Analysis of Biodeterioration on Ancestral Halls with Different Visitor Flow Rates Using Amplicon Sequencing and Conventional Culturing Methods

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SUMMARY

Wooden buildings are facing biodeterioration because of natural weathering. Ancestral halls are traditional Chinese wooden architecture with high artistic value. The research analyzed nine ancestral halls with different visitor flow rates in Guangdong Province under subtropical monsoon climates. With amplicon sequencing and conventional culturing methods, a common core harmful microorganism group which are proven to be capable of degrading cellulose and lignin was found, and some of them originated from human activities, including *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Acinetobacter*, *Toxicocladosporium*, *Cladosporium*, *Aspergillus*, and *Epicoccum*, allowing us to apply unified antimicrobial methods to different ancestral halls in Guangdong Province. Additionally, the microbial community is similar between damaged and undamaged points, predicting the potential risk of taking paint coating as the only method for antimicrobial preservation. Hence, we evaluated the effect of four representative biocides and determined the feasibility of low-concentration Isothiazolinone. This research adds significant reference for the protection of wooden buildings.

INTRODUCTION

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[1,2]. The colonization by microorganisms of the wood surface results in forming of a biofilm, an extracellular polysaccharide matrix

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[3,4,5,6]. 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[7,8]. What's more, studies show that cellulolytic microorganisms such as *Bacillus*, *Aspergillus*, *Cladosporium*, and *Penicillium* are capable of destroying Polychrome wood artifacts[1]. 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[9,10].

Currently, the surface of the wooden ancestral halls is often covered with a coating of paint for longer preservation, which could reduce microbial growth[11]. 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[2]. 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*[12,13]. 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[12].

The diversity of microbiota colonized on the surface of wooden cultural relics can be affected by humans. On the one hand, human activities can affect the original microbial community structure, resulting in damage to the surfaces of cultural relics. 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[14]. Once the invaders adapt to the environmental conditions, they will successfully colonize the surface and destroy the original community structure[14]. In addition, human activities may bring in various organic substances, which can change the nutritional balance on the surface of the original cultural relics and result in the community structure[15]. On the other hand, visitor flow rate sometimes may affect microbial diversity. For instance, Nattaphon Suphaphimol found that the fungal diversity was different between the two temples with distinct visitor flow rates in Chiang Mai, Thailand, and microorganisms like Aspergillus, Malassezia, and Toxicocladosporium which

belong to human skin were found on the one with higher visitor flow rate, the Buak Krok Luang temple[16]. However, at the same time, Elsa Fuentes compared the Granite Buildings located in rural and urban areas and found that the diversity of fungi in the two places was extremely similar[17]. Tianxiao Li et al.[4] studied the microbiome on the steles of Lingyan Temple and determined that changes in the air microbiome due to natural rainfall, plant respiration, and numerous visitors also affected the composition of the stele surface microbiota, but the contribution of human traffic to changes in the microbiome was unclear.

Biocides are 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[18]. 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[19,20,21]. Currently, Phytostats have arisen attention for their environmental friendliness, but whether they could exert obvious antibacterial effects and how to apply them to practical use are still in question[22,23].

The ancestral hall is traditional Chinese wooden architecture, especially prosperous in the southern areas. According to the Third National Census of Cultural Relics, among more than 25,000 immovable cultural relics registered in Guangdong Province, ancestral hall buildings account for about one-third. Here, we investigated the microbiome of nine ancestral halls in Guangdong Province, including the Chen Clan Ancestral Hall, Guan Clan Ancestral Hall, Sansheng Gongwang Temple, Lin Clan Ancestral Hall, Donglin Liang Ancestral Hall, Liede Lin Clan Ancestral Hall, Qingxi Ancestral Hall, Ming Min Ancestral Hall, and Fuchen Gong Temple. Among them, the oldest ones were built during the Ming Dynasty (AD1368 to AD1644), and the latest ones were built during the Qing Dynasty (AD1636 to AD 1912). All of them were open to the public. An ancestral hall was 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. For example, the Qingxi Ancestral Hall, Ming Min Ancestral Hall and the Fuchen Gong Temple, which we found in the Kongmei Village in a rural district, still maintain as important and sacred places for praying and memorizing ancestors. The visitors are usually limited to the local villagers and only come here at special festivals or anniversary days. More often, the ancestral halls not only retain their original functions but also become popular activity centers and even important cultural relics today. For instance, the Lin Clan Ancestral Hall, the Donglin Liang Ancestral Hall and the Liede Lin Clan Ancestral Hall, are not only places for praying and memorizing, but also open to the local citizens for gathering and relaxing on usual days; the Chen Clan Ancestral Hall, which worked as the Chen Clan's Industry College when built in 1894, is housing the Guangdong

Folk Art Museum now; the Guan Clan Ancestral Hall and the Sansheng Gongwang Temple, which are located in Lingnan Impression Garden and used to belonged to the ancestral hall complex in Lianxi Village, has been rated as a national AAAA-level tourist attraction now. With exquisite architectural features, profound historical value and rich humanistic feelings, the ancestral halls have influenced cultural and architectural developments worldwide.

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. Microbial weathering often contains the effects of mechanical and chemical weathering and has become an unavoidable issue for wooden architecture protection at present. But there are not many studies on the microbial weathering of ancestral halls now.

It is important to determine the types of microorganisms on the surface of ancestral halls and clarify the mechanism of biological degradation for the long-term preservation of cultural relics. In this research, we analyzed the microbiota from different ancestral hall surfaces based on 16S and 18S amplicon sequencing. Considering that the climate condition will affect microbial diversity, we selected all the sampling ancestral halls in the coastal areas of Guangdong Province to ensure that they are in the same subtropical monsoon climate[24]. First of all, we compared the differences and similarities of the microbial diversity at damaged points under different visitor flow rates and explored the microorganisms' ability of biological degradation and nutrient cycling. It was found that the ancestral halls under different visitor flow rates shared the same core group of harmful microorganisms, which provided the possibility that the ancestral halls in Guangdong Province were allowed to achieve basic long-term antimicrobial protection with a uniform prevention measure. Additionally, we innovatively compared the difference in microbial diversity between damaged sites and undamaged sites on the surface of the ancestral hall and predicted the potential risk of depending on a single protection method (for instance, adding a coating of paint on the surface) for long-term preservation. At the same time, based on the conventional culturing method, we isolated the microbial strains on the surface of Chen Clan Ancestral Hall, and verify their ability to degrade lignin and cellulose. Furthermore, we selected 4 different biocides, including the commercial types and environment-friendly types, to test the antimicrobial sensitivity of the microbiota from Chen Clan Ancestral Hall. This research is the largest study on the microbial weathering of ancestral halls and adds significant reference value for the protection of wooden buildings which are located in Guangdong Province or under subtropical monsoon climate conditions.

RESULTS

Characteristics of the samples

Nine ancestral halls with different visitor flow rates were selected for sample collection. According to the distances from the central area, they can be divided into three groups, the urban group (Chen Clan Ancestral Hall, Guan Clan Ancestral Hall, Sansheng Gongwang Temple), which is in the central city of Guangzhou; the suburban group (Lin Clan Ancestral Hall, Donglin Liang Ancestral Hall, Liede Lin Clan Ancestral Hall), which is in the outskirts of Guangzhou; the rural district group (Qingxi Ancestral Hall, Ming Min Ancestral Hall, Fuchen Gong Temple), which is in the country areas of Jieyang(Figure 1 and Table 1).

