Potential use of high-throughput sequencing of bacterial communities for postmortem submersion interval estimation

Jing He¹ · Juanjuan Guo¹ · Xiaoliang Fu¹ · Jifeng Cai¹

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Abstract

Microorganisms play vital roles in the natural decomposition of carcasses in aquatic systems. Using high-throughput sequencing techniques, we evaluated the composition and succession of microbial communities throughout the decomposition of rat carcasses in freshwater. A total of 4,428,781 high-quality 16S rRNA gene sequences and 2144 operational taxonomic units were obtained. Further analysis revealed that the microbial composition differed significantly between the epinecrotic (rat skins) and the epilithic (rocks) samples. During the carcass decomposition process, Proteobacteria became the dominant phylum in the epinecrotic, epilithic, and environmental (water) samples, followed by Firmicutes in the epinecrotic samples and Bacteroidetes in the epilithic and water samples. Microbial communities were influenced by numerous environmental factors, such as dissolved oxygen content and conductivity. Our study provides new insight about postmortem submersion interval (PMSI) estimation in aquatic environments.

Keywords PMSI · Epinecrotic · Epilithic · Forensic · Microorganism

Introduction

The postmortem interval (PMI) is the time span between death and postmortem examination. PMI estimations are of vital importance in most forensic investigations. In the terrestrial environment, succession of saprophytic organisms, such as bacteria, fungi, and necrophagous insects on decomposed

carcasses, is often used as a factor in PMI estimation. However, carcass decomposition in aquatic environments is more complicated due to multiple factors, such as water temperature, salinity, tide effects, the depth at which the carcass is located, carcass mobility in the water, the original bacterial and chemical composition of the water, and scavengers [1, 2]. Thus, PMI estimation remains an essential and difficult task in forensic science.

Postmortem submersion interval (PMSI) is the amount of time from submersion of remains to their discovery [3–5]. It has been suggested that water temperature is the most important factor for estimating PMSI [6]. However, the reliability of the PMSI estimation using Reh's table showed that only 59% of the cases could be reliably estimated [1]. Recently, some researchers have developed an aquatic decomposition score method for PMSI estimation based on accumulated degree days. However, this model of PMSI estimation has some limitations [7–9].

Microorganisms, including the internal microbiota of the carcass as well those of the surrounding environment, play an important role in the natural decomposition of carcasses in aquatic systems [1]. Numerous reports have found the presence of microorganisms on carcasses in all stages of decomposition in aquatic systems [5, 10]. For instance, it has been suggested that microorganisms drive carcasses decomposition

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✉ Jifeng Cai
cjf_jifeng@163.com

Jing He
402588969@qq.com

Juanjuan Guo
459879644@qq.com

Xiaoliang Fu
fuxiaoliang8893@163.com

¹ Department of Forensic Medicine, School of Basic Medical Sciences, Central South University, Changsha 410013, Hunan, People's Republic of China

[11]. Metabolic activities of microorganisms constitute a major part of the decomposition process [12]. Therefore, epinecrotic microbial communities could be used for PMSI estimation by linking successional changes of microbial composition, which could potentially serve as a biological indicator of PMSI [5, 10]. Bacterial sequencing could be useful for accurately estimating PMSI in aquatic environments [1, 3–5, 13], similar to terrestrial habitats [12].

In aquatic systems, microbes mainly thrive in the form of biofilms [4, 14, 15]. A biofilm is an assemblage of surface-associated microorganisms enclosed in an extracellular polymeric substance [5, 16–18]. Biofilms encapsulate various microorganisms, including non-biofilm forming bacteria, eukaryotes, and other abiotic substances such as sediment particles. They also play important roles in aquatic environments by contributing to energy flow and nutrient cycling [19–21]. Biofilms formed on decomposing carcasses are generally defined as epinecrotic biofilms [22–25], while those formed on inorganic substrates (e.g., rocks) are defined as epilithic biofilms [26–29]. Succession of epinecrotic biofilms has been used in PMSI estimation using an automated ribosomal intergenic spacer analysis in a swine decomposition model [5]. Only a few studies have evaluated aquatic biofilm microorganisms compared to those of terrestrial environments using high-throughput sequencing [12, 30].

