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Research Paper

Microbial DNA in human nucleic acid extracts: Recoverability of the microbiome in DNA extracts stored frozen long-term and its potential and ethical implications for forensic investigation

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ABSTRACT

Human DNA samples can remain unaltered for years and preserve important genetic information for forensic investigations. In fact, besides human genetic information, these extracts potentially contain additional valuable information: microbiome signatures. Forensic microbiology is rapidly becoming a significant tool for estimating post-mortem interval (PMI), and establishing cause of death and personal identity. To date, the possibility to recover unaltered microbiome signatures from human DNA extracts has not been proven. This study examines the microbiome signatures within human DNA extracts obtained from six cadavers with different PMIs, which were stored frozen for 5–16 years. Results demonstrated that the microbiome can be co-extracted with human DNA using forensic kits designed to extract the human host's DNA from different tissues and fluids during decomposition. We compared the microbial communities identified in these samples with microbial DNA recovered from two human cadavers donated to the Forensic Anthropology Center at Texas State University (FACTS) during multiple decomposition stages, to examine whether the microbial signatures recovered from "old" (up to 16 years) extracts are consistent with those identified in recently extracted microbial DNA samples. The V4 region of 16 S rRNA gene was amplified and sequenced using Illumina MiSeq for all DNA extracts. The results obtained from the human DNA extracts were compared with each other and with the microbial DNA from the FACTS samples. Overall, we found that the presence of specific microbial taxa depends on the decomposition stage, the type of tissue, and the depositional environment. We found no indications of contamination in the microbial signatures, or any alterations attributable to the long-term frozen storage of the extracts, demonstrating that older human DNA extracts are a reliable source of such microbial signatures. No shared Core Microbiome (CM) was identified amongst the total 18 samples, but we identified certain species in association with the different decomposition stages, offering potential for the use of microbial signatures co-extracted with human DNA samples for PMI estimation in future. Unveiling the new significance of older human DNA extracts brings with it important ethical-legal considerations. Currently, there are no shared legal frameworks governing the long-term storage and use of human DNA extracts obtained from crime scene evidence for additional research purposes. It is therefore important to create common protocols on the storage of biological material collected at

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crime scenes. We review existing legislation and guidelines, and identify some important limitations for the further development and application of forensic microbiomics.

1. Introduction

Human DNA can remain unchanged for years and extracts stored at −20 °C or −80 °C can preserve important genetic information for long periods of time, which can be submitted as evidence in court [1]. This makes such extracts particularly valuable in (older) cases in which other evidence has been destroyed and further analyses are required. Forensic genetics uses various techniques for human DNA extraction, including traditional methods such as organic extraction (phenol-chloroform) [2] and extraction with Chelex 100 Resin [3], depending on the type of biological material. Over time and with the possibility of automating multiple steps in DNA analysis, many laboratories have switched to solid phase extraction (*e.g.*, ion exchange columns, magnetic beads) in which DNA is selectively bound to a substrate such as silica particles. In this way, the DNA is retained while the proteins and other cellular components are washed away, releasing the DNA in a purified form. The extracted human DNA is typically stored at −20 °C, or even at −80 °C in order to prevent the activity of nucleases and preserve DNA for genetic profile typing [4].

Previous research has not clarified whether human DNA extracts also preserve the microbiome – which comprises "all of the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the human gut)" [5]. While the field of forensic microbiomics is still in development, the huge potential of microbiome analysis to contribute to forensic investigations has already been demonstrated, and includes potential applications for postmortem interval (PMI) estimation [6], human identification [7], biological sex determination [8], and even manner and cause of death [9]. The potential to recover microbiome signatures from human DNA extracts held in storage opens up new possibilities for the development and validation of forensic microbiomics methods [10], and ultimately, new opportunities for the investigation of older criminal cases for example by providing new estimation of the PMI, or new information on the identity of an individual or the cause of death.

This study examines microbial signatures recovered from human DNA extracts obtained from six cadavers which were stored frozen for between 5 and 16 years. The type and number of microbial communities identified in these samples are compared with those from human cadavers donated to the Forensic Anthropology Center at Texas State University (FACTS), retrieved using a commercial DNA extraction kit. The study aims to understand: (1) whether the microbiome recovered from human DNA extracts is consistent with the human postmortem microbiome, or whether the presence of microbial communities in these extracts could result from contamination, (2) the effects of prolonged periods of storage at −20 °C on the survival of the microbiome in human DNA extractions, (3) whether the microbial differences that are known to characterise the different stages of decomposition can be observed in these old human DNA extractions, and (4) whether the burial environment, the geographical location where the body was found, the tissue type, and/or the cause of death influence the recovered microbiome. In addition, this study examines some important legal frameworks and guidelines for the storage and re-use of DNA extracts. Re-analysis of such samples and broad comparative studies offer important ways to further develop the field of forensic microbiomics and to generate new information for old forensic cases, yet legislation on how long samples and DNA extractions can be retained differs considerably by country [10,11] and often depends on the outcome or conclusion of criminal proceedings.

1.1. The human postmortem microbiome

The human postmortem microbiome develops from the microbial communities already present in the body before death, and from communities that colonize the body after death [8]. Human cadavers host large numbers of microorganisms and provide an attractive habitat for several microbial communities. These include the thanatomicrobiome (microbial communities and fungi located in human internal organs and cavities, that succeed antemortem microbes within 48 h postmortem [12]) and the epinecrotic microbial and fungal communities which reside on the external surfaces of the decomposing body [8,13–15]. The signature of the thanatomicrobiome and epinecrotic microbial communities is affected by the microbes present in the antemortem microbiome of the deceased and individual biological and lifestyle factors [16]. The antemortem microbiome is known to vary depending on anatomical location on the body (body site) [17], sex [18,19], age [20,21], geographical origin of the individual [22], health condition [23,24], body size [18,19,25] and lifestyle including diet [19,26,27], alcohol consumption [27,28], physical activity [28] and smoking habits [29–31]. By far the largest and most diverse of the antemortem microbial communities resides in the gut [31]. Bacterial decomposition of the body commences in the intestines and spreads from there to the other organs, generally following a certain order: the brain, stomach, bowel, liver, and pancreas are affected before muscles, tendons and bones [8]. The rate of decomposition is determined by several abiotic and biotic variables, including temperature (the higher the temperature, the quicker the decomposition, within limits), cause of death (in septic conditions, putrefaction occurs more rapidly), or the presence of clothing or blankets. Other factors that influence the rate of decomposition include submersion in water, drying of fluids due to ventilation of the environment, body size, and presence of external trauma with consequent loss of blood [32].