Long-term weathering has caused damage to the paint coating. We investigated the microbial colonization of these peeling paint surfaces and the obvious microbial stain was found in most of them. For instance, the surface of the wooden railing (GUAN_2) is covered with dark green biofilm; the surface of the wooden door (SAN_3), the surface of the wooden pillar (DONG_1), and the surface of 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); and yellow plaques can be found on the wooden screen (QING_2). The microorganism colonization leads to aesthetic damage and structural damage to the ancestral hall.

To determine the environmental conditions of the sampling sites, we measured the temperature, relative humidity, light, moisture contents of the wood, relative altitude from ground level, and the direction of the wood face at all sampling points (Table S1).

Determining the diversity of microbial communities from samples using amplicon sequencing

Most of the samples showed high-quality sequencing results, except for sample MING_3, in which the concentration of DNA fragments could not meet the required concentration of sequencing even after increasing the amount of amplified DNA and adding two rounds of PCR cycles, and had to be discarded in the following analysis. After quality control and optimization of the DNA fragments, 20,000 to 120,000 raw data were generated at each sample, and the corresponding Operational Taxonomic Unit (OTU) numbers produced ranged from 78 to 472 for bacteria and 11 to 108 for fungi and eukaryotes. The Shannon index evaluates the biodiversity of the sample: the Shannon index of the 18S amplicon is below 2 in most samples, indicating that there are only a few types of fungi on the surface of the ancestral hall; however, the abundance of bacteria in various samples is quite random, some are relatively simple, in

which the Shannon index is below 0.5, and some have rich microbial species, in which the Shannon index is up to 5 or more. The alpha diversity index of different samples did not produce an obvious distribution bias of microbial diversity due to geographical differences. And the coverage index is equal to 1 for all samples, indicating the uniformity of the sampling (Table S2 and Table S3).

According to the amplicon sequencing results, the overall taxonomic composition of the colonizing microbiome on the surface of ancestral halls was determined, and organized at the phylum level: Firmicutes and Proteobacteria were the major bacteria species with high content in urban, suburban, and rural districts; the two phyla together accounted for more than 84% of the microbiota in every ancestral hall (Figure 2A and Figure S1A). At the same time, in some ancestral halls, we could also observe species including Bacteroidetes (Urb_SAN1.38%, Sub_LIN7.36%, RD_QING8.02%, RD_MING3.20%), Actinobacteria (Sub_LIN1.21%, RD_QING3.46%, RD_FU2.67%, RD_MING9.24%), and Cyanobacteria Chloroplast (RD_QING1.32%, RD_MING1.57%) with a content of more than 1%. Ascomycota is the most important eukaryotic microorganism in urban, suburban, and rural districts, with an abundance of more than 90% at almost every ancestral hall (except RD_FU whose abundance is 74.52%) (Figure 2B and Figure S1B).

Almost all ancestral halls shared some similarities in the dominant microorganisms. The main 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 and Figure S1C). In the genus-level of eukaryotes, abundances of *Toxicocladosporium*, *Cladosporium*, *Aspergillus* (except Urb_CHEN, Sub_LIE, whose abundance is less than 1%), *Epicoccum* (RD_QING, RD_FU, whose abundance is less than 1%) were significant in all ancestral halls (Figure 2D and Figure S1D).

The dominant genera in each ancestral hall also have differences. Urb_GUAN, Urb_SAN, Sub_LIE, and RD_MING showed a high abundance of Massilia (3.68%, 32.93%, 17.22%, 13.96%). Sub_LIE also showed a high abundance of *Exiguobacterium* (9.03%) and *Lysinibacillus* (10.55%). In addition, both Urb _GUAN and RD_QING contain a relatively high abundance of *Pantoea* (6.70%, 3.61%) (Figure 2C and Figure S1C). Sub_DONG, RD_FU, and RD_MING showed a high abundance of *Hortaea* (11.75%, 1.57%, 6.21%). Sub_LIN showed a high abundance of *Didymella* (24.55%). RD_FU showed a high abundance of *Sarcosphaera* (10.98%) and *Rhizophlyctis* (7.40%) (Figure 2D and Figure S1D).

Based on the microbial information of the damage points of different ancestral halls, we selected one ancestral hall in each group (the urban/suburb/rural district): Chen Clan Ancestral Hall, Lin Clan Ancestral Hall, and Qingxi Ancestral Hall. We took two pairs of samplings from damaged and undamaged external 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. To make sure that the differences between damaged and undamaged sampling points result from microbial colonization on the surface, we monitored the environmental data including the temperature, relative humidity, water content of the wood, and light, and ensure the conditions of a pair are consistent.

The collinear relationship diagram showed that: for bacterial genera, *Paenibacillus*, *Bacillus*, and *Acinetobacter* were easier to find at the damaged points, while *Pantoea*, *Curtobacterium*, and *Cellulomonas* were easier to find at the undamaged points; the probability of *Pseudomonas* to be found in the damaged points and the undamaged points were basically the same (Figure 2E and Figure S1E). For eukaryotic microorganisms, the genera with high abundance at the damaged points and the undamaged points showed extremely high commonality. The relative abundance of *Toxicocladosporium* and *Cladosporium* in the damaged and undamaged points was similar, and the total content was close to 80%, which were the main genera in both damaged and undamaged points with an extremely high possibility to be found. Differences also existed. *Didymella* and *Epicoccum* were easier to find at the damaged points, and Aspergillus was more likely to be found at the undamaged points (Figure 2F and Figure S1F).

Genetic function prediction for the microbial communities based on KEGG

We predicted cellulose degradation for all sequencing samples based on KEGG analysis and found that it was possible to reconstruct a complete cellulose degradation process for all the studied sites (Figure 3A). The complete pathway of cellulose metabolism can be found on all the samples: cellulose is decomposed into cellulose dextrin through two metabolic pathways K01179 and K01188, and then further decomposed into cellobiose.

We also summarized the relative abundance of five microbial biological cycle functions related to the basic nutrient cycle, to judge the microorganisms' ability to long-term colonization on the surfaces of ancestral halls (Figure 3B, Figure 3C and Figure 3D). Among the microbial groups that colonized the surface of the ancestral hall, the two basic cycles of carbon fixation of prokaryotic carbon and nitrogen metabolism were the most abundant. Simultaneous sulfur metabolism, photosynthetic carbon fixation, and methane metabolism also

existed in this microbiota, which showed that the microbiota existing on the damaged surface of the ancestral hall has the possibility of realizing an independent nutrient supply. allowing microorganisms of the ancestral hall to colonize the surface for a long time.

In the functional analysis of the nitrogen cycle, we found that the microbial groups on the surface of the damaged points have complete corresponding metabolic pathways including nitrogen fixation, nitrate reduction, and denitrification processes. Some ancestral halls (RD_MING, Urb_CHEN) also have the function of nitrification. It is particularly worth noting that our amplicon dataset retrieves a large number of annotated sequences (K02586, K02588, and K02591) associated with genes involved in nitrogen fixation. It is manifested by the phenomenon that the nine ancestral halls all contain these pathways, and the content is extremely high in Qingxi Ancestral Hall and Mingmin Ancestral Hall when compared with other ones, indicating that the surfaces of all ancestral halls have the basic potential to achieve nitrogen fixation (Table S3). It showed that these microbial groups have a relatively strong ability to fix nitrogen and produce ammonium salt, and the presence of ammonium salt will promote microorganisms' degradation of wood cellulose, which will make it easier for the microbiome to obtain essential nutrients from wood, and further damage the wooden structure of ancestral halls[25].