Here, we applied high-throughput sequencing to study the composition and succession of microbial communities throughout the decomposition process of rat carcasses in an aquatic environment. We evaluated the changes of epinecrotic, epilithic, and aquatic microbial communities during decomposition. We also aimed to assess differences in microbial community succession and predicted functions of epinecrotic and epilithic samples. Our study provides new insight about PMSI estimation in aquatic environment.

Materials and methods

The study and all protocols were approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (approval No: 201503465).

Experiments were conducted in Changsha, Hunan Province, China (28.13° N, 112.56° E) in July 2017. Six-week-old Sprague-Dawley rat cadavers (180–220 g) (Changsha Tianqin Biotechnology Co., Changsha, China) were used as a decomposition model. Nine rats were sacrificed and the carcasses were placed in three 60 cm × 40 cm × 40 cm glass containers, at three rats per container. A fourth container was used as a control (fresher water without any rat carcasses). The containers were pre-filled with freshwater collected from the Xiangjiang River, the largest river in Hunan Province, China. The bottoms of the containers were covered with a layer of small rocks, which were pre-washed with the

freshwater from the Xiangjiang River. The containers were equipped with filters and oxygen (Fig. S1). The oxygen was supplied via an oxygen pump (3 W, 2.5 L/min flow rate). The containers holding cadavers were placed apart in a ventilated basement with a 12 h:12 h dark:light cycle. Indoor air temperature and humidity were kept at 23 °C and 99% relative humidity.

Water chemistry

Water properties, including dissolved oxygen (mg/L), pH, conductivity (mS/cm), and temperature (°C), were measured on each sampling day using a Horiba Multi Water Quality Checker (Kyoto, Japan) that was placed 10 cm below the water surface.

Sample collection

Three types of specimens were collected: epinecrotic (skins of rat carcasses), epilithic (surfaces of the rocks in the container), and environmental (water from the tank). A diagram for the experimental set up is illustrated in Fig. 1. Epinecrotic specimens were sampled at multiple time points: 10 min before sacrifice, immediately after sacrifice, and then daily until the rat carcasses were completely decayed, which typically occurred within 6 days (total 7 samples). Epilithic and water specimens were sampled 10 mins after rat carcasses were placed in containers, and then daily, similar to what was described for the epinecrotic samples. Skins of three rat carcasses and three rocks from each container were rubbed gently for 60 s using sterile cotton applicators. For skin sampling, the hair was not removed so that the microbial communities between skin and hair biofilm communities were not altered. Three water samples of 50 ml were collected in each container on each sampling day as environmental samples. The water samples were collected at a depth of 10 cm from the top and used directly for DNA isolation (no centrifuge or filtration). Consecutive collections were performed a few centimeters apart from the previous sampling area to avoid disturbed bacterial communities in a previously sampled area. Carcasses were immediately submerged after sample collection. After sampling, the tip of the applicator was cut off and placed in a 1.5-ml centrifuge tube. All samples were immediately frozen at –20 °C until further processing.

DNA extraction and PCR amplification

Microbial DNA was extracted from each sample using the FastDNA® Spin Kit for Soil (MP Biomedicals, USA) [31–33] following the manufacturer's instructions. DNA quality was checked using 1% agarose gel electrophoresis. DNA samples that did not meet the standard were discarded. PCR reactions were conducted using the 338F/

Fig. 1 Diagram showing the experimental set up

806R primer set [34], which targeted the V3-V4 region of the 16S rRNA gene (forward primer: 5'-ACTC CTACGGGAGGCAGCAG-3', reverse primer: 5'-GGAC TACHVGGGTWCTAAT-3'). PCR tests were carried out in 20 μ L reaction volumes containing 2 μ L 10X buffer, 2 μ L 2.5 mM dNTPs, 0.8 μ L 5 μ M primer (each), 0.2 μ L rTaq polymerase, 0.2 μ L bovine serum albumin (BSA), and 10 ng template DNA. Thermal cycling conditions were as follows: 95 $^{\circ}$ C for 3 min, followed by 28 cycles at 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 45 s, with a final extension at 72 $^{\circ}$ C for 10 min.