The natural fluctuations in the number and types of microorganisms associated with a cadaver have great potential to be harnessed for the development of precise and accurate methods for estimation of the PMI [33–36]. Currently, PMI is generally estimated based on changes to the body, including cooling, lividity, and rigor mortis, followed by gross morphological changes. These are generally divided into five stages of decomposition: fresh, bloat, active decay, advanced decay, and partially or completely skeletonized remains (*e.g.,* Galloway et al. and Megyesi et al. [37,38]). These methods are widely used in forensic anthropology, even though they tend to offer broad estimates only, and they suffer from problems with accuracy and precision [32]. During the early stages of decomposition, the cadaveric ecosystem consists mostly of the bacteria that live in and on the human body before death. After death, the physical and chemical barriers of the immune system that limit bacterial migration break down, facilitating movement of bacteria into nearby tissues [39]. Internal organs are normally considered to be sterile in healthy adults, but within 24 h postmortem, microbes start to proliferate [40]. Due to their persistent sterility for up to five days postmortem, the liver and pericardial fluids are therefore optimal sampling sites to evaluate the degree of postmortem microbial migration [41]. Endogenous microbial populations succeed in a predictable way over the course of the decomposition [42,43]. The thanatomicrobiome has been found to be less influenced by external factors than the epinecrotic microbial community [15], leading researchers to target shifts in the thanatomicrobiome in developing a microbiome-based PMI estimation method [6,34,44–47].

Several studies on human and animal decomposition have identified common trends in microbiome shifts during decomposition, including a drop in microbial diversity associated with the initial stages of

decomposition [16,34,35,48,49] and the presence of four predominant phyla during decomposition: Bacteroidetes, Acidobacteria, Actinobacteria and Firmicutes [34,35,49,50]. Postmortem microbial succession patterns are strongly influenced by oxygen availability, which causes a shift from aerobic to anaerobic taxa during the bloat stage [51]. Microbes proliferate in blood, liver, spleen, heart and brain in a time-dependent manner and their relative abundances vary in different body organs and at specific postmortem intervals [8]. For example, reproductive organs start to decay later than other internal organs during decomposition [52]. The composition of the postmortem microbiome also differs by biological sex, with *Pseudomonas sp.* only having been identified in female cadavers, and *Rothia sp*. only in male cadavers [8]. Organ thanatomicrobiome analyses suggest that facultative anaerobes, such as *Lactobacillus*, predominate in the "short PMI" timeframe, while in a "long PMI" timeframe a predominance of obligate anaerobes like *Clostridium* is observed [53]. Notably, Firmicutes (*e.g.*, *Clostridium, Peptoniphilus,* and *Bacillus*) represent a stable and constant biomarker in microbial communities derived from different body locations [33]. Even the manner of death (natural, accidental, suicide, homicide, undetermined) has been shown to influence the thanatomicrobiome in various organs [33,39,52].

2. Materials and methods

2.1. Ethical approval

This study was submitted and approved in Italy by the Novara Intercompany Ethics Committee (CE 24/21) and in the United Kingdom by the Northumbria University Ethics Committee (submission ref. 24514 and 29218). The study included samples collected from (1) deceased individuals who have been subjected to forensic genetic analysis by one of the authors at the request of the Judicial Authority, and whose genetic material is stored at the Medical Forensic Laboratory of the Department of Health Sciences at the University of Eastern Piedmont in Novara, and (2) deceased individuals whose body was donated to the Forensic Anthropology Center at Texas State University (FACTS) for forensic taphonomic research. Before submitting the study to the two ethics committees (Novara Intercompany Ethics Committee and Northumbria University Ethics Committee), we considered the existing ethical frameworks for using biological samples (or their derivatives) taken from deceased subjects. In particular, we examined the issue of consent to participate in the study. Two main frameworks are relevant in this context: a framework for samples belonging to juridical cases and a framework for samples collected from donated cadavers.

For biological material (and/or samples derived from it through laboratory analysis) taken for judicial purposes from deceased subjects and stored at the Forensic Medical Laboratory of the Department of Health Sciences of the University of Eastern Piedmont, the "Provision relating to the processing of particular categories of data, pursuant to art. 21, paragraph 1 of Legislative Decree 10 August 2018, n. 101" (Annex I, point 5.3) of the Italian Guarantor for the protection of personal data, applies. This provision stipulates that obtaining the informed consent of the deceased is not possible and therefore not required. Permission from the legal next-of-kin is also not required, although in practical implementation of the provision the legal next-of-kin will be informed, and in the case that they indicate that sampling and/or analysis is against the wishes of the deceased, the research will not be further pursued.

The sampling and analysis of tissues from deceased persons whose bodies were donated to FACTS, was conducted in accordance with the Texas Uniform Anatomical Gift Act (Health and Safety Code Chapter 692A) [54]. Whole body donations studied during decomposition outdoors at the Forensic Anthropology Research Facility (FARF; the human taphonomy facility managed by FACTS located in San Marcos, Texas) are acquired for scientific research purposes, through the expressed and documented willing of the donors and/or their legal next of kin. The body donation program complies with all legal and ethical standards associated with the use of human remains for scientific research in the USA.

Finally, we examined the legal framework for processing personal data (biological sex and age) collected during sampling for forensic investigative purposes, as well as for data that may derive from subsequent laboratory activity (the DNA profile). For such additional considerations, see Suppl. Material.

2.2. Chemicals and materials

Nucleospin FLB and Nucleomag 96 Blood kit was purchased from Macherey-Nagel (Düren - Nordrhein-Westfalen, Germany). Platinum Hot Start PCR Master Mix 2x was purchased from ThermoFisher Scientific (Waltham, MA USA). 1x Accuprime Pfx Supermix and SequalPrep™ Normalisation kit was purchased from Invitrogen™ (ThermoFisher Scientific, Waltham, MA USA). QIAamp PowerFecal Pro DNA Kit was purchased from QIAGEN (Hilden, Germany). 16S rRNA primers and agarose were purchased from Sigma-Aldrich (UK). PhiX Control v3 and MiSeq Reagent Kits v2 were purchased from Illumina (Illumina Inc., Cambridge, UK).

2.3. Samples

A total of eighteen samples were collected from 8 human cadavers (5 males and 3 females), derived from Italian court cases (n = 6) and donated to the FACTS (n = 2). The six cadavers (five males, one female) from Italian court cases were discovered in different locations in Northwest Italy (Piedmont and Liguria regions). The cadavers were found in different stages of decomposition, including one in active decomposition, two in advanced decomposition, and one partially skeletonised. Two were found in charred condition (Table 1). The time intervals reported for these cases in Table 1 indicates either the PMI or the time between the discovery and sampling of the subject, as well as the human DNA extraction from the tissue/biological fluid, and ranged between four days and seven years. Several tissues/body fluids, including muscles, blood and organs, were sampled at the scene for the purpose of obtaining human DNA for forensic genotyping. Storage duration of the DNA extracts at −20 °C prior to the start of the present study is reported in Table 1.

The two cadavers donated to the FACTS were placed to decompose outdoors at the FARF. The cadavers were placed unclothed in flexed supine body positions to decompose naturally in shallow oval-shaped pits, one of which remained open during decomposition and one of which was covered immediately with soil. A metal cage was placed over the open pit experiment to protect the remains from large scavengers. Other than this, the pits and cadavers were exposed to the natural elements, including weather, insects and small scavengers. Oral and tooth swabs were collected at different decomposition stages (Table 1). Swabs were stored frozen at −20 °C. DNA extractions were made for the purpose of the present study and the extracts were processed immediately. Therefore, no storage period at −20 °C is reported for these extractions.