At the same time, there is no methane-producing metabolic pathway to be found in all samples; a certain number of methane oxidation-related gene pathways such as k10944, k10945, and k10946 can be found in most ancestral halls (Urb_CHEN, Urb_GUAN, Urb_SAN, Sub_DONG, Sub_LIE), but the contributions are relatively minor in our amplicon dataset.

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 sample using conventional culturing methods (Table 2). The isolated and dominant microorganisms shown in the amplicon sequencing are consistent. All isolates were tested for cellulose and lignin degradation ability except 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 (data could not be shown). 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 S2A-E); *Rhodotorula muciaginosa* strains and *Cystobasidium* sp. could not degrade both lignin and cellulose (data could not be shown).

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 four samples of Urb_CHEN was detected by the agar disk-diffusion method.

For the mix strains, 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 zones 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). For the isolated strains, we further tested the antimicrobial effect of Isothiazolinone and Sodium hypochlorite. 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, the mix strains of each sample were then detected by Isothiazolinone respectively at a concentration gradient (from 1% to 0.01%) and we tried 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 in the other three samples 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, we consider that the fungal community of CHEN_D1 contained a higher content of *Epicoccum* (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 the antimicrobial sensitivity following the decreasing concentration of Isothiazolinone. Unfortunately, the isolate of *Epicoccum* was not obtained during the isolation process, so the antimicrobial sensitivity of Isothiazolinone to this fungus hasn't been further studied yet.

DISCUSSION

This research is the first to study the problem of microbial weathering in the long-term preservation of Chinese ancestral halls. We clarify the characteristics of the microbial communities at nine ancestral halls in the subtropical monsoon climate of Guangdong, compare the sensitivity of the microbiome from Chen Clan Ancestral Hall to different biocides, and discuss the feasibility of low - concentration Isothiazolinone being the most suitable choice for wooden cultural relics.

Microbial weathering has always been an important issue in the conservation of cultural relics, but there has been little discussion of microbial diversity on the surface of wooden cultural relics. The limited research on wooden cultural relics mainly focuses on buried or underwater cultural heritage, buried or waterlogged wood, such as the Wreck Archaeology of " South China Sea I "[26,27]. However, this research mainly focuses on the microbial degradation mechanism of ancestral halls, a type of traditional wooden cultural relics that were under the open air and has been suffering from natural weathering for a long time.

Wooden structures consist of 40-60% of cellulose, 15-30% of hemicellulose, and 17-35% of lignin[28], which are a rich source of nutrition for microorganisms. The amplicon sequencing results showed that the microbiota on the surface of damaged ancestral halls with different traffic flows was dominated by Firmicutes, Proteobacteria, and Ascomycota, and may contain Bacteroidetes and Actinobacteria. At the genus level, it can be further clarified that the common group of harmful microorganisms in all the damaged sites includes *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Acinetobacter*, *Toxicocladosporium*, *Cladosporium*, *Aspergillus*, and *Epicoccum*, and is also likely to contain *Massilia*, *Pantoea*, and *Hortaea*. Firmicutes and Proteobacteria are the major components of biofilms that could retain essential nutrients and water required by microorganisms[24]. The colonization of actinomycetes produces pigments,

organic acids, and polysaccharides[29] 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. Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Ascomycota are also considered the main phyla for cellulose degradation [30]. *Cladosporium* and *Aspergillus* were isolated from lithographs and have been shown to have cellulose-degrading abilities [31]. *Cladosporium* is also considered to be the main chromogen in artworks[32]. In the previous literature, it has been demonstrated that *Cladosporium* is the main microbial disease of canoes preserved in the Oceanographic Museum of China[33]. *Bacillus*, *Paenibacillus* sp., *Acinetobacter*, and *Pseudomonas* have all been shown to utilize lignin as the sole carbon source[34,35]. *Pantoea* has been proven to mediate lignin degradation through the production of laccase and lignin peroxidase [36].

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 long-term persistence of microbiota on the surface of wooden ancestral halls[37].

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[38]. 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, and five strains of bacteria and five strains of fungi exhibit cellulose-degrading activity. 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[39]. *Microbacterium oleivorans* strain cannot utilize lignin and cellulose directly but may be able to utilize cellobiose for nutrients[40].

With the development of the increasingly prosperous cultural tourism industry, the visitor flow rate shows a significant contribution to the composition of microbial communities on the surface of cultural relics. Nowadays, ancestral halls have become important local scenic spots with their aesthetic significance and enlightening function. The microbiota brought by human activities will affect the original microbial community structure on the surface and may lead to further biodeterioration of cultural relics. Amplicon sequencing results indicated

that *Aspergillus* and *Toxicocladosporium* were the dominant fungal genera on the surface of all the damaged sites of the ancestral halls. *Aspergillus* and *Epicoccum* were found to belong to the most common fungal genera on human feet[41]. The type strain of *Toxicocladosporium* was isolated from moldy paint in Suriname and was found to cause skin disease[42]. *Acinetobacter* and *Pseudomonas* are considered human skin bacterial residents[41]. At the same time, the microorganisms on the surface of the Chen Clan Ancestral Hall obtained based on the conventional cultivation method, such as *Bacillus cereus*, which is also a common microorganism in human hands[41]; and *Staphylococcus* is a pathogenic bacteria of human bloodstream infection that can from biofilm[43]. Many of the genera detected at the damaged sites on the surface of the ancestral hall showed extremely high correlations with human skin microbiota, which shows that the harm caused by human activities can be long-term and visitors should avoid touching the cultural relics.

However, the Shannon index also indicated that the microbial diversity on the surface of damaged sites of ancestral halls did not change significantly with the increase of human flow, and the dominant microbial groups on the surfaces of all ancestral halls had obvious similarities in species. This may be because the original microbial community on the surface has formed a stable biofilm, which facilitates the adsorption and adaptation of the invader microorganisms to the surface of cultural relics. Additionally, the microbiota carried by humans shows great adaptability to the surfaces of ancestral halls due to the ability to degrade lignocellulose. Therefore, it can also adapt to the environment on the surface of the ancestral hall with only a small amount of microorganisms[5]. The discovery allows most of the ancestral halls in Guangdong to use a unified approach for long-term antimicrobial preservation.

It is worth noting that, *Pseudomonas*, *Toxicocladosporium*, *Cladosporium*, and other potential microbial diseases that cause wood to suffer from microbial deterioration also exist on the surface of the ancestral hall with intact paint. And it is also easier to find *Pantoea*, *Curtobacterium*, *Cellulomonas*, *Didymella*, and *Epicoccum* where the paint surface is not damaged and the wood is not exposed. 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[44]. However, our research shows that on the surface of the undamaged ancestral hall, in other words, the coating of paint, there also exists microbial communities that cause microbial degradation of cultural relics. On the one hand, the similarity of microbial species may be caused by the spread of microorganisms which is due to the adjacent location of the damaged sites and the undamaged sites. On the other hand, the microorganisms may have the effect of degrading paint. For example, *Pantoea*, *Pseudomonas*, *Cladosporium* and *Epicoccum* have been proven to be able to degrade paint as well as wood[45,46], so they can colonize the surface of ancestral

halls with complete paint. Generally speaking, due to the slow rate of microbial degradation of paint, it is rare to observe obvious microbial stains on the paint surface. Above all, taking the method of adding a coat of paint on the surfaces of wooden cultural relics as the only antimicrobial preservation measure should be prudent.

In order to control the degradation of microorganisms on the surface of cultural relics, more and more biocides have been applied to the protection of cultural relics. However, at the same time, more and more biocides have been proven to be harmful to the environment and human health. Therefore, new biocides represented by plant essential oil extracts have aroused attention due to their environmental friendliness[47]. Unfortunately, cinnamon essential oil and thyme essential oil show a weak antimicrobial effect in this research. 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[48]. The research tried to reduce this hazard 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 four samples obtained from Chen Clan Ancestral Hall, the sizes of the inhibition zones were all greater than 10mm, and some were even greater than 15mm, showing moderate or even highly sensitive[49]. 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.

