

Microbes and cultural heritage: from biodiversity to applications

Edited by

Virginia Helena Albarracín, Erasmo Gámez-Espinosa, Fadwa Jroundi, Sandra Gabriela Gomez De Saravia and Mai Bingjie

Published in

Frontiers in Microbiology



FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-8325-7834-6
DOI 10.3389/978-2-8325-7834-6

Generative AI statement

Any alternative text (Alt text) provided alongside figures in the articles in this ebook has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Microbes and cultural heritage: from biodiversity to applications

Topic editors

Virginia Helena Albarracín — CONICET Center for Electron Microscopy (CIME), Argentina

Erasmó Gámez-Espinosa — Center for Research and Development in Paint and Coating Technology. CONICET, Argentina

Fadwa Jroundi — University of Granada, Spain

Sandra Gabriela Gomez De Saravia — National University of La Plata, Argentina

Mai Bingjie — Shanghai University, China

Citation

Albarracín, V. H., Gámez-Espinosa, E., Jroundi, F., Gomez De Saravia, S. G., Bingjie, M., eds. (2026). *Microbes and cultural heritage: from biodiversity to applications*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-7834-6

Table of contents

- 05 **Editorial: Microbes and cultural heritage: from biodiversity to applications**
Virginia Helena Albarracín, Erasmo Gámez-Espinosa, Mai Bingjie, Sandra Gabriela Gomez de Saravia and Fadwa Jroundi
- 08 **Microbes on the “peachy spots” of ancient Kaihua paper: microbial community and functional analysis**
YanJun Zhou, Yan Shi, Yanyan Huang and Jiang Zhong
- 19 **Microbial diversity in earthen site of exhibition Hall of pit no. 1 at the terracotta warriors Museum in Emperor Qinshihuang’s mausoleum site museum and its correlation with environmental factors**
Cen Wang, Lilong Hou, Nan Jiang, Yu Wang, Xiaofen Mao, Ping Zhou, Yin Xia, Yuanyuan Wang, Chuyue Chen, Xinyu Yang, Qiang Luo and Jiao Pan
- 34 **Insight into the microbial diversity and community in the sacrificial pits of Sanxingdui site (Sichuan, China)**
Ruru Chen, Zhenbin Xie, Qing Xiao, Chong Wang and Rui Wen
- 52 **Reducing microbial contamination on historical leather artifacts at the Auschwitz-Birkenau State Museum using ethanol in the form of mist**
Krzysztof Kraśnicki, Natalia Pydyn, Aleksandra Papis, Krystyna Guzińska, Dorota Kaźmierczak, Urszula Maciołek and Anna Wawrzyk
- 69 **Microbial community shifts during salt mitigation treatments of historic buildings using mineral poultices: a long-term monitoring of salt and associated biofilms**
Johannes Tichy, Beate Sipek, Martin Ortbauer, Lukas Fürnwein, Monika Waldherr, Alexandra Graf, Katja Sterflinger and Guadalupe Piñar
- 92 **Identifying keystone taxa and metabolisms of epilithic biofilms is crucial to the conservation of stone heritage from biodeterioration**
Chenchen Ma, Xiaoying Zhang, Fasi Wu and Xiaobo Liu
- 105 **Analysis and prevention of microbial degradation of shadow puppetry artifacts preserved in the National Shadow Puppetry Museum in Chengdu**
Yu Wang, Yuanyuan Wang, Zhiqian Guan, Zeao Wang, Yangbo Duan, Chen Min, Yuhang Zhong, Lilong Hou and Jiao Pan
- 116 **Echoes of 1816: microbial footprints in heritage artifacts from Argentina’s museum of independence**
Daniel Gonzalo Alonso-Reyes, Fátima Silvina Galván, Natalia Noelia Alvarado, María Cecilia D’Arpino, Luciano José Martinez, Hernán José Esquivel, Cecilia Aymara Gallardo, María Julia Silva Manco and Virginia Helena Albarracín

- 133 Multifunctional protective strategies for wooden cultural heritage: antimicrobial efficacy of polyacrylic resins, siloxane coupling agents, and silver nanoparticles**
Andreea Ștefania Dumbravă, Viorica Maria Corbu, Ioana Cristina Marinaș, Radu Pericleanu, Liliana Marinescu, Ludmila Motelica, Doina Roxana Trusca, Nicoleta Ianovici, Tatiana Eugenia Șesan, Irina Gheorghe-Barbu, Denisa Ficăi, Ovidiu Cristian Oprea, Anton Ficăi and Mariana Carmen Chifiriuc
- 157 Difference and environmental drivers of bacterial communities on wall paintings of the Maijishan and Mogao Grottoes, China**
Wenxia Ma, Qiqi Chen, Fasi Wu, Dongpeng He, Yulong Duan, Yongqiang Yue, Ji-Dong Gu, Xiaoyan Yang and Huyuan Feng
- 172 Extensive biofilm covering on sgraffito wall art: a call for proactive monitoring**
Irit Nir, Anath Sharaby, Hana Barak, Mariela J. Pavan, Lonia R. Friedlander, Victor Multanen and Ariel Kushmaro
- 183 Correction: Extensive biofilm covering on sgraffito wall art: a call for proactive monitoring**
Irit Nir, Anath Sharaby, Hana Barak, Mariela J. Pavan, Lonia R. Friedlander, Victor Multanen and Ariel Kushmaro



OPEN ACCESS

EDITED AND REVIEWED BY
Gulnaz T. Javan,
Alabama State University, United States

*CORRESPONDENCE
Virginia Helena Albarracín
✉ valbarracin@csnat.unt.edu.ar
Fadwa Jroundi
✉ fadwajroundi@gmail.com;
✉ fadwa@ugr.es

RECEIVED 13 March 2026
ACCEPTED 18 March 2026
PUBLISHED 01 April 2026

CITATION
Albarracín VH, Gámez-Espinosa E,
Bingjie M, Gomez de Saravia SG and
Jroundi F (2026) Editorial: Microbes and
cultural heritage: from biodiversity
to applications.
Front. Microbiol. 17:1829794.
doi: 10.3389/fmicb.2026.1829794

COPYRIGHT
© 2026 Albarracín, Gámez-Espinosa,
Bingjie, Gomez de Saravia and Jroundi.
This is an open-access article distributed
under the terms of the [Creative
Commons Attribution License \(CC BY\)](#).
The use, distribution or reproduction in
other forums is permitted, provided the
original author(s) and the copyright
owner(s) are credited and that the
original publication in this journal is
cited, in accordance with accepted
academic practice. No use, distribution
or reproduction is permitted which does
not comply with these terms.

Editorial: Microbes and cultural heritage: from biodiversity to applications

Virginia Helena Albarracín^{1*}, Erasmo Gámez-Espinosa²,
Mai Bingjie³, Sandra Gabriela Gomez de Saravia² and
Fadwa Jroundi^{4*}

¹Laboratorio de Microbiología Ultraestructural y Molecular, Centro Integral de Microscopía Electrónica (CIME), Facultad de Agronomía, Zootecnia y Veterinaria, Universidad Nacional de Tucumán-CONICET NOA SUR, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Tucumán, Argentina, ²CIDEPINT Research and Development Center in Paint and Coating Technology, Faculty of Engineering, CICPBA-CONICET-UNLP, La Plata, Argentina, ³Key Laboratory of Silicate Cultural Relics Conservation, Ministry of Education, School of Cultural Heritage and Information Management, Shanghai University, Shanghai, China, ⁴Department of Microbiology, University of Granada, Granada, Spain

KEYWORDS

biofilm formation, conservation strategies, cultural heritage, historical sites, innovative microbial management, microbial colonization, microbiome analysis in heritage, preservation of cultural heritage

Editorial on the Research Topic

Microbes and cultural heritage: from biodiversity to applications

Cultural heritage (CH) objects are not inert witnesses of the past; they are dynamic ecological niches shaped by continuous interactions between materials, environmental conditions, and living microorganisms. Manuscripts, wooden artifacts, stone monuments, wall paintings, leather objects, and archaeological remains provide substrates for microbial colonization. These microorganisms can drive aesthetic alteration, material weakening, salt-mediated damage, enzymatic degradation, and pigment formation, threatening historical integrity. At the same time, they provide insights into biodiversity, ecological adaptation, and innovative conservation strategies.

The Research Topic “*Microbes and cultural heritage: from biodiversity to applications*” brings together 11 contributions that collectively advance the field of heritage microbiology. Rather than focusing solely on descriptive surveys of microbial diversity, these studies integrate ecological perspectives with conservation science. By combining modern analytical approaches with material and environmental studies, the contributions demonstrate that understanding microbial biodiversity is essential not only for diagnosing biodeterioration processes but also for designing sustainable and informed preservation strategies. Taken together, the studies illustrate the ongoing transition of heritage microbiology from a descriptive discipline toward a more predictive and application-oriented field.

Cultural heritage as a substrate-specific microbiome

A central theme emerging from this Research Topic is that each heritage material hosts a distinct, substrate-driven microbiome. Material composition,

physicochemical properties, microclimate, and human contact shape microbial community assembly. As a result, cultural heritage objects function as highly specialized microbial ecosystems shaped by both environmental and anthropogenic factors.

Zhou et al. investigated the characteristic “peachy spots” on ancient Kaihua paper. Their results revealed that bacteria, rather than fungi, were strongly associated with stain formation. Acid-producing and pigment-forming genera such as *Methylobacterium* and *Rubrobacter* were enriched in affected areas, suggesting that metabolic activity linked to acidification and pigment synthesis may drive discoloration processes in historical paper. This work highlights the importance of linking microbial identity with functional potential in order to clarify deterioration mechanisms. Similarly, Alonso-Reyes et al. conducted the first comprehensive survey on microbial communities associated with historical artifacts from Argentina’s Museum of Independence. The study revealed structured microbial biofilms dominated by Gram-positive taxa, with particularly high diversity detected on a nineteenth-century albumen photograph. The protein-rich composition of photographic material likely created favorable conditions for microbial growth. In addition, the presence of human-associated microorganisms on frequently handled objects underscored the influence of visitor interaction on museum microbiomes. Archaeological contexts further expand this perspective. Chen et al., analyzed microbial communities associated with soils from the Sanxingdui sacrificial pits. Distinct bacterial and fungal taxa were linked to processes such as organic matter degradation and potential bronze biocorrosion. Differences between surrounding soils and artifact-associated samples indicated that burial activities and material composition shape localized microbial assemblages. Together, these studies demonstrate that cultural heritage materials support highly specialized microbial communities whose composition reflects both environmental context and substrate chemistry.

Keystone taxa and functional mechanisms of biodeterioration

Beyond identifying microbial diversity, several contributions address the functional mechanisms that drive biodeterioration. Understanding these mechanisms is essential for developing effective conservation strategies. Ma, Zhang et al. investigated epilithic biofilms colonizing the Leizhou Stone Dog monuments. Their analysis identified phototrophic bacteria and nitrifying microorganisms as keystone taxa of these biofilms. The study suggested that metabolic activity related to nitrogen cycling and phototrophic growth plays an important role in the deterioration of stone surfaces. These findings emphasize that the intensity of biodeterioration depends not only on microbial diversity but also on the metabolic functions performed by dominant taxa.

Wall paintings represent another vulnerable substrate. In a comparative study of the Majijishan and Mogao Grottoes, Ma, Chen et al. reported that bacterial communities differed markedly between sites. These differences were primarily driven by variations in substrate composition and microclimatic conditions. Humid environments favored proliferation of Actinobacteria, whereas arid

conditions selected xerotolerant microorganisms. The absence of a shared core microbial community between the two sites highlights the strong influence of environmental filtering in shaping wall-painting microbiomes. Complementing this work, Nir et al. documented extensive microbial colonization on sgraffito wall art. Their observations revealed complex biofilm composed of bacteria, cyanobacteria, and fungi interacting with plaster and cement substrates. The heterogeneous distribution of microbial growth across the surface further illustrated how local environmental conditions influence colonization patterns. Together, these studies highlight the need for long-term monitoring and site-specific evaluation when assessing biodeterioration risks. Organic materials were also examined from a functional perspective. Wang et al. studied traditional shadow puppetry artifacts composed primarily of collagen-rich hide. The authors identified microorganisms capable of degrading collagen, a process that can lead to severe structural damage in leather-based heritage objects. Importantly, the study also explored antimicrobial compounds capable of inhibiting dominant degraders, illustrating how functional microbial analysis can inform targeted conservation strategies.

Environmental drivers and treatment-induced microbial shifts

Environmental conditions and conservation treatments themselves can influence microbial dynamics on heritage surfaces. Several contributions in this Research Topic highlight the importance of considering these ecological responses. Tichy et al. monitored microbial changes associated with salt-removal treatments applied to historic buildings. Their results showed that shifts in salt composition altered the structure of microbial biofilms, with bacterial populations responding particularly strongly to these changes. The mineral composition also influenced both salt retention and microbial diversity. These findings demonstrate that conservation treatments may modify microbial ecosystems and therefore require careful ecological evaluation. In the earthen exhibition hall of the Terracotta Warriors Museum, Wang, Hou et al. examined how environmental factors shape fungal communities. Several genera, including *Penicillium*, *Aspergillus*, and *Talaromyces* were associated with specific microclimatic conditions. The authors also tested volatile allicin as a potential antifungal treatment, showing inhibitory effects on several strains. Such findings illustrate how environmentally compatible biocontrol strategies may help mitigate microbial growth on sensitive heritage materials.

From diagnosis to innovation: microbial-informed conservation

Several studies in this Research Topic emphasize translational applications and the development of sustainable conservation strategies informed by microbiological knowledge. Kraśnicki et al. evaluated the effectiveness of ethanol mist treatment for

disinfecting historical leather artifacts from the Auschwitz-Birkenau State Museum. The treatment significantly reduced microbial contamination while preserving the structural integrity of the leather. This study demonstrates that carefully designed disinfection strategies can successfully balance microbial control with material conservation. Dumbrovă et al. explored protective coating for wooden objects incorporating silver nanoparticles within polymeric materials. These coatings showed antimicrobial and anti-biofilm properties while maintaining compatibility with the treated surfaces. Such innovations highlight the growing convergence between microbiology, materials science, and conservation technology.

Together, these applied studies signal an important shift in conservation practice. Rather than relying on broad sterilization approaches, modern strategies increasingly aim to manage microbial communities in ways that are compatible with both the material substrate and the surrounding environment.

Toward a visionary and sustainable heritage microbiology

The contributions gathered in this Research Topic demonstrate that heritage microbiology has become an integrative discipline linking biodiversity research with conservation practice. Cultural heritage materials can be understood as dynamic microbial ecosystems shaped by environmental conditions, material properties, and human activity.

Climate change adds further urgency to this field of research. Intensified rainfall–drought cycles, salt crystallization events, and fluctuating humidity increasingly reshape microbial ecosystems on cultural heritage surfaces. Microbial communities respond rapidly to these stresses, often shifting toward halotolerant, stress-adapted, or pigment-producing taxa that can accelerate deterioration processes.

At the same time, microorganisms associated with heritage materials are not merely agents of decay. They can also provide valuable information about historical production techniques, storage conditions, and environmental exposures experienced by objects over time. In this sense, microbial communities may serve as biological archives that complement traditional historical and archaeological evidence.

Ultimately, the contributions presented in this Research Topic illustrate the evolution of heritage microbiology from a discipline focused primarily on documenting biodeterioration toward one capable of predicting, monitoring, and managing

microbial processes in cultural heritage environments. Through continued interdisciplinary collaboration among microbiologists, conservators, material scientists, and archaeologists, this emerging field will play an increasingly important role in safeguarding humanity's material legacy.

Author contributions

VA: Writing – original draft, Writing – review & editing. EG-E: Writing – review & editing. MB: Writing – review & editing. SG: Writing – review & editing. FJ: Writing – original draft, Writing – review & editing.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors VA and FJ declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.



OPEN ACCESS

EDITED BY

Yingnan Bian,
Enlight Medical, China

REVIEWED BY

Teresa Rinaldi,
Sapienza University of Rome, Italy
Hana Stiborova,
University of Chemistry and Technology in
Prague, Czechia

*CORRESPONDENCE

Jiang Zhong
✉ jzhong@fudan.edu.cn

RECEIVED 27 October 2023

ACCEPTED 26 December 2023

PUBLISHED 11 January 2024

CITATION

Zhou Y, Shi Y, Huang Y and Zhong J (2024)
Microbes on the “peachy spots” of ancient
Kaihua paper: microbial community and
functional analysis.
Front. Microbiol. 14:1326835.
doi: 10.3389/fmicb.2023.1326835

COPYRIGHT

© 2024 Zhou, Shi, Huang and Zhong. This is
an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Microbes on the “peachy spots” of ancient Kaihua paper: microbial community and functional analysis

YanJun Zhou¹, Yan Shi¹, Yanyan Huang² and Jiang Zhong^{1,2*}

¹Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai, China, ²Institute for Preservation and Conservation of Chinese Ancient Books, Fudan University, Shanghai, China

Kaihua paper is a type of precious hand-made paper in China that has been used throughout Chinese history. Due to its extraordinary whiteness and fine texture, it was adopted by the imperial palace in the reign of Emperor Kang Xi and Yong Zheng of the Qing Dynasty of China in the 17th and 18th century. It is stained by a special type of yellowish-brown spot after years of storage, which is called a “peachy spot.” The formation of such spots remains unclear, although complicated physicochemical processes or microbial activities might be involved. We performed nondestructive sampling and high-throughput sequencing on peachy spot surfaces, unstained areas, and air samples in the stack room to analysis the the bacterial and fungal communities, and performed prediction of functional genes of the bacterial communities. The results showed that peachy spot formation was mainly related to bacterial communities rather than fungal communities. Significantly more potential acid- producing, acidophilic or cellulase-producing bacteria, such as, *Streptococcus*, *Staphylococcus*, and *Lysinibacillus*, and pigment-producing bacteria, such as *Methylobacterium* and *Rubrobacter*, were identified in the peachy spot samples. Prediction of the functional genes of the bacterial community also suggested the production of acidic substance pigments. These findings provide new insights into the pigment formation mechanism in ancient paper and open an opportunity to develop new strategies to preserve the ancient paper documents.

KEYWORDS

microbial community, traditional Chinese handmade paper, biodeterioration, Kaihua paper, functional prediction

1 Introduction

Paper, as a carrier of human thought, art and history, is critical in human society. Paper relics, like all other cultural heritage materials, are nonrenewable and will degenerate over time. Thus, preserving paper relics is a cultural and technological challenge.

Microbial deterioration of paper materials has always been a focus in the preservation of paper relics (Cappitelli et al., 2010). Early studies on the biodegradation of cultural heritage have been carried out for approximately a century (Sterflinger et al., 2018), using mostly culture-dependent methods and microscopic observations (Mitchell and McNamara, 2010). Fungi, especially filamentous fungi, are considered the most important organisms in microbial hazards. Due to technical limitations, only culturable microbes, which represent only a small

part of the total microbes, were studied (Amann et al., 1995). These culturable strains were probably not the dominant microbes that caused paper damage. In recent years, new methods have been applied to study the microbial community on various historical relics, including paper, parchment, storeroom objects, and photographic materials (Sclocchi et al., 2016; Migliore et al., 2017; Liu et al., 2018; Piñar et al., 2020; Branysova et al., 2022). Genomic analysis based on high-throughput sequencing is becoming a powerful approach to study the diversity and impact of microorganisms on historical objects (Kraková et al., 2018; Sterflinger et al., 2018; Beata, 2020).

Traditional Chinese handmade paper was made of plant fiber, following slightly different processes depending on the geographical area and historical period of production. The sources of plant fiber include bark, straw, and bamboo. Moreover, paper is often coated with gelatin or other substances to make it impermeable to ink (Area and Cheradame, 2011). These diverse organic and inorganic components in paper provide substrates for microbial growth and reproduction under favorable conditions, which in turn destroys the structure of the paper (Sterflinger, 2010). Therefore, different types of paper may favor different microorganisms that settle on its surface, resulting in different structures of microbial communities and ultimately different kinds of damage.

Kaihua paper is a unique type of ancient paper preserved to this day that is famous for its delicate and white and thin characteristics. It was one of the most valuable royal papers in the Qing Dynasty, especially during the reign of the three emperors, Kangxi, Yongzheng and Qianlong (1662–1795 A.D.). The plant fiber used to make Kaihua paper was from the family *Thymelaeaceae*. Ancient Kaihua paper commonly developed isolated or coalescent yellowish-brown spots, which are called “peachy spots.” Such spots affect the paper greatly and may lead to irreversible loss of historical content. Morphologically, peachy spots resemble the well-studied “foxing spots,” a common damage to paper cultural relics (Meynell and Newsam, 1978). Among the many hypotheses about foxing formation, metals and microorganisms are considered the two major causes (Choi, 2007). However, the role of microorganisms in the formation of “peachy spots” on Kaihua paper remains unknown.

In this study, culture-independent high-throughput sequencing was used to identify bacterial and fungal communities on Kaihua paper affected by peachy spots. The potential roles of bacterial and fungal groups in the formation of peachy spots were explored by correlation analysis and prediction of functional genes of the microbial community.

2 Materials and methods

2.1 Tested paper samples

Three sets of ancient books made of Kaihua paper preserved in Fudan University Library were selected for this study (Figure 1). These books came from the reign of Emperor Kang Xi and Yong Zheng and Qian Long of the Qing Dynasty of China in the 18th century and have been kept in the same stack room for rare ancient books for a long time without being touched. The temperature of the stack room is kept at 16–20°C and the humidity at 50–60%. Although the books are in relatively good condition, there are obvious peachy spots on each set of books.

2.2 Sampling method

The sampling areas most suitable for the study were chosen and evaluated carefully, and the sampling protocol was designed to minimize impact to the paper. A sterilized polyester fiber swab (Swvip™, Cleanmo, Shenzhen, China) was selected as the sampling tool (swab head size: 4.0 mm x 11.0 mm, total length: 70.0 mm). The swab head was fully moistened with 20 µL of sterilized wetting solution (0.15 M NaCl and 0.1% Tween-80, dissolved in ultrapure water) and used to gently wipe the preselected sampling position 20 times to collect the microorganisms on the paper surface. Then, the swab head was peeled off using sterile tweezers and immediately transferred into the PowerBead extraction tube in the MOBIO PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA). For each set of books, three samples of peachy spots and one sample of unstained area of the inner page were collected.

To exclude interference from airborne microorganisms of the stack room at the time of sampling, the indoor air sedimentation sample were independently collected as the control. Three sterilized polyester fiber swabs were moistened as other sample swabs and allowed to stand on the sampling bench during the sampling, which lasted about 1 h. At the end of the sampling, the heads of three swabs were peeled off and transferred into a single extraction tube for DNA extraction and amplification process, following the same procedure as the other samples to keep consistency.

Samples were stored in a –20°C freezer, and DNA extraction was performed within 12 h.

2.3 DNA extraction and sequencing

For DNA extraction, each sample was processed using the MOBIO PowerSoil DNA Isolation Kit following the manufacturer's instructions with minor modifications. The C6 solution provided by the kit was replaced with sterile TE buffer to minimize DNA degradation.

Sequences of the 16S rDNA and ITS rDNA were amplified for bacteria and fungi, respectively, using primer sets of 515F (GTGCCAGCMGCCGCGGTAA)/806R (GGACTACHVGGGTWCTAAT) for the V4 region of 16S rDNA (Kuczyński et al., 2011) and ITS5-1737F (GGAAGTAAAAGTCGTAACAAGG)/ITS2-2043R (GCTGCGTTCTTCATCGATGC) for region 1 of ITS rDNA (Bellemain et al., 2010). The amplification was conducted according to the following program: initial denaturation at 94°C for 5 min, 20 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and extension for 90 s at 72°C, final extension at 72°C for 10 min. Then the same amplification was performed once again to add the barcode sequences to the primers for sample distinction after sequencing.

The concentration, total amount and purity of the amplification products were determined, and then the qualified amplification products were sequenced on the Illumina MiSeq (300-bp paired-end reads) platform (Illumina Inc., San Diego, United States) by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

2.4 Data processing

The QIIME platform (Caporaso et al., 2010) was used to filter the acquired sequences to remove low quality (Q value less than 20), short

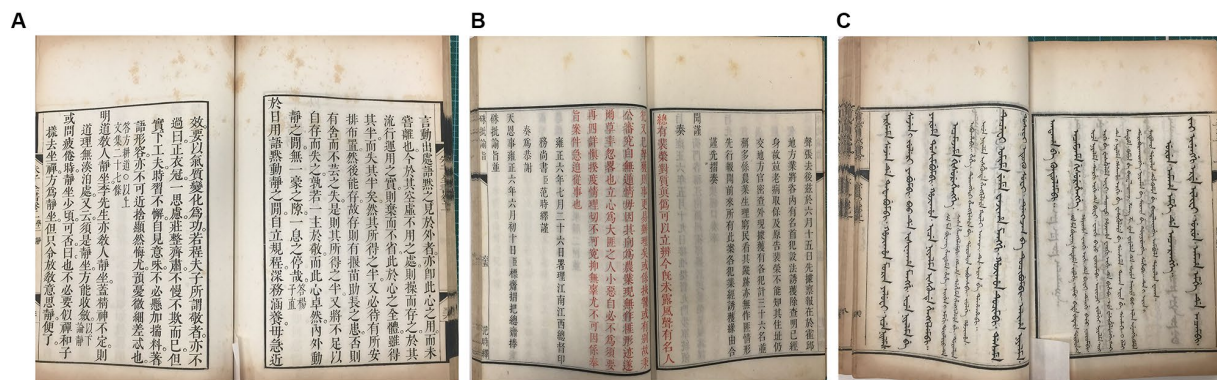


FIGURE 1

Representative images of the “peachy spots” of Kaihua paper from three sets of ancient books. (A) *Zhu Zi Quan Shu* dated approximately 1713–1717 A.D. (B) *Zhu Pi Yu Zhi* dated approximately 1713–1717 A.D. (C) *Yu Zhuan Xing Li Jing Yi* in Manchu dated approximately 1732–1738 A.D.

length (shorter than 250 bp) and chimeric sequences. The sequences were split according to their taxonomy and assigned to operational taxonomic units (OTUs) at the 97% similarity level using MOTHUR¹ (Schloss et al., 2009). The representative sequences of these OTUs were classified within the SILVA database release 119 for bacteria and UNITE for fungi (Abarenkov et al., 2010).

2.5 Microbial community analyses and functional gene prediction

The QIIME platform was used to analyze the bacterial and fungal community structure and composition on the normalized OTU dataset. Rarefaction curves were created to assess sampling efficiency. Three alpha diversity indexes, Chao 1, PD value and Shannon index, were calculated to reflect the overall diversity and complexity of the microbial community.

Multivariate analyses on the OTU dataset were performed using the UniFrac unweighted metric in QIIME. Principal coordinates analysis (PCoA) was performed to show the differences between the sample sets. Species abundance clustering heatmaps were used to analyze the microbial composition structure.

Tax4Fun was implemented to predict the functional differences between unstained areas and peachy spots. Tax4Fun is an R package that links 16S rRNA gene sequences with the functional annotation of sequenced prokaryotic genomes, which is realized with a nearest-neighbor identification based on a minimum 16S rRNA sequence similarity (Aßhauer et al., 2015). It was used to predict the functional genes from the 16S rDNA information of “peachy spots” groups and samples from unstained area based on the Silva database, and the KEGG functional annotation abundance table was obtained. Then STAMP software (Parks et al., 2014) was used to screen for genes with significant differences in abundance between “peachy spots” samples and unstained samples. Only differences with p values less than 0.05 were reported.

2.6 Statistical analyses

All statistical analyses were conducted in the R environment (v3.4.3; <http://www.r-project.org/>), and all statistical tests were considered significant at p values less than 0.05. Two-way nested ANOSIM (analysis of similarity) was used to test for significant differences in microbial composition (Clarke and Warwick, 1994). Wilcoxon's test was used to compare the relative abundance between the unstained areas and peachy spots.

3 Results

3.1 Sequencing quality, rarefaction curve and alpha diversity

Samples were taken from the surface of three sets of ancient books made of Kaihua paper: *Zhu Zi Quan Shu*, *Zhu Pi Yu Zhi*, and *Yu Zhuan Xing Li Jing Yi* in Manchu. Sequencing of the amplicon libraries of 16S rDNA and ITS rDNA yielded a total of 1,656,407 raw reads belonging to bacteria and fungi. After quality trimming and assigning reads to their respective samples, 789,811 high-quality 16S rDNA sequences and 810,781 high-quality ITS rDNA sequences were obtained for 13 samples, including 9 samples of peachy spots, 3 samples of unstained areas, and 1 stack room air sample (Table 1).

The rarefaction curves confirmed the efficiency of the sampling (Figures 2A, 3A). In the bacterial part, the alpha diversity index of the peachy spots was significantly higher than that of the unstained group ($p < 0.05$), and the alpha diversity indexes of the three groups of peachy spot samples were very close to each other, with no significant differences. The diversity of the bacterial community at the peachy spots was significantly higher than that of the unstained area of the same book (Figures 2B–D). However, no consistent difference was found in the diversity of the fungal community between peachy spots and unstained areas (Figures 3B–D). In addition, there was a significant within-group difference in the Chao1 index and PD value in the KTa group (Figures 3B,D), indicating that the fungal community structure varied greatly between spots in the same book. It seemed that peachy spots on Kaihua paper were more related to bacteria than fungi.

¹ <http://www.mothur.org/>

TABLE 1 Summary of sample information and high-quality sequences.

Group	Sample	Origin	Sampling area	High-quality sequence (16S)	High-quality sequence (ITS)
KTa	KTa1	Zhu Zi Quan Shu	“peachy spots”	84,153	73,858
	KTa2	Zhu Zi Quan Shu	“peachy spots”	62,210	75,413
	KTa3	Zhu Zi Quan Shu	“peachy spots”	51,495	62,134
KTb	KTb1	Zhu Pi Yu Zhi	“peachy spots”	69,066	51,474
	KTb2	Zhu Pi Yu Zhi	“peachy spots”	53,649	55,645
	KTb3	Zhu Pi Yu Zhi	“peachy spots”	54,788	54,764
KTc	KTc1	Yu Zhuan Xing Li Jing Yi in Manchu	“peachy spots”	68,239	48,333
	KTc2	Yu Zhuan Xing Li Jing Yi in Manchu	“peachy spots”	74,378	74,693
	KTc3	Yu Zhuan Xing Li Jing Yi in Manchu	“peachy spots”	25,497	69,569
K	Ka	Zhu Zi Quan Shu	unstained area	67,128	59,748
	Kb	Zhu Pi Yu Zhi	unstained area	84,958	83,747
	Kc	Yu Zhuan Xing Li Jing Yi in Manchu	unstained area	50,844	50,514
Control	Control		indoor air	43,406	50,889

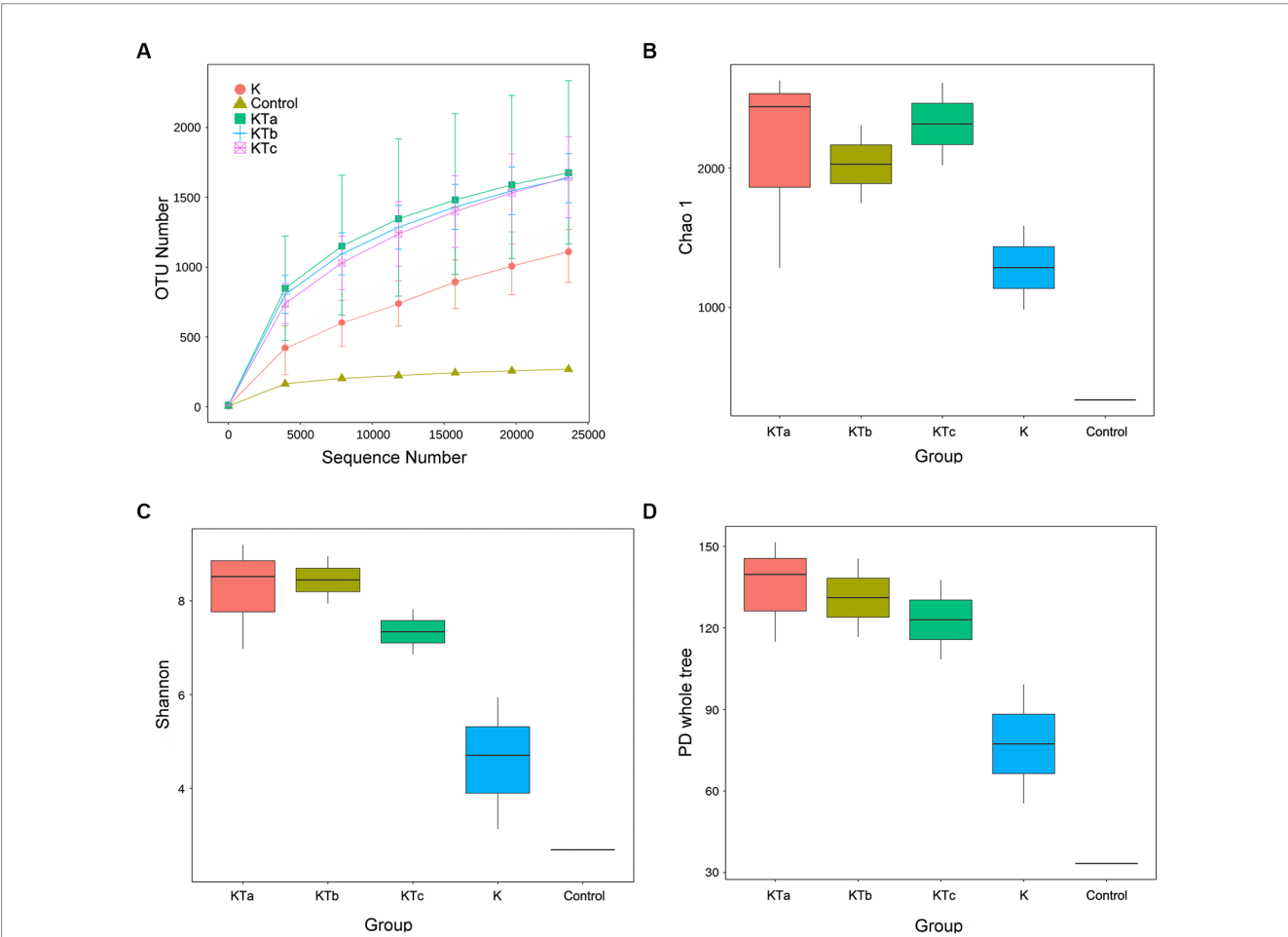


FIGURE 2 Rarefaction curves and alpha diversity index of bacterial communities of 9 “peachy spot” samples, 3 unstained samples and 1 air sample. (A) Rarefaction curves; (B) Chao1 index; (C) Shannon index; (D) PD value. The alpha diversity of the KT group (KTa, KTb and KTc) was significantly higher than that of the K group ($p < 0.05$).

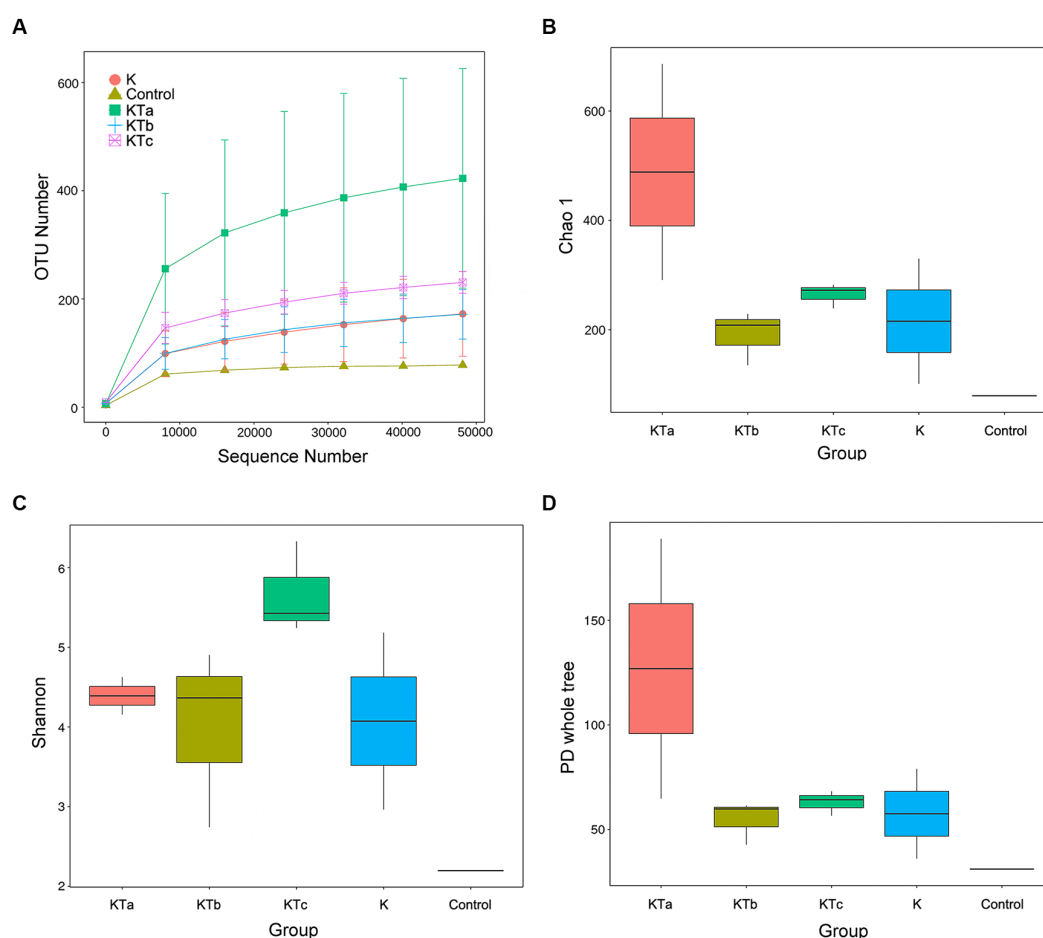


FIGURE 3
Rarefaction curves and alpha diversity index of fungal communities of 9 “peachy spot” samples, 3 unstained samples and 1 air sample. (A) Rarefaction curves; (B) Chao1 index; (C) Shannon index; (D) PD value.

3.2 Microbial community structure on the “peachy spots”

Species annotation was performed on all bacterial and fungal OTUs. Most identified bacteria belonged to the phyla *Bacillota*, *Pseudomonadota*, and *Actinomycetota* (Supplementary Table S1). All identified fungi belonged to 7 phyla. The phylum *Ascomycota* occurred with the highest abundance in all samples, followed by *Basidiomycota* and *Zygomycota* (Supplementary Table S2).

The top 35 most abundant genera of either bacteria or fungi were used for cluster analysis (Figure 4). The heatmap showed that the bacterial communities of high abundance were quite similar within peachy spot groups KTa, KTb, and KTc and were clearly different from the unstained group (K group) and the air deposition sample (control) (Figure 4A). Peachy spot groups had significantly higher proportions of *Bacillota*, such as *Methylobacterium*, *Christensenellaceae*, *Streptococcus*, *Lysinibacillus*, *Staphylococcus* and *Lactobacillus*, than the unstained group and air deposition sample (Figure 4A). In addition, *Rubrobacter* of *Actinobacteria* was also significantly aggregated in peachy spots, whereas the proportion of other genera of *Actinobacteria* and *Pseudomonadota* was relatively low (Figure 4A). Among the unstained paper samples, *Paenibacillus* and *Bacillus* were more

prominent (Figure 4A). In the air deposition sample, there were significantly more *Escherichia-Shigella* (Figure 4A).

Furthermore, the clustering of the species abundance of fungi varied greatly. No significant similarity was found between different peachy spot groups or between peachy spot groups, the unstained paper group, and the air deposition sample (Figure 4B), indicating that fungi might not be the main cause of peachy spots. *Aspergillus*, which was frequently found on ancient books, aggregated in the air sample but not in the paper samples (Figure 4B). In addition, the proportions of *Cercospora* and *Fusarium* were also higher in the air deposition sample than in all paper samples (Figure 4B). In the unstained paper group, there were high abundances of *Cladosporium*, *Neurospora* and *Talaromyces* (Figure 4B). These genera include multiple species that are common plant endophytes.

To reveal the differences in community structure between peachy spot areas and unstained areas, we conducted PLS-DA to compare the composition of both bacteria and fungi in all samples. The results showed that the microbial community (both bacteria and fungi) on the paper surface could be completely separated from that of the indoor air of the stack room (Figure 5). The bacterial community of peachy spot samples could also be separated from the unstained paper samples (Figure 5A),

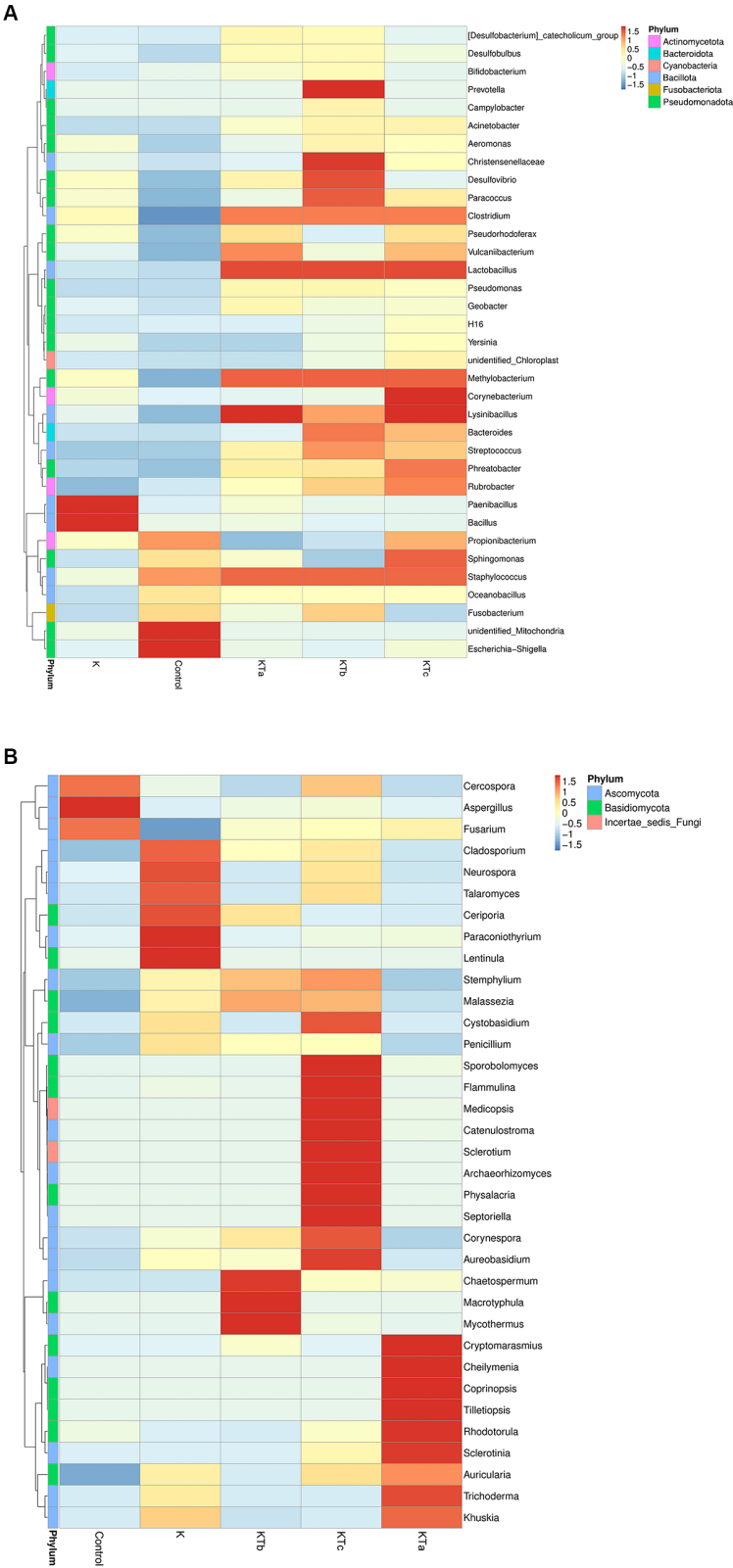


FIGURE 4
Heatmap of grouping clustering in bacterial communities (A) and fungal communities (B). Thirty-five genera with the highest proportion were used for this cluster analysis.