2.4. DNA extracts isolated for human identification purposes in Italian court cases

Eleven tissue samples (SG01-02-04-05-08-10-14-15-18-21-22) were collected during forensic investigations from six cadavers found in various locations in North-West Italy (Piedmont and Liguria regions) (Table 1). The tissues were collected either at the scene, from the burial environment or during autopsy using sterile scalpels. The blood sample (SG02) was collected using a sterile swab. DNA extraction was carried out using Nucleomag 96 Blood kit combined with KingFisher mL (ThermoFisher Scientific, Waltham, MA USA). Quantitation was conducted in different ways depending on sample type (tissues or blood). Details on the methods used to quantify the material and to obtain full

Table 1
Samples and associated biological and sampling information: age, sex, decomposition stage, type of tissue or body fluid, deposition type, sampling dates, time elapsed between the death/disappearance/discovery of the subject and the sampling of tissue/biological fluid for each forensic sample and DNA storage length at −20 °C.

Sample code	Age	Sex and individual number	Decomposition stage	Tissue/body fluids	Deposition type	Disappearance/death/discovery	Time elapsed between death/discovery and sampling	Sampling date	Storage at −20 °C
Cadavers from North-west Italy									
SG01 SG02	38	Male 1	Charred	Heart Blood	Car accident near Turin (TO)	Death: 11/12/2004	4 days post-mortem	15/12/2004	16 years
SG04 SG05	40	Male 2	Partial skeletonization	Spleen Quadriceps	Mountain near Turin (TO)	Disappearance: November 2004Discovery: March 2005	6 days post-discovery (March 2005)	05/04/2005	15 years
SG08	–	Male 3	Active decay	Liver	River bank, near Cuneo (CN)	Discovery: March 2007	Not available	March 2007	13 years
SG10	–	Male 4	Advanced decay	Quadriceps	Found exposed near the seaside in Savona (SV), then buried in mountain near SV	Discovery: 03/07/2003	7 years post-death	10/05/2010	11 years
SG14 SG15 SG18	37	Female 1	Advanced decay	Quadriceps	Little river bank, near Asti (AT)	Disappearance: 23/01/2014Discovery: 18/10/2014	6 days post-discovery	24/10/2014	6 years
SG21 SG22	66	Male 5	Charred	Quadriceps Heart	Homicide near Cuneo (CN), found in a car	Discovery: 14/11/2015	4 days post-discovery	18/11/2015	5 years
Body donors studied at the Forensic Anthropology Research Facility (FARF)									
SG103	91	Female A	Fresh	Buccal swab	Open pit	Date of death: 18/04/2015	10 days post-mortem	28/04/2015	–
SG104			Active Decay	Buccal swab			16 days post-mortem	04/05/2015	–
SG105			Fresh	Tooth 3.2			5 years, 2 months, 12 days post-mortem	28/04/2015[a]30/06/2020[b]	–
SG106			Full skeletonization	Tooth 4.3			5 years, 2 months, 12 days post-mortem	03/12/2015[a] 30/06/2020[b]	–
SG107	67	Female B	Fresh	Buccal swab	Buried	Date of death: 05/05/2015	2 days post-mortem	07/05/2015	–
SG108			Fresh	Tooth 3.1			5 years, 1 month, 25 days post-mortem	07/05/2015[a]30/06/2020[b]	–
SG109			Full skeletonization	Tooth 4.1			5 years, 1 month, 25 days post-mortem	17/8/2017[a] 30/06/2020[b]	–

[a] date of tooth extraction;
[b] date of tooth swabbing.

genetic profiles can be found in the Suppl. Material.

2.5. Samples collected from donated human cadavers

A total of seven tooth and buccal swabs (SG103-104-105-106-107-108-109) were collected from the two cadavers at different stages during decomposition (Table 1). Microbial DNA was extracted using the QIAamp PowerFecal Pro DNA Kit following the protocol reported in Procopio et al. [55]. Biological material from the buccal mucosa of both body donors studied at FARF was collected using a sterile swab which was rubbed along the inside of the left and right buccal mucosa (10 strokes each side), during the fresh and active stages of decomposition. In addition, biological material was collected from the surface of extracted single-rooted teeth during fresh and skeletonized decomposition stages, by gently rubbing all surfaces of the extracted tooth for a duration of 30 s using sterile swabs.

2.6. Microbiome analysis

All 18 samples were quantified (or re-quantified, in the case of the samples from the Italian court cases) immediately prior to the execution of the microbiome analyses using NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) (Table S1). NanoDrop was the method chosen for quantification as we expected the presence of PCR inhibitors in the DNA extracts; therefore, we excluded the use of amplification-based quantification methods to overcome any potential limitation. Subsequently, microbial communities were targeted by amplifying the 16 S rRNA locus [56] using forward 515FB (GTGYCAGCMGCCGCGGTAA) and reverse 806RB (GGACTACNVGGGTWTCTAAT) primers [49,57,58] in order to test if any microbial DNA was present prior to any sequencing approach. 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 following Procopio et al. [49] 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 or 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 (run in TAE buffer at 100–120 V).

After confirming that microbial DNA was present in all 18 samples, extracts were sent to the NUOmics DNA Sequencing Research Facility (Northumbria University, Newcastle, UK) for the amplification and sequencing of the hypervariable region V4 of the 16 S ribosomal RNA gene using the Illumina Miseq Next Generation Sequencer (Illumina Inc., Cambridge, UK), following the gold standards suggested by the Earth Microbiome Project to target and sequence the highly variable V4 region

of the 16 S rRNA gene for microbial identification. We followed the method proposed by Kozich et al. [59] and adopted and described in detail in Procopio et al. [55].

Paired-end reads from each sample were sequenced with forward and reverse reads in separate files and processed by means of the microbiome bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology 2), v.2021.2 [60,61]. Denoising and quality control, including removal of chimeras, were achieved by means of the DADA2 [61] plugin and to avoid low quality sequences reads were truncated (250 bp for forward, 240 bp for reverse reads). The classifier adopted for the taxonomic assignment was Silva v.138 (99% OTUs full-length sequences) [62].

2.7. Statistical analysis

Statistical analyses were performed within the computing environment R (https://www.R-project.org/). All the taxon abundances were calculated and graphically plotted with the aid of the PHYLOSEQ v.1.22.3 package [63,64]. Prior to performing formal analyses and creating the figures, pre-processing steps were applied to the Amplicon Sequence Variants (ASV) counts: ASVs with fewer than 5 reads were filtered out and abundances were standardized to the median sequencing depth according to McMurdie and Holmes [64]; ASVs recognized as mitochondrial or chloroplast sequences were also excluded. The Indicator Species Analysis (ISA), (a classification-based method to access the statistical significance of the relationship between species occurrence/abundance and groups of sites) was performed using the multipatt function from the indicspecies v.1.7.9 R package, with 999 permutations [65] in order to assess whether ASVs were significantly associated with a particular stage of decomposition.

3. Results

Quantification and optical density results obtained with NanoDrop at the time of the study are reported in Suppl. Table S1. The samples extracted with the kit specifically designed for the microbiome (SG103-SG109) resulted in lower DNA abundances than the others. The 16 s rRNA gene was successfully amplified in all 18 samples, as shown by the signal they provided on the agarose gel (Fig. 1A and B). The raw data generated for this study have been deposited at the NCBI Sequence Read Archive (SRA-NCBI; https://www.ncbi.nlm.nih.gov/sra) under Project accession number PRJNA773228 and BioSample accession numbers SAMN22451803-SAMN22451820.