Illumina MiSeq sequencing

PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, USA) and quantified using QuantiFluorTM-ST (Promega, USA). Purified amplicons were pooled at equal concentrations and paired-end sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, USA) according to protocols provided by the Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China).

Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered using Trimmomatic, and merged using FLASH. Operational taxonomic units (OTUs) were clustered with a 97% similarity

cutoff using Usearch (version 7.0), and chimeric sequences were identified and removed using UCHIME. Taxonomy was assigned to each OTU based on an open-reference data search, using an RDP Classifier algorithm against the Silva 16S rRNA database with a confidence threshold of 97%. 'ut's with fewer than 30,000 sequences per sample were excluded.

Alpha diversity was estimated by four phylogenetic diversity metrics: Chao1, Shannon, Simpson, and Coverage. The four phylogenetic diversity metrics were analyzed using Mothur (version 1.30.1) software. Analyses of OTU taxa among three containers in the epinecrotic, epilithic, and water samples were performed using the Student's *t* test. Principal coordinates analyses (PCoA) were performed on the basis of the weight UniFrac distances. The Welch's *t* test was used to identify bacterial community taxon that showed significant differences in abundance between epinecrotic and epilithic communities [35]. A non-metric multidimensional scaling (NMDS) classification was conducted to explain the clustering of bacterial community composition variation, which was based on the weighted UniFrac distance of OTUs. Partial least squares discriminate analysis (PLS-DA) was performed to examine differences in the bacterial communities between the epinecrotic and epilithic samples. Linear discriminant analysis effect size (LefSe) was used to identify taxa differences between the epinecrotic and epilithic bacterial communities. High LDA scores represent significantly higher abundances of certain taxa.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict metagenomic functional content based on the 16S rRNA sequences. The OTU table was used as the input for metagenome imputation and was first rarefied to an even sequencing depth prior to the PICRUSt analysis. The resulting OTU table was normalized by 16S rRNA gene copy number. Gene content was predicted for each sample. Then the predicted functional composition profiles were collapsed into level 3 of Kyoto Encyclopedia of Genes and Genomes (KEGG) data pathways. Environmental factors were determined by the variance inflation factor (VIF) as previously described [36]. The environmental factors with VIF > 20 were considered useless environmental factors [36, 37]. The Mantel test was used to examine whether variation in environmental factors significantly correlated with differences in the weighted UniFrac distance among epinecrotic communities.

Results

Changes in water properties during rat carcass decomposition

Water temperature, pH, dissolved oxygen, and conductivity during decomposition were recorded and are shown in Fig. S2. The water temperature ranged from 26 to 28 °C. Water pH was stable within the range of 7.40–8.06. Conductivity increased, while dissolved oxygen decreased as time progressed. No significant changes in temperature, pH, dissolved oxygen, or conductivity were observed for the control (water without any rat carcasses) during the same period of time (data not shown).

Physical changes of rat carcasses during decomposition

The rat carcasses progressed through five stages of decomposition: submerged fresh, early floating, early floating decay, advanced floating decay, and sunken remains [3, 38, 39]. The submerged fresh stage began at the time of death. Rat carcasses remained fresh and displayed no visible external signs of decomposition. The early floating stage began within 48 h and was characterized by the carcass floating on the water surface with a continuous odor and slightly flabby skin. Over the course of the early floating stage, carcasses displayed minor signs of decomposition, such as disarticulated soft tissues. Decay stage began on the third day and was characterized by disintegration of soft tissues. The carcasses released a strong odor and their eyes became disjoined. Carcass skins were covered with a thin and sticky microbial biofilm. The advanced decay stage was marked by further tissue deterioration. The advanced floating decay stage began 5 days

postmortem and was characterized by the disappearance of leg bones and other major tissue deterioration. The odor of decomposition was still apparent but less strong compared to the late stage of decay. Major tissue mass lost during this period may contribute to this phenomenon. Carcasses entered the sunken remains stage by day 6 of the experiment. This stage was characterized by remaining small bones of the carcasses.