CONCLUSION

In conclusion, the microbial contamination on the surface of nine ancestral halls in the subtropical monsoon climate of Guangdong Province's coastal areas, which is different in visitor flow rate, was analyzed using the conventional culturing method together with amplicon sequencing. The microbial community that makes the ancestral halls long-term suffer microbial deterioration was detected, with *Bacillus* sp., *Pseudomonas* sp., *Paenibacillus* sp., *Acinetobacter* sp., *Toxicocladosporium* sp., *Cladosporium* sp., *Aspergillus* sp., and *Epicoccum* sp. being the main members. And a proportion of the species isolated and purified in this community showed lignin or cellulose degradation capacity. Human activity has caused more similar microbial colonization, leading to more serious biodeterioration, which allows the ancestral halls located in Guangdong Province to adopt the same approach to avoid microbial deterioration. There is also a risk of much more biodeterioration if only ancestral halls are protected through painting. Biocide is a common means of microbial control. In this study,

Isothiazolinone at low concentrations was confirmed to be an effective inhibitor. However, when used properly, it should consider the impact on the environment, cultural heritage, and humans.

LIMITATIONS OF STUDY

Due to climatic constraints, the results of our study are only applicable to the ancestral halls in the subtropical monsoon climate of Guangdong Province's coastal areas. Besides, some of the microorganisms in the samples are uncultivable, which limits the research on the capacity for biological degradation. Furthermore, there are not many studies available to date on the natural weathering of wooden artifacts in the open air, which raises the difficulty of the study.

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AUTHOR CONTRIBUTIONS

J.R.L and J.L conceived the experiments. M.L, X.S, T.Y and Y.L collected the microbiological samples. M.L, X.S, Q.W, T.Y, L.Y and P.A performed the experiments. M.L and X.S analyzed the data. M.L, X.S, and Q.W wrote the paper. J.R.L and J.L performed the review and editing of the original draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Main figure titles and legends

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 communities from samples using amplicon sequencing

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

(E and F) Distribution of Genera level taxonomy of microbiome in damaged and undamaged surfaces among the samples of Urb_CHEN, Sub_LIN, and RD_QING based on 16S amplicon and 18S amplicon. (E) Bacterial genera. (F) Fungal genera.

See also Figure S1.

Figure 3. Genetic function prediction for the microbial communities based on KEGG

(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 of three samples, while Urb_CHEN, Sub_LIN, and RD_QING had four samples and RD_MING had two samples.

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

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 disks on each BPM plate were loaded with water, Isothiazolinone at the concentration of 1%(v/v) from left to right. (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 disks on each PDA plate were loaded with water, Isothiazolinone at the concentration of 1% (v/v), and NaClO at the concentration of 1% (w/v) from left to right. (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 diameter by using Isothiazolinone at the concentration gradient. Vertical lines indicate standard deviations of three replicate tests for each. (C) A bacterial community for two days. (D) Fungi community for four days.
See also Table 4.

Table

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 1: Abbreviation of the sampling ancestral halls and their samples

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

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.

See also Figure S2.

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

Isolates	Inhibition zone diameter (mm)	
	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.

[a] "/" means that the 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.

Table4: The inhibition zone shown on the BMP agar medium for bacteria (two days) or PDA agar medium for fungi (three days) using different biocide: Isothiazolinone at the concentration of 1% (v/v) and NaClO at the concentration of 1% (w/v). Every plate was placed with three disks (6-mm diameter) as parallel controls.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Pseudomonas sp.	This paper	N/A
Pantoea dispersa strain	This paper	N/A
Paenibacillus sp.	This paper	N/A
Bacillus cereus strain	This paper	N/A
Bacillus megaterium strain	This paper	N/A
Staphylococcus hominis strain	This paper	N/A
Curtobacterium sp.	This paper	N/A
Microbacterium oleivorans strain	This paper	N/A
Rhodotorula mucilaginosa strain	This paper	N/A

Cystobasidium sp.	This paper	N/A
Aureobasidium pullulans strain	This paper	N/A
Cladosporium sp.	This paper	N/A
Trichoderma sp.	This paper	N/A
Daldinia sp.	This paper	N/A
Aspergillus sydowii strain	This paper	N/A
Biological samples		
Microbial samples on the surface of ancestral halls	This paper	N/A
Chemicals, peptides, and recombinant proteins		
M9 salts	Sigma-Aldrich	Cat#M6030
CTAB	Sangon Biotech, China	(Moller et al. 1992)
2×Taq Master Mix	Sangon Biotech, China	Cat#B639295
2×Hieff® Robust PCR Master Mix	Yeasen, China	Cat#10105ES03
TBE buffer(Tris, boric acid, EDTA)	Sangon Biotech, China	Cat#B548102
Ethidium bromide (EB)	Sangon Biotech, China	A600195; CAS: 1239-45-8
Sodium carboxymethylcellulose (CMC-Na)	Sangon Biotech, China	Cat#A501427; CAS: 9004-32-4
guaiacol	Sangon Biotech, China	Cat#A600490; CAS:90-05-1
Isothiazolinone	This paper	CAS:96118-96-6
NaClO	Linser	CAS:7681-52-9
Thymus Vulgaris Essential Oil	This paper	CAS:8007-46-3
Cinnamon Essential Oil	This paper	CAS:8007-80-5
Critical commercial assays		
E.Z.N.A™ Mag-Bind Soil DNA Kit	OMEGA	Cat#M5635-02
Qubit dsDNA HS assays	ThermoFisher	Cat#Q32854
Deposited data		
16S amplicon Sequencing date	This paper: Sequence Read Archive (SRA)	[BioProject PRJNA925150]: [Accessions: SRR23110177-SRR23110205]

18S amplicon Sequencing date	This paper: Sequence Read Archive (SRA)	[BioProject PRJNA925313]: [Accessions: SRR23117924-SRR23117952]
16S rRNA sequencing data from all bacterial isolates	This paper; Genbank	accession numbers from OQ283719 to OQ283726
ITS rRNA sequencing data from all fungal isolates	This paper; Genbank	accession numbers from OQ283737 to OQ283743
Oligonucleotides		
Primers: the 27F-forward primer: AGAGTTTGATCCTGGCTCAG	This paper	N/A
Primers: the 1492R-forward primer: GGTTACCTTGTTACGACTT	This paper	N/A
Primers: the ITS1-forward primer: TCCGTAGGTGAACCTGCGG	This paper	N/A
Primers: the ITS4-reverse primer: TCCTCCGCTTATTGATATGC	This paper	N/A
Software and algorithms		
Illumina MiSeq system	Illumina MiSeq, USA	N/A
PEAR (version 0.9.8)	[50]	https://cme.h-its.org/exelixis/web/software/pear/index.htm
Usearch (version 11.0.667)	Robert Edgar	http://drive5.com/uparse/
Mothur (version 3.8.31)	[51]	http://mothur.org
R vegan package (version 3.6.0)	R vegan package (version 3.6.0)	https://www.r-project.org
STAMP (version 2.1.3)	[52]	https://beikolab.cs.dal.ca/software/STAMP
PICRUSt (v1.1.4)	PICRUSt (v1.1.4)	http://picrust.github.io/picrust/
Other		
Hieff NGS™ DNA Selection Beads	Yeasen	Cat#12601ES56
Qubit® 4.0	Thermo，USA	N/A
SMART SENSOR AS817 Humidity	SMART SENSOR	Cat#AS817
SMART SENSOR AS803 Digital Lux Meter	SMART SENSOR	Cat#AS803
SMART SENSOR AS981 Moisture Meter	SMART SENSOR	Cat#AS981
Sterile swabs	This paper	N/A

Filter paper	Sangon Biotech，China	Cat#F503315
Thermal instrument	T100 Thermal Cycle 1861096, USA	N/A
Thermal instrument	Applied Biosystems 9700, USA	N/A

MATERIALS AND METHODS

Sample sites.

