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# Microbial decomposition in experimental single and mass graves: New insights on post-burial interval estimation

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ABSTRACT

Soil microbiomes are increasingly recognized as valuable indicators in forensic investigations, but microbial dynamics in mass graves remain poorly understood. This study investigates differences in microbial succession between individual graves (IG) and mass graves (MG) with human body donors and evaluates the potential of soil microbiome data to predict post-burial interval (PBI). Using ASV-level assessment, we analysed soil samples collected over time from both grave types in a controlled decomposition experiment. At the final timepoint (M18), MG and IG soils exhibited significantly different microbial compositions, with specific taxa, some associated with specific decomposition stages, enriched in each context. A regression model trained on IG samples predicted PBI with a mean error of 2.68 months when adjusted for seasonal variation but performed poorly on MG samples (RMSE = 7.12 months), highlighting ecological complexity and reduced generalisability. These findings underscore the importance of studying MG-specific microbial processes and caution against applying models developed from single-body burials to mass grave contexts. As mass graves are encountered in humanitarian and criminal investigations and establishing the duration of burial can be an important component of forensic reconstruction, our findings highlight the value of further research into context-specific microbiome models and their integration alongside existing methods for detection and time estimation in complex burial environments.

## 1. Introduction

The post-mortem microbiome or necrobiome, i.e., the microbial species associated with decomposition [1], holds great promise in its usefulness for investigating temporal patterns of decomposition. Within the necrobiome, endogenous microbial communities from the remains (cadaveric microbiome) interact with naturally occurring microorganisms in the soil (soil microbiome) throughout decomposition [2]. Although these communities are initially distinct, the influx of decomposition fluids and gases from the cadaver(s) alters the local soil environment, leading to changes in nutrient availability, oxygen levels, and pH that drive microbial succession in both habitats [3,4]

Microbial succession has traditionally been investigated in the context of estimating the post-mortem interval (PMI). The principle behind using the necrobiome in this way is based on the predictable manner of microbial succession in decomposing bodies, where various stages of decomposition can be identified by characteristic microbial profiles [5,6]. Microbial community composition and/or abundance can therefore function as a “microbial clock” [7] to estimate PMI. Essentially, these studies have shown that microbial community composition and function shift systematically in line with decomposition stages and with insect access [7,8].

Despite advances in next-generation sequencing for PMI estimation, comparatively fewer studies have examined how burial environments

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change over time in response to decomposition. Post-burial interval (PBI) refers to the length of time a body has been interred and focuses on the ecological and microbial changes that develop in grave soil after burial [9]. Several recent studies show that soil microbial succession can provide temporal signals linked to burial duration. One study demonstrated that shifts in grave soil bacterial communities could be used to estimate time since burial using random forest models, with nitrogen content and soil temperature improving accuracy [10]. Another investigation reported linear microbial succession patterns in buried cadavers over a two-month period [5], and a separate study identified specific taxa associated with burial periods greater than 120–150 days [11]. Work in forensic entomology has also noted that PBI remains underrepresented compared with PMI and that burial environments alter succession patterns relative to surface decomposition [12]. Together, these studies show that while PMI frameworks capture early post-mortem change, there is a clear need for PBI focused approaches that describe microbial transformations in soil over extended burial periods.

However, even within PMI-focused research, knowledge gaps remain regarding the post-mortem microbiome [13]. One issue is that most of the current research relies on animal models to infer post-mortem microbiome behaviour in human decomposition. Ethical, legal, and logistical constraints limit access to human cadavers, making animal models such as mice [7,14], pigs [15–17], and rats [18] essential for controlled studies. Although these models have advanced our understanding of decomposition, they may not accurately reflect how human cadaveric microbes interact with burial environments, particularly over long intervals. The need for human-based research to understand decomposition related changes is of particular importance especially following a study suggesting that animal models, while useful, may not accurately represent human decomposition [19].

Several studies have however extended microbial succession investigations to human cadavers, moving beyond animal models. For example, succession of skin microbial communities in humans has been documented during the first 12 h post-mortem [20]. Investigations of soil microbial communities beneath human remains have also shown that taxonomic shifts and environmental predictors (e.g., pH, conductivity) can be incorporated into PMI estimation models [21]. Yet, broad systematic reviews show that while microbial succession appears promising for PMI estimation, most human-based studies remain limited in sampling scope, often focusing on the human body rather than the surrounding soil [22–25]. These human-based studies also remain largely confined to single deposition or surface contexts and rarely focus on long-term decomposition or complex burial environments, including graves with multiple bodies. Hence, while microbial succession research shows strong potential for PMI estimation, its utility for predicting PBI, particularly in human burials, remains underexplored.

Some research, however, has explored human post-mortem microbial changes in various burial conditions [26] or interaction relation to insect activity [27]. Microbial profiles in buried remains differ from surface depositions as burial tends to slow decomposition rates, sometimes by up to eightfold, due to restricted insect access and differences in surface and subsurface temperature and moisture [28–31]. Yet, despite these differences, long-term grave soil microbial succession remains poorly characterised. This represents a critical gap for applications exploring the age of a grave based on how long soil from the grave site has been displaced.

As noted, there is little to no research available on the post-mortem microbiome within complex burials, such as mass graves. A mass grave has been defined as a single deposition environment containing at least two bodies [32]. Mass graves can be used to conceal remains after human rights violations and/or criminal activity [32,33]. The presence of multiple remains in a mass grave results in complex decomposition processes, with potential interactions between the microbial communities associated with different individuals. These factors may produce microbial signatures that differ substantially from those observed in single graves, highlighting the need to understand how multiple cadavers collectively shape soil microbiome dynamics.

Only a small number of studies have attempted to address decomposition research questions in mass grave contexts. For example, a preliminary study using rabbits demonstrated that carcass position influences decomposition rate [34]. To the best of our knowledge, microbial community profiles associated with multiple human cadavers in a mass grave have never been characterised. Given the complexity of simultaneous decomposition processes, documenting necrobiome development in mass graves is essential for advancing fundamental understanding of soil changes driven by the presence of multiple decomposing cadavers. It could also serve to improve the applicability of microbial models of PBI and grave age estimations involving multiple remains.

Unknowns regarding decomposition processes in mass graves, also pose challenges to forensic archaeological excavation, documentation, and sampling strategies. Differences in gross decomposition stage are a known complicating factor in the excavation and collection of evidence from mass graves and have been hypothesized to result from differential microbial development in these environments [35]. Differential decomposition, often characterized by advanced (skeletonized) stages of decomposition at the periphery of the mass of bodies in a grave, and relatively fresh stage decomposition at the core of the mass, affects visibility and the identification of remains and materials. This influences critical decisions during excavation, such as which areas of the grave need to be prioritized during digging, recording, and sampling. Equipment, protective clothing, and packaging and handling of remains must also be adapted depending on the condition of the remains [35–38].

Documenting taphonomic evidence and establishing the post-mortem interval (PMI) and post-burial interval (PBI) is also key to corroborating witness statements and other evidence in mass grave investigations [39]. Clarifying the relationship between microbial decomposition processes in mass graves and key taphonomic features, such as differential decomposition, would therefore provide valuable information with which to optimize excavation, documentation, and sampling strategies, help to maintain standards across the grave, and support processes of criminal prosecution.

Thus, as part of a larger interdisciplinary project exploring mass and single graves through taphonomic experiments with human body donors [40], this research compared post-mortem microbial communities in soil from individual and mass graves buried for 18 months. Microbial community dynamics in grave soil were analysed at multiple time points throughout burial until excavation of the remains after 18 months. While the burial date and time was consistent for all donors, the time elapsed between time-of-death and time-of-burial varied between donors. Therefore, this study models PBI by analysing microbial community changes in soil as a function of burial duration. Hence, this study evaluates the potential of the soil microbiome as a tool for estimating time since burial and consequently, the age of a grave. However, it is important to note here that in the period between time-of-death and time-of-burial, all bodies in this study were stored frozen. We also demonstrated in a previous study [41] that freezing did not significantly alter the cadaveric microbiome, ensuring comparability between the microbiome at death and at burial.

The outcome of this research is intended to add to existing knowledge on human decomposition in individual and mass grave contexts and develop robust microbiome models for estimating post-burial interval (PBI). It also aims to assess the transferability of single-grave PBI models to mass graves and provide comparative data for future mass grave taphonomic experiments. Finally, this research seeks to suggest improvements to sampling strategies in mass graves, particularly for microbiome research.

