectively, see Fig. 5 for reference), and these groups are adapted to survive better with low pHs and worse with high pHs [56]. This explains the shift in the Acidobacteria populations observed with increasing pHs in this study. Acidobacteria abundances started to increase again after six months, even though the pH started to be more favourable after four months; this may be related to their slow growth rate in comparison with other bacteria [58]. Additionally,

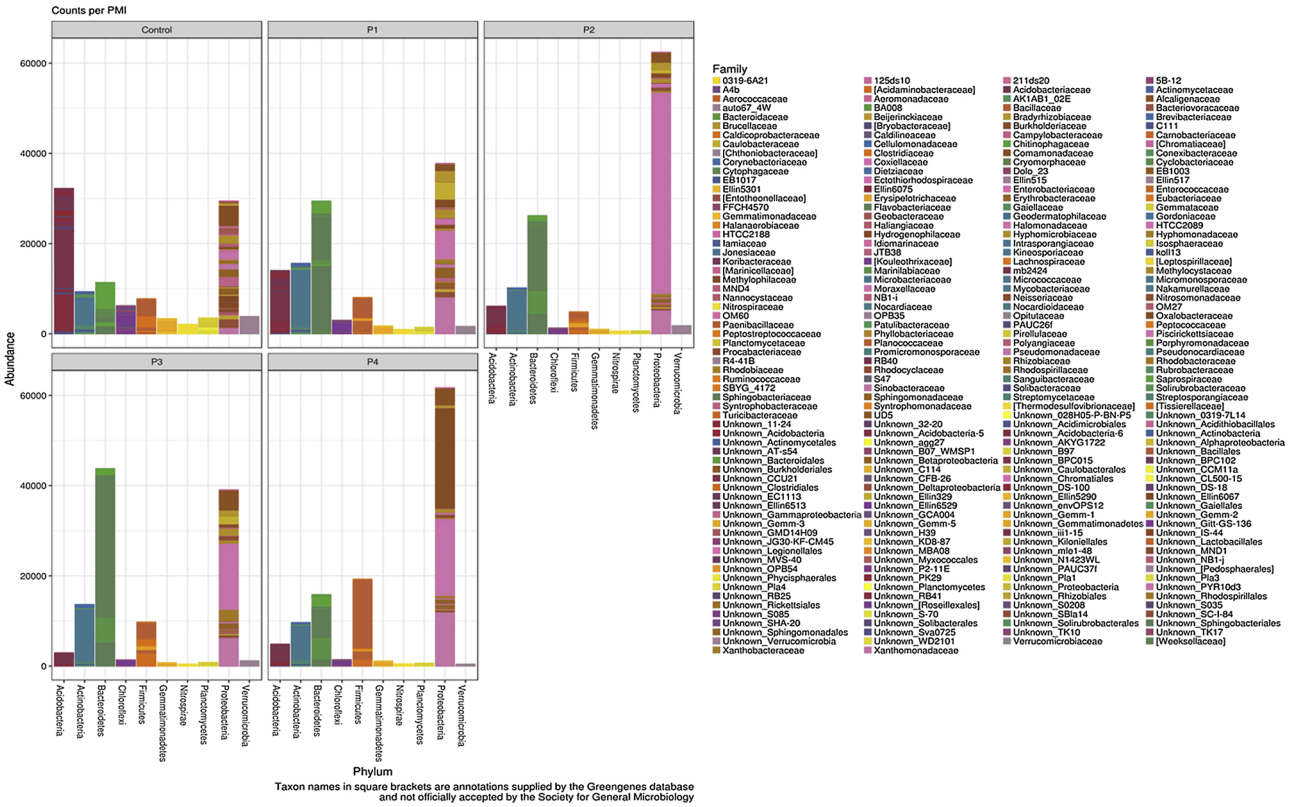

Fig. 5. Microbial abundances at different PMIs resolved at the family level.

Acidobacteria are oligotrophic bacteria [55], which means that they prefer environments with low levels of nutrients in contrast with copiotrophic bacteria which prefer environmental conditions rich in nutrients. Their recurrence after six months indicates from a microbial point of view the almost complete consumption of the organic matter that was introduced with the carcass, as was visually confirmed by the quite complete skeletonization of the body recovered after six months.

