Insights into the Microbial Weathering of Chinese Wooden Ancestral Halls in Guangdong Province

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Abstract

Wooden buildings are facing biodeterioration because of the natural weathering. Ancestral halls are traditional Chinese wooden architecture with high artistic value. The research analyzed the microbial diversity of nine ancestral halls in Guangdong Province under subtropical monsoon climates. With amplicon sequencing, we found a common core microorganism group, including *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Acinetobacter*, *Toxicocladosporium*, *Cladosporium*, *Aspergillus*, and *Epicoccum*. And a total of 7 different species of bacteria and 9 different species of fungi were isolated from Chen Clan Ancestral halls, most of which were proven to be capable of degrading cellulose or lignin. The comparison between damaged and undamaged sites (whether the paint coating is flaked off) showed that the microbial composition of these two sites is similar, but with a different abundance. At the phylum level, Firmicutes and Ascomycota were significantly more abundant in damaged surfaces; and *Cladosporium*, as the predominant genus, may play a crucial role at the deterioration of wooden ancestral halls. The similarity of their microbial composition also indicates that using paint as the only method for protecting bare wood is not enough. Apparent correlation between microorganisms on wooden surfaces and humans showed that the increase of visitor flow rates could have an influence on the composition of microbiota on cultural relic. We evaluated the effect of four representative biocides and determined the feasibility of low-concentration Isothiazolinone. This research facilitates a basic understanding of the microbial weathering of ancestral halls and add significant reference for the protection of wooden buildings.

1. Introduction

Bare wooden architecture under open-air conditions is a common habitat for many microorganisms, including bacteria and fungi, due to its porous structure and organic components(Cennamo et al., 2018, De Windt et al., 2014). Wooden structures consist of 40-60% of cellulose, 15-30% of hemicellulose, and 17-35% of lignin(Velasco-Rodriguez et al., 2022), which are a rich source of nutrition for microorganisms. The colonization by microorganisms of the wood surface results in forming of a biofilm, an extracellular polysaccharide matrix (EPS) consisting of cell debris and cell excretions such as proteins, polysaccharides, and lipids. As the biofilms provide a protective microenvironment for microorganisms, allowing more of them to grow and reproduce on the surface, the wooden architecture faces further biodeterioration(Coelho et al., 2021, Li et al., 2022, Negi and Sarethy, 2019, Skipper et al., 2022). Meanwhile, the pigments produced by the microorganisms and the various colors of microbial communities cover the surface of wooden cultural relics, leading to severe aesthetic damage(Kim et al., 2020, Sterflinger and Pinar, 2013). What's more, studies show that cellulolytic microorganisms such as *Bacillus*, *Aspergillus*, *Cladosporium*, and *Penicillium* are capable of destroying Polychrome wood artifacts(Cennamo et al., 2018). Enzymes such as cellulase, hemicellulase, and laccase produced during the metabolism can degrade cellulose and lignin and destroy the main structure of wooden cultural relics(Huang et al., 2021, Zhang et al., 2021).

The ancestral hall is traditional Chinese wooden architecture, especially prosperous in the southern areas. Among the ancestral halls we investigated, the oldest ones were built during the Ming Dynasty (AD1368 to AD1644), and the latest ones were built during the Qing Dynasty (AD1636 to AD1912). An ancestral hall was first built for the people who share the same ancestors to remember and honor predecessors and

inherit family traditions. It has special educational and memorial meanings for every clan. With exquisite architectural features, profound historical value and rich humanistic feelings, the ancestral halls have influenced cultural and architectural developments worldwide until now. However, as most of the ancestral halls are exposed in the open air for long years without much protection, most of them are now facing both structural damage and aesthetic damage due to microbial weathering, physical weathering, and chemical weathering. But there are not many studies on the microbial weathering of ancestral halls now.

Currently, the surface of the wooden ancestral halls is often covered with a coating of paint for preservation, which could reduce microbial growth(Gobakken et al., 2010). The presence of paint, especially paint containing urushiol (a component with an antimicrobial effect), has been considered to play a good role in the protection of cultural relics (Kim et al., 2021). However, the protective effect of the coating is found to be temporary. Studies show that when applying acrylics, a kind of paint that is widely used, to eight different commercial hardwood surfaces and putting them under the open air, the painting layer would be degraded as early as a year, and even the most resistant one will be cracking and peeling within five years(De Windt et al., 2014). Apart from that, the increased relative humidity can further cause the swelling of the coating; the mechanical stresses and physical weathering can lead to the coating's mottling, cracking, and flaking, and eventually, the wooden surface will be exposed(Phulpoto et al., 2020). Therefore, there are still discussion over the effectiveness and limitations of covering ancestral halls with a coating of paint for antimicrobial preservation. Hence, it is important to compare the microbial composition between damaged and undamaged sites (whether the paint coating is flaked off).

The diversity of microbiota colonized on the surface of wooden cultural relics can be affected by environmental factors. For instance, human activities can affect the microbial community structure. Gibbons observed that the built environment surfaces were composed of 25 to 30% human skin-associated microbial taxa, even in the absence of direct human interaction (Gibbons, 2016); with the prosperity of tourism, more and more cultural heritages are open to people, and the microorganisms carried on the skin or hair may fall on the surface of cultural relics and challenge the balance of the former microbial ecology equilibrium(Pasquarella et al., 2015). What’s more, Zhang et al. also pointed out that the environmental temperature, relative humidity, and pH were closely related to the variation in and distribution of microbial communities from the Sandstone of Beishiku Temple (Zhang et al., 2023).

Simply adding a coating of paint cannot completely avoid the microbial weathering. Microorganisms that cause biodeterioration and biodegradation are often found on the surface of the painting, including bacteria such as *Bacillus*, *Pseudomonas*, *Enterobacter*, *Actinomycetes*, and various fungi such as *Aspergillus*, *Trichoderma*, *Altemaria*, *Cladosporium*, and *Acremonium*(Phulpoto et al., 2020, Hyvarinen et al., 2002). Apart from the method of adding a coating of paint, the biocide is also a common way to prevent microorganisms. As of present, the chemical biocides for wooden cultural relics mainly include organic biocides, inorganic biocides, and plant essential oils(Kakakhel et al., 2019). Some commercialized biocides such as Isothiazolinone, benzalkonium chloride, and sodium hypochlorite are considered to have good bacteriostatic effects but may cause harm to the environment and human health at high concentrations(Kampf, 2018, Kozirog et al., 2016, Silva et al., 2020); plant essential oils have arisen attention for their environmental friendliness, but whether they could exert obvious antibacterial

effects and how to apply them to practical use still require further study (Antonelli et al., 2020, Palla et al., 2020).

Given the significance of learning the mechanism of microbial weathering, it is important to determine the taxonomy of microbial community and clarify the biological degradation process of cultural relics. Considering that the climate conditions will affect microbial diversity, we selected all the sampling sites in the coastal areas of Guangdong Province to ensure that they are in the same subtropical monsoon climate(Ding et al., 2022). As a result, this study's objectives were to: (1) analyze the microbiota from different ancestral hall surfaces and their potential functions; (2) compare the microbial diversity of damaged surfaces and undamaged surfaces to reveal the protective effects of paint; (3) characterize the microbial communities and their correlation with environmental factors; (4) verify the results of amplicon sequencing through strain isolation experiments; (5) test the antimicrobial sensitivity of microorganisms under four biocides. This research will facilitate a basic understanding of the microbial weathering of ancestral halls and add significant reference for the protection of wooden buildings which are located in Guangdong Province or under subtropical monsoon climate conditions.

2. Materials and methods

2.1. Sample sites

Sampling was carried out at nine wooden ancestral halls in Guangdong Province, located in the southeast coastal area of China (Figure 1). The ancestral halls chosen are all in the typical subtropical monsoon climate but differ in the city location, in order to observe the commonalities of the microbiome in different areas under the same climate condition. According to the distinct visitor flow rate, we divided the nine ancestral halls into three groups: the urban group (Urb_CHEN, Urb_GUAN, Urb_SAN), which is in the central city of Guangzhou and has an average hourly population of 10,000 people; the suburban group (Sub_LIN, Sub_DONG, Sub_LIE), which is in the outskirts of Guangzhou and has an average hourly population of 100 people; the rural district group (RD_QING, RD_FU, RD_MING), which is in the country areas of Jieyang and has an average hourly population less than 10.

