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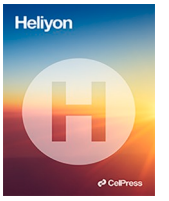

Research article

Estimating postmortem interval based on oral microbial community succession in rat cadavers

Xiaoxue Wang [1], Cuiyun Le [1], Xiaoye Jin, Yuhang Feng, Li Chen, Xiaolan Huang, Shunyi Tian, Qiyan Wang, Jingyan Ji, Yubo Liu, Hongling Zhang, Jiang Huang [**], Zheng Ren [*]

Department of Forensic Medicine, Guizhou Medical University, Guiyang, 550004, Guizhou, China

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ABSTRACT

The accurate estimation of the postmortem interval has been one of the crucial issues to be solved in forensic research, and it is influenced by various factors in the process of decay. With the development of high-throughput sequencing technology, forensic microbiology has become the major hot topic in forensic science, which provides new research options for postmortem interval estimation. The oral microbial community is one of the most diverse of microbiomes, ranking as the second most abundant microbiota following the gastrointestinal tract. It is remarkable that oral microorganisms have a significant function in the decay process of cadavers. Therefore, we collected outdoor soil to simulate the death environment and focused on the relationship between oral microbial community succession and PMI in rats above the soil. In addition, linear regression models and random forest regression models were developed for the relationship between the relative abundance of oral microbes and PMI. We also identified a number of microorganisms that may be important to estimate PMI, including: *Ignatzschineria*, *Morganella*, *Proteus*, *Lysinibacillus*, *Pseudomonas*, *Globicatella*, *Corynebacterium*, *Streptococcus*, *Rothia*, *Aerococcus*, *Staphylococcus*, and so on.

1. Introduction

In forensic medicine, the postmortem interval (PMI) is the period between the examination of the carcass and the occurrence of death [1]. Moreover, the accurate estimation on the postmortem interval has been one of the crucial issues to be solved in forensic research, which is crucial in determining the nature of the cases, delineating the scope of the investigations, identifying and excluding suspects, etc. [2]. Nevertheless, traditional methods of estimating PMI, such as algor mortis, livor mortis, rigor mortis, etc. [3–6], are mainly dependent on the subjective experience of forensic scientists to make a rough estimate of the time frame [7]. As time passes, the degree of decay in the cadavers deepens, causing the identification of the postmortem phenomenon to be challenging. In addition, the decay process is complex [8] and could be influenced not only by factors intrinsic to the cadavers themselves (bacteria and other microorganisms inherent to the cadavers [9,10]) but also by external factors (temperature [11–14], humidity and precipitation

* Corresponding author.
** Corresponding author.
E-mail addresses: mmm_hj@126.com (J. Huang), mmm_rz@163.com (Z. Ren).
[1] These authors contributed equally to this work.

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[15–17], insects [18–21], etc.). Therefore, it is more challenging to estimate PMI.

Fortunately, as high-throughput sequencing (HTS) technology progresses, the application of forensic microorganisms has gained more attention worldwide [22,23]. It is worth noting that HTS technology could directly sequence the DNA of the microbial community on cadavers to obtain biological information on the species and use a series of bioinformatics analysis tools to investigate microbial community structure and metabolism, the relative abundance of the dominant species, etc. [24]. Hence, the links between microorganisms and PMI could be further explored, providing new research options for the estimation of PMI. Scientists have devoted their efforts to finding more accurate and efficient ways to estimate PMI by applying the changes in microbial communities on cadavers. For instance, Metcalf et al. [25] developed a microbial clock to accurately predict PMI based on the rat decomposition model. Liu et al. [26] constructed an artificial neural network model for the estimation of PMI using the microbiome of murine remains in aseptic conditions. Furthermore, a random forest model was conducted by Zhang et al. [27] to predict PMI on the basis of the succession of microbial communities in buried rat cadavers. Compared with directly observing postmortem phenomena, mathematical models constructed using biological information from the microbial community are unaffected by subjective consciousness, which is more reliable in estimating PMI. In summary, it is feasible to apply microorganisms for estimating PMI and could be expected to be a prospective tool.

The oral cavity connects the human body to the external environment [28]. Additionally, oral microorganisms play an essential role in the human microbiome and human health [29]. Moreover, the oral microbial community is one of the most diverse microbiomes, ranking as the second most abundant microbiota following the gastrointestinal tract. It has been found that the oral microbiota contains nearly 1,000 bacterial species, with the most common phyla including Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria and TM7 [30]. Although it has been demonstrated that oral microorganisms have a significant function in the decay process of cadavers [31], few studies have explored the features of the microbial community in the oral cavity of carcasses that are exposed on top of the soil during decomposition. Nevertheless, in outdoor crime scenes, remains of bodies scattered on the surface are also common in forensic practice [32]. Consequently, our study focused on the association between the succession of microbial communities in the oral cavity of rats above soil during decay and PMI. In the meantime, we targeted the identification of the species that are relevant to PMI at different taxonomic levels and the development of linear regression models to estimate PMI. In addition, we built random forest (RF) models to estimate PMI based on genus-level microbiome data.