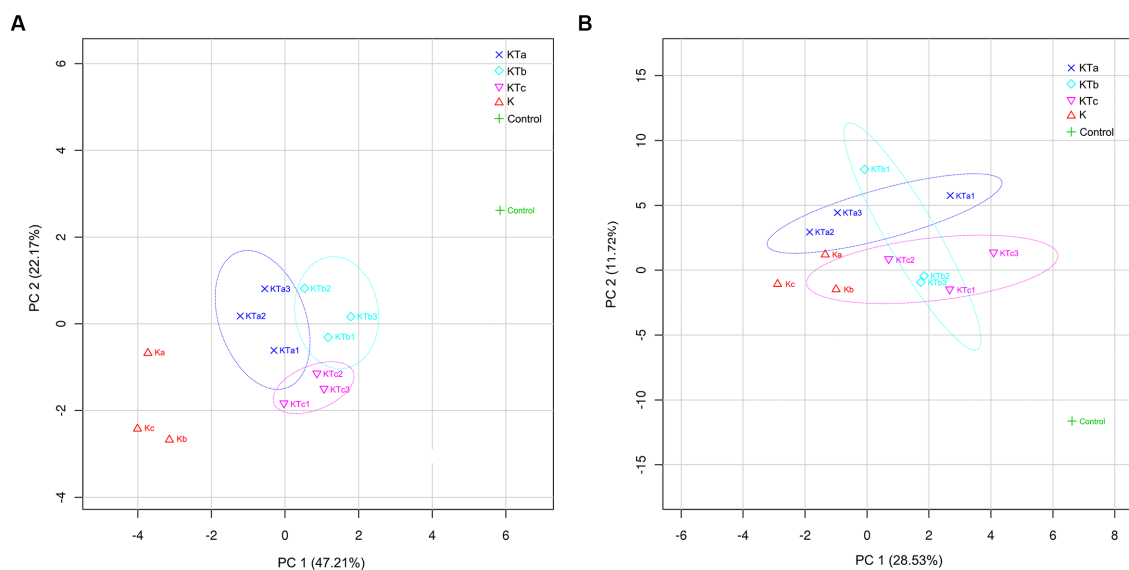


FIGURE 5
PLS-DA (partial least squares discrimination analysis) of bacterial communities (A) and fungal communities (B).

whereas the fungal community of these two sets of samples overlapped (Figure 5B). Moreover, the bacterial community structures of the peachy spot groups were relatively close to each other, and the difference within each group was less than that of fungi (Figure 5). These results also suggested that the formation of peachy spots may be directly related to the bacterial community but not to the fungal community.

3.3 Functional gene prediction

To elucidate the potential contribution of the bacteria to the formation of peachy spots, we defined the peachy spot samples in all books as the peachy spot group (KT) and the samples from unstained area of the inner page in all books as the unstained paper group (K). The R package Tax4Fun was used to predict functional genes in the bacterial community from the 16S rDNA data of the two groups. STAMP was used to screen for genes with significant differences in abundance between groups.

The abundance of some gene ortholog groups, such as mineral absorption, riboflavin metabolism, carotenoid biosynthesis, and lysine biosynthesis, was significantly higher in the peachy spot group than in the unstained paper group (Figure 6). This also suggested that the bacterial community at the peachy spots area might be associated with the accumulation of pigments and the increased production of acidic substances.

4 Discussion

Ancient paper was made of many organic and inorganic compounds that can be used by bacteria and fungi. Traditionally, groups of fungi, including *Penicillium*, *Aspergillus*, *Trichoderma*, *Chaetomium* and *Alternaria*, were considered important for paper damage (Arai, 2000; Nitiu et al., 2020), as they are relatively easy to

observe and culture. However, they may not necessarily be the key microbes that cause paper damage.

Most previous studies have also linked fungi to the cause of paper stains. Microscopic observation of the presence of mycelia has been used as a criterion to determine whether a stain is due to mold or mildew damage. The presence of UV fluorescence is used as a standard to define “foxing spots” (Meynell and Newsam, 1978; Koochakzadeh and Alizadeh Gharetapeh, 2021), but fluorescence is also considered by other researchers as a factor in the formation of “foxing” spots (Bicchieri et al., 2001; Figueira et al., 2020). The peachy spots on the Kaihua paper resemble foxing spots by description, although in our previous tests, no UV fluorescence was observed at the spots (data not shown).

In this study, we used high-throughput sequencing technology to determine the bacterial and fungal communities on the surface of peachy spots and compared them with unstained surfaces as well as air samples from a stack room. The results showed that the abundance of *Penicillium* was not significantly higher in the position of peachy spots than that without it. The abundance of *Aspergillus* in the air deposition sample of the stack room was even higher than that of peachy spot samples. This implied that these typical filamentous fungi might have come from the stack room environment where the ancient books were stored but had not stably established on the surface of Kaihua paper and thus were not related to the formation of peachy spots. *Cercospora* and *Fusarium* were also found to be relatively abundant in the air deposition sample, both of which have multiple species that are pathogenic to plants (Daub and Ehrenshaft, 2000; Rampersad, 2020; Sinha et al., 2023). These fungi may come from other contaminated ancient books or old wooden bookshelves and furniture and pose a potential threat to paper (Şimşekli et al., 1999; Gravesen, 2000; Piñar et al., 2013). In addition, high abundances of *Cladosporium*, *Neurospora* and *Talaromyces* were found in the unstained paper samples, and multiple species of these genera belong to plant endophytes (Tian et al., 2009; Yamagiwa et al., 2011; Nitiu et al., 2020).

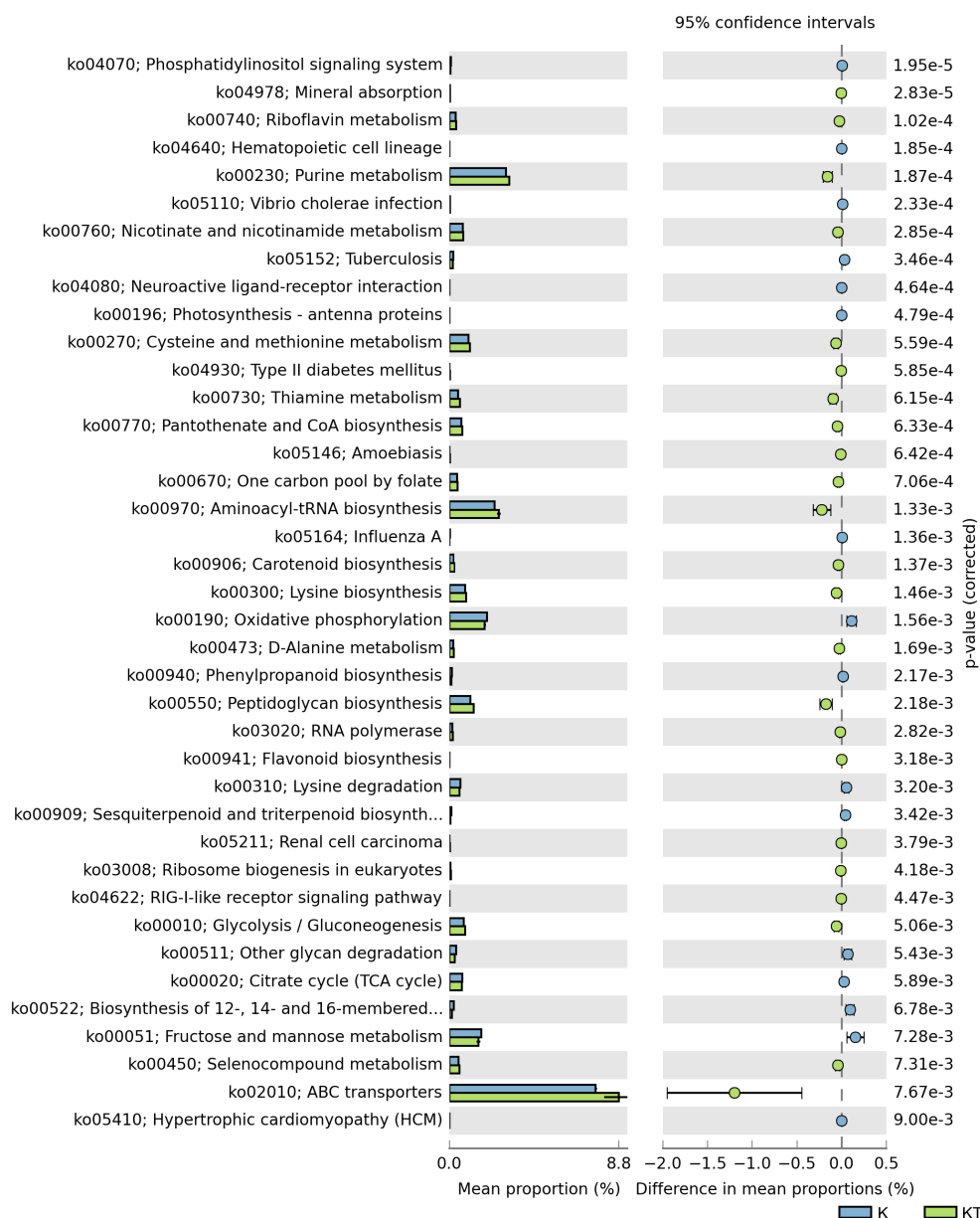


FIGURE 6

Predicted KEGG ortholog groups with significant differences ($p < 0.05$) in relative abundance between the "peachy spots" and unstained paper.

The results suggested that the formation of peachy spots on Kaihua paper might be more related to bacteria than fungi. The role of bacteria in the conservation and preservation of paper relics has long been neglected or underestimated. In recent years, more attention has been given to bacterial communities, where the bacterial genera *Bacillus*, *Clostridium*, *Pseudomonas*, *Micrococcus*, *Actinomyces* and *Cellulophaga* were often found to be related to paper damage (Kraková et al., 2012; Dunca et al., 2014; Lech, 2016; Szulc et al., 2018; Raeisnia et al., 2022; Pavlović et al., 2023). In this study, we found significantly more *Actinomyces* of the genus *Rubrobacter* in peachy spot groups. Species of the genus *Rubrobacter* has previously isolated from deteriorated monuments (Piñar et al., 2013) and the carotenoid pigments they produce has been found to have a causal relationship with the rosy discoloration of ancient wall paintings (Imperi et al.,

2007; Ettenauer et al., 2014). And *Actinomyces* have been reported to possibly utilize insoluble matrix components and penetrate materials in parchment and canvas paintings and even increase the water solubility of the matrix for further utilization (Pavić et al., 2015; Lech, 2016).

Nevertheless, other bacteria commonly present on paper relics were not abundant (below 1% for *Clostridium* and *Pseudomonas*) or even absent (*Micrococcus* and *Cellobacter*) in peachy spot samples. Instead, bacteria with acid-producing, acidophilic or cellulase-producing potential, including *Lactobacillus*, *Streptococcus*, *Staphylococcus*, and *Lysinibacillus*, and bacteria with pigment-producing potential, such as *Methylobacterium* (van Aken et al., 2004; Vadivukkarasi et al., 2018), were found to be highly abundant in peachy spots.

The result of functional gene prediction with Tax4fun supported the hypothesis regarding the role of bacteria in peachy spot formation. The abundance of predicted bacterial gene orthologs involved in the biosynthesis and metabolism of riboflavin, carotenoids and flavonoids was significantly higher in the peachy spot groups than in the unstained paper group. Some bacteria have the capability to produce organic acids and various enzymes to hydrolyze the paper components (Pavić et al., 2015), making the material easier to be colonized by more microbes, and the addition of pigment-producing bacteria may further help the stain formation. It is also possible that the chemical composition of peachy spot areas promoted the survival of certain bacteria and shaped the structure of the bacterial community.

The results obtained in the present study also showed that bacteria and fungi not only coexist in the microenvironment of the paper surface but also form relatively stable communities in each group. This may be because the paper itself continues to exert selective pressure on microorganisms. Studies of purple spots on ancient parchment hypothesized a succession model, starting from halophilic archaea, followed by halotolerant bacteria and actinomycetes, and then fungi (Migliore et al., 2019). Similarly, peachy spots on Kaihua paper may be the result of synergy and competition between different kinds of archaea, bacteria, and fungi. Their succession caused stains and the aging of Kaihua paper after a long time. Some archaea have been found to produce pink pigments in previous study (Mazzoli et al., 2018; Tichy et al., 2023). Although archaea were not investigated in this study, they could have played a role in the biodeterioration of Kaihua paper. The accumulation of bacteria with potential acid- and pigment-producing ability may be a relatively late phenomenon in the process of peachy spot formation. The results also remind us that without timely intervention, the bacteria at peachy spots will continue to destroy precious cultural relics.

Although studies based on DNA sequencing provide clues for the peachy spots, they may not tell the current microbial activities on the paper. Part of the detected DNA may be the remains of dead cells that have not been degraded completely. This gives us an opportunity to investigate the microbial community involved in the formation of peachy spots, but it also increases the difficulty of analyzing the process. One solution is to analyze RNA, which is much less stable than DNA. However, we found that the concentration of RNA recovered from the paper surface using noninvasive methods was too low to study. Due to the limitation of sample numbers and the lack of integrated analysis of multiple omics, the exact mechanism of peachy spot formation needs to be further studied.

In summary, through nondestructive sampling and high-throughput sequencing, it was found that the bacterial community on the peachy spots on Kaihua paper significantly differed from that on the unstained paper and the air in the stack room. No such difference was found in the fungal community. Bioinformatics analysis and functional gene prediction indicated that the formation of peachy spots may be mainly related to bacteria rather than fungi. The bacterial community may be mostly responsible for acidic substance and pigment accumulation in paper. The mechanisms of peachy spot formation are rather complex, and therefore, an increase in sample size and multiomics joint analysis will be useful to reveal the key microorganisms and their succession process that cause this phenomenon.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA1031546, PRJNA1032408.

Author contributions

YZ: Data curation, Investigation, Visualization, Writing – original draft. YS: Data curation, Writing – review & editing. YH: Investigation, Methodology, Writing – original draft. JZ: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was financially supported by the National Key R&D Program of China (2019YFC1520400). The funding body had no role in the design of the study, the collection, analysis, and interpretation of data, or in writing the manuscript.

Acknowledgments

We gratefully thank the Fudan University Library and the Institute for Preservation and Conservation of Chinese Ancient Books of Fudan University for providing the ancient books for this study. We thank Academician Yuliang Yang (Institute for Preservation and Conservation of Chinese Ancient Books) and Guanghui Yang (Institute for Preservation and Conservation of Chinese Ancient Books) for their support and useful comments on research methods.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1326835/full#supplementary-material>

References

- Abarenkov, K., Nilsson, R. H., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., et al. (2010). The UNITE database for molecular identification of fungi—recent updates and future perspectives. *New Phytol.* 186, 281–285. doi: 10.1111/j.1469-8137.2009.03160.x
- Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169. doi: 10.1128/mr.59.1.143-169.1995
- Arai, H. (2000). Foxing caused by Fungi: twenty-five years of study. *Int. Biodeterior. Biodegrad.* 46, 181–188. doi: 10.1016/S0964-8305(00)00063-9
- Area, M. C., and Cheradame, H. (2011). Paper aging and degradation: recent findings and research methods. *Bioresources* 6, 5307–5337. doi: 10.15376/biores.6.4.5307-5337
- Ařhauer, K. P., Wemheuer, B., Daniel, R., and Meinicke, P. (2015). Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* 31, 2882–2884. doi: 10.1093/bioinformatics/btv287
- Beata, G. (2020). The use of omics tools for assessing biodeterioration of cultural heritage: a review. *J. Cult. Herit.* 45, 351–361. doi: 10.1016/j.culher.2020.03.006
- Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., and Kausrud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiol.* 10:189. doi: 10.1186/1471-2180-10-189
- Bicchieri, M., Pappalardo, G., Romano, F. P., Sementilli, F. M., and de Acutis, R. (2001). Characterization of foxing stains by chemical and spectrometric methods. *Restaurator* 22, 1–19. doi: 10.1515/REST.2001.1
- Branysova, T., Demnerova, K., Durovic, M., and Stiborova, H. (2022). Microbial biodeterioration of cultural heritage and identification of the active agents over the last two decades. *J. Cult. Herit.* 55, 245–260. doi: 10.1016/j.culher.2022.03.013
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303
- Cappitelli, F., Pasquariello, G., Tarsitani, G., and Sorlini, C. (2010). Scripta manent? Assessing microbial risk to paper heritage. *Trends Microbiol.* 18, 538–542. doi: 10.1016/j.tim.2010.09.004
- Choi, S. (2007). Foxing on paper: a literature review. *J. Am. Inst. Conserv.* 46, 137–152. doi: 10.1179/019713607806112378
- Clarke, K. R., and Warwick, R. M. (1994). *Change in marine communities: An approach to statistical analysis and interpretation*. Plymouth, UK: Plymouth Marine Laboratory, Natural Environment Research Council.
- Daub, M. E., and Ehrenshaft, M. (2000). The photoactivated cercospora toxin cercosporin: contributions to plant disease and fundamental biology. *Annu. Rev. Phytopathol.* 38, 461–490. doi: 10.1146/annurev.phyto.38.1.461
- Dunca, S. I., Tanase, C., Padurariu, C., Balaes, T., Ardelean, E., and Puica, N. M. (2014). Study of the contaminating microbiota of old paper supports. *Eur. Sci. J. ESJ* 10, 237–251.
- Ettenuer, J. D., Jurado, V., Pinar, G., Miller, A. Z., Santner, M., Saiz-Jimenez, C., et al. (2014). Halophilic microorganisms are responsible for the rosy discolouration of saline environments in three historical buildings with mural paintings. *PLoS One* 9:e103844. doi: 10.1371/journal.pone.0103844
- Figueira, F., Matos, M., Nunes, A., Afonso, M., Rocha, A. C., Campelo, J., et al. (2020). Considerations about foxing stains in three paper collections ranging from the 16th to the 20th century. *Conserv. Património* 35, 45–57. doi: 10.1080/14786419.2016.1180600
- Gravesen, S. (2000). Microbiology on indoor air '99—what is new and interesting? An overview of selected papers presented in Edinburgh, august, 1999. *Indoor Air* 10, 74–80. doi: 10.1034/j.1600-0668.2000.010002074.x
- Imperi, F., Caneva, G., Cancellieri, L., Ricci, M. A., Sodo, A., and Visca, P. (2007). The bacterial aetiology of rosy discoloration of ancient wall paintings. *Environ. Microbiol.* 9, 2894–2902. doi: 10.1111/j.1462-2920.2007.01393.x
- Koochakzadeh, A., and Alizadeh Gharetapeh, S. (2021). Paper foxing stains on a historic manuscript from the early Qajar era: abiotic or biotic foxing? *Heritage* 4, 1366–1374. doi: 10.3390/heritage4030074
- Kraková, L., Chovanová, K., Selim, S. A., Šimonovičová, A., Puřkarová, A., Maková, A., et al. (2012). A multiphase approach for investigation of the microbial diversity and its biodegradative abilities in historical paper and parchment documents. *Int. Biodeterior. Biodegrad.* 70, 117–125. doi: 10.1016/j.ibiod.2012.01.011
- Kraková, L., Šoltys, K., Otlewska, A., Pietrzak, K., Purkrtová, S., Savická, D., et al. (2018). Comparison of methods for identification of microbial communities in book collections: culture-dependent (sequencing and MALDI-TOF MS) and culture-independent (Illumina MiSeq). *Int. Biodeterior. Biodegrad.* 131, 51–59. doi: 10.1016/j.ibiod.2017.02.015
- Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., and Knight, R. (2011). Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr. Protoc. Bioinformatics Chapter* Chapter 10:10.7.1. doi: 10.1002/0471250953.bi1007s36
- Lech, T. (2016). Evaluation of a parchment document, the 13th century incorporation charter for the city of Krakow, Poland, for microbial hazards. *Appl. Environ. Microbiol.* 82, 2620–2631. doi: 10.1128/aem.03851-15
- Liu, Z., Zhang, Y., Zhang, F., Hu, C., Liu, G., and Pan, J. (2018). Microbial community analyses of the deteriorated storeroom objects in the Tianjin museum using culture-independent and culture-dependent approaches. *Front. Microbiol.* 9, 311–312. doi: 10.3389/fmicb.2018.00802
- Mazzoli, R., Giuffrida, M. G., and Pessione, E. (2018). Back to the past: “find the guilty bug—microorganisms involved in the biodeterioration of archeological and historical artifacts”. *Applied Microbiology & Biotechnology*. 102, 6393–6407. doi: 10.1007/s00253-018-9113-3
- Meynell, G. G., and Newsam, R. J. (1978). Foxing, a fungal infection of paper. *Nature* 274, 466–468. doi: 10.1038/274466a0
- Migliore, L., Perini, N., Mercuri, F., Orlanducci, S., Rubecchini, A., and Thaller, M. C. (2019). Three ancient documents solve the jigsaw of the parchment purple spot deterioration and validate the microbial succession model. *Sci. Rep.* 9:1623. doi: 10.1038/s41598-018-37651-y
- Migliore, L., Thaller, M. C., Vendittozzi, G., Mejia, A. Y., Mercuri, F., Orlanducci, S., et al. (2017). Purple spot damage dynamics investigated by an integrated approach on a 1244 a.D. parchment roll from the secret Vatican archive. *Sci. Rep.* 7, 9521–9512. doi: 10.1038/s41598-017-05398-7
- Mitchell, R., and McNamara, C. J. (2010). *Cultural heritage microbiology: Fundamental studies in conservation science*. Washington, DC: ASM Press.
- Nitiu, D. S., Mallo, A. C., and Saparrat, M. C. (2020). Fungal melanins that deteriorate paper cultural heritage: an overview. *Mycologia* 112, 859–870. doi: 10.1080/00275514.2020.1788846
- Parks, D. H., Tyson, G. W., Philip, H., and adn Beiko, R. G. (2014). Stamp: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494
- Pavić, A., Ilić-Tomić, T., Pačevski, A., Nedeljković, T., Vasiljević, B., and Morić, I. (2015). Diversity and biodeteriorative potential of bacterial isolates from deteriorated modern combined-technique canvas painting. *Int. Biodeterior. Biodegrad.* 97, 40–50. doi: 10.1016/j.ibiod.2014.11.012
- Pavlović, J., Puřkarová, A., Planý, M., Farkas, Z., Rusková, M., Kvalová, K., et al. (2023). Colored stains: microbial survey of cellulose-based and lignin rich papers. *Int. J. Biol. Macromol.* 241:124456. doi: 10.1016/j.jbiomac.2023.124456
- Piñar, G., Piombino-Masali, D., Maixner, F., Zink, A., and Sterflinger, K. (2013). Microbial survey of the mummies from the capuchin catacombs of Palermo, Italy: biodeterioration risk and contamination of the indoor air. *FEMS Microbiol. Ecol.* 86, 341–356. doi: 10.1111/1574-6941.12165
- Piñar, G., Sclocchi, M. C., Pinzari, F., Colaizzi, P., Graf, A., Sebastiani, M. L., et al. (2020). The microbiome of Leonardo da Vinci's drawings: a bio-archive of their history. *Front. Microbiol.* 11:593401. doi: 10.3389/fmicb.2020.593401
- Raeisnia, N., Arefian, E., and Amoozegar, M. A. (2022). Microbial community of an 11th century manuscript by both culture-dependent and -independent approaches. *Microbiology* 91, 313–323. doi: 10.1134/S0026261722300117
- Rampersad, S. N. (2020). Pathogenomics and management of *fusarium* diseases in plants. *Pathogens*. 9:340. doi: 10.3390/pathogens9050340
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/aem.01541-09
- Sclocchi, M. C., Kraková, L., Pinzari, F., Colaizzi, P., Bicchieri, M., řaková, N., et al. (2016). Microbial life and death in a foxing stain: a suggested mechanism of photographic prints defacement. *Microb. Ecol.* 73, 815–826. doi: 10.1007/s00248-016-0913-7
- řimşekli, Y., Gücin, F., and Asan, A. (1999). Isolation and identification of indoor airborne fungal contaminants of food production facilities and warehouses in Bursa, Turkey. *Aerobiologia* 15, 225–231. doi: 10.1023/A:1007623831010
- Sinha, S., Navathe, S., Singh, S., Gupta, D. K., Kharwar, R. N., and Chand, R. (2023). Genome sequencing and annotation of *Cercospora sesami*, a fungal pathogen causing leaf spot to *Sesamum indicum*. *Three biotech* 13:55. doi: 10.1007/s13205-023-03468-4
- Sterflinger, K. (2010). Fungi: their role in deterioration of cultural heritage. *Fungal Biol. Rev.* 24, 47–55. doi: 10.1016/j.fbr.2010.03.003
- Sterflinger, K., Little, B., Pinar, G., Pinzari, F., de los Rios, A., and Gu, J. D. (2018). Future directions and challenges in biodeterioration research on historic materials and cultural properties. *Int. Biodeterior. Biodegrad.* 129, 10–12. doi: 10.1016/j.ibiod.2017.12.007
- Szulc, J., Otlewska, A., Ruman, T., Kubiak, K., Karbowska-Berent, J., Kozięlec, T., et al. (2018). Analysis of paper foxing by newly available omics techniques. *Int. Biodeterior. Biodegrad.* 132, 157–165. doi: 10.1016/j.ibiod.2018.03.005
- Tian, C., Beeson, W. T., Iavarone, A. T., Sun, J., Marletta, M. A., Cate, J. H., et al. (2009). Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa*. *Proc. Natl. Acad. Sci. U. S. A.* 106, 22157–22162. doi: 10.1073/pnas.0906810106
- Tichy, J., Waldherr, M., Ortbauer, M., Graf, A., Sipek, B., Jembrih-Simbuerger, D., et al. (2023). Pretty in pink? Complementary strategies for analysing pink biofilms

- on historical buildings. *Sci. Total Environ.* 904:166737. doi: 10.1016/j.scitotenv.2023.166737
- Vadivukkarasi, P., Jayashree, S., and Seshadri, S. (2018). Occurrence and ecological significance of *Methylobacterium*. *Trop. Ecol.* 59, 575–587.
- van Aken, B., Peres, C. M., Doty, S. L., Yoon, J. M., and Schnoor, J. L. (2004). *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* x *nigra* DN34). *Int. J. Syst. Evol. Microbiol.* 54, 1191–1196. doi: 10.1099/ijs.0.02796-0
- Yamagiwa, Y., Inagaki, Y., Ichinose, Y., Toyoda, K., Hyakumachi, M., and Shiraishi, T. (2011). *Talaromyces wortmannii* FS2 emits β -caryphyllene, which promotes plant growth and induces resistance. *J. Gen. Plant Pathol.* 77, 336–341. doi: 10.1007/s10327-011-0340-z



OPEN ACCESS

EDITED BY

Marika Pellegrini,
University of L'Aquila, Italy

REVIEWED BY

Yulong Duan,
Northwest Institute of Eco-Environment and
Resources, Chinese Academy of Sciences
(CAS), China
Taha Menasria,
University of Batna 2, Algeria

*CORRESPONDENCE

Qiang Luo
✉ bmyluoqiang@126.com
Jiao Pan
✉ jiaopan@ustb.edu.cn

RECEIVED 29 January 2024

ACCEPTED 29 April 2024

PUBLISHED 20 September 2024

CITATION

Wang C, Hou L, Jiang N, Wang Y, Mao X,
Zhou P, Xia Y, Wang Y, Chen C, Yang X,
Luo Q and Pan J (2024) Microbial diversity in
earthen site of exhibition Hall of pit no. 1 at
the terracotta warriors Museum in Emperor
Qinshihuang's mausoleum site museum and
its correlation with environmental factors.
Front. Microbiol. 15:1378180.
doi: 10.3389/fmicb.2024.1378180

COPYRIGHT

© 2024 Wang, Hou, Jiang, Wang, Mao, Zhou,
Xia, Wang, Chen, Yang, Luo and Pan. This is
an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Microbial diversity in earthen site of exhibition Hall of pit no. 1 at the terracotta warriors Museum in Emperor Qinshihuang's mausoleum site museum and its correlation with environmental factors

Cen Wang¹, Lilong Hou², Nan Jiang³, Yu Wang¹, Xiaofen Mao⁴,
Ping Zhou⁴, Yin Xia⁴, Yuanyuan Wang¹, Chuyue Chen²,
Xinyu Yang², Qiang Luo^{4*} and Jiao Pan^{1*}

¹Key Laboratory of Archaeomaterials and Conservation, Ministry of Education, Institute for Cultural Heritage and History of Science and Technology, University of Science and Technology Beijing, Beijing, China, ²Ministry of Education Key Laboratory of Molecular Microbiology and Technology, Department of Microbiology, College of Life Sciences, Nankai University, Tianjin, China, ³Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China, ⁴Emperor Qinshihuang's Mausoleum Site Museum, Key Scientific Research Base of Ancient Polychrome Pottery Conservation of State Administration of Culture Heritage, Shaanxi Xi'an, China

Introduction: Earthen sites are essential cultural relic resources, and site museums are a fundamental component of China's cultural heritage protection. The mausoleum of the Qin Shi Huang Emperor is one of the largest, most peculiar, and richest imperial tombs in the world. The exhibition hall of the burial pit No. 1 of the Terra Cotta Warriors is the earliest exhibition hall built and opened to the public. However, after years of excavation and open exhibitions, the earthen site of the Emperor Qinshihuang's Mausoleum Site Museum has deteriorated to varying degrees due to changes in the modern environment. There is an urgent need to control microbial diseases and protect the earthen site.

Methods: We analyzed the physical and chemical properties and bioindicators of the collected soil samples. We also established a metagenomic library and conducted a correlation analysis between microbial community composition and environmental factors. Cultivable fungi obtained from air and soil samples were identified, and allicin volatile gas fungistasis test was conducted.

Result: Research has found that four different areas of the exhibition hall have different types of microbial diseases owing to their different environments. The main pathogenic fungi in earthen site may lead to potential microbial diseases that affect important cultural relics such as the Terra Cotta Warriors. *Penicillium*, *Aspergillus* and *Talaromyces* showed relatively specific growth in relation to environmental factors and showed a better raw growth advantage. Allicin gas had an inhibitory effect on 12 types of fungi, therefore allicin gas had a potent inhibitory effect on the growth of the most culturable fungal hyphae.

Discussion: This study provides basic data for the study of microbial diversity in the exhibition hall of Pit No. 1 at the Terracotta Warriors Museum in Emperor Qinshihuang's Mausoleum Site Museum. It provides a reference for future protection work, which is of great significance.

KEYWORDS

the terracotta warriors museum, microbial diversity, earthen site, environmental factor, function of microorganisms

1 Introduction

Earthen sites are historical sites built with soil as the main building material and are important evidence of ancient human activity (Xiaoying et al., 2016; Kong et al., 2019; Xia Yin et al., 2023). There are nearly 1,000 earthen sites in China according to the statistics of China's early key cultural relics protection institutions (Luo Xilian et al., 2018). Meanwhile, more than 80 Chinese earth sites are on the World Heritage List (2019) published by UNESCO (Briseghella et al., 2020). However, these earthen sites are often extremely fragile, and many are being degraded as a result of inappropriate excavation processes and unsuitable protective environments (Richards et al., 2020; Yumin et al., 2020). Due to the diversity of topography, hydrological environments, climatic environments and built forms of earthen sites, the protection of different earthen sites should vary according to their characteristics (Chonggen et al., 2020). Site museums are a fundamental part of cultural heritage conservation in China (Gu et al., 2013). For example, a cultural museum has been built on the site in order to protect the Jinsha Site Museum (Zhu and Kan, 2009; Wei et al., 2019). The same is true of the Emperor Qinshihuang's Mausoleum Site Museum. The protection of the burial pits of the Terracotta Warriors belongs to the *in-situ* protection of the site, which is the protection of the earthen site by most of the site museums in China (Zhang et al., 2009). Prior to excavation, the cultural relic artifacts as well as the earthen site were rarely affected by the external atmospheric environment and remained in a very stable preservation environment for many years (Zhuangbo et al., 2022). However, exposed sites are susceptible to damage from the surrounding environment, such as changes in temperature and humidity (Xilian et al., 2019; He et al., 2021; Zhang et al., 2022). The impact of human activities such as visits and archaeological research on earthen sites can lead to destabilization of microbial populations. Therefore the protection of soil sites should be emphasized.

Currently, research focuses on the effects of physical and chemical factors on the degradation of earthen sites (Ma et al., 2020; Xudong et al., 2020; Germinario and Oguchi, 2021). Instead, little attention has been paid to the interaction of biological factors, especially microorganisms, in the degradation of earthen sites. Characterizing the microbial communities living in artifacts, particularly the identification of the most destructive microorganisms, will provide a basis for standardized microbiological control of protected earthen sites (Yang et al., 2020). Currently, only 1% of microorganisms can be cultured under laboratory conditions, so that methods for genome characterization is an important tool for determining the diversity and composition of microbial communities and functions (Sauro et al., 2018), such as metagenomics data assembly and analyses. It is an experimentally validated method for studying microbial communities in environments provides a powerful method, and has been successfully used to determine the composition and diversity of microbial communities from different environments (Alam et al., 2021), such as samples from river mud,

groundwater and soil (Elena et al., 2019; Zaouri et al., 2020; Jiaxian et al., 2021). He Jintao had use the metagenomics data assembly to analyze the assembly processes of microbial communities in stone biodeterioration of the West Lake UNESCO world heritage area in China (Jintao et al., 2022).

Understanding how environmental factors affect the soil microbial community can also be achieved at the same time as accurately understanding how the microbial community affects the earthen sites (Carini et al., 2020). For example, carbon and nitrogen in the soil may affect the growth and metabolism of microorganisms through a variety of direct or indirect effects, and even change the structure of the microbial community in the soil as a whole, while microorganisms may be involved in nitrogen metabolism and decomposition in the soil (Jiang et al., 2013). There are two sources of fungal diseases on the surface of soil sites. On the one hand, many of the soils were not specially treated during the construction process, and the earthen sites contain many fungal spores, on the other hand the dispersal of mold spores brought by fallout and visitor activities (Xingqun et al., 1999). These spores cause damage to the earthen sites when the museum soil is moist and at the right temperature, and the fungal spores utilize the organic matter in the soil as a nutrient resuscitation for survival. There are a number of earth sites have been affected by various microorganisms or have undergone microbial degradation. Microbial diseases have emerged at archaeological sites in Liangzhu City, and there is a strong correlation between microbial diversity and the physical and chemical properties of the soil. The presence of *Acidibacter* may cause the continuous degradation of organic matter in the soil, such that the soil loses its original structure, becomes loose, and easily falls off (Jing et al., 2019). With an increase in the degree of degradation of the Jinsha soil site, the soil microbial community and functional diversity also flourished, and many types of bacteria are involved in the soil degradation process (Tongtong et al., 2017). The microbial community on the surface of the Lidu wine cellar site was determined and analyzed, and its dominant fungi mainly include filamentous fungi and yeast (Wu Fasi et al., 2012). The corrosive fungi on the surface of the Western Zhou Tomb Site in Dahekou, Yicheng, Shanxi Province, mainly belong to *Pseudomonas* and *Alternaria* (Yin et al., 2013).

The Qin Shi Huang Emperor mausoleum, built from 247 to 208 BC, is one of the largest, most peculiar, and richest imperial mausoleums in the world. It fully reflects the artistic wisdom of the Han working people in ancient China more than 2000 years ago and is a precious resort for the Chinese nation. On March 4, 1961, the mausoleum of the Qin Shi Huang Emperor was announced by the State Council as the first batch of major historical and cultural sites to be protected at the national level. The Qin Shi Huang Mausoleum and the Terra Cotta Warriors Pit were approved by UNESCO for inclusion on the World Heritage List in December 1987. The No. 1 Pit exhibition hall is a typical semi-open museum. It is the first exhibition hall built and opened to the public at the Emperor Qinshihuang's Mausoleum Site Museum and is the largest core exhibition hall.

However, after years of excavation and open exhibition, the earthen sites and unearthed cultural relics of the Emperor Qinshihuang's Mausoleum Site Museum have also deteriorated to varying degrees due to changes in the modern environment, resulting in the ruins unable to maintain their original state (Qiang et al., 2021). A large number of microorganisms and various coarse and fine particles to gather in the exhibition hall of Pit 1. The aggregation of flocs promotes the adsorption of these microorganisms and particles on the surface of the site, causing the accumulation of dark sediment on the surface and leading to further destructive effects such as biological corrosion, biodegradation, and pigment pollution produced by microorganisms. Luo et al. isolated and identified four main fungi in flocs: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium oxalate*, and *Alternaria alternata*. The diversity of fungal communities in the flocs was analyzed, with *Alternaria* and *Aspergillus* occupying an absolute advantage (Qiang et al., 2021). There is an urgent need to study and control microbial diseases and protect the earthen site of the Emperor Qinshihuang's Mausoleum Site Museum.

Choosing plant-based antifungal agents from actual plants is safer and more environmentally friendly (Al-Qahtani et al., 2022). Plant volatile oil, which is widely found in roots, bark, stems, leaves, flowers, and fruits, is composed of terpenes, ketones, epoxides, esters, acids, alcohols, and sulfides and has good antibacterial, antifungal, antiviral, and antioxidant effects (Basile et al., 2009; Thotathil et al., 2023). Yang's treatment with allicin interfered with the biosynthesis, glucose catabolism, and oxidative stress of the cell membrane and cell wall of *Trichosporon alfredii*, proving that allicin had a favorable effect on fungal inhibition (Yang et al., 2023).

In July 2022, we investigated the soil at the Terra Cotta Warriors archaeological site. We divided the exhibition hall of Pit 1 of the Terra Cotta Warriors into four areas: excavation, canopy, the surface under the restoration, and the surface of the restoration. The locations are shown in Figure 1. We found a large amount of flocculent material in the restoration area, consistent with previous research results. Soil samples will be collected to analyze soil physical and chemical properties and bioindicators. A metagenomic library was established, and the species and functional diversity of each sample were analyzed. At the same time, a correlation analysis was conducted between the composition of microbial communities and environmental factors. The main culturable fungi obtained from the two samples were isolated, purified, and identified. A volatile gas fungistasis experiment was conducted to test whether allicin could inhibit the growth of these fungi. Our research provides basic data for the long-term protection of the exhibition hall of Pit 1 of the Terra Cotta Warriors in the Emperor Qinshihuang's Mausoleum Site Museum and puts forward protection suggestions for microbial diseases. This provides relevant data on the impact of microorganisms on earthen site protection and the planning of earthen site protection strategies.

2 Materials and methods

2.1 Sample collection

Pit No. 1 at the Terracotta Warriors Museum is divided into four areas: excavation area (EX), canopy area (CE), restoration area (RE), and restoration area surface (RS). The excavation area (EX) is the pit

in which the Terracotta Warriors archaeological excavations are carried out, in which the soil may come into direct contact with the Terracotta Warriors and other artifacts. The rammed earth walls in the canopy area (CE) effectively supported the huge roof of the trellis, ensuring that it would not collapse, and played an important role in the protection of the Terracotta Warriors. Scaffolding wood filled with green paste mud, with moisture, anticorrosive effect. The restoration area (RE) and the surface of the restoration area (RS) are the areas where the terracotta warriors were restored and exhibited next to the terracotta pits. The samples of RE were collected from the soil about 10 cm below the top soil layer (RS). Gray flocs were found in all four areas, and many flocs accumulated on the restoration area surface. Three parallel samples were collected from each area using sterile spoons and placed in sealed bags. Soil samples were collected from each area at specific locations, times, and temperatures, as listed in Table 1. Some were transported to the laboratory using ice bags, whereas others were transported to the laboratory using dry ice. Those transported in ice bags were stored in a 4°C refrigerator, while those transported with dry ice were stored in a −80°C refrigerator.

2.2 Isolation and purification of cultivable fungi(microbial isolation, cultivation, and identification)

Twelve soil samples were diluted 103 times, and the diluted soil suspension was separately applied to the PDA (Potato dextrose agar medium) medium, with three replicates for each medium of each sample. After 3 days of culture at 28°C, a single fungal colony was selected, inoculated in the new PDA medium, and cultured at 28°C for 3 days again until pure strains were obtained for subsequent experiments. All pure microorganisms were stored at −80°C.

2.3 Metagenomics data assembly and analyses

Total microbial genomic DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN, Inc., Netherlands) following the manufacturer's instructions. The quantity and quality of extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. The extracted microbial DNA was processed to construct metagenome shotgun sequencing libraries with insert sizes of 400 bp using the Illumina TruSeq Nano DNA LT Library Preparation Kit. Each library was sequenced using the Illumina HiSeq X-ten platform (Illumina, United States) using the PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China) (Huang et al., 2021).