Sampling was carried out at nine wooden ancestral halls in Guangdong Province, located in the southeast coastal area of China. 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.

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

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.

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.

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 status. Then we separated the colonies according to their colors and morphologies and repeated the isolating process until obtaining the purebred strains.

Total community genomic DNA extraction was performed using the CTAB Extraction Method[53]. We measured the concentration of the DNA using a Qubit 4.0 (Thermo, USA) to ensure that adequate amounts of high-quality genomic DNA had been extracted.

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: the 27F-forward primer and the 1492R-reverse primer. 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 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 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 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 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 bacterial 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.

The PCR products were checked using electrophoresis in 2% (w/v) agarose gels in TBE buffer (Tris, boric acid, EDTA) stained with ethidium bromide (EB) and visualized under UV light.

Gene library construction, quantification, and sequencing

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. After sequencing, the two short Illumina readings were assembled by PEAR software (version 0.9.8)[50] 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.

Statistical analysis

The α-diversity indices (including Chao1, Simpson, and Shannon indices) were quantified in terms of OTU richness. To assess sample adequacy, rarefaction curves of the observed numbers of OTUs were constructed, and all α diversity indices were calculated with Mothur[51] software (version 3.8.31). The OTU rarefaction curve and rank abundance curves were plotted in R (version 3.6.0). To estimate the diversity of the microbial community of the sample, we calculated the withinsample (alpha) diversity by T-test for two groups and made multiple group comparisons using the ANOVA test. 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. These analyses were visualized using R vegan package (version 2.5-6), and finally, the inter-sample distances were presented as scatterplots. Difference comparison is used to identify features with significantly different abundances between groups using STAMP (version 2.1.3)[52] and LefSe (version 1.1.0). Correlation coefficients and p-values between communities/OTUs were calculated using SparCC (version 1.1.0), and correlation matrix heatmaps were drawn using the R corrplot package (version 0.84). R graph package (version 2.0.0) is used to build network graphs.

Function prediction

Functional prediction analysis of bacteria and archaea 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 and archaeal metabolic functions.

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): 9mmol 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. 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. We 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.

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. Then we 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.