We chose two ancestral halls from each group and took three samplings from the damaged external surfaces each, including the Urb_GUAN (GUAN_1, GUAN_2, GUAN_3), the Urb_SAN(SAN_1, SAN_2, SAN_3), Sub_DONG(DONG_1, DONG_2, DONG_3), Sub_LIE (LIE_1, LIE_2, LIE_3), RD_FU(FU_1, FU_2, FU_3), and RD_MING(MING_1, MING_2, MING_3). And we also chose an ancestral hall from each group and took two pairs of samplings from damaged and undamaged external surfaces each, including Urb_CHEN (CHEN_D1, CHEN_U1, CHEN_D2, CHEN_U2), Sub_LIN (LIN_D1, LIN_U1, LIN_D2, LIN_U2), and RD_QING (QING_D1, QING_U1, QING_D2, QING_U2). The damaged sites are adjacent to their undamaged counterparts with the same environmental conditions, both being sampled to achieve a direct comparison of microbiome colonization.

2.2. Environmental data recording

Relative humidity and temperature (RH and C) measurements were taken at each recording site using SMART SENSOR AS817 Humidity & Temperature Meter. Light (lux) measurements were taken at each sampling spot using SMART SENSOR AS803 Digital Lux Meter. The moisture contents of the wood sampled were obtained using SMART SENSOR AS981 Moisture Meter. The number of hourly visitors

was recorded by counting at the entrance of the ancestral hall for an arbitrary hour of the day as well as asking for the official statistics from the staff in charge.

Information regarding details of the sampling location, such as the relative altitude from ground level and the direction of the wood face (aspect), was recorded along with the environmental factors above. The data and the photographs were taken to allow accurate resampling at a future date if necessary.

2.3. Surface sampling

Sampling was performed using sterile swabs. To obtain samples a sterile swab was dipped in sterile M9 salts and wiped over a 5 cm square region of the surface. Fresh M9 salts were used for each sample to prevent cross-contamination between sampling sites. At all stages of sampling, nitrile gloves were worn to prevent contamination of the samples with skin microbiota. Samples were stored at 4°C for further culturing or sequencing.

2.4. Amplicon Sequencing Analysis

The 16S rRNA V3–V4 amplicon was amplified using 2×Hieff® Robust PCR Master Mix (Yeasen,10105ES03, China). Two universal bacterial 16S rRNA gene amplicon PCR primers were used: the Nobar_341F (5'-CCTACGGGNGGCWGCAG-3') and the Nobar_805R (5'-GACTACHVGGGTATCTAATCC -3'). The reaction was set up as follows: microbial DNA (10ng/µl) 2µl; amplicon PCR forward primer (10µM) 0.5µl; amplicon PCR reverse primer (10 µM) 0.5µl; 2×Taq Master Mix (Sangon Biotech, B639295, China) 12.5µl; 9.5µL doble distilled water (ddH_2O) (total 25µl). The plate was sealed and PCR was performed in a thermal instrument (T100 Thermal Cycle 1861096, USA) using the following program: 1 cycle of denaturing at 95 °C for 3 min, the first 5 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1min 30 s, then 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1min 30 s and a final extension at 72 °C for 10 min.

The 18S rRNA V4 amplicon was amplified using 2×Hieff® Robust PCR Master Mix (Yeasen,10105ES03, China). Two universal fungal 18S rRNA V4 gene amplicon PCR primers were used: the 18SV4-forward primer (5'-GGCAAGTCTGGTGCCAG-3') and the 18SV4-reverse primer (5'-ACGGTATCTRATCRTCTTCG-3'). The reaction was set up as follows: microbial DNA (10ng/µl) 2µl; amplicon PCR forward primer (10µM) 1µl; amplicon PCR reverse primer (10 µM) 1µl; 2×Hieff® Robust PCR Master Mix (Yeasen,10105ES03, China) 15µl (total 30µl). The plate was sealed and PCR was performed in a thermal instrument ((Applied Biosystems 9700, USA) using the following program: 1 cycle of denaturing at 94 °C for 3 min, the first 5 cycles of denaturing at 94 °C for 30 s, annealing at 45 °C for 20 s, elongation at 65 °C for 30 s, then 20 cycles of denaturing at 94°C for 20 s, annealing at 55 °C for 20 s, elongation at 72 °C for 30 s and a final extension at 72 °C for 5 min.

Samples were delivered to Sangon BioTech (shanghai) for library construction using universal Illumina adaptor and index. Before sequencing, the DNA concentration of each PCR product was determined using a Qubit® 4.0 Green double-stranded DNA assay and it was quality controlled using a bioanalyzer (Agilent 2100, USA). Depending on coverage needs, all libraries can be pooled for one run. The amplicons from each reaction mixture were pooled in equimolar ratios based on their concentration. According to the manufacturer's instructions, sequencing was performed using the Illumina MiSeq

system (Illumina MiSeq, USA). Sequence processing, OTU clustering, Representative tags alignment, and biological classification were performed. After sequencing, the two short Illumina readings were assembled by PEAR software (version 0.9.8) according to the overlap and fastq files were processed to generate individual fasta and qual files, which could then be analyzed by standard methods. The effective tags were clustered into operational taxonomic units (OTUs) of≥97% similarity using Usearch software (version 11.0.667). Chimeric sequences and singleton OTUs (with only one read) were removed, after which the remaining sequences were sorted into each sample based on the OTUs. The tag sequence with the highest abundance was selected as a representative sequence within each cluster. Bacterial and fungal OTU representative sequences were classified taxonomically by blasting against the RDP Database and UNITE fungal ITS Database, respectively.

2.5. Statistical analysis

The α-diversity indices (such as Chao1, Simpson, and Shannon indices) were quantified in terms of OTU richness. Beta diversity evaluates differences in the microbiome among samples and is normally combined with dimensional reduction methods such as principal coordinate analysis (PCoA), non-metric multidimensional scaling (NMDS), or constrained principal component analysis (PCA) to obtain visual representations.

2.6. Function prediction

Functional prediction analysis of bacteria using PICRUSt (v1.1.4) software, by comparing existing sequencing data with a microbial reference genome database of known metabolic functions, enabling the prediction of bacterial metabolic functions.

2.7. Isolation, and identification of microorganisms

Microorganisms were isolated from the swabs by adding 4mL of M9 salts to the swab holder and vortexing at 180rpm at 30°C for 3h. The resulting suspension was then plated out onto non-selective media—BPM plates (Beef extract Peptone Medium) for bacteria growth and PDA plates (Potato Dextrose Agar Medium, adding 0.1g/L chloramphenicol to avoid bacteria growth) for fungi growth, and cultured for 2-7days at 28°C following various growing conditions. Then we separated the colonies according to their colors and morphologies and repeated the isolating process until obtaining the purebred strains. The purebred strains were then sent for sequencing to identify their genus and species.

Total community genomic DNA extraction was performed using the CTAB Extraction Method(Moller et al., 1992).

The 16S rRNA V3–V4 amplicon was amplified using 2×Taq Master Mix (Sangon Biotech, B639295, China). Two universal bacterial 16S rRNA gene amplicon PCR primers were used: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The reaction was set up as follows: microbial DNA (10ng/μl) 2μL; amplicon PCR forward primer (10μM) 0.5μL; amplicon PCR reverse primer (10 μM) 0.5μL; 2×Taq Master Mix (Sangon Biotech, B639295, China) 12.5μL; 9.5μL ddH2O (total 25μL. The plate was sealed and PCR was performed in a thermal instrument (T100 Thermal Cycle 1861096, USA) using the following program: 1 cycle of denaturing at 95 °C for 3 min, 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1min 30 s and a final extension at 72 °C for 10 min.