From the 18 samples, the sequencing provided a total of 1453,775 useful reads, after DADA2 denoising step, with a variable distribution in the samples, from a minimum of 500 to a maximum of 202,701 (Suppl. Fig. S1). The numbers of identified ASVs ranged between 11 and 516 per sample. An initial number of 1864 ASVs was identified, subsequently reduced to 1822 after removal of contaminants, and to 1388, after ASVs with fewer than five reads were removed. Among the 1388 ASVs, 22 were Archaea, four were unassigned, and 1362 were Bacteria.

First, an MDS/PCoA on unweighted-UniFrac distance was drawn in order to identify possible groupings among different decomposition stages or body sites (Fig. 2). Samples taken at similar decomposition stages cluster closer together than samples collected at different stages of decomposition. In particular, samples in advanced decomposition and fully skeletonised were clearly separated from the other decomposition stages on the first principal component. Charred, fresh and active decay samples clustered on the same side of the PCoA but were separated on their second principal component. Samples were not clustered in a clear way according to the anatomical location on the body (*e.g.,* quadriceps samples belonging to different individuals at different decomposition stages did not cluster together).

Phylum abundances were then evaluated to identify the most prevalent phyla on the whole sample (Suppl. Fig. 2). Firmicutes species were the most abundant, and together with Proteobacteria they represented 79.8% of the overall population. Actinobacteriota and Bacteroidota shared similar abundances (~8%), followed by Chloroflexi (~1%) and Acidobacteriota (~1%).

Phylum abundances examined according to the decomposition stage showed the presence of specific phyla at specific post-mortem stages, *i.e.,* Actinobacteriota were only found in fresh and in fully skeletonised remains, Cyanobacteria only in fully skeletonised remains, and Asgardarchaeota only in charred cadavers). Others (*i.e.*, Firmicutes and Proteobacteria) were found in all samples (Fig. 3). Evaluation based on the two different modes of deposition (*i.e.,* open pit *versus* shallow burial)

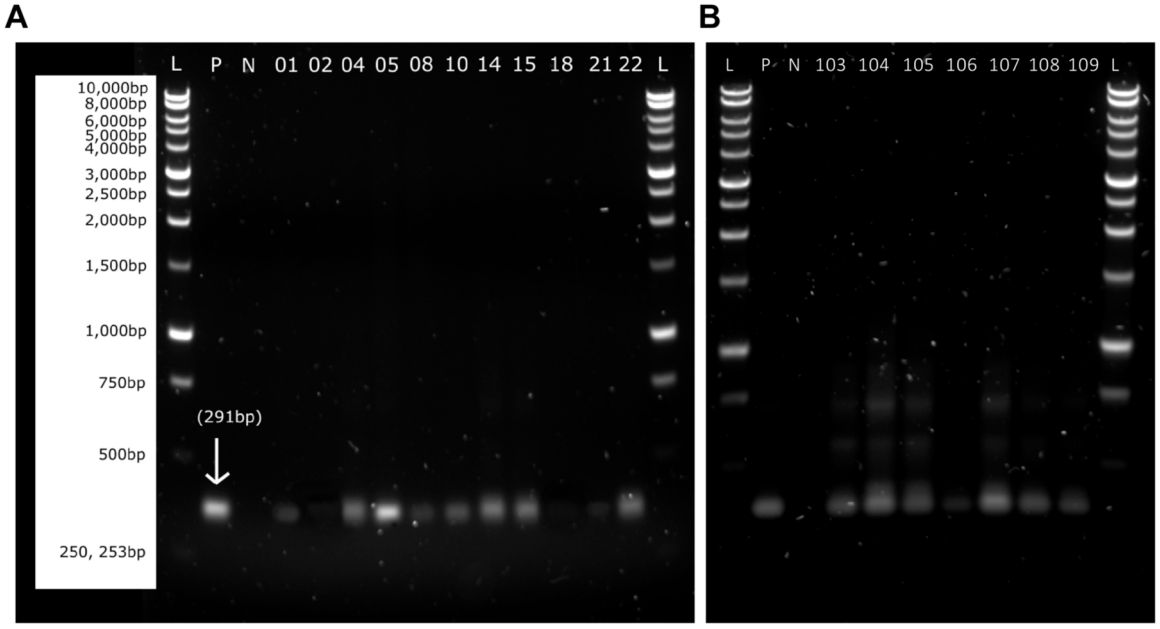

Fig. 1. Agarose gel electrophoresis of the PCR product of the 16s rRNA gene for DNA extracts. A) Lanes L: Promega 1 kb DNA ladder; lane P: positive control (E. coli, BL21(DE3)); lane N: negative control; lanes 1–22: showing amplified 16s rRNA genes from samples SG01 - SG22. B) Lanes L: Promega 1 kb DNA ladder; lane P: positive control (E. coli, BL21(DE3)); lane N: negative control; lanes 103–109: showing amplified 16 s rRNA genes from samples SG103 - SG109.