2. Materials and methods

2.1. Experimental design and sample collection

Our research was carried out in accordance with strict ethical standards defined in the 1964 Declaration of Helsinki and subsequent amendments. Additionally, the animals involved in this experiment were 21 male Sprague Dawley (SD) rats purchased from Beijing Huafu Kang Biotechnology Co. Each of these 21 SD rats was adaptively fed for 7 days, fasted for 12 h before they were sacrificed, and finally humanely euthanized by cervical dislocation. We collected outdoor soil in Guizhou to simulate the death environment, and the soil was left untreated in its original condition. Then, we conducted the rat sample collection indoors, with 3 rats removed at hour 0 as negative control samples and the remaining 18 rats placed equally on the approximately 20 cm thick soil we collected. Seven time points were set for collecting samples from the oral cavity of the cadavers: 0, 12, 24, 48, 72, 120, and 168 h. The temperature and humidity during decomposition of the rat remains varied slightly, with an average temperature of 25 °C and a relative humidity of approximately 70 %. Before collecting samples, we performed and recorded estimates of visual body assessment on the head and torso position of rat cadavers based on the works of Megyesi et al. [33] and Tullis et al. [34]. At each time point, oral swabs were collected from three rats, and the samples were taken by wiping the mouths of each rat with sterile cotton swabs moistened with physiological saline solution. In addition, the sampled areas of the oral cavity included the palate, tongue, inner cheek mucosa, and tooth surface. All samples were preserved in a freezer at −80 °C until further processing.

2.2. DNA extraction, PCR amplification and sequencing

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. For specific PCR amplification steps and parameters, we recommend the method of Wang et al. [35]. We purified the amplified product by applying Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China). The amplification products were quantified based on the instructions of the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China) [36].

2.3. Data analysis

Microbiome bioinformatics were performed with QIIME2 2019.4 [37]. Raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin [38]. Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin [39]. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft [40]. Classification of ASVs by applying the classify-sklearn naive Bayes taxonomy classifier in the feature-classifier plugin [41] on the SILVA Release 132

Database [42].

First, the results of the visual estimates were shown in Table S1. Alpha-diversity metrics (Chao1 [43], Shannon [44], Simpson [45], etc.) was calculated based on the ASV table using QIIME2, and the Shannon indices were plotted as Rarefaction Curve, and displayed in Fig. S1. Beta diversity metrics was assessed using Bray–Curtis distances and visualized by principal coordinate analysis (PCoA) with the ape package 5.7-1 of R software v4.2.2. In addition, statistical tests were carried out through permutational multivariate analysis of variance (PERMANOVA) and visualized from the vegan package 2.6–4 using R software v4.2.2. Next, we used linear discriminant analysis effect size (LEfSe) to find taxa that were abundantly different among groups with default parameters [46]. R software v4.2.2 was used to cluster and draw a heatmap with the top 30 genera in terms of abundance. The relationship between oral microorganisms and PMI in rat remains was further explored based on the relative abundance of the top 10 species at different taxonomic levels, and taxonomic composition analysis was executed by utilizing QIIME2 (2019.4) to invoke the "qime taxon bar graph" command. Meanwhile, the top 10 species based on relative abundance at different taxonomic levels were visualized with the correlation matrix by applying the PerformanceAnalytics package 2.0.4 of R software v4.2.2. Eventually, we created linear regression plots on the basis of species selected at different taxonomic levels. We used the package "randomForest" to construct a predictive model of PMI based on the relative abundance of microorganisms at the genus level of the samples and the PMI data. After 100 iterations, the microbial communities at the genus level were ranked according to the degree of importance of the features using the random forest model. In addition, we obtained the minimum error to obtain the biomarkers based on 10-fold cross-validation. We built a random forest regression model based on biomarkers to estimate PMI. Meanwhile, we calculated the mean absolute error (MAE) and the goodness of fit (R^2) to evaluate the performance of the model. For the detailed algorithms of MAE and R^2, we refer to the methods of Liu et al. [26] and Zhang et al. [27].

3. Result

Within 7 days, a total of 21 rat oral samples were collected, and all samples were successfully sequenced, with a total of 2,231,703 raw reads obtained. To ensure that the reads are of sufficiently high quality, we removed low-quality reads with adapters from the raw sequencing data (raw reads), and obtained a total of 2,062,198 clean reads and 6,963 bacterial ASV sequences for subsequent analysis. The rarefaction curve based on the Shannon index of each sample reached a saturation plateau at a sequencing depth of 4,000 as appropriate, as shown in Fig. S1.

3.1. Analysis of oral microbial community diversity during decomposition of rat cadavers

During our research, we attempted to avoid the influence of scavengers from the external environment on the rat remains. Based on the researches by Megyesi et al. [33] and Tullis et al. [34], a visual assessment of rat carcasses was carried out, and the results were shown in Table S1. We identified carcasses on Days 0, 0.5 and 1 as the fresh stage; carcasses on Days 2, 3 and 5 as the early decay stage;