The raw sequencing reads were processed to obtain quality-filtered reads for further analysis. Sequencing adapters were removed from sequencing reads using Cutadapt (v1. 2. 1). Low-quality reads were trimmed using a sliding-window algorithm. Sequencing reads were aligned to the host genome using Burrow-Wheeler Aligner (BWA) to remove host contamination. Once quality-filtered reads were obtained, they were assembled *de novo* to construct a metagenome for each sample using the Iterative De Bruijn graph

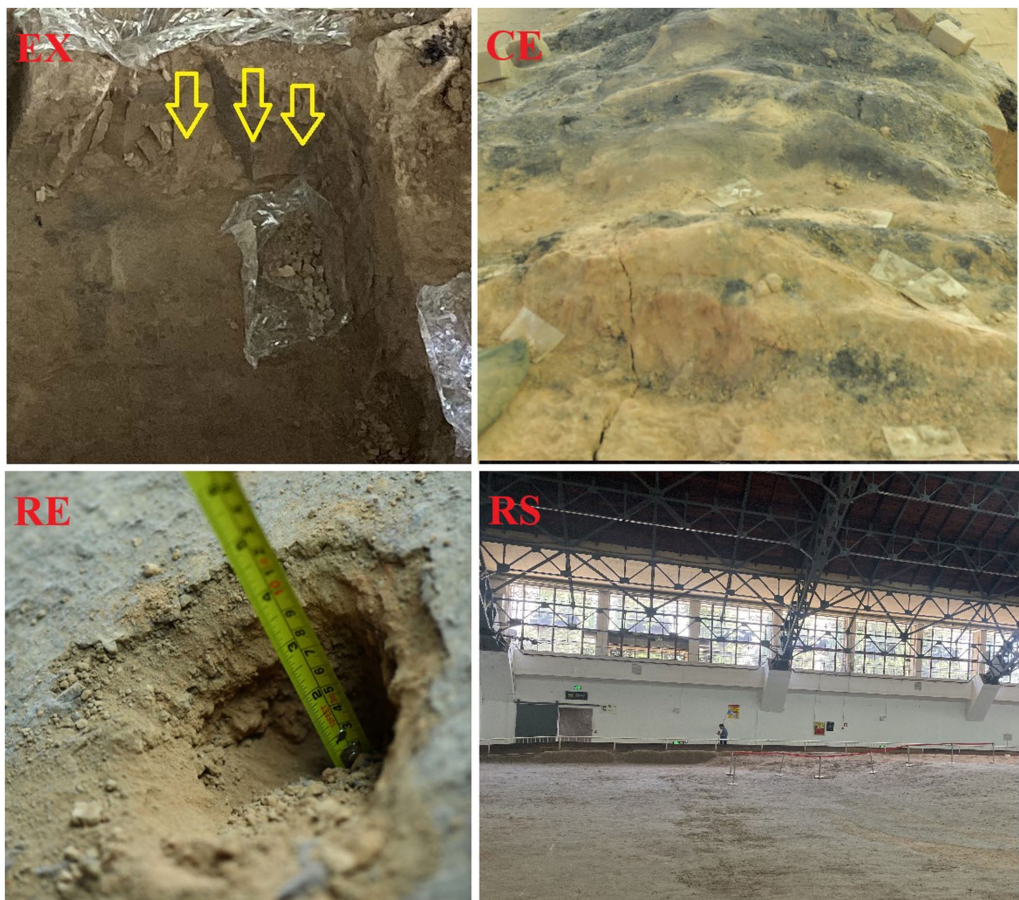


FIGURE 1
Environmental images of soil sampling locations. Excavation Area (EX), Canopy Area (CE), Restoration Area (RE), Restoration Area Surface (RS).

TABLE 1 Environmental information of soil sample collection locations.

Sampling location	EX	CE	RE	RS
Location description	T24, G10 (West Section)	T24, Q9 (West Section)	Open space on the northwest side of the restoration area	Open space on the northwest side of the restoration area
Time	10:30	10:00	9:30	8:40
Real-time temperature	23.9°C	30.9°C	28.2°C	28.2°C
Real-time relative humidity	79.8%	56.6%	21.02%	21.02%

assembler for sequencing data with highly uneven depth. All coding regions (CDS) of the metagenomic scaffolds longer than 300 bp were predicted using MetaGeneMark. The CDS sequences of all samples were clustered using Cluster Database at High Identity with Tolerance (CD-HIT) with 90% protein sequence identity to obtain a non-redundant gene catalog. The gene abundance in each sample was estimated using soap coverage based on the number of aligned reads. The lowest common ancestor taxonomy of the nonredundant genes was obtained by aligning them against the NCBI NT database using BLASTN (e-value <0. 001). Similarly, functional profiles of non-redundant genes were obtained by annotation against the KEGG, EggNOG, and CAZy databases using the DIAMOND alignment algorithm at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) (Huang et al., 2021).

2.4 Analysis of species diversity and functional diversity of microorganisms in soils of different regions

Analysis was performed using GenesCloud, a free online platform for data analysis.¹ QIIME was used to determine the composition and abundance distribution of each sample at each classification level (domain, phylum, class, order, family, genus, and species), integrate the species annotation information of contig sequences with the abundance table in each sample, and obtain the species abundance

1 <https://www.genescloud.cn>

table of each sample at each classification level. Based on the sequencing results, the composition of the microbial communities in the soil samples was analyzed at the genus level to conclude.

The common and unique functional groups of each sample were analyzed. Based on the composition spectrum of the underlying functional units annotated in each functional database for each sample, the R software was used to calculate and visually present the number of common and unique functional class groups for each sample using Venn diagrams. Principal coordinate analysis (PCoA) was used to analyze the functional beta diversity of each sample. This was done to obtain the spatial distribution characteristics of the sample functional composition and quantify the differences between the samples.

2.5 Analysis of the physical and chemical properties of the environmental factor and association analysis

Twelve soil samples of approximately 100 g each were sent to the Institute of Applied Ecology, Chinese Academy of Sciences, for chemical, physical, and bioindicator determination.

The soil texture and structure, water content, trace element content, heavy metal content, organic carbon content, microbial carbon content, phosphorus-related hydrolase, and carbon-related hydrolase were mainly measured as environmental factors for the correlation analysis. Soil pH (water) was determined with a glass electrode (pH 700 Bench Meter, Eutech Instruments) at a ratio of 1:2.5. Soil total carbon (C), nitrogen (N), and sulfur (S) were measured using an automatic element analyzer (Analyzer vario MICRO cube, Elementar, Germany). The trace metals were extracted using 0.1 M BaCl₂ and determined using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Microbial biomass carbon (MBC) was obtained using the chloroform fumigation direct extraction method. α -glucosidase activity (α) and β -glucosidase activity (β) were obtained by the p-nitrophenol method. Cellobiohydrolase activity (CBH), xylosidase activity, phosphomonoesterase I (ALP) and II (ACP) were determined by the p-nitrophenylphosphate method, and phosphodiesterase (PDEs) by the bisnitrophenylphosphate method.

An environmental factor association analysis heat map was plotted using heat map tools on the GenesCloud platform.² The tool was developed using the pheatmap package (V1.0.8), which was slightly modified to improve the layout style. The data were normalized by z-scores. The package uses popular clustering distances and methods implemented in the dist and hclust functions in R. Based on the species annotations of each sample in the database at the genus level and the obtained soil physicochemical property analysis results, the correlation between each sample species and the environment was visually presented using a heatmap.

2.6 Antimicrobial experiment of volatile allicin gas

The antimicrobial effect of allicin volatile gas was determined using a dichotomy plate with culturable fungi that were isolated and purified

as the experimental object (Huang et al., 2019). A PDA medium was poured on one side of a split plate. After medium solidification, a fungal coating with a diameter of approximately 8 mm was applied to the culture medium. The air concentration of allicin was 625 μ L/L when 50 μ L allicin was added to the other side; the volume of the 90 mm culture dish was about 80 mL. An equal amount of sterile distilled water was used as a negative control to observe the inhibitory effect.

2.7 Bacterial growth culture and heavy metal ion stress growth culture

During the excavation of the Terracotta Warriors, many copper weapons were unearthed, so there are high levels of copper in the soil, especially in the excavated areas (the average value is 48.760 mg/kg). Macro-genomic sequencing analysis showed that *Cupriavidus* were found in the excavation area where a large number of copper artifacts had been buried. Therefore, we carried out further analyses of the *Cupriavidus alkaliphilus*.

For the particular bacterium *C. alkaliphilus*, which was isolated and obtained from the excavation area and account for 0.15% of species diversity, even in the excavation areas the percentage reaches 6.75%. We performed the growth curve of this strain. One ring of activated *C. alkaliphilus* plaque was inoculated into the 50 mL LB liquid medium, incubated at 37°C, 180 rpm for 16 h, and reserved for use. The sterilized LB liquid medium was divided into 18 100 mL sterilized triangular bottles, each filled with 20 mL of liquid, sealed with air-permeable sealing film, and labeled and numbered; one bottle was labeled as the control and stored at 4°C, and the other 17 bottles were labeled as 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 h. Each bottle was inoculated with the above-mentioned bacterial seed solution at an inoculum of 10% (V/V), and shake cultivation was performed for 16 h at 37°C and 180 rpm and the corresponding bottles were taken out according to the labels at the corresponding time and then preserved in the refrigerator at 4°C for the measurement of the OD₆₀₀ nm values by photoelectrophoresis turbidimetry. Three parallel groups were established at each time point. The measurements were performed until a plateau was reached.

The LB liquid medium was prepared by adding CuSO₄·5H₂O so that the Cu²⁺ concentration gradient was 5, 7.5, 10, 20, 25, and 30 mg/L (in terms of pure Cu²⁺), and 17 triangular flasks of each concentration were labeled as 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 h. In addition, the LB liquid medium without any heavy metal ions was used as the control, and the pre-cultured bacterial solution was inoculated into the above medium at an inoculum of 10% (V/V) and placed on a shaker (180 rpm) at a constant temperature (37°C) for the shake flask culture. The corresponding flasks were taken out at the corresponding times according to their labels and stored in the refrigerator at 4°C. Three parallel groups were established at each time point. Measurements were taken until a plateau was reached.

2.8 Measuring bacterial growth by the photo turbidimetric method and plotting growth curves and growth histograms

After sampling the bacterial growth culture solution, the wavelength of the UV-visible spectrophotometer was adjusted to 600 nm. The OD_{600nm} value of the bacterial solution of *C. alkaliphilus*

² <https://www.Genescloud.cn>

at different incubation times was determined, and the average value of each sample was taken thrice. The range of the spectrophotometer was 0.0000–3.0000; if the mass concentration of the bacterial suspension was too large, it was diluted at appropriate times, and the incubation time, dilution times, and OD_{600nm} value were recorded. The incubation time of *C. alkaliphilus* served as the horizontal coordinate, the average OD_{600nm} value of the measured bacterial solution was used as the vertical coordinate, and the growth curve of *C. alkaliphilus* was drawn. Using the Cu²⁺ concentration as the horizontal coordinate and the OD_{600nm} value as the vertical coordinate, the histogram of the maximum amount of growth of Cu²⁺-*C. alkaliphilus* was plotted.

3 Results

3.1 Cultivable fungi isolation, cultivation, and identification

In July 2022, we collected three soil samples from the excavation, canopy, restoration area, and restoration area surface in the exhibition hall of the Terra Cotta Warriors Burial Pit No. 1 of the Emperor Qinshihuang's Mausoleum Site Museum. A total of 12 samples were collected. Following separation and purification, 28 cultivable fungi were identified and classified. Of these, 16 were identified in previous experiments (Qiang et al., 2021) and may be the main pathogenic fungi, as shown in Table 2. The plate photographs and photomicrographs of the fungi isolated from the soil are presented in Figure 2.

3.2 Fungal community analysis

The microbial diversity results obtained from the above-mentioned 12 samples were analyzed. The results are shown in Figure 3, where they rank among the top 20 in terms of genus-level

abundance. *Penicillium*, *Aspergillus*, *Lipomyces*, *Alternaria*, and *Fusarium* accounted for the largest proportion. Among the 20 genera present in the largest proportions, *Penicillium*, *Aspergillus*, *Alternaria*, *Fusarium*, *Talaromyces*, and *Trichoderma* were obtained in isolation and purified. The analysis of the microbial community composition in the 12 soil samples showed that the top ten microorganisms in abundance were all bacteria, as shown in the Figure 3. Among these, *Nocardioide*s, *Amycolatopsis*, *Arthrobacter*, *Pseudonocardia*, and *Streptomyces* accounted for the largest proportion. The abundance of fungi and bacteria in the soil suggests the potential harm to soil sites and even to the Qin Terracotta Warriors.

TABLE 2 Identification results of cultivable fungi isolated and purified from soil samples.

QWS-1	<i>Penicillium chrysogenum</i>	100.00%	MK841451.1
QWS-2	<i>Simplicillium aogashimaense</i>	100.00%	MT860448.1
QWS-3	<i>Penicillium corylophilum</i>	99.89%	MH876147.1
QWS-4	<i>Aspergillus niger</i>	99.82%	KT192262.1
QWS-5	<i>Aspergillus versicolor</i>	99.80%	KP940477.1
QWS-6	<i>Aspergillus flavus</i>	99.42%	MT509808.1
QWS-7	<i>Aspergillus sydowii</i>	99.69%	KJ524908.1
QWS-8	<i>Aspergillus puulaauensis</i>	99.68%	AP024448.1
QWS-9	<i>Alternaria alternata</i>	99.69%	KT207688.1
QWS-10	<i>Fusarium proliferatum</i>	98.27%	MG274295.1
QWS-11	<i>Fusarium fujikuroi</i>	99.90%	CP023090.1
QWS-12	<i>Fusarium solani</i>	99.77%	MT579869.1
QWS-13	<i>Fusarium incarnatum</i>	100.00%	MT560229.1
QWS-14	<i>Talaromyces funiculosus</i>	99.77%	MH876669.1
QWS-15	<i>Trichoderma longibrachiatum</i>	100.00%	MT520608.1
QWS-16	<i>Trichoderma harzianum</i>	99.27%	MN611195.1

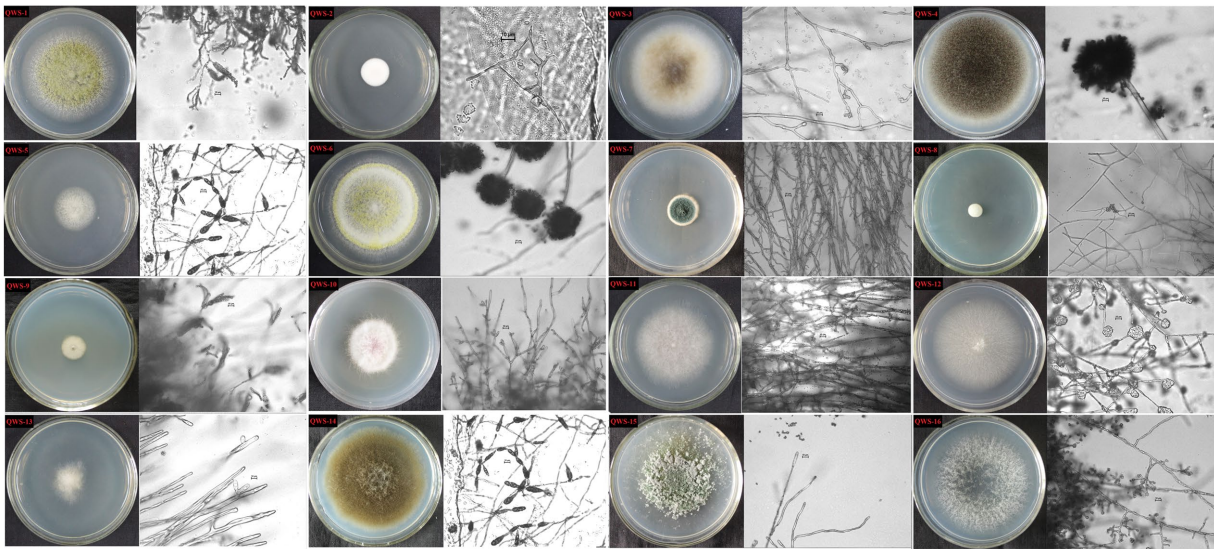


FIGURE 2 Plate photos and micrographs of isolated and purified fungi (Inner diameter of the medium is 5cm, The scale in the micrograph is 10µm).

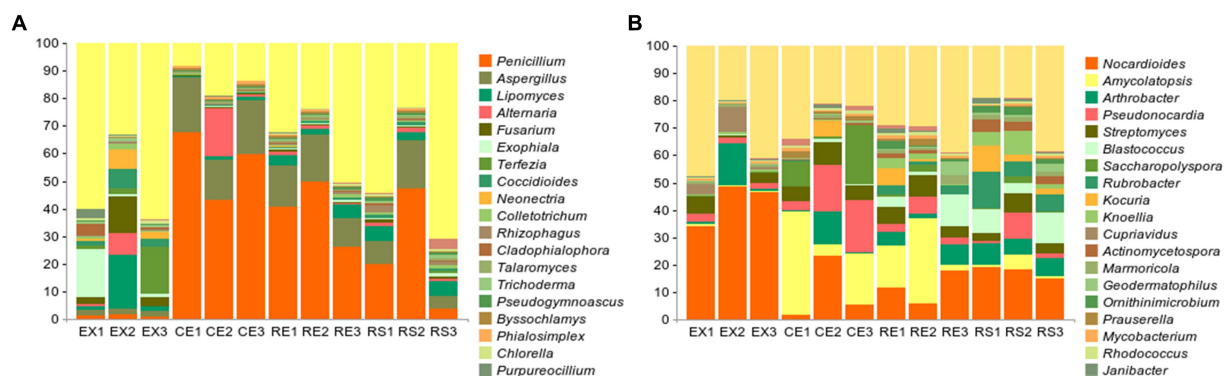


FIGURE 3
Microbial diversity results of soil samples (A represents fungal genus-level diversity results, B represents bacterial genus-level diversity results).

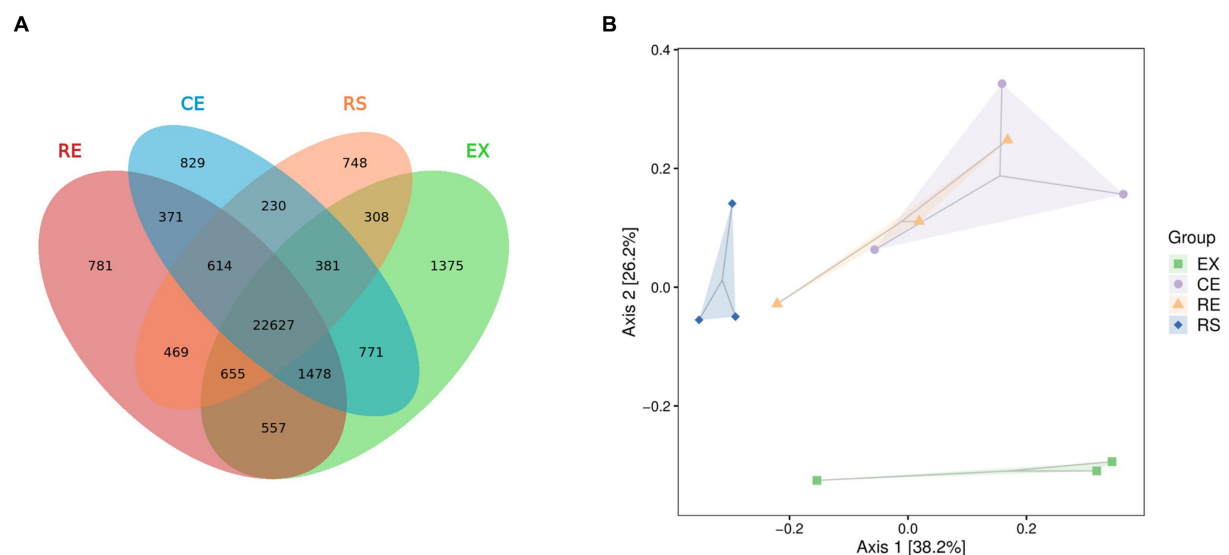


FIGURE 4
Analysis of the number and beta diversity of common and unique species groups in soil samples from four regions. (A is the Venn diagram, B is the PCoA diagram).

Venn plots of the number of common and unique species groups in the collected samples from the four regions were drawn, and PCoA analysis on their beta diversity was performed to quantify the differences between the samples. From Figure 4 shows that the excavation area contains more unique species, whereas the excavation area (EX) and canopy area (CE) contain more of the same species. There were significant differences in species between the excavation area (EX) and surface area (RS) of the restoration area and other groups. The species group differences between the two samples in the canopy area (CE) and restoration area (RE) were relatively small, and the similarity was relatively high.

3.3 Analysis of the functional diversity of microorganisms

The KEGG Metabolic Pathway Database is the core database of KEGG. This database systematically categorizes metabolic pathways into seven major categories: metabolism, genetic information

processing, environmental information processing, cellular processes, organic systems, human diseases, and drug development. By integrating the annotated the KO database of soil microorganisms with the gene abundance matrix, the top three pathways ranked in abundance were found to be related to metabolism, genetic information processing, and cellular processes. The corresponding KO abundance of each protein was obtained, as shown in Figure 5A.

The EggNOG database is managed by the European Molecular Biology Laboratory, which uses the Smith-Waterman comparison algorithm to annotate the functions of orthologous groups of genes. A relative abundance diagram is shown in Figure 5B. The top three are amino acid transportation and metabolism, energy production and conversion, replication, recombination, and restoration area. Using the EggNOG annotation of soil sample microorganisms, the bias and composition of microbial protein production in excavated soil can be analyzed, providing data support for various mechanisms and functions of research.

The CAZy database focuses on carbohydrate-active enzymes that degrade, modify, and generate glycosidic bonds. This is a professional

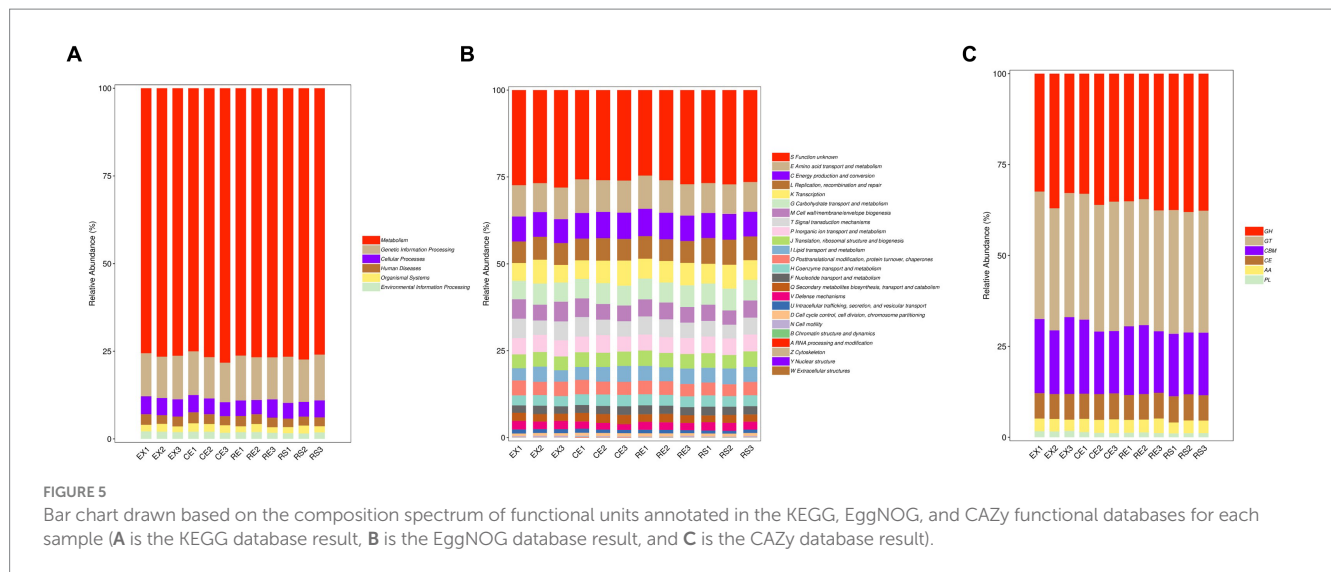


FIGURE 5

Bar chart drawn based on the composition spectrum of functional units annotated in the KEGG, EggNOG, and CAZy functional databases for each sample (A is the KEGG database result, B is the EggNOG database result, and C is the CAZy database result).

database used to study related enzymes. Based on the similarity of amino acid sequences in protein domains and their corresponding functions, CAZy divides these carbohydrate-active enzymes into six major functional protein modules: glycoside hydrolases, glycosyl transferases, polysaccharide lyases, carbohydrate esterases, auxiliary oxidoreductases, and carbohydrate-binding modules. A relative abundance diagram is shown in Figure 5C. The top three modules were glycoside hydrolase, glycosyltransferase, and carbohydrate-binding modules. By annotating the CAZy database of soil microorganisms in excavation pits, the bias and composition of microbial carbohydrate enzyme systems can be fully analyzed, providing data support for the study of pathways such as microbial degradation and metabolism.

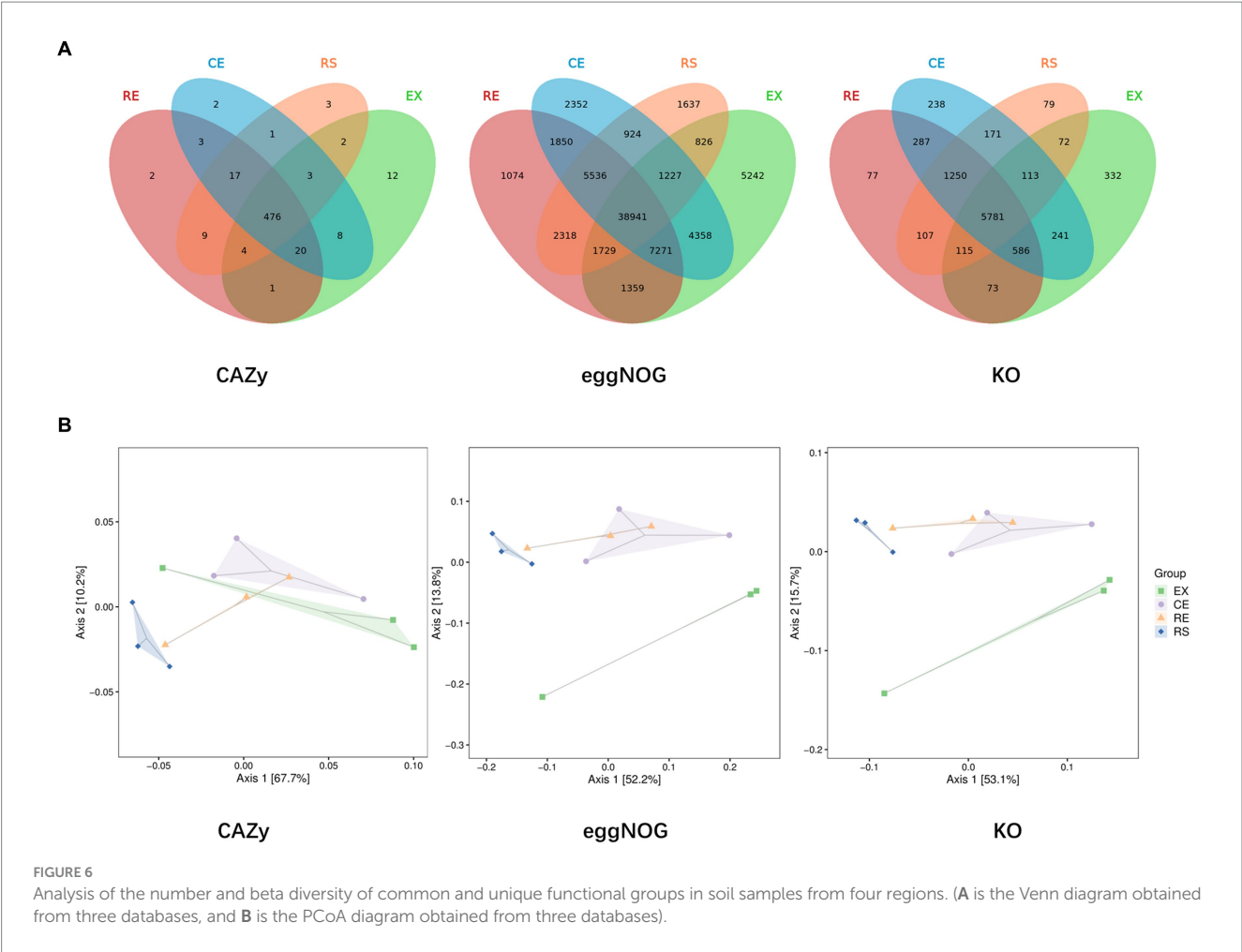
Venn plots of the number of common and unique functional groups in the collected samples from the four regions were drawn, and PCoA analysis on their beta diversity was performed to quantify the differences between the samples, as shown in Figure 6. The excavation area contains a relatively large number of unique species and functional components. The species group differences between the two samples in CE and RE were relatively small, and the similarity was relatively high.

3.4 Correlation analysis between microorganisms and soil physical and chemical properties

The soil macroelement content was the lowest in the remediation area, but no significant differences among the sampling points were found, as shown in Figure 7A. The soil moisture content, pH, and microbial biomass carbon content in the excavation area were significantly higher than those at the other sampling points, indicating that soil conditions, such as humidity and pH, are more conducive to microbial growth in the area, as shown in Figures 7B,C. The activity of soil carbon-related hydrolase is the highest in the surface sampling area. In particular, α - and β -glucosidases indicate that the hydrolysis of soil organic matter in this area increases, which is not conducive to the stability of soil mass, as shown in Figure 7D. This result was consistent with the metagenomic sequencing results from the CAZy database. Similar to carbon-related hydrolase, the activity of soil phosphorus-related hydrolase was the highest at the surface sampling point, followed by the excavation area, which proves that the hydrolysis of soil organic matter in this area

increased, which is not conducive to land stability, as shown in Figure 7E. The proportion of silt particles in the soil at each sampling point was highest, followed by those of sand and clay particles. The proportion of silt particles in the soil of the excavation area was the highest, whereas that of clay particles decreased. The risk of soil erosion in this area was high, as shown in Figure 7F. Available nutrients in the soil are crucial for the growth of soil microorganisms. There was no significant difference in the alkali-hydrolyzed nitrogen content of the soil at each sampling point. The soil of the excavation and canopy area had the highest contents of available phosphorus and potassium, which could provide effective nutrients for microbial activities, as shown in Figure 7G. Due to the alkalinity of the soil, there is a carbonation reaction when adding acid, which increases the determination of soil organic carbon content. The results showed that the total carbon content of the soil was 11.88–15.27%, whereas the organic carbon content was only 3.39–11.32%. In particular, in the excavation area, the organic carbon content was only 21.9% of the total carbon content, as shown in Figure 7H.

The following environmental factors most closely related to microbial growth activities were screened: α -glucosidase activity (α) and β -glucosidase activity (β). The correlation between water content (H_2O), soil pH (pH), microbial biomass carbon (MBC), available phosphorus (P), and available potassium (K) was analyzed with the genera of dominant fungi and bacteria, and a heat map was drawn (Figure 8). Soil α -glucosidase activity, cellobiose hydrolase, alkaline phosphomonoesterase, microbial biomass carbon, and organic carbon in the total carbon ratio significantly correlated with the content of dominant fungi and were mostly positively correlated. There was a significant and mostly negative correlation between the soil moisture content and dominant fungal content. *Penicillium*, *Aspergillus*, *Talaromyces*, *Filamentomyces*, and *Phialosimplex* differed from the other genera. Soil α -glucosidase activity, available phosphorus, available potassium, water content, microbial biomass carbon, and powder and copper content significantly correlated with the content of dominant bacteria, with most of them being negatively correlated. Soil β -glucosidase activity, phosphodiesterase, sand content, cadmium ion concentration, and selenium ion concentration significantly correlated with the content of dominant bacteria, with most of them being positively correlated. The relationship between *Nocardioides* and *Cupriavidus* and soil properties was more specific. The correlation analysis of soil property factors with dominant fungi and bacteria provided data support for analyzing the



function of microorganisms in the soil of the Emperor Qinshihuang's Mausoleum Site Museum and their impact on soil sites.

3.5 Antimicrobial experiment of allicin volatile gas

Dichotomy was used to determine the antifungal effect of allicin volatile gas. Sterile distilled water was used as the negative control. The inhibitory effect was observed when the air concentration of allicin was 625 $\mu\text{L/L}$. As shown in Figure 9, under normal growth conditions of fungal hyphae in the negative control group, allicin gas had a potent inhibitory effect on the growth of the most culturable fungal hyphae. Allicin gas had a potent inhibitory effect on 12 types of fungi.

3.6 Growth curve and growth volume histogram of *Cupriavidus alkaliphilus*

The culture of *C. alkaliphilus* was carried out by traditional shaking cultivation, and its growth was measured by the phototurbidimetric method at OD₆₀₀ nm using an uninoculated pure medium as a negative control. The growth curves and amounts of *C. alkaliphilus* at different Cu²⁺ concentrations are shown in Figure 10. With an increase in Cu²⁺ concentrations, the acclimatization period of *C. alkaliphilus* was

significantly prolonged, and its growth in the culture containing Cu²⁺ was lower than that in the culture without copper ions.

4 Discussion

We analyzed the physical and chemical properties and bioindicators of soil samples from earthen sites in the exhibition hall of the Terra Cotta Warriors burial pit No. 1 of the Emperor Qinshihuang's Mausoleum Site Museum. A metagenomic library was established, the species diversity of the microbial community was analyzed, and functional diversity was predicted. Simultaneously, we analyzed the correlation between the microbial community composition and environmental factors. This study provides basic data for the prevention and treatment of microbial diseases in soil sites in the exhibition hall of the Terra Cotta Warriors burial pit No. 1 of the Emperor Qinshihuang's Mausoleum Site Museum and is of great significance for protecting the soil site museum.

4.1 Analysis of microbial diseases in earth site of Terra Cotta Warriors Pit No.1

The mausoleum of Emperor Qin Shi Huang was built more than 2,000 years ago. The Terra Cotta Warriors were discovered in March 1974, and excavations began in July. The No. 1 Pit has been

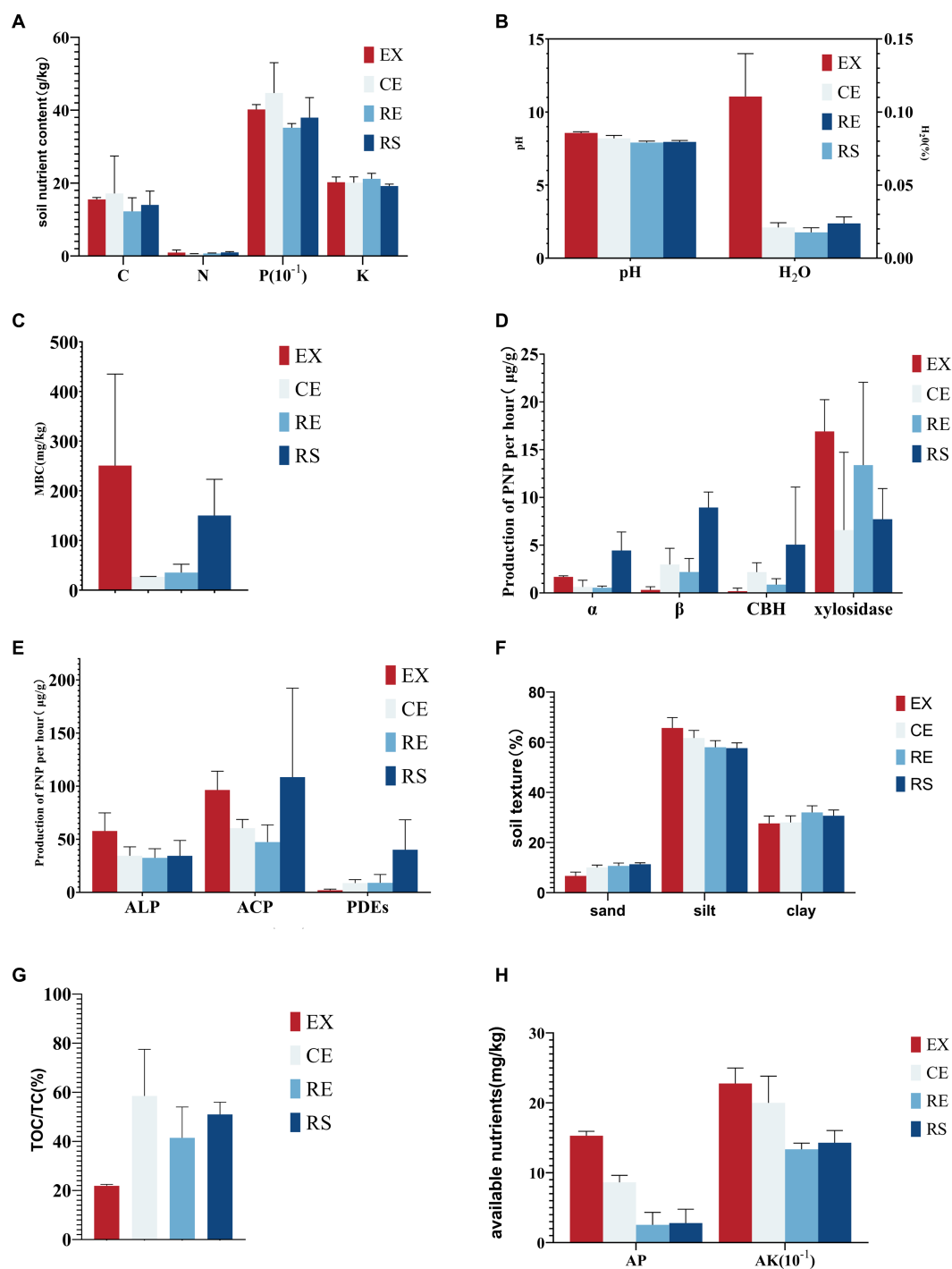


FIGURE 7

Results of the chemical, physical, and bioindicator determination of soil samples (A is the content of soil macroelements, B is the soil water content and pH, C is the microbial biomass, D is the activity of soil carbon-related hydrolase, E is the activity of soil phosphorus-related hydrolase, F is the soil texture, G is the proportion of soil organic carbon to the total carbon content, and H is the content of soil available nutrients).

excavated three times on large scales (Ma et al., 2016). The exhibition hall of the no. 1 Pit is the earliest semi-open exhibition hall built and opened to the public at the Emperor Qinshihuang's Mausoleum Site Museum. The exhibition hall is a steel-frame building. Apart from the necessary measures to avoid sunlight and rain, there are no other environmental protection measures. Sunlight can directly illuminate the site hall through glass

windows, causing significant changes in temperature and humidity in areas exposed to sunlight, may also cause the growth of some photosynthetic microorganisms (Dexing, 2013; Binyaseen, 2022). Owing to the large space in the exhibition hall, effective temperature and humidity controls cannot be implemented. With an increasing number of tourists and the entry of archaeological excavation personnel, many microorganisms and various coarse

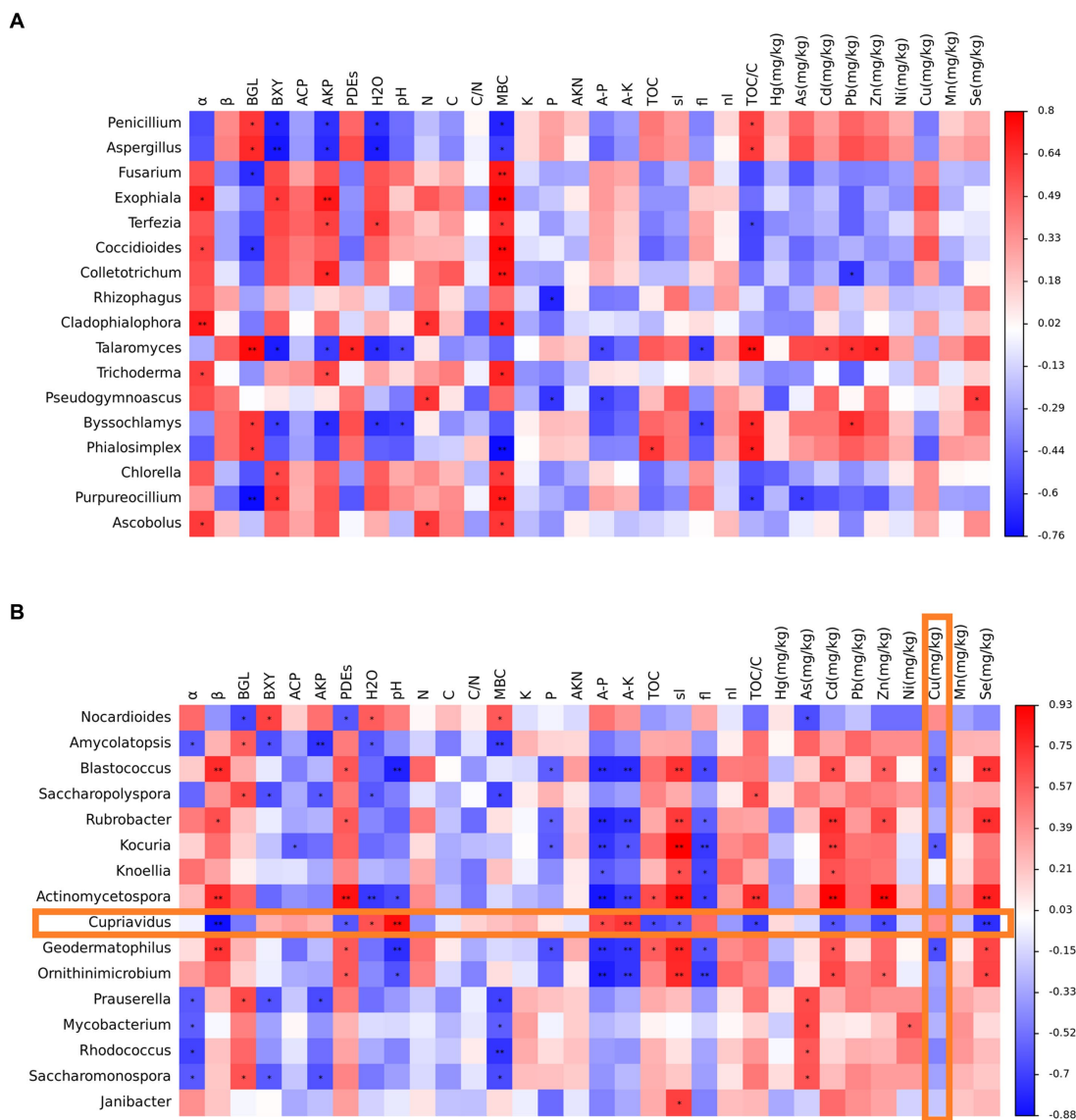


FIGURE 8

Analysis results of the correlation between dominant fungi and dominant bacteria with relevant environmental factors (A represents fungal results, B represents bacterial results, *: $p < 0.05$, **: $p < 0.01$).

and fine particles have gathered in the exhibition hall of Pit 1. The aggregation of flocs further accumulates dark sediments on the surface of the earthen site, leading to further microbial contamination. Early scholars conducted targeted research. Zhang et al. speculated that the surface sediment of the site and cultural relics came from clothing fibers worn by many tourists. Li et al. suggested that dust discharge from nearby industries, power plants, and civilian furnaces also adds to the dust from Pit 1. Furthermore, several poplar and willow trees are planted around the South Ring Road of Pit 1 (Li Hua and Du Weisha, 2019). In the canopy and restoration areas, especially on the surface of the restoration area, these flocs can be observed to gather in large quantities, whereas less flocs accumulates in the excavation area, because of its low elevation and state of excavation. Therefore, PCoA revealed that the species composition of the excavation area differed significantly from that of the other three areas.