For a more detailed description we examined variation at the family level (Table 2 and Fig. 5). Within the phylum of Proteobacteria, one of the most significant variations between control and gravesoil was the increase of the Pseudomonadaceae members, particularly after two months. Also, Xanthomonadaceae increased notably in the gravesoil samples compared with the control, with larger variations especially recorded after one and six months post-mortem. Comamonadaceae showed an increased abundance in particular after six months, despite being already relatively abundant in the control. Caulobacteraceae, on the contrary, increased particularly at the first time-point and decreased during the advanced decomposition stages. Finally, Alcaligenaceae increased continuously with increasing PMIs. Focusing on Bacteroidetes instead, we found members of the Flavobacteriaceae family which increased after one, two and particularly after four months, and then were reduced (despite remaining more abundant than in the control) after six months. Sphingobacteriaceae were particularly abundant after the first month and then decreased progressively reaching almost basal levels after six months, leaving space for the arrival of Porphyromonadaceae (that were almost absent in the control) in particular from two months onwards. Saprospiraceae, a family of bacteria present in the control, decreased after the first month and then almost disappeared after prolonged PMIs. Finally, within the Firmicutes phylum, we noticed variations in the abundance of Paenibacillaceae between control and gravesoil, with the samples collected at the last time point being the sample with the greater abundances of bacteria belonging to this family in comparison with other samples. Despite the decline of some copiotrophic taxa after six months and the partial re-establishment of the initial conditions within the soil microbiota, our results suggest that six months are not sufficient to completely recover the starting conditions in this burial environment. Some phyla far from their complete repopulation within the soil, such as Acidobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia (Fig. 5) and several taxa specifically associated with the presence of the pig carcass were still persistent after six months, as described in the following section of the paper.

Several of these families were already known to be associated with decomposition studies; for example, Cobaugh et al. [26] showed slight increases of Xanthomonadales during the initial decomposition stages, and a rapid increase in their abundances during the advanced decay stages. Metcalf et al. [27] showed that Alcaligenaceae increased in the gravesoil under exposed mouse remains during their advanced decay stage of decomposition, in accordance with our findings here. However, they showed also that Sphingobacteriaceae increased during the advanced decay stage of decomposition [27], in contrast with the results presented here.

Overall, the results obtained here showed the existence of several families in which abundances increase or decrease at specific decomposition time stages in a statistically significant way, although these patterns were sometimes in accordance with other studies and sometimes not. The presence of very different experimental designs across all the studies cited here (including humans, pigs and mice, exposed or buried, within different types of soil and different environmental conditions) makes the interpretation of the results more challenging. However, the similarities for some specific taxa found across these different works, such as for Xanthomonadaceae and Alcaligenaceae, show the potential and the importance of the research in this field allowing the identification of specific signatures that may be promising

Table 2
Abundances of the families which showed an increased beta variability between different samples with ANOVA p values and with false discovery rate (FDR) correction (Benjamini and Hochberg) q values.

Phylum	Firmicutes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria
Families	Paenibacillaceae	Saprospiraceae	Porphyromonadaceae	Sphingobacteriaceae	Flavobacteriaceae	Alcaligenaceae	Comamonadaceae	Caulobacteraceae	Xanthomonadaceae	Pseudomonadaceae
C1	780	930	11	1075	1096	164	4575	171	1393	1765
P1	157	432	831	14132	9466	1508	1749	3856	8186	6287
P2	87	100	4431	3965	14527	2203	968	782	5313	44565
P3	79	133	5082	5202	30906	4480	696	1837	6352	14556
P4	67	90	4360	1437	6266	3759	22242	194	12006	16885
ANOVA *p* value	0.048	< 0.0001	0.0008	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
C1 vs P1 *q* value	0.0235	0.0154	> 0.05	< 0.0001	0.0002	0.0003	0.0119	< 0.0001	0.0003	0.0145
C1 vs P2 *q* value	0.0187	0.0005	0.0004	0.0002	< 0.0001	< 0.0001	0.0038	0.0013	0.0069	< 0.0001
C1 vs P3 *q* value	0.0187	0.0005	0.0004	< 0.0001	< 0.0001	< 0.0001	0.0036	< 0.0001	0.0021	< 0.0001
C1 vs P4 *q* value	0.0187	0.0005	0.0004	> 0.05	0.0037	< 0.0001	< 0.0001	> 0.05	< 0.0001	< 0.0001

for forensic scenarios.

3.4. Identification of endogenous mammalian microbial communities and basal communities

Decomposing mammal carcasses contain specific microbial communities associated with their skin and within their gut, and the identification of some of these specific mammalian microbes within the soil may have important forensic relevance. In fact, it would be interesting to make comparisons with studies undertaken on human corpses to evaluate the extent of the suitability of swine bodies in simulating human burials, as we might expect commensal biota to differ in pigs and humans for several reasons, including differences in diet, human consumption of drugs such as antibiotics, levels of hygiene, differences in immune systems etc. Also, it may be important to understand which of the basal microbes also present within the control samples varies depending on the decomposition stage, despite the presence of microbes introduced by the carcass. We wanted to test these research questions, such as are pigs suitable as proxies for humans? Do the control microbiota change after cadaver burial? Furthermore, we also wanted to assess if the variations observed between the control soil and the gravesoils were due to the introduction of new bacterial species from the mammals and/or to the colonisation of the carcass by new bacterial species attracted by the decomposing bodies which were not present in the control soils. For this reason, we excluded all the OTUs previously identified within the control keeping only OTUs which were exclusively present within the gravesoil (Supporting Table S4). In summary, we found 2331 different OTUs present within the basal microbiome and 478 taxa specifically associated with the gravesoil only (not identified in the control).