Comparison of microbial communities among the epinecrotic, epilithic, and environmental samples at OTU level

After sequence trimming, quality filtering, and removal of chimeras, a total of 4,428,781 high-quality sequences (with an average length of 440 bases) were retained. These high-quality sequences were clustered into 2144 unique OTUs, with an average of 1009 OTUs in the epinecrotic samples (rat skins), 999 OTUs in the epilithic samples (rocks), and 526 OTUs in the environmental samples (water) (Table 1). The rock samples had the highest number of OTUs (1384) at the beginning of the experiment, but decreased to 886 at the end of the experiment (day 6). The number of OTUs of rat skin samples also decreased from 1075 to 682, while the water sample OTUs remained stable (Table 1). Alpha diversity analysis further confirmed that the microbial communities decreased over decomposition, but alpha diversity indexes of samples collected immediately after death were higher than those of samples collected from live rats (Table 1). The OTUs in the control samples (water without rat carcass) did not significantly change, remaining at 440, through the entire experimental period. Student's *t* test showed that the OTUs were significantly different between the epinecrotic (rat skins) and the epilithic (rock) bacterial communities ($p < 0.05$), as well as between the epilithic and the environmental (water) samples ($p < 0.05$). No significant difference was observed between replicated samples obtained from each container ($p > 0.05$).

Comparison of microbial communities among the epinecrotic, epilithic, and environmental samples at phylum to species level

We classified $96.12 \pm 0.41\%$ of high-quality sequences to the phylum level, $92.04 \pm 0.76\%$ to the family level, $90.68 \pm 0.55\%$ to the genus level, and $33.56 \pm 1.18\%$ to the species level. Our results revealed bacteria belonging to 28 phyla (27 in rat skin, 28 in rock, and 23 in water samples), 273 families (248 in rat skin, 264 in rock, and 212 in water samples), 742 genera (673 in rat skin, 687 in rock, and 553 in water samples), and 1315 species (Table S1). All raw sequences were deposited in the Sequence Read Archive (accession number SRP139022).

Table 1 Operational taxonomic unit (OTU)–based diversity indexes in rat skin, rock and water samples during decomposition (data were presented as average of each sample type)

Sample	Sample region	Sample time	OTUs	Shannon	Simpson	Ace	Chao1
D00	Skin	Prior to death (0 day)	1075	2.99	0.18	517.78	503.00
D01	Skin	1 day	1254	2.84	0.33	560.50	564.79
D02	Skin	2 days	1187	2.82	0.34	588.64	553.54
D03	Skin	3 days	1186	3.22	0.17	549.73	555.76
D04	Skin	4 days	990	3.35	0.11	526.07	506.88
D05	Skin	5 days	688	2.29	0.24	346.38	314.12
D06	Skin	6 days	682	2.96	0.12	334.03	334.23
S01	Stone	1 day	1384	4.87	0.02	867.20	863.57
S02	Stone	2 days	1121	4.83	0.02	847.02	861.34
S03	Stone	3 days	1077	4.88	0.02	740.05	743.87
S04	Stone	4 days	702	3.90	0.06	622.49	632.86
S05	Stone	5 days	825	3.92	0.06	566.36	561.90
S06	Stone	6 days	886	3.60	0.12	680.68	665.38
W01	Water	1 day	447	3.82	0.06	518.82	509.25
W02	Water	2 days	573	4.56	0.03	623.35	622.10
W03	Water	3 days	743	3.90	0.05	718.17	755.25
W04	Water	4 days	398	3.13	0.20	409.79	411.04
W05	Water	5 days	401	3.63	0.05	377.15	396.85
W06	Water	6 days	592	3.37	0.08	398.95	381.79