4.2 Correlation between dominant fungi and bacteria in Terra Cotta Warriors Pit No. 1 and environmental factors

Using heat maps, the GenesCloud tool was used to visualize the correlation between dominant species and environmental factors in each sample. Because all four areas are located in the same exhibition hall, some of the physical properties of the soil are relatively similar. In our study, α -glucosidase activity (α), β -glucosidase activity (β), the water content, soil pH, microbial biomass carbon, available phosphorus, and available potassium are closely related to microbial growth activities. The relationship between *Cupriavidus* and soil properties was more specific. Many copper weapons were unearthed during the excavation of the Terra Cotta Warriors; therefore, there was high copper content in the soil, especially in the excavation area. *Cupriavidus* sp. was identified as a *Cupriavidus alkaliphilus* in later identification experiments, and it

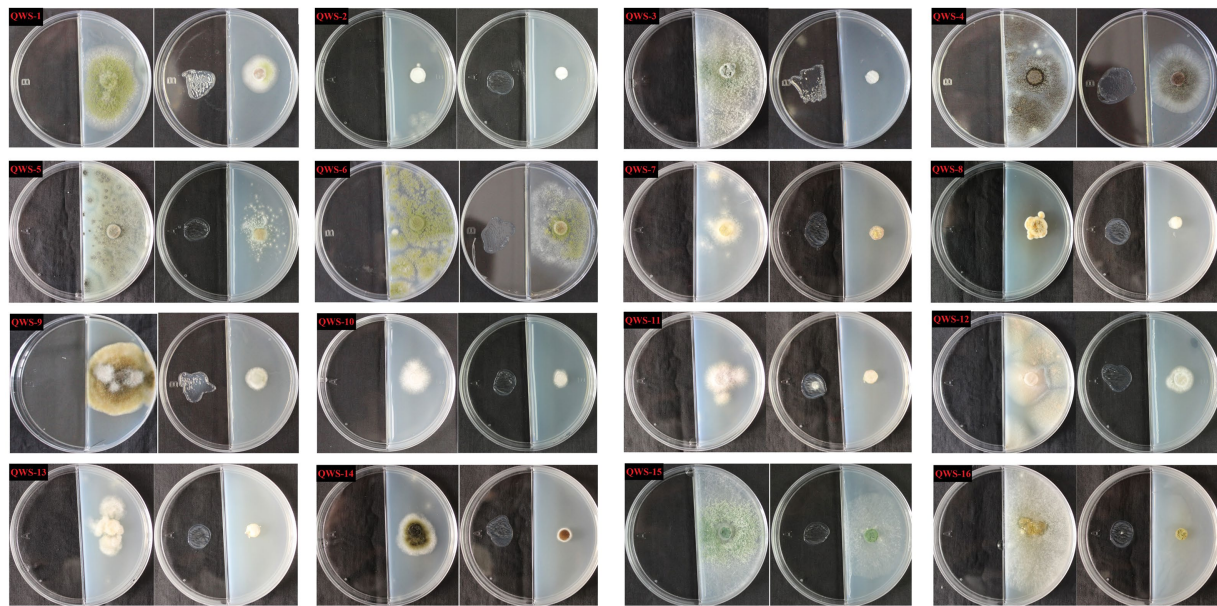


FIGURE 9
Antifungal effect of volatile alliin gas determined by dichotomy (Inner diameter of the medium is 9 cm) Fungal moss is 8mm in diameter).

has genes for resistance to nickel, zinc, arsenate, chromate, cobalt, cadmium, and copper, as well as genes for the production of secondary metabolites, such as bacteriocins, non-ribosomal synthesizing peptides, iron carriers, and polyketides (Rojas et al., 2016). A large group of genes essential for resisting and detoxifying several heavy metals has also been identified (Kutralam-Muniasamy and Pérez-Guevara, 2019). Several genes are involved in the resistance to and detoxification of heavy metals in copper-greedy bacteria. Therefore, *C. alkaliphilus* can potentially harm the metal cultural relics unearthed in the burial pits of the Terra Cotta Warriors.

4.3 Possible potential hazards of *Cupriavidus alkaliphilus* on copper artifacts

In our experiments, *C. alkaliphilus* was cultured using media containing different concentrations of Cu^{2+} . When lower Cu^{2+} were present, *C. alkaliphilus* had a shorter logarithmic period of growth and a relatively faster growth rate. The acclimatization period of *C. alkaliphilus* appeared to be significantly prolonged with increasing concentrations of Cu^{2+} . The growth level of *C. alkaliphilus* was lower in cultures containing Cu^{2+} stress compared to those without Cu^{2+} ions. The presence of *C. alkaliphilus* could potentially be harmful to metallic artifacts excavated from the burial pits of the Terracotta Warriors.

4.4 The application value of alliin as an antibacterial agent in the protection of cultural relics

Choosing plant-based antifungal agents from actual plants is safer and more environmentally friendly. In recent years, domestic and international research has revealed multiple known

and novel structural types of active plant-derived antibacterial ingredients. It is ideal for developing plant-based antibacterial agents and has received increasing attention from scholars. Alliin ($\text{C}_6\text{H}_{10}\text{OS}_2$) is an organosulfur compound with the scientific name of diallyl thiosulfinate, is the main bioactive component of garlic and exhibits broad-spectrum antibacterial and antifungal activity. In this study, we used the dichotomy method to determine the antifungal effect of volatile alliin gas, which has a potent inhibitory effect on the mycelial growth of most fungi. However, owing to the odor of alliin itself, we still need to further improve this method by reducing the concentration of alliin. However, because alliin has a potent inhibitory effect on the mycelial growth of most fungi, it can be regarded as a reference for the prevention of microbial diseases in soil sites and cultural relics.

In the different areas of the exhibition hall of Pit 1, there are terracotta warriors that are excavated, restored and conserved. Therefore, the dominant fungi found in the earthen ruins may be the cause of microbial damage to the precious cultural relics of the Terra Cotta Warriors, with potential risks. Therefore, fungi discovered at soil sites must be taken seriously by conservationists. This study is of great significance for the protection of the Terra Cotta Warriors and other precious cultural relics in the Emperor Qinshihuang's Mausoleum Site Museum.

5 Conclusion

The problem of microbial diseases in the earthen sites in the exhibition hall of burial pit No. 1 of the Terra Cotta Warriors in the Emperor Qinshihuang's Mausoleum Site Museum is cannot be ignored. The microbial diseases in different areas of the exhibition hall differ because of the different environments. The main pathogenic fungi in the earthen sites may cause microbial

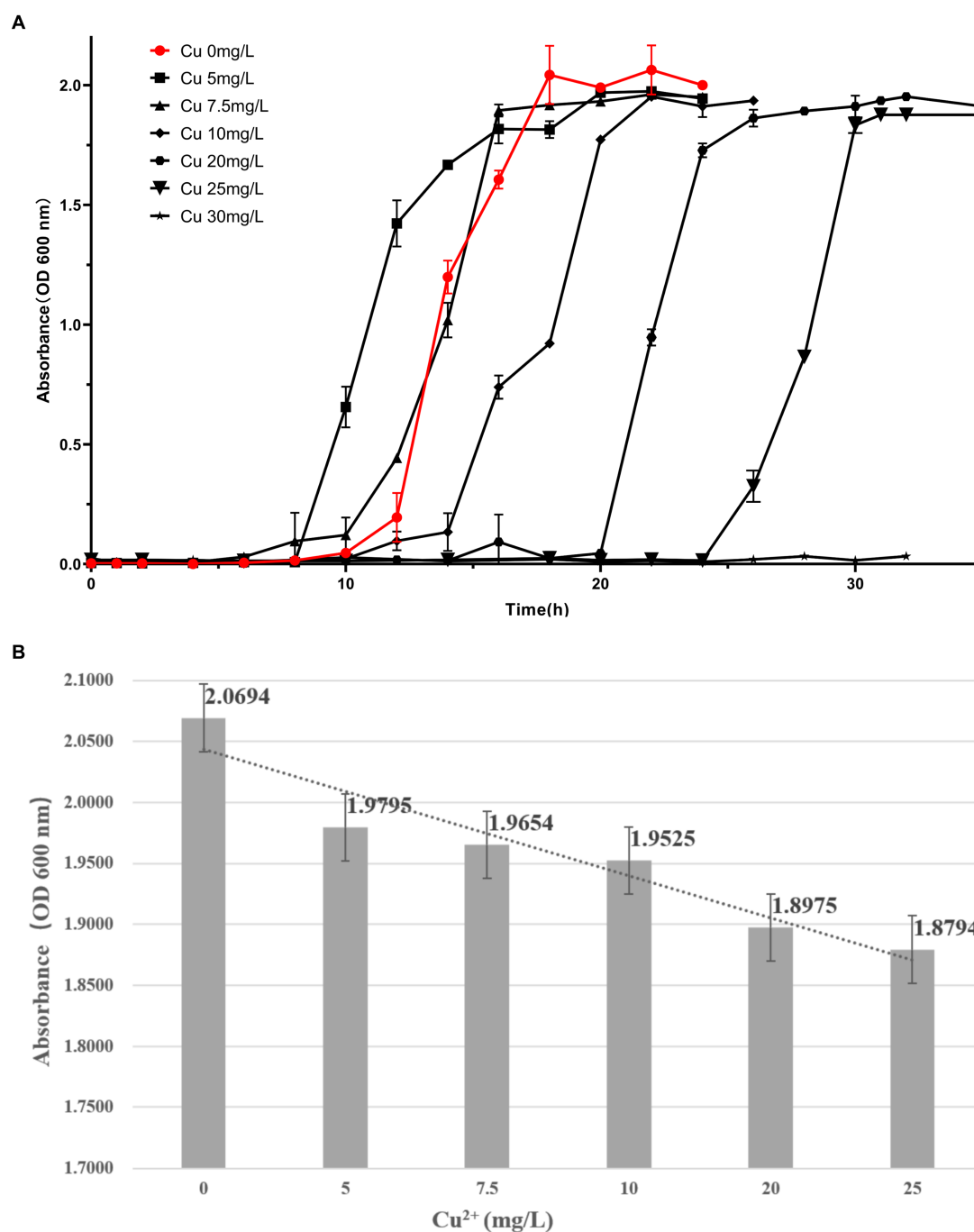


FIGURE 10
Growth curves and histograms of copper ion stress in the growth of *Cupriavidus alkaliphilus*.

diseases that affect essential cultural relics such as the Terra Cotta Warriors. *Cupriavidus alkaliphilus* is abundant in the soil of the excavation areas of exhibition halls and may pose a potential hazard to excavated metallic artifacts. Allicin is expected to become an effective bacteriostatic agent to prevent cultural relics, such as the earthen sites and Terra Cotta Warriors, from being persecuted by disease microorganisms. This study provides basic data on the microbiological problems of earthen sites in the exhibition hall of the Terra Cotta Warriors Burial Pit No. 1 in the Emperor Qinshihuang's Mausoleum Site Museum, which is of

great significance for the protection of the sites as well as for the protection of the Terra Cotta Warriors and other precious cultural relics in the museum.

Data availability statement

The raw sequence data (amplicon and metagenomic datasets) were deposited into the NCBI Sequence Read Archive (SRA) database BioProject (Accession Number: PRJNA1047174).

Author contributions

CW: Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. LH: Methodology, Software, Validation, Writing – review & editing. NJ: Data curation, Software, Validation, Writing – review & editing. YuW: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. XM: Investigation, Writing – review & editing. PZ: Investigation, Writing – review & editing. YX: Investigation, Writing – review & editing. YuaW: Data curation, Writing – review & editing. CC: Methodology, Validation, Writing – review & editing. XY: Software, Validation, Writing – review & editing. QL: Project administration, Supervision, Writing – review & editing. JP: Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by “Youth Open Projects supported by the Qinshihuang’s Mausoleum Site Museum” and the Fundamental Research Funds for the Central Universities from University of Science and Technology Beijing.

References

- Alam, K., Abbasi, M. N., Hao, J., Zhang, Y., and Li, A. (2021). Strategies for natural products discovery from uncultured microorganisms. *Molecules* 26:2977. doi: 10.3390/molecules26102977
- Al-Qahtani, W. H., Dinakarkumar, Y., Arokiyaraj, S., Saravanakumar, V., Rajabathar, J. R., Arjun, K., et al. (2022). Phyto-chemical and biological activity of *Myristica fragrans*, an ayurvedic medicinal plant in southern India and its ingredient analysis. *Saudi J. Biol. Sci.* 29, 5, 3815–3821. doi: 10.1016/j.sjbs.2022.02.043
- Basile, A., Sorbo, S., Spadaro, V., Bruno, M., Maggio, A., Faraone, N., et al. (2009). Antimicrobial and antioxidant activities of coumarins from the roots of *Ferulago campestris* (Apiaceae). *Molecules* 14, 939–952. doi: 10.3390/molecules14030939
- Binyaseen, A. M. (2022). Improving thermal performance of existing uninsulated R. C. Domes through passive cooling measures using polyurethane foam in double skin layer in hot climate. *Case Stud. Constr. Mater.* 16:e00866. doi: 10.1016/j.cscm.2021
- Briseghella, B., Colasanti, V., Fenu, L., Nuti, C., Spacone, E., and Varum, H. (2020). Seismic analysis by macroelements of Fujian hakka tulou, chinese circular earth constructions listed in the UNESCO world heritage list. *Int. J. Architect. Herit.* 14, 1551–1566. doi: 10.1080/15583058.2019.1618973
- Carini, P., Delgado-Baquerizo, M., Hinkley, E. S., Holland-Moritz, H., Brewer, T. E., Rue, G., et al. (2020). Effects of spatial variability and relic DNA removal on the detection of temporal dynamics in soil microbial communities. *MBio* 11, e02776–e02719. doi: 10.1128/mBio.02776-19
- Chonggen, P., Keyu, C., Danting, C., Sancai, X., Jian, G., and Dayong, Z. (2020). Research progress on in-situ protection status and technology of earthen sites in moist environment. *Constr. Build. Mater.* 253. doi: 10.1016/j.conbuildmat.2020.119219
- Dexing, L. (2013). Diseases and prevention of Terra cotta warriors newly unearthed in pit 1 of the terracotta warriors. *Relics and Museology* 4, 77–84.
- Elena, Z., Goerlitz David, S., Tighe Scott, W., Wagner Nicole, Y., Yu, B., Hall Brenda, L., et al. (2019). Antarctic relic microbial mat Community revealed by metagenomics and Metatranscriptomics. *Front. Ecol. Evol.* 7:1. doi: 10.3389/fevo.2019.00001
- Germinario, L., and Oguchi, C. T. (2021). Underground salt weathering of heritage stone: lithological and environmental constraints on the formation of sulfate efflorescences and crusts. *J. Cult. Herit.* 49, 85–93. doi: 10.1016/j.culher.2021.02.011
- Gu, Z. L., Luo, X. L., MengX, Z., Wang, Z. S., Ma, T., Yu, C., et al. (2013). Primitive environment control for preservation of pit relics in archaeology museums of China. *Environ. Sci. Technol.* 47, 1504–1509. doi: 10.1021/es303981m
- He, X., Wang, S., and Zhang, B. (2021). A semi-theoretical model for water condensation: dew used in conservation of earthen heritage sites. *Water* 13:52. doi: 10.3390/w13010052
- Huang, X., Han, Y., Du, J., Guo, P., Wang, Y., Ma, K., et al. (2021). Inhibitory effect of Cinnamaldehyde on Main destructive microorganisms of Nanhai no. 1 shipwreck. *Appl. Sci.* 11:5262. doi: 10.3390/app11115262
- Huang, F., Zheng, X., Ma, X., Jiang, R., Zhou, W., Zhou, S., et al. (2019). Theabrownin from Pu-erh tea attenuates hypercholesterolemia via modulation of gut microbiota and bile acid metabolism. *Nat. Commun.* 10:4971. doi: 10.1038/s41467-019-12896-x
- Jiang, X. Y., Cao, L. X., and Zhang, R. D. (2013). Effects of addition of nitrogen on soil fungal and bacterial biomass and carbon utilisation efficiency in a city lawn soil. *Soil Res.* 52, 97–105. doi: 10.1071/SR13210
- Jiaxian, S., Mc Farland Alexander, G., Young Vincent, B., Hayden Mary, K., and Hartmann Erica, M. (2021). Toward accurate and robust environmental surveillance using metagenomics. *Front. Genet.* 12. doi: 10.3389/fgene.2021.600111
- Jing, L., Tao, Y., Yue, L., Chengyan, Z., Yuan, Y., Yuxia, Z., et al. (2019). Diversity and speculative metabolism of bacteria from soil of Jinsha earthen site. *Microbiology China* 46, 3205–3215. doi: 10.13344/j.microbiol.china.190080
- Jintao, H., Nan, Z., Muhammad, A., Xiaoqiang, S., Chao, S., Qiang, L., et al. (2022). From surviving to thriving, the assembly processes of microbial communities in stone biodeterioration: a case study of the West Lake UNESCO world heritage area in China. *Sci. Total Environ.* 805:150395. doi: 10.1016/j.scitotenv.2021
- Kong, D., Wan, R., Jianxun, C., Yanlin, J., Weiming, H., and Wang, Y. (2019). The study on engineering characteristics and compression mechanisms of typical historical earthen site soil. *Constr. Build. Mater.* 213, 386–403. doi: 10.1016/j.conbuildmat.2019.04.028
- Kutralam-Muniasamy, G., and Pérez-Guevara, F. (2019). Comparative genome analysis of completely sequenced Cupriavidus genomes provides insights into the biosynthetic potential and versatile applications of Cupriavidus alkaliphilus ASC-732. *Can. J. Microbiol.* 65, 575–595. doi: 10.1139/cjm-2019-0027
- Li Hua, H., and Du Weisha, T. (2019). Comparison of site environments of emperor Qin’s terracotta warriors and horses museum and Han Yangling museum. *Sci. Conserv. Archaeol.* 31, 53–60. doi: 10.16334/j.cnki.cn31-1652/k.2019.02.008
- Luo Xilian, G., Zhaolin, T. W., Yin, X., and Tao, M. (2018). Experimental study of a local ventilation strategy to protect semi-exposed relics in a site museum. *Energ. Buildings* 159, 558–571. doi: 10.1016/j.enbuild.2017.12.001
- Ma, Y., Fuller, B., Sun, W., Hu, S., Chen, L., Hu, Y., et al. (2016). Tracing the locality of prisoners and workers at the mausoleum of Qin Shi Huang: first emperor of China (259–210 BC). *Sci. Rep.* 6:26731. doi: 10.1038/srep26731
- Ma, D., Luo, W., Qin, Y., Chen, D., Huang, F., and Wang, C. (2020). Study on the casting cores to identify the manufacturing place of Chinese bronze vessels excavated in the Qiaojia Yuan tombs from spring and autumn period. *Archaeol. Anthropol. Sci.* 12, 1–10. doi: 10.1007/s12520-020-01169-0

Acknowledgments

We gratefully acknowledge the technical assistance of Xingzhong Liu, Dongsheng Wei of Nankai University, Lijun Chen of Shenyang Institute of Applied Ecology, Chinese Academy of Sciences, Hong Guo of University of Science and Technology Beijing, Maosheng Shen of the Emperor Qinshihuang’s Mausoleum Site Museum.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

- Qiang, L., Shumei, Y., Hua, L., Du Weisha, J. B., Ying, Y., Xiaoxi, L., et al. (2021). Research on the flocculent materials and Fungi on the surface at the terracotta warrior pit no. 1 site. *Relics Museology* 3, 101–107.
- Richards, J., Viles, H., and Guo, Q. (2020). The importance of wind as a driver of earthen heritage deterioration in dryland environments. *Geomorphology* 369:107363. doi: 10.1016/j.geomorph.2020.107363
- Rojas, F. U., Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., et al. (2016). Draft genome sequence of heavy metal-resistant *Cupriavidus alkaliphilus* ASC-732T, isolated from *Agave* rhizosphere in the northeast of Mexico. *Genome Announc.* 4:e01013. doi: 10.1128/genomeA.01013-16
- Sauro, F., Cappelletti, M., Ghezzi, D., Columbu, A., Hong, P. Y., Zowawi, H. M., et al. (2018). Microbial diversity and biosignatures of amorphous silica deposits in orthoquartzite caves. *Sci. Rep.* 8:17569. doi: 10.1038/s41598-018-35532-y
- Thotathil, V., Sidiq, N., Fakhroo, A., and Sreerama, L. (2023). Phytochemical analysis of *Anastatica hierochuntica* and *Aerva javanica* grown in Qatar: their biological activities and identification of some active ingredients. *Molecules* 28:3364. doi: 10.3390/molecules28083364
- Tongtong, F., Wang, Y., Qinya, G., Qiang, L., Xiaoxuan, P., Xudong, Z., et al. (2017). Identification and analysis of the microbe in Lidu ancient earthen cellar. *Microbiol. China* 44, 49–58. doi: 10.13344/j.microbiol.china.160404
- Wang, Y., Wenjing, H., Yeqing, H., Xinduo, H., Cen, W., Kaixuan, M., et al. (2022). Microbial diversity of archaeological ruins of Liangzhu City and its correlation with environmental factors[J]. *Int. Biodeterior. Biodegradation* 175:105501. doi: 10.1016/j.ibiod.2022.105501
- Wei, G., Sheng, Y., Liang, Z., Heng, X., Jing, Z., Bin, W., et al. (2019). Consolidating effect of hydroxyapatite on the ancient ivories from Jinsha ruins site: surface morphology and mechanical properties study. *J. Cult. Herit.* 35, 116–122. doi: 10.1016/j.culher.2018.06.002
- Wu Fasi, S., Bomin, H. D., Chen Gangquan, Y., Zongren, Z. W., and Wanfu, W. (2012). Composition of fungi community at an archaeological excavation site in Yicheng, Shanxi. *Sci. Conserv. Archaeol.* 24, 77–83. doi: 10.16334/j.cnki.cn31-1652/k.2012.03.005
- Xia Yin, F., Fei, W. J., Wah, Y. C., and Zaolin, G. (2023). Salt enrichment and its deterioration in earthen sites in emperor Qin's mausoleum site museum, China. *Indoor Built Environ.* 32, 1862–1874. doi: 10.1177/1420326X231175347
- Xiaoying, Z., Yin, X., Lili, Z., Shangxin, Z., Shumei, Y., Qianli, R., et al. (2016). Soluble salt content and safety level in the Qin mausoleum complex earthen site pits. *Sci. Conserv. Archaeol.* 28, 19–25. doi: 10.16334/j.cnki.cn31-1652/k.2016.04.003
- Xilian, L., Bin, C., Wei, T., Juan, L., and Zhaolin, G. (2019). Experimental study on local environmental control for historical site in archaeological museum by evaporative cooling system. *Renew. Energy* 143, 798–809. doi: 10.1016/j.renene.2019.05.036
- Xingqun, Z., Zhijun, Z., and Xiaorong, Z. (1999). Investigation of the fungal flora of the Qin terracotta warriors and its harm. *Relics Museol.* 1, 76–80.
- Xudong, W., Bo, Z., Qiangqiang, P., Qinglin, G., Wenwu, C., and Fengjie, L. (2020). Experimental studies on sacrificial layer in conservation of earthen sites. *J. Cult. Herit.* 41, 74–83. doi: 10.1016/j.culher.2019.07.003
- Yang, X., Bai, S., Wu, J., Fan, Y., Zou, Y., Xia, Z., et al. (2023). Antifungal activity and potential action mechanism of Allicin against *Trichosporon asahii*. *Microbiol. Spectr.* 11:e0090723. doi: 10.1128/spectrum.00907-23
- Yang, S., Wu, L., Wu, B., Zhang, Y., Wang, H., and Tan, X. (2020). Diversity and structure of soil microbiota of the Jinsha earthen relic. *PLoS One* 15:e0236165. doi: 10.1371/journal.pone.0236165
- Yin, X., Man, L., Zhang Shangxin, F., Qianli, H. J., Shumei, Y., Sun Zhichao, X., et al. (2013). Prevention and treatment of soluble salt and fungal damage to earthen ruins in site museums. *Sci. Conserv. Archaeol.* 25, 114–119. doi: 10.16334/j.cnki.cn31-1652/k.2013.018
- Yumin, D., Wenwu, C., Kai, C., and Kewen, Z. (2020). Study on damage assessment of earthen sites of the Ming Great Wall in Qinghai Province based on fuzzy-AHP and AHP-TOPSIS. *Int. J. Architect. Herit.* 14, 903–916. doi: 10.1080/15583058.2019.1576241
- Zaouri, N., Jumah, M. R., Cheema, T., and Hong, P. -Y. (2020). Metagenomics-based evaluation of groundwater microbial profiles in response to treated wastewater discharge. *Environ. Res.* 180:108835. doi: 10.1016/j.envres.2019.108835
- Zhang, P., Chen, Z. L., Yang, H. Y., and Wang, H. (2009). On utilization of underground space to protect historical relics model, Tunn. Undergr. Space Technol. 24, 245–249. doi: 10.1016/j.tust.2008.09.001
- Zhang, Y., Zhang, Z., Zhao, W., and Li, Q. (2022). Crack segmentation on earthen heritage site surfaces. *Appl. Sci.* 12:12830. doi: 10.3390/app122412830
- Zhu, L. D., and Kan, A. (2009). Research on the 3D modeling and spatial patterns of archaeological stratigraphy: a case study of the Jinsha site. *Int. Conf. Inf. Sci. Eng.* 17, 5406–5409. doi: 10.1109/ICISE.2009.999
- Zhuangbo, F., Xilian, L., Junqi, W., and Shijie, C. (2022). Energy-efficient preservation environment control for enclosed exhibition hall of earthen relics. *Energ. Buildings* 256:111713. doi: 10.1016/j.enbuild.2021.111713



OPEN ACCESS

EDITED BY

Sandra Gabriela Gomez De Saravia,
National University of La Plata, Argentina

REVIEWED BY

Alejandra Teresa Fazio,
University of Buenos Aires, Argentina
Silvia Elena Rastelli,
National University of La Plata, Argentina

*CORRESPONDENCE

Rui Wen
✉ rwen80@163.com

RECEIVED 31 August 2024

ACCEPTED 13 November 2024

PUBLISHED 10 December 2024

CITATION

Chen R, Xie Z, Xiao Q, Wang C and
Wen R (2024) Insight into the microbial
diversity and community in the sacrificial pits
of Sanxingdui site (Sichuan, China).
Front. Microbiol. 15:1489025.
doi: 10.3389/fmicb.2024.1489025

COPYRIGHT

© 2024 Chen, Xie, Xiao, Wang and Wen. This
is an open-access article distributed under
the terms of the [Creative Commons
Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use,
distribution or reproduction in other forums is
permitted, provided the original author(s) and
the copyright owner(s) are credited and that
the original publication in this journal is cited,
in accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Insight into the microbial diversity and community in the sacrificial pits of Sanxingdui site (Sichuan, China)

Ruru Chen¹, Zhenbin Xie², Qing Xiao², Chong Wang² and
Rui Wen^{1*}

¹School of Cultural Heritage, Northwest University, Xi'an, China, ²Sichuan Institute of Cultural Relics and Archaeology, Chengdu, China

Introduction: The Sanxingdui site (Sichuan, China) is the typical representative of the ancient Shu culture, which lasts from the late Neolithic to early Western Zhou. The sacrificial pits are located in the core region of Sanxingdui site, and numerous artifacts are unearthed including ivory, seashells, bronzes, pottery, jade, stone, gold, bone, and horn products. The function of the pits and buried artifacts has always been the focus, but the microbiome around artifacts attracts less attention. Recently, the microbiome in buried ivory soil has just been identified; however, the microbiome around other artifacts has never been studied. In term of the unique perspective for interpretation the archaeological issues, the study was carried out for: (1) the microbial diversity and community of soil in the lower layer of artifacts in sacrificial pits, (2) the potential biodeterioration behavior of organic and inorganic relics, and (3) the impact of sacrificial and burial activities in different sacrificial pits on microbiome.

Methods: There were 45 soil samples around different artifacts in three sacrificial pits and 12 raw soil samples inside or outside the sacrificial pit sampling from Sanxingdui site. The microbial genomes were then identified and analyzed using the next-generation high-throughput sequencing.

Results: The represented bacterial phyla were Proteobacteria, Actinobacteriota, GAL15, Chloroflexi, Acidobacteriota, Methyloirabitolota, Thermoplasmata, Crenarchaeota, Gemmatimonadota, and Firmicutes, and the represented fungal phyla were Ascomycota, Mortierellomycota, and Basidiomycota. Further microbial functional analysis found that the bacterial genera *Sphingopyxis*, *Limnobacter*, and *Streptomyces* and the fungal genera *Cladosporium*, *Acremonium*, and *Mortierella* were concerned with the degradation of organic matter, while the genera *Pseudomonas*, *Arthrobacter*, *Variovorax*, *Aspergillus*, and *Penicillium* might be related to the biocorrosion of bronzes. In addition, the microbial composition and principal co-ordinate analysis (PCoA) demonstrated the significant differences in microbial composition and structure between the raw soil samples and the soil samples around the artifacts and also between the soil samples in different sacrificial pits.

Discussion: It is important to understand the biodeterioration of the buried artifacts and the sacrificial activities in Sanxingdui site according to the results of microbial diversity and community. The combination of microbiology and archaeology will shed light on the archaeological issues related to the ancient human activities and behaviors.

KEYWORDS

Sanxingdui site, high-throughput sequencing, microbiome, biodeterioration, burial activity

1 Introduction

Microbes are ubiquitous and are isolated from various archaeological relics and cultural heritage, including bones (Kazarina et al., 2019), dental calculi (Gancz et al., 2023), mural (He et al., 2021), wooden relics (Liu et al., 2018; Cao et al., 2024), historical textiles (Brzozowska et al., 2018), stone relics (Liu et al., 2020), metallic antiquities (Junco et al., 1992), and pottery (Ren et al., 2023). The combination of archaeology and microbiology promises to provide an innovative perspective for the analysis and interpretation of archaeological issues. On the one hand, microbial diseases of cultural heritage have always been a research focus in the field of archaeological heritage conservation (Li et al., 2017; He et al., 2021; Zhang et al., 2023). The degradation and deterioration caused by microbial activity are non-negligible and irreversible during the long-term burial or open environments, resulting in the destruction of economic, artistic, historical, and cultural value. The organic matter is attacked and broken down by microbial metabolic pathways and enzyme action, which is converted into simple organic acids, amino acids, glucose and carbonates, etc., accelerating the energy flow and nutrient cycle in the ecosystem (Yang et al., 2024). The most common mechanism of biodeterioration on inorganic and metallic is concerned with the formation of bacterial and fungi biofilms, which are composed of cells, spores, fat globules, and dirt matter, and gradually hardened into the sticky layers through the action of

cellular excretions (Swaroop et al., 2016). Numerous microbes are inhabited in the biofilms, causing the physical and chemical damage of metal, stone, or pottery relics via the release of various bioactive natural products.

On the other hand, microbial community in the soil of archaeological sites can serve as certain indicators of ancient human activities. Some studies indicate that human consumption and behavior habits leave traces on microbiome in archaeological evidence (Margesin et al., 2016; Siles et al., 2018). Therefore, the differences of microbial composition and structure at different ancient human-impacted soil regions can be regarded as the unique record of environmental changes and anthropogenic activities during their formation, providing necessary information to archaeological research over long time scales. Recently, the microbial community inhabiting in soil from three archaeological human-impacted layers at Monte Iato settlement (Sicily, Italy) has been investigated and proved the distinction on microbial C-source utilization patterns and structures reflecting different past human activities (Siles et al., 2018). But the microbiome of archaeological soil has received less attention than other remains. It is extremely necessary to identify the soil microbial community of buried environments not only for assessing microbial biocorrosion of burial artifacts but also for speculating on human activities and burial behaviors at the time.

The Sanxingdui site (Figure 1) is located in Sanxing Village, Sanxingdui Town, Guanghan City, Sichuan Province, China, on the

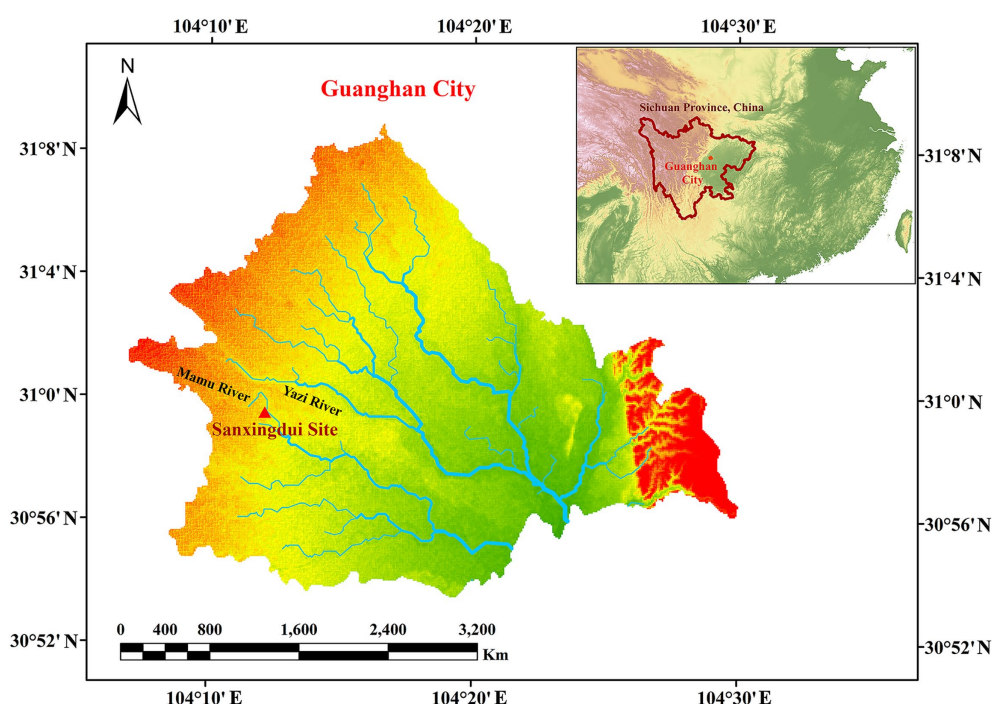


FIGURE 1
Map of Sanxingdui site.

south bank of the Yazi River and in the western suburbs of Guanghan City. The site is regarded as an outstanding representative of the ancient Shu culture for the late Neolithic to early Western Zhou, selecting as one of the “Top 100 Archaeological Discoveries of the Century” in China. The sacrificial area is situated on the south bank of the Mamu River, covering an area of approximately 13,000 cm². In 1986, two sacrificial pits, named No. 1 pit (K1) and No. 2 pit (K2), were discovered in the first archaeological excavation. Until 2019, six newly discovered sacrificial pits (numbered K3 to K8) were found and excavated in 2020. The brief excavation reports of the sacrificial area and sacrificial pit K4 have been published, respectively, confirming that the ¹⁴C dating of K4 is 3,148–2,966 cal. BP. The artifacts unearthed from the sacrificial pits include ivory, seashells, bronzes, pottery, jade, stone, gold, bone, and horn products. The distribution and artifacts buried in K3, K4, and K8 are shown in Figure 2. The deposits of K3 and K4 are

stratigraphically divided into rammed earth, ash layer, and artifact layer, while deposit of K8 is more complex and divided into rammed earth, ash layer, separation layer, and artifact layer. In the artifact layer of K3, K4, and K8, the upper is the ivory, and the lower is the other artifacts involving bronzes, gold, jade, pottery, etc. The microbial community of the ivory soil has been detected in previous study (Sun et al., 2024). But the microbial composition and structure in the lower soil of artifact layer have received little attention.

The aim of the study was to identify the microbial community in the lower soil of artifact layer via high-throughput sequencing and then to discuss the effect of sacrificial ceremony, sacrifice selection, burial activities, and other activities at that time on microbial composition and structure, simultaneously, to further evaluate the possible biodeterioration of the buried artifacts, providing a basis for the long-term preservation.

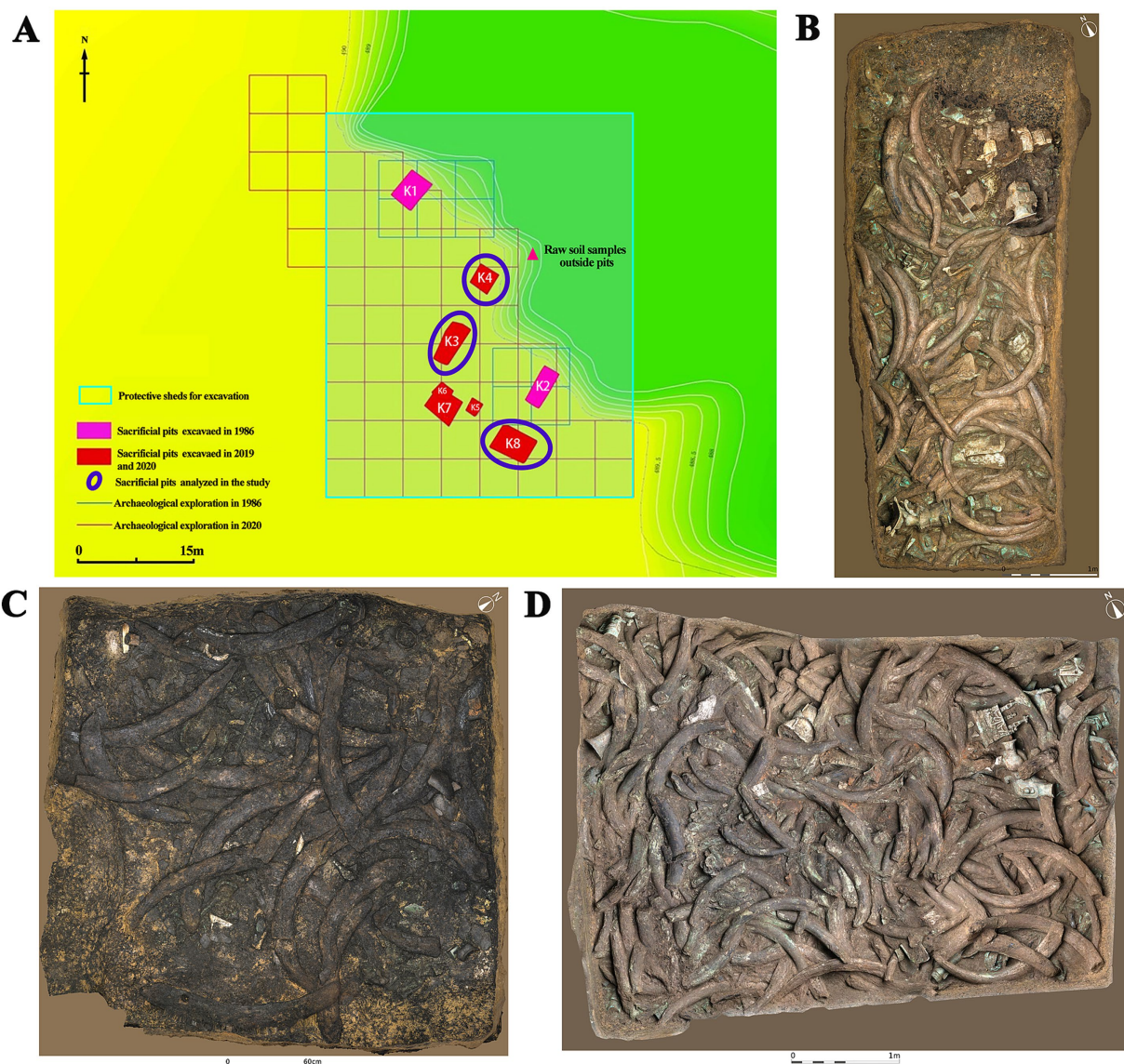


FIGURE 2
Distribution of sacrificial pits and images of artifacts in analyzed sacrificial pits. (A) Distribution of sacrificial pits. (B) Sacrificial pit K3. (C) Sacrificial pit K4. (D) Sacrificial pit K8.

2 Materials and methods

2.1 Archaeological site and sample collection

Archaeological excavations of the six new sacrificial pits K3 to K8 were conducted in October 2020. The sacrificial pits K3, K4, and K8 were selected as the subject of this study. The shape, size, and some physical–chemical properties of the three sacrificial pits were introduced in the previous reports (Sun et al., 2024). A brief introduction to soil moisture content and pH value of buried environment was as follows. The soil moisture content in K3 was 22.64%, and the pH was 7.16. The soil moisture content of K4 was 22.08%, and the pH was 7.18. The soil moisture content of K8 was 20.20%, and the pH was 7.19. During the excavations, five soil samples (numbered “1–5,” test sample) were collected from the lower of artifact layer in K3, K4, and K8, respectively, for a total of 15 samples. In addition, the raw soil samples (numbered “0,” control group A) at the bottom of each pits in K3, K4, and K8 and one raw sample (numbered “S_0,” control group B) outside the sacrificial pits were also analyzed as control samples to comparative analysis. Soil samples were gathered using sterile scalpel and collected into Corning centrifuge tubes with -30°C storage temperature. As shown in Tables 1, 19 samples were categorized according to the excavated sacrificial pits and the type of artifacts near its location. All samples were sequenced three replicates to minimize errors and finally a total of 57 samples.

2.2 DNA extraction and amplicon generation

Total genomic DNA was extracted from 0.5 g soil samples using the FastDNA® Spin Kit for Soil (MP Biomedicals, LLC, USA) as recommended by the manufacturer's instructions. The concentration and purification of DNA samples were detected with NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). The integrity of DNA was examined using 1% agarose gel electrophoresis with the voltage of 5 V/cm for 20 min by the electrophoresis system JY-600C (Beijing Junyi Electrophoresis Co., Ltd., China).

Subsequently, PCR amplification was conducted using ABI GeneAmp® 9700 (Thermo Fisher Scientific Inc., USA). The primers of bacteria were designed based on the V4 hypervariable regions of 16S rRNA with 515F 5'-GTGYCAGCMGCCGCGTAA-3' and 806R 5'-GGACTACNVGGGTWTCTAAT-3' (Peiffer et al., 2013). The primers of fungi were amplified with the rRNA ITS1 hypervariable regions of ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2R 5'-GCTGCGTTCTTCATCGATGC-3' (He et al., 2021). After quality check of amplicons employing 2% agarose gel, the purification was conducted with the AxyPrep DNA Gel Extraction Kit (Axygen Scientific Inc., USA) according to the manufacturer's instructions. The amplicons were quantified using Quantus™ Fluorometer (Promega Co., USA) for subsequent sequencing.

2.3 Library preparation and sequencing

The DNA libraries were established utilizing the NEXTFLEX Rapid DNA-Seq Kit (BIOO Scientific Co., USA) in accordance with the manufacturer's instructions. The purified amplicons were pooling

in equimolar amounts, and then, the DNA libraries were validated by the Agilent Fragment Analyzer 5400 (Agilent Technologies, Inc., USA) with the approximate size 450 bp and 480 bp for bacteria and fungi, respectively. After passing the test, sequencing was performed on an Illumina MiSeq PE300 platform (Illumina, Inc., USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.4 Data processing

The raw sequencing files were quality-filtered and merged by fastp (version 0.19.6) (Chen et al., 2018) and FLASH (version 1.2.11) (Magoč and Salzberg, 2011), respectively, to select high quality data and remove the adapters. Reads were excluded with the score < 20 . The optimized sequences were clustered into operational taxonomic units (OTUs) employing UPARSE (version 7.1) (Edgar, 2013) at the 97% sequence similarity (Li et al., 2017).