The microbiome associated exclusively with the presence of the carcass showed a wide variety of different families (Fig. 6), with several of them being unique for the gravesoil and never being identified in the control soil (Table 3).

Indicator species analysis undertaken to investigate the presence of specific species associated with the basal microbiome, or with the presence of the carcasses (significance level = 5%), showed that overall 873 species contributed to the differences observed between the control and the gravesoil (p values $\leq$ 0.05) and 187 species contributed significantly to discriminate mammals-associated bacteria from basal microbes (Supporting Table S5).

Interestingly, only Bacteroidaceae have been previously mentioned in the literature in studies of the decomposition of carcasses in the soil. In particular, members of this family have been previously found in the abdominal cavity during the bloating stage of decomposing mice [27]. After the rupture of the corpse, they decreased dramatically within the body and increased in the soil underneath; we found here that their survival within the gravesoil lasts at least for six months from the deposition of the body (Supporting Table S2) in particular for *Bacteroides*. This particular finding is in accordance with Cobaugh et al. [26] and proves that non-endogenous soil species can survive for prolonged times outside their original environment (e.g., gut) probably due to the reduced concentrations of oxygen that characterise the burial environment that accommodates the presence of anaerobic bacteria such as *Bacteroides*.

It has to be noted that during this experiment we did not have control on the freezing procedure adopted prior to the beginning of the burials (and in particular on the period of time the pigs have been kept frozen) due to the previously mentioned ethical and legal reasons; despite we are aware that this procedure may have had an impact on the survival of gut bacteria, a study done on frozen foods showed that microorganisms can survive during freezing, storage and thawing [59] and a more recent one provided evidence for the repair of eventual DNA lesions in bacteria at −15 °C, resulting in an overall survival and functionality of microbes found in icy environments [60]. Our results indicated the existence of several taxa specifically associated with the