Changes in the bacterial communities were observed inside the same type of samples during carcass decomposition. In the epinecrotic samples, Proteobacteria was the dominant phylum during the first 4 days of decomposition, but Firmicutes became the dominant phylum on days 5 and 6 (Fig. 2a). At the family level (Fig. 2b), the abundance of Paenibacillaceae was the highest in samples

obtained prior to death ($37.51 \pm 2.64\%$), but gradually decreased thereafter. Clostridiaceae increased in abundance and became the most abundant taxon on days 1, 4, 5, and 6. The abundance of Comamonadaceae increased on day 2 and became the dominant family, but subsequently decreased from day 4. The abundance of Enterobacteriaceae gradually increased.

Fig. 2 Bacterial community structure variation during decomposition at the phylum and family levels. Relative abundance of bacterial phyla during decomposition in the epinecrotic samples (a), epilithic samples (c), and water samples (e). Relative abundance of bacterial families

during decomposition in the epinecrotic samples (b), epilithic samples (d), and water samples (f). Sample names refer to samples as described in Table 1

Fig. 3 A two-dimensional principal coordinate analysis (PCoA) plot of weight UniFrac distance matrices for epilithic and epinecrotic samples during decomposition. The bacterial community of the epinecrotic samples from rat carcasses (roundness) and epilithic samples (plus sign) is represented (a). A non-metric multidimensional scaling (NMDS) plot of weighted UniFrac distance matrices for epilithic and epinecrotic samples during decomposition. The bacterial community of the epinecrotic

samples from rat carcasses (roundness) and epilithic samples (multiple sign) are represented (b). The Partial Least Squares Discriminant Analysis (PLS-DA) between epilithic and epinecrotic samples is presented on OTU levels. The space distance of the sample point is the distance between the samples (c). Sample names refer to samples as described in Table 1

In the epilithic samples, Proteobacteria was the dominant phylum from days 2 to 6 (Fig. 2c). The family of Comamonadaceae became the dominant taxon on days 2 and 3, but gradually decreased thereafter. The abundance of Moraxellaceae was dominant on days 4 ($21.56 \pm 4.47\%$), and 6 ($33.65 \pm 5.03\%$) (Fig. 2d).

In the water samples, Proteobacteria was the dominant phylum from days 2 to 6 (Fig. 2e). The family of Comamonadaceae became the dominant taxon from days 2 to 5, but gradually decreased (Fig. 2f). Clostridiaceae became the most abundant family on day 6 ($23.89 \pm 4.52\%$).

Statistical analysis of the microbial communities between the epinecrotic and the epilithic samples

PCoA and two-dimensional NMDS were applied to analyze possible similarities and differences of microbial communities between the epinecrotic and the epilithic samples. Both analyses revealed that the epinecrotic and the epilithic samples

from the early decomposition stage formed similar clusters, while samples from the later stages of decomposition were disparate (Fig. 3a, b). PLS-DA further suggested that the epinecrotic communities were closer to each other, but the epilithic communities were more separated (Fig. 3c).

At the phylum level, Welch's *t* test showed that the abundances of several phyla, including Cyanobacteria, Chlorobi, Gracilibacteria, and Bacteroidetes, were significantly different between the epinecrotic and the epilithic bacterial communities (Fig. 4a, $p < 0.001$). These phyla were more abundant in

Fig. 4 Epinecrotic and epilithic community differences during decomposition at the phylum, family, and genera levels. Data were analyzed using the Student's *t* test. a Significant differences during decomposition at the phylum level. b Significant differences during decomposition at the family level. c Significant differences during decomposition at the genera level. "D" indicates the epinecrotic communities. "S" indicates the epilithic communities. Sample names refer to samples as described in Table 1. * p value < 0.05 . ** p value < 0.01 . *** p value < 0.001