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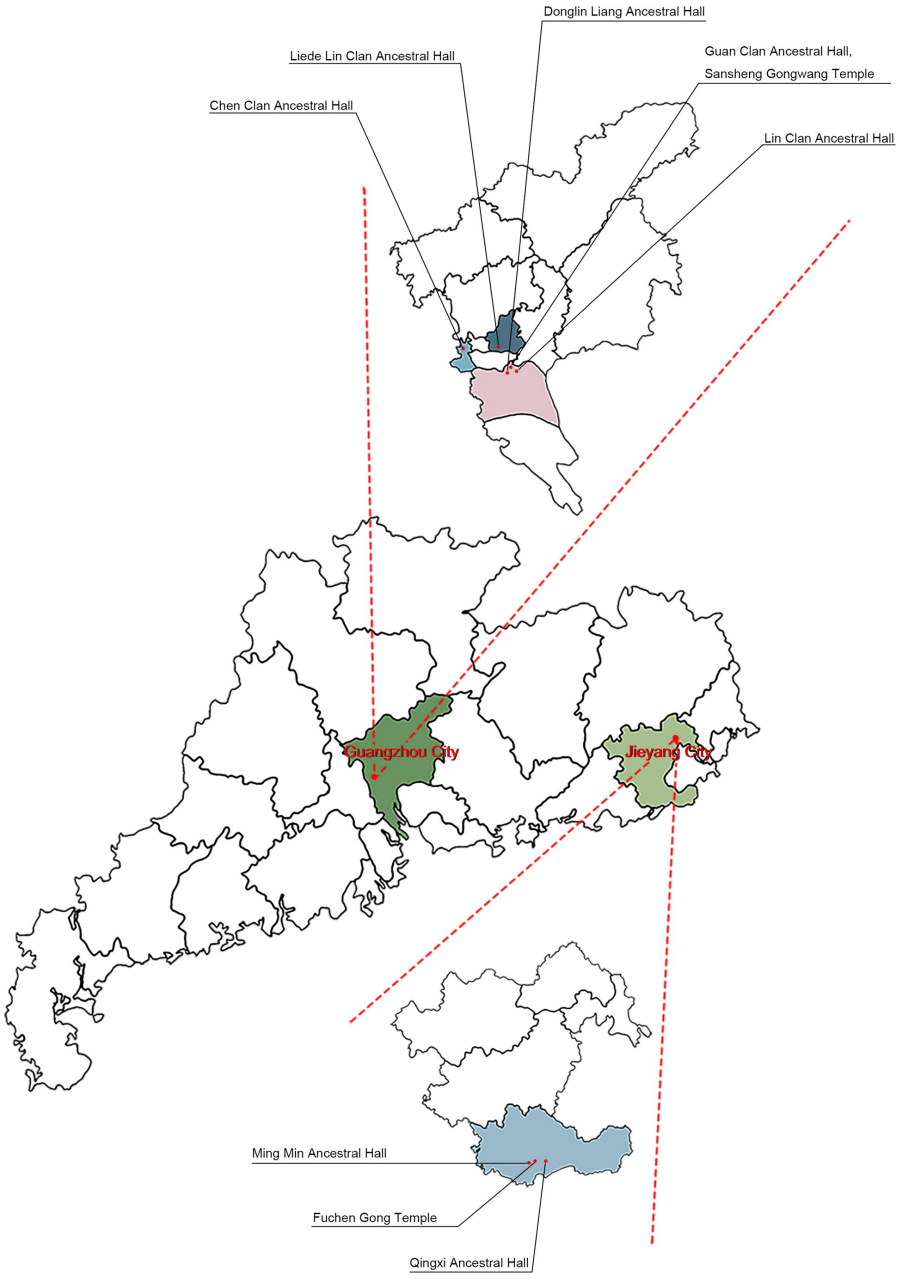
Donglin Liang Ancestral Hall
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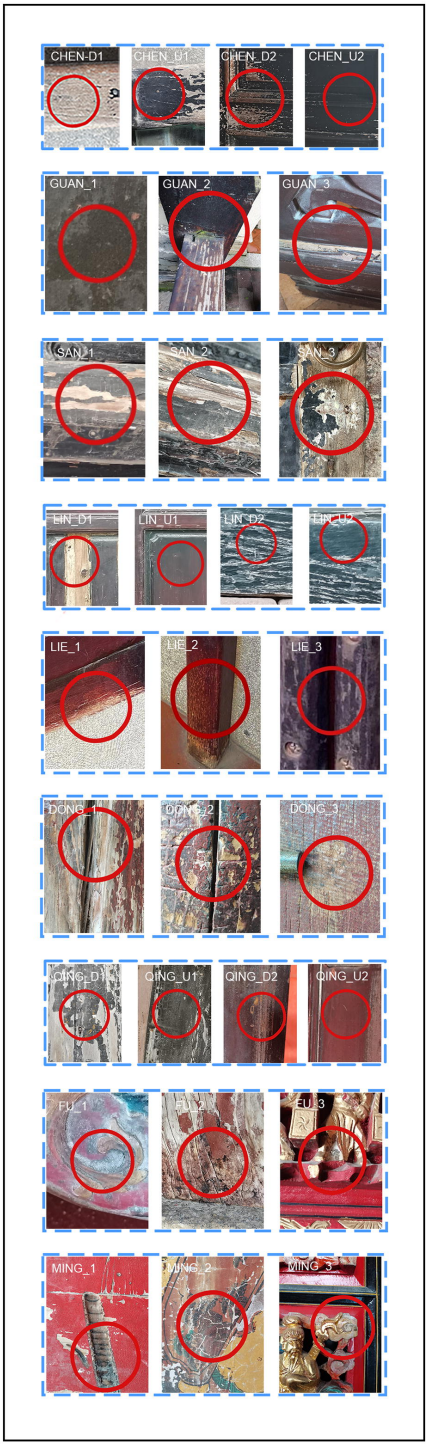
CHEN-D1
CHEN_D1
CHEN_D2
CHEN_U2
GUAN_1
GUAN_2
GUAN_3
SAN_1
SAN_2
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LIN_D1
LIN_U1
LIN_D2
LIN_U2
LIE_1
LIE_2
LIE_3
DONG_1
DONG_2
DONG_3
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FU_1
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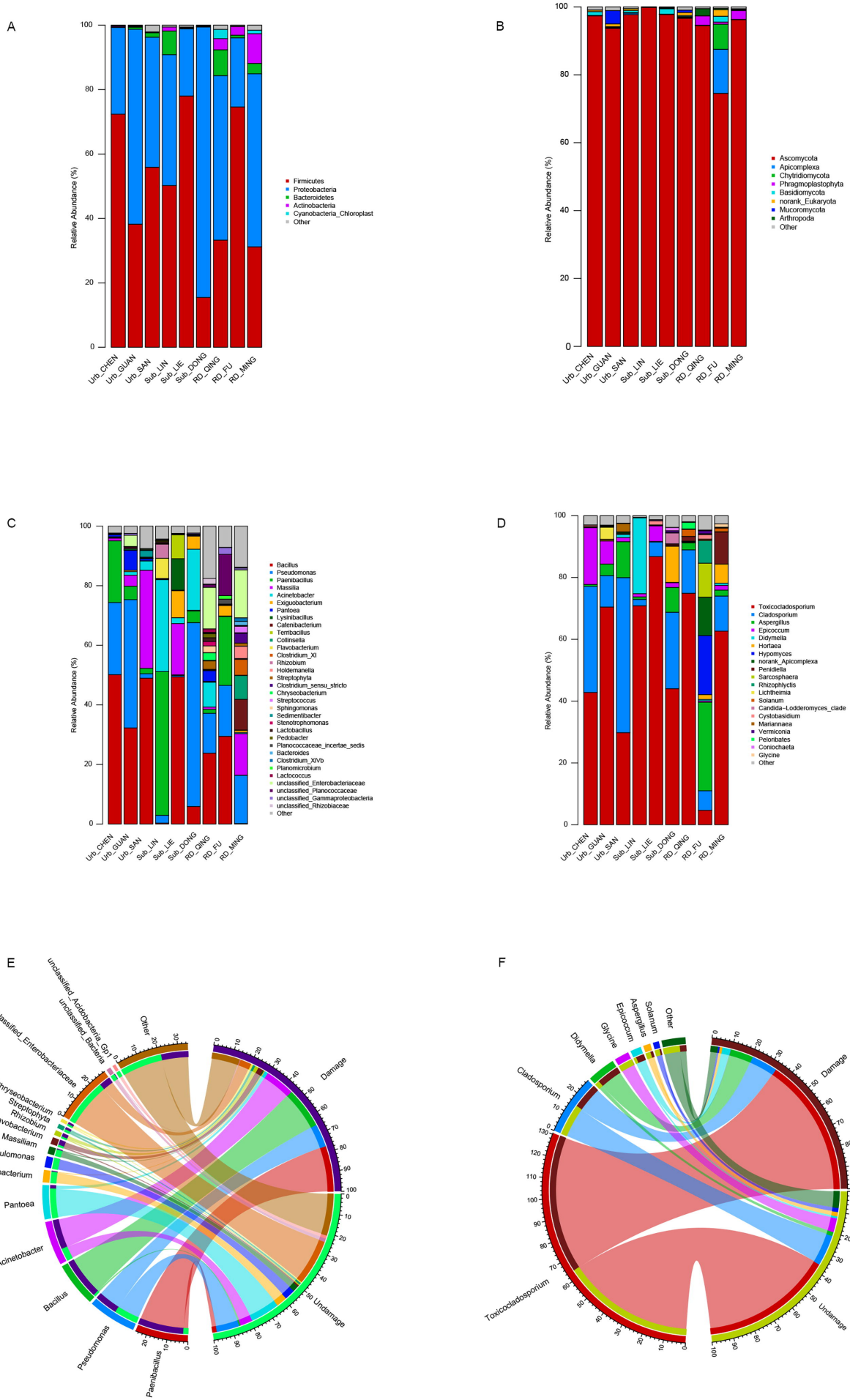
MING_3