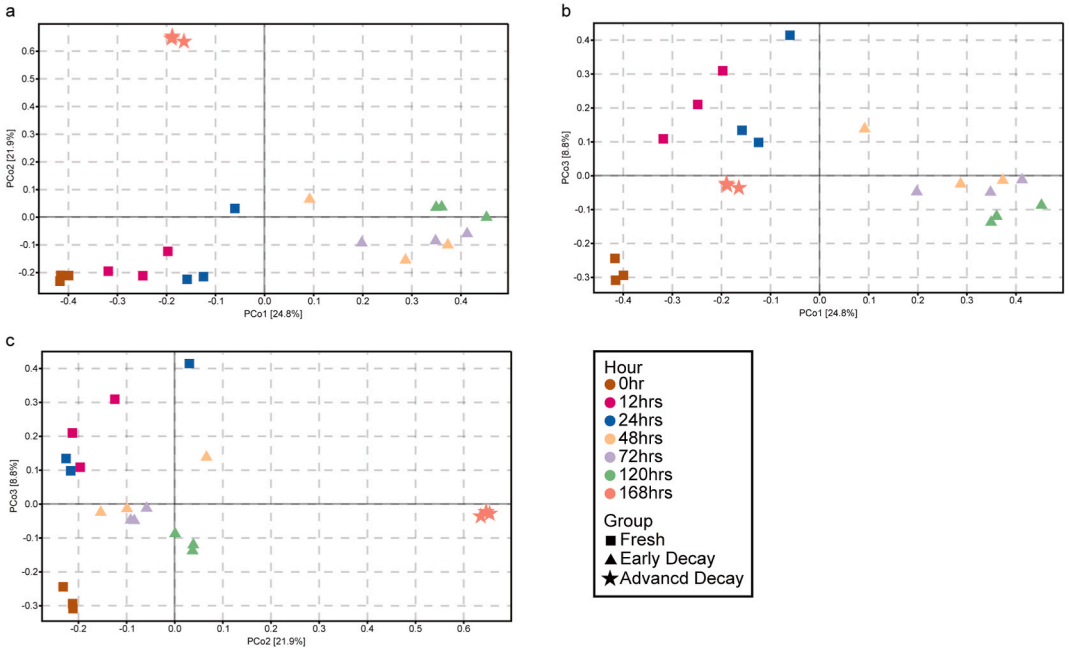

Fig. 1. Principal coordinates analysis of oral samples (different colours represent different PMIs and different shapes represent different decay stages). **a** PCoA results based on PCo1 and PCo2; **b** PCoA results based on PCo1 and PCo3; **c** PCoA results based on PCo2 and PCo3.

and carcasses on Day 7 as the advanced decay stage.

To explore the differences in microbial communities during the decay of rat cadavers, we plotted a principal coordinates analysis (PCoA) scatter plot utilizing the Bray–Curtis distance, as illustrated in Fig. 1. The various colours represent the rat oral swab samples we collected at different time points, and the various shapes indicate the different stages of decay. The first three principal components we selected explained 24.8 %, 21.9 %, and 8.8 % of the variation among these microbial communities. For PCo1 (Fig. 1a and b), the 21 oral samples could be classified as early decay and non-early decay; for PCo2 (Fig. 1a and c), these samples could be divided into advanced decay and non-advanced decay; and for PCo3 (Fig. 1b and c), the samples were separated into fresh and non-fresh, while samples at hour 0 were distinguished from samples from other time points in the fresh period, which indicated a large variability of the microorganisms of the carcasses during the 24 h postmortem. Moreover, the clustering of the above time points was consistent with the results of the visual evaluation analyses. Furthermore, to better visualize the differences in the microbial communities of rat cadavers among the groups at different decay stages, the results of the PRERMANOVA based on Bray–Curtis distances are depicted in Table S2 and Fig. 2, which revealed significant variation ($p < 0.05$) at different decay stages.

3.2. Selection of the biomarkers in oral cavity of rat carcasses during decomposition

We utilized LEfSe to find the differential species in the microbial communities at different decay stages, as presented in Fig. 3 and Table S3. In Fig. 3, the various colours represent the different stages of decay. The branch diagram indicates the taxonomic hierarchy from the phylum to genus level (from the inner to outer circles), with the node sizes representing the average relative abundance of taxonomic units. In particular, hollow nodes depict taxonomic units that were not significantly different between groups. Conversely, colored nodes indicate that these taxonomic units showed significant intergroup variability and higher relative abundance in the grouped samples represented by that color. Combining Fig. 3 and Table S3, we noted a number of microorganisms with high linear discriminant analysis (LDA) values, for example, at the phylum level, Actinobacteria and Bacteroidetes; at the class level, Actinobacteria, Bacilli, Gammaproteobacteria, and Bacteroidia; at the order level, Micrococcales, Pasteurellales, Lactobacillales, Corynebacteriales, Flavobacteriales, Enterobacteriales, and Cardiobacteriales; at the family level, Moraxellaceae, Micrococcaceae, Aerococcaceae, Pasteurellaceae, Corynebacteriaceae, Staphylococcaceae, Flavobacteriaceae, Enterobacteriaceae, Wohlfahrtiimonadaceae, and Enterococcaceae; and at the genus level, *Vagococcus*, *Ignatzschineria*, *Savagea*, *Escherichia−Shigella*, *Morganella*, *Peptostreptococcus*, *Proteus*, *Lysinibacillus*, *Myroides*, *Pseudomonas*, *Sphingomonas*, *Globicatella*, *Rodentibacter*, *Corynebacterium*, *Corynebacterium_1*, *Streptococcus*, *Psychrobacter*, *Jeotgalicoccus*, *Rothia*, *Aerococcus*, *Bosea*, and *Staphylococcus*. Surprisingly, we observed that Actinobacteria (phylum), Actinobacteria (class), Micrococcales (order), Micrococcaceae (family), and *Rothia* (genus) not only had the highest LDA values in the fresh stage but also belonged to the same taxonomic branch from a classification point of view.