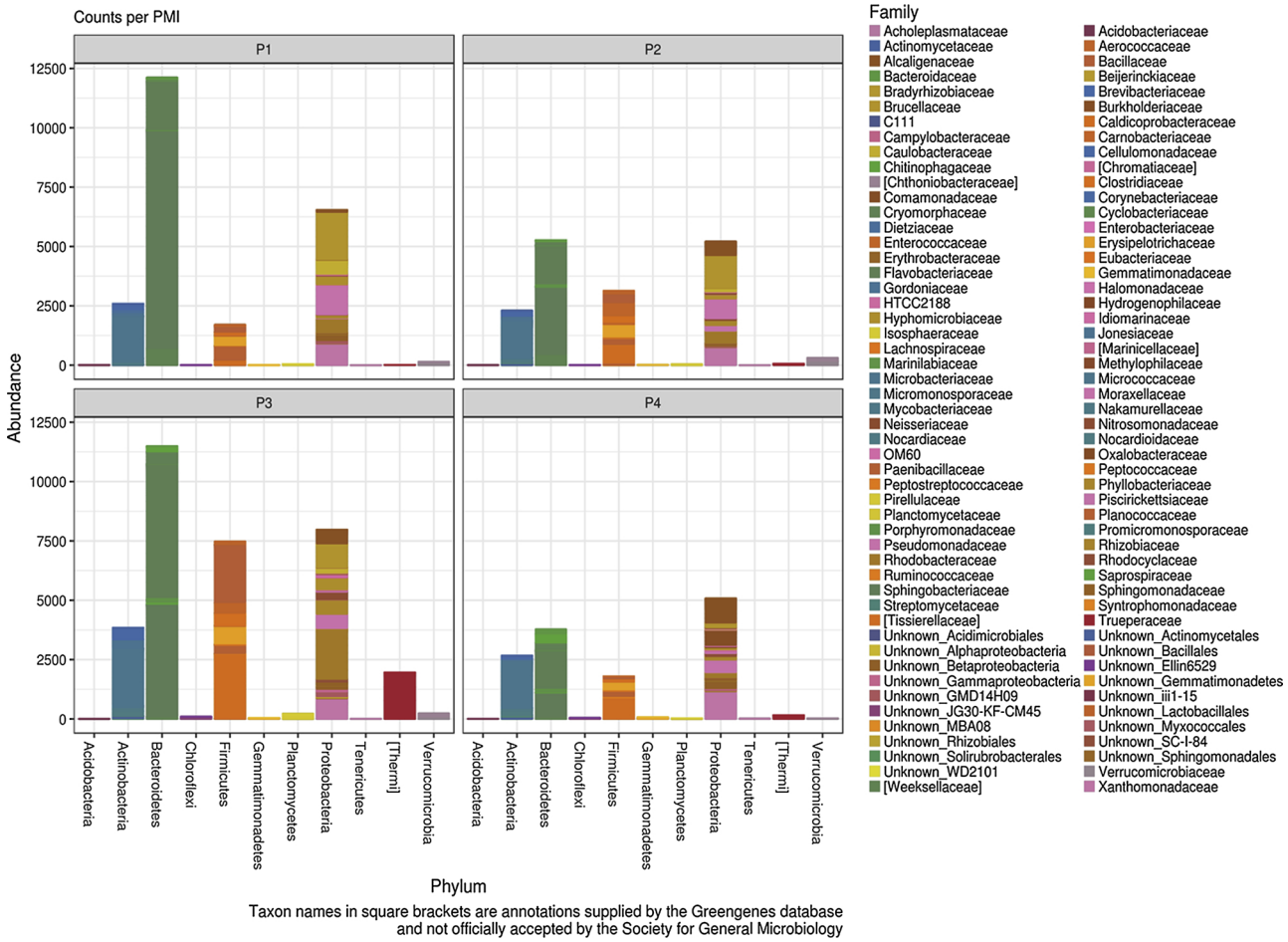

Fig. 6. Microbiome associated uniquely with the mammalian carcasses and not identified within the control sample. Abundances per each OTU have been reported into families (see legend) and aggregated into phyla (X axis).

Table 3
List of families identified uniquely in gravesoil and not in control soil.

Families uniquely present in gravesoil	
Acholeplasmataceae	Enterococcaceae
Actinomycetaceae	Gordoniaceae
Bacteroidaceae	Lachnospiraceae
Campylobacteraceae	Neisseriaceae
Dietziaceae	Ruminococcaceae

carcass, supporting the idea that the freezing procedure did not kill completely the microbiome associated with pigs. Moreover, our findings on the presence of Bacteroidaceae uniquely in the soil with the carcasses and not in the control further confirms this hypothesis, indicating that the freezing procedure did not significantly affect the survival of gut microbes.

To explore the shifts in the basal communities at different decomposition stages we evaluated which families showed statistically significant differences in abundance at different PMIs (Student's *t*-test with Benjamini and Hochberg False Discovery Rate (FDR) correction, Supporting Fig. S5-S8 and Supporting Table S6). This particular analysis may be useful to look for more generic biomarkers that may be applicable to a broader range of forensic contexts in a more general way (e.g. not only to pigs but also to humans), excluding specific bacteria that could have been brought by the animal and that could suffer from individual variability.

The highest statistical power was associated with Geobacteraceae, which were significantly less abundant in all the gravesoil samples in comparison with the control soil. Additionally, other significant variations were observed at the first time point with a decrease in the abundance of Phycisphaerales, at the second time point with an increase of Alcaligenaceae and a decrease of Cytophagaceae, and at the third time point with an increase of Flavobacteriaceae and a decrease of Cytophagaceae, Gemmataceae, Geobacteraceae, Marinicellaceae, Piscirickettsiaceae and Planctomycetaceae (Supporting Table S6).

Despite the general trend of decline of basal species observed within the gravesoil, we identified an interesting opposite trend for Alcaligenaceae and Flavobacteriaceae (Supporting Table S6 and Fig. 7). Alcaligenaceae belong to the class of β-Proteobacteria and were shown to increase in abundance during advanced decomposition stages in the soil underneath decomposing mice [27]. This is consistent with our results and the idea that, regardless of the type of corpse used for the study and the type of soil, members of a microbial family may become in future useful targets to estimate the PMI of the victim or to infer the former presence of bodies in an advanced state of decomposition which have been moved from the burial place to a separate location. Belonging to the phylum Bacteroidetes, Flavobacteriaceae on the contrary has never been found to be correlated with decomposition studies and so further work on larger datasets may clarify their role during the active decay stage of buried carcasses to exclude external effects (e.g. seasonality, humidity, soil type) on the recorded increase in their abundances over time.

In addition to the most significant families that were differentially abundant in different samples, other families deserve to be mentioned here due to their statistical power. In particular, a significant increase in