## 2. Materials and methods

### 2.1. Ethics, donor information and sampling for microbiome analysis

Nine human donors were included in this research at the Forensic Anthropology Center at Texas State University (FACTS), which manages an outdoor human taphonomic research facility in San Marcos, Texas, USA: the Forensic Anthropology Research Facility (FARF). All procedures were performed in compliance with relevant laws and institutional guidelines, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and have been approved by the appropriate institutional committees. All donations complied with FACTS guidelines for body donations and with legal and ethical regulations for research on human remains in the USA [42]. Documented informed consent was signed and obtained from all participants and/or next-of-kin, and ethical approval was obtained from Northumbria University (ref: 21514) for the extraction and analysis of microbial DNA obtained from samples collected in this experiment. Further details of the demographics of the human donors, on their clinical information and on the assigned graves for the experiment are detailed in Table 1.

### 2.2. Grave descriptions

The experiment was carried out in an area of the Forensic Anthropology Research Facility (FARF) that had not been used previously for decomposition studies, to prevent the influence of pre-existing microbial populations associated with human decomposition on analysis and subsequent results. The mass grave (surface dimensions: 2 m x 3 m; base dimensions: 2 m x 2.5 m; depth 0.75 m) was dug alongside three single graves (0.6 m x 2 m x 0.75 m) in an area of FARF that measures approximately 5 m x 19 m (Supplementary Fig 1) and is located at 29°93’47’’ N, 7°98’13’’ W. The graves were separated from each other by 2 m and were dug perpendicularly to the natural flow of water to prevent cross contamination.

**Table 1**
Donor demography details and grave allocations.

| Human Donor ID | Code | Grave Assigned | Sex | Age | Clinical information |
|---|---|---|---|---|---|
| 2021.003 | D3 | Mass Grave (MG) | F | 90 | No chemotherapy/radiation treatment; Antibiotic use unknown. |
| 2020.011 | D11 | Individual Grave 1 (IG1) | F | 51 | Chemotherapy 1 month before death; Antibiotic use unknown. |
| 2021.012 | D12 | Individual Grave 2 (IG2) | M | 69 | Edentulous; No chemotherapy/radiation treatment; Antibiotic use for UTI 3 months prior to death. |
| 2021.013 | D13 | Mass Grave (MG) | M | 70 | No chemotherapy/radiation treatment; Antibiotic use (Zosyn) of unknown timing. |
| 2021.014 | D14 | Mass Grave (MG) | M | 54 | Amputee (right leg); No chemotherapy/radiation treatment; Antibiotic use unknown. |
| 2021.020 | D20 | Mass Grave (MG) | F | 66 | No chemotherapy/radiation treatment or antibiotics. |
| 2021.021 | D21 | Mass Grave (MG) | F | 66 | Edentulous; No chemotherapy/radiation treatment; Antibiotic use unknown. |
| 2021.022 | D22 | Mass Grave (MG) | M | 78 | Edentulous; No chemotherapy/radiation treatment; Taken antibiotics for a UTI at some point in last few months prior to death. |
| 2021.023 | D23 | Individual Grave 3 (IG3) | F | 77 | No chemotherapy/radiation treatment; Antibiotic use unlikely. |

Six of the nine human donors were buried in the experimental mass grave (MG) and the remaining three were buried in individual graves (IG – IG1, IG2 and IG3), as in Fig. 1.

Two additional pits, with corresponding dimensions to the experimental mass and individual graves (Control Mass Grave 1 [CMG1] and Control Individual Grave 1 [CIG1]), but without body donors, were created as control graves. All graves in this study were machine-dug with a toothed-bucket backhoe to simulate conditions often found in forensic casework wherein victims are often buried in shallow clandestine graves, less than 1 m deep [43]. Bodies in the experimental graves were dressed in cotton t-shirts and shorts and were buried with small items, such as jewellery and wallets, while the control graves were filled only with clothing and the aforementioned items.

### 2.3. Soil sampling

To assess soil microbial community differences between single and mass graves, and to test the accuracy of soil microbiome analysis for PMI estimation in single and mass graves, grave soil samples were collected before burial, at selected time points during burial, and during excavation and recovery. Samples were collected monthly during burial for the first three months, and then at three-month intervals for the remaining experimental period. Hence, the soil sampling frequency occurred as shown in Table 2, where M refers to month. A comprehensive description of environmental conditions, including soil temperature and moisture content, is also detailed in a geophysical imaging study of the experiment [44].

Samples at M0 were taken by combining soil obtained from three corners of each grave to represent the microbial community throughout the grave. This was done at surface level (0 cm) and repeated at 10 cm depth intervals until the bottom of the mass, single and control graves. At M18, the graves were excavated manually by removing 10 cm spits of grave soil, using clean gloves at each layer. Following excavation of each layer, the sampling area was “cleaned” with a bleached trowel by gently scraping the surface to remove any loose soil. Soil was collected in sterile 1.5 mL Eppendorf tubes by inserting the Eppendorf tube diagonally into the soil to avoid cross contamination when using collection tools. An overview of the layers and sampling locations at M18 is illustrated in Supplementary Figure 2.

During burial all samples were collected using an auger to core downwards vertically at a predetermined depth and location (A, B and C) in each grave to ensure minimal disruption to the soil and cadavers. The sampling points A, B and C were targeted at depths of approximately 60 cm, 33 cm and 50 cm respectively. Actual depths for each sample obtained are listed in Supplementary Table 1. Finally, control samples were collected at all timepoints throughout the experiment from an area outside of the experimental plot (located approximately three meters away perpendicularly from the south side of IG1) to account for seasonality changes or any other change to the soil microbiome not influenced by decomposition. This is also depicted in Supplementary Figure 1.

### 2.4. DNA extraction and sequencing

Microbial DNA extraction was performed using the DNeasy 96 Powersoil Pro kit (Qiagen, Germany) by following the manufacturer’s protocol, using approximately 0.25 g of soil. Additionally, a previously published sample loading protocol [45] was used to avoid cross-contamination when loading samples onto the 96 well plate. Sample sequencing was carried out at the NU-OMICS DNA sequencing facility (Northumbria University in Newcastle, UK) on an Ilumina MiSeq platform. Library preparation and sequencing of all samples followed the Schloss wet-lab MiSeq SOP [46]. Targeting and sequencing of the V4 region (using 515 F and 806 R primers) of the 16S rRNA for bacterial