The ITS amplicon was amplified using 2×Taq Master Mix (Sangon Biotech, B639295, China). Two universal ITS amplicon PCR primers were used: the ITS1-forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and the ITS4-reverse primer (5'-TCCTCCGCTTATTGATATGC-3'). The reaction was set up as follows: microbial DNA (10ng/μL) 2μL; amplicon PCR forward primer (10μM) 0.5μL; amplicon PCR reverse primer (10 μM) 0.5μL; 2×Taq Master Mix (Sangon Biotech, B639295, China) 12.5μL; 9.5μL ddH2O (total 25μL). The plate was sealed and PCR was performed in a thermal instrument (T100 Thermal Cycle 1861096, USA) using the following program: 1 cycle of denaturing at 95 °C for 3 min, 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1min and a final extension at 72 °C for 10 min.

2.8. Identification of cellulolytic microbes and ligninolytic microbes

Sodium carboxymethyl cellulose (CMC-Na) was used to screen cellulolytic strains and guaiacol was used to screen ligninolytic microbes. Detailed information on each medium is below. CMC-degrading agar medium for bacteria (per liter): CMC-Na 15.0g, $Na_2HPO_4 \cdot 7H_2O$ 12.8g, KH_2PO_4 3.0g, NaCl 0.5g, NH_4Cl 1.0g, agar 15.0g; CMC-degrading agar medium for fungi (per liter): CMC-Na 2.0g, KH_2PO_4 1.0g, $MgSO_4$ 0.5g, $(NH_4)_2SO_4$ 2.0g, NaCl 0.5g, agar 18.0g; lignin-degrading agar medium for bacteria (per liter): 9mM guaiacol, yeast extract 50mg, $Na_2HPO_4 \cdot 7H_2O$ 12.8g, KH_2PO_4 3.0g, NaCl 0.5g, NH_4Cl 1.0g，agar15.0g; lignin-degrading agar medium for fungi (per liter): 9mM liquid guaiacol, $MgSO_4$ 0.5g, KH_2PO_4 1.0g, Na_2HPO_4 0.2g. No additional carbon source but CMC-Na was added to the CMC-degrading agar medium, to make sure that the only carbon source during the microbial growth came from the degradation of cellulose. A small amount of yeast extract was added to the lignin-degradation agar medium as an auxiliary carbon source component. Incubated the plates at 28°C for 48 hours (bacteria) or 96 hours (fungi) and examined the diameter of the degradation circles, which was measured in millimeters with the scale ruler and noted.

2.9. The disk-diffusion test of biocides

Isothiazolinone, NaClO, Thymus Vulgaris Essential Oil, and Cinnamon Essential Oil were used to carry out the test. Antimicrobial sensitivity of the microorganisms isolated from the four samples of Urb_CHEN was detected by agar disk-diffusion procedure. The bacterial suspension (at the log phase) was plated on BPM agar medium and the spore suspension was plated on PDA agar medium. The disks (6-mm diameter) were soaked in biocides for an hour before being placed on the plates. The isolated strains were detected by the four biocides respectively at the concentration of 1%, to verify whether the biocide has an effect on the isolation itself. The mix strains of each sample were detected by the four biocides respectively at a concentration gradient (1%, 0.5%, 0.25%, 0.125%, 0.1%, 0.05%, 0.01%), to observe the antimicrobial effect trend on the whole microbiome. Incubated the plates at 28°C for 48 hours (bacteria) or 96 hours (fungi) and examined the diameter of the inhibition zone, which was measured in millimeters with the scale ruler and noted.

3. Results

3.1. Characteristics of the samples

Nine ancestral halls with different visitor flow rates were selected for sample collection, and the

environmental factors of the sampling sites were measured. (Table S1, Table 1 and Figure 1). The ancestral hall, as a wooden building, is often protected by coving a coating of paint on the surface. However, long-term weathering had caused damage to parts of the surface, where the paint flaked off, bare wood was exposed, and obvious microbial stain could be found, and we defined them as "damaged sites". For instance, the wooden railing (GUAN_2) is covered with dark green biofilm; the surface of the wooden door (SAN_3), the wooden pillar (DONG_1), and the shrine (FU_3) can be observed to have white biofouling; obvious black biofilms can be seen on the threshold (FU_2) and the sacrificial table (MING_1); yellow plaques can be found on the wooden screen (QING_2).

3.2. Taxonomic Analysis of the diversity of microbial community

After quality filtering, a total of 1467 and 367 OTUs were obtained from the 16S and 18S sequences, respectively. Despite three repetitions of PCR on MING_3 sample, DNA concentration still cannot meet the requirements of library construction for amplicon. The alpha diversity of prokaryotic and eukaryotic communities, including Shannon index, Chao index, Ace index, Simpson index and Shannoneven index, was listed, and it evaluates the biodiversity of the sample, which shows that the fungi types are few and the bacterial abundance is various. The coverage index is equal to 1 for all samples, indicating the uniformity of the sampling (Table S2 and Table S3).

We visualized the dominant prokaryotes and eukaryotes among the samples of damaged surfaces. And the results showed that all ancestral halls shared some similarities in the dominant organisms. At the phylum level, the two dominant prokaryotic phyla, Firmicutes and Proteobacteria, account for more than 84% of the total OTU relative abundance among all 9 ancestral halls (Figure 2A, Figure S1A, and Table S4). Ascomycota is the dominant eukaryotic phylum, with an abundance of more than 90% at 8 of the 9 ancestral halls (except RD_FU whose abundance is 74.53%) (Figure 2B, Figure S1B and Table S5). At the genus level, the four dominant bacterial genera are *Bacillus* (except Sub_LIN, RD_MING, whose abundances are less than 0.5%) *Pseudomonas* (except Sub_LIE, whose abundance is less than 0.1%), *Paenibacillus* (except Sub_LIE, RD_MING, whose abundance is less than 0.5%), *Acinetobacter* (except Urb_CHEN, RD_FU, RD_MING, whose abundance is less than 0.5%) (Figure 2C, Figure S1C and Table S6). The dominant eukaryotic genera, *Toxicocladosporium*, *Cladosporium*, *Aspergillus* (except Urb_CHEN, Sub_LIE, whose abundance is less than 0.5%) and *Epicoccum* (RD_QING, RD_FU, whose abundance is less than 1%), had significant abundance in all 9 ancestral halls (Figure 2D, Figure S1D and Table S7).

3.3. Comparison of microbial abundance between damaged sites and undamaged sites

There are conflicts in discussion over whether species are significantly associated with damaged or undamaged surfaces and whether they play an important role in biodeterioration(Skipper et al., 2022). Therefore, the investigation into the role of microorganisms in biodeterioration shall take a pair of damaged and undamaged samples that are from closely related sites and exposed to the same climatic conditions.

We selected three ancestral halls, the Chen Clan Ancestral Hall, the Lin Clan Ancestral Hall and the Qingxi Ancestral Hall, and took two pairs of samplings from damaged and undamaged surfaces each. The damaged sites are adjacent to their undamaged counterparts with the same environmental conditions, both being sampled to achieve a direct comparison of microbiome colonization. Difference comparison

is used to identify features with significantly different abundances between groups using STAMP (version 2.1.3)(Parks et al., 2014). STAMP was used to calculate PICRUSt2 results; Welch's t test was used for two-group comparisons; Welch's inverted confidence interval method was used to calculate the confidence interval; the Benjamini–Hochberg false discovery rate method was used to calculate the adjusted p value.

For prokaryote microorganisms, there were nine phyla shared among all the damaged and undamaged samples; and four of them, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, showed relatively high abundance (Figure S2A). 38 bacterial genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Chryseobacterium*, and *Curtobacterium*, were abundant among all the samples (Figure S1E and Figure S2C). Firmicutes was more abundant in damaged samples, while Proteobacteria and Actinobacteria were more abundant in undamaged samples (Figure S3). For eukaryotic microorganisms, there were seven phyla shared among all the damaged and undamaged samples, including Chlorophyta, Ascomycota and Phragmoplastophyta, and Ascomycota showed the highest abundance (Figure S2B). 13 fungal genera, including *Epicoccum*, *Aspergillus*, and *Cladosporium*, were differentially abundant among all the samples, with *Toxicocladosporium* having the highest abundance (Figure S1F and Figure S2D). Ascomycota was more abundant in damaged samples, while Phragmoplastophyta was more abundant in undamaged samples (Figure S4). *Cladosporium* was more abundant in damaged samples than in undamaged samples (except for the Lin Clan Ancestral Hall) (Figure S5). The results above suggested that these organisms were specific for the most deteriorated samples.