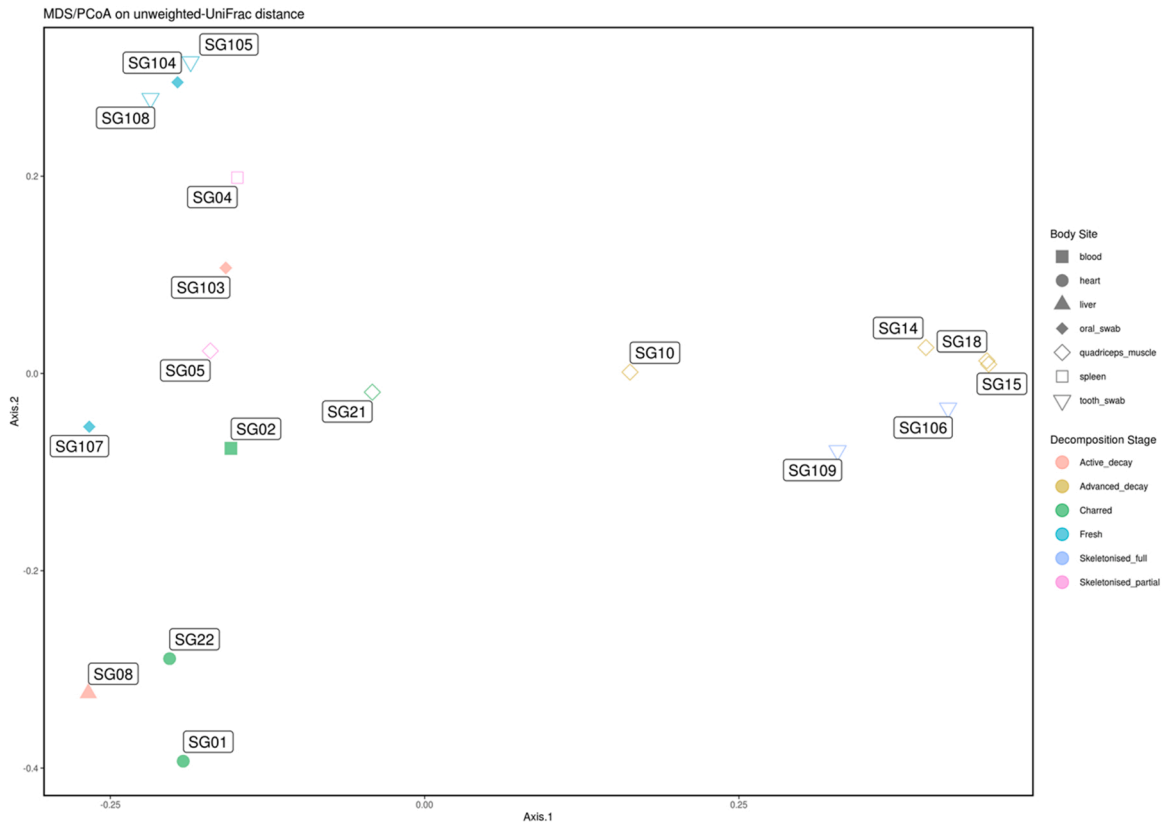

Fig. 2. PCoA (Principal Coordinates Analysis) on unweighted-UniFrac distance. Different shapes represent different body sites used for the DNA extraction, whereas different colours represent different decomposition stages.

revealed an increased abundance of taxa belonging to the Bacillales order in "Female B" (buried) in comparison with in "Female A" (open pit). No other major differences were observed (Suppl. Fig. S3).

This study did not reveal a Core Microbiome (CM) shared amongst all 18 samples. One ASV ("d46e2205", see Suppl. Table S2 for the full identifiers) belonging to the genus of *Escherichia-Shigella* was identified in all but one sample (SG107). The same ASV was shared across all samples derived from Italian forensic casework. Among the fresh DNA extracts from body donors, the CM was instead characterised by the *Enterococcus* identified by the ASV "9908fffa" (see Suppl. Table S2 for the full identifiers).

The indicator species analysis (ISA), run with a significance level of 0.05, identified several ASVs were closely associated with specific groups of interests (Suppl. Data S1). By grouping the samples according to the time in which DNA was extracted (*e.g.,* fresh *vs.* frozen extracts) we found 50 ASVs (p-value <0.05) characterising specifically the group of fresh extracts, including six ASVs with a p-value < 0.01 ("9908fffa", "68a9a8d8", "e93b3133", "0ed3a683", "ff9d93d7" and "581a5501"). When considering the older frozen DNA extracts, we instead found only two ASVs ("43fddf15" and "1a4f8fdc", p-value <0.05). When samples were grouped based on the stage of decomposition, ISA showed 25 species associated with the "fresh" stage of decomposition with a p-value < 0.05 (including 21 with a p-value <0.01), 21 species associated with the "advanced decay" stage (of which 10 had a p-value <0.01), nine species associated with "partial skeletonization" (p-value <0.05), 99 ASVs associated with "full skeletonization" (p-value <0.05) and two associated with the "charred" remains (one with p-value <0.05, one with p-value <0.01).

4. Discussion

4.1. Laboratory analyses

An important aim of this study was to investigate the presence and survival rate of microbial signatures in old human DNA extracts that were stored frozen for extended periods of time, obtained from bodies in different stages of decomposition. DNA quantification results, extracted using Nucleomag 96 Blood kit, unsurprisingly returned higher DNA concentrations in the Italian samples than for the more recent extracts, which were processed with the QIAamp PowerFecal Pro DNA Kit. Whereas the Italian extracts contained both human and microbial DNA, the PowerFecal Pro DNA kit specifically targets microbial DNA, resulting in notably lower concentrations of total DNA. Almost all the ratios calculated for the optical density are in the range of what is considered to be normal for forensic samples and this reflect the nature of the samples included in the study.

All samples included in the study yielded successful amplification of the 16s rRNA gene, despite the fact that some differences in the intensity of the amplicons were visible on the agarose gel (*i.e.,* SG02-18-21 bands were quite faint and SG05 band was the most intense one). The samples with the lightest bands include two extracts from two charred individuals (SG02 from blood and SG21 from quadriceps), and one from a body in advanced stage of decomposition (SG18). It is possible that the weak signal of the SG18 band was caused by technical issues associated with the gel, because two biological replicates extracted from the same tissue type from the same individual (SG14-15) both gave a stronger signal. Other charred samples from the heart muscle (in particular SG01) also resulted in a relatively weak amplification in comparison with the overall sample, likely as a result of the charred condition of the remains.

In order to test if the microbiome recovered from old human DNA extracts is consistent with the expected human postmortem microbiome,