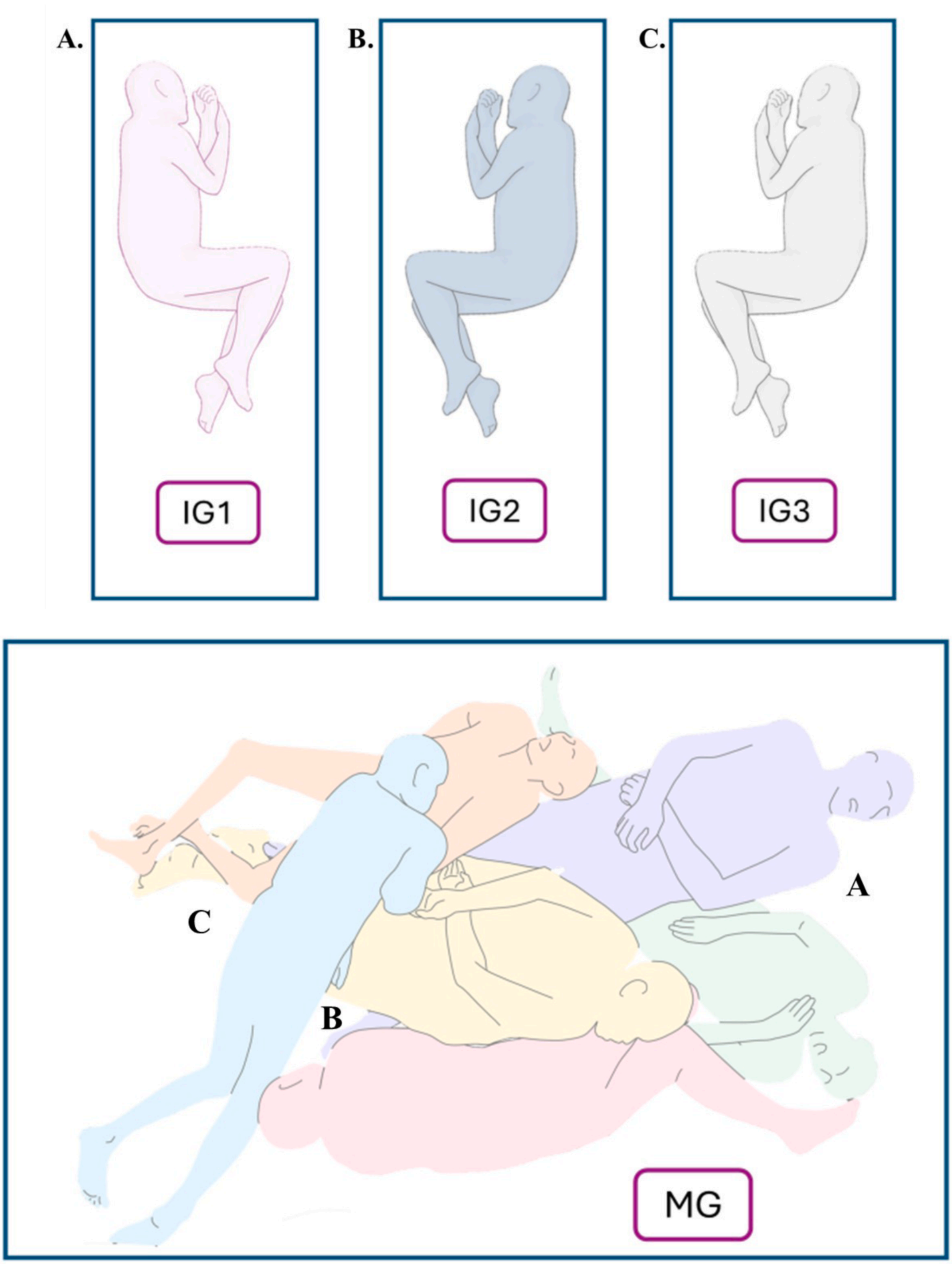

**Fig. 1.** Individual graves (A) IG1, (B) IG2 and (C) IG3 after donor deposition (above). Mass grave (below) showing the position of the donors and grave IDs. Points A, B and C refer to soil sampling locations during the decomposition period at the specified time points.

identification were done following the gold standards suggested by the Human Microbiome Project. Raw reads were processed in QIIME 2 (version 2022.11), and the taxonomic classifier adopted was the QIIME2 release SILVA-138–99 database. Sequences identified as chloroplasts or mitochondria were removed prior to downstream analyses and resulting qiime2 artefacts were imported into R for further analysis.

### *2.5. Statistical analysis*

To identify taxa discriminating between grave types, analyses were restricted to samples collected at M18 at a depth of 70 cm (a total of 41 samples: MG = 15; IG = 26). ASVs (amplicon sequence variants) present in fewer than five samples were excluded to reduce sparse features. LEfSe (Linear Discriminant Analysis Effect Size) analysis was conducted using microbiomeMarker (version 1.80). A random forest classifier was trained with 10-fold cross-validation for model evaluation, and a two-class summary function was used to summarise prediction outputs and assess the model's ability to discriminate between MG and IG samples.

To evaluate whether post-burial interval (PBI) could be predicted from microbial community profiles, all samples collected across the

**Table 2**
Sample collection timepoints, with reference to actual period of collection.

| Sample Collection Month | Actual Month |
|---|---|
| M0 | May 2021 |
| M1 | June 2021 |
| M2 | July 2021 |
| M3 | August 2021 |
| M6 | November 2021 |
| M9 | February 2022 |
| M12 | May 2022 |
| M15 | August 2022 |
| M18 | November 2022 |

entire experimental period were included but limited to those obtained in the 30 – 40 cm depth range, as collection at timepoints M1-M15 only occurred in this range. The dataset was further refined toward taxa most reflective of decomposition dynamics by calculating the coefficient of variation for each ASV across samples. Taxa below the third-quartile threshold were excluded to retain highly variable ASVs across the experimental period for modelling. The random forest regression model for predicting PBI was trained with this refined dataset using only IG samples with 5-fold cross-validation. All random forest analysis were carried out using caret (version 6.0–94).

Finally, to minimise the influence of background soil microbial communities unrelated to decomposition, a weighted subtraction method was applied. For each taxon, its abundance in the grave soil was decreased according to its proportional abundance in seasonality-matched control soils, ensuring that only decomposition-associated microbial shifts were retained. Negative adjusted values were set to zero to maintain biological interpretability.

## 3. Results

### 3.1. Sequencing and data filtering

A total of 342 samples were included in the sequencing project, comprising 14 controls (extraction and sequencing controls) and 328 soil samples. Sequencing efforts resulted in a total of 11,662,251 reads across all samples, with the number of reads per sample ranging from a minimum of 0 (from sequencing controls) to a maximum of 100,105. The average number of reads per sample was 26,090, while the median number was 19,434. No ASVs were observed with a total sum of one or less, and no singletons (ASVs with only a single read detected across all samples) were identified. The sequences were mapped to a total of 7847 ASVs. After quality filtering, 1779 ASVs were retained.

### 3.2. Baseline grave soil: comparison of IGs and MG

Baseline analysis of pre-deposition (M0) and control soils showed no significant differences in alpha diversity across grave locations (Shannon index; Kruskal–Wallis, $p = 0.79$; Fig. 2A), indicating similar microbial richness and evenness across sites prior to decomposition. In contrast, microbial diversity differed significantly with depth ($p = 0.0025$; Fig. 2B), with topsoil exhibiting higher diversity than deeper layers.

Beta diversity patterns supported these findings. PCoA of Bray–Curtis dissimilarities showed strong separation by depth (PERMANOVA, $R^2 = 0.581$, $p = 0.001$), while grave type explained a smaller proportion of variation ($R^2 = 0.084$). Topsoil communities clustered more tightly across grave types compared with deeper samples, which showed greater heterogeneity. Control soils remained tightly clustered across all timepoints, indicating minimal temporal change in undisturbed soil microbial communities.

However, microbial diversity varied significantly with soil depth (Fig. 2B; $p = 0.0025$), with topsoil exhibiting greater diversity than deeper layers, likely due to environmental gradients.

### 3.3. Soil microbial communities after 18 months in IG and MG

At the M18 timepoint, alpha diversity differed significantly across grave types (Kruskal–Wallis, $p < 2.2 \times 10^{-16}$). Pairwise Dunn tests, in Fig. 3, showed both overlapping and distinct diversity patterns among the grave types, indicating differences in microbial richness and evenness according to grave-type.

To assess decomposition-driven community changes, beta diversity analysis focused on samples collected at 70 cm depth. This depth was selected because it maximises the detection of decomposition-associated microbial shifts (as cadavers were directly on soil at this depth), while minimising confounding variation associated with soil depth. PCoA of Bray–Curtis dissimilarities (Fig. 4) showed clear separation between mass grave (MG) and individual grave (IG) samples. PERMANOVA confirmed that grave type explained 23.3% of the observed variation ($R^2 = 0.233$), demonstrating distinct microbial communities between both burial contexts (MG and IG).

Examination of the ten most abundant taxa across all samples (Fig. 5) revealed highly similar taxonomic profiles among individual graves (IG1–IG3), whereas MG samples showed markedly different relative abundances across phylum, order, family, and genus levels. These compositional differences further support the divergence in microbial community structure between grave types.

To further identify taxa significantly associated with each grave type, a LEfSe analysis was performed (Fig. 6). An LDA threshold of 4.5 was applied to reduce noise from soil bacteria naturally present in the environment and prioritise the most strongly discriminating taxa likely to be functionally relevant to decomposition. The results highlighted taxa that were differentially enriched in MG or IG samples, offering further insight into the microbial signatures potentially driven by grave type.

### 3.4. Classification prediction at T18: MG vs IGs

To evaluate whether soil microbial communities could reliably distinguish burial type, the random forest classifier was applied to M18 samples at 70 cm depth. Across all samples, 1779 ASVs were initially detected. ASVs present in fewer than five samples were excluded, representing approximately one-third of the samples in the smaller group (MG: $n = 15$, IG: $n = 26$), leaving 352 ASVs for analysis.