3.4. The relationship of the abundance of microbial community and environmental factors

To evaluate the effects of environment on the abundance and diversity of the microbial community, the Spearman correlation heatmap was used to reflect the impact of environmental factors, including relative humidity, temperature, light, the moisture content of the wood and the visitor flow rate. Take the visitor flow rate as an example. For the prokaryotes, the abundance of *Sedimentibacter*, *Trichococcus*, *Terribacillus*, *Pantoea*, *Proteiniphilum* was positively correlative with visitor flow rate, and the abundance of *Streptophyta*, *Sphingomonas*, *Flavobacterium*, *Lactococcus* was negatively correlative with visitor flow rate (figure S6). For the eukaryotes, the abundance of *Rhizopus* and *Lichtheimia* was positively correlative with visitor flow rate, and the abundance of *Solanum*, *Glycine* was negatively correlative with visitor flow rate (figure S7).

Apart from the visitor flow rate, other environmental factors also contributed to the composition of microbial community. For instance, the relative humidity was significantly correlative with the abundance of several fungi; temperature and the moisture content of the wood can also affect the abundance of some bacteria. The abundance and diversity of the microbial community is the consequence of cooperative interaction among all the environmental factors.

3.5. Genetic function prediction for the microbial community

Based on KEGG analysis, we are able to construct an integrated cellulose decomposition pathway for all the sample sites, which is cellulose decomposing into cellulose dextrin through the two metabolic pathways K01179 and K01188, and then further decomposing into cellobiose (Figure 3A).

We also summarized the relative abundance of five microbial biological cycle functions related to the

basic nutrient cycle. The results of KEGG annotation showed that the two basic cycles of carbon fixation of prokaryotic carbon and nitrogen metabolism had relatively high abundance in all samples; simultaneous sulfur metabolism, photosynthetic carbon fixation, and methane metabolism also existed in this microbiota, which suggested that the bacterial community of the samples was undergoing an active metabolism process (Figure 3B, Figure 3C and Figure 3D).

3.6. Verification of the degrading ability of microorganisms from Urb_CHEN based on conventional culturing methods

A total of 7 different species of bacteria and 9 different species of fungi were isolated from the Urb_CHEN samples using conventional culturing methods (Table 2). All isolates were tested for cellulose and lignin degradation ability (except for the *Staphylococcus* strain; no follow-up experiments were carried out because of its pathogenicity).

Among all the bacteria, *Pseudomonas* sp., *Pantoea dispersa* strain, *Paenibacillus* sp., *Bacillus cereus* strain, and *Priestia megaterium* strain showed both cellulose-degradation ability and lignin-degradation ability (Figure 4A-E); *Curtobacterium* sp. could degrade lignin but not cellulose (Figure 4F); *Microbacterium oleivorans* strain could not degrade both lignin and cellulose. As for the fungi, *Aureobasidium pullulans* strain., *Cladosporium* sp., *Trichoderma* sp., *Daldinia* sp., and *Aspergillus sydowii* strain could degrade cellulose but not lignin, which is manifested by the obvious hydrophytic zones on plates (Table 3 and Figure S8A-E); *Rhodotorula muciaginosa* strains and *Cystobasidium* sp. could not degrade both lignin and cellulose.

3.7. Antimicrobial sensitivity test of the microorganisms from Urb_CHEN

Using biocides is an important means to inhibit biodeterioration on the surface of cultural relics. Four biocides were selected: Isothiazolinone, Sodium hypochlorite, Thymus Vulgaris Essential Oil, and Cinnamon Essential Oil. The antimicrobial sensitivity of the microorganisms from the samples of Urb_CHEN was detected by the agar disk-diffusion method.

For the samples, we found that Isothiazolinone had a complete antimicrobial effect on fungi, and it is also the only biocide to show a significant inhibitory effect on bacteria (for the four samples CHEN_D1, CHEN_U1, CHEN_D2, and CHEN_U2, the diameters of each inhibition zone were 25.50±0.50mm, 29.00±1.73mm, 28.17±1.76mm, and 48.33±2.08mm). Sodium hypochlorite showed little antibacterial function but exhibited an inhibitory effect on fungi (for the four samples CHEN_D1, CHEN_U1, CHEN_D2, and CHEN_U2, the diameters of each inhibition zones were 26.67±3.06mm, 24.00±3.46mm, 24.33±2.52mm, and 19.33±1.15mm). Thymus Vulgaris Essential Oil and Cinnamon Essential Oil did not suppress any microbial growth when at a concentration of 1%(v/v). To get further insights into the effect of Isothiazolinone and Sodium hypochlorite, we further tested their antimicrobial effect on the isolated strains. Compared to Sodium hypochlorite at a concentration of 1% (w/v), it was observed that Isothiazolinone at a concentration of 1% showed significant antibacterial effects on all bacteria (Figure 5a and Table 4) and most of the fungi (Figure 5b and Table 4).

However, considering that the use of high-concentration Isothiazolinone may have harmful effects on the environment and human health, the concentration should be appropriately controlled while used as the biocide. Therefore, all samples were then detected by Isothiazolinone at a concentration gradient (from

1% to 0.01%) to observe the antimicrobial effect trend. It was found that with the decrease in the concentration of Isothiazolinone, the antimicrobial sensitivity of the bacterial population gradually decreased. For the bacterial strains, even if the concentration was reduced to 0.05%, there still existed an obvious inhibition zone (Figure 5c). For the fungal strains, when the concentration of Isothiazolinone was greater than 0.25%, except for CHEN_D1, all the fungi showed a significant inhibitory effect with a diameter of the inhibition zone greater than 6cm, and even if the concentration was reduced to 0.01%, there still existed an obvious inhibition zone (Figure 5d). The results above show that the microbiota on the surface of Chen Clan Ancestral Hall is extremely sensitive to Isothiazolinone. Combined with the results of the amplicon sequencing, it is observed that *Epicoccum* had a higher abundance in the fungal community of CHEN_D1 (35.61%) than other samples, which may be the key factor to the insignificant antimicrobial effect of Isothiazolinone on CHEN_D1, resulting in the irregular changes of antimicrobial sensitivity following the decreasing concentration.

4. Discussion

The amplicon sequencing results showed that the damaged surfaces of ancestral halls shared a common group of microorganisms: at the phylum level, the microbiota was dominated by Firmicutes, Proteobacteria, and Ascomycota; at the genus level, the microbiota was dominated by *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Acinetobacter*, *Toxicocladosporium*, *Cladosporium*, *Aspergillus*, and *Epicoccum*.

Among the microbiota derived from our samples, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Ascomycota are considered the main phyla for cellulose degradation (Liu et al., 2021). Firmicutes and Proteobacteria are the major components of biofilms which could retain essential nutrients and water required by microorganisms(Ding et al., 2022). The colonization of actinomycetes produces pigments, organic acids, and polysaccharides(Duan et al., 2017) through secondary metabolism, which not only threatens the aesthetics of ancestral halls and causes acid corrosion of wooden structures, but also promotes the formation of biofilms on the surface. Phulpoto et al in their previous study found that Firmicutes and Actinobacteria were the most dominant phyla in paint degradation followed by the Proteobacteria.(Phulpoto et al., 2023). Comparing the damaged and undamaged sites: Firmicutes and Ascomycota were more abundant in damaged samples, while Proteobacteria, Actinobacteria and Phragmoplastophyta were more abundant in undamaged samples. We hypothesized that it is their different degradation ability of cellulose and paint as well as their various adaptability to environment that led to the current distribution on damaged and undamaged surfaces. And it should also be noted that changes in absolute abundances cannot be concluded from the relative abundance data(Li et al., 2020).