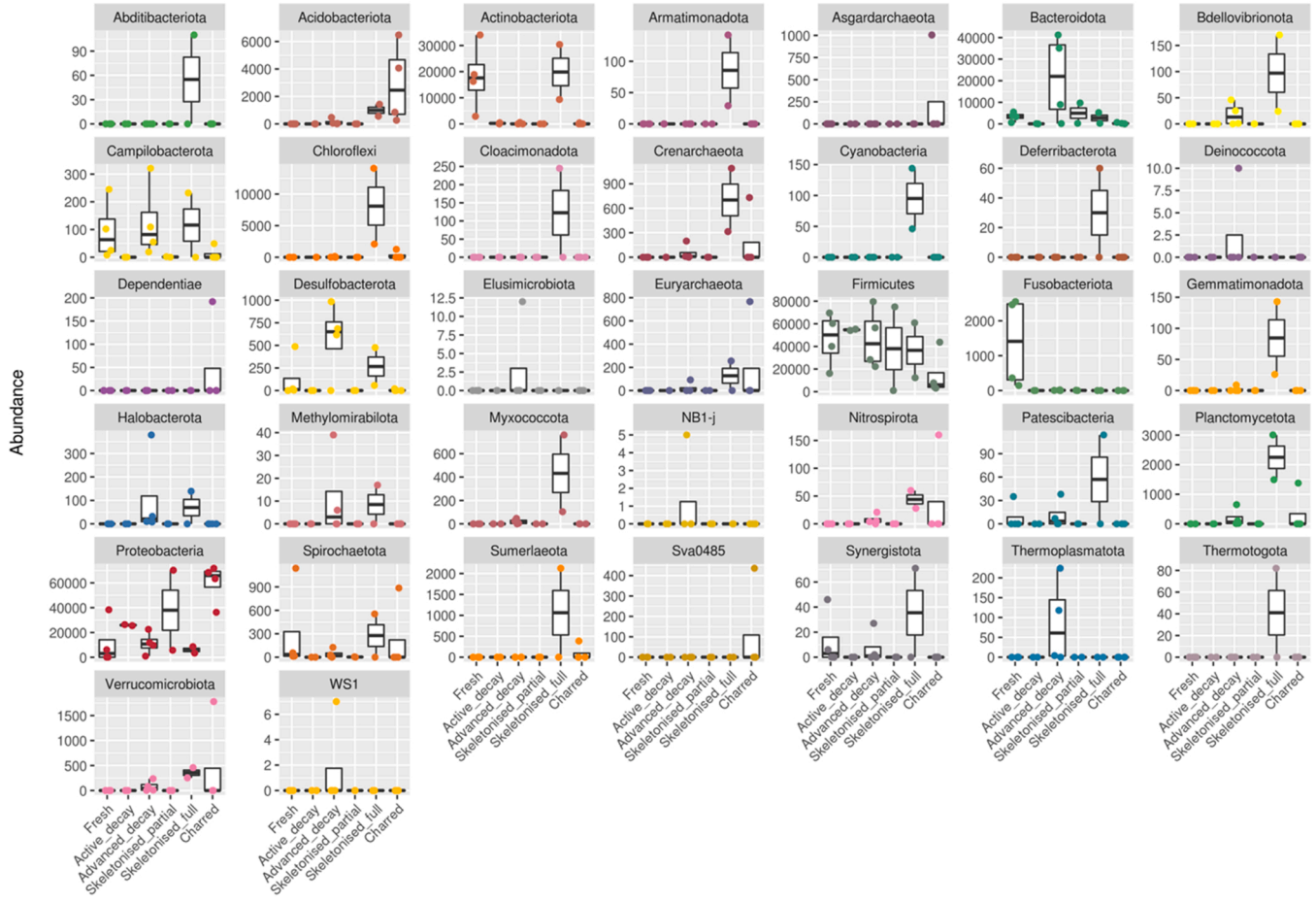

Fig. 3. Phylum abundances arranged by decay stage, built on data from 18 samples divided as follows: fresh (n = 4), active decay (n = 2), advanced decay (n = 4), skeletonised partial (n = 2), skeletonised full (n = 2), charred (n = 4). Taxonomic classification based on Silva v.138.

or whether the microbial communities found in these samples could have resulted from contamination during sample processing or during frozen storage, we compared Italian casework extracts with extractions from samples collected during human decomposition experiments at FARF. The separation of the samples in the PCoA along the first axis shows that newly extracted tooth swabs from skeletonised samples (FARF) share a similar microbiome profile with extracts taken from quadriceps from two individuals in an advanced stage of decomposition (Italian judicial cases). Samples are further separated along the second dimension, in particular those taken from fresh cadavers and those from charred individuals. The fact that all samples but one (SG107) shared one specific taxa further shows that there are some recurring taxa both in "fresh" and in "old" microbial DNA samples, and suggests that the microbiome sequenced from "old" extracts did not originate from recent contamination.

Based on our findings, the prolonged storage of DNA extracts at −20 °C does not impede the successful sequencing of microbial ASVs. According to the number of reads obtained in the library, "fresh" and "old" extracts are similar. The samples which generated the lowest number of reads and ASVs obtained (SG01-02; stored frozen for 16 years), and two other samples that yielded only a small number of reads (SG21-22; stored frozen for 5 years), consisted of charred samples. In these cases, the low yield appears to be associated with the condition of the cadaver rather than the duration of frozen storage, as other non-charred samples which were stored for extended durations (SG04-05) resulted in a relatively high number of reads (*e.g.*, SG04 yielded the highest number).

Among the most prevalent phyla in the study, Firmicutes are the most abundant in all samples, with the exception of charred samples, where their abundance was notably lower. Firmicutes are a large group of bacteria which typically characterise the human bacterial flora [66] and are commonly found in association with soil and aquatic environments [67,68]. High abundances of Firmicutes have frequently been reported in decomposition studies [44,49,50,69–71], particularly in cadavers with large PMIs. The majority of the recovered Firmicutes consisted of Clostridia, followed by Bacilli. Clostridia are well-known obligate anaerobes that are abundant in soil but are also symbiotic bacteria in the human gut. They are able to colonise human tissues rapidly after death [72] and are highly abundant in decomposing remains with both short and long PMIs [73]. Bacilli are primarily aerobic, although several taxa in this phylum are anaerobic but aerotolerant [74]. They are commonly found in the gastrointestinal tract and have a proteolytic effect in the cadaver [75]. Bacilli have been reported as one of the most abundant classes in decomposition studies that examined the different stages of decomposition [71,74,76].

The second most prevalent phyla are Proteobacteria, which are facultative or obligate anaerobes and decomposers that are reported to be one of the most abundant taxa in and on decaying carcasses and cadavers [33,36,44,50,77]. The Proteobacteria identified in this study were found to be equally represented by Alphaproteobacteria and Gammaproteobacteria. Alphaproteobacteria are usually abundant, particularly during active decomposition in grave soil samples but also during advanced decomposition in both soil and skin samples [34]. Gammaproteobacteria have been identified in several studies of bodies with large PMIs, and have been found in different anatomical locations and various depositional environments [8,42,44]. Interestingly, most of the Proteobacteria identified in this study were found in the charred samples.

Amongst the less abundant phyla, we identified nearly equal abundances of Bacteroidota and Actinobacteriota. Bacteroidota have been found in high concentrations in soils during cadaveric and carcass decomposition [34,49,78] but also in and on cadavers [12,44,79]. In this study, they were most abundant in samples collected during advanced decomposition. Actinobacteriota have previously been found in fresh cadavers in oral samples [47,80] but also in partially and fully skeletonised remains [36] and in grave soils [81]. This concurs with our study, in which Actinobacteriota were found to be most abundant in fresh oral swabs and in oral swabs taken from skeletonised remains.

While some of these phyla were associated with most of the stages of decomposition represented in our sample, certain phyla were predominantly associated with specific stages. Fusobacteriota were found mostly in fresh samples, Sumerlaeota mostly in fully skeletonised samples, and Acidobacteriota mostly in charred samples. The ISA results highlighted that several taxa (identified at genus level) were statistically significantly associated with certain individuals or decomposition stages. Interestingly, the group of charred samples are differentiated from the other samples by the presence of the taxa *Pyrinomonas* (p-value 0.0017) and *Cupriavidus* (p-value 0.0472) genera which were identified in all four charred samples. *Pyrinomonas* (from the Greek "pyrino", born of fire, and "monas", unit) are thermophilic bacteria that have been found in geothermally heated savanna soils from volcanic fumaroles [82,83]. To our knowledge, no studies have analysed the microbiome of burnt remains, and *Pyrinomonas* has not previously been observed in association with cadaveric remains. *Cupriavidus* is a genus that includes bacteria that are predators of other soil bacteria and fungi. These are highly resistant to metals and their growth is stimulated by the presence of copper [84]. *Cupriavidus* bacteria have been found both in soil and in clinical samples [85], but they have not been reported in fire-related contexts before. Besides these two indicator species, we observed additional taxa that are highly abundant in the burnt samples, including *Vulcaniibacterium* (Gammaproteobacteria, Xanthomonadaceae), *Escherichia-Shigella* (Gammaproteobacteria, Enterobacteriaceae), *Pseudomonas* (Gammaproteobacteria, Pseudomonadaceae), *Methylobacterium-Methylorubrum* (Alphaproteobacteria, Rhizobiales), *Aeribacillus* (Bacilli, Bacillaceae), *Romboutsia* (Clostridia, Peptostreptococcaceae), *Paraclostridium* (Clostridia, Peptostreptococcaceae) and *Clostridium_sensu_stricto_1* (Clostridia, Clostridiaceae). Some of these taxa have been frequently reported to be associated with decomposing remains and carcasses (*i.e.,* Pseudomonadaceae, Xanthomonadaceae, Enterobacteriaceae [1,44,49,86–88]). *Vulcaniibacterium*, similarly to *Pyrinomonas*, has been isolated from a geothermally heated soil samples [88], and their presence in charred samples is therefore not completely unexpected. *Pseudomonas* are able to produce heat resistant enzymes, including lipases and peptidases [89,90]. *Methylobacterium-Methylorubrum* spp. and *Aeribacillus* spp. are reported to be resistant to exposure to high temperatures [91,92]. Bacteria of the Clostridia class are obligate anaerobes which are able to produce resilient endospores; *Clostridium* genus spores in particular are able to survive extreme and extended heating conditions [93]. *Clostridium* species have also been frequently reported in decomposition studies, and they are considered to be ubiquitous post-mortem communities both in early and in advanced decomposition stages [73]. In sum, the taxa represented in the charred samples consist of bacterial species that are capable of surviving extreme conditions including low oxygen levels and high temperatures (*e.g.*, a burning car). These results suggest that burnt cadavers contain a distinct microbiome, and they warrant further study of the microbiome in burnt remains as well as investigation of the potential for developing specific microbiomic PMI estimation methods for burnt remains.