The classifier distinguished mass grave (MG) from individual grave (IG) samples with high accuracy, achieving a ROC score of 0.983 and an AUC of 0.906 (Fig. 7A). Model optimisation selected an mtry value of 2, which specifies the number of variables considered at each split in the random forest. This value produced the highest classification performance, as reflected in the ROC score, indicating that the model was highly effective at distinguishing MG from IG samples. Feature importance analysis identified the top 20 ASVs that contributed most strongly to classification (Fig. 7B), highlighting key microbial taxa that differentiate MG and IG soil communities.

### 3.5. Post burial interval (PBI) estimation

To assess whether soil microbial communities could predict post-burial interval (PBI), samples collected across all timepoints were analysed, focusing on only samples in the 30–40 cm depth range, as collection at timepoints M1-M15 only occurred in this range. A total of 184 samples, excluding nine soil controls, were included. PCoA, in Supplementary Figure 3, and statistical analysis indicated significant differences in microbial community composition across PBIs (PERMANOVA, $R^2 = 0.04532$, $p = 0.001$).

Following community-level differences observed across PBI, temporal changes in relative abundance of dominant taxa were examined. Across taxonomic ranks (phylum, order, family and genus), mean

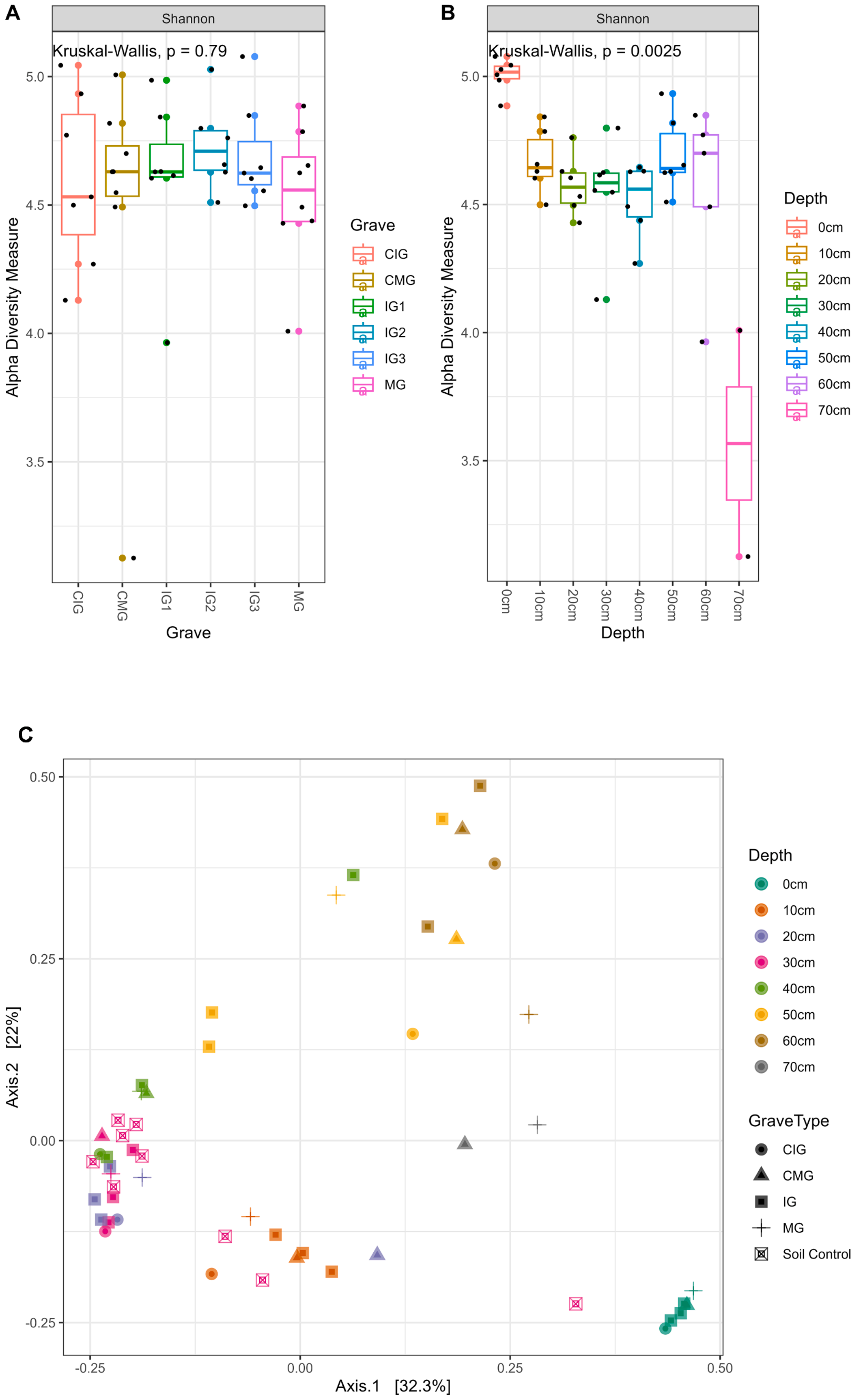

**Fig. 2.** Baseline soil microbial diversity prior to body deposition in graves. (A) Alpha diversity (Shannon index) across grave types showed no significant differences (Kruskal-Wallis, p = 0.79). (B) Alpha diversity decreased significantly with soil depth (Kruskal-Wallis, p = 0.0025). (C) PCoA (Bray-Curtis) shows sample clustering primarily by depth ($R^2$=0.581, p = 0.001), with some influence from grave type ($R^2$=0.084), however, soil control samples clustered together.

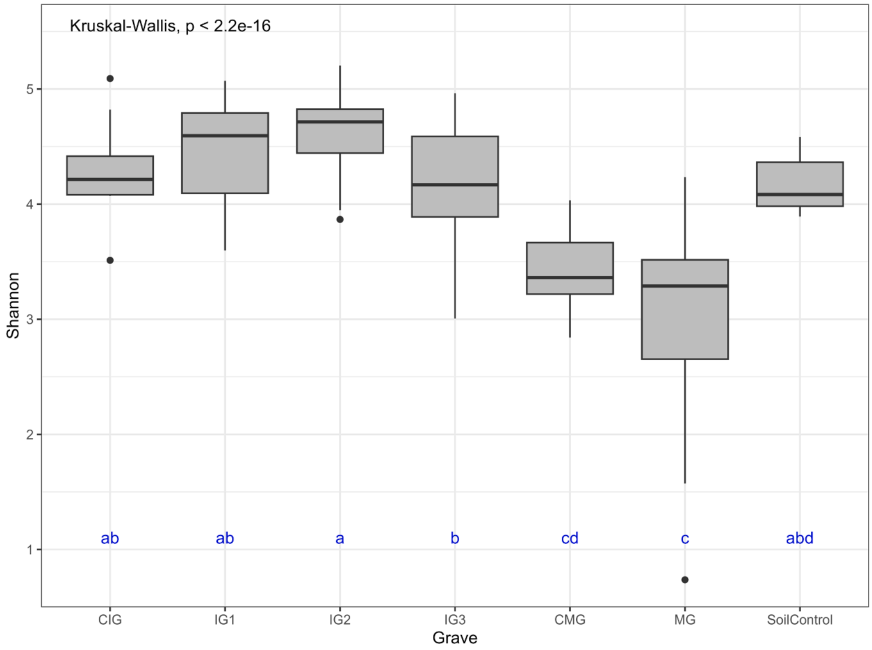

**Fig. 3.** *Alpha diversity (Shannon index) at M18 across grave types and soil control.* Boxplots show the Shannon diversity index of soil samples collected at 18 months post-burial (M18). Samples were primarily from 70 cm depth, except for control graves where samples from all depths were included due to limited availability at 70 cm. Following a significant Kruskal-Wallis test ($p < 2.2 \times 10^{-16}$), groups sharing at least one letter do not differ significantly from each other based on post hoc comparisons (Dunn post hoc test); groups with no letters in common differ significantly at $p < 0.05$.