At the genus level, among the microbiota derived from our samples, *Bacillus*, *Paenibacillus*, *Acinetobacter*, and *Pseudomonas* are proved to be capable of utilizing lignin as the only carbon source(Mendes et al., 2021, Xiong et al., 2020); *Aspergillus* as well as *Cladosporium* were isolated from lithographs and proved to have cellulose-degrading abilities (Coronado-Ruiz et al., 2018); *Pseudomonas*, *Cladosporium* and *Epicoccum* have been proven to be able to degrade paint as well as wood(Sanmartin et al., 2015, Shirakawa et al., 2002). In our study, *Cladosporium* was more abundant in damaged samples than in undamaged samples. We obtained the isolation of it from Chen Clan Ancestral Hall and had proven its ability to degrade cellulose; it is also considered to be the main chromogen in

artworks(Sabatini et al., 2018) and the main microbial disease of canoes preserved in the Oceanographic Museum of China(Zhang et al., 2019). Therefore, *Cladosporium* may play a crucial role at the deterioration of ancestral halls; and it is suggested that using paint as the only method for protecting the bare wood is not enough.

As is shown in the KEGG analysis, it is possible to reconstruct a complete reaction process of cellulose degradation on all the sampling surfaces, and microorganisms can obtain the basic nutrients and energy needed for survival through carbon fixation, nitrogen fixation, and sulfur cycle. This finding may be the key reason to ensure the essential nutrient cycle of the cultural relics' microbiome(Ding et al., 2020).

At the same time, we further screened and cultivated eight bacterial isolates and seven fungal isolates from Chen Clan Ancestral Hall. The seven bacterial isolates obtained were consistent with the main bacterial group of Chen Clan Ancestral Hall shown in the result of amplicon sequencing, while only two of the fungal isolates appeared in the main fungal group of the amplicon sequencing result. This may be due to the inability of amplicon sequencing to identify less abundant species(Liu et al., 2018). Additionally, in the test of the lignocellulose-degrading ability of the isolated strains, it can be found that six strains of bacteria show lignin-degrading activity; five strains of bacteria and five strains of fungi exhibit cellulose-degrading activity. *Pantoea* has been proven to mediate lignin degradation through the production of laccase and lignin peroxidase (Atiwesh et al., 2022). *Microbacterium oleivorans* strain cannot utilize lignin and cellulose directly but may be able to utilize cellobiose for nutrients(Lian et al., 2016). Although *Rhodotorula* and *Cystobacter* show no lignocellulose-degrading ability, the metabolism production of carotenoids will damage the aesthetics of ancestral halls and protect biofilms against low-wavelength radiation(Cojoc et al., 2019).

Environmental factors may affect the diversity and distribution of microorganisms. For example, with the increasingly prosperous cultural tourism industry, the microbiota brought by human activities will affect the microbial diversity on cultural relics. The microorganisms on the surface of ancestral halls, including prokaryotes such as *Sedimentibacter*, *Trichococcus*, *Terribacillus*, *Pantoea*, *Proteiniphilum* and eukaryotes such as *Rhizopus*, *Lichtheimia*, are positively correlated with visitor flow rate. Researches show that *Trichococcus* populations dominate the microbial community within urban sewer infrastructure(VandeWalle et al., 2012); one of the sources of the *Pantoea* is human feces(Asai et al., 2019); *Rhizopus* and *Lichtheimia* are the most common fungi that cause mucormycosis(Gomes et al., 2011). Among the microorganisms derived by isolation from the Chen Clan Ancestral Hall, *Bacillus cereus* is a common microorganism in human hands(Adamczyk et al., 2020); *Staphylococcus* is a pathogenic bacteria of human bloodstream infection that can form biofilm(Szczuka et al., 2015). Therefore, the microbial diversity on the surface of ancestral halls is likely to be influenced by the contamination caused by tourists frequenting.

New biocides represented by plant essential oil extracts have aroused attention due to their environmental friendliness(Cappitelli et al., 2020). Unfortunately, cinnamon essential oil and thyme essential oil show a weak antimicrobial effect in this research. As for the mature commercial biocides, compared to sodium hypochlorite, Isothiazolinone is a more effective biocide and exhibits a pronounced effect. However, excessive use of Isothiazolinone is considered to be harmful to the environment and human health(Romani et al., 2022). Therefore, we tried to reduce the risk by reducing the concentration of

Isothiazolinone. We found that when the concentration of Isothiazolinone is set at 0.05% (v/v) (for bacteria) and 0.01% (v/v) (for fungi) and applied to the sensitivity test on the microbiota of the samples, the sizes of the inhibition zones were all greater than 10mm, and some were even greater than 15mm, which means showing a moderate or even high sensitivity(Fu et al., 2022). The results show that it is feasible to reduce environmental damage while achieving an antimicrobial effect by using low-concentration Isothiazolinone. But even so, the harmful effects of Isothiazolinone should still be carefully concerned.

5. Conclusion

In conclusion, the microbial community on the surface of nine ancestral halls in the subtropical monsoon climate of Guangdong Province's coastal areas was analyzed using the conventional culturing method together with amplicon sequencing. A core microorganism group was detected, including *Bacillus* sp., *Pseudomonas* sp., *Paenibacillus* sp., *Acinetobacter* sp., *Toxicocladosporium* sp., *Cladosporium* sp., *Aspergillus* sp., and *Epicoccum* sp.. A proportion of the species isolated from the samples showed lignin or cellulose degradation capacity. The comparison between damaged and undamaged sites showed that the microbial composition of these two sites is similar, but with a different abundance. At the phylum level, Firmicutes and Ascomycota were significantly more abundant in damaged surfaces; and *Cladosporium*, widely located on surfaces of ancestral halls, was identified as the predominant source of cellulose-decomposing genus. Based on the similarity of their microbial composition, we suggest that using paint as the only method for protecting bare wood is not enough. The microorganisms on the surface of ancestral halls, including prokaryotes such as *Sedimentibacter*, *Trichococcus*, *Terribacillus*, *Pantoea*, *Proteiniphilum* and eukaryotes such as *Rhizopus*, *Lichtheimia*, are positively correlated with visitor flow rate, which showed that the increase of visitor flow rates could have an influence on the composition of microbiota on cultural relics, but the abundance and diversity of the microbial community is the consequence of cooperative interaction among all the environmental factors. Low-concentration Isothiazolinone is confirmed to be an effective inhibitor; but when used in practical, its impact on the environment and human health should be concerned.

Data accessibility

Raw sequencing reads about 16S amplicon sequencing and 18S amplicon sequencing are deposited at the NCBI Sequence Read Archive under the BioProject PRJNA925150 (SRR23110177 to SRR23110205) and PRJNA925313 (SRR23117924 to SRR23117952). DNA sequences of the bacterial and fungal isolates are deposited at the NCBI GenBank (accession numbers: OQ283719 to OQ283726, and OQ283737 to OQ283743). The datasets supporting this article have been uploaded as part of the supplemental material.

Author's contributions

Ma.L.: methodology, validation, formal analysis, investigation, resources, data curation, visualization, and writing – original draft; **X.S.:** methodology, validation, formal analysis, investigation, resources, data curation, visualization, and writing – original draft; **Q.W.:** investigation, visualization, and writing – original draft; **T.Y.:** investigation and resources; **Mu.L.:** investigation, and resources; **P.A.:** investigation; **J.R.L.:** conceptualization, validation, formal analysis, writing – review and editing, supervision, and funding acquisition; **J.L.:** conceptualization, validation, formal analysis, writing – review and editing, and funding acquisition.

Competing interests

We declare we have no competing interests.

Ethics

Permission was obtained from the Villager Committees and the staff in charge of the ancestral halls before sampling was carried out.

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Figures and tables

Figure 1. Characteristics of the samples.