To further explore the changes in microbial communities in the oral cavity of rat cadavers, we plotted a heatmap based on the data of the top 30 microorganisms in terms of relative abundance at the genus level after normalizing the rows, as shown in Fig. 4. At the same time, we clustered the stages of decay in the samples using Pearson's correlation coefficient and found that the clustering results were consistent with the PCoA results. According to the clustering results on the left of the heatmap, the top 30 species at the genus level all belonged to the four phylum levels Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes at the different PMIs. In the fresh period, Actinobacteria, Proteobacteria, and Firmicutes constituted the main phylum in the oral cavity of the rat remains. However, Bacteroidetes were mainly found in the early decay stage. Firmicutes and Proteobacteria were important phyla of advanced decay, but their microbial communities at the genus level differed from those of the fresh phase. Combining phylum-level microbial taxonomic composition results (Fig. S2), as the time of death passed, Proteobacteria exhibited a trend of decreasing and then increasing; Firmicutes displayed a form of increasing, then decreasing, and finally increasing slightly; the relative abundance of Bacteroidetes showed a reduction followed by increasing and ending with reducing relative abundance; and the relative abundance for Actinobacteria was relatively high at hour 0 and hour 24, with decreasing relative abundance at the remaining hours. In addition, it was clear that *Sphingomonas*, *Globicatella*, *Rodentibacter*, *Corynebacterium*, *Corynebacterium_1*, *Streptococcus*, *Psychrobacter*, *Jeotgalicoccus*, *Rothia*, *Aerococcus*, *Bosea*, and *Staphylococcus* were more predominant in the oral samples at the fresh stage; *Escherichia−−Shigella*, *Morganella*, *Peptostreptococcus*, *Proteus*, *Lysinibacillus*, *Myroides*, and *Pseudomonas* were more abundant in the early decay stage; both *Ignatzschinaria* and *Savagea* only appeared at stage of the advanced decay, and *Vagococcus* also had the highest relative abundance

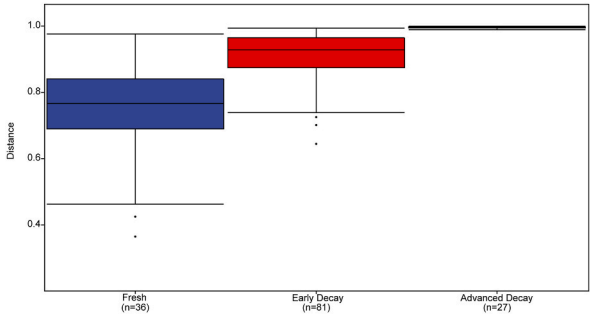

Fig. 2. The boxplot shows the significant variation between groups at different stages of decay.

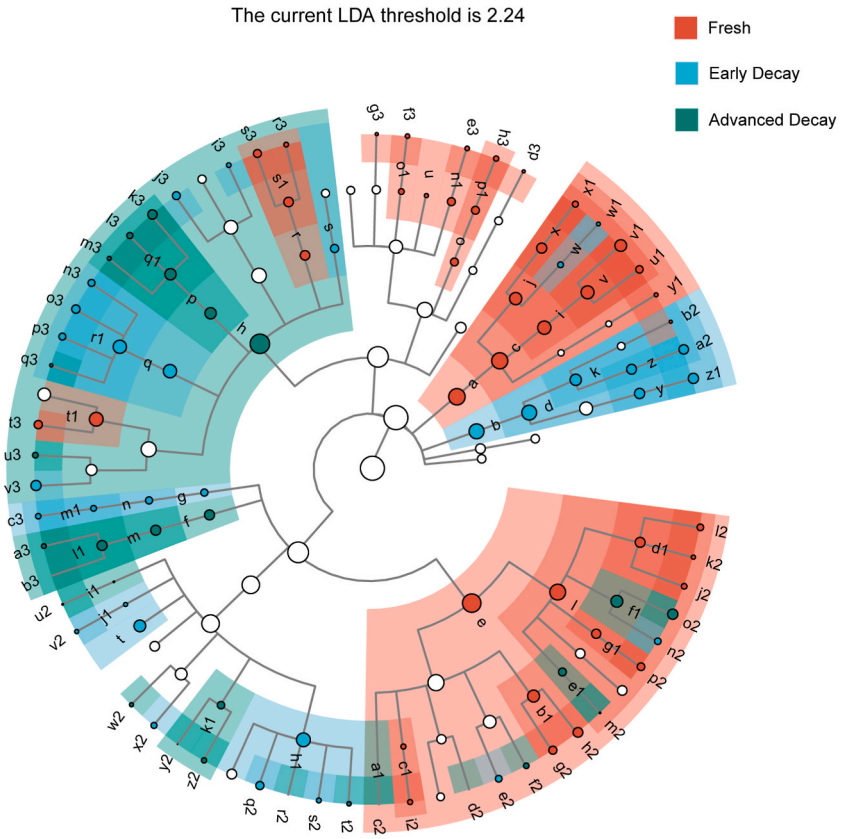

Fig. 3. The branching diagram based on linear discriminant analysis effect size.

at this stage. Interestingly, these microbes had the same stage of decomposition in the LEfSe analysis. This also indicated that our selected bacterial communities above were significantly different and enriched at different stages of decay, which could make these microorganisms ideal biomarkers for the estimation of PMI.