A
Relative Abundance (%)
Urb_CHEN
Urb_GUAN
Urb_SAN
Sub_LIN
Sub_LIE
Sub_DONG
RD_QING
RD_FU
RD_MING
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Cyanobacteria_Chloroplast
Other
B
Ascomycota
Apicomplexa
Chytridiomycota
Phragmoplastophyta
Basidiomycota
norank_Eukaryota
Mucoromycota
Arthropoda
C
Bacillus
Pseudomonas
Paenibacillus
Massilia
Acinetobacter
Exiguobacterium
Pantoea
Lysinibacillus
Catenibacterium
Terribacillus
Collinsella
Flavobacterium
Clostridium_XI
Rhizobium
Holdemanella
Streptophyta
Clostridium_sensu_stricto
Chryseobacterium
Streptococcus
Sphingomonas
Sedimentibacter
Stenotrophomonas
Lactobacillus
Pedobacter
Planococcaceae_incertae_sedis
Bacterodes
Clostridium_XIVb
Planomicrobium
Lactococcus
unclassified_Enterobacteriaceae
unclassified_Planococcaceae
unclassified_Gammaproteobacteria
unclassified_Rhizobiaceae
D
Toxicocladosporium
Cladosporium
Aspergillus
Epicoccum
Didymella
Hortaea
Hypomyces
norank_Apicomplexa
Penidiella
Sarcosphaera
Rhizophlyctis
Lichtheimia
Solanum
Candida−Lodderomyces_clade
Cystobasidium
Mariannaea
Vermiconia
Peloribates
Coniochaeta
Glycine
E
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unclassified_Bacteria
Other
unclassified_Enterobacteriaceae
Chryseobacterium
Streptophyta
Rhizobium
Flavobacterium
Massiliam
Cellulomonas
Curtobacterium
Pantoea
Acinetobacter
Bacillus
Pseudomonas
Paenibacillus
Damage
Undamage
F
Other
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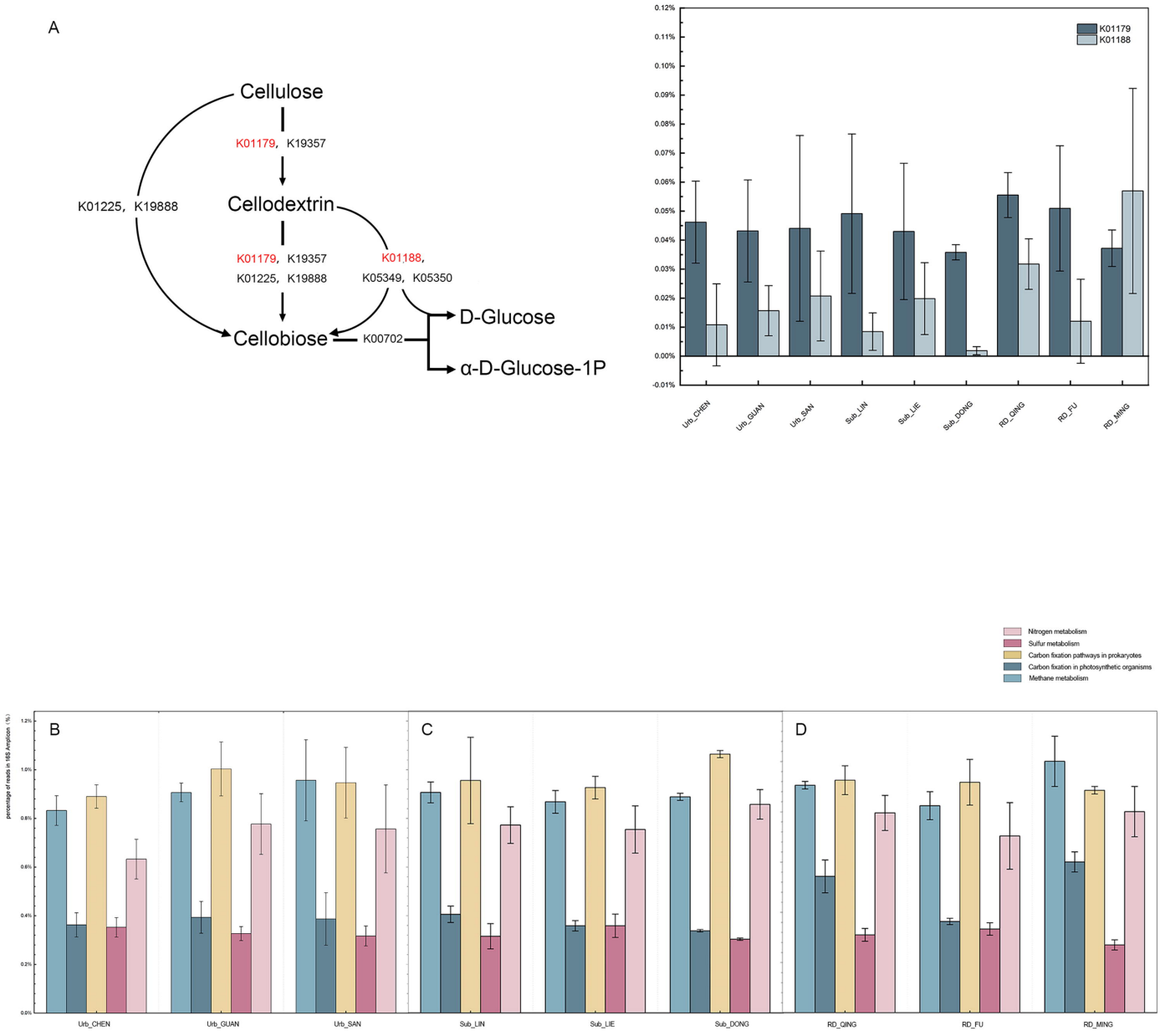
Aspergillus
Epicoccum
Glycine
Didymella
Cladosporium
Toxicocladosporium
Damage
Undamage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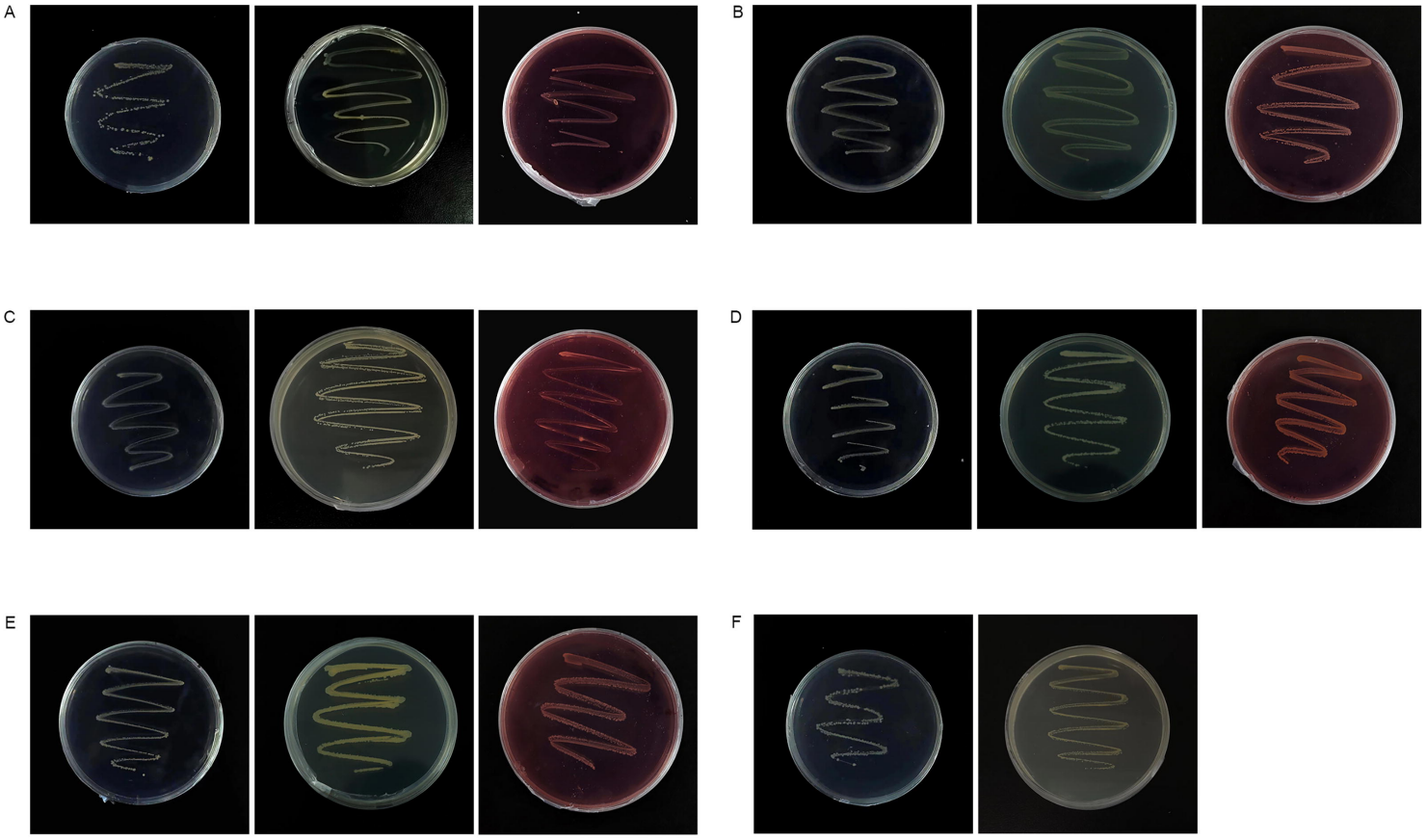
Cellodextrin
K01225, K19888
K01179, K19357
K01225, K19888
K01188,
K05349, K05350
Cellobiose
K00702
D-Glucose
α-D-Glucose-1P
K01179
K01188
Urb_CHEN
Urb_GUAN
Urb_SAN
Sub_LIN
Sub_LIE
Sub_DONG
RD_QING
RD_FU
RD_MING
Nitrogen metabolism
Sulfur metabolism
Carbon fixation pathways in prokaryotes
Carbon fixation in photosynthetic organisms
Methane metabolism
B
C
D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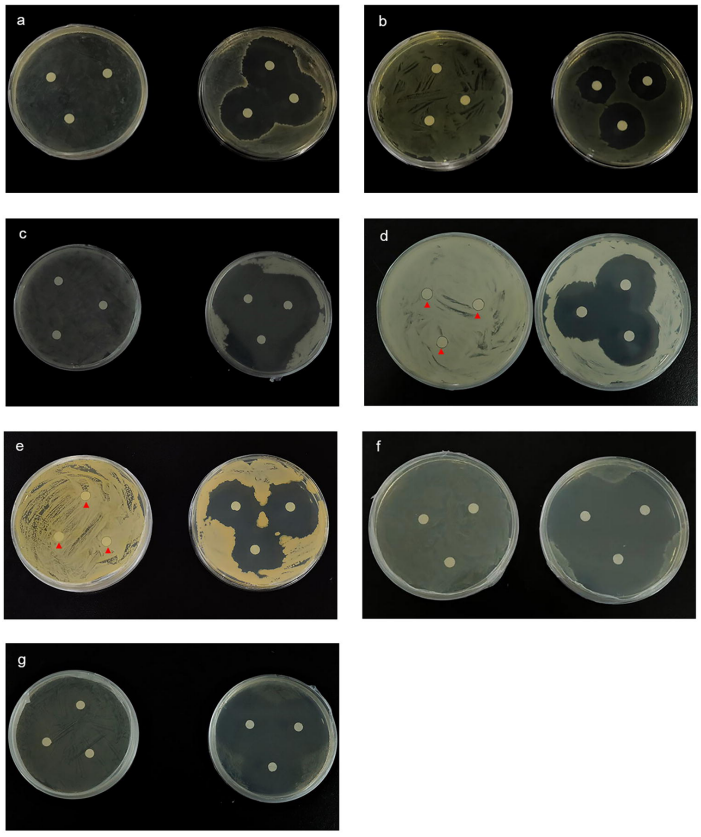
A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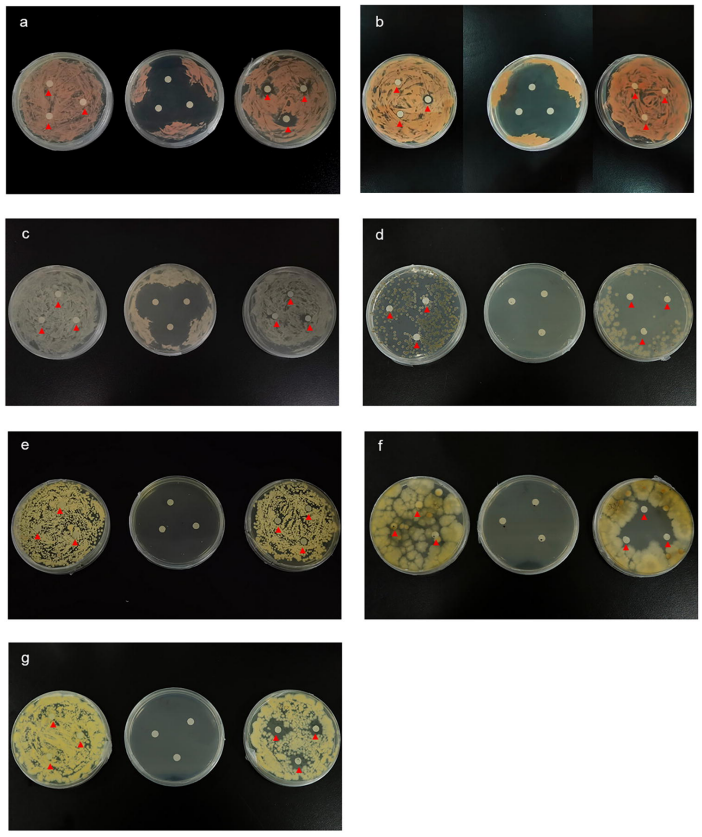
B