Due to the limited size of the sample and the number of replicates in each category, as well as the multiple variables potentially affecting the results (*i.e.*, diverse body recovery environments, cause of death, type of tissue sampled), it is not possible to assess whether the identified microbial signatures are consistent with signatures described in the literature as associated with specific stages of decomposition [34,51,69,94]. While our results demonstrate that certain taxa were indeed associated predominantly with specific decomposition stages, the potential influences of the burial environment in this case are as yet unclear. For instance, phylum Sumerlaeota was found almost uniquely in the samples from two skeletonised cadavers. Sumerlaeota bacteria are not yet fully understood, due to a paucity of studies so far, but there is evidence to suggest they are associated with several different soil types and marine sediments [95]. They are characterised by the presence of multiple peptidases which allow them to degrade organic matter and adapt to both aerobic and anaerobic conditions [95]. So far, they have not been reported to be involved in the process of cadaveric decomposition, and considering the type of samples (tooth swabs) and the environment from which the skeletonised samples were collected (FARF, *i.e.*, the same environment and soil) it is possible that the recovered Sumerlaeota result from the local burial environment rather than the stage of cadaver decomposition. Similarly, Fusobacteriota were identified only in fresh samples (oral and tooth swabs). These bacteria are known to be involved with dental pathologies and infections [96] and therefore their presence in these fresh samples is unsurprising and may be associated with the type of tissue, rather than the stage of decomposition of the remains.

Sample SG08, which generated amongst the lowest number of reads and had a very low variety of ASVs, was obtained from the liver of "Male 3", a cadaver which was recovered in active decomposition stage in the proximity of a river bank in March in northern Italy. In this case, the cool river bank, particularly during the winter months in temperate climates (such as in Piedmont) may have reduced or hindered microbial growth, affecting the number of taxa identified in this extract. Microorganisms and microbial activity are strongly influenced by temperature (microbial activity increases as temperature increases and slows with decreasing temperatures), and it is known that temperature affects the microbial decomposition of cadavers in soil [97]. By comparison, samples SG14-15-18 show a notably higher variety of bacteria than SG08. These samples were also collected from a cadaver ("Female 1") found in the proximity of a river bank during winter, however both the environment and the type of tissue sampled differed considerably from those collected from "Male 3". In fact, the cadaver of "Female 1" was found in an advanced stage of decomposition after a prolonged period of time from when this individual was reported missing (nine months), and was almost fully submerged in a wet slime/mud characteristic of the river bank environment. Because the internal organs had liquefied and were not distinguishable, only muscle tissue was sampled. The collected muscle tissue sample was contaminated with the surrounding mud and decomposition liquids, in contrast to the liver sample collected from "Male 3". The difference in the variety of bacteria identified in both cases is likely due to the combined effects of temperature/seasonality, humidity, PMI/decomposition stage and the type of tissue sampled. SG10 also comprised a muscle sample, collected from "Male 4", a cadaver in advanced stage of decomposition. In this case the cadaver was recovered from a coffin buried in a cemetery in the Ligurian Apennines, an area which is characterised by low temperatures. SG10 revealed a lower number of identified microbial species and read counts than SG14-15-18, which may have been related to the cold environment which is likely to have led to slower microbial growth than in the river bank environment. Furthermore, "Male 4" was sampled seven years after burial, so the expected microbial presence, variety and activity was lower than the one expected for "Female 1".

We compared the two donated cadavers to assess the potential effect of exposure of the body *versus* burial of the body. "Female A" was placed in a pit that remained open, and "Female B" was buried in a shallow grave. No major differences in the microbial composition of the tooth swabs of these individuals were identified, although Bacillales order were more abundant in "Female B" than in "Female A" (Suppl. Data S1). Bacillales belong to Firmicutes phylum and include *Bacillus*, *Listeria* and *Staphylococcus* genera. Several bacteria belonging to *Bacillus* spp. were found to be notably more present in the buried donor. *Bacillus* are ubiquitous in soil and represent a large percentage of bacterial residents

in the soil habitat [98]. While the lack of replicates impedes attaching significant conclusions to this observation, the presence of a high abundance of *Bacillus* spp. in the swab taken from the buried individual, is therefore not unexpected. We did not observe any other major differences between the microbiome of these two samples. These results generally concur with the findings of other analyses conducted on the same cadavers (including an isotope study [99,100] and a bone proteomics study [101]), which showed that the difference in mode of placement (shallow open pit *vs.* shallow burial) did not play a significant role in biomolecule preservation and changes in the isotopic signatures of different tissues.

Our findings emphasise the fact that the presence and recovery of specific microbial taxa is dependent on a number of important variables, including the stage of decomposition of the cadaver, modification of the remains (*e.g.*, burning), the type of tissue sampled, and the environment in which decomposition took place. It is essential to understand the effects of these variables in order to evaluate recovered microbiomes and to further develop and validate microbiomics approaches for forensic purposes.

4.2. Legislative frameworks for the storage of DNA extracts from forensic casework

The results obtained from this work underscore the previously noted importance [4,102] of the storage and preservation of what was obtained from the biological traces found at the crime scene (*i.e.,* extracts of DNA, RNA, proteins). The potential value of such samples for both the methodological and technical advancement of microbiomics, and application of microbiomics in forensic practice are significant. A lack of consistency in the long-term preservation of such samples, as well as variation in legislative frameworks for their use for additional research purposes, risks undermining this largely unexplored source of information.

On a global level there is no shared vision regarding storage of such extracts: a shared framework or legislation on this topic is lacking and guidelines differ considerably between countries. The National Institute of Standards and Technology (NIST), through the Technical Working Group on Biological Evidence Preservation, suggested best practice guidelines for evidence handlers in order to ensure the integrity, to prevent the loss, and to reduce the premature destruction of biological evidence after collection [10]. They recommend retaining indefinitely any biological evidence collected during homicide investigations for open and/or charges filed cases. For other types of crime and case statuses they advise different retention lengths (at a minimum, for the duration of the statute of limitations) on a case-by-case basis. According to this document (page IV), biological evidence consists of any "sample of biological material – hair, tissue, bones, teeth, blood, semen, or other bodily fluids – or evidence items containing biological material". It is not clear if this definition encompasses DNA extracts, and therefore it is not clear whether the listed recommendations apply to these samples or not. However, in the paragraph referring to the packaging and storing of biological evidence, specific recommendations on the storage of extracted DNA are provided, with the indication that the best way for long-term storage of DNA extracts (*e.g.,* $>$ 72 h) is the frozen preservation (*e.g.*, ≤ -10 °C).