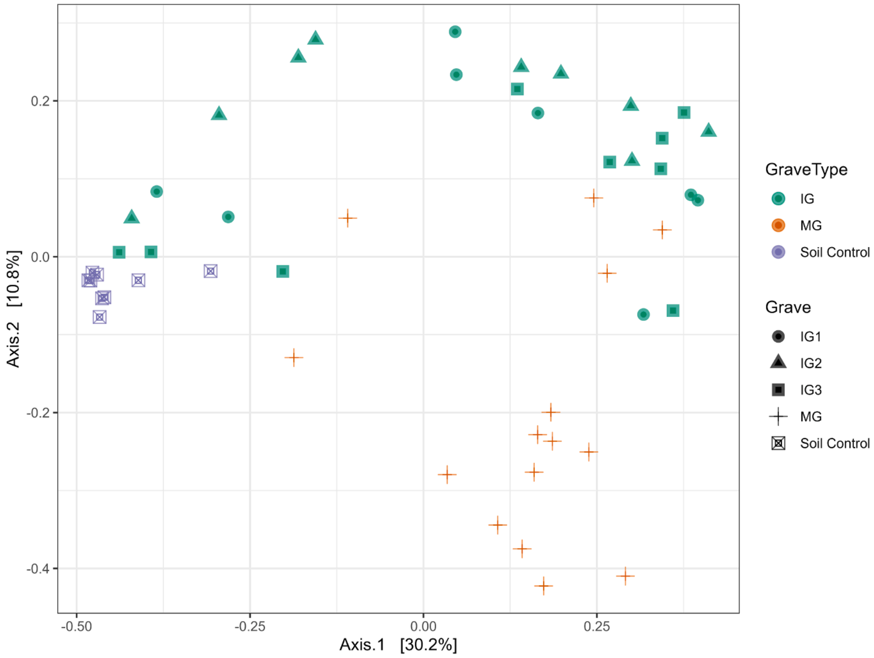

**Fig. 4.** Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis dissimilarities, showing the clustering of soil microbial communities from mass grave (MG), individual graves (IG1, IG2, IG3), and soil control samples collected at 70 cm depth and 18 months post-burial (M18). PERMANOVA analysis indicated that 23.3% of the observed variation in microbial composition ($R^2 = 0.233$) was attributable to differences between grave types.

relative abundances varied over the 18-month period in both IG and MG soil samples. Mean values were calculated across replicate samples (and graves for IG) at each PBI, and variability across replicates was quantified as standard deviation. Fig. 8 shows the five most abundant taxa by taxonomic rank; additional taxa, split by specific grave are shown in Supplementary Figure 4.

To assess whether soil microbial communities could predict post-burial interval (PBI), 397 highly variable ASVs were included in the analysis. These ASVs were selected based on their coefficient of variation across samples, with a threshold set at the third quartile of the distribution (10.5503) to exclude taxa that remained stable over time. This ensured that only taxa showing substantial temporal variability, and therefore likely reflecting decomposition-related dynamics, were retained (see Methods). Visualization of selected ASVs over PBI (Supplementary Figure 5) showed distinct abundance peaks at different intervals, confirming their variability over time.

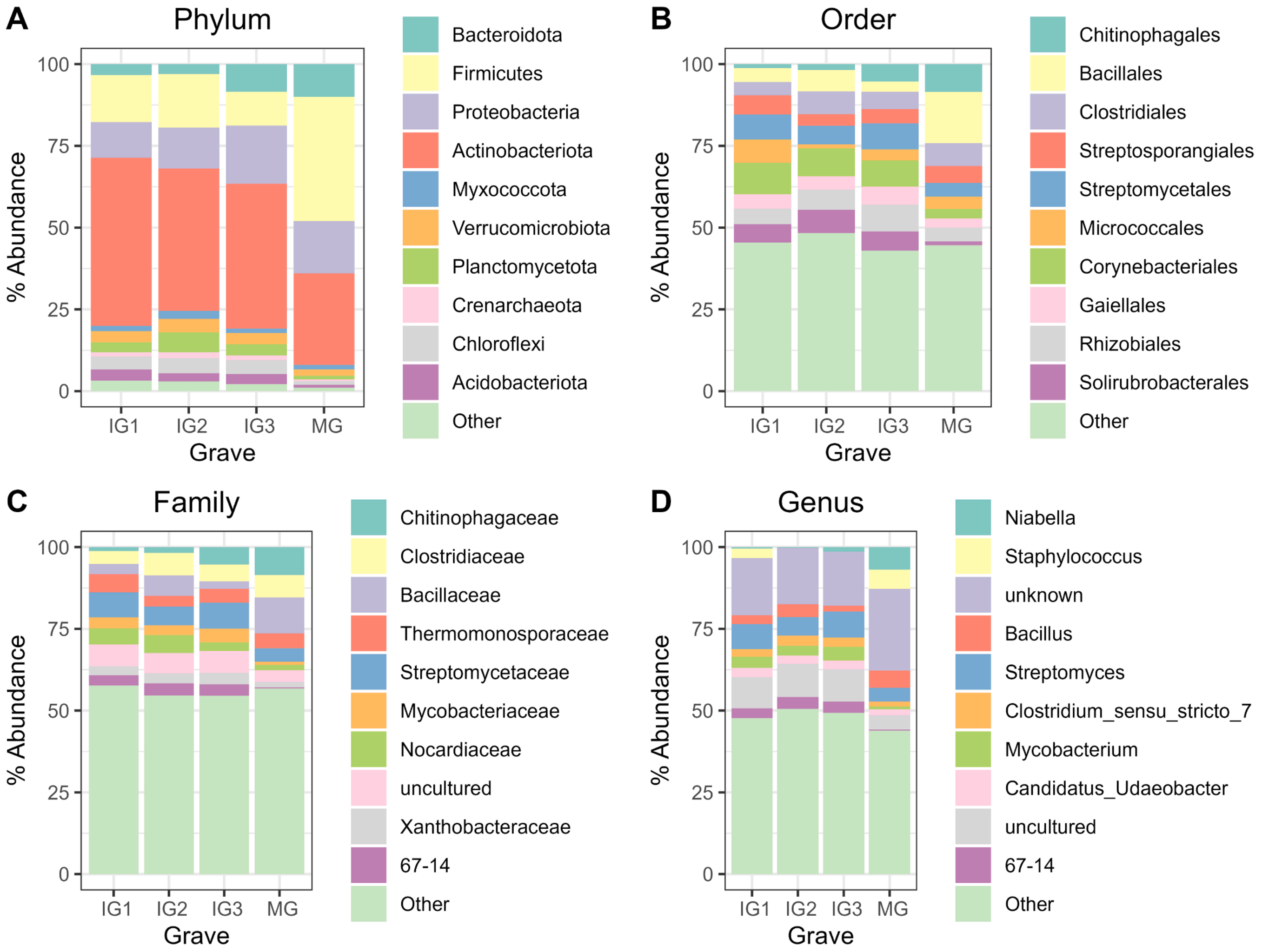

**Fig. 5.** Relative abundance of the top 10 microbial taxa at - (A) Phylum, (B) Order, (C) Family and (D) Genus levels - across individual graves (IG1, IG2, IG3) and the mass grave (MG).