The location of nine ancestral halls and the sampling sites. Sample CHEN_D1, CHEN_U1, LIN_D2, LIN_U2, and FU_2 on wooden thresholds. Sample CHEN_D2, CHEN_U2, GUAN_1, SAN_3, LIN_D1, LIN_U1, DONG_3, LIE_1, and MING_2 on wooden doors. Sample GUAN_2 on a wooden railing. Sample GUAN_3, QING_D2, and QING_U2 on wooden folding screens. Sample SAN_1 and SAN_2 on Wooden beams. Sample LIE_2 on a wooden table corner. Sample LIE_3 on a wooden bolt. Sample DONG_1, DONG_2, QING_D1, and QING_U1 on wooden pillars. Sample FU_1 on a Wooden incense burner. Sample FU_3 and MING_3 on shrines. Sample MING_1 on a sacrificial table.

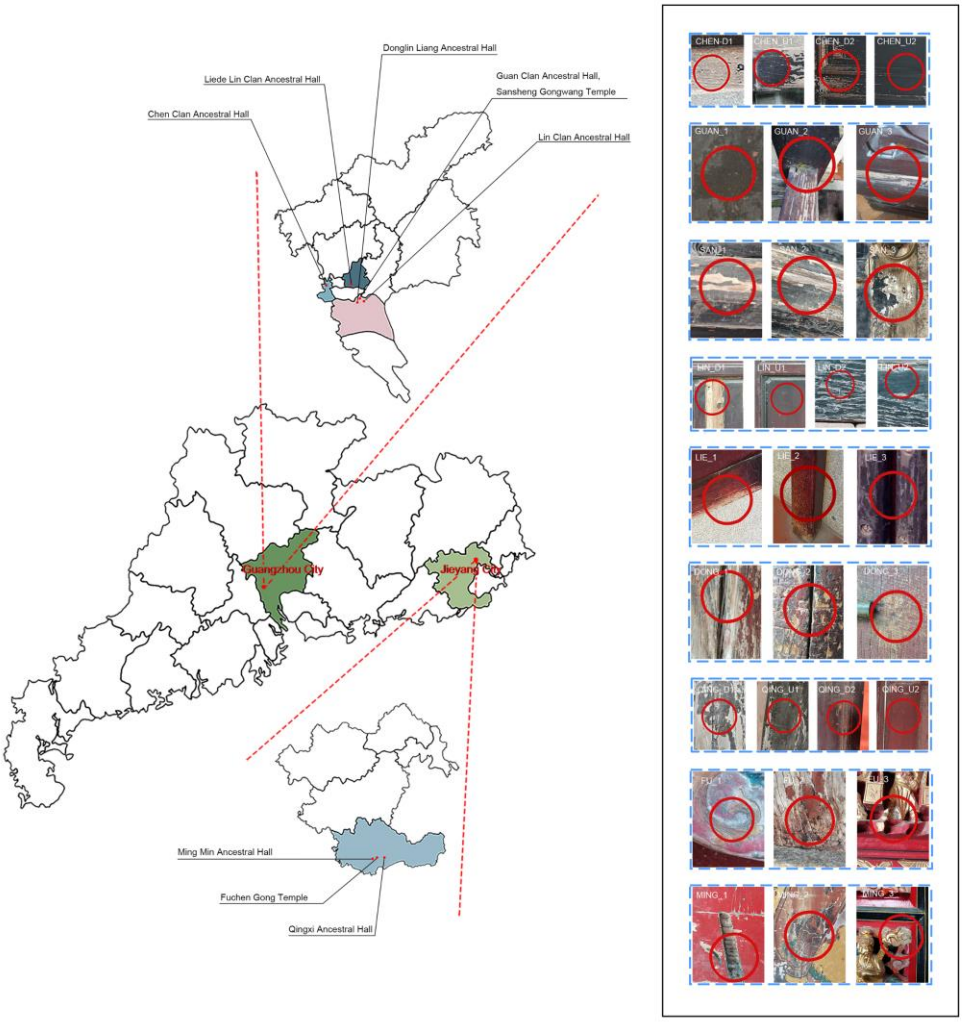

Figure 2. Determining the diversity of microbial community.

(A, B, C and D) Percentage of amplicon reads that mapped to each ancestral hall. Phylum level taxonomy and genus level taxonomy of microbiome on the damaged surfaces of nine ancestral halls under the same climate conditions based on 16S amplicon sequencing and 18S amplicon sequencing. (A) Bacterial phyla. (B) Fungal phyla. (C) Bacterial genera. (D) Fungal genera.

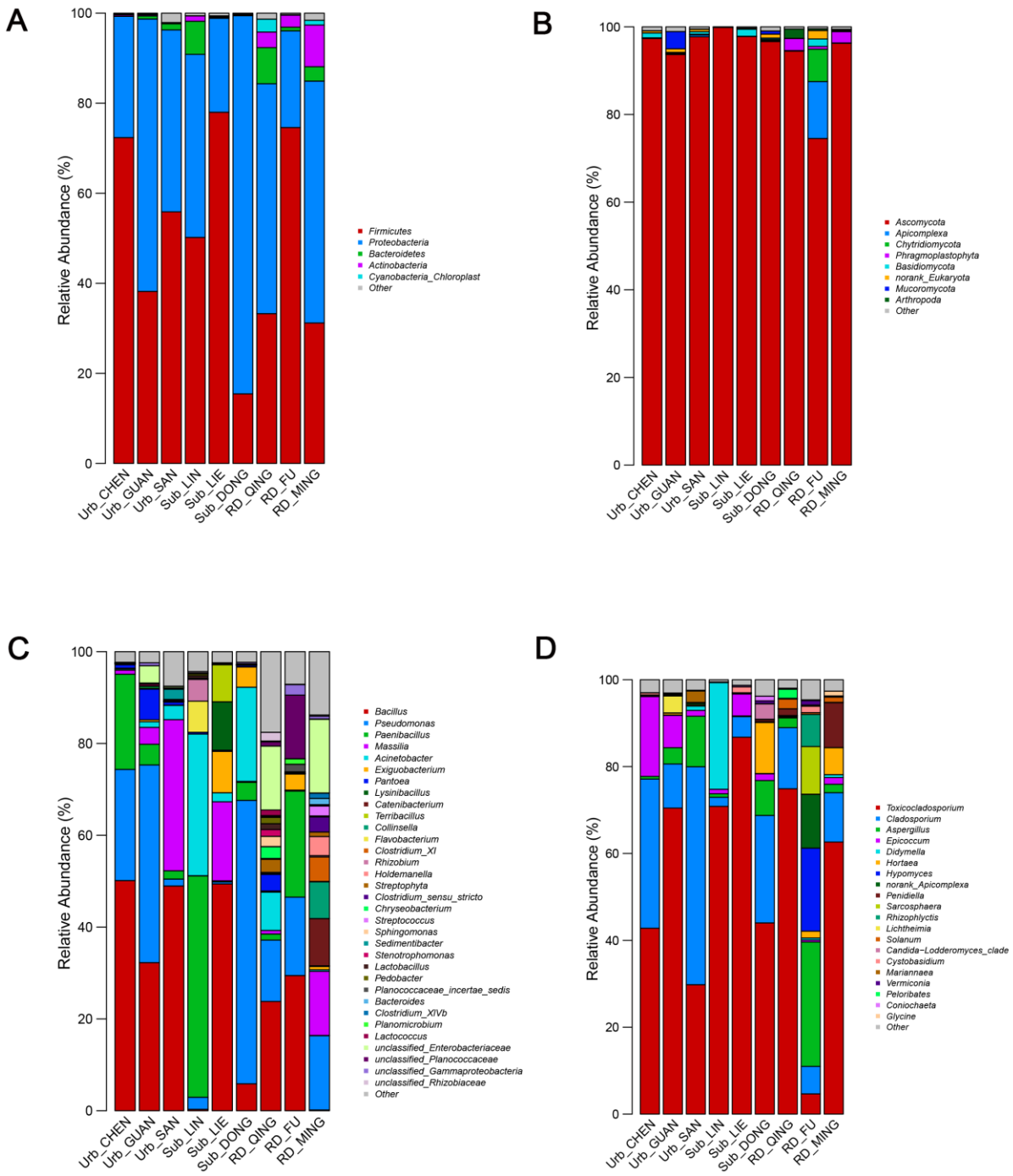

Figure 3. Genetic function prediction for the microbial community.