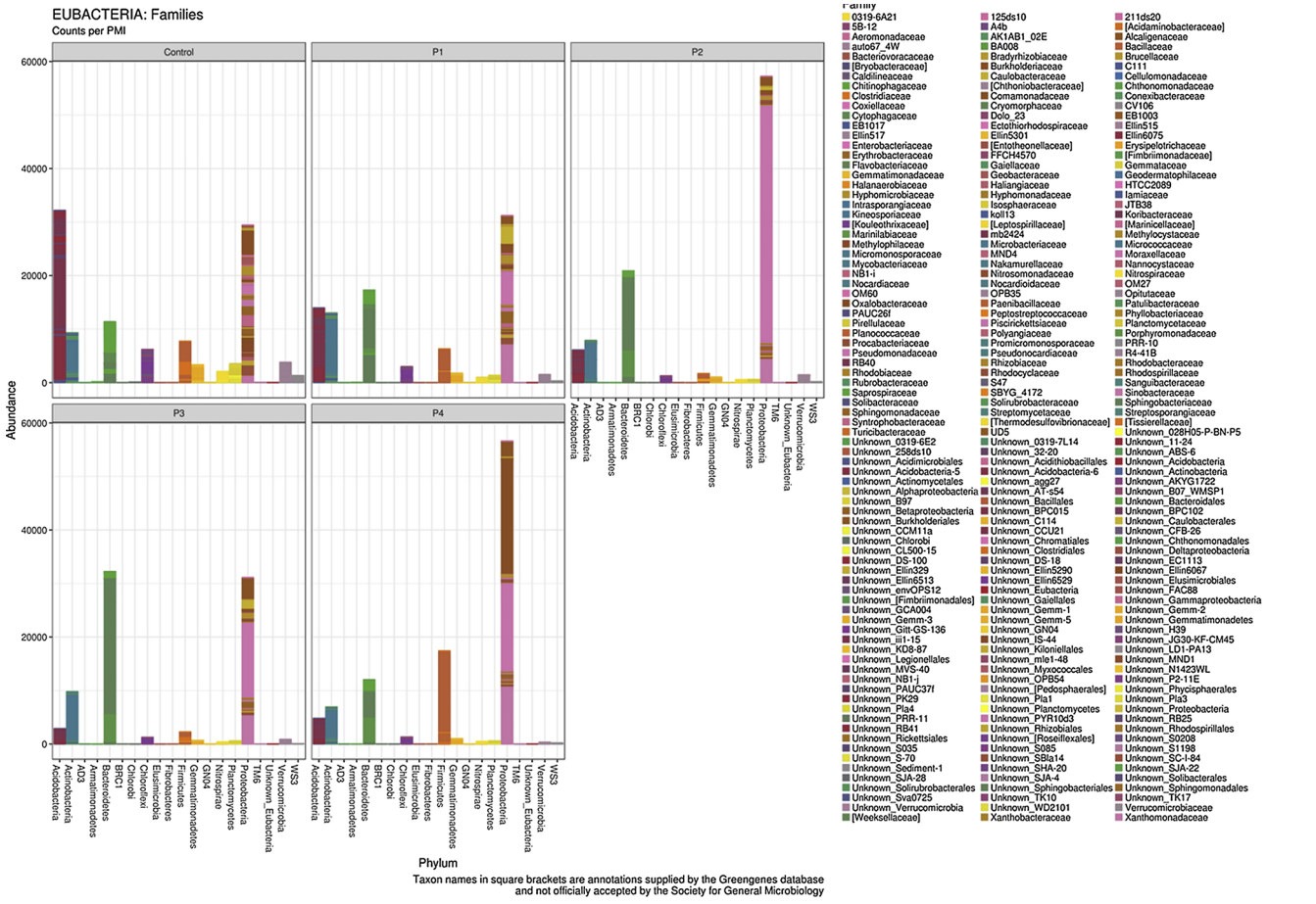

Fig. 7. Abundances of the basal microbiota in the control and after increasing PMIs.

the Porphyromonadaceae (Bacteroidetes phylum) was observed during all the phases of the decomposition from two months onwards, Bacillaceae (Firmicutes phylum) increased particularly after six months, Pseudomonadaceae (Proteobacteria phylum) increased after two months and remained quite abundant thereafter, and Comamonadaceae (Proteobacteria phylum) increased after six months (Fig. 7).

Taken together, these results suggest that basal soil microbes, in addition to the mammalian-associated microorganisms, can contribute to the identification of specific biomarkers that can assist scientists in the evaluation of the decomposition grade of mammalian corpses. More specifically the analysis of combined basal and introduced biota could avoid some biases introduced by the inter-individual variability that potentially may exist between different carcasses and that may further complicate the interpretation of the results. In this regard, an improved experimental design should consider more bodies for each phase of decomposition, in addition to suitable soil controls at each sampling site in order to explore and subtract the biodiversity associated to a given site at a given time.

4. Conclusions

In this study we have shown that shifts in the microbial populations within the gravesoil collected from experimental shallow burials show trends comparable with other studies, demonstrating the great potential of this approach for forensic research. Future analyses on the microbial families which showed consistency within different experimental designs, such as Xanthomonadaceae and Alcaligenaceae, will be useful to increase the actual knowledge in PMI estimation from soil samples. We were able to show a general reduction in taxonomic richness of soil microbes with increasing PMIs, in accordance with other studies, and we also have provided the first substantial characterisation of specific taxa associated with gravesoil collected from burial environments, also focusing specifically on mammals-associated microbes and on basal communities, to overcome the limits due to inter-individual and inter-species variability. Of course, this work was intended to be exploratory, and for this reason the results showed here obtained from our limited dataset might need further studies in order to be corroborated and validated. Overall, this study showed promising results that strongly motivate additional research, and will help to frame the design of studies in the future. Random Forest models [27] may also be used hereafter on this dataset to discover if the changes observed here may be useful to estimate the PMI of the bodies.