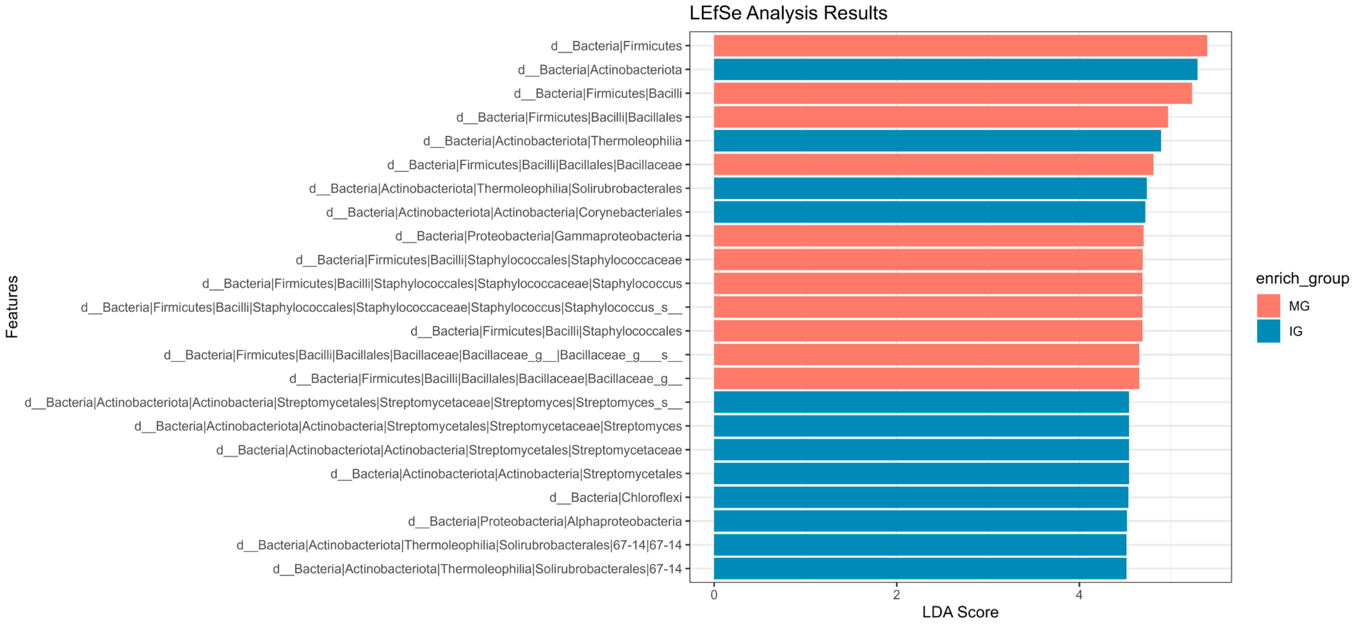

**Fig. 6.** LEfSe results showing taxa enriched in mass graves (MG) and individual graves (IG).

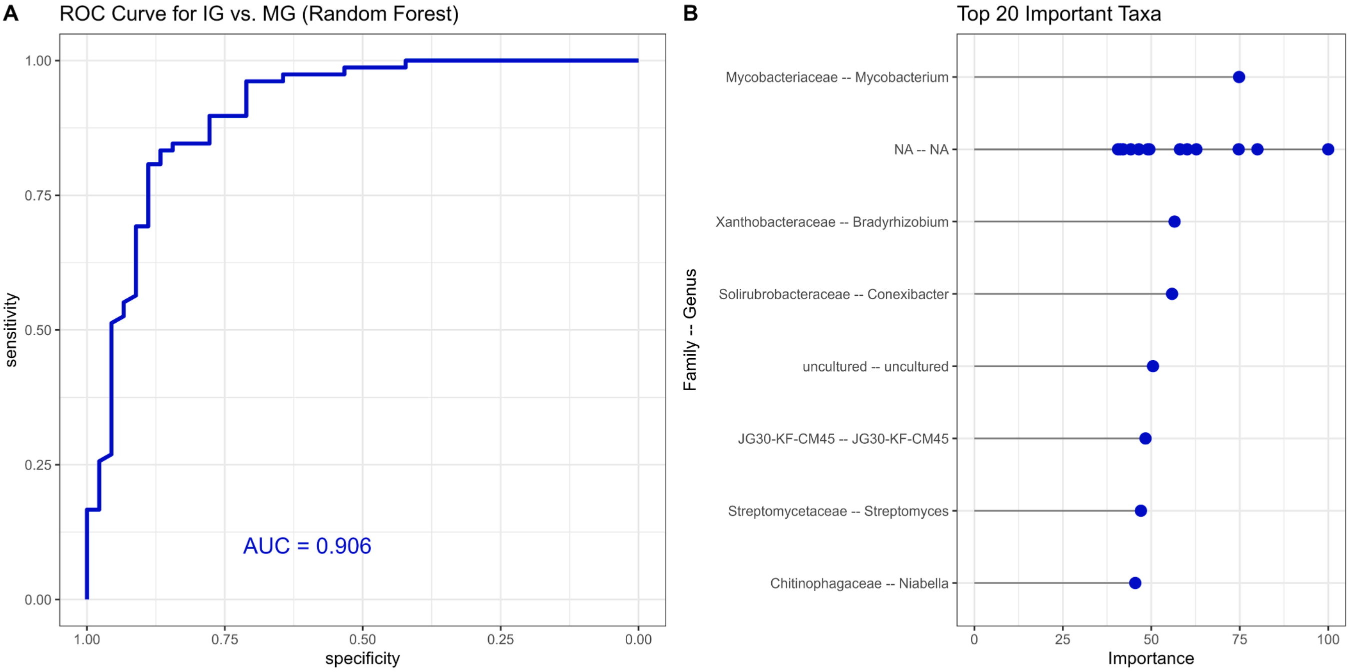

**Fig. 7.** Random Forest classification of MG and IG soil microbiomes at M18 and 70 cm depth. (A) ROC curve with an AUC of 0.906. (B) Importance plot showing the top 20 ASVs for classification.

A random forest regression model trained on individual grave (IG) samples predicted PBI with strong accuracy. Model optimisation selected an *mtry* value of 199, which provided the best predictive performance. For IG samples, the model achieved a root mean square error (RMSE) of 2.88 months and predicted PBI values closely aligned with observed values (Fig. 9A). When the IG-trained model was applied to mass grave (MG) samples, predictive accuracy declined, yielding an RMSE of 6.95 months. Comparison of actual versus predicted PBI values for MG samples (Fig. 9B) demonstrated greater dispersion and deviation from the identity line, indicating reduced accuracy compared to IG predictions (Fig. 9).

### 3.6. *Accounting for seasonal variations*

Using a refined dataset generated through the weighted subtraction method described in the Methods, the random forest regression model was retrained on individual grave (IG) samples. This adjustment improved predictive accuracy for IG samples, reducing the RMSE from 2.88 months to 2.68 months. For mass grave (MG) samples, the adjustment slightly reduced model performance, with RMSE increasing from 6.95 months to 7.12 months. These results indicate that accounting for taxa variations arising from changing seasons only slightly improved PBI predictions in IGs but did not enhance predictions for MG samples.

## 4. Discussion

### 4.1. *Initial assessment: baseline analysis at M0*

This baseline characterization focused on both alpha and beta diversity to explore how microbial communities were influenced by grave type and soil depth. Alpha diversity, as measured by the Shannon index, revealed that microbial richness and evenness were similar across the sites selected for the different graves, prior to cadaver deposition. This indicates that before burial, the microbial communities in the soil across the experimental area were consistent, suggesting that the location chosen for each grave had no significant influence on the initial microbiome composition of the soil.

Soil depth, however, had a significant impact on microbial diversity. Topsoil showed greater microbial diversity compared to deeper layers, reflecting the influence of environmental gradients. Research on microbial diversity underscores the results reported here that microbial biomass and turnover is reduced in deeper soils in comparison to surface soil [47–50]. This depth-related variation highlights the importance of sampling at consistent depths in subsequent PBI analyses, as it ensures that observed microbial changes over time reflect decomposition and burial processes rather than differences associated with soil depth.

### 4.2. *Assessment of soil post-decomposition (M18)*

Individual (IG) and control individual (CIG) graves displayed higher alpha diversity compared to the mass grave (MG) and its control (CMG). Notably, the soil control samples exhibited alpha diversity levels similar to both individual and all control graves. This variation in alpha diversity across grave types and controls primarily reflects differences in overall sample richness, which may be influenced by factors unrelated to cadaveric decomposition and therefore does not directly indicate decomposition-driven change.

One potential explanation may be due to grave size differences between mass and individual graves, and their controls. Disturbing a larger volume of soil is likely to result in more extensive soil mixing, which can alter resource availability and soil conditions. Experimental soil disturbance or mixing studies have shown that such changes can reduce microbial alpha diversity by giving competitive advantage to taxa that can exploit changing resource availability or adapt to the altered environment [51,52]. Another study showed that the effects of mixing can persist even after a year following the initial stress [53]. It is therefore plausible that disturbance affecting a larger volume of soil in MG and CMG may have favoured a smaller group of microbial taxa (compared to IG and CIG), leading to reduced community evenness and lower Shannon diversity.