Fig. 5 Histogram of the LDA scores computed for features differentially abundant between epilithic and epinecrotic samples. “D” represents epinecrotic samples; “S” represents epilithic samples

the epilithic compared to the epinecrotic samples. Firmicutes, however, was less abundant in the epilithic compared to the epinecrotic samples. Moraxellaceae, Flavobacteriaceae, and Chromatiaceae were significantly more abundant in the epilithic samples (Fig. 4b, $p < 0.001$). The abundances of *Flavobacterium*, *Hydrogenophaga*, *Acidaminobacter*, *Rheinheimera*, and *Acinetobacter* were higher in the epilithic compared to epinecrotic samples (Fig. 4c). In addition, using linear discriminant analysis effect size (LefSe), we found that the abundances of 21 taxonomic clades were significantly higher in the epilithic compared with the epinecrotic samples (Fig. 5 and Table S2). The representative differentially abundant bacterial taxon included *Chryseobacterium* ($p < 0.001$) and *Lysobacter* ($p < 0.001$). All of these bacterial communities had high LDA scores ($\text{LDA} > 3.4$).

Analysis of predicted microbial pathways during carcass decomposition

A total of 237 biochemical pathways were identified using KEGG pathway analysis. The pathways with higher abundances were similar between the epilithic, epinecrotic, and water samples (Fig. 6). Many metabolic pathways were enriched in the KEGG analysis, suggesting that microbial metabolism during carcasses decomposition was particularly active. These pathways included ATP binding cassette (ABC) transporters, two-component system, purine metabolism, ribosome, pyrimidine metabolism, arginine and proline metabolism, butanoate metabolism, pyruvate metabolism, and propanoate metabolism.

Effect of environmental factors on epinecrotic bacterial communities

The VIF values of conductivity (VIF = 8.05), pH (VIF = 5.41), temperature (VIF = 3.63), and dissolved oxygen (VIF = 12.36) were all lower than 20. Therefore, all of these factors were considered useful environmental factors. The Mantel test showed that conductivity ($R^2 = 0.17$, $p < 0.05$) had a major effect on epinecrotic bacterial community variation. Dissolved oxygen ($R^2 = 0.08$, $p < 0.05$) had a small, but statistically significant, impact on epinecrotic bacterial communities. Temperature ($R^2 = 0.06$, $p > 0.05$) and pH ($R^2 = 0.01$, $p > 0.05$) had insignificant effects on epinecrotic bacterial communities.

Discussion

Consistent with previous studies [1, 4], our data showed that several phyla, including Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria, were abundant in our epinecrotic samples. These microbes varied significantly during the decomposition process. Dominant microorganisms found in rat carcass skin samples are known to be common to vertebrates and to aquatic environments. These microbes include *Clostridium*, *Paenibacillus*, *Hathewayia*, *Aquabacterium*, and *Enterococcus* (Fig. S3). *Clostridium* is

Fig. 6 The highest abundance of biochemical pathway variation during decomposition. Relative abundance of biochemical pathways during decomposition in the epinecrotic samples (a), epilithic samples (b), and water samples (c). Sample names refer to samples as described in Table 1

common in the human intestinal flora, as well as in many other environments [40–42]. *Paenibacillus* has also been isolated from many environmental samples, such as marine sediment [43], soil [44, 45], and hot springs [46]. *Aquabacterium* was found in drinking water systems [47]. *Enterococcus* has also been investigated in a number of habitats and is a common coliform [48–51].

The dynamic shifts in microbiological composition during the decomposition process may be useful for estimating PMSI. For example, we found that the abundance of Proteobacteria decreased while Firmicutes increased during decomposition. This is consistent with a previous study by Benbow et al. [4], who showed a similar trend during swine decomposition in freshwater. Changes in nutrients during decomposition may be a contributing factor to this trend. Degradation products of decomposition could be used as nutrients for the formation and development of microbial biofilms, so the dominant bacterial species vary at different stages of decomposition. Changes in environment, such as dissolved oxygen levels, may also contribute to changes in microbiological composition. Clostridiaceae and Comamonadaceae were the most abundant families and displayed different succession patterns. A significant increase in Clostridiaceae may indicate mid-late submersion interval. An increase in Comamonadaceae would suggest an early submersion interval. Detection of Clostridiaceae in combination with Comamonadaceae could help narrow the PMSI estimation. Thus, using a combination of staggered bacterial populations (the succession of microorganisms) may greatly improve PMSI estimation. Analysis using Random forests further confirmed this conclusion. In addition, Random forests analysis showed that several other bacterial families, including Methylocystaceae, Phyllobacteriaceae, and Nocardiaceae, could be good PMSI indicators.