3.3. Building linear regression models and random forest regression models

For further insight into the relationship between oral microorganisms and time to death in rat carcasses, we analyzed the taxonomic composition and visualized the correlation matrices based on the top 10 species in relative abundance at different biological classification levels, as demonstrated in Figs. S2–S11. Taking into consideration the top 10 microbiome data in relative abundance at five levels (from the phylum level to the genus level) and the results of correlation analyses with PMI, we concluded that the best correlations with PMI from the phylum level to the genus level were for Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae and *Rothia*. This finding was the same as the result in the LEfSe analysis. Therefore, we performed linear regression models based on the relative abundance data of the microorganisms selected above. At the phylum level (Fig. 5a), we detected a strong negative correlation between Actinobacteria and PMI ($p = 0.0008$, $R^2 = 0.4522$). At the class level (Fig. 5b), we observed that there was a relatively strong negative correlation between Actinobacteria and PMI ($p = 0.0009$, $R^2 = 0.4514$). At the order level (Fig. 5c), we noticed a negative correlation between Micrococcales and PMI ($p = 0.0146$, $R^2 = 0.2755$). At the family level (Fig. 5d), we found a negative correlation between Micrococcaceae and PMI ($p = 0.0138$, $R^2 = 0.2790$). At the genus level (Fig. 5e), the regression results indicated that the relative abundance of *Rothia* exhibited a negative correlation with PMI ($p = 0.0210$, $R^2 = 0.2498$). We summarize in Table S4 the linear regression equations based on the relative abundance of the species at different taxonomic levels associated with PMI. In conclusion, we found that Actinobacteria (phylum) had the best linear fit for the estimation of PMI.

Moreover, to observe whether significant differences existed among microorganisms at the same time point, we carried out the Kruskal–Wallis (K–W) test on the microbiome data, and the outcomes of the test are listed in Tables S5–S9. Combined with the test results, we found that the linear regression models could not well reflect the complexity among microorganisms. Hence, we utilized the random forest regression model to estimate PMI. First, we used the relative abundance data of microorganisms at the genus level for random forest modelling (Fig. S12), and the R^2 and MAE values of the model were 98.69 % and 7.62 ± 1.54 h. Thus, we ranked the contributions of microorganisms from high to low with a random forest algorithm, and we excluded microbes with low contributions based on 10-fold cross-validation (Fig. S13). Finally, we obtained 30 biomarkers (Fig. S14) that might play the crucial roles in the estimation of PMI, including: *Ignatzschineria*, *Morganella*, *Proteus*, *Lysinibacillus*, *Pseudomonas*, *Globicatella*, *Corynebacterium*,

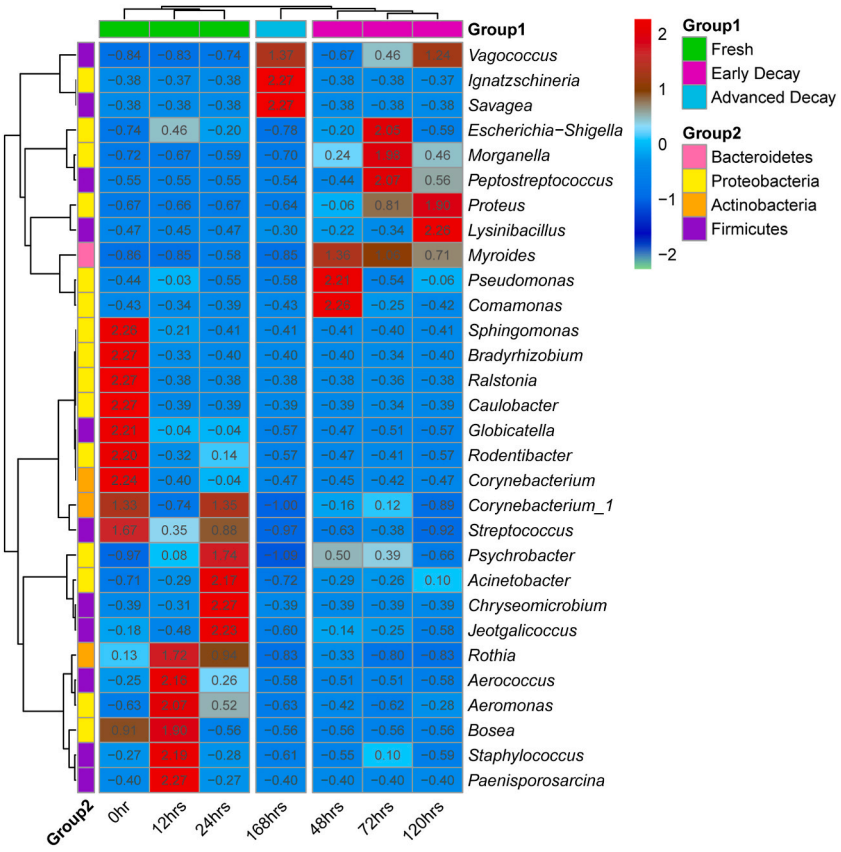

Fig. 4. The heatmap of top 30 genera of relative abundance of oral microorganisms in rat cadavers.

Streptococcus, *Rothia*, *Aerococcus*, *Staphylococcus*, and so on. In addition, we built the random forest model again with the data of these 30 biomarkers (Fig. 6). We noticed that compared to building a random forest regression model based on all microorganisms at the genus level, the random forest regression model utilizing the biomarkers had better R^2 values as well as smaller MAE values, i.e., 98.76 % and 6.93 ± 1.19 h, respectively.