Acknowledgements

The authors are grateful to the Royal Society for funding both a Ph.D. studentship (N.P.) and University Research Fellowship (M.B.) under grants RG130453 and UF120473, respectively. We also acknowledge the technical support of Dr. Marco Chiapello and Dr. Samuele Voyron from the University of Turin for helpful advices during R scripts coding, and both Dr. Serena Doni and Dr. Grazia Masciandaro from the CNR based in Pisa for the physical-chemical analyses of soil. We thank also Dr Bart van Dongen from the University of Manchester and Prof. Tim Thompson from Teesside University for the precious advice they have recommended during the final stage of preparation of this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigen.2018.12.002.

References

[1] A.S. Khodakova, R.J. Smith, L. Burgoyne, D. Abarno, A. Linacre, Random whole metagenomic sequencing for forensic discrimination of soils, PLoS One. 9 (2014) e104996.
[2] L.A. Dawson, S. Hillier, Measurement of soil characteristics for forensic applications, Surf. Interface Anal. 42 (2010) 363–377.
[3] V. Chazottes, C. Brocard, B. Peyrot, Particle size analysis of soils under simulated scene of crime conditions: the interest of multivariate analyses, Forensic Sci. Int. 140 (2004) 159–166.
[4] R. Sugita, Y. Marumo, Validity of color examination for forensic soil identification, Forensic Sci. Int. 83 (1996) 201–210.
[5] K. Pye, S.J. Blott, D.J. Croft, J.F. Carter, Forensic comparison of soil samples: assessment of small-scale spatial variability in elemental composition, carbon and nitrogen isotope ratios, colour, and particle size distribution, Forensic Sci. Int. 163 (2006) 59–80.
[6] A. Ruffell, P. Wiltshire, Conjunctive use of quantitative and qualitative X-ray diffraction analysis of soils and rocks for forensic analysis, Forensic Sci. Int. 145 (2004) 13–23.
[7] H. Habtom, S. Demanèche, L. Dawson, C. Azulay, O. Matan, P. Robe, R. Gafny, P. Simonet, E. Jurkevitch, Z. Pasternak, Soil characterisation by bacterial community analysis for forensic applications: a quantitative comparison of environmental technologies, Forensic Sci. Int. Genet. 26 (2017) 21–29.
[8] S. Giampaoli, A. Berti, R.M. Di Maggio, E. Pilli, A. Valentini, F. Valeriani, G. Gianfranceschi, F. Barni, L. Ripani, V.R. Spica, The environmental biological signature: NGS profiling for forensic comparison of soils, Forensic Sci. Int. 240 (2014) 41–47.
[9] E.M. Jesmok, J.M. Hopkins, D.R. Foran, Next-generation sequencing of the bacterial 16S rRNA gene for forensic soil comparison: a feasibility study, J. Forensic Sci. 61 (2016) 607–617.
[10] A. Konopka, What is microbial community ecology? ISME J. 3 (2009) 1223–1230.
[11] A.O. Olakanye, T.K. Ralebitso-Senior, Assessing subsurface decomposition and potential impacts on forensic investigations, in: T.K. Ralebitso-Senior (Ed.), Forensic Ecogenomics, Elsevier, 2018, pp. 145–176.
[12] J.L. Pechal, T.L. Crippen, M.E. Benbow, A.M. Tarone, S. Dowd, J.K. Tomberlin, The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing, Int. J. Legal Med. 128 (2014) 193–205.
[13] L.G. Roberts, J.R. Spencer, G.R. Dabbs, The effect of body mass on outdoor adult human decomposition, J. Forensic Sci. 62 (2017) 1145–1150.
[14] A. Sutherland, J. Myburgh, M. Steyn, P.J. Becker, The effect of body size on the rate of decomposition in a temperate region of South Africa, Forensic Sci. Int. 231 (2013) 257–262.
[15] A. Spicka, R. Johnson, J. Bushing, L.G. Higley, D.O. Carter, Carcass mass can influence rate of decomposition and release of ninhydrin-reactive nitrogen into gravesoil, Forensic Sci. Int. 209 (2011) 80–85.
[16] S. Niederegger, X. Steube, P. Tiltmann, G. Mall, Decomposition rate of intact and injured piglet cadavers, Rechtsmedizin. 27 (2017) 8–15.
[17] L.N. Bates, D.J. Wescott, Comparison of decomposition rates between autopsied and non-autopsied human remains, Forensic Sci. Int. 261 (2016) 93–100.