The most abundant sequence of each OTU was selected as a representative sequence, which was annotated the taxonomy via RDP Classifier (version 2.13). The bacterial sequences were submitted to the SILVA 138/16 s rDNA database¹ with the confidence threshold of 70%. Similarly, the fungal sequences were analyzed at the same confidence threshold against the UNITE 8.0/its_fungi database.²

2.5 Statistical analysis

The bioinformatic analysis of all samples was carried out via the Majorbio Cloud platform.³ Alpha diversity (α -diversity, diversity within-sample) of microbiome was assessed by Mothur (version 1.30.2) with the indices of the observed richness (Sobs), the Chao1 estimator (Chao 1), the Shannon diversity index (Shannon), the Simpson diversity index (Simpson), and Pielou evenness index (Pielou_e). The Kruskal–Wallis *H*-test was performed to examine the significant differences between the different samples ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). The beta diversity (β -diversity, similarity and difference between groups of samples) was visualized with principal co-ordinate analysis (PCoA) based on the Bray–Curtis distance via the vegan package in R3.3.1. The differences in microbial taxa of groups were performed by stats package in R3.3.1 and scipy package in python v1.0.0.

3 Results

3.1 Evaluation of microbial sequencing data

In total, 3,922,031 bacterial sequences and 4,655,448 fungal sequences were obtained from high-throughput sequencing for analysis after verifying, with the average length 256 bp of former and 240 bp of latter. Subsequently, the low-abundance OTUs with < 10 copies in all samples were filtered to improve the reliability of microbial composition estimates (Bokulich et al., 2013; Nikodemova et al., 2023). Meanwhile, the number of effective sequences from all

¹ <https://www.arb-silva.de/>

² <https://unite.ut.ee/>

³ <https://cloud.majorbio.com>

TABLE 1 Soil samples of sacrificial pits in Sanxingdui site.

Group	Name	Type	Sampling location	Brief description
K3_0	K3_0	Control group A	Raw soil at the bottom of southeast of K3	---
K3	K3_1	Test group	Soil near a bronze figure and a jade object	---
	K3_2	Test group	Soil from the inner abdomen of a bronze <i>dakouzun</i>	Near bronze
	K3_3	Test group	Soil at the top of a bronze figure	Near bronze
	K3_4	Test group	Soil near a bronze eye-shaped object	Near bronze
	K3_5	Test group	Soil at the bottom of a bronze figure with a <i>Zun</i> -vessel on its head	Near bronze
K4_0	K4_0	Control group A	Raw soil at the bottom of southwest of K4	---
K4	K4_1	Test group	Soil near a pottery sherd	Near pottery
	K4_2	Test group	Soil near a stone ware	---
	K4_3	Test group	Soil near a bronze ware	Near bronze
	K4_4	Test group	Soil near a bronze ware	Near bronze
	K4_5	Test group	Soil near a pottery sherd	Near pottery
K8_0	K8_0	Control group A	Raw soil at the bottom of northeast of K8	---
K8	K8_1	Test group	Soil inside a bronze <i>lei</i>	Near bronze
	K8_2	Test group	Soil inside a bronze <i>dakouzun</i>	Near bronze
	K8_3	Test group	Soil inside a bronze <i>lei</i>	Near bronze
	K8_4	Test group	Soil near a bronze <i>dakouzun</i>	Near bronze
	K8_5	Test group	Soil near a bronze ware	Near bronze
S_0	S_0	Control group B	Soil from the eastern slope outside pit K4, vertical depth 1 m	---

samples was rarefied to the number of the sample with minimum sequences, to minimize the effect of sequencing depth on the subsequent bioinformatics analysis.

To ensure the sequencing quantity and depth were sufficient and representative, the rarefaction curves of bacteria and fungi are given in [Supplementary Figure S1](#), which tended to flatten out, suggesting that the sequencing data were able to comprehensively and truthfully reflect the microbial composition and community. Meanwhile, the Good's coverage ([Supplementary Figures S1E,F](#)) over 99.3% also indicated the sequencing data of all samples were enough for the follow-up analysis.

3.2 Alpha diversity of microbiome

The α -diversity of microbiome was characterized with Sobs, Chao 1, Shannon, Simpson, and Pielou_e indices, to assess the community richness, diversity, and evenness. The larger the values of Sobs and Chao 1, the higher the community richness. The larger the Shannon value and the smaller the Simpson value, the higher the community diversity. Similarly, the closer the Pielou_e value is to 1, the higher the species evenness. As shown in [Table 2](#), the Sobs and Shannon indices of control group S_0 were largest for both bacteria and fungi, which meant the outside raw soil samples had the highest community richness and diversity, while the Pielou_e index on fungi of control group S_0 was smallest, meaning the lowest community evenness. As for the test groups, group K4 indicated the best biodiversity. The order of bacterial richness, diversity, and evenness in the lower of artifact layer was K4 > K8 > K3, while fungi was different with the order of K4 > K3 > K8.

In terms of bacteria ([Figure 3](#)), almost all α -diversity indices displayed a highly significant difference ($p < 0.001$) between group S_0

and groups K3, K4, and K8. Group K3_0 and group K4_0 also indicated a difference ($p < 0.05$) or a highly significant difference ($p < 0.001$) in Sobs, Shannon, and Pielou_e indices from the group K3 and group K4, respectively. Furthermore, group K3 and group K4 also shown the statistical difference ($p < 0.05$) on Sobs, Shannon, and Pielou_e indices. The results demonstrated that the raw soil samples outside sacrificial pits (control group B), the raw soil samples inside sacrificial pits (control group A), and the test groups exhibited statistical difference in bacterial richness, diversity, and evenness, following the order: control group B > control group A > test group. Referring to fungi, the Shannon and Pielou_e indices did not perform the statistical difference, which meant the fungal diversity and evenness of all samples were closed, but the Sobs index of control group S_0 also shown the highly significant differences ($p < 0.001$) from other groups.

It was clearly observed that the statistical difference in α -diversity of bacteria was much higher than that of fungi. In addition, the statistical difference between different samples from the same sacrificial pit in bacteria and fungi was still remained ([Supplementary Figures S2, S3](#)), especially for control groups K3_0, K4_0, and K8_0 and the test samples from corresponding sacrificial pits. The results probably were caused by the different sampling locations and the nearby artifacts in spite of the same sacrificial pit.

3.3 OTU characterization of microbiome

Through the trimmer and filtering methods, a total of 3,174 bacterial OTUs and 733 fungal OTUs were detected with high reliability and accuracy. According to Venn plot ([Figure 4](#)), there were 2,212 bacterial OTUs and 402 fungal OTUs in the control group S_0, which were much higher than other groups. In test groups, the order

TABLE 2 α -diversity index of soil samples.

Sacrificial pit	Name	Bacteria					Fungi				
		Sobs	Chao 1	Shannon	Simpson	Pielou_e	sobs	Chao 1	Shannon	Simpson	Pielou_e
K3_0	K3_0	620	674.52	4.75	0.03	0.74	18	18.000	2.338	0.128	0.81
K3	K3_1	281	335.38	3.05	0.10	0.54	42	43.200	1.909	0.217	0.51
	K3_2	443	563.01	3.57	0.07	0.59	34	38.667	1.253	0.412	0.36
	K3_3	252	287.28	2.82	0.13	0.51	40	39.667	2.553	0.134	0.71
	K3_4	236	255.83	2.74	0.21	0.50	31	31.333	2.075	0.224	0.60
	K3_5	320	376.94	3.60	0.06	0.62	22	23.667	1.810	0.308	0.59
Average of K3	/	306	363.69	3.15	0.11	0.55	34	35.307	1.920	0.259	0.55
K4_0	K4_0	1,063	1160.70	5.19	0.02	0.74	23	23.667	1.845	0.273	0.58
K4	K4_1	875	1028.27	4.88	0.02	0.72	14	13.667	1.776	0.237	0.68
	K4_2	487	591.53	3.87	0.05	0.63	44	44.333	2.263	0.167	0.60
	K4_3	397	514.33	3.55	0.06	0.59	70	71.222	2.616	0.114	0.62
	K4_4	332	394.45	3.14	0.10	0.54	42	42.500	2.303	0.165	0.62
	K4_5	622	696.97	4.34	0.04	0.67	19	19.333	1.055	0.566	0.35
Average of K4	/	543	645.11	3.96	0.06	0.63	38	38.211	2.003	0.250	0.58
K8_0	K8_0	414	480.97	3.06	0.12	0.51	30	30.000	1.831	0.290	0.54
K8	K8_1	457	548.47	3.58	0.09	0.58	17	17.000	2.189	0.188	0.78
	K8_2	741	906.49	4.39	0.04	0.66	39	38.667	2.928	0.090	0.80
	K8_3	286	347.91	2.48	0.23	0.44	27	27.000	1.104	0.498	0.34
	K8_4	396	487.84	3.59	0.09	0.60	37	37.667	1.240	0.504	0.33
	K8_5	312	345.94	3.57	0.06	0.62	19	18.667	1.155	0.555	0.39
Average of K8	/	439	527.33	3.52	0.10	0.58	28	27.800	1.723	0.367	0.53
Outside pits	S-0	1767	1911.62	5.92	0.01	0.79	267	275.063	2.656	0.290	0.47

All the values in the table are averages.

of the bacterial OTUs was following K4 > K8 > K3, and the order of the fungal OTUs was K3 > K8 > K4.

More importantly, Figure 4 also displays the number of unique (in side) or shared (in core) OTUs in different groups, which revealed the similarity and overlap across sacrificial pits. Only 203 bacterial OTUs and 8 fungal OTUs were common to all groups, while 580 bacterial OTUs and 35 fungal OTUs were shared in groups K3, K4, and K8, which indicated the test groups K3, K4, and K8 exhibited higher overlap with each other rather than with control groups. Simultaneously, there were also numerous OTUs shared between two of the sacrificial pits or only appeared in one pits, suggesting the heterogeneity of the test groups from different sacrificial pits on OTU level. The similar overlap and differences were observed as well in OTU results of individual samples (Supplementary Figure S4). Even though samples collected from the same sacrificial pit, the OTUs of different samples still showed differences. The results above probably indicated the impact of excavation location and buried artifacts on soil microbiome.

3.4 Identification and composition of microbial community

To analyze the community composition and structure, the microbial OTUs were further classified. The bacterial OTUs were

divided into 42 phyla, 405 families, and 667 genera. As shown in Figure 5, there were 18 dominant bacterial (relative abundance >1%) phyla in groups. The top bacterial (relative abundance >2%) phyla were Proteobacteria, Actinobacteriota, GAL15, Chloroflexi, Acidobacteriota, Methylomirabilota, unclassified_k_norank_d_Bacteria, Thermoplasmatota, Crenarchaeota, Gemmatimonadota, Firmicutes, and Nitrospirota, which were detected in all groups, and the combined abundance of these bacteria exceeded 80%. The phyla above were common to the samples of human archaeological remains. The results indicated that the test and control groups were similar at the phylum level in bacteria but differed in relative abundance.

The differences in bacterial community and composition among different pits were further noticed at the genus level. There were 65 dominant bacterial (relative abundance >1%) genera in samples. It should be noted that some norank and unclassified genera, such as genera *norank_f_norank_o_norank_c_norank_p_GAL15*, *unclassified_d_Bacteria*, and *norank_f_norank_o_Gaiellales*, were identified in all groups but lacking the accurate identification information. Thus, the bacteria only identified to order level were filtered out. The bacterial composition showed the evident differences between different groups. In group S_0, the top bacterial genera (relative abundance >2%) were genera *Arthrobacter* (4.65%), *Streptomyces* (3.07%), as well as genera *norank_f_Nitrososphaeraceae* (5.75%), *norank_f_Vicinamibacteraceae* (3.48%),

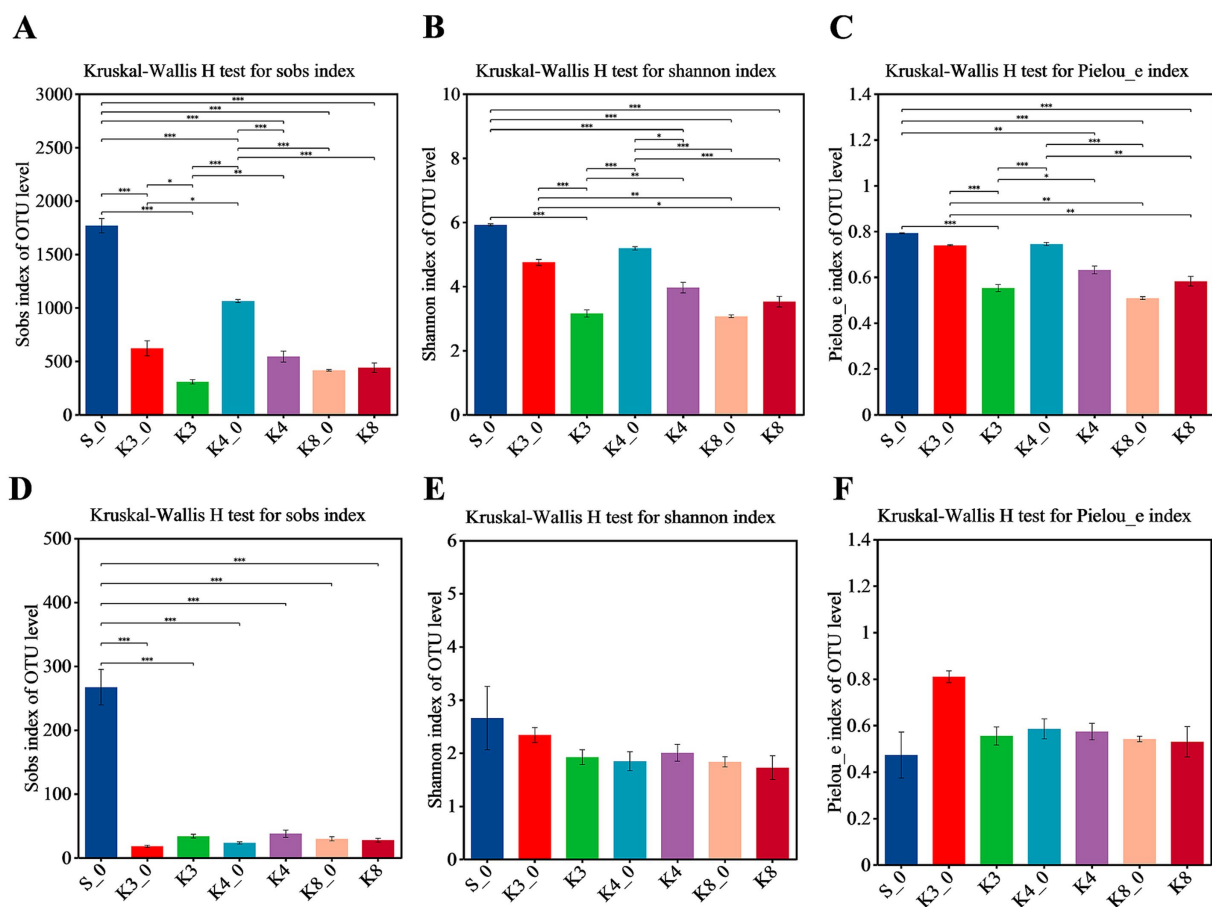
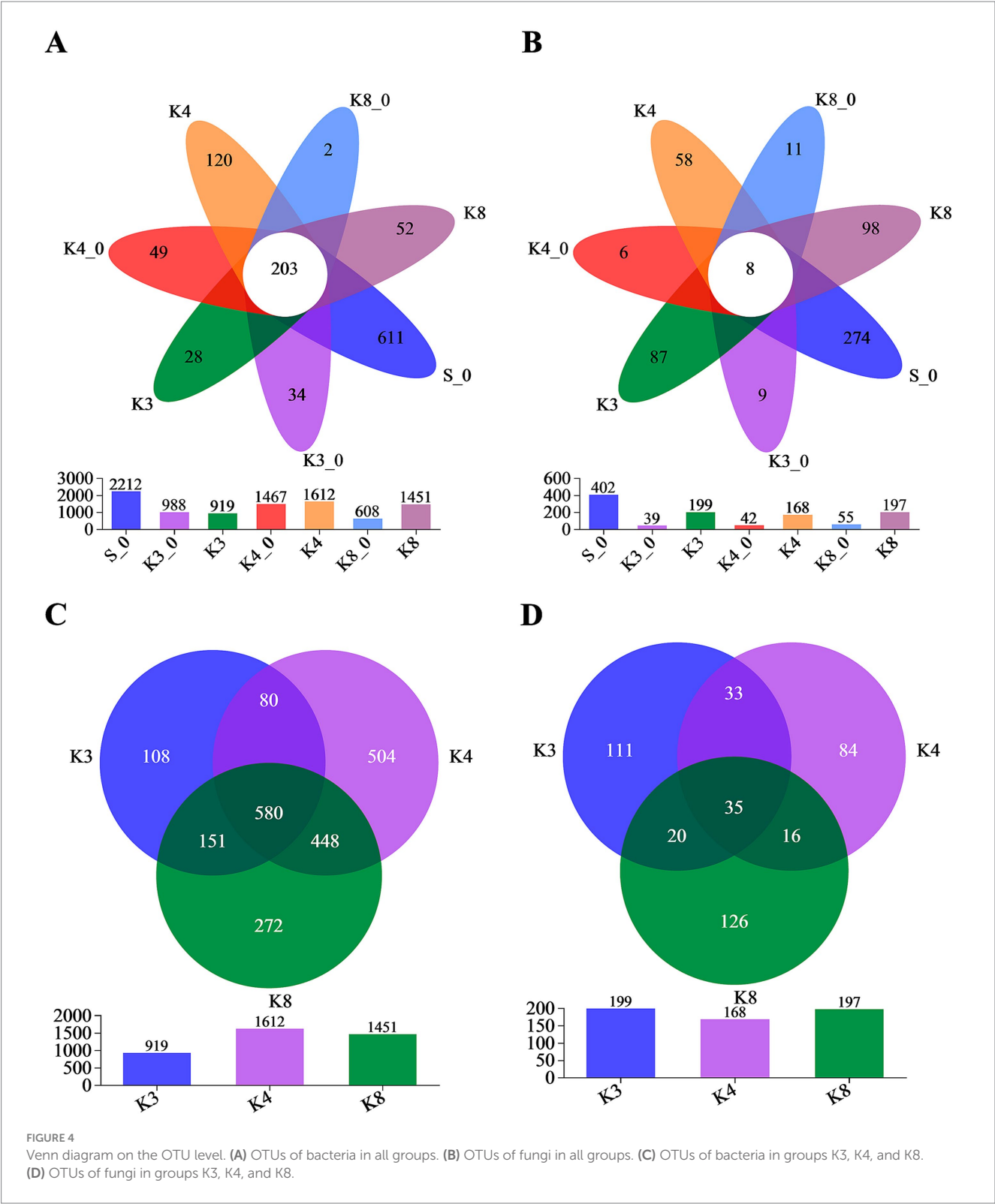


FIGURE 3
α-diversity shown by Sobs, Shannon, and Pielou_e. (A–C) Bacteria. (D–F) Fungi.

norank_f_Roseiflexaceae (3.66%), and *norank_f_Gemmatimonadaceae* (2.04%). In group K3_0, the genera *Sphingomonas* (3.20%), *Nitrospira* (2.41%), and *Candidatus_Udaeobacter* (2.02%) were found. Meanwhile, there were six top bacterial genera including genera *Cupriavidus* (7.72%), *Sphingopyxis* (5.19%), *Variovorax* (4.91%), *Limnobacter* (3.28%), *Candidatus_Methylomirabilis* (3.26%), and *Caulobacter* (2.56%) in group K3. Similarly, eight top bacterial genera including the genera *Limnobacter* (9.20%), *Cupriavidus* (5.86%), *Pseudomonas* (4.20%), *Bacillus* (3.55%), *Methylothera* (2.63%), *Variovorax* (2.57%), *Sphingopyxis* (2.49%), and *Streptomyces* (2.53%) were detected in group K4. In group K4_0, the top bacterial genera were genera *Sphingopyxis* (4.42%), *norank_f_Gemmatimonadaceae* (2.93%), *Pseudomonas* (2.65%), *Variovorax* (2.63%), *Pedobacter* (2.37%), *Luteimonas* (2.32%), *unclassified_f_Comamonadaceae* (2.30%), and *Nitrospira* (2.13%). Obviously, group K3 and group K4 had more precise dominant bacterial genera with higher abundance than group K3_0 and group K4_0, respectively. As for group K8, the top bacterial genera were genera *Candidatus_Methylomirabilis* (2.95%), *Bacillus* (2.45%), *Arthrobacter* (2.29%), *Variovorax* (2.57%), and *norank_f_Gemmatimonadaceae* (3.60%), while group K8_0 included more bacterial genera, such as genera *Hansschlegelia*, *Nocardioides*, *Pseudonocardia*, *Luteimonas*, *Variovorax*, *Rhodococcus*, and *Olivibacter*, accounting for 28.01, 11.97, 10.78, 7.12, 6.88, 5.42, and 2.13%, respectively.

The fungal OTUs belonged to 12 phyla, 182 families, and 298 genera. There were only four dominant fungal phyla (relative abundance >1%) in groups, which included Ascomycota, Mortierellomycota, Basidiomycota, and an unclassified_k_Fungi (Figure 6). It is worth noting that Mortierellomycota was not detected in groups K3_0 and K8, although it was more abundant in groups K3 and K4. At the genus level, the number of dominant fungal genera (relative abundance >1%) was 46. Similar to the bacterial results, the fungal genera in groups displayed the remarkable differences between groups. In group S_0, the top fungal genera (relative abundance >2%) were genera *unclassified_f_Nectriaceae* (28.92%), *Neocosmospora* (22.23%), *Microascus* (5.76%), *Nectria* (4.31%), *Fusarium* (3.97%), *Penicillium* (2.91%), *Ilyonectria* (2.32%), *Purpureocillium* (2.06%), and *Thelonectria* (2.02%). The top fungal genera in group K3 were genera *Mortierella* (16.93%), *Simplicillium* (11.33%), *Purpureocillium* (10.40%), *unclassified_f_Nectriaceae* (10.96%), *Rhodotorula* (7.78%), *Fusarium* (6.61%), *Robbauera* (5.72%), *Cladosporium* (4.52%), *Aspergillus* (4.05%), *Cutaneotrichosporon* (2.80%), *Vishniacozyma* (2.79%), and *Penicillium* (2.35%), while the different fungi such as genera *Cephalotrichum* (18.19%), *unclassified_f_Cordycipitaceae* (12.71%), *Infundichalara* (7.00%), *Engyodontium* (6.28%), *Dichotomopilus* (5.87%), *Xenochalara* (4.85%), and *Alternaria* (2.68%) were found in group K3_0. In group K4, the top fungal genera were more concentrated in genera *Ilyonectria*, *Mortierella*, *Simplicillium*,



Cladosporium, *Acremonium*, and *Lecanicillium*, with the relative abundance of 34.29, 24.87, 10.29, 10.20, 7.70, and 4.51%, respectively. The control group K4_0 shown the accordant fungi with group K4 in addition to genera *Cephalotrichum* (42.38%) and *Aspergillus* (3.45%). In group K8, genera *Purpureocillium* (31.76%), *Clonostachys* (14.96%), *Rhodotorula* (6.65%), *Cladosporium* (4.63%),

unclassified_f_Plectosphaerellaceae (3.95%), *Simplicillium* (3.61%), *Penicillium* (3.45%), and *Aspergillus* (2.41%) were the top fungal genera. Apparently, the types and relative abundance of different fungi in group K3 and group K8 differed obviously from group K3_0 and group K8_0, respectively. In contrast, the groups K4 and K4_0 displayed a certain similarity in fungal community composition.

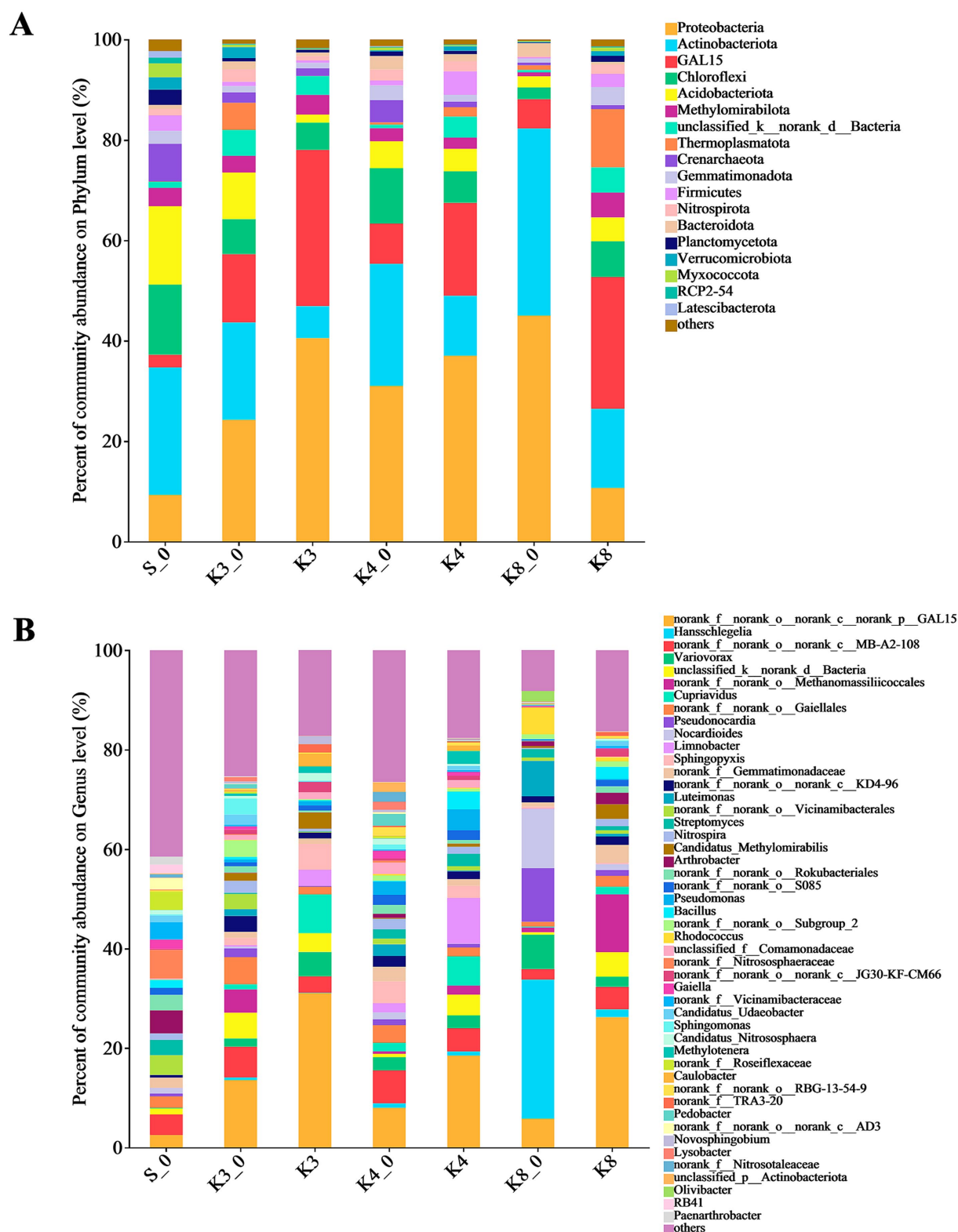
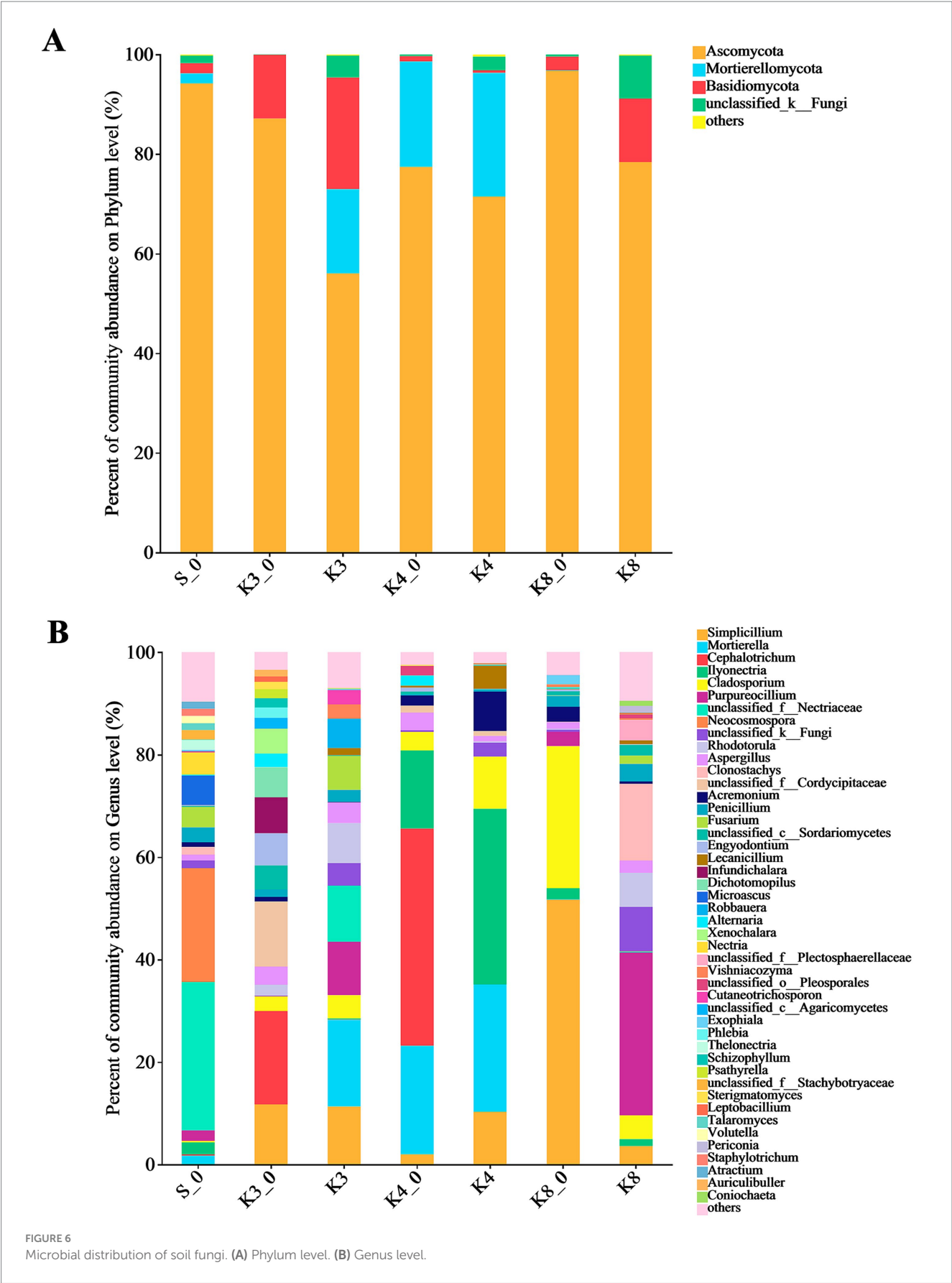


FIGURE 5
Microbial distribution of soil bacteria. (A) Phylum level. (B) Genus level.

3.5 Beta diversity of microbiome

The similarities and differences of bacterial and fungal communities were evaluated via clustering using Bray–Curtis

distance-based principal co-ordinate analysis (PCoA) on the OTU level. The PCoA result of bacteria (Figure 7A) revealed that the sacrificial pits of K3, K4, and K8 shown significant difference in taxa biodiversity. Especially, group K4 exhibited the evident



differences from groups K3 and K8. Group K4 also shown a dispersed distribution, which was closer to the control groups. As for fungi (Figure 7B), the group K4, as well as group K4_0, also performed significant difference with other groups, whereas it is hard to distinguish the samples of groups K3 and K8, suggesting that the fungal community between group K3 and group K8 was similar. The microbial distribution and structure were also performed by hierarchical clustering analysis (HCA, Supplementary Figures S5, S6), which were consistent with the PCoA results. Compared to group K4, group K3 and group K8 show higher similarity in bacteria and fungi communities and taxa biodiversity.

In addition, it should be noted that the location of samples and the material of artifacts around the samples, including pottery and bronze, also affect the microbial diversity. As shown in Figures 7C,D, the samples near pottery and bronze displayed the

differences in bacterial biodiversity, but the distinction in fungi was not observed.

3.6 Differences in microbial taxa and composition

Based on the microbial results above, the differences in community and composition were further investigated. The top 20 bacterial and fungal genera are exhibited in Figure 8. As for bacteria, there were 15 genera manifesting the highly significant difference ($p < 0.001$) in different groups. The genus *norank_f_norank_o_norank_c_norank_p_GAL15* was dominant in groups K3, K4, and K8. The genera *Candidatus_Methylophilus*, *Sphingopyxis*, and *Cupriavidus* were dominant in group K3. The genera *Limnobacter*, *Pseudomonas*, and *Bacillus* were more

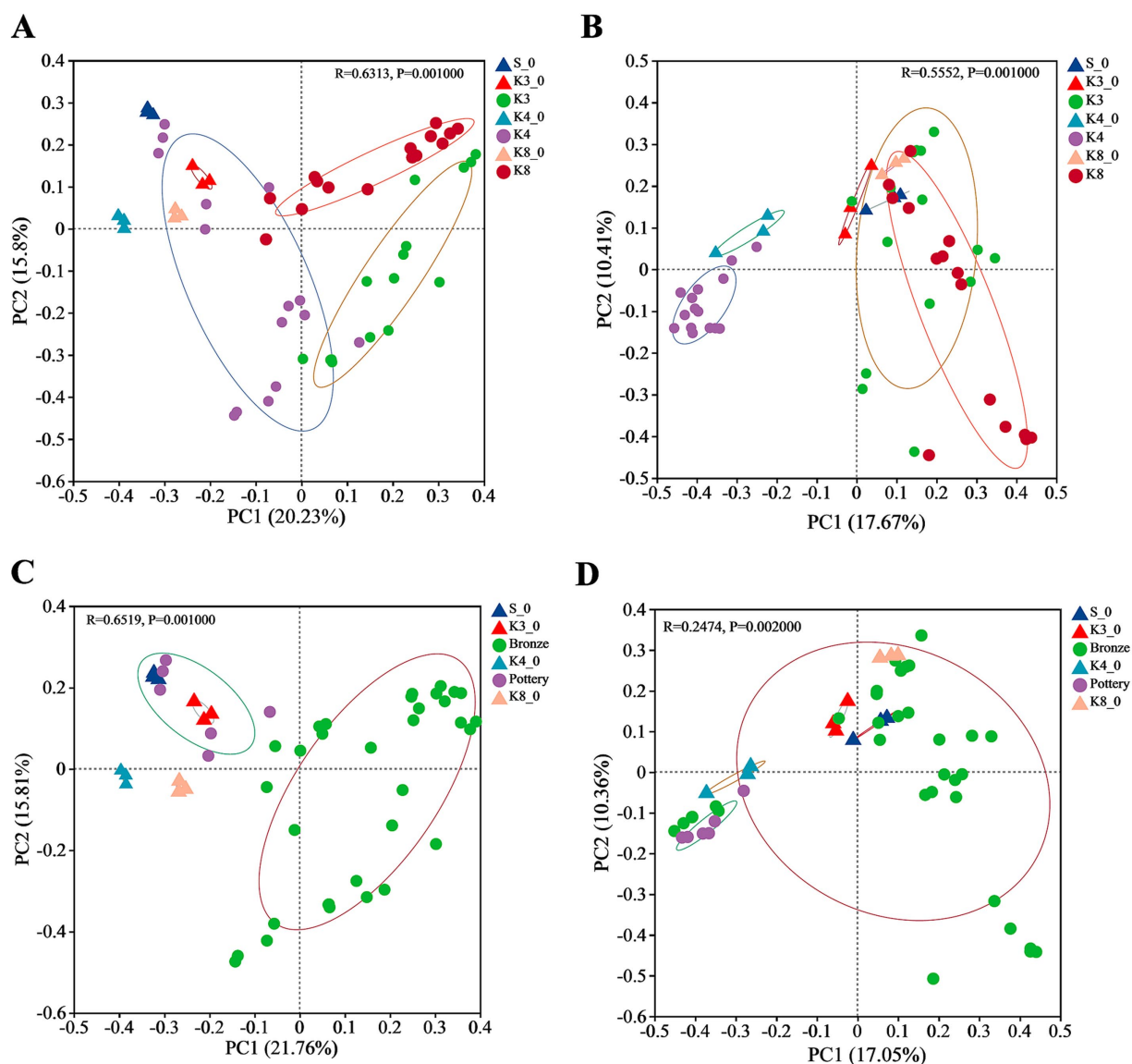


FIGURE 7
PCoA of bacteria and fungi in different groups. (A) Bacteria in all groups. (B) Fungi in all groups. (C) Bacteria in soil samples near bronzes and pottery sherds. (D) Fungi in soil samples near bronzes and pottery sherds.

abundant in group K4. The genera *norank_f__Gemmatimonadaceae* and *Candidatus_Methyloirabilis* were more abundant in group K8.

Similarly, 15 genera of fungi performed the highly significant difference ($p < 0.001$). The genera *Robbauera*, *Fusarium*, and *Rhodotorula* were more abundant in group K3. The genera *Ilyonectria* and *Mortierella* were dominant in group K4. In group K8, the abundance of genera *Purpureocillium* and *Clonostachys* increased markedly. From the results of difference analysis, it could be seen that there were statistical difference in the abundance of different genera in each sacrificial pit.

4 Discussion

Combining the results of α -diversity and OTU characteristics, it has been known that the group S_0 shown the superior biodiversity than test groups in sacrificial pits K3, K4, and K8 and also exhibited the significant statistical difference in community richness, evenness, and diversity simultaneously. Moreover, the microbial composition of group S_0 exhibited a richer genera, which might be consistent with the fact that the soil that has not been disturbed by humans. In contrast, although there were differences in biodiversity between sacrificial pits K3, K4, and K8, the differences were smaller than those between group S_0. The more direct result was that the shared OTUs among groups K3, K4, and K8 increased significantly (Figures 5C,D). However, in terms of microbial community and structure, groups K3 and K8 were more similar and quite different from group K4 according to the PCoA results. Further comparing the microbiome in the same sacrificial pit, it could be found that the soil samples located in different artifacts exhibited the different microbial composition and structure with the relatively dispersed PCoA plots. In sacrificial pits K3, K4, and K8, we found that group K4 has shown the most homogeneous microbial community with the highest value of community evenness and most concentrated PCoA plots. An interesting phenomenon was observed in fungi that the order of fungal diversity was $K4 > K3 > K8$ based on the α -diversity, but with the different OTUs order of $K3 > K8 > K4$, which could be explained by the community evenness index of Pielou_e (Table 1). The Pielou_e values of fungi in group K4 were closer except for sample K4_5, also with the highest average of 0.58, resulting in the most shared fungal OTUs in group K4. The results demonstrated that the buried environment and the excavation location were affected microbial diversity. Even within the same sacrificial pit, the microbial composition and diversity were not exactly the same.

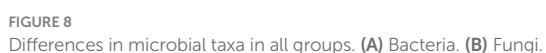
4.1 Microbial function and feature in sacrificial pits

A great number of bacteria and fungi exist in soil (Janssen et al., 2002). It is one of the most crucial aim to identify and compare the microbial community and composition of soil in different sacrificial pits. In recent years, several investigations have been conducted in the human archaeological remains (Philips et al., 2017). Some of the most common soil bacteria are considered to be Firmicutes, Actinobacteriota, Actinobacteria, Proteobacteria, Acidobacteriota,

Methyloirabilota, Chloroflexi, Bacteroidota, and Nitrospirota (Kazarina et al., 2019; Wei et al., 2022). The fungal phyla of Ascomycota and Basidiomycota are dominated in human archaeological remains, followed by Mortierellomycota, Chytridiomycota, or Zygomycota (Margesin et al., 2016; Siles et al., 2018; Voyron et al., 2022). All bacterial and fungal communities collectively reflect the serious impact of ancient anthropogenic activities on soil environment.

In our results of bacteria, Proteobacteria were the most widespread phylum in the test groups, followed by Actinobacteriota, GAL15, Chloroflexi, Acidobacteriota, etc. (Figure 5). Proteobacteria are the largest and phenotypically most diverse phylogenetic lineage, which contain strictly anaerobic and aerobic species, facultative aerobes, and microaerophiles to participate the carbon, nitrogen, and sulfur cycles on Earth (Kerstens et al., 2006). In Sanxingdui sacrificial pits, Proteobacteria were dominated via Alphaproteobacteria and Gammaproteobacteria. The former included the genera *Caulobacter*, *Hansschlegelia*, *Sphingomonas*, and *Sphingopyxis*. The latter contained genera *Cupriavidus*, *Limnobacter*, *Variovorax*, *Methylothera*, *Pseudomonas*, and *Luteimonas*. Among them, genus *Hansschlegelia*, the most abundant in group K8_0, represented a novel lineage of autotrophic methanol-utilizing bacteria as the carbon and energy source in plant and soil (Ivanova et al., 2007; Wen et al., 2011). Genus *Sphingopyxis* has been reported in catacombs (Alias-Villegas et al., 2013), which is also widespread in diverse ecological environments, including soil, sediment, water, and heavy metal contaminated sites (Sharma et al., 2021). It has attracted considerable attention not only for excellent ability to survive under extreme environments but also for the ability to degrade organic matter. Therefore, although the content of copper ion was high in the soil of sacrificial pits, genus *Sphingopyxis* was still found dominantly in K3, K4, and K4_0, probably indicating the presence and biodegradation of organic matter. Genus *Limnobacter* is an obligately aerobic and grew on various organic substrates, but it has been designated chemolithoheterotrophs, which is able to grow by oxidation of thiosulfate to sulfate (Spring et al., 2001). The capabilities might indicate the presence of sulfide or degradation of organic matter in sacrificial pits. Genus *Variovorax* generally inhabits soil and water and plays an important role in the regulation of plant growth and development (Finkel et al., 2020). Meanwhile, the genus is found in the ancient apple seeds (Milanesi et al., 2019) and decaying woods (Ghio et al., 2012). Genus *Variovorax*, hence, is considered as a possible source of hydrolytic enzymes to degrade cellulose. Moreover, it should be noted that the genus is often detected after bushfire (Smith et al., 2008). Therefore, the presence in all groups except group S_0 might indicate the impact of high temperature processes in the ash layer on bacteria.