The European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) provided an audit of legislative frameworks within the European Union for the collection, retention and use of forensic DNA profiles, however the document refers to either biological samples (intended as the biometric material obtained from a suspect, also known as "reference sample") or to genetic profiles obtained from those. No specific guidelines on the preservation of DNA extracts derived from "casework samples" (*e.g.,* evidence obtained at the crime scene) are provided [11], although such samples are mentioned in the document.

The European Network of Forensic Science Institutes (ENFSI) DNA Working Group created a DNA Database Management Review and Recommendations document which states that "the cell material of crime scene stains from which a DNA profile has been generated is usually stored" [103]. In this case again, the definition seems to be quite generic and it leaves room for interpretation; specifically, there is no clear guidance on the duration of this storage, nor if DNA extracts should or should not be considered as "cell material". The document provides guidelines on how to deal with "reference samples", their preservation and storage, but lacks information on how to deal with casework samples. ENFSI suggests considering the possibility of performing additional DNA testing when there are doubts about the identity of the donor of the trace, implying a need to preserve DNA material obtained from casework samples (*e.g.*, in cases where the original biological sample was exhausted during previous attempts of DNA extraction).

In Italy the question is still open. The law n. 85 of 30 June 2009, (Law n. 85, 2009), that introduced the national DNA database, and the Decree of the President of the Republic 7 April 2016, n. 87 (Regulation containing provisions for the implementation of law no. 85) highlight technical and procedural criteria, and procedures for the sampling and storage of biological samples ("reference samples"). However, there is no mention at all of the preservation of the biological material obtained from the evidence ("casework samples"). The only reference in the Italian legislation to preservation is given in art. 10 paragraph 2 of Law 85/2009, which considers the possibility of reopening unsolved cases without, however, any mention of methods and duration. In this regard, art. 13 (Law n. 85, 2009) regulates the deletion of profiles and the destruction of biological samples following acquittal with a final sentence or if the crime does not exist. In all other cases, as provided for in paragraph 4 art. 13, the profile remains in the database no more than 40 years, while the biological sample is stored no more than 20 years. With regards to DNA extracts, section II of the DPR, chapter IV art. 24, states that they must be destroyed after complete typing, therefore precluding the possibility of obtaining a new typing in the future. "Casework samples", which in Italy are instead known as "biological evidence", are only mentioned in art. 6 (Law n. 85, 2009) but again, there is no mention of measures for preservation and future usage modalities of DNA extracts derived from them.

Recently in the United Kingdom the National Police Chiefs' Council (NPCC) has agreed to the guidelines adopted by Police Forces in England and Wales, in which this question has been covered in more detail. In the NPCC Forensic Retention Guidance v. 1.0 the preservation of the generated material, which is the material created during the examination of an evidence, is discussed. This encompasses different kinds of samples such as slide mounted fibres, scanning electron microscope stubs, but also DNA extracts. According to this document, generated material is retained or returned by the Forensic Unit, the existence and location of this material is documented on the case file and the retention period depends on the offence (case category)[3] and can range from a minimum of 1 year to a maximum of 30 years [104].

In sum, there is a distinct lack of consistency in legislation and guidelines internationally, which significantly impacts the capability to perform additional research on old cases using new technologies and therefore also limits the potential to solve them.

5. Conclusions

This study shows that it is possible to recover microbial signatures from "old" human DNA extracts comparable to those from recently extracted microbial DNA samples from decomposing cadavers. The microbial communities we identified from DNA extracts from casework samples collected during different stages of decomposition are consistent with those found in other studies with similar post-mortem intervals and with the recent FACTS samples we included in this study.

[3] Major crime (MoPI group 1 offences), Serious crime (MoPI group 2 offences), and Non-serious/volume crime (MoPI group 3 offences).

We can conclude that long-term storage of DNA extracts at −20 °C does not affect the survival of the microbial signature during a time-frame of between 5 and 16 years. By contrast to the decomposition stage, the type of tissue, and the environmental conditions in which the decomposition took place, the duration of frozen storage does not seem to affect microbial DNA yield and successful sequencing. The microbial signatures contained in material related to older casework therefore represent an important source of medico-legal and scientific information pertaining to long time frames and PMI estimation.

We found a high abundance of Firmicutes and Proteobacteria in our study, which is consistent with other decomposition studies conducted on various PMIs and different anatomical locations and depositional environments. We also identified phyla that are predominantly associated with specific stages of body decomposition, such as Fusobacteriota taxa in the fresh stage and Sumerlaeota taxa in the skeletonised stage. Furthermore, this study identified for the first time the presence of *Pyrinomonas* and *Cupriavidus* genera in charred samples. These taxa have previously been found in association with volcanic soils and in clinical samples, but they have hitherto never been reported in cadaveric samples. This observation expands on the very limited current knowledge on burnt and charred remains, and opens important new avenues for research.

Considering the potential future use of older human DNA extracts to advance microbiomics approaches, such as for PMI estimation, we argue that there is a need for the scientific community and legislators to define a common standard and protocol for retaining case materials for the purposes of new analyses and future investigation, prosecution or appeals. The distinct differences between countries at present impede the comparative studies that are needed in order to further develop the forensic applications of microbiomics. Amongst the inconsistencies found, the use of different terminologies to indicate reference and casework samples, limits the potential for comparative studies and complicates correct interpretation of the few existing guidelines.

For this reason, it is essential to clarify and standardise the terminology that has to be used in forensic genetics laboratories when referring to different types of evidence, samples and DNA extracts. Within individual national frameworks, the lack of clear guidelines on the precise legal status of and appropriate procedures for retaining and storing extracts, means that the potential for their use to yield new case evidence and advance forensic microbiomics, currently remains largely unused. To maximise the chances of obtaining new relevant information from old samples for the resolution of cold cases it is highly important that appropriate scientific bioethics and jurisprudence frameworks are developed in tandem with rapidly progressing technological innovations.

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Author contributions

Giulia Sguazzi: Investigation; Roles/Writing - original draft; **Hayley L. Mickleburgh**: Conceptualization; Methodology; Roles/Writing - original draft; Writing - review & editing; **Stefano Ghignone**: Data curation; Formal analysis; Software; **Samuele Voyron**: Data curation; Formal analysis; Software; **Filippo Renò**: Funding acquisition; **Mario Migliario**: Funding acquisition; **Federica Sellitto**: Investigation; **Flavia Lovisolo**: Investigation; **Giulia Camurani**: Investigation; Roles/Writing - original draft; **Nengi Ogbanga**: Investigation; Roles/Writing - original draft; **Sarah Gino**: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision;Roles/Writing - original draft; Writing - review & editing; **Noemi Procopio**: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Roles/Writing - original draft; Writing - review & editing.

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Declaration of Interests

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2022.102686.

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