### 4.3. *Microbial community differences in IG and MG at T18*

The plot in Fig. 6 highlights the taxa that contributed most to the observed differences, with certain features being distinctly enriched in either MG or IG communities. It also identifies taxa with observable

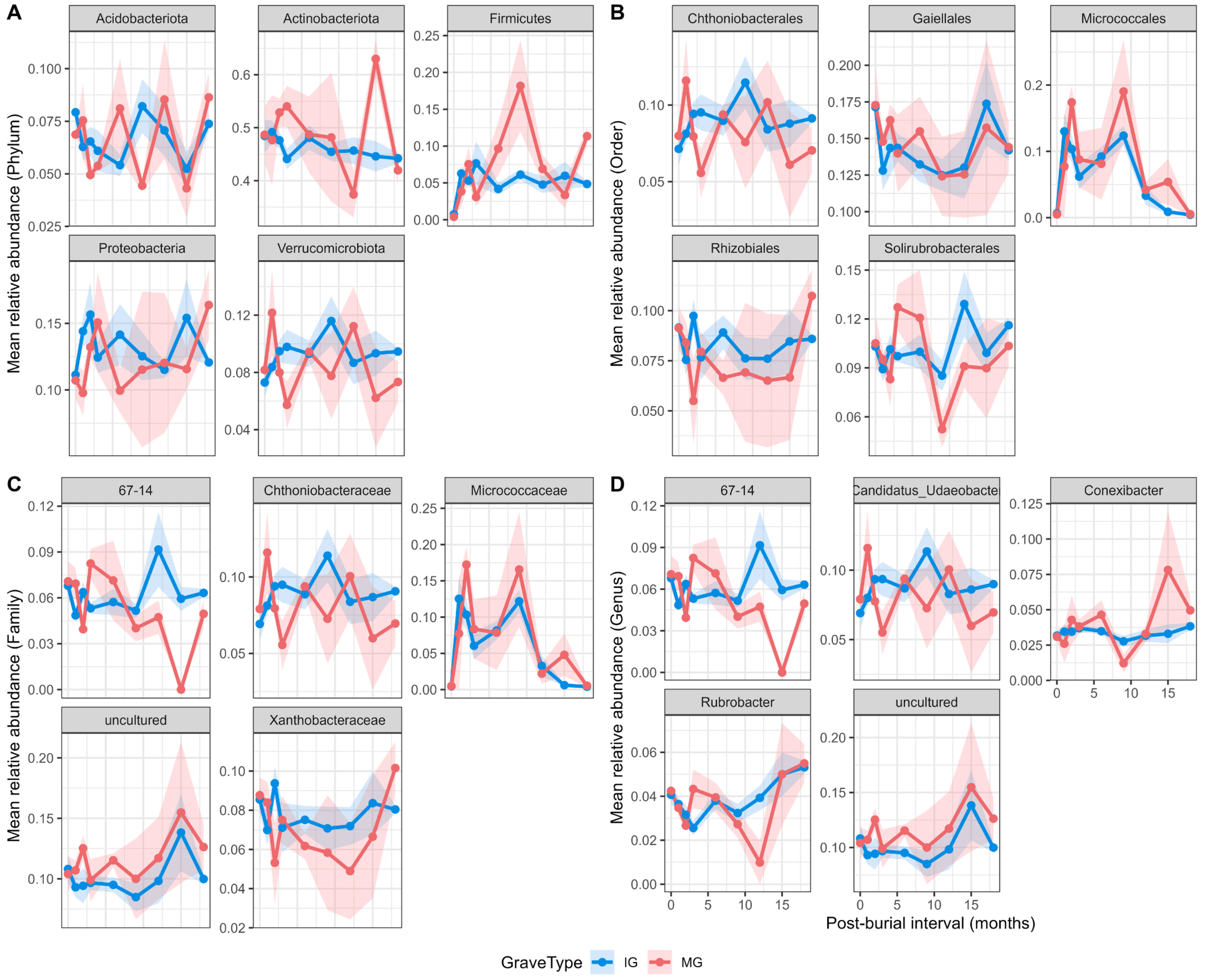

**Fig. 8.** Mean relative abundance of the top five most abundant taxa at phylum (A), order (B), family (C) and genus (D) levels across an 18-month post-burial interval (PBI) period for individual and mass graves. Lines represent mean values of replicates in corresponding grave type (IG - including IG1,2 and 3; or MG) at each PBI, and shaded ribbons indicate standard deviation from the mean value.

differences in relative abundance, as seen in Fig. 5, which has been explored in relation to soil and decomposition studies. For example, one taxon identified by both LEfSe and relative abundance analysis, *Bacillales*, has been reported to be depleted during advanced decay [3]. However, while this is reflected in IG samples, MG soil appears to have increased abundance of *Bacillales*.

Additionally, *Firmicutes* and *Staphylococcus* has also been linked, in previous research [54–57], to early decomposition stages, and were identified in this study as an enriched taxa for mass grave samples. The persistence of these early decomposition taxa at later timepoints in MGs likely reflects the slower overall progression of decomposition in mass burial contexts.

Previous work investigating differential decomposition in mass graves has shown that bodies located at the edges of a grave tend to decompose more rapidly than those within the dense center, likely due to increased oxygen availability, greater contact with surrounding soil, and easier access for insects and other necrophagous fauna [34,58,59]. This results in heterogeneous decomposition rates within the same mass grave, but potentially an overall slower progression of decay when compared to single graves. Although direct comparative studies between both grave types are limited, the results of this study align with what has been established regarding differential decomposition in mass graves. It is possible that the more constrained overall burial environment in mass graves (e.g., reduced oxygen diffusion, limited soil-body contact, restricted insect access) may delay microbial succession and tissue breakdown relative to single grave environments. Consequently, this study may also provide insights on the rate of decomposition in mass graves when compared to single graves, which are by far more studied.

It has also been reported that increased fat slows degradation in cadavers by inhibiting heat dispersion, even though presence of an increased amount of fat provides a nutrient-rich environment that supports the proliferation of decomposing microbes, resulting in an initially rapid decomposition, but an ultimately delayed skeletonization process [56,60]. This may further explain the observations as the presence of multiple bodies in a mass grave collectively increases the total fat available, potentially slowing skeletonization or decomposition process, and resulting in the observation of microbial taxa associated with early decomposition stages. However, the limited availability of studies, particularly on microbial decomposition communities, to conclusively support these observations underscore the need for further research between mass and single graves.

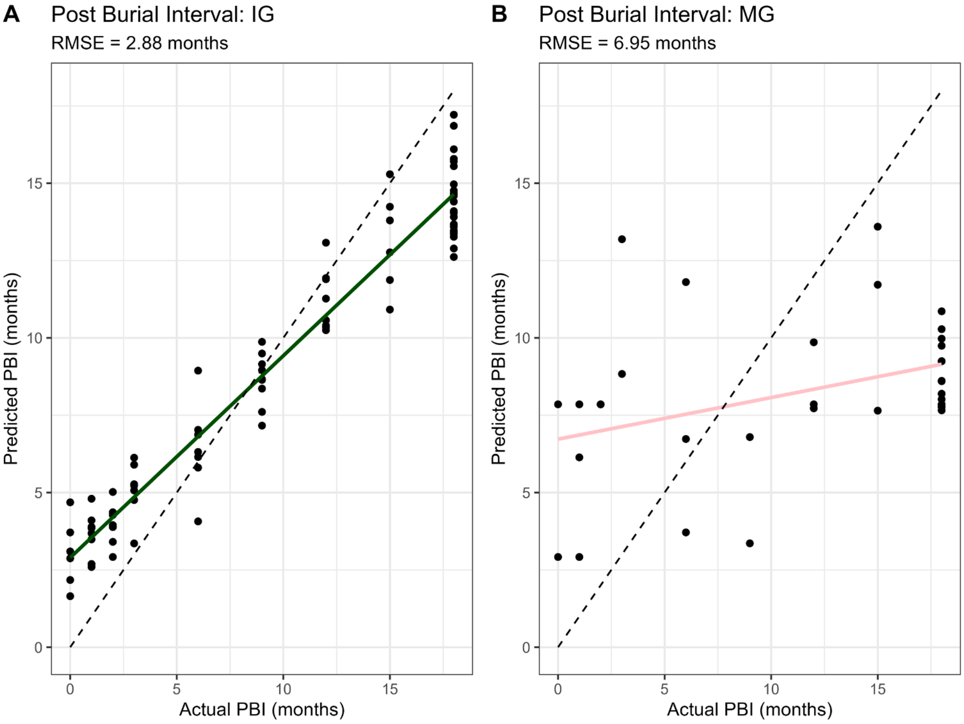

**Fig. 9.** Predicted versus actual Post Burial Intervals (PBIs) for (A) Individual Graves and (B) Mass Grave. The solid lines represent linear fits, while the dashed lines indicate the ideal fit, where predicted values equal actual values.