Development and colonization of microbial communities in a microbial biofilm are related to resource substrate types [26, 27, 52, 53]. We assessed differences in microbial community succession between epinecrotic and epilithic samples. Our findings revealed that two microbial biofilms had significant differences in microbial composition. The relative abundances of *Cyanobacteria* and *Aquabacterium* were higher in the epilithic biofilms than in the epinecrotic biofilms. Algae were the dominant microorganisms in the epilithic biofilms, with only a small population in the epinecrotic biofilms. This finding agreed with a previous study, which demonstrated that the abundance of algae was higher than bacteria in epilithic biofilms [54]. Our study also found that heterotrophs, such as *Clostridium*, *Aquabacterium*, and *Enterococcus*, were dominant in the epinecrotic biofilms, while autotrophs, such as some species of *Pseudomonas*, *Aquaspirillum*, and *Flavobacterium*, were predominantly

present in the epilithic biofilms. These findings are in agreement with previous studies [53, 55], which illustrated that heterotrophs or detritivores are the dominant microorganisms in epinecrotic biofilms, while autotrophs are the dominant microorganisms in epilithic biofilms. The reason for such differences between epinecrotic and epilithic biofilms may be related to the fact that the carcass substrates can provide nutrients to the epinecrotic biofilms, while the epilithic (such as rocks) substrates cannot provide nutrients to the epilithic biofilms. Due to differences in composition of microbial biofilms on different substrates, our study offers a novel approach towards forensic investigations from a unique microbiological perspective.

Nutrient cycling during carcass decomposition in terrestrial environments has been investigated, but those in aquatic settings are still poorly understood [11, 56]. Our study showed that the pathways of high abundance were similar in epinecrotic, epilithic, and water samples (such as ATP binding cassette transporters, two-component system, and purine metabolism). The valine, leucine, and isoleucine degradation pathway was not found in the epinecrotic and epilithic communities, and the oxidative phosphorylation pathway was not found in the water microbial communities. The reason for this phenomenon is unknown and further studies are required to better understand this complex issue.

A previous study revealed that epinecrotic biofilms may be utilized as a microbial clock for PMSI estimation [5]. However, microbial biofilms are influenced by numerous environmental factors such as temperature and oxygen content. For example, bacterial biofilm communities were affected by ocean acidification [57], different seasons [58], and physical and chemical properties of water [5]. These properties could in turn be used for PMSI estimation. In our study, the carcass decomposition experiments were conducted in a closed environment, with constant ambient and water temperature and relative humidity. Our results showed that the microbial biofilm communities were mainly affected by conductivity and, to a lesser degree, by dissolved oxygen. One possible explanation for the heavy influence of conductivity to carcass decomposition is that the experiment was conducted in relatively small containers, where the conductivity could be influenced by the carcass decomposition (Fig. S2b). Thus, conductivity may have affected carcass decomposition. More investigation in an open environment (like rivers) is needed to confirm our conclusions.

There are several limitations in our study, one of which is its small sample size. Other limitations include the effect of rat hair and lack of flow in the water containers. While the containers without water flow simplified the number of variables, the conditions did not accurately reflect the natural environment. More studies are required to address these issues.

Conclusions

In this study, we found that the microbial communities of epinecrotic, epilithic, and aquatic samples vary greatly during carcass decomposition. Our results suggest that detection of both Clostridiaceae and Comamonadaceae together could help improve PMSI estimation. Our study provides new insight about PMSI estimation and considering microbial biofilms as indicators of decomposition.

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Compliance with ethical standards

The study and all protocols were approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (approval No: 201503465).

Conflict of interest The authors declare that they have no conflicts of interest.

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