Actinobacteriota, the second abundant bacterial phylum in our results, are generally distributed across both terrestrial and aquatic environments, as well as in the microbiome of higher eukaryotes. The phylum shows the unrivalled metabolic versatility, producing highly diverse bioactive natural products, but it must be noted that there were lots of unclassified genera with high abundance in Actinobacteriota in our results, such as genera *g__norank_f__norank_o__norank_c__MB-A2-108* and *g__norank_f__norank_o__Gaiellales*. Therefore, it is difficult to analyze their properties and functions in the ecosystem. Furthermore, the genera *Streptomyces*, *Rhodococcus*, *Nocardioides*, and



Pseudonocardia were identified in sacrificial pits, which are found in ancient wall painting, ancient seeds, wooden shipwreck, tomb, or cave (Stakhov et al., 2008; Juanjuan et al., 2016; Liu et al., 2018; Sakr et al., 2020). Among them, genus *Streptomyces* can cause the deterioration of complex natural polymers via producing a great deal of enzymes (Sakr et al., 2020). The genus was dominant in groups K4 and S_0, probably indicating the presence of natural polymers. The phylum *Methylomirabilota* plays a key role in global carbon and nitrogen cycle as the methane oxidizing bacteria. The genus *Candidatus_Methylomirabilis* participates in the cycle by oxygenic denitrification and aerobic methane oxidation pathways (Sun et al., 2024). The dominant abundance in groups K3 and K8 indicated that a certain amount of organic matter probably was present in sacrificial pits.

In the part of fungi, Ascomycota were the most abundant fungal phylum, with the genera *Cladosporium*, *Aspergillus*, *Penicillium*, *Simplicillium*, *Purpureocillium*, *Lecanicillium*, and *Acremonium*. Among them, the genera *Cladosporium* and *Simplicillium*, belonging to family Cordycipitaceae, were dominant in all groups except for group S_0, suggesting the effect of human burial behavior and buried artifacts. The genus *Cladosporium* is isolated in ancient wall painting and beeswax drops on ancient papers (He et al., 2021; Pavlović et al., 2022), showing the proteolytic and lipolytic abilities in the process of organic degradation. In addition, previous studies have indicated that the genus *Cladosporium* is capable of hydrolyzing cellulose by the production of cellulases (Leung et al., 2016; Li et al., 2019). The genus *Simplicillium* is identified on the polychrome pottery figurines (Zhang et al., 2023), resulting in the fading of the blue (azurite) and green (malachite) pigments through the absorption of copper (II) ion. Moreover, the genus has been proved to degrade tempera paintings and artifacts made with dammar varnish and rock-art paintings (Jurado et al., 2008). So genus *Simplicillium* is destructive to both organic and inorganic materials. The genera *Lecanicillium* and *Acremonium* belong to the order Hypocreales. Genus *Lecanicillium* contains some entomopathogenic species, and some are capable to degrade organic compounds (Fenice, 2016; Pozdnyakova et al., 2019). For instance, *Lecanicillium muscarium* is a potent producer of extracellular cold-resistant, chitin-hydrolyzing enzymes for the degradation of chitin-rich materials (Fenice, 2016), but the degradative properties of the genus *Lecanicillium* have been poorly studied. The genus *Acremonium* is the environmental saprophytes in soil and decaying plant material and is able to secrete a lot of cellulolytic enzymes with high activity (Amiri et al., 2011; Hu et al., 2015). The genus is found in ancient wall paintings (He et al., 2021), and the dominant proportion in group K4 probably implied the degradation of cellulose organic matter.

The genera *Robbaurera*, *Rhodotorula*, *Vishniacozyma*, and *Cutaneotrichosporon*, belonging to Basidiomycota, were found in K3 and K8, especially in group K3 with the dominant abundance. Among them, genus *Vishniacozyma* has been identified as the core composition during the spontaneous fermentation of ice wine, probably relating to the fermentation process (Chen et al., 2020). As the pigmented yeast, genus *Rhodotorula* is strict aerobic yeasts with peculiarly metabolic characteristics. The genus is very important for food industries because it can produce glycogen, lipids, and carotenoid pigments during the growth phase (Hernández-Almanza et al., 2014), but it should be noted that the genus does not ferment carbohydrates (Dworecka-Kaszak and Kizerwetter-Świda, 2011). In addition, genus *Mortierella*, belonging to Mortierellomycota, was the core part in

groups K3, K4, and K4_0. The genus is the widespread filamentous fungi that can decompose various organic compounds such as cellulose, hemicellulose, and chitin (Ozimek and Hanaka, 2021).

4.2 The biocorrosion of bronzes in sacrificial pits

The problem of biocorrosion could not be ignored during thousands of years of burial. Considering that the bronzes were the most unearthed artifacts, the biocorrosion (also known as microbiologically influenced corrosion, MIC) of bronzes was the major concern. Some microbial taxa known to corrode metal material or solubilizing metals from metal-bearing substrates were found. As for bacteria, the genus *Pseudomonas* is repeatedly identified in archaeological remains and cultural heritage (Li et al., 2018; Siles et al., 2018; He et al., 2021; Pavlović et al., 2022). Numerous studies have shown that the genus *Pseudomonas* contains nitrogen-fixing bacteria and nitrate-reducing bacteria (NRB) (Dou et al., 2022; Wu et al., 2023). The NRB can reduce nitrate under biocatalysis by transporting the electrons into the cell cytoplasm and further cause the biocorrosion. The genus *Arthrobacter* is an aerobic culturable bacteria in soil and is also thought to be able to decompose various organic matter (Yao et al., 2015). More importantly, the genus also contained some species with excellent tolerance to excess heavy metal, which formed biofilms and colonized on the surface of copper, resulting in the pitting corrosion (Swaroop et al., 2016). The *Variovorax* sp. has also been isolated from bacterial biofilms of copper pipes, probably leading to the biodeterioration (Reyes et al., 2008), which might be the reason why the genus was significant abundance in all sacrificial pits and almost absent in control group S_0. In addition, the genus *Cupriavidus* also shows the prominent resistance to high concentrations of copper ion, and its growth initiation is strongly stimulated by copper in some instances (Makkar and Casida, 1987; Gillan et al., 2017). So it was reasonable that genus *Cupriavidus* was present in groups K3, K4, and K8 in spite of the high concentration of copper ion from bronzes.

Many fungal genera are also capable of corrosion metals. Recent study has demonstrated that the conidia and hyphae of some fungi adhere to the surface of the metal. They penetrate into the surface layer of metal and go deep into the bulk, finally causing corrosion damage with pitting, ulcers, and cavities (Belov et al., 2023). The genus *Penicillium* was found in all groups and dominant in groups K3 and K8, which has been proven to be able to solubilize several metals including zinc, stannum, aluminum, plumbum, and copper (Siegel et al., 1983). The genus *Aspergillus* exhibits the marked tolerance to copper and also shows the evident biocorrosion of copper metal through proton- and ligand-mediated dissolution mechanisms, causing obvious mass loss and surface etching (Zhao et al., 2020). This was a good explanation for why the genus was the dominant fungi in sacrificial pits. Genus *Rhodotorula*, dominant in groups K3 and K8, unexpectedly, was also identified in biofilms that causes MIC in copper pipes, probably meaning that the genus was involved in the corrosion of bronzes (Reyes et al., 2008) in sacrificial pits. In addition, the genus *Cladosporium* was also involved in the biodeterioration of metal (Iverson, 1987).

As we all know, the MIC process of metals is related to the formation of microbial biofilms, which can form on any metal

surface. The bacterial and fungal colonization in biofilms can modify the localized environment at the metal surface and cause corrosion. Generally, there are two typical MIC processes, namely, the extracellular electron transfer MIC (EET-MIC) and metabolite MIC (M-MIC) (Dou et al., 2022). The former mainly utilizes EET to transport the electrons in the corrosion process of sulfate-reducing bacteria (SRB) and NRB because metal oxidation occurs extracellularly while sulfate or nitrate reduction occurs intracellularly with biocatalysis (Di, 2022). The genus *Pseudomonas* and genus *Arthrobacter* are usually considered to corrode the copper by EET-MIC. For example, *Pseudomonas aeruginosa* utilizes copper as an electron donor, and the terminal electron acceptor is nitrate, causing and accelerating the corrosion of bronzes (Dou et al., 2022). The *Arthrobacter sulfureus* is electrochemically active and is able to transfer electron through the membrane-associated C-cytochromes or through conductive pili (Swaroop et al., 2016). As for the other biocorrosion mechanism, the M-MIC often is caused by corrosive metabolites such as organic acids secreted by bacteria and fungi. The genus *Variovorax* in biofilm may destroy copper by releasing acid metabolites. Moreover, more and more research studies (Rao et al., 2002; Anjum et al., 2009; Amiri et al., 2011) also demonstrate that the fungi such as genera *Penicillium* and *Aspergillus* can generate varieties of organic acid metabolites including citric acid, lactic acid, oxalic acid, malic acid, and gluconic acid to dissolve and leach metals effectively, especially copper.

4.3 The effect of burial activity and artifact material on microbiome

By comparing the results of microbial diversity of different groups, it was clear to observe that the microbial composition and structure of raw soil samples exhibited a significant difference from the test samples in sacrificial pits K3, K4, and K8, meaning the microbial community could be modified in the archaeological site because of the buried man-made structure and artifacts. The results were consistent with previous studies (Junco et al., 1992). Compared to the control group S_0, there were many dominant bacteria and fungi, such as genera *Caulobacter*, *Sphingomonas*, *Sphingopyxis*, *Limnobacter*, *Cladosporium*, *Acremonium*, *Simplicillium*, and *Mortierella* in test samples in sacrificial pits. Some of them were related to the degradation of organic matter, indicating that there might be a certain amount of organic matter ever buried in the sacrificial pits, such as proteins, lipids, and carbohydrates. Unfortunately, as for organic residues, only some textile traces were found on the surface of the bronzes. Although a large amount of ivory was preserved, the collagen in it was almost completely degraded (Honglin et al., 2022). More investigations are needed to identify and demonstrate the presence of different organics in sacrificial pits in the future. The other dominant bacterial genera *Pseudomonas*, *Arthrobacter*, and *Variovorax* and fungal genera *Aspergillus* and *Penicillium* in sacrificial pits were in connection with the biocorrosion of bronzes, also revealing the impact of the buried bronzes on microbiome. In addition, the phylum GAL15 was the primary bacteria in all sacrificial pits. The abundance of bacteria GAL15 in different groups followed the distribution pattern: raw soil outside pits (group S_0) < raw soil in pits (group K3_0, K4_0, and K8_0) < test soil in artifact layer (groups K3, K4, and K8). These

results demonstrated that the abundance of phylum GAL15 might be related to the buried artifacts, especially for the bronzes in sacrificial pits, which were consistent with previous findings in ivory layer of Sanxingdui site (Sun et al., 2024). Unfortunately, none of family or genus was matched in this phylum in in-deep analysis.

In addition, the proportion of buried artifacts of different materials also affected the microbial community. The results of β -diversity (Figure 7) indicated that the bacterial and fungal communities of group K3 and group K8 were much closer, and both differed significantly from those of group K4. This phenomenon might result from the material of the buried artifacts. Based on the excavation brief of sacrificial area of Sanxingdui site (Honglin et al., 2022), there are 2,686 numbered artifacts in K3, of which bronzes are the largest in number at 1,171, accounting for 43.60%. As for K8, there are 5,210 artifacts unearthed as the publication of the brief report, with 3,822 bronzes, accounting for 73.36%. Furthermore, the brief excavation report of K4 has been published separately (Danyang et al., 2024), which records the exact number of artifacts in artifact layer. There are 1,118 artifacts found in artifact layer of K4, with 468 pottery sherds and 348 bronzes. The proportions of pottery and bronzes were 41.86 and 31.13%, respectively. The large number of bronzes in K3 and K8, therefore, had a significant impact on the microbial composition and structure, causing the evidence differences from K4. Recent studies have revealed that the copper contents in soil near bronzes in sacrificial pits of Sanxingdui site increased several times (Jianguo et al., 2023). Many metals are essential for life, such as copper, sodium, potassium, zinc, and iron, while all metals can exert toxicity when presented above certain threshold concentrations (Gadd, 2010). The high content of copper ion in soil could inhibit the growth of certain microorganisms, which led to the significant difference of bacterial and fungal communities between different sacrificial pits, also between the sacrificial pits and soil outside pits. The PCoA results of bronzes and pottery sherds (Figures 7C,D) suggested the impact of copper ion was more obvious on bacteria than fungi, with the more concentrated distribution of the same type samples. Meanwhile, the bacterial community of all raw soils either inside pits or outside pits was closer to group K4, because of the lower proportion of the bronzes in K4.

5 Conclusion

In summary, the bacteria were classified into 42 phyla, 405 families, 667 genera, and 3,174 OTUs, while the fungi were annotated into 12 phyla, 182 families, 298 genera, and 733 OTUs. Some microbes associated with the deterioration of organics and bronzes were found in sacrificial pits, indicating that biodeterioration was the important cause for the degradation and corrosion of sacrificial offering or buried artifacts. Simultaneously, the analysis of the microbial profiles of the soil samples from Sanxingdui site indicated the significant differences in microbial diversity and community between raw soil and soil around artifacts in sacrificial pits. The differences of α -diversity, OTU characterization, microbial composition, and community between raw soil groups and test groups revealed the huge impact of human sacrificial and burial activities on microbiome. The microbial diversity in artifact layer in the sacrificial pits decreased, while the bacteria and fungi resistant

to heavy metals and degrading organic matter increased. The microbial diversity analysis, therefore, may be an available method to speculate and understand the ancient remains and human activities.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in <https://www.ncbi.nlm.nih.gov/sra>, PRJNA1188358.

Author contributions

RC: Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Data curation. ZX: Funding acquisition, Project administration, Resources, Writing – review & editing. QX: Formal analysis, Software, Writing – review & editing. CW: Data curation, Investigation, Writing – review & editing. RW: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by National Key R&D Program of China (2022YFF0903800), Sanxingdui Archaeological Excavation Cultural Relics Protection and Restoration Project, and “111 project” (D18004).

References

- Alias-Villegas, C., Jurado, V., Laiz, L., and Saiz-Jimenez, C. (2013). *Sphingopyxis italica* sp. nov., isolated from Roman catacombs. *Int. J. Syst. Evol. Microbiol.* 63, 2565–2569. doi: 10.1099/ijso.0.046573-0
- Amiri, F., Yaghmaei, S., and Mousavi, S. M. (2011). Bioleaching of tungsten-rich spent hydrocracking catalyst using *Penicillium simplicissimum*. *Bioresour. Technol.* 102, 1567–1573. doi: 10.1016/j.biortech.2010.08.087
- Anjum, F., Bhatti, H. N., Ghauri, M. A., Bhatti, I. A., Asgher, M., and Asi, M. R. (2009). Bioleaching of copper, cobalt and zinc from black shale by *Penicillium notatum*. *Afr. J. Biotechnol.* 8, 5038–5045.
- Belov, D. V., Belyaev, S. N., and Yunin, P. A. (2023). Biocorrosion of copper under the impact of microscopic Fungi. *Russ. J. Phys. Chem. A* 97, 1812–1824. doi: 10.31857/s004453723120051
- Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276
- Brzozowska, I., Bogdanowicz, A., Szczesny, P., Zielenkiewicz, U., and Laud, A. (2018). Evaluation of bacterial diversity on historical silk velvet textiles from the Museum of King John III's palace at Wilanów. *Poland. Int. Biodeterior. Biodegrad.* 131, 78–87. doi: 10.1016/j.ibiod.2017.02.017
- Cao, J., Liu, X., Wang, J., Chen, H., Liu, D., Li, J., et al. (2024). Analysis of microbial diversity and its degradation function in wooden piles at Shahe ancient bridge site in Xi'an and protection measures. *Herit. Sci.* 12. doi: 10.1186/s40494-024-01157-w
- Chen, Y., Zhang, W., He, Y., Wang, B., Xiao, J., Zhou, X., et al. (2020). Microbial community composition and its role in volatile compound formation during the spontaneous fermentation of ice wine made from Vidal grapes. *Process Biochem.* 92, 365–377. doi: 10.1016/j.procbio.2020.01.027
- Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560
- Danyang, X., Yue, F., Rui, W., Hong, X., and Honglin, R. (2024). Brief excavation report of sacrificial pit K4 of Sanxingdui site, Guanghan City, Sichuan. *Sichuan Cultural Relics.* 4–20+22+121.
- Di, W. (2022). Identifications of different microbiologically influenced corrosion (MIC) mechanisms and MIC mitigation using enhanced biocide treatment. Doctor of Philosophy Dissertation/Thesis, Ohio University.
- Dou, W., Pu, Y., Gu, T., Chen, S., Chen, Z., and Xu, Z. (2022). Biocorrosion of copper by nitrate reducing *Pseudomonas aeruginosa* with varied headspace volume. *Int. Biodeterior. Biodegrad.* 171:105405. doi: 10.1016/j.ibiod.2022.105405
- Dworecka-Kaszak, B., and Kizerwetter-Świda, M. (2011). Pseudomycelium forming *Rhodotorula* - unusual picture of biofilm. *Mikologia Lekarska* 18, 74–78.
- Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604
- Finice, M. (2016). The Psychrotolerant Antarctic fungus *Lecanicillium muscarium* CCFEE 5003: a powerful producer of cold-tolerant Chitinolytic enzymes. *Molecules* 21:447. doi: 10.3390/molecules21040447
- Finkel, O. M., Salas-González, I., Castrillo, G., Conway, J. M., Law, T. F., Teixeira, P. J. P. L., et al. (2020). A single bacterial genus maintains root growth in a complex microbiome. *Nature* 587, 103–108. doi: 10.1038/s41586-020-2778-7
- Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156, 609–643. doi: 10.1099/mic.0.037143-0
- Gancz, A. S., Farrer, A. G., Nixon, M. P., Wright, S., Arriola, L., Adler, C., et al. (2023). Ancient dental calculus reveals oral microbiome shifts associated with lifestyle and disease in Great Britain. *Nat. Microbiol.* 8, 2315–2325. doi: 10.1038/s41564-023-01527-3
- Ghio, S., Lorenzo, G. S. D., Lia, V., Talia, P., Cataldi, A., Grasso, D., et al. (2012). Isolation of *Paenibacillus* sp. and *Variovorax* sp. strains from decaying woods and

Acknowledgments

We are grateful to thank Majorbio Co. Ltd. (Shanghai, China) for providing technical support. All the staffs from Sichuan Provincial Cultural Relics and Archaeology Research and the Sanxingdui Joint Excavation Team are thanked for permission to take samples. We also thank Shiwei Wang from the College of Life Science, Northwest University for the comments to analyze data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1489025/full#supplementary-material>

characterization of their potential for cellulose deconstruction. *Int J Biochem Mol Biol* 3, 352–364.

Gillan, D. C., Van Camp, C., Mergeay, M., Provoost, A., Thomas, N., Vermard, L., et al. (2017). Paleomicrobiology to investigate copper resistance in bacteria: isolation and description of *Cupriavidus necator* B9 in the soil of a medieval foundry. *Environ. Microbiol.* 19, 770–787. doi: 10.1111/1462-2920.13645

He, D., Wu, F., Ma, W., Zhang, Y., Gu, J.-D., Duan, Y., et al. (2021). Insights into the bacterial and fungal communities and microbiome that causes a microbe outbreak on ancient wall paintings in the Majishan grottoes. *Int. Biodeterior. Biodegrad.* 163:105250. doi: 10.1016/j.ibiod.2021.105250

Hernández-Almanza, A., Cesar Montanez, J., Aguilar-González, M. A., Martínez-Ávila, C., Rodríguez-Herrera, R., and Aguilar, C. N. (2014). *Rhodotorula glutinis* as source of pigments and metabolites for food industry. *Food Biosci.* 5, 64–72. doi: 10.1016/j.fbio.2013.11.007

Honglin, R., Yu, L., Hao, Z., Zhenbin, X., Haichao, L., Chong, W., et al. (2022). Sacrificial area of the Sanxingdui site in Guanghan City, Sichuan. *Archaeology (China)*. 15–33+12.

Hu, J., Zhu, R., and Gao, M. (2015). Application of *Acremonium* cellulase in saccharification of corncobs. *Industrial Microbiol.* 45, 1–6.

Ivanova, E., Doronina, N., and Trotsenko, Y. (2007). *Hansschlegelia plantiphila* gen. Nov. sp. nov., a new aerobic restricted facultative methylotrophic bacterium associated with plants. *Syst. Appl. Microbiol.* 30, 444–452. doi: 10.1016/j.syapm.2007.03.001

Iverson, W. P. (1987). “Microbial corrosion of metals” in *Adv. Appl. Microbiol.* ed. A. I. Laskin (San Diego: Academic Press), 1–36.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M., and Sait, M. (2002). Improved Culturability of soil Bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Appl. Environ. Microbiol.* 68, 2391–2396. doi: 10.1128/aem.68.5.2391-2396.2002

Jianguo, D., Wenli, Z., Min, G., and Yuxin, X. (2023). Study on the buried layer soil around ancient ivory in Sanxingdui. *J. Sichuan Univ. Sci. Engin.* 36, 20–27. doi: 10.11863/j.suse.2023.03.03

Juanjuan, C., Xuping, Z., Xunxun, S., Hui, T., and Liping, Z. (2016). Separation of Actinomycetes and phylogenetic analysis in Dachangliang ancient site and Majuangou I cultural stratum of Nihewan. *Genom. Appl. Biol.* 35, 913–917. doi: 10.13417/j.gab.035.000913

Junco, A. S. D., Moreno, D. A., Ranninger, C., Ortega-Calvo, J. J., and Sáiz-Jiménez, C. (1992). Microbial induced corrosion of metallic antiquities and works of art a critical review. *Int. Biodeterior. Biodegrad.* 29, 367–375. doi: 10.1016/0964-8305(92)90053-Q

Jurado, V., Sanchez-Moral, S., and Saiz-Jimenez, C. (2008). Entomogenous fungi and the conservation of the cultural heritage: a review. *Int. Biodeterior. Biodegrad.* 62, 325–330. doi: 10.1016/j.ibiod.2008.05.002

Kazarina, A., Gerhards, G., Petersone-Gordina, E., Kimsis, J., Pole, I., Zole, E., et al. (2019). Analysis of the bacterial communities in ancient human bones and burial soil samples: tracing the impact of environmental bacteria. *J. Archaeol. Sci.* 109:104989. doi: 10.1016/j.jas.2019.104989

Kerstens, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., and Stackebrandt, E. (2006). “Introduction to the Proteobacteria” in *The prokaryotes: Volume 5: Proteobacteria: Alpha and Beta subclasses*. eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt (New York, NY: Springer New York), 3–37.

Leung, H. T. C., Maas, K. R., Wilhelm, R. C., and Mohn, W. W. (2016). Long-term effects of timber harvesting on hemicellulolytic microbial populations in coniferous forest soils. *ISME J.* 10, 363–375. doi: 10.1038/ismej.2015.118

Li, Q., Cao, L., Tan, H., Sun, J., Cui, Y., and Xu, R. (2018). Bacterial communities in the waterlogged wooden cultural relics from the “Nanhai no. 1” shipwreck. *Acta Microbiol Sin.* 58, 1439–1452. doi: 10.13343/j.cnki.wsxb.20170488

Li, Q., Zhang, B., Wang, L., and Ge, Q. (2017). Distribution and diversity of bacteria and fungi colonizing ancient Buddhist statues analyzed by high-throughput sequencing. *Int. Biodeterior. Biodegrad.* 117, 245–254. doi: 10.1016/j.ibiod.2017.01.018

Li, H., Zou, W., Bai, G., and Xu, J. (2019). Screening and identification of high-level cellulase-producing fungi. *Food Ferment. Industries* 45, 54–58. doi: 10.13995/j.cnki.11-1802/ts.018431

Liu, Z., Fu, T., Hu, C., Shen, D., Macchioni, N., Sozzi, L., et al. (2018). Microbial community analysis and biodeterioration of waterlogged archaeological wood from the Nanhai no. 1 shipwreck during storage. *Sci. Rep.* 8:7170. doi: 10.1038/s41598-018-25484-8

Liu, X., Koestler, R. J., Wscheid, T., Katayama, Y., and Gu, J.-D. (2020). Microbial deterioration and sustainable conservation of stone monuments and buildings. *Nat. Sustain.* 3, 991–1004. doi: 10.1038/s41893-020-00602-5

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Makkar, N. S., and Casida, L. E. (1987). *Cupriavidus necator* gen. Nov., sp. nov.: a nonobligate bacterial predator of Bacteria in soil. *Int. J. Syst. Evol. Microbiol.* 37, 323–326. doi: 10.1099/00207713-37-4-323

Margesin, R., Siles, J. A., Cajthaml, T., Öhlinger, B., and Kistler, E. (2016). Microbiology meets archaeology: soil microbial communities reveal different human activities at archaic Monte Iato (sixth century BC). *Microb. Ecol.* 73, 925–938. doi: 10.1007/s00248-016-0904-8

Milanesi, C., Faleri, C., Cresti, M., Andreolli, M., Lampis, S., Vallini, G., et al. (2019). Apple seeds in an excavated Roman amphora remained intact for 2000 years despite exposure to a broadly-degrading microbial community. *J. Archaeol. Sci. Rep.* 25, 472–485. doi: 10.1016/j.jasrep.2019.04.024

Nikodemova, M., Holzhausen, E. A., Deblois, C. L., Barnett, J. H., Peppard, P. E., Suen, G., et al. (2023). The effect of low-abundance OTU filtering methods on the reliability and variability of microbial composition assessed by 16S rRNA amplicon sequencing. *Front. Cell. Infect. Mi.* 13. doi: 10.3389/fcimb.2023.1165295

Ozimek, E., and Hanaka, A. (2021). *Mortierella* species as the plant growth-promoting Fungi present in the agricultural soils. *Agriculture* 11. doi: 10.3390/agriculture11010007

Pavlović, J., Sclocchi, M. C., Planý, M., Ruggiero, D., Puškárová, A., Bučková, M., et al. (2022). The microbiome of candle beeswax drops on ancient manuscripts. *Int. Biodeterior. Biodegrad.* 174:105482. doi: 10.1016/j.ibiod.2022.105482

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. USA* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Philips, A., Stolarek, I., Kuczkowska, B., Juras, A., Handschuh, L., Piontek, J., et al. (2017). Comprehensive analysis of microorganisms accompanying human archaeological remains. *GigaScience* 6, 1–13. doi: 10.1093/gigascience/gix044

Pozdnyakova, N. N., Varese, G. C., Prigione, V., Dubrovskaya, E. V., Balandina, S. A., and Turkovskaya, O. V. (2019). Degradative properties of two newly isolated strains of the ascomycetes *Fusarium oxysporum* and *Lecanicillium aphanocladii*. *Int. Microbiol.* 22, 103–110. doi: 10.1007/s10123-018-0032-z

Rao, D. V., Shivannavar, C. T., and Gaddad, S. M. (2002). Bioleaching of copper from chalcopyrite ore by fungi. *Indian J. Exp. Biol.* 40, 319–324

Ren, Y., Wu, C., Wang, L., Guo, J., Zhao, X., Chai, Y., et al. (2023). Fading mechanism of the blue and green pigments on Chinese polychrome pottery of the Tang dynasty: first discovery of *Simplicillium aogashimaense* strain on artifacts. *J. Cult. Herit.* 62, 181–186. doi: 10.1016/j.culher.2023.05.028

Reyes, A., Letelier, M. V., De la Iglesia, R., González, B., and Lagos, G. (2008). Microbiologically induced corrosion of copper pipes in low-pH water. *Int. Biodeterior. Biodegrad.* 61, 135–141. doi: 10.1016/j.ibiod.2007.06.001

Sakr, A. A., Ghaly, M. F., Edwards, H. G. M., Ali, M. F., and Abdel-Halim, M. E. F. (2020). Involvement of Streptomyces in the deterioration of cultural heritage materials through biomineralization and bio-pigment production pathways: a review. *Geomicrobiol J.* 37, 653–662. doi: 10.1080/01490451.2020.1754533

Sharma, M., Khurana, H., Singh, D. N., and Negi, R. K. (2021). The genus *Spingopyxis*: systematics, ecology, and bioremediation potential - a review. *J. Environ. Manag.* 280:111744. doi: 10.1016/j.jenvman.2020.111744

Siegel, S. M., Siegel, B. Z., and Clark, K. E. (1983). Bio-corrosion: Solubilization and accumulation of metals by fungi. *Water Air Soil Pollut.* 19, 229–236. doi: 10.1007/BF00599050

Siles, J. A., Öhlinger, B., Cajthaml, T., Kistler, E., and Margesin, R. (2018). Characterization of soil bacterial, archaeal and fungal communities inhabiting archaeological human-impacted layers at Monte Iato settlement (Sicily, Italy). *Sci. Rep.* 8:1903. doi: 10.1038/s41598-018-20347-8

Smith, N. R., Kishchuk, B. E., and Mohn, W. W. (2008). Effects of wildfire and harvest disturbances on Forest soil bacterial communities. *Appl. Environ. Microbiol.* 74, 216–224. doi: 10.1128/aem.01355-07

Spring, S., Kämpfer, P., and Schleifer, K. H. (2001). *Limnobacter thiooxidans* gen. Nov., sp. nov., a novel thiosulfate-oxidizing bacterium isolated from freshwater lake sediment. *Int. J. Syst. Evol. Microbiol.* 51, 1463–1470. doi: 10.1099/00207713-51-4-1463

Stakhov, V. L., Gubin, S. V., Maksimovich, S. V., Rebrikov, D. V., Savilova, A. M., Kochkina, G. A., et al. (2008). Microbial communities of ancient seeds derived from permanently frozen Pleistocene deposits. *Microbiol. J.* 77, 348–355. doi: 10.1134/s0026261708030156

Sun, S., Xu, Z., Ren, M., Li, S., Xie, Z., Luo, Y., et al. (2024). Identification of microbial diversity in buried ivory soil at the Sanxingdui site in Guanghan City, China, using high-throughput sequencing. *Front. Microbiol.* 15:1384650. doi: 10.3389/fmicb.2024.1384650

Swaroop, B. S., Victoria, S. N., and Manivannan, R. (2016). *Azadirachta indica* leaves extract as inhibitor for microbial corrosion of copper by *Arthrobacter sulfureus* in neutral pH conditions—a remedy to blue green water problem. *J. Taiwan Inst. Chem.* 64, 269–278. doi: 10.1016/j.jtice.2016.04.007

Voyron, S., Tonon, C., Guglielmo, L., Celi, L., Comina, C., Ikeda, H., et al. (2022). Diversity and structure of soil fungal communities unveil the building history of a burial mound of ancient Japan (Tobitsuka Kofun, Okayama prefecture). *J. Archaeol. Sci.* 146:105656. doi: 10.1016/j.jas.2022.105656

Wei, X., Ling, X., Yang, L., Zhang, J., Cui, M., He, Z., et al. (2022). Analysis of microbial community structure and diversity in burial soil of Yangguanzhai cemetery. *Front. Microbiol.* 13:845870. doi: 10.3389/fmicb.2022.845870

Wen, Y., Huang, X., Zhou, Y., Hong, Q., and Li, S. (2011). *Hansschlegelia zhihuaiae* sp. nov., isolated from a polluted farmland soil. *Int. J. Syst. Evol. Microbiol.* 61, 1114–1117. doi: 10.1099/ijs.0.021543-0

Wu, X., Wang, X., Meng, H., Zhang, J., Lead, J. R., and Hong, J. (2024). *Pseudomonas fluorescens* with nitrogen-fixing function facilitates nitrogen recovery in reclaimed coal mining soils. *Microorganisms* 12. doi: 10.3390/microorganisms12010009

Yang, M., Liu, N., Wang, B., Li, Y., Li, W., Shi, X., et al. (2024). Stepwise degradation of organic matters driven by microbial interactions in China's coastal wetlands: evidence from carbon isotope analysis. *Water Res.* 250:121062. doi: 10.1016/j.watres.2023.121062

Yao, Y., Tang, H., Su, F., and Xu, P. (2015). Comparative genome analysis reveals the molecular basis of nicotine degradation and survival capacities of *Arthrobacter*. *Sci. Rep.* 5. doi: 10.1038/srep08642

Zhang, Y., Ruff, S. E., Oskolkov, N., Tierney, B. T., Ryon, K., Danko, D., et al. (2023). The microbial biodiversity at the archeological site of Tel Megiddo (Israel). *Front. Microbiol.* 14:1253371. doi: 10.3389/fmicb.2023.1253371

Zhao, J., Csetenyi, L., and Gadd, G. M. (2020). Biocorrosion of copper metal by *aspergillus Niger*. *Int. Biodeterior. Biodegrad.* 154:105081. doi: 10.1016/j.ibiod.2020.105081



OPEN ACCESS

EDITED BY

Sandra Gabriela Gomez De Saravia,
National University of La Plata, Argentina

REVIEWED BY

Paula Sampaio,
University of Minho, Portugal
Natalia Bellotti,
National Scientific and Technical Research
Council (CONICET), Argentina

*CORRESPONDENCE

Natalia Pydyn
✉ pydyn.natalia@gmail.com

RECEIVED 13 February 2025

ACCEPTED 24 March 2025

PUBLISHED 10 April 2025

CITATION

Kraśnicki K, Pydyn N, Papis A, Guzińska K,
Kaźmierczak D, Maciołek U and Wawrzyk A
(2025) Reducing microbial contamination on
historical leather artifacts at the
Auschwitz-Birkenau State Museum using
ethanol in the form of mist.
Front. Microbiol. 16:1576114.
doi: 10.3389/fmicb.2025.1576114

COPYRIGHT

© 2025 Kraśnicki, Pydyn, Papis, Guzińska,
Kaźmierczak, Maciołek and Wawrzyk. This is
an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic practice.
No use, distribution or reproduction is
permitted which does not comply with these
terms.

Reducing microbial contamination on historical leather artifacts at the Auschwitz-Birkenau State Museum using ethanol in the form of mist

Krzysztof Kraśnicki¹, Natalia Pydyn^{1*}, Aleksandra Papis¹,
Krystyna Guzińska², Dorota Kaźmierczak², Urszula Maciołek³
and Anna Wawrzyk^{1,4}

¹Auschwitz-Birkenau State Museum, Oświęcim, Poland, ²Lukasiewicz Research Network-Lodz Institute of Technology, Lodz, Poland, ³Analytical Laboratory, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Skłodowska University, Lublin, Poland, ⁴Department of Basic Biomedical Science, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, Sosnowiec, Poland

Purpose: The aim of the study was to evaluate the biocidal efficacy and determine the influence of 90% ethyl alcohol applied in the form of a mist on the surface of model and historical leather.

Materials and methods: The main object of the study were historical leather shoes from the collections of the Auschwitz-Birkenau State Museum (A-BSM) in Oświęcim (Poland). Microorganisms found before on historical leather objects in A-BSM were inoculated onto samples of model and historical leather. Ethanol mist was applied with an airbrush with optimized parameters for 15 s at a concentration of 90%. To increase the biocidal effectiveness, the samples were sealed in a tight package and stored for 22 hours. The effect of disinfection was assessed using culture-dependent methods. Changes on the surfaces was assessed using SEM, FTIR, and XPS techniques.

Results: On surfaces inoculated with microorganisms in the following quantities: $10^5 - 10^8$ CFU bacteria and 10^5 CFU fungi, a reduction of 99.51% to 99.99% was observed. Ethanol disinfection had no negative effect on the surface morphology and collagen structure.

Conclusions: Disinfection with ethanol applied in the form of mist can be effectively used to eliminate microbiological contamination of historical objects made of leather in A-BSM.

KEYWORDS

ethanol, biodeterioration, microorganisms, cultural heritage, disinfection

1 Introduction

The German Nazi concentration and extermination camp Auschwitz-Birkenau was established in 1940. It was an important part of the drama of World War II and the result of the genocidal policy of Nazi Germany. The huge number of victims, the vast area from which people were brought, the industrial methods of killing people in gas chambers made

A-BSM the most recognizable symbol of the Holocaust and the site of Genocide in the world (Ambrosewicz-Jacobs et al., 2008).

In order to preserve the memory of the victims of the Nazi regime, the A-BSM was established in the former camp area. The institution's activities focus on securing the remains of the former camp, including personal items found after the camp was liberated, which are often the only evidence of the presence of the people who were imprisoned there or killed in gas chambers. The stolen property consists largely of items containing leather elements. These include shoes, suitcases, clothing and equipment, as well as orthopedic supplies. Leather shoes are one of the largest groups of objects in the Auschwitz Museum Collections (Figure 1).

The Museum's activities focus on ensuring proper storage conditions and conducting necessary conservation work on the remains of the camp and its facilities. All facilities and their elements require protection against deterioration processes in a way that causes the least possible damage to their surfaces.

The metabolic activity of microorganisms is the one of the factors causing the destruction of museum collections. Contamination of historic objects is not only the result of microflora floating in the air, although this microflora, including bacteria and fungi, may affect the state of preservation of such objects. There are also other factors that contribute to the degradation of historic objects, including primary contamination. Highly porous materials are more susceptible to microbial colonization because of their capacity to absorb more water and retain it in for longer periods of time. Surface roughness affects the trapping of moisture and concentrates it in micro-fissures where growth is usually more abundant. It also enables the accumulation of particles - soot, organic and inorganic debris, pollens, spores, and salts (Jacob et al., 2018). Microflora transferred by air movement onto historical objects gradually inhabits the microstructural discontinuities of the surface and, through the emission of extracellular enzymatic substances, causes their hydrolysis to simple organic compounds. The biodeterioration of objects is initiated by the naturally occurring enzymatic activity of microorganisms due to the secretion of enzymes from groups such as cellulases, amylases, and proteases. As a result of microbiological decomposition, discolouration, and biological deposits may appear on historical objects, leading to structural weakening and disruption of the continuity of the surface (Szostak-Kotowa, 2004).

As a result of the conducted studies on the identification of microorganisms occurring on historical objects in A-BSM several species of bacteria were identified, including: *Bacillus atrophaeus*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus simplex*, *Bacillus subtilis*, *Enterobacter* sp., *Micrococcus luteus*, *Pantoea agglomerans*, *Psychrobacillus psychrodurans*,

Staphylococcus aureus, *Staphylococcus epidermidis*, and the following species of fungi were identified: *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium chrysogenum*, *Rhizopus nigricans*, *Stephanoascus ciferrii*, and *Trichoderma viride* (Wawrzyk et al., 2018).

Microorganisms, due to complex adaptation mechanisms to changing environmental conditions, are characterized by a wide range of tolerance to changes in pH, temperature and humidity, and a small amount of organic substances is enough for them to survive and develop. Materials of animal or plant origin are particularly susceptible to decomposition, i.e., those made of leather, wood, paper, silk, or wool. The mechanism of biological decomposition of organic materials is based on metabolic activity due to the action of ligninocellulolytic enzymes in the case of wood and paper, cellulases, keratinases, and esterases in the case of leather materials and clothing (Mazzoli et al., 2018).

Therefore, a very important stage preceding the conservation of historical objects is their examination and if there is such need—decontamination. Currently, various disinfection methods are used in museums. These include: washing with alcohol, spraying with quaternary ammonium compounds or fogging with ethylene oxide. Many of the biocidal substances used leave noticeable changes on the surface of the disinfected object, which is not permissible in the case of historical materials (Sequeira et al., 2012; Gutarowska et al., 2017). The effect of disinfection may be the opposite of the expected one and cause accelerated degradation processes (Ortiz et al., 2013).

Considering the conservation works, special attention should be paid to the selection of disinfection methods for historic objects that determine the safety of people performing renovation works. The disinfectant cannot pose a greater threat than the microorganisms it is used to eradicate. The disinfection commonly used in museums so far is often harmful to human health, e.g. ethylene oxide (ETO), highly effective and safe for most museum objects, but at the same time toxic and carcinogenic to humans (Wawrzyk et al., 2018).

Historical objects, depending on the material they are made of, are inhabited by microorganisms with varying sensitivity to disinfectants. Ethanol is a commonly known disinfectant in medicine. Liquid ethanol in concentrations ranging from 60% to 90% has biocidal properties against bacterial, fungal, and viral cells. Its action is based on the denaturation of microbial proteins, leading to the disruption of cell wall continuity and the spilling of cytoplasm into the extracellular environment (Gerba et al., 2024).

In medicine, ethanol is used in liquid form. In the case of museum objects, prolonged contact of aqueous solutions with historical surfaces is not recommended. Increased moisture level could be an initiator of accelerated degradation processes which would lead to irreversible changes in structure by dissolving compounds sensitive to contact with aqueous solutions. The molecular structure of ethanol, due to the presence of a hydroxyl group, makes it a substance with a high redox potential, capable to react with many organic substances, which is not desirable in the case of surfaces of historical objects (Dai et al., 2025). A concentration of 90% ethanol was introduced to accelerate evaporation from the surface and thus shorten the time of action on the surface in this study. Thanks to that, the humidity of the object

Abbreviations: A-BSM, Auschwitz-Birkenau State Museum; ATCC, American Type Culture Collection; ATR, Attenuated Total Reflectance; CFU, Colony Forming Unit; ETO, Ethylene Oxide; FTIR, Fourier Transform Infrared spectroscopy; MCT, Mercury Cadmium Telluride; MEA, Malt Extract Agar; PCA, Plate Counting Agar; PE, Polyethylene; SDA, Sabouraud Dextrose Agar; SEM, Scanning Electron Microscopy; UHV, Ultra-High Vacuum; UV-C, Ultra Violet C; XPS, X-ray photoelectron Spectroscopy.



FIGURE 1

Objects made of leather from the collections of the A-BSM in Oświęcim (A) shoes (B) orthopedic prosthesis (C) suitcase. Photo: Sebastian Mrozek.

would not increase, otherwise soaking of the surface could make them vulnerable to damage. The effectiveness of application of 90% ethanol in the mist form to disinfect textiles was demonstrated by us before (Wawrzyk et al., 2023).

Due to the biocidal effectiveness of liquid ethanol demonstrated in the use of disinfection of abiotic surfaces, the effect of ethanol was tested using a mist application.

The effectiveness of ethanol was studied using strains isolated from the Museum's objects and strains from the American Type Culture Collection (ATCC). The bacteria used were: *Pantoea agglomerans*, *Staphylococcus aureus*, and molds: *Alternaria alternata*, *Aspergillus niger*, *Penicillium chrysogenum*, and *Aspergillus flavus*. The species were selected based on their frequency of occurrence, potential harmful effects on human health, and adverse effects on objects. Fungi of the genera *Aspergillus*, *Alternaria*, and *Penicillium* can cause chronic inflammation in humans resulting from continuous exposure to mold spores, and can also contribute to severe respiratory infections (Hyde et al., 2018). *Staphylococcus aureus* may pose a threat to human health through direct contact with contaminated surfaces causing allergies and purulent leather infections (Tong et al., 2015). Microorganisms with the ability to enzymatically decompose cellulose and produce acids that contribute to the progress of bio-deterioration on objects (*A. alternata*, *A. flavus*, *A. niger*, and *P. chrysogenum*) were also selected for research (Di Carlo et al., 2016). Endotoxin produced by *Pantoea agglomerans* and other Gram-negative bacteria present in leather dust is considered to be the main cause of byssinosis, initiating acute and chronic inflammation of endothelial cells (Dutkiewicz et al., 2016).