4. Discussion

The accurate estimation of the postmortem interval has consistently been a challenge for forensic scientists to resolve and is influenced by various factors in the corruption process [47]. Nonetheless, the appearance of forensic microbiology offered new ideas for exploring the estimation of PMI and was considered a promising tool in forensic investigations [48,49]. Relevant studies have already focused on the epinecrotic bacterial communities of the main external anatomical sites (eye, nose, ear, rectum), including the oral cavity, in human and animal models [50–53]. The oral cavity was the second most abundant microbiota and had a substantial role in the decay of cadavers [54,55]. Field corpse scenes are commonly found in forensic science practice. However, there have been few studies on the characterization of microbial communities in the oral cavity of cadavers exposed above the soil during decomposition. Therefore, in this study, we collected outdoor soil to simulate the death environment and focused on analysing the microbiome data in the oral cavity area of rat cadavers above the soil during decomposition. In addition, we selected some microorganisms that might be important for the estimation of PMI. In addition, linear regression models and the random forest model were constructed based on the relative abundance data of microorganisms to estimate the PMI.

In forensic science, the stages of decomposition include fresh, early decay and advanced decay [56,57]. This is consistent with the division results following the visual estimation analysis in our study. In this research, according to the visualization of the principal coordinates analysis, we observed differences in the microbial communities at different stages of decay, as previously reported by Zhang et al. [27]. However, the degree of explanation of the principal components was not as good as we expected, and we presumed that this might be caused by the short period of time we collected the samples. Unfortunately, with the deepening decomposition, we could not collect samples from the corresponding parts at the later stage of sampling (after 168 h). In addition, the subsequent principal components explained less variance compared to the first three, so we did not repeat them. Hence, the use of humans [8,58] or large animals (e.g., pigs [59–62]) will be an area for further improvement in our future studies.

Our study demonstrated that most of the oral microbial communities during rat carcass decomposition belonged to four phyla:

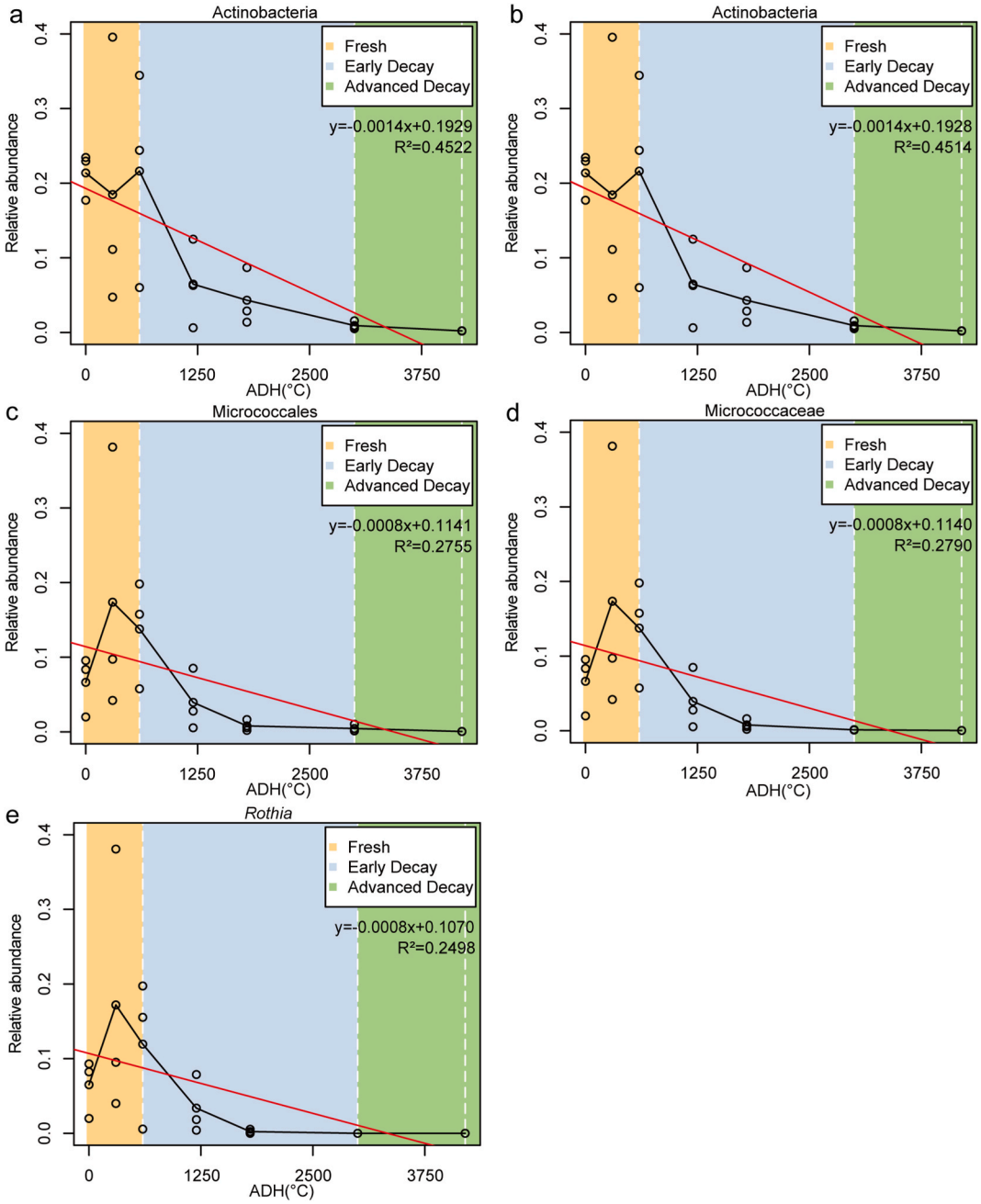

Fig. 5. The relative abundance changes of microorganisms at different taxonomic levels showed a strong negative correlation with ADH (accumulation degree hours). The red line represents the line constructed by the linear regression model; The black line is the connection of the mean value at each time point. **a** Relative abundance changes of Actinobacteria at the phylum level over time; **b** Relative abundance changes of Actinobacteria at class level over time; **c** Relative abundance changes of Micrococcales at order level over time; **d** Relative abundance changes of Micrococcaceae at family level over time; **e** Relative abundance changes of Rothia at genus level over time.

Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. Proteobacteria abundance increased overall throughout decomposition, while Actinobacteria, Firmicutes, and Bacteroidetes decreased, which was consistent with previous studies based on different mammals [8,50–53,58,63,64]. Despite the different sample sources (oral [8,63] and proximal large intestine [65]) we found a similar trend of declining abundance in the Bacteroidetes. Combined with earlier studies [63], we suggested that the reason for the enrichment of Proteobacteria over time could be due to the rapid depletion of oxygen, resulting in the predominance of Proteobacteria. However, the results of our study were inconsistent with previous findings for trends of Proteobacteria and Firmicutes. Hyde et al. [8] documented an increase in the abundance of Firmicutes in oral samples, but two human cadavers collected before and after bloating made the

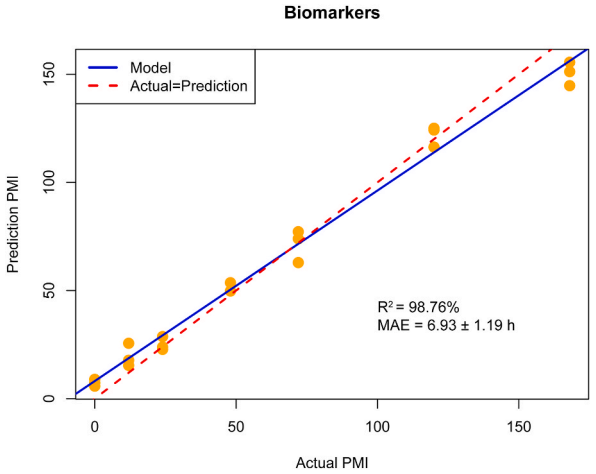

Fig. 6. Random forest regression model based on 30 biomarkers. Each orange dot represents an oral sample. The red dotted line indicates that the predicted values are equal to the actual values. The solid blue line represents the fit of the samples.

results discontinuous. In previous research, it was observed that Firmicutes increased with decomposition, replacing Proteobacteria as the most abundant phylum in oral samples [58], we considered that this might be related to whether the insects were colonised in the carcasses or not, i.e., carcasses that were open to colonisation by insects were dominated by Firmicutes, whereas carcasses not colonised by insects were dominated by Proteobacteria [60,66].

The linear regression modelling led to the discovery that the Actinobacteria phylum had the best fit compared to microorganisms at the other four taxonomic levels (Actinobacteria, Micrococcales, Micrococcaceae, *Rothia*) and showed a negative correlation over time. Actinobacteria are gram-positive bacteria, one of the most diversified prokaryotes, and are widespread in both terrestrial and aquatic ecosystems, with the ability to promote the decay of the remains of animals and plants [67,68]. Meanwhile, *Rothia* had been shown early to be one of the major microbial communities of oral saliva and to have an essential driving role in the early stage of cadaver decomposition [69].