a
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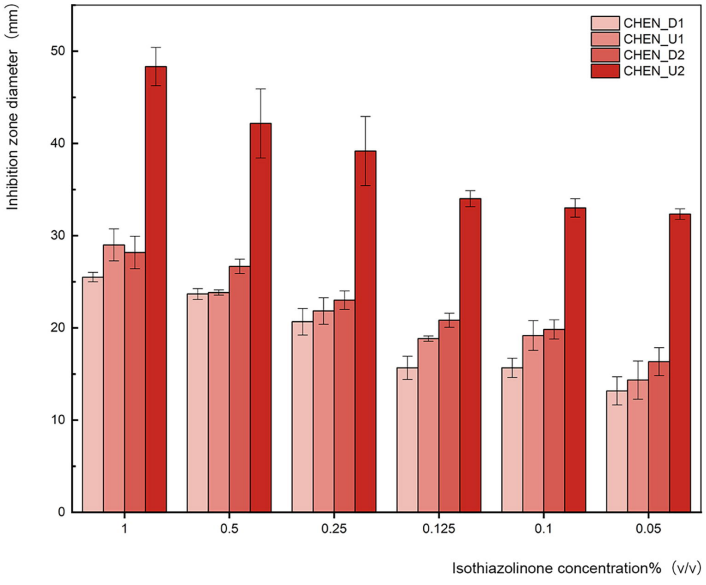
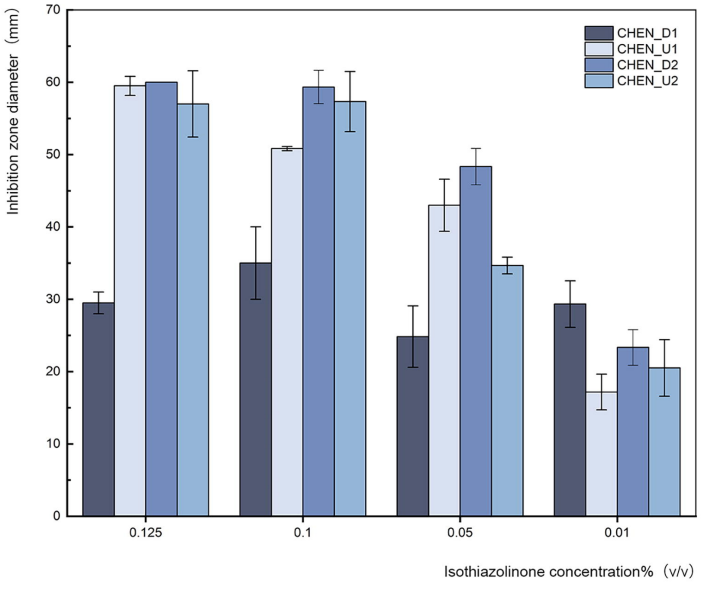
C


Inhibition zone diameter（mm）
0
10
20
30
40
50
CHEN_D1
CHEN_U1
CHEN_D2
CHEN_U2
1
0.5
0.25
0.125
0.1
0.05
Isothiazolinone concentration%（v/v）

D


Inhibition zone diameter（mm）
0
10
20
30
40
50
60
70
CHEN_D1
CHEN_U1
CHEN_D2
CHEN_U2
0.125
0.1
0.05
0.01
Isothiazolinone concentration%（v/v）