Conservation and disinfection works carried out on historical objects cannot adversely affect their surface structure. Very precise, accurate analyses of the surfaces should be carried out before and after the use of the implemented disinfection techniques (Wawrzyk et al., 2020b). Comprehensive physicochemical analysis—FTIR (Fourier Transform Infrared Spectroscopy), SEM (Scanning Electron Microscopy), and XPS (X-ray photoelectron Spectroscopy) allowed for the assessment of possible changes in the collagen structure and determination of the condition of the layers covering the historical leather objects and thus to determine whether the disinfection using ethanol in the mist form negatively affected the surfaces of the tested leather surfaces.

2 Materials and methods

2.1 Microbiological examination of historical objects

For quantitative studies of microbial contamination of collagen objects in A-BSM, 16 children's shoes were used. Swabs were taken from the outer surfaces of footwear made of degraded collagen materials, vegetable-tanned grain leather that showed visible signs of biodegradation. Material for analysis was collected using sterile swabs from surfaces limited by a 25 cm² template, and the swab was then placed in a transport tube. The number of microorganisms was determined using the culture method with serial dilutions. After transport to the laboratory, each collected sample was suspended in 1 ml of sterile 0.85% physiological saline solution and shaken thoroughly for 1 min. 100 µl of the sample was plated on plates with certified solid, ready-made microbiological media (BTL, Poland). Two different media were used: standard agar for determining the total number of bacteria Plate Counting Agar (PCA) and agar Malt Extract Agar (MEA) with malt extract for determination of the number of filamentous fungi and yeasts. The plates were incubated for 10 days at 26°C, and then the bacterial colonies and the microscopically identified mold fungi that grew on solid media were counted. The results were presented as the number of colony-forming units per 100 cm² of the tested surface.

2.2 Preparation of inoculum and leather samples

A diagram showing the biocidal effectiveness of ethanol in the form of mist research action plan is shown in the Figure 2.

Microorganisms from recognized culture collections (ATCC—American Type Culture Collection) and isolated from the surface of leather objects in the A-BSM collections were used to study the biocidal efficacy of ethanol. The microorganisms were isolated in previous studies, deposited in cryobanks of the A-BSM laboratory and, after multiplication, used in this work. The mold fungi selected for the study were inoculated on Sabouraud agar medium with 4% glucose (BTL, Poland). The plates were incubated at 25 ± 2°C

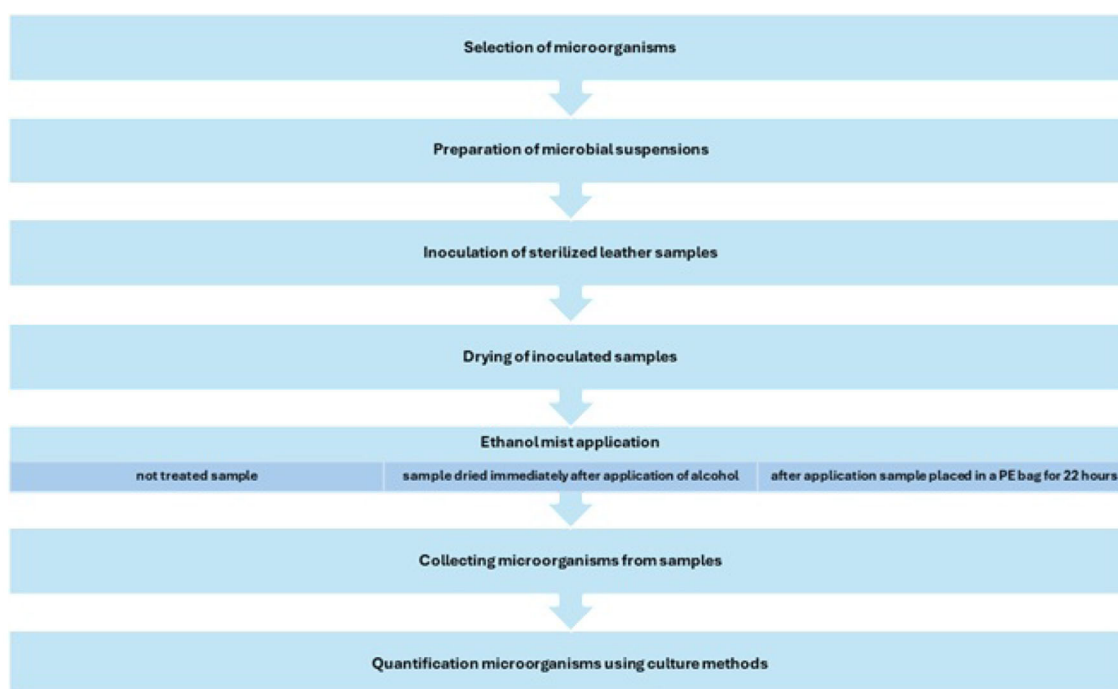


FIGURE 2
Diagram of biocidal effectiveness of ethanol in the form of mist research.

TABLE 1 Concentrations of microbial suspensions applied to model and historic leather samples.

Strain	Concentration of microbial suspension [CFU/ml]
<i>Alternaria alternata</i> (A-BSM cryobank)	5.5×10^5
<i>Aspergillus flavus</i> (ATTC 9643)	1.85×10^6
<i>Aspergillus niger</i> (A-BSM cryobank)	2.30×10^6
<i>Pantoea agglomerans</i> (A-BSM cryobank)	4.59×10^8
<i>Penicillium chrysogenum</i> (A-BSM cryobank)	1.88×10^6
<i>Staphylococcus aureus</i> (ATTC 6538)	4.73×10^8

for 5 days. Suspensions were prepared from the cultures in sterile water to obtain a density of approximately $10^5 - 10^6$ CFU/sample. The suspension obtained was diluted tenfold each time and the number of CFU (Colony Forming Unit) was determined using the plate count method. The bacteria were inoculated on Plate Count Agar (BTL, Poland). The plates were incubated at $30 \pm 2^\circ\text{C}$ for 2 days. Suspensions were prepared from the cultures in sterile water, diluted in peptone water and determined using the plate count method to obtain a density of approximately $10^5 - 10^8$ CFU/ml on the sample.

The concentrations of the microbial suspension were used as described in Table 1.

Historical leather samples of leather shoe dated to the first half of the 20th century from A-BSM collection were used to optimize

the process. Studies were also performed on modern vegetable-tanned pig grain leather, referred in this article as model leather. Historical and model leather samples measuring $40 \text{ mm} \times 40 \text{ mm}$ were prepared and were sterilized with a UV-C lamp before experiment. The volume of 0.6 ml microbial suspension was applied using a pipette onto the model and historic leather samples, and then the samples were dried in a laminar flow cabinet until a constant mass was obtained.

2.3 Application of ethanol in the mist form

The method of applying the ethanol in the mist form to the surfaces of historical objects was established, optimized and described before (Wawrzyk et al., 2023). The aforementioned publication contains information on the process of optimizing the application of ethanol mist, but the research was conducted on textile material. The cotton used in those studies is a cellulose material that has different properties and reacts differently with biocides than the collagen materials included in this publication. Briefly, the Paasche VL 0819 and VE 0707 dual-action airbrush was selected, which allowed the most even spraying of 90% ethanol in the form of a mist, assessed visually as the degree of surface wetting, at a working pressure of 0.2 MPa and using a PA HEAD VLH-5 nozzle with a 1.05 mm diameter tip. Ethanol was applied to the material in the form of a mist from a distance of 16 cm from the sample for 12 seconds on a surface of 16 cm^2 . As part of the optimization of disinfection parameters after applying ethanol mist, the samples were additionally stored in PE (Polyethylene) foil at $21^\circ\text{C} \pm 1^\circ\text{C}$ for $22 \pm 1 \text{ h}$ to extend the effect of ethanol vapors.

The distance between airbrush and sample was optimized to obtain the most uniform application possible with little wetting and the smallest possible losses resulting from ethanol dispersion.

2.4 Evaluation of the biocidal effectiveness of ethanol in the form of mist

After application of ethanol, the samples were allowed to dry for approximately 5 h in a vertical laminar airflow cabinet. Inoculated skin samples without ethanol mist application were used as a control. The microorganisms were then washed out by shaking for 5 min at 250 rpm in 100 ml of peptone water with the addition of 2% Tween. Ten-fold dilutions were made and inoculated onto media. Fungi were incubated on SDA (Sabouraud Dextrose Agar) medium for 5 days at 25°C, and bacteria on PCA medium for 48 h at 30°C. Then, the number of microorganisms was determined using the formula:

$$M = \frac{\Sigma C}{v(n_1 + 0.1n_2)d} \cdot 100 \quad (1)$$

Where:

M - number of bacteria/fungi on the sample [cfu/sample];

C - sum of colonies on all plates from the counted dilution;

v - volume of inoculation applied to each plate in ml;

n₁ - number of plates from the counted dilution;

n₂ - number of plates from the second counted dilution;

d - dilution index corresponding to the counted dilution.

If no colonies were detected on two plates, the result was given as <100 or on a logarithmic scale <2. This means that no microorganisms were present or were below the detection limit.

From the obtained number of microorganisms, the percentage and logarithmic reduction were calculated:

The percentage reduction was calculated using the formula:

$$Reduction, \% = (W - T) / W \cdot 100 \quad (2)$$

The logarithmic reduction was calculated using the formula:

$$Reduction \log_{10} = \log W - \log T \quad (3)$$

Where:

W - number of bacteria on the model sample without alcohol application examined at the same time as the samples with ethanol application.

T - number of bacteria on the model or historical sample after ethanol exposure.

2.5 Analysis of the chemical composition of the surface after decontamination

2.5.1 Scanning electron microscopy

The examination using an electron microscope enabled imaging of the surface topography of the analyzed leather samples before and after the disinfection process. The device used in this

study was a scanning electron microscope SEM: FEI Quanta 3D FEG with a vacuum sputtering device Polaron SC7640 (Quorum Technologies, Great Britain).

2.5.2 Fourier transform infrared spectroscopy

In order to investigate whether ethanol caused adverse changes on the surface of the disinfected leather, Fourier-transform infrared spectroscopy (FTIR) was applied, using a Nicolet 8700 FTIR spectrometer (Thermo Scientific, USA) with a Smart Orbit™ diamond ATR (Attenuated Total Reflectance) module and MCT (Mercury Cadmium Telluride) detector cooled with liquid nitrogen. Before and after the application of ethanol in the form of mist, spectra were collected from a 2–3 μm thick layer in the range of 4,000–650 cm⁻¹ with a resolution of 4 cm⁻¹. The ATR spectra were subjected to ATR correction, scaled normalization, and baseline. Spectra equivalent to transmission spectra were obtained. The OMNIC 3.2 software (Thermo Scientific, USA) was used for the tests.

2.5.3 X-Ray photoelectron spectroscopy

The determination of the chemical composition of the surface layer of the samples before and after the decontamination process with 90% ethanol solution was carried out using the XPS technique. For this purpose, a multi-chamber UHV (Ultra-High Vacuum) analytical system (Prevac, Poland) was used for the measurement. The photoelectrons were excited by X-ray radiation with the characteristic line Al Kα and energy of 1486.7 eV, generated by the VG Scienta SAX 100 lamp with an aluminum anode together with the VG Scienta XM 780 monochromator. The operating parameters of the X-ray lamp used: U = 12 kV, I_e = 30 mA. The photoelectrons were recorded using the Scienta R4000 hemispherical analyzer. The pressure in the analysis chamber during the measurements was maintained at a level lower than 1.0E-8 mbar. Basic parameters of the analyzer for the survey spectrum: operating mode: sweep, transition energy: 200 eV, measured range of photoelectron binding energy: 0–1350 eV, measurement step: 0.5 eV, collection time in a single step: 200 ms. In order to compensate for the electric charge generated during the measurement, the samples were bombarded with a beam of low-energy electrons. The recorded spectra were processed using the Casa XPS Version 2.3.16 PR16 program. All spectra were calibrated by determining the position of the C1s carbon line for the energy of 284.5 eV.

3 Results

3.1 The number of microorganisms on leather objects

The total number of bacteria on the historical children's shoes from A-BSM collections was 1.3 × 10³ CFU/100 cm². Fungi contaminated these surfaces to similar extent as bacteria — 1.26 × 10³ CFU/100 cm².

TABLE 2 Evaluation of the biocidal effectiveness of ethanol against selected strains for model and historic leather.

Strain	Sample type	Mass of ethanol applied [g]	Cell count [cfu/sample]	log [cfu/sample]	Reduction level	
					log	%
<i>Alternaria alternata</i> (A-BSM cryobank)	Model	0	2.05×10^4	4.31	-	-
	Model	0.20	1.0×10^2	2.0	2.31	99.51
	Historical	0.16	1.0×10^2	2.0	2.31	99.51
<i>Aspergillus flavus</i> (ATTC 9643)	Model	0	1.9×10^5	5.26	-	-
	Model	0.18	9.5×10^1	2.0	3.26	99.95
	Historical	0.21	9.5×10^1	2.0	2.36	99.95
<i>Aspergillus niger</i> (A-BSM cryobank)	Model	0	6.0×10^4	4.78	-	-
	Model	0.11	2.04×10^2	2.0	2.48	99.66
	Historical	0.22	1.02×10^2	2.0	2.78	99.83
<i>Pantoea agglomerans</i> (A-BSM cryobank)	Model	0	6.77×10^4	4.83	-	-
	Model	0.21	1.02×10^2	2.00	2.83	99.85
	Historical	0.16	1.02×10^2	2.00	2.83	99.85
<i>Penicillium chrysogenum</i> (A-BSM cryobank)	Model	0	1.04×10^5	5.02	-	-
	Model	0.19	1.04×10^2	2.0	2.98	99.90
	Historical	0.13	1.04×10^2	2.0	2.98	99.90
<i>Staphylococcus aureus</i> (ATTC 6538)	Model	0	1.25×10^6	6.1	-	-
	Model	0.13	1.25×10^2	2.00	4.10	99.99
	Historical	0.12	1.25×10^2	2.00	4.10	99.99

3.2 Biocidal efficacy of ethanol in the form of mist

For each of the tested strains, a reduction of microbial counts of more than 99.5% was demonstrated. The highest reduction of 4.1 log was observed for the *Staphylococcus aureus* strain. The lowest reduction of 2.31 log was observed for the *Alternaria alternata* strain. The results are presented in the Table 2.

3.3 Analysis of surface topography and morphology

3.3.1 Scanning electron microscopy

In order to visualize the changes in surface topography and morphology under the influence of 90% ethanol in the form of mist applied to model and historical leather, dried immediately after application and after 22 h of storage in a foil package, examinations were performed under a SEM microscope. The model leather I and historic leather I samples were control samples that were not subjected to the decontamination process. The model leather II and historic leather II samples were prepared by applying a 90% ethanol solution to the surface, and the measurement was performed immediately after the biocidal agent had been applied and dried. For the model leather III and historic leather III samples, the measurement was performed 22 h after disinfection with ethanol in the form of a mist after

22 h of storage in ethanol vapors. Microscopic examinations conducted using scanning electron microscopy on both model (Figure 3) and historical (Figure 4) leather samples did not reveal any significant differences in the structure of the tested material in the analyzed samples. Moreover, the disinfection method used did not have a significant effect on the surfaces of the tested materials. No noticeable changes in the morphology of the observed surfaces were found. It can therefore be concluded that the disinfection technique used did not adversely affect the physicochemical properties of the collagen fibers contained in the disinfected samples.

3.3.2 Fourier transform infrared spectroscopy

The model leather I and historic leather I samples were control samples that were not subjected to the decontamination process. The model leather II and historic leather II samples were prepared by applying a 90% ethanol solution to the surface, and the measurement was performed immediately after the biocidal agent had been applied and dried. For the model leather III and historic leather III samples, the measurement was performed 22 hours after disinfection with ethanol in the form of a mist after 22 hours of storage in ethanol vapors. For all samples, the measurement was performed on the top and bottom side of the sample.

As a result of the measurements, the spectra of the model material samples and the spectra of the historic material were obtained. The spectra for the model material samples are shown

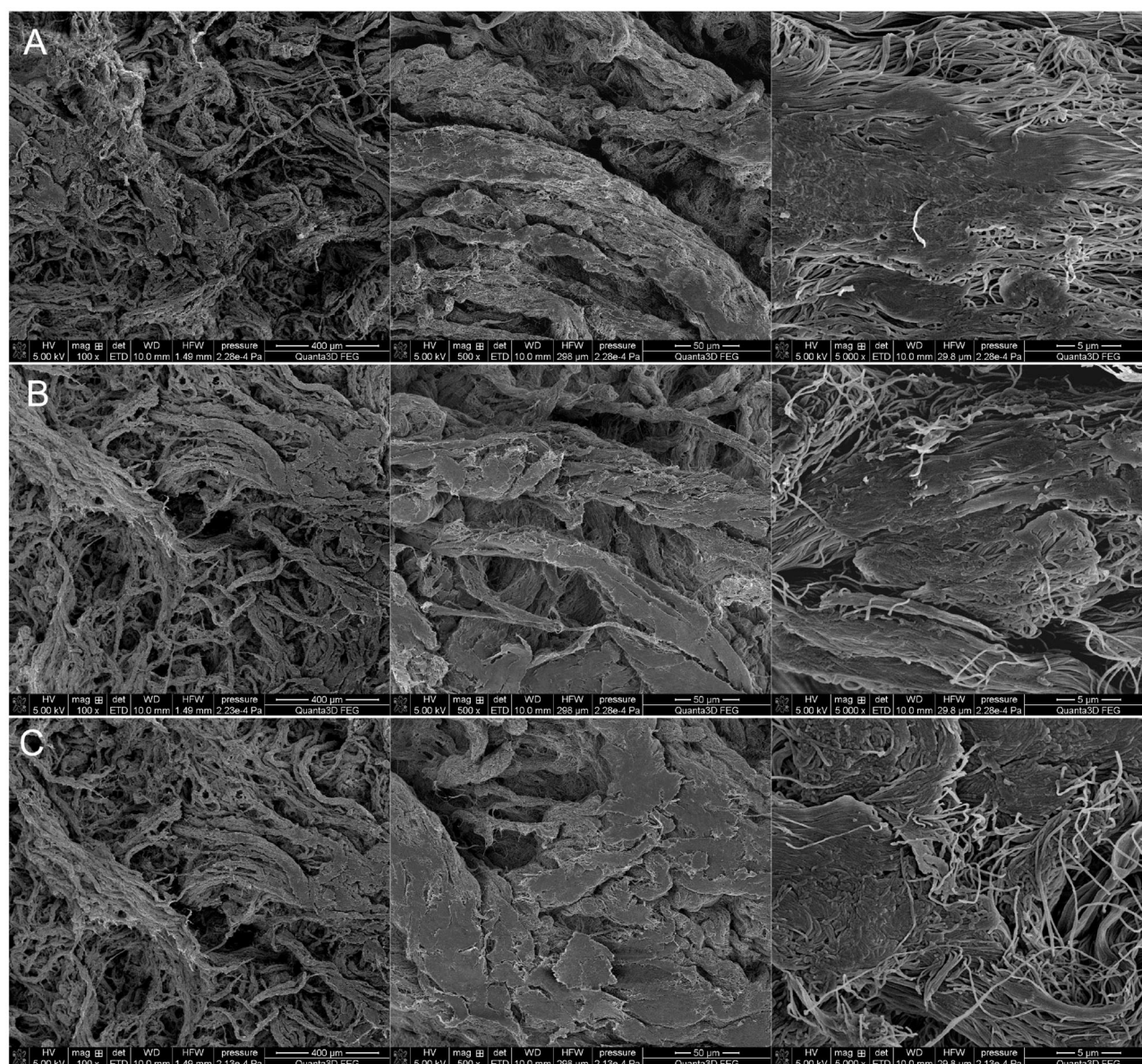


FIGURE 3

Scanning Electron Microscopy (SEM) microscopic images of model leather at magnifications of 100, 500, and 5,000x: (A) model leather—control, (B) model leather—after application of 90% ethanol in the form of mist and direct drying, (C) model leather—after application of 90% ethanol in the form of mist and drying after 22 h of storage in ethanol vapors.

in Figures 5, 6. FTIR-ATR studies of model leather samples I, II, III showed slight differences in the intensity of the IR spectral bands. No changes in the band positions were observed due to the use of ethanol. Figures 7, 8 show the spectra obtained for the historic leather samples. For the historical leather top sample after the use of ethanol exposed to long-term exposure to ethanol vapors, a slight change in the band intensity was observed in relation to the reference sample—new signals were observed in the area of $3,500\text{ cm}^{-1}$, $3,400\text{ cm}^{-1}$, $1,200\text{--}1,100\text{ cm}^{-1}$ (Figure 7). The peaks of historical leather I, historical leather II and historical leather III, located at the same wavenumber value, overlap, which indicates no significant differences between the samples treated with ethanol and not treated (Figures 7, 8).

3.3.3 Analysis of the chemical composition of the surfaces

In order to fully illustrate the effect of disinfection with a 90% ethanol solution applied in the form of a mist on the leather surfaces, an additional study was performed using the XPS technique to determine the elemental composition of the outer layers of the tested samples. Analysis of the spectral lines visible in the spectra enabled the detection of elements contained in the tested samples. The outer surface of the model leather contained the following elements: carbon, nitrogen, oxygen, sodium, silicon, sulfur, calcium, and phosphorus (Figure 9). The samples of historic leather contained carbon, nitrogen, oxygen, sodium, aluminum, magnesium, silicon, sulfur, calcium, and iron on their surface

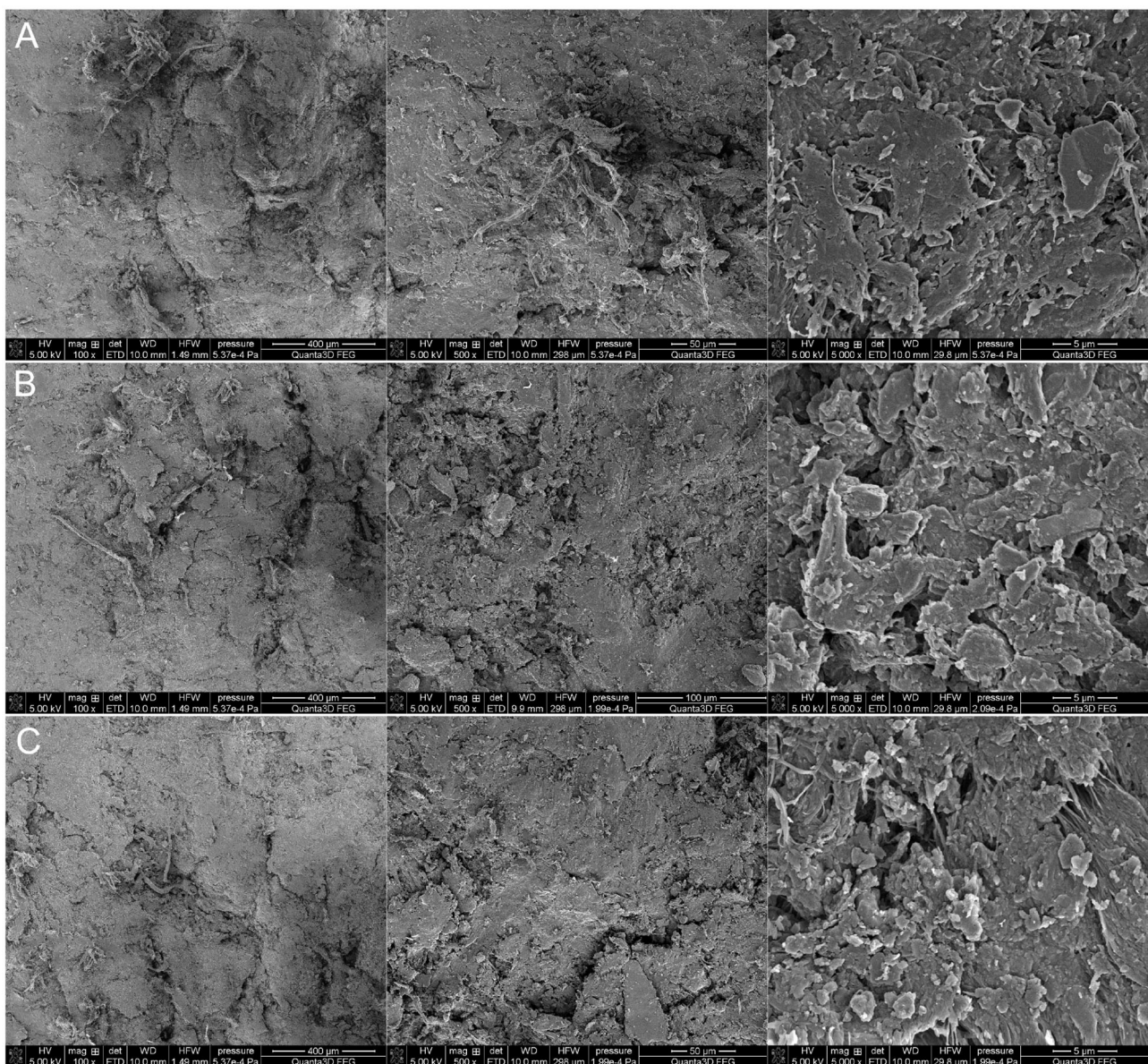


FIGURE 4

Scanning Electron Microscopy (SEM) microscopic images of historical leather at 100, 500, and 5,000x magnifications: (A) historical leather—control, (B) historical leather—after application of 90% ethanol in the form of mist and direct drying, (C) historical leather—after application of 90% ethanol in the form of mist and drying after 22 h of storage in ethanol vapors.

(Figure 10). In model leather samples, the vast majority of the elemental composition expressed in mass percentages is carbon and oxygen, which is due to the structure of the leather, mostly composed of collagen (Figure 11). XPS studies performed in a narrow range of binding energies showed the presence of the following chemical bonds characteristic of carbon: C-C, C-O-C, C=O, O-C=O. Calcium present in the samples occurs in the form of carbonates, while silicon in the form of silicon(IV) oxide. No significant differences were found between not treated and decontaminated model leather samples (Figure 9).

In the case of historic samples, among elements found in model leather, aluminum, iron and sulfur were also determined, which most likely constitute a secondary layer of the tested historic

surface. The average analysis depth for an XPS measurement is about 5 nm, so it mainly determines changes in the secondary layer of the object. Analysis of samples of historic leather materials also did not show any significant changes in the composition of elements after disinfection with a 90% ethanol solution in the form of mist (Figure 12).

XPS studies performed in a narrow range of binding energies showed the presence of the following chemical bonds characteristic of carbon: C-C, C-O-C, C=O, O-C=O, C-C, C-O-C, C=O, O-C=O, C-C/C-HC-C/-C-N-, C-OH, C-O-C, C=O, COOR, C-C/C-H, C-C/-C-S/-C-N-, C-OH, C-O-C, C=O, and COOR. Calcium present on the surface of the tested samples occurs in the form of carbonates, while silicon in the form of SiO₂.

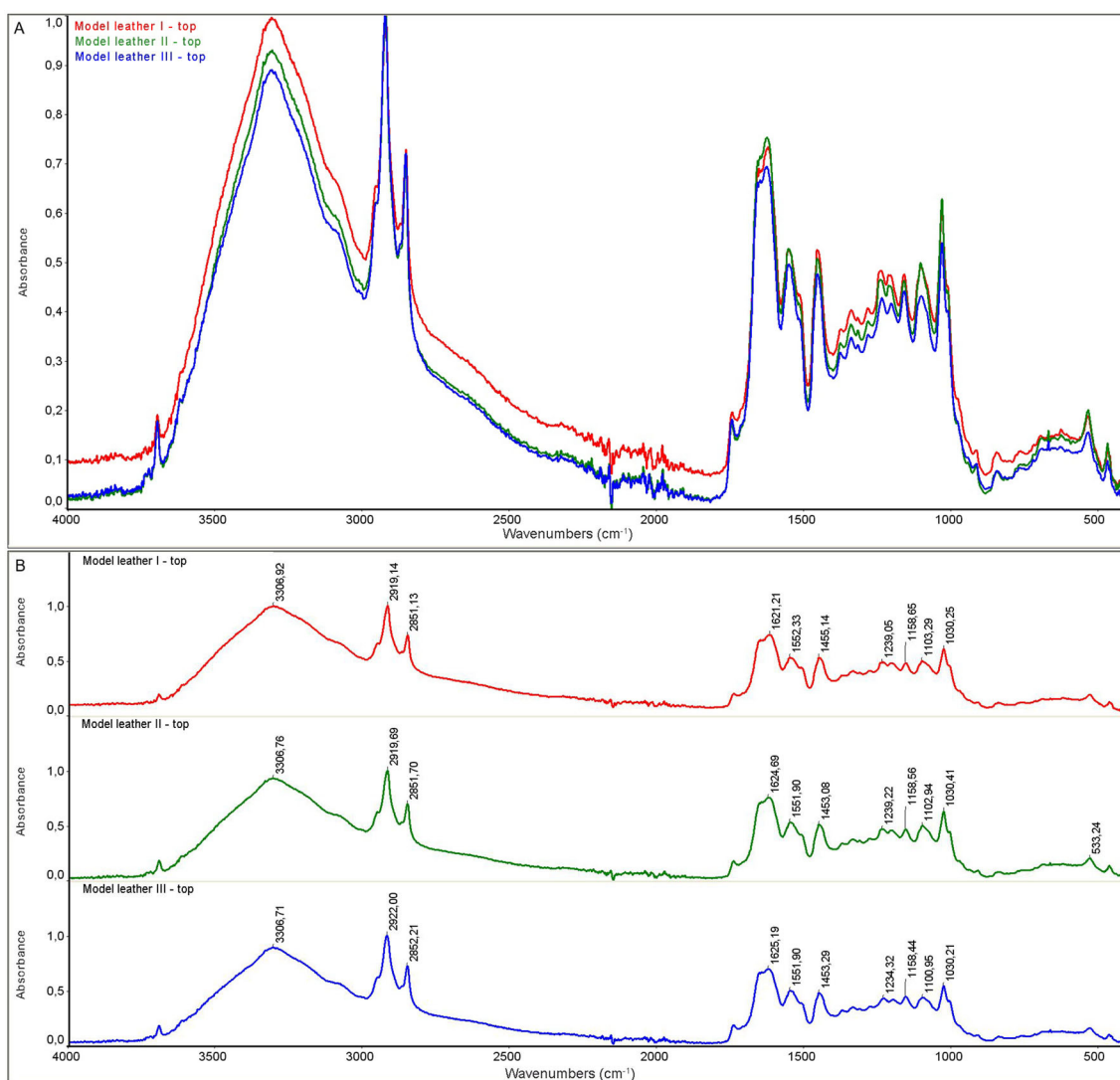


FIGURE 5

ATR spectra of the model leather samples, upper side: (A) superimposed, (B) with characteristic peaks marked; model leather I (red)—not subjected to the decontamination process; model leather II (green)—after applying ethanol and dried; model leather III (blue)—after 22 h of storage in ethanol vapors.

4 Discussion

Leather is a common building material for historical objects found in A-BSM, such as shoes or suitcases, which are often the only remnant of their owners, victims of the concentration camp, and therefore their historical value is priceless.

Microorganisms that may be harmful to human health and have a destructive effect on the surfaces of historical objects are identified on objects found in A-BSM. The presence of bacteria and fungi, native to the museum environment, on the surfaces of historical objects may pose a threat to the health of people exposed to professional contact with such items (Skóra et al., 2015). Biodeterioration of leather objects involves the decomposition of tannins, fats and proteins and may result in the appearance of discolorations, stains, indentations and a weakening of the strength (Rybitwa et al., 2020). In the current study, we detected

an average of 1.26×10^3 cfu/100cm² of bacteria and 1.30×10^3 cfu/100cm² of fungi on the leather surfaces of historical children's shoes. In addition, in 2020 we published data on microbiological contamination of leather objects. On the surfaces of leather shoes, the average number of bacteria ranged between 1.96×10^6 and 7.61×10^6 cfu/100cm², while fungi colonized these objects in an average number of 2.33×10^4 – 2.56×10^4 cfu/100cm². Thirty species of bacteria and 24 species of fungi were identified, including microorganisms that could adversely affect the state of preservation of the objects (e.g., *P. agglomerans*, *A. pullulans*) and the health of workers carrying out conservation work (e.g., *B. cereus*, *S. epidermidis*) (Rybitwa et al., 2020). For comparison, public utility areas are characterized by presence of microorganisms in average number ranging from 10^1 to 10^4 cfu/m² (Wawrzyk et al., 2020a). Uneven distribution of microorganisms on historical leather objects may result from various factors such as the chemical

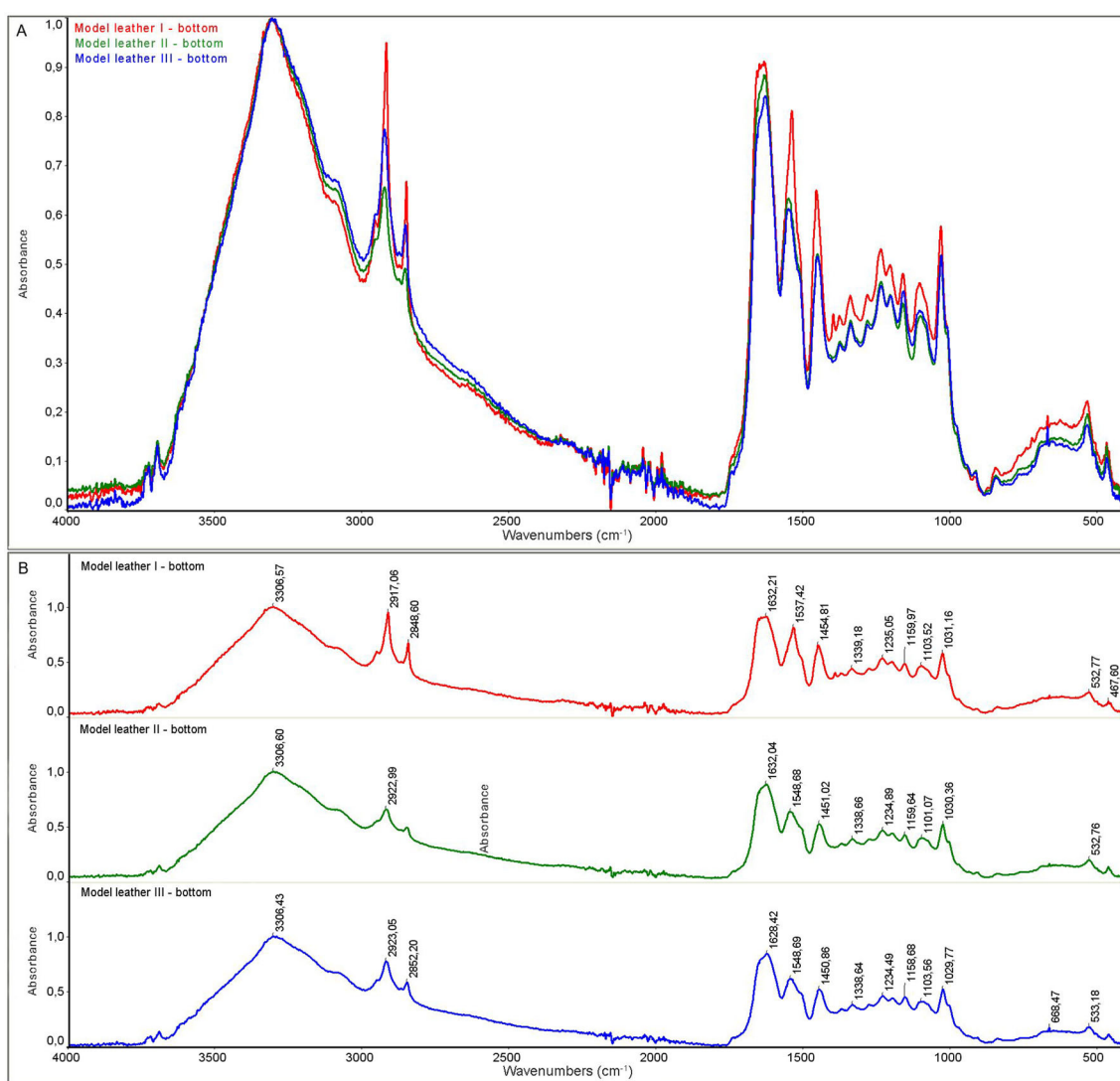


FIGURE 6

ATR spectra of the model leather samples, back side: (A) superimposed, (B) with characteristic peaks marked; model leather I (red)—not subjected to the decontamination process; model leather II (green)—after applying ethanol and dried; model leather III (blue)—after 22 h of storage in ethanol vapors.

composition of the leather, its degree of preservation, biocides used in the past and environmental conditions. Well-preserved leather (e.g., impregnated with fats, waxes) is less susceptible to colonization, because microorganisms have a harder time breaking down hydrophobic protective substances. Damaged, dried out or cracked leather creates microenvironments that are conducive to the deposition of dust and moisture, which facilitates the development of microorganisms.

Based on the results of previous studies (Rybitwa et al., 2020), to analyze the biocidal effectiveness of ethanol in the form of mist, we selected microorganisms occurring on the surfaces of leather objects in A-BSM, potentially harmful to objects and human health, such as *Aspergillus flavus*, *Aspergillus niger*, *Pantoea agglomerans*, and *Penicillium chrysogenum*. Bacteria of the *Staphylococcus* genus were also previously identified on leather objects (Rybitwa et al., 2020), so for our research we used a health-threatening

representative of this group, namely *Staphylococcus aureus*. In addition, the mold fungus *Alternaria alternata*, often found on historical objects in A-BSM (Koziróg et al., 2014; Rajkowska et al., 2014; Wawrzyk et al., 2020b, 2018), which produces acids contributing to the progress of biodeterioration (Di Carlo et al., 2016) and may also cause allergies in humans, was selected for our study.

Biodegradation is a major problem in the protection of cultural heritage, including historical leather objects, and traditional biocides often have limited effectiveness due to microbial resistance (Vadrucci et al., 2020). A commonly used method of disinfecting microbiological contamination of abiotic surfaces is spraying with 70% ethanol. It should be noted that this method is rarely used in museums, because contact of aqueous solutions with historical surfaces may adversely affect their surface, and ultimately negatively affect its properties. As a result of wetting, the leather

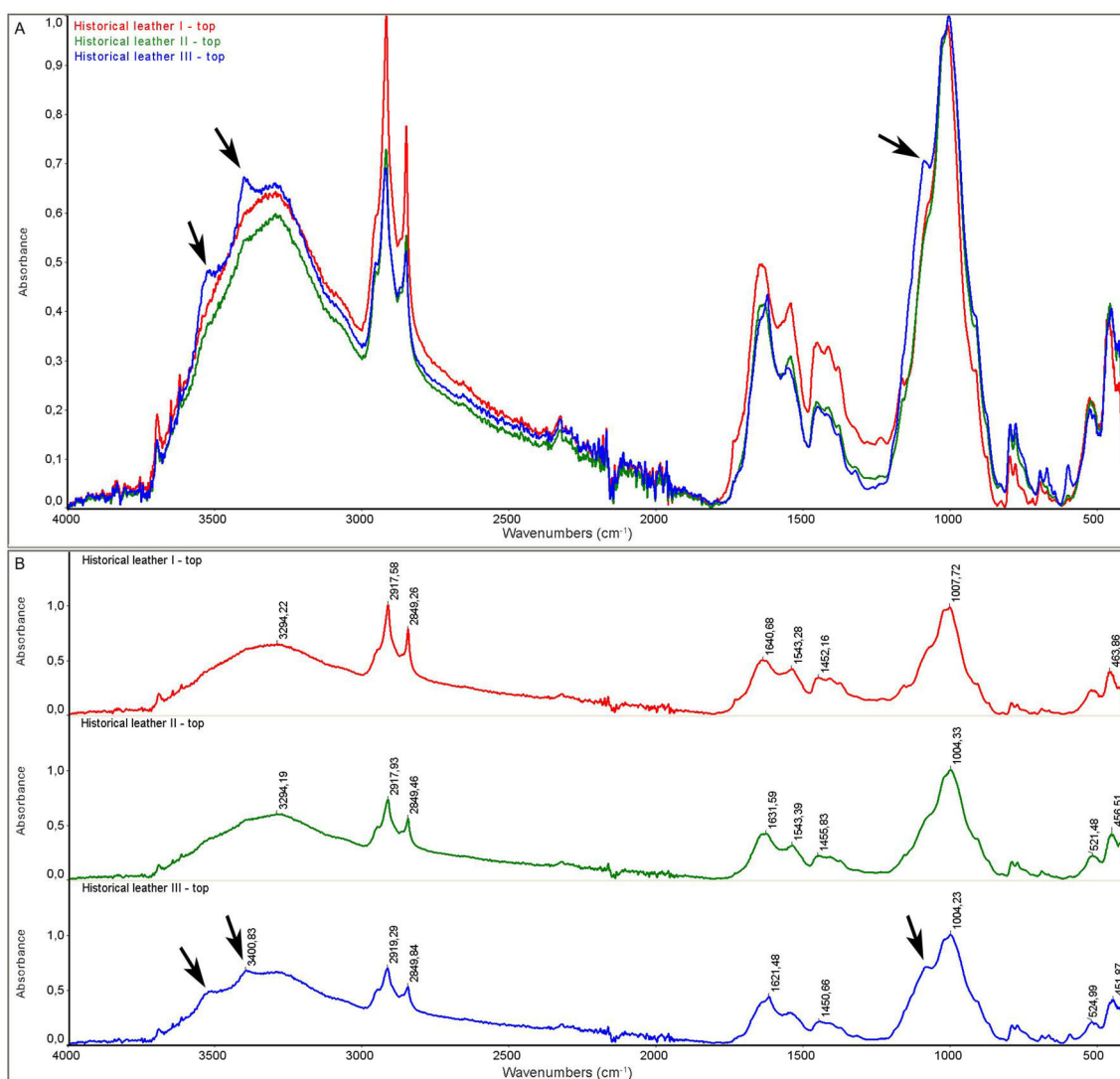


FIGURE 7

ATR spectra of historical leather samples, top side: (A) superimposed, (B) with characteristic peaks and new ones marked with arrows; historical leather I (red)—not subjected to the decontamination process; historical leather II (green)—after applying ethanol and dried; historical leather III (blue)—after 22 h of storage in ethanol vapours.

surface may shrink, stiffen, which leads to increased potential fragility or a noticeable change in morphology. In earlier attempts described in the literature, in order to use ethanol on historical leather, tests were performed using 70%–80% alcohol. However, promising results were obtained only as a result of applying a compress for several hours and soaking the leather sample for 10 min in an aqueous alcohol solution. Satisfactory effectiveness was achieved, although the author does not recommend this method for use on cultural heritage objects (Scheerer, 2012). Therefore, it is important to constantly search for alternative techniques that will eliminate microorganisms and, at the same time, be safe for objects containing collagen materials, enabling safe work of conservators (Dignard and Mason, 2020).