Then, we performed a random forest model using all microbiome data at the genus level of oral samples and evaluated the performance of the model, i.e., the R^2 and MAE values were 98.69 % and 7.62 ± 1.54 h, respectively. Nevertheless, the microbiome data did not all have an effect on the estimation of PMI [27]. Therefore, with the aim of further avoiding the model over-fitting and improving the accuracy of the model, we utilized 10-fold cross-validation [70] to obtain the number of feature variables with the minimum error for further analyses. Finally, we gained the minimum error at a number of feature variables of 30. In addition, we ranked the importance of microbiome data in PMI estimation based on percent of increase in mean squared error (%IncMSE) and visualized the top 30 biomarkers, including: *Ignatzschineria*, *Morganella*, *Proteus*, *Lysinibacillus*, *Pseudomonas*, *Globicatella*, *Corynebacterium*, *Streptococcus*, *Rothia*, *Aerococcus*, *Staphylococcus*, and so on. *Morganella* is anaerobic or facultatively anaerobic and involved in the formation of biogenic amines, which we hypothesized might explain its higher content during the period of early decay [71]. *Proteus* belonged to the facultative anaerobes, which could be related to the fact that the oral cavities of the rats were in the closed state when they died [64]. And the discovery of *Globicatella* may be due to postmortem hypoxia in rats [72]. Previous studies showed that *Corynebacterium*, *Streptococcus*, *Rothia*, and *Staphylococcus* were common genera observed in the human oral cavity during the early stage of decomposition [69]. Of these, *Staphylococcus* was an ideal candidate for the estimation of PMI by using machine learning methods [73]. *Aerococcus* is a genus of bacteria found in dust and air [74] and has been increasingly recognized as a significant human pathogen [75]. We speculated that the enrichment of *Aerococcus* was due to the collapse of the immune system caused by the death of the rats, which allowed airborne *Aerococcus* to colonise the oral cavity. Furthermore, *Ignatzschineria*, *Lysinibacillus*, and *Pseudomonas* generally appeared in the middle to late stages of decay [69]. Among them, the relative abundance of *Ignatzschineria* was significantly higher, which might be related to the presence of necrophagous insect species on carcasses in the late stage of decomposition [76]. Moreover, we reconstructed a random forest regression model based on the data of the selected 30 biomarkers, and the performance of the model was evaluated as follows: the R^2 and MAE values were 98.76 % and 6.93 ± 1.19 h, respectively. Meanwhile, we could notice that the random forest regression model constructed with biomarkers data had a larger R^2 value and a lower MAE value compared to using all microbiome data, which indicated that the estimation of PMI with biomarkers data might be more accurate. Specifically, a random forest model constructed using biomarkers from the oral cavity within 168 h of decomposition, based on the use of soil to simulate the environment of death, allowed us to estimate PMI within 6.93 ± 1.19 h. However, forensic science experts have already begun to explore the possibility of predicting PMI with the help of machine learning (ML) to predict PMI with higher accuracy [77]. For example, Johnson et al. collected human nasal and ear canal samples in temperate forests and constructed the k-nearest neighbour (KNN) regressor model to predict PMI using phylum-level microbiome data over a time period of 800 accumulated degree days (ADD) with an MAE of 55 ADD [73]. Cao et al. sampled the caecum of rats in an artificial climate chamber and used the microbial community to build a partial least squares (PLS) model to predict PMI, which resulted in a root mean square error (RMSE) of 1.96 d over 9 days [78]. The random forest algorithm has been widely applied to predict PMI due to possessing the advantages of high learning capacity,

robustness, and feasibility [79]. In 2013, Metcalf et al. utilized microbiological data from the skin of postmortem mice for the first time in the laboratory environment to build a random forest model to predict PMI, which allowed them to predict PMI during the 48-day decomposition period with an MAE value of 3.30 ± 2.52 d and pioneered new methods for estimating PMI using microbiome data [25]. Next, the academics explored the relationship between microbiome data and PMI in further depth by utilizing the random forest algorithm, including different experimental subjects [61,80], different collection sites [27,81,82], and different environments [83–86], all of which broadened our research ideas. Recently, Liu et al. reported that artificial neural networks (ANNs) had higher accuracy for PMI prediction [26]. However, because of our limited sample size, we did not construct an ANN model. Consequently, increasing the sample size to build models that could be more accurate in predicting PMI would be one of the goals of our endeavours. However, it should be noted that gaps exist between our study and forensic practice, including the extension of sampling time, the use of human samples, the influence of surrounding environmental conditions, the feasibility of the technology and further verification by using actual cases. The method of estimating PMI with microbiome data is still in the exploratory stage, which makes it necessary to improve the reliability of this method by comparing it with other more common approaches to PMI estimation [53]. Therefore, the ways in which we should relate our findings to forensic science practice need to be further explored.

5. Conclusion

In conclusion, our study collected outdoor soil to simulate the death environment and focused on analyzing microbiome data from the oral cavity of rat carcasses on soil during decay. The phylum Actinobacteria fit best in the linear regression model and showed a negative correlation over time. In addition, a random forest regression model was developed to estimate PMI, with an MAE of 6.93 ± 1.19 h. Meanwhile, we selected some microorganisms that might be important for the estimation of PMI, including: *Ignatzschineria*, *Morganella*, *Proteus*, *Lysinibacillus*, *Pseudomonas*, *Globicatella*, *Corynebacterium*, *Streptococcus*, *Rothia*, *Aerococcus*, *Staphylococcus*, and so on. However, there exist some shortcomings in our study, such as short sampling time, sample types, and limitations of the surrounding environmental conditions, which were because our study is a basic study that lacks further validation and technical feasibility for practical applications. In summary, our study suggested that oral microbiology could be used as a complementary method to traditional forensic medicine for the estimation of PMI.

Statements and declarations

Conflict of interest

The authors declare that there is no conflict of interest.

Ethics statement

The present research was conducted following the guidelines of the Ethics Committee in Guizhou Medical University (Approval number: 2201523).

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Xiaoxue Wang: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cuiyun Le:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaoye Jin:** Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Yuhang Feng:** Validation, Investigation, Data curation. **Li Chen:** Validation, Investigation, Data curation. **Xiaolan Huang:** Validation, Investigation, Data curation. **Shunyi Tian:** Validation, Investigation, Data curation. **Qiyan Wang:** Methodology, Investigation, Conceptualization. **Jingyan Ji:** Methodology, Investigation, Conceptualization. **Yubo Liu:** Methodology, Investigation, Conceptualization. **Hongling Zhang:** Methodology, Investigation, Conceptualization. **Jiang Huang:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Zheng Ren:** Writing – review & editing, Supervision, Resources,

Methodology, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31897.

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