(A) Reconstruction of the cellulose degradation pathways at the microbiome on the surfaces of ancestral halls based on KEGG Orthology, some of which that marked in red were detected in our study.

(B, C and D) The major functions driving geomicrobiological energy metabolism of microbiome communities on urban (A), sub-urban (B), and rural (C) ancestral halls. Totally five functions are summarized through KEGG Pathway. All abundances are reported as the percentage of amplicon reads. Vertical lines indicate the standard deviation.

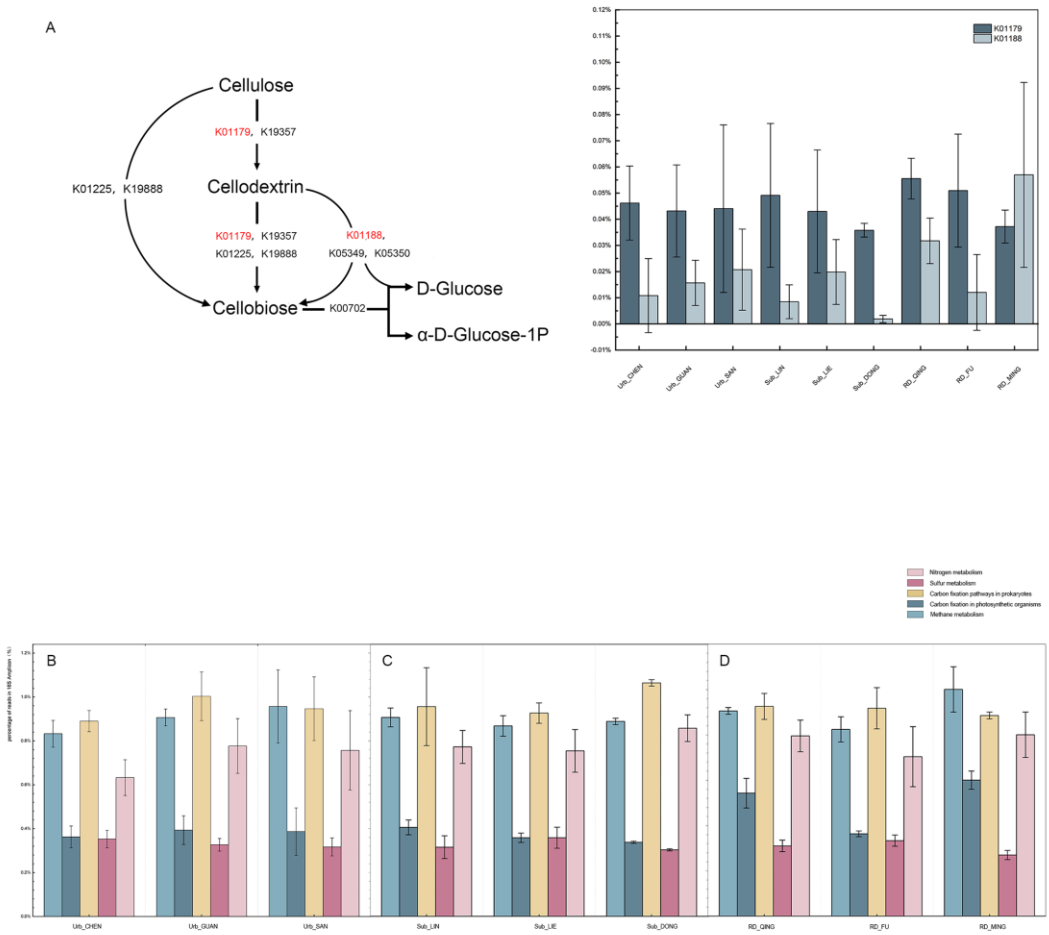

Figure 4. Verification of the degrading ability of microorganisms from Urb_CHEN based on conventional culturing methods

The three mediums for each isolate from left to right are respectively the lignin-degrading agar medium, the LB medium, and the CMC-degrading agar medium. Bacterial isolates that can use guaiacol on lignin-degrading agar medium and cellulose on CMC-degrading agar medium for two days. (A)*Pseudomonas* sp. (B) *Pantoea dispersa* strain. (C) *Paenibacillus* sp. (D) *Bacillus cereus* strain. (E) *Priestia megaterium* strain. Bacterial isolates that can only use guaiacol on lignin-degrading agar medium for two days. (F) *Curtobacterium* sp. *Microbacterium oleivorans* strain which cannot grow on lignin-degrading agar medium or CMC-degrading agar medium does not be shown.

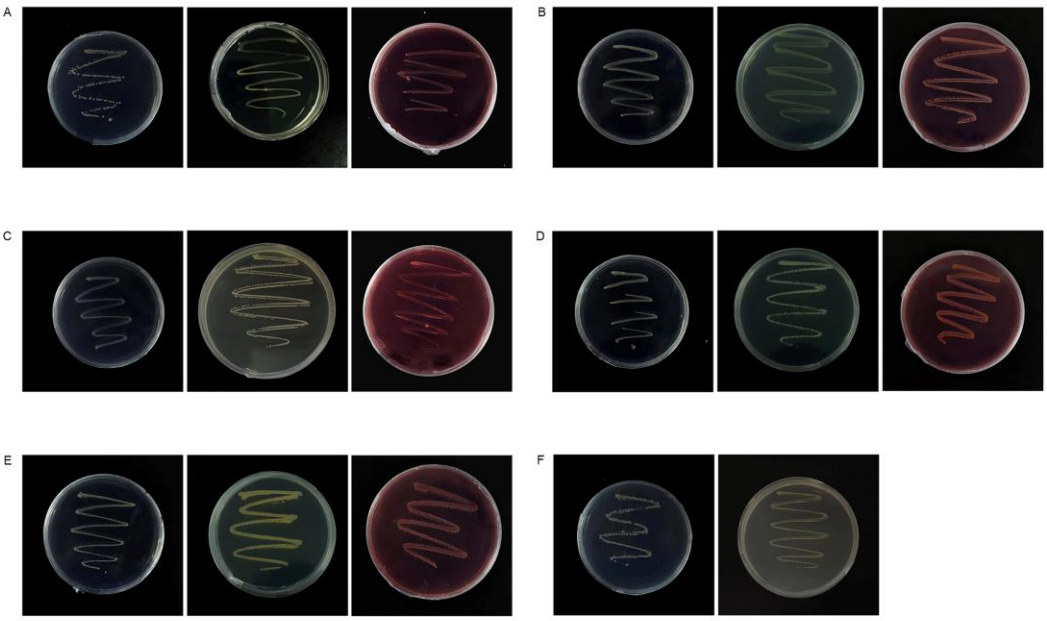

Figure 5. Antimicrobial sensitivity test of the microorganisms from Urb_CHEN

[a] Antimicrobial sensitivity test of the bacterial isolates using agar disk-diffusion method for two days. The left BPM plates were loaded with water; the right BPM plates were loaded with Isothiazolinone at the concentration of 1%(v/v). (A) *Pseudomonas* sp. (B) *Pantoea dispersa* strain. (C) *Paenibacillus* sp. (D) *Bacillus cereus* strain. (E) *Priestia megaterium* strain. (F) *Curtobacterium* sp. (G) *Microbacterium oleivorans* strain.

[b] Antimicrobial sensitivity test of the fungal isolates using agar disk-diffusion method for three days. The PDA plates from left to right were loaded with water, Isothiazolinone at the concentration of 1% (v/v), and NaClO at the concentration of 1% (w/v). (A) *Rhodotorula mucilaginosa* strain. (B) *Cystobasidium* sp. (C) *Aureobasidium pullulans* strain. (D) *Cladosporium* sp. (E) *Trichoderma* sp. (F) *Daldinia* sp. (G) *Aspergillus sydowii* strain.

The Red triangles on both [a] and [b] are used for locating partially indistinct disks. Three disks on the same plates were treated identically and used as a cross-reference.

[c and d] Standard curve for the change of Inhibition zone diameters by using Isothiazolinone at the concentration gradient. Vertical lines indicate standard deviations. [c] Bacterial community for two days. [d] Fungi community for four days.