[18] D.O. Carter, D. Yellowlees, M. Tibbett, Cadaver decomposition in terrestrial ecosystems, Naturwissenschaften 94 (2007) 12–24.
[19] R.A. Parkinson, K.-R. Dias, J. Horswell, P. Greenwood, N. Banning, M. Tibbett, A.A. Vass, Microbial Community analysis of human decomposition on soil, in: K. Ritz, L. Dawson, D. Miller (Eds.), Criminal and Environmental Soil Forensics, Springer, 2009, pp. 379–394.
[20] J. Pinheiro, Decay process of a cadaver, in: A. Schmitt, E. Cunha, J. Pinheiro (Eds.), Forensic Anthropology and Medicine, Humana Press, 2006, pp. 85–116.
[21] D.W. Hopkins, P.E.J. Wiltshire, B.D. Turner, Microbial characteristics of soils from graves: an investigation at the interface of soil microbiology and forensic science, Appl. Soil Ecol. 14 (2000) 283–288.
[22] R.C. Bergmann, T.J.U. Thompson, An RNA-based analysis of changes in biodiversity indices in response to Sus scrofa domesticus decomposition, Forensic Sci. Int. 241 (2014) 190–194, https://doi.org/10.1016/j.forsciint.2014.06.001.
[23] A.O. Olakanye, T. Thompson, T.K. Ralebitso-Senior, Changes to soil bacterial profiles as a result of Sus scrofa domesticus decomposition, Forensic Sci. Int. 245 (2014) 101–106.
[24] M. Chimutsa, A.O. Olakanye, T.J.U. Thompson, T.K. Ralebitso-Senior, Soil fungal community shift evaluation as a potential cadaver decomposition indicator, Forensic Sci. Int. 257 (2015) 155–159.
[25] E.R. Hyde, D.P. Haarmann, J.F. Petrosino, A.M. Lynne, S.R. Bucheli, Initial insights into bacterial succession during human decomposition, Int. J. Legal Med. 129 (2015) 661–671.
[26] K.L. Cobaugh, S.M. Schaeffer, J.M. DeBruyn, Functional and structural succession of soil microbial communities below decomposing human cadavers, PLoS One. 10 (2015) e0130201.
[27] J.L. Metcalf, L.W. Parfrey, A. Gonzalez, C.L. Lauber, D. Knights, G. Ackermann, G.C. Humphrey, M.J. Gebert, W. Van Treuren, D. Berg-Lyons, A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system, Elife. 2 (2013) e01104.
[28] J.L. Metcalf, Z.Z. Xu, S. Weiss, S. Lax, W. Van Treuren, E.R. Hyde, S.J. Song, A. Amir, P. Larsen, N. Sangwan, Microbial community assembly and metabolic function during mammalian corpse decomposition, Science 351 (80-) (2016) 158–162.
[29] S.J. Finley, J.L. Pechal, M.E. Benbow, B.K. Robertson, G.T. Javan, Microbial signatures of cadaver gravesoil during decomposition, Microb. Ecol. 71 (2016) 524–529.
[30] N. Procopio, A. Williams, A.T. Chamberlain, M. Buckley, Forensic proteomics for the evaluation of the post-mortem decay in bones, J. Proteomics. 177 (2018) 21–30.
[31] G. Tomasello, A. Sorce, P. Damiani, E. Sinagra, F. Carini, The importance of intestinal microbial flora (microbiota) and role of diet, Prog. Nutr. 19 (2017) 342–344.
[32] S. Matuszewski, S. Konwerski, K. Frątczak, M. Szafałowicz, Effect of body mass and clothing on decomposition of pig carcasses, Int. J. Legal Med. 128 (2014) 1039–1048.
[33] B. Singh, K.J. Minick, M.S. Strickland, K.G. Wickings, T.L. Crippen, A.M. Tarone, M.E. Benbow, N. Sufrin, J.K. Tomberlin, J.L. Pechal, Temporal and spatial impact of human cadaver decomposition on soil bacterial and arthropod community structure and function, Front. Microbiol. 8 (2018) 2616.
[34] P.C.Y. Woo, S.K.P. Lau, J.L.L. Teng, H. Tse, K.-Y. Yuen, Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories, Clin. Microbiol. Infect. 14 (2008) 908–934.
[35] A.E. Parada, D.M. Needham, J.A. Fuhrman, Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples, Environ. Microbiol. 18 (2016) 1403–1414.
[36] A. Apprill, S. McNally, R. Parsons, L. Weber, Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton, Aquat. Microb. Ecol. 75 (2015) 129–137.
[37] J. Zhang, K. Kobert, T. Flouri, A. Stamatakis, PEAR: a fast and accurate Illumina Paired-End reAd mergeR, Bioinformatics 30 (2013) 614–620.