### *4.4. PBI estimation*

Microbial communities have been explored for PMI estimation, with several studies showing predictable microbial shifts during cadaver decomposition that can be modelled to estimate time since death [3–5,7,14,21,25,61–65]. More recent work suggests that microbial community profiling in grave soils also holds potential for estimating PBI and the age of a grave, as soil microbial succession can capture long-term decomposition-associated changes [5,9,11]. However, existing PBI studies often focus on short-term burial experiments or single graves, leaving limited insight into whether soil microbial succession can reliably estimate burial duration in human graves, especially in mass grave settings. This study therefore explored whether soil microbiomes from individual and mass graves could be used to predict post-burial interval over an extended 18-month period.

The regression model trained on longitudinal soil microbiome data from individual graves produced encouraging results, with a mean prediction error of approximately 2.88 months, reduced to 2.68 months after correcting for seasonal effects. These findings suggest that when sampling depth is consistent and stable taxa are excluded, microbial community dynamics can provide a reasonably accurate temporal signal for estimating PBI in single-body burials.

However, some important limitations temper the broader applicability of this model to mass graves. Model performance declined markedly when applied to MG soil samples, with error increasing to 6.95 months and further to 7.12 months after seasonal adjustment. This disparity likely reflects ecological differences between IG and MG contexts, where decomposition is shaped by greater cadaveric biomass, altered oxygen diffusion, and varying nutrient and moisture conditions. As such, microbiome-based time estimation models trained on IG data may not generalize to MG scenarios without careful recalibration or model retraining on context-specific data. This highlights the need for prediction models built specifically for MG contexts using MG-derived microbial data. Future research should therefore focus on developing MG-specific prediction models, although this will require a larger MG sample size at each sampling timepoint to support reliable model training, validation, and feature selection.

Another limitation of this study was related to the sample collection during the decomposition period, that is the period between M0 and M18. Sampling at a depth above the bodies that was sufficient to capture the decomposition taxa without disturbing the bodies, was often not feasible due to the overlying clay-rich soil becoming too hard to penetrate using a coring device. This limitation may have contributed to a higher root mean square error (RMSE) in PBI estimations. Since initial analyses revealed that microbial profiles were more strongly influenced by depth than by horizontal spatial positioning, only samples within a narrow depth range (30–40 cm) were included in the model. While this controlled for vertical heterogeneity, it may have reduced the ability to detect decomposition-driven microbial change.

Future studies may benefit from lateral sampling of soil at the level of or just beneath cadaver-level rather than coring vertically from above. This targets the zone most influenced by gravitational movement and pooling of decomposition fluid exudates while minimizing disturbance. A decomposition study on soil chemistry at FACTS [66] demonstrated that decomposition products migrate deeper in soil than the level generally sampled, with chemical changes extending more than 5 cm below sampled level. This suggests that soil collected deeper and laterally to the body may improve the characterisation of the soil necrobiome, as well as the resolution and robustness of microbiome-based time prediction models.

Finally, sample availability, particularly for the MG, hampered model generalisability. A robust predictive model for mass graves will require longitudinally sampled data, with substantial replicates across diverse decomposition scenarios. In addition, including experiments where bodies are added sequentially over time to the graves, would benefit application in real-world mass contexts, since establishing whether burials took place during one event or multiple provides crucial evidence for prosecution purposes. Sequential burial or a single burial event including bodies in widely varying stages of decomposition, may reveal different patterns of microbial change than those seen when all bodies are at the same stage of decomposition and are buried simultaneously, requiring flexible prediction models that can adjust to these changing conditions.

### 4.5. *Conclusions and implications for future applications*

This study provides, for the first time, data on the microbial changes that occur in grave soils during human decomposition over an 18-month period. The results are consistent with findings from earlier studies on individual graves and experimental models that microbial succession in burial environments can provide a temporal signal of decomposition [5,7,63]. The study extends forensic microbiome research to mass grave contexts and with human body donors, which remain underrepresented in the literature compared to single burials or animal studies. The findings also support earlier research indicating that mass graves display significant ecological complexity during body decomposition [34,35].

Significant differences were observed in microbial diversity and community composition between mass and individual grave soils at the end of the 18-month period, with Firmicutes, Staphylococcus, and Bacillales being significantly enriched in mass grave soils compared to individual grave soils. Individual grave soils showed profiles more consistent with advanced stages of decomposition. A machine learning model based on soil microbiome data was able to classify mass and individual grave samples with high accuracy. These results suggest that soil microbiomes reflect burial context and can potentially help differentiate between graves containing single or multiple bodies. In terms of potential for forensic casework, such classification could help distinguish burial contexts in cases where graves are disturbed or robbed of human remains and evidence and could be used to complement geophysical or chemical detection methods and corroborate witness statements. However, these findings should be interpreted with caution as this study represents the first investigation of microbial differences between mass and individual graves, and further validation is necessary before application in forensic practice.

The results also highlight the potential and limitations of microbiome-based post-burial interval (PBI) estimation. Models trained on individual grave soil samples predicted PBIs with relatively good accuracy (average error ~2.7 months after adjusting for seasonal influences), but their performance declined substantially when applied to mass grave samples (error ~7 months). This indicates that PBI estimation models developed for single burials are not directly transferable to mass graves without recalibration. The greater ecological complexity of mass graves due to the presence of multiple decomposing cadavers, as well as variations in oxygen and nutrient availability, and differences in tissue composition (e.g., fat content), seems to alter microbial succession by comparison to individual graves.

Despite these limitations, the potential for microbiome-based methods in forensic casework remains significant, particularly as a complementary approach that can corroborate other evidence and increase the precision of PBI estimation. Traditional methods rely heavily on morphological and taphonomic assessment of remains. Gross decomposition scoring systems, such as the accumulated degree-days (ADD) method, are widely used but have shown variable accuracy, particularly in buried contexts where decomposition is slowed and more heterogeneous [26,30]. Forensic entomology, which uses insect colonization and development stages to estimate time since death, can be highly accurate, particularly in the first few weeks after death, for surface remains but can be less reliable in burials due to restricted or delayed insect access [67]. Chemical and geochemical analyses, including changes in soil chemistry such as pH, and elevated nitrogen or phosphate concentrations, have been tested as proxies for decomposition but are strongly affected by local soil type, moisture, and climate [6,31]. Histological and molecular approaches, such as muscle protein degradation or RNA decay, have also been investigated but show inconsistent preservation in buried remains and become less reliable as decomposition advances [26].

Microbiome analysis therefore potentially offers a distinct line of evidence by the fact that microbial succession could be used to predict time since burial and is sensitive to the burial environment [5,7,63].

The results from this study further indicate that while microbiome profiles could provide additional support in PBI estimation, models must be built on data from comparable burial contexts, i.e., mass burial trained models for mass burial contexts. Future research should expand on these initial findings by investigating the persistence of microbial signals after excavation, exploring microbial communities beyond bacteria (e.g., fungi, eukaryotes), and assessing sequential or mixed burial scenarios that reflect other commonly encountered real-world cases. Such studies would be directly relevant to humanitarian and criminal investigations, where establishing whether multiple deposition events occurred is important.

In sum, this work provides an important first step toward understanding microbial succession in mass graves and its forensic relevance. While the results underline the potential of soil microbiome analysis for detecting graves, distinguishing burial contexts, and supporting time-since-burial estimation, they also demonstrate the need for methodological refinement, larger datasets, and validation across diverse scenarios.

## *CRediT authorship contribution statement*

**Noemi Procopio:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Daniel J. Wescott:** Conceptualization. **Darren Smith:** Writing – review & editing, Supervision. **Andrew Nelson:** Writing – review & editing, Supervision. **Hayley L. Mickleburgh:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Nengi Ogbanga:** Writing – original draft, Investigation, Formal analysis, Data curation. **Timothy P. Gocha:** Investigation.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for this journal and was not involved in the editorial review or the decision to publish this article.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2026.103517.

## Data availability

The raw sequencing data (FASTQ files) generated in this study are available in the NCBI Sequence Read Archive (SRA), under BioProject accession number PRJNA925138.

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