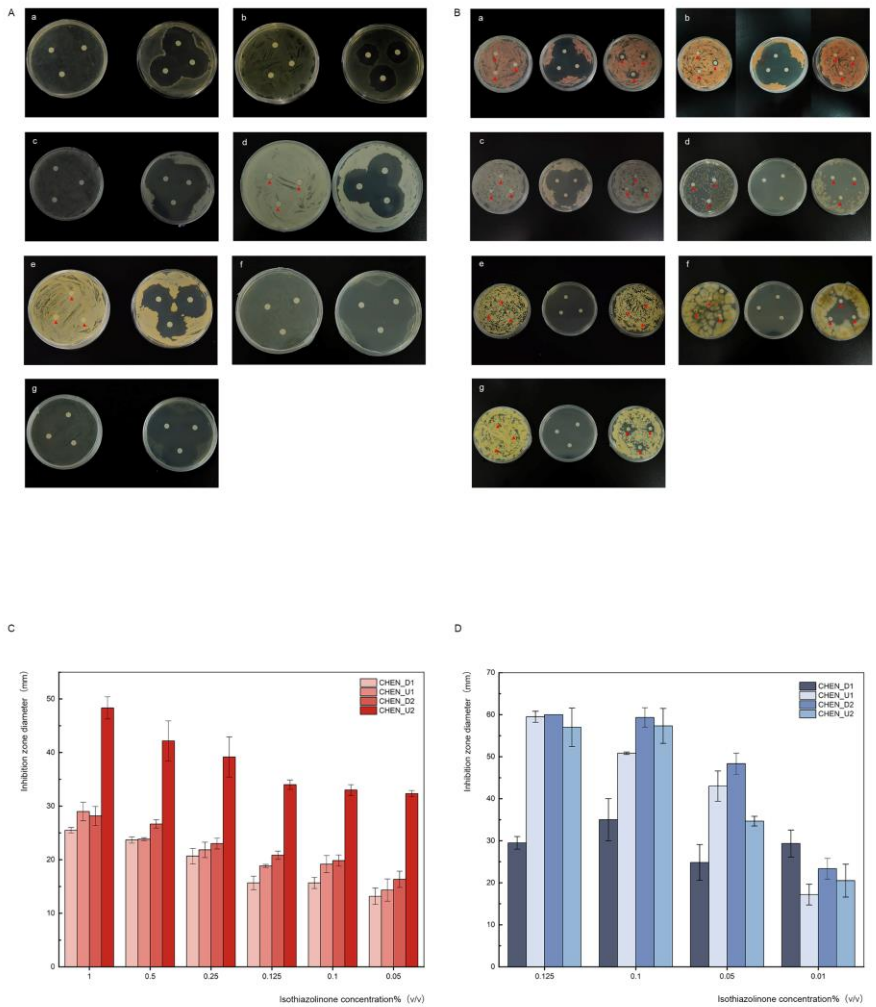

Table 1: Abbreviation of the sampling ancestral halls and their samples

Ancestral hall name	Label	Samples	
Urban			
Chen Clan Ancestral Hall	Urb_CHEN	Damaged sites	CHEN_D1 CHEN_D2
		Undamaged sites	CHEN_U1 CHEN_U2
Guan Clan Ancestral Hall	Urb_GUAN	Damaged sites	GUAN_1 GUAN_2 GUAN_3
Sansheng Gongwang Temple	Urb_SAN	Damaged sites	SAN_1 SAN_2 SAN_3
Sub-urban			
Lin Clan Ancestral Hall	Sub_LIN	Damaged sites	LIN_D1 LIN_D2
		Undamaged sites	LIN_U1 LIN_U2
Donglin Liang Ancestral Hall	Sub_DONG	Damaged sites	DONG_1 DONG_2 DONG_3
Liede Lin Clan Ancestral Hall	Sub_LIE	Damaged sites	LIE_1 LIE_2 LIE_3
Rural district			
Qingxi Ancestral Hall	RD_QING	Damaged sites	QING_D1 QING_D2
		Undamaged sites	QING_U1 QING_U2
Ming Min Ancestral Hall	RD_MING	Damaged sites	MING_1 MING_2 MING_3
Fuchen Gong Temple	RD_FU	Damaged sites	FU_1 FU_2 FU_3

Table 2: Molecular identification of strains isolated from the four samples of Urb_CHEN

	Nucleotide Blast reference strains			
	Clost relative strain	**Accession number**	**Phylum**	**Similarity (%)**
Bacteria				
CHEN_B1	*Pseudomonas* sp.	MN043751.1	Proteobacteria	99.79%
CHEN_B2	*Pantoea dispersa* strain	MT921704.1	Proteobacteria	99.86%
CHEN_B3	*Paenibacillus* sp.	JF768727.1	firmicutes	99.72%
CHEN_B4	*Bacillus cereus* strain	MN691548.1	Firmicutes	98.21%
CHEN_B5	*Priestia megaterium* strain	MF431767.1	Firmicutes	100.00%
CHEN_B6	*Staphylococcus hominis* strain	KY992547.1	Firmicutes	100.00%
CHEN_B7	*Curtobacterium* sp.	MK704290.1	Actinobacteria	99.72%
CHEN_B8	*Microbacterium oleivorans* strain	JQ660049.1	Actinobacteria	99.65%
Fungi				
CHEN_F1	*Rhodotorula mucilaginosa* strain	ON954707.1	Basidiomycota	99.84%
CHEN_F2	*Cystobasidium* sp.	AF444619.1	Basidiomycota	99.32%
CHEN_F3	*Aureobasidium pullulans* strain	KT352844.1	Ascomycota	98.23%
CHEN_F4	*Cladosporium* sp.	OK242732.1	Ascomycota	99.82%
CHEN_F5	*Trichoderma* sp.	ON248245.1	Ascomycota	99.84%
CHEN_F6	*Daldinia* sp.	MK311339.1	Ascomycota	99.83%
CHEN_F7	*Aspergillus sydowii* strain	OP237096.1	Ascomycota	99.65%

[a] "CHEN_B1 ~ CHEN_B8" and "CHEN_F1 ~ CHEN_F7" indicate strains were identified by 16S and ITS rDNA gene. CHEN_B2, CHEN_B4, CHEN_B5, CHEN_B6, CHEN_B8, CHEN_F1, CHEN_F3, and CHEN_F7 could be annotated to species. Others could only be annotated to the genus.

Table 3: Hydrolytic zones of the fungal isolates on CMC-degrading agar medium for three days

Fungi	Hydrophytic zones diameter (mm)
CHEN_F1	No degradation
CHEN_F2	No degradation
CHEN_F3	5.00±0.00
CHEN_F4	8.08±1.18
CHEN_F5	23.67±3.51
CHEN_F6	9.67±0.76
CHEN_F7	5.33±0.58

[a] Data are represented as mean ± SEM.

Table 4: The inhibition zone shown on the BMP agar medium for bacteria (two days) or PDA agar medium for fungi (three days) using different biocides: Isothiazolinone at the concentration of 1% (v/v) and NaClO at the concentration of 1% (w/v).

	Inhibition zone diameter (mm)	
Isolates	**Isothiazolinone**	**NaClO**
CHEN_B1	33.73±0.76	/
CHEN_B2	26.00±2.65	/
CHEN_B3	45.33±1.15	/
CHEN_B4	35.33±0.58	/
CHEN_B5	32.00±1.00	/
CHEN_B6	45.33±0.58	/
CHEN_B7	42.00±2.00	/
CHEN_F1	36.00±1.00	14.67±2.02
CHEN_F2	23.83±1.04	No inhibition
CHEN_F3	39.33±0.58	No inhibition
CHEN_F4	Complete inhibition	44.67±2.31
CHEN_F5	Complete inhibition	9.00±1.00
CHEN_F6	Complete inhibition	27.33±1.15
CHEN_F7	Complete inhibition	17.67±2.52

[a] Data are represented as mean ± SEM.

[b] "/" means that our research did not carry out the relative experiments. Under the condition of 1% (w/v) concentration, NaClO showed no inhibition effect on the mixed strains; therefore, there is no need to test for the bacterial isolates.