[38] T. Rognes, T. Flouri, B. Nichols, C. Quince, F. Mahé, VSEARCH: a versatile open source tool for metagenomics, PeerJ. 4 (2016) e2584.
[39] D. McDonald, M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, G.L. Andersen, R. Knight, P. Hugenholtz, An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea, ISME J. 6 (2012) 610–618.
[40] D. McDonald, J.C. Clemente, J. Kuczynski, J.R. Rideout, J. Stombaugh, D. Wendel, A. Wilke, S. Huse, J. Hufnagle, F. Meyer, The biological observation matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome, Gigascience 1 (2012) 7.
[41] R.C. Team, R: A Language and Environment for Statistical Computing, (2013).
[42] P.J. McMurdie, S. Holmes, phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data, PLoS One 8 (2013) e61217.
[43] J. Oksanen, F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. Minchin, R.R.B. O'Hara, G.L. Simpson, P. Solymos, M.H. Stevens, H.E. Szoecs, H. Wagner, Vegan: Community Ecology Package. R Package Version 2.4-6, (2018).
[44] M. Dufrêne, P. Legendre, Species assemblages and indicator species: the need for a flexible asymmetrical approach, Ecol. Monogr. 67 (1997) 345–366.
[45] M. De Cáceres, P. Legendre, Associations between species and groups of sites: indices and statistical inference, Ecology 90 (2009) 3566–3574.
[46] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Stat. Soc. Ser. B (1995) 289–300.
[47] B. Bandeira, J.-L. Jamet, D. Jamet, J.-M. Ginoux, Mathematical convergences of biodiversity indices, Ecol. Indic. 29 (2013) 522–528.
[48] L.E. Van Belle, D.O. Carter, S.L. Forbes, Measurement of ninhydrin reactive nitrogen influx into gravesoil during aboveground and belowground carcass (Sus domesticus) decomposition, Forensic Sci. Int. 193 (2009) 37–41.
[49] A.L. Emmons, J.M. DeBruyn, A.Z. Mundorff, K.L. Cobaugh, G.S. Cabana, The persistence of human DNA in soil following surface decomposition, Sci. Justice 57 (2017) 341–348.
[50] L.F.W. Roesch, R.R. Fulthorpe, A. Riva, G. Casella, A.K.M. Hadwin, A.D. Kent, S.H. Daroub, F.A.O. Camargo, W.G. Farmerie, E.W. Triplett, Pyrosequencing enumerates and contrasts soil microbial diversity, ISME J. 1 (2007) 283–290.
[51] J.L. Pechal, T.L. Crippen, A.M. Tarone, A.J. Lewis, J.K. Tomberlin, M.E. Benbow, Microbial community functional change during vertebrate carrion decomposition, PLoS One 8 (2013) e79035.
[52] J. Meyer, B. Anderson, D.O. Carter, Seasonal variation of carcass decomposition and gravesoil chemistry in a cold (Dfa) climate, J. Forensic Sci. 58 (2013) 1175–1182.
[53] D.O. Carter, D. Yellowlees, M. Tibbett, Moisture can be the dominant environmental parameter governing cadaver decomposition in soil, Forensic Sci. Int. 200 (2010) 60–66.
[54] S.L. Forbes, Decomposition chemistry in a burial environment, in: M. Tibbett, D.O. Carter (Eds.), Soil Analysis in Forensic Taphonomy, CRC Press, Taylor & Francis, New York, 2008, pp. 203–223.
[55] N. Fierer, M.A. Bradford, R.B. Jackson, Toward an ecological classification of soil bacteria, Ecology 88 (2007) 1354–1364.
[56] A.K. Bartram, X. Jiang, M.D.J. Lynch, A.P. Masella, G.W. Nicol, J. Dushoff, J.D. Neufeld, Exploring links between pH and bacterial community composition in

soils from the Craibstone Experimental Farm, FEMS Microbiol. Ecol. 87 (2014) 403–415.
[57] A. Quaiser, T. Ochsenreiter, C. Lanz, S.C. Schuster, A.H. Treusch, J. Eck, C. Schleper, Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics, Mol. Microbiol. 50 (2003) 563–575.
[58] N.L. Ward, J.F. Challacombe, P.H. Janssen, B. Henrissat, P.M. Coutinho, M. Wu, G. Xie, D.H. Haft, M. Sait, J. Badger, Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils, Appl. Environ. Microbiol. 75 (2009) 2046–2056.
[59] M.L. Speck, B. Ray, Effects of freezing and storage on microorganisms in frozen foods: a review, J. Food Prot. 40 (1977) 333–336.
[60] M. Dieser, J.R. Battista, B.C. Christner, DNA double-strand break repair at −15 C, Appl. Environ. Microbiol. 79 (2013) 7662–7668.