The primary objective of the study was to test the biocidal efficacy of ethanol in the form of mist applied to historical leather materials, as well as its effect on modern leather materials.

In previous studies, we demonstrated almost 100% effectiveness of disinfection of textile, cotton historical objects using 90% ethanol in the form of mist, without damaging their surfaces (Wawrzyk et al., 2023, 2024). We also used 90% ethanol in the form of mist to reduce autochthonous microflora occurring on paint layers in historical buildings located in the A-BSM area. Thanks to this approach, the quantitative reduction of bacteria was achieved in the range of 70.4%–>99%, and fungi in the range of 78.2%–>99% (Wawrzyk et al., 2025). In order to optimize the disinfection process and achieve a greater reduction in the number of microorganisms, the method used in the conservation of historic objects for paper disinfection was used: the time of ethanol vapor exposure was extended to 22 h (Karbowska-Berent et al., 2018). Our studies showed >99% reduction in the number of all tested fungi (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, and *Alternaria alternata*) and bacteria (*Pantoea*

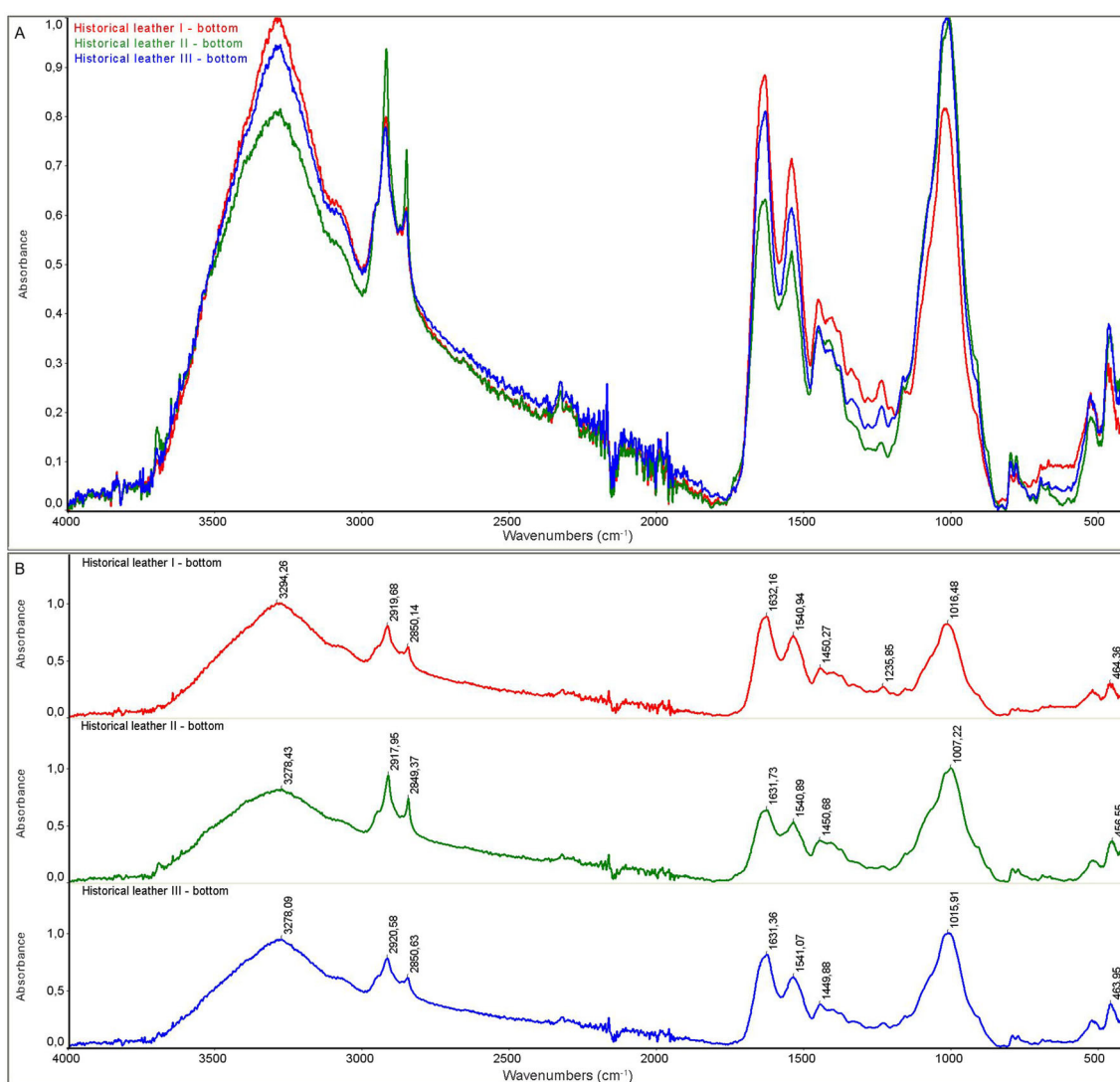


FIGURE 8

ATR spectra of historical leather samples, back side: (A) superimposed, (B) with characteristic peaks marked; historical leather I (red)—not subjected to the decontamination process; historical leather II (green)—after applying ethanol and dried; historical leather III (blue)—after 22 h of storage in ethanol vapours.

agglomerans, *Staphylococcus aureus*) present on both model and historic leather after disinfection with ethanol in the form of mist.

However, in museums, the implementation of innovative disinfection techniques requires a thorough examination of the potential consequences associated with such a process. In order to assess the possible harmfulness of the disinfection method using 90% ethanol in the form of mist on the surfaces of historical objects, a number of analyses were performed. The use of SEM microscopy allows for the detection of surface damage and collagen structure cracks, which could lead to mechanical weakening of the object, through the loss of specific material properties and constitute a place for initiation of the adhesion of microorganism cells, leading to further biodeterioration. Scanning Electron Microscopy (SEM) is a proven method for imaging cracks on surfaces in other areas of science (Wawrzyk et al., 2021). The images obtained during SEM observations did not show significant changes for samples in this regard after using ethanol as a disinfection method.

Collagen-based materials are very sensitive to degradation and are mainly subject to hydrolysis and oxidation. Namely, acid hydrolysis and photo-oxidation are the most common causes of degradation of leather products. Several studies on parchment have described typical features of hydrolysis, oxidation and gelatinization observed using ATR-FTIR spectroscopy, which is widely used in collagen degradation studies. ATR-FTIR spectroscopy can detect changes in the amide I and amide II bands, with an increase in the distance between wavenumbers and the intensity ratio indicating oxidation, while changes in the AI/AII ratio can indicate the type of hydrolysis (Vyskočilová et al., 2019). FTIR measurements of examined samples did not reveal any negative effect of disinfection with 90% ethanol in the form of mist on the structure of disinfected leather samples, both model and historical ones. Obtained results indicate that the ethyl alcohol used in the study evaporated to a large extent from the tested surfaces. Leather products

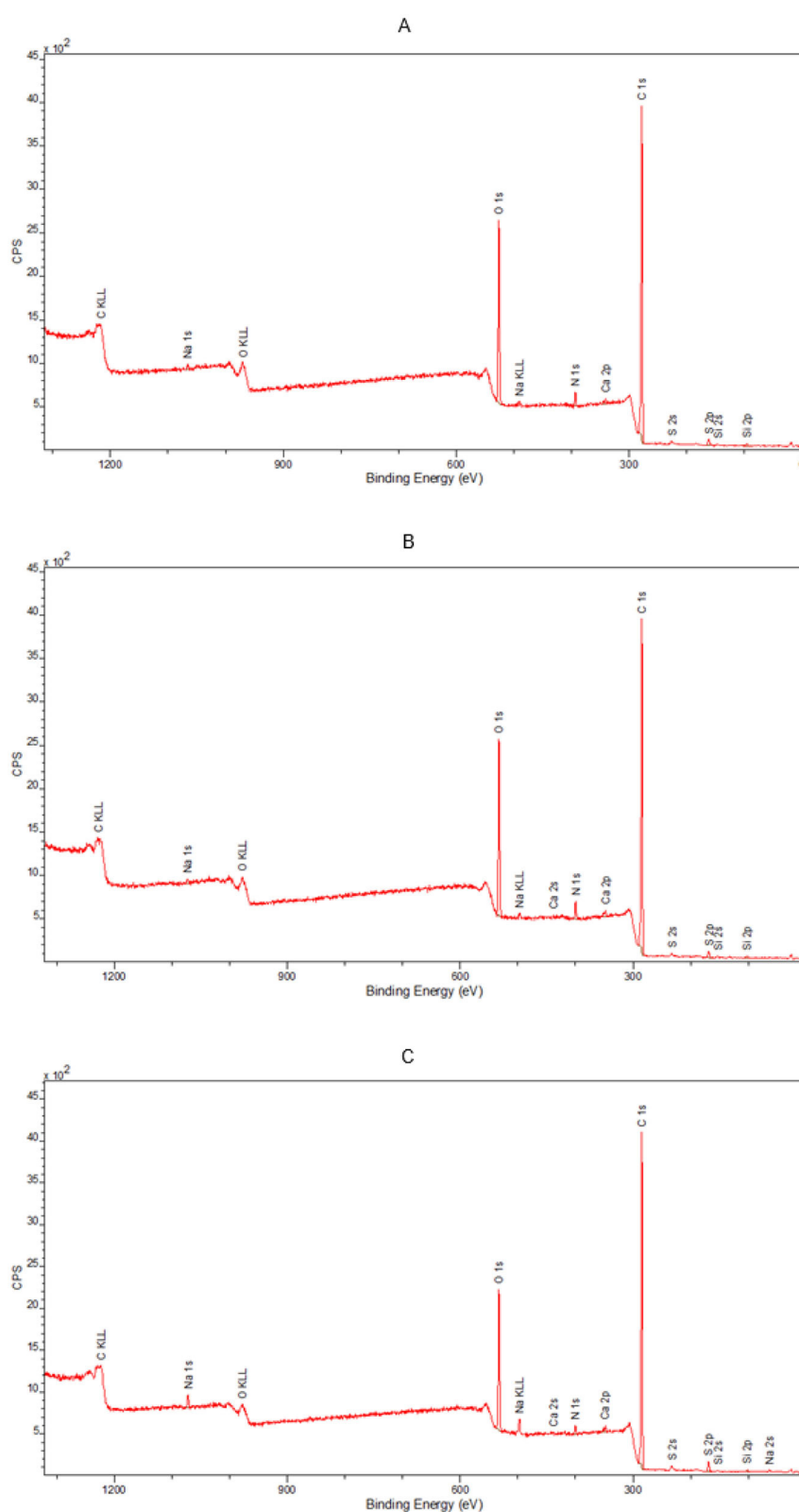


FIGURE 9

Broadband spectrum obtained by XPS spectroscopy for samples: (A) model leather I; (B) model leather II; (C) model leather III, used to calculate the elemental composition of the surface of the tested material.

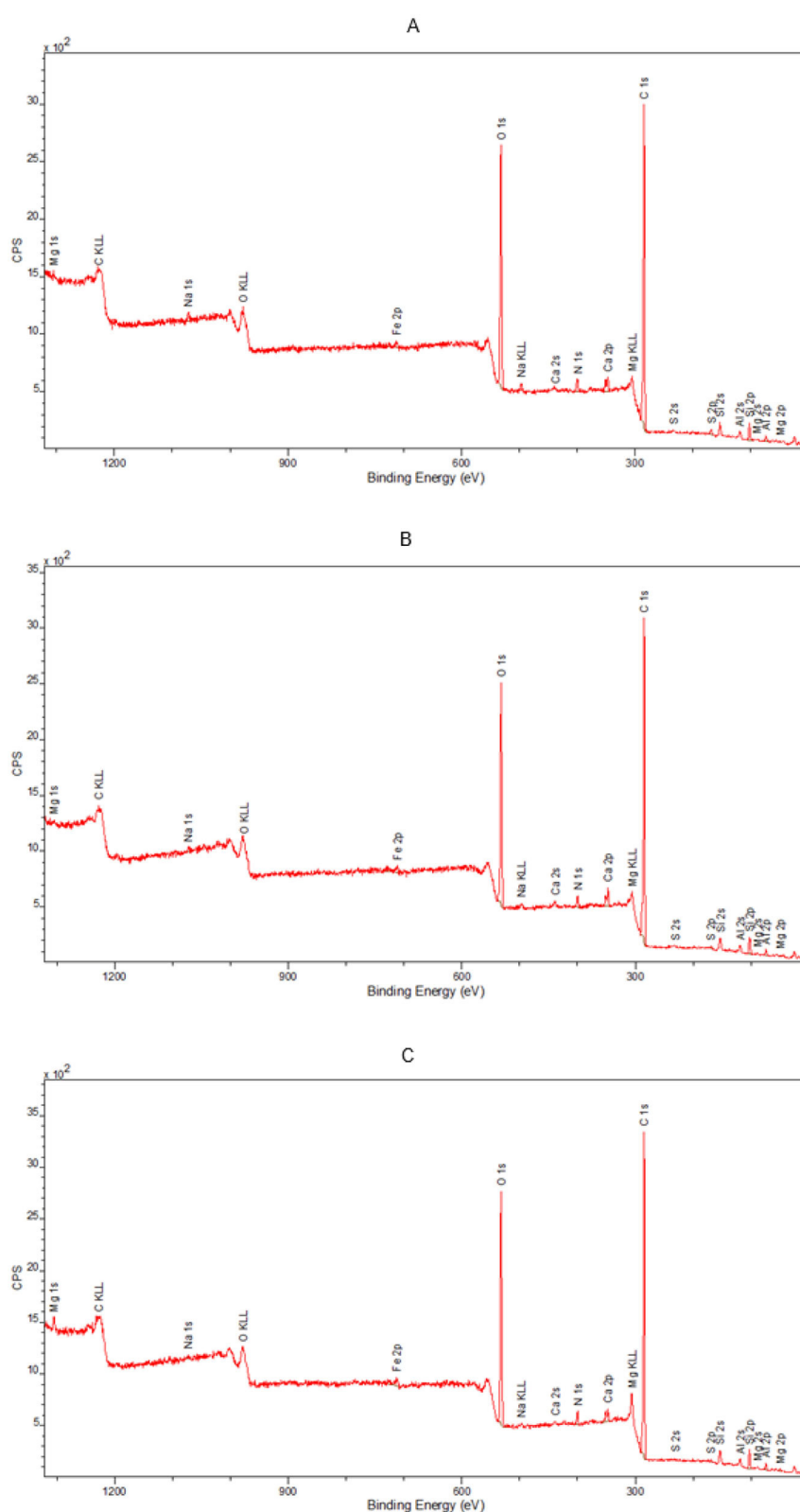


FIGURE 10

Broadband spectrum obtained by XPS spectroscopy for the samples: (A) historic leather I; (B) historic leather II; (C) historic leather III, used to calculate the elemental composition of the surface of the tested material.

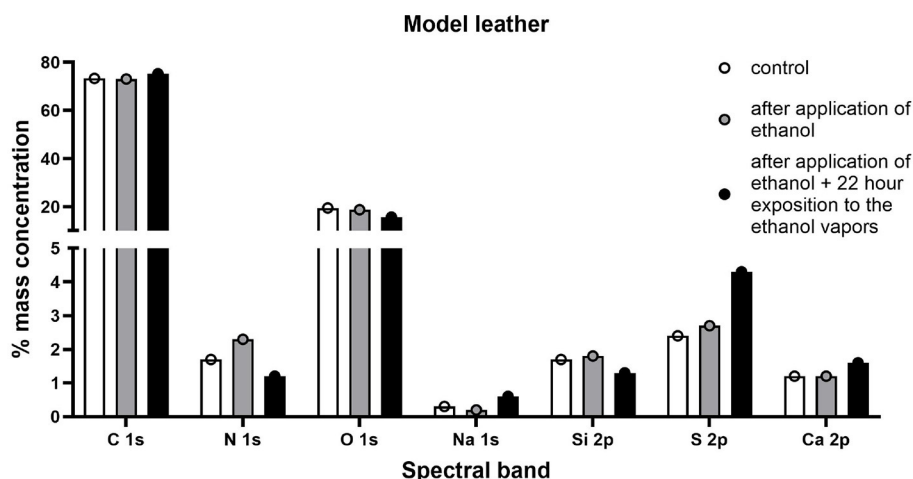


FIGURE 11
Percentage distribution of individual elements included in the outer layer of the model leather samples subjected to the test.

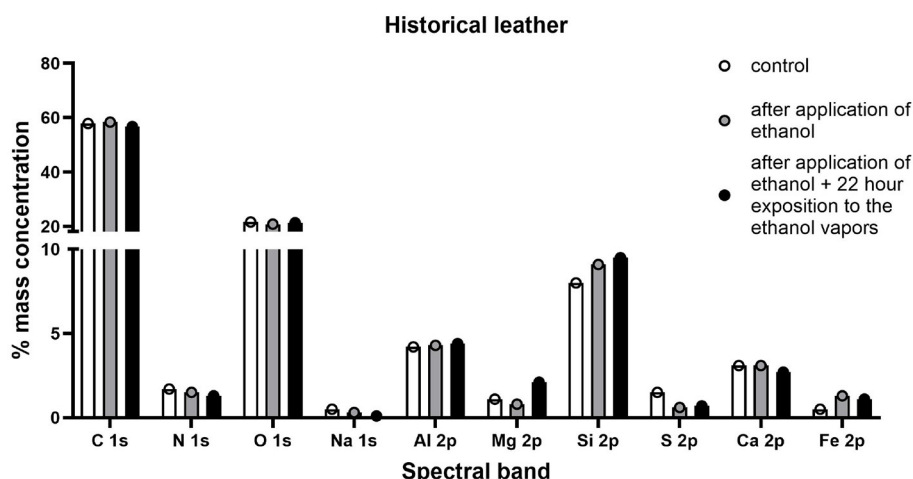


FIGURE 12
Percentage distribution of individual elements included in the outer layer of the tested historical leather samples.

consist of collagen fibers, which do not react significantly with ethanol.

Complementary to FTIR measurements, XPS tests confirm the lack of influence of disinfection of leather samples with a 90% ethanol solution in the form of mist on the chemical structure of the outer surface layer. The slight changes in the percentage content of individual elements could have been caused by the presence of ethanol on the surface of the samples, which did not evaporate completely.

The obtained results allow to demonstrate the lack of contraindications to the use of this technique for disinfection of historical leather materials. Disinfection with ethanol applied in the form of mist after optimizing the concentration, exposure time and selection of the appropriate application technique can be effectively used to eliminate microbiological contamination of historical objects made of leather. What is important, like every method dedicated to use on historical material, it requires validation for specific objects.

The ethanol mist method does not require large financial outlays, complicated equipment, or special precautions compared to other methods used to protect cultural assets. Minimizing mechanical damage reduces the risk of physical damage to the leather surface. The mist form can allow for even distribution of the substance, reaching hard-to-reach places. It is a quick and easy-to-use method in museum, so it can be practically implemented in current conservation tasks. Ethanol evaporates quickly, which can reduce the risk of long-term exposure to moisture on the material, and therefore can be used to disinfect materials that should not be exposed to aqueous solutions.

Ethanol effectively eliminates many types of microorganisms, although as a disinfectant has limited effectiveness compared to other waterless disinfection techniques because it requires the presence of water to effectively denature proteins and its rapid evaporation may leave microbial spores intact, whereas methods such as gas disinfection (e.g., ethylene oxide) or

UV radiation are more effective against a broad spectrum of microorganisms, including spores. However, ethanol is less toxic and less harmful to humans compared to methods using ethylene oxide or ionization, which can be effective in eliminating pathogens on surfaces, but have limited use in decontamination of heritage objects.

To conclude, we proved that disinfection of historical leather objects with the 90% ethanol in the mist form is biocidally effective, for tested bacteria and fungi. Moreover, we were first to demonstrate that this technique does not damage the leather object's surface structure, as examined by SEM, FTIR, and XPS analyses. 90% ethanol evaporates quickly, so there is no threat of soaking of the surface. This disinfection method is also cheap, easy to use, convenient and highly available. What is important, it requires validation before use on different historic materials.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

KK: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. NP: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. AP: Funding acquisition, Resources, Supervision, Writing – review & editing. KG: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. DK: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. UM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. AW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

References

- Ambrosewicz-Jacobs, J., Oleksy, K., Yehuda, B., Wiesel, E., Birenbaum, H., and Bartoszewski, W. (2008). *Remembrance, Awareness, Responsibility: International Conference on the Occasion of the 60th Anniversary of the Museum's Founding. Oświęcim 2007. Pamięć, świadomość, odpowiedzialność*. Oświęcim: The Auschwitz-Birkenau State Museum Publishing House.
- Dai, Q., Zeng, L., Cen, Q., Du, W., Ning, P., Li, K., et al. (2025). Key process of ethanol hydrogen donor promoting the conversion of organic matter in sludge to bio-oil. *Water Res.* 268:122753. doi: 10.1016/j.watres.2024.122753
- Di Carlo, E., Chisesi, R., Barresi, G., Barbaro, S., Lombardo, G., Rotolo, V., et al. (2016). Fungi and bacteria in indoor cultural heritage environments: microbial-related risks for artworks and human health. *Environ. Ecol. Res.* 4, 257–264. doi: 10.13189/eer.2016.040504
- Dignard, C., and Mason, J. (2020). *Caring for leather, skin and fur, Canadian conservation institute notes*. Available online at: <https://cool.culturalheritage.org/byform/mailling-lists/cdl/2012/0927.html> (accessed March 2, 2025).
- Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M., and Milanowski, J. (2016). *Pantoea agglomerans*: a mysterious bacterium of evil and good. part iii. deleterious effects: infections of humans, animals and plants. *Ann. Agric. Environ. Med.* 23, 197–205. doi: 10.5604/12321966.1203878
- Gerba, C. P., Boone, S., Nims, R. W., Maillard, J.-Y., Sattar, S. A., Rubino, J. R., et al. (2024). Mechanisms of action of microbicides commonly used in infection prevention and control. *Microbiol. Molec. Biol. Rev.* 88, e00205–e00222. doi: 10.1128/mmmr.00205-22
- Gutarowska, B., Pietrzak, K., Machnowski, W., and Milczarek, J. M. (2017). Historical textiles-a review of microbial deterioration analysis and disinfection methods. *Textile Res. J.* 87, 2388–2406. doi: 10.1177/0040517516669076
- Hyde, K. D., Al-Hatmi, A. M., Andersen, B., Boekhout, T., Buzina, W., Dawson, T. L., et al. (2018). The world's ten most feared fungi. *Fungal Divers.* 93, 161–194. doi: 10.1007/s13225-018-0413-9
- Jacob, J. M., Schmult, M., and Villa, F. (2018). Biofilms and lichens on eroded marble monuments. *APT Bull.* 49, 55–60.
- Karbowska-Berent, J., Górniak, B., Czajkowska-Wagner, L., Rafalska, K., Jarmilko, J., and Koziół, T. (2018). The initial disinfection of paper-based historic

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Acknowledgments

The authors of the article would like to thank the Deputy Director of the Auschwitz-Birkenau State Museum in Oświęcim, Rafał Pióro, for making it possible to carry out the research, providing access to historical materials for research and substantive support. The authors would also like to thank the Head of Preventive Conservation research of Getty Conservation Institute in Los Angeles, Michał Łukomski, for substantive cooperation during the implementation of this research.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

- items-observations on some simple suggested methods. *Int. Biodeterior. Biodegrad.* 131, 60–66. doi: 10.1016/j.ibiod.2017.03.001
- Koziróg, A., Otlewska, A., Piotrowska, M., Rajkowska, K., Nowicka-Krawczyk, P., Hachułka, M., et al. (2014). Colonising organisms as a biodegradation factor affecting historical wood materials at the former concentration camp of Auschwitz II-Birkenau. *Int. Biodeterior. Biodegrad.* 86, 171–178. doi: 10.1016/j.ibiod.2013.08.004
- Mazzoli, R., Giuffrida, M. G., and Pessione, E. (2018). Back to the past: “find the guilty bug—microorganisms involved in the biodeterioration of archaeological and historical artifacts”. *Appl. Microbiol. Biotechnol.* 102, 6393–6407. doi: 10.1007/s00253-018-9113-3
- Ortiz, P., Antúnez, V., Ortiz, R., Martín, J., Gómez, M., Hortal, A., et al. (2013). Comparative study of pulsed laser cleaning applied to weathered marble surfaces. *Appl. Surf. Sci.* 283, 193–201. doi: 10.1016/j.apsusc.2013.06.081
- Rajkowska, K., Otlewska, A., Koziróg, A., Piotrowska, M., Nowicka-Krawczyk, P., Hachułka, M., et al. (2014). Assessment of biological colonization of historic buildings in the former Auschwitz II-Birkenau concentration camp. *Ann. Microbiol.* 64, 799–808. doi: 10.1007/s13213-013-0716-8
- Rybitwa, D., Wawrzyk, A., Wilczyński, S., and Łobacz, M. (2020). Irradiation with medical diode laser as a new method of spot-elimination of microorganisms to preserve historical cellulosic objects and human health. *Int. Biodeterior. Biodegrad.* 154:105055. doi: 10.1016/j.ibiod.2020.105055
- Scheerer, S. (2012). *Mold on leather*. Available online at: <https://www.canada.ca/en/conservation-institute/services/preventive-conservation/guidelines-collections/caring-leather-skin-fur.html> (accessed March 2, 2025).
- Sequeira, S., Cabrita, E. J., and Macedo, M. F. (2012). Antifungals on paper conservation: an overview. *Int. Biodeterior. Biodegrad.* 74, 67–86. doi: 10.1016/j.ibiod.2012.07.011
- Skóra, J., Gutarowska, B., Pielech-Przybylska, K., Stępień, Ł., Pietrzak, K., Piotrowska, M., et al. (2015). Assessment of microbiological contamination in the work environments of museums, archives and libraries. *Aerobiologia* 31, 389–401. doi: 10.1007/s10453-015-9372-8
- Szostak-Kotowa, J. (2004). Biodeterioration of textiles. *Int. Biodeterior. Biodegrad.* 53, 165–170. doi: 10.1016/S0964-8305(03)00090-8
- Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler Jr, V. G. (2015). *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* 28, 603–661. doi: 10.1128/CMR.00134-14
- Vadrucci, M., De Bellis, G., Mazzuca, C., Mercuri, F., Borgognoni, F., Schifano, E., et al. (2020). Effects of the ionizing radiation disinfection treatment on historical leather. *Front. Mater.* 7:21. doi: 10.3389/fmats.2020.00021
- Vyskočilová, G., Ebersbach, M., Kopecká, R., Prokeš, L., and Přihoda, J. (2019). Model study of the leather degradation by oxidation and hydrolysis. *Heritage Sci.* 7, 1–13. doi: 10.1186/s40494-019-0269-7
- Wawrzyk, A., Dymel, M., Guzińska, K., Cywiński, P., Papis, A., Konka, A., et al. (2023). Optimization of the process of eliminating microorganisms harmful to human health and threatening objects isolated from historical materials from the Auschwitz-Birkenau state museum in Poland (A-BSM) collection with the use of ethanol in the form of mist. *Materials* 16:2700. doi: 10.3390/ma16072700
- Wawrzyk, A., Gutarowska, B., Rybitwa, D., Pietrzak, K., Machnowski, W., Wrzosek, H., et al. (2018). Vaporised hydrogen peroxide (VHP) and ethylene oxide (ETO) methods for disinfecting historical cotton textiles from the Auschwitz-Birkenau state museum in Oswiecim, Poland. *Int. Biodeterior. Biodegrad.* 133, 42–51. doi: 10.1016/j.ibiod.2018.05.016
- Wawrzyk, A., Łobacz, M., Adamczuk, A., Sofińska-Chmiel, W., Wilczyński, S., and Rahnama, M. (2021). The use of a diode laser for removal of microorganisms from the surfaces of zirconia and porcelain applied to superstructure dental implants. *Microorganisms* 9:2359. doi: 10.3390/microorganisms9112359
- Wawrzyk, A., Poskrobko, J., Guzińska, K., Kaźmierczak, D., Papis, A., Jastrzębiowska, N., et al. (2024). Analysis of the surface of historic fabric from the Auschwitz-Birkenau state museum after treatment with ethanol mist used to eliminate microorganisms harmful to human health. *Materials* 17:2323. doi: 10.3390/ma17102323
- Wawrzyk, A., Pydyn, N., Papis, A., Bormann, M., Kraśnicki, K., et al. (2025). Identification of microorganisms harmful to human health inhabiting historical surfaces using maldi tof mass spectrometry and DNA sequencing and their elimination using ethanol in the mist form. *J. Cult. Heritage*.
- Wawrzyk, A., Rahnama, M., Rybitwa, D., Wiecek, K., Michalczewski, G., Podsiadły, E., et al. (2020a). Decontamination of microbiologically contaminated abiotic porous surfaces in an oral surgery clinic using vaporised hydrogen peroxide (VHP). *J. Environ. Health Sci. Eng.* 18, 639–653. doi: 10.1007/s40201-020-00490-z
- Wawrzyk, A., Rybitwa, D., Rahnama, M., and Wilczyński, S. (2020b). Microorganisms colonising historical cardboard objects from the Auschwitz-Birkenau state museum in Oświęcim, Poland and their disinfection with vaporised hydrogen peroxide (VHP). *Int. Biodeterior. Biodegrad.* 152:104997. doi: 10.1016/j.ibiod.2020.104997



OPEN ACCESS

EDITED BY

Erasmus Gámez-Espinosa,
National University of La Plata, Argentina

REVIEWED BY

Scott G. Mitchell,
Spanish National Research Council (CSIC),
Spain

Guillermo P. Lopez,
National University of La Plata, Argentina

*CORRESPONDENCE

Johannes Tichy
✉ j.tichy@akbild.ac.at

RECEIVED 31 March 2025

ACCEPTED 07 May 2025

PUBLISHED 22 May 2025

CITATION

Tichy J, Sipek B, Ortbauer M, Fürnwein L,
Waldherr M, Graf A, Sterflinger K and
Piñar G (2025) Microbial community shifts
during salt mitigation treatments of historic
buildings using mineral poultices: a long-term
monitoring of salt and associated biofilms.
Front. Microbiol. 16:1603289.
doi: 10.3389/fmicb.2025.1603289

COPYRIGHT

© 2025 Tichy, Sipek, Ortbauer, Fürnwein,
Waldherr, Graf, Sterflinger and Piñar. This is an
open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Microbial community shifts during salt mitigation treatments of historic buildings using mineral poultices: a long-term monitoring of salt and associated biofilms

Johannes Tichy^{1*}, Beate Sipek², Martin Ortbauer²,
Lukas Fürnwein³, Monika Waldherr³, Alexandra Graf³,
Katja Sterflinger¹ and Guadalupe Piñar¹

¹Institute for Natural Sciences and Technology in the Art, Academy of Fine Arts Vienna, Vienna, Austria, ²Institute for Conservation – Restoration, Academy of Fine Arts Vienna, Vienna, Austria, ³Department of Applied Life Sciences/Bioengineering/Bioinformatics, FH Campus Wien, Vienna, Austria

Increased heavy rainfall followed by periods of drought due to climate change is leading to more frequent salt-crystallization cycles. This not only leads to increased salt-weathering on architectural surfaces of cultural heritage monuments, but also creates an ideal ecological niche for the formation of biofilms by salt-loving microorganisms. These biofilms, characterized by a distinctive pink coloration, cause additional esthetic alterations to affected surfaces. In this study, mineral poultices prepared with different clay minerals (sepiolite, kaolinite and vermiculite) were developed and tested for a long-term (1 year) application on salt-weathered surfaces, thus contributing to their preservation. The poultices were tested on the surfaces of two historic buildings: the St. Virgil's Chapel in Vienna and the Mauerbach Charterhouse in Lower Austria, both showing salt efflorescence and a uniform pink biofilm. First, the poultices were tested to evaluate their salt retention capacity, salt-weathering resistance and processability. The retention properties of the poultices were examined by measuring their salt content throughout the treatment using high performance liquid chromatography (HPLC) and continuous flow analysis (CFA). Salt content was also measured on the wall surfaces before and after treatment. Second, the effect of the desalination treatments on salt-associated pink biofilms was also evaluated. The shifts within the biofilm communities during and after the treatment were monitored by qPCR and long-read archaeal- and bacterial-16S rRNA amplicon analysis using the Nanopore sequencing technology. The results demonstrate that both the selected clay minerals and the salt composition in the treated areas significantly influenced the salt storage capacity of the poultices and their resistance to salt weathering. Fluctuations in salt load and ionic composition during and after treatment affected biofilm composition, with bacterial communities proving more sensitive than archaea to these changes. Both qPCR and metataxonomic results show that the effects of the poultices on the colonizing biofilms depend not only on the composition of their microbial members, but also on external abiotic factors such as the chemical composition and concentration of the salt mixtures on the surfaces. In addition, the biodiversity within the biofilms shows to be affected differently depending on the mineral clay used.

KEYWORDS

salt-weathering, mineral poultices, pink biofilms, archaea, bacteria, long-term monitoring, metataxonomics

1 Introduction

Time and climate change are two uncontrollable factors that challenge the preservation of cultural heritage. Particularly in Central Europe, increasing heavy rainfalls, alternating with periods of intense drought, promotes the formation of salt efflorescence, which endangers cultural heritage monuments (Leissner et al., 2015). This phenomenon not only creates new ecological niches for halophilic microorganisms, facilitating microbial growth on monument surfaces, but also complicates restoration efforts in maintaining the integrity of inorganic surfaces. It has been reported that the process of microbial colonization in built cultural heritage heavily contaminated by salt very often leads to the phenomenon of pinkish discolouration (Imperi et al., 2007; Piñar et al., 2014; Tichy et al., 2023). This discolouration is due to halophilic/halotolerant microbial communities producing carotenoid pigments, mostly bacterioruberin (BR), which are dispersed into the cultural heritage environment, causing the characteristic pinkish appearance (Imperi et al., 2007; Ettenauer et al., 2014; Tescari et al., 2018b; Cojoc et al., 2019; Basile et al., 2025). The main players in the discolouration phenomenon are halophilic bacteria (Saiz-Jimenez and Laiz, 2000; Schabereiter-Gurtner et al., 2001) and archaea (Rölleke et al., 1998; Piñar et al., 2001), usually embedded into a biofilm located either on top of the inorganic surface or within the salt efflorescence itself. Halophilic communities, besides causing esthetic damage through the pigment production, may also contribute to the damage of architectural surfaces by changing the inorganic surface properties through the process of biodegradation (Schröer et al., 2021).

Salt crystallization exerts a destructive force on construction materials (Arnold and Zehnder, 1991; Charola, 2000). To prevent further degradation of affected cultural heritage surfaces, remediation or mitigation of harmful salts is required (Charola and Bläuer, 2015). For the removal of salt efflorescence, a classical approach includes the application of poultices (Verges-Belmin and Siedel, 2005; Lubelli and Van Hees, 2010; Lenz, 2017), which function based on diffusion and advection principles (Pel et al., 2010). While the physical effects of desalination and stabilization treatments on architectural surfaces are well-documented, even over the long term (Zehnder, 2007; Heritage et al., 2013; Laue et al., 2021), less attention has been given to their biological impact. Few studies have monitored microbial communities over extended periods following salt reduction or bio-cleaning treatments (Bosch-Roig et al., 2015; Pinna et al., 2018; Ranalli and Zanardini, 2021; Pavlović et al., 2022). Long-term microbial monitoring after salt reduction is essential, as variations in salt composition and ion concentration shifts can influence microbial communities on deteriorated surfaces (Adamiak et al., 2015; Tichy et al., 2023). Additionally, for inorganic surfaces such as stone or mortar, it is difficult to distinguish between damage caused by either physical and/or chemical processes and that caused by microbiological activity (Steiger et al., 2011). Therefore, when assessing treatment effectiveness, both salt composition and concentration changes, as well as their impact on microbial communities, must be monitored

before, during, and after cleaning, desalination, or consolidation treatments.

In this study, a long-term desalination treatment was applied to salt-contaminated surfaces at two historic sites: St. Virgil's Chapel and the Charterhouse Mauerbach. Both locations have been comprehensively analyzed for salt composition and associated microbial biofilms (Tichy et al., 2023). To mitigate salt-induced damage, we applied mineral poultices composed of different clay minerals—sepiolite, kaolinite, and vermiculite—for 1 year and monitored the treatment over time. These minerals were selected due to their distinct physicochemical properties relevant for salt extraction (Bergaya and Lagaly, 2013). Sepiolite, a fibrous clay with high specific surface area and porosity, facilitates capillary transport and salt uptake. Kaolinite, a plate-like clay with lower cation exchange capacity, offers dimensional stability and ease of handling. Vermiculite, with its high ion-exchange capacity and swelling behavior, can enhance ion mobility and retention. Such properties suggest that these clays can support both effective salt removal and adaptability to surface conditions in heritage conservation applications. In addition, natural clays, especially kaolinite and vermiculite used in this study, show antimicrobial properties (Williams and Haydel, 2010; Londono et al., 2017) and could be functionalised for the release of biocidal agents (Gallo et al., 2020; Pastor et al., 2020) in order to obtain better control of the microbiomes associated with salt-weathered surfaces in heritage buildings. However, the raw materials used in this study were not modified with biocides. Similar clay materials have been used in desalination studies targeting construction materials and artworks (Verges-Belmin and Siedel, 2005; Lubelli and Van Hees, 2010), but a comparative evaluation of different clays over long treatment periods remains limited. Therefore, to assess their potential for a long-term application, this study tested the salt retention capacity of each poultice, its resistance to salt weathering and its handling properties. In addition, the treatment effects on surface ion concentrations and on the colonizing biofilms were monitored by metataxonomic profiling and quantitative PCR.

2 Materials and methods

2.1 Description of the locations of treated surfaces

The treatment was applied to two historic buildings. First of all, the Chapel of St. Virgil (1,220/30 AD) Vienna (Austria), has been the subject of numerous microbial investigations (Ripka et al., 2006; Piñar et al., 2009; Ettenauer et al., 2010) (Figure 1a). A more recent study also included climatic and salt chemistry analyses (Tichy et al., 2023). The chapel, located underground, exhibits severe salt damage, primarily due to halite (NaCl) efflorescence. This results from water infiltration following heavy rainfalls, which dissolves and transports de-icing salts used in winter. The surface of the rendering is rough, highly porous and structurally weakened due to progressive

weathering. The poultices were tested on a previously investigated vault, designated hereafter as V2 (Tichy et al., 2023).

The second site, the Charterhouse in Mauerbach (Lower Austria), is a former monastery built in 1314, also previously studied (Tichy et al., 2023). Here, water ingress is caused by capillary forces (rising damp). The investigated plastered surfaces of the walls are smooth with negligible porosity. Poultices were applied in two rooms: M4, which showed no visible salt efflorescence but contained gypsum, and M6, which exhibited extensive salt efflorescence, primarily thenardite (Na_2SO_4) (Tichy et al., 2023).

2.2 Poultice compositions and application

Three clay minerals with distinct properties were selected for the preparation of mineral poultices for desalination tests in the two historical sites. The selected minerals were kaolinite, sepiolite and vermiculite. The poultices remained on the surfaces for a total period of 1 year. The clay minerals were combined with quartz sand and perlite as additives. The addition of perlite serves the purpose of reducing the overall weight of the mixture, which is an important factor for the application on salt-damaged plaster structures. The mixtures consisted of one volume part of each clay mineral, one volume part of perlite, and two to three volume parts of quartz sand, with the addition of 1–1.5 volume parts of water, to create a mixture that was easily applicable. The proportion of quartz sand and water was dependent upon the properties of the clay mineral.

Three test areas (each 400 cm²) were selected in each of the test locations of the two sites (Figure 1b). The poultices were applied in a 1 cm-thick layer over a polyamide mesh (2 mm mesh size) using a spatula. The intermediate layer of polyamide was inserted to facilitate the removal of the poultice and thus protect the historic surfaces.

2.3 Sampling

Sampling of the different applied poultices was carried out at 1, 6, and 12 months of treatment. At each time point, for each location and poultice type, a 5 × 5 × 1 cm section of the poultice was aseptically removed from the wall, placed into a petri dish (Figure 1c) and transported on ice to the laboratory. Samples were stored at −20°C

until further biological analysis. Before analysis, the poultices were homogenized using a sterile scalpel to obtain a fine granular mass.

Sampling of the walls after 12 months of treatment with the different poultices, hereafter referred to as “treated” (T) samples, was following the same procedure described for untreated walls (“NT” samples) in Tichy et al. (2023). Briefly, the treated pink biofilm surface of 5 × 5 cm was scratched with a sterile scalpel, collecting the fine obtained powder in a falcon tube, transported on ice to the laboratory and stored at −20°C until further analysis.

2.4 DNA extraction, library preparation and sequencing with Nanopore sequencing platform

The DNA extraction, from poultices and wall surfaces, was done in triplicate for each sample with the FastDNA Spin Kit for soil (MP Biomedicals, Illkirch, France), as explained in Tichy et al. (2023). DNA extracts of the poultices were pooled following a volume reduction through a vacuum concentrator at 25°C (Savant SpeedVac DNA 130, Thermo Fisher Scientific, Waltham, United States).

Full-length archaeal and bacterial 16S rRNA amplicons were obtained through a first PCR round, using the primers 27F/1942R for bacteria and SSU1ArF/SSU1000ArR for archaea, as explained by Tichy et al. (2023) with the modification that only 30 cycles were used in this study for the archaeal amplification. Afterwards the amplification products were cleaned up with NucleoSpin Gel and PCR Clean-up XS (Ref: 740611, MACHEREY-NAGEL, Düren/Germany) according to the instructions made by the manufacturer.

Then long-amplicon bacterial 16S rRNA gene library preparation and sequencing was done according to the protocol of the Oxford Nanopore Barcoding Kit (SQK-PBK004, nested-pcr-protocol-FFP_9038_v108_rev S_14Aug2019-minion-1) with all extracted samples. The used primers were 27F and 1492R configured with an adapter predefined by Oxford Nanopore Technologies (Forward primer: 5'-TTTCTGTTGGTG CTGATATTGC-27F-3'; Reverse primer: 5'-ACTTGCCTGTCGCTCTATCTTC-1492R-3') and synthesized by Eurofins Genomics. Moreover, this was also done identically for the archaeal amplicons with the primers SSU1ArF/SSU1000ArR including the Oxford Nanopore Technologies specific adapters (Forward primer: 5'-TTTCTGTTGGTG CTGATA



FIGURE 1

Example of the application of mineral poultices in St. Virgil's Chapel: (a) view from above of St. Virgil's Chapel; (b) application of mineral poultices [from left to right: sepiolite; kaolinite; vermiculite]; (c) aseptically sampling procedure of poultices [5 × 5 cm²].

TTGC-SSU1ArF-3'; Reverse primer: 5'-ACTTGCCTGT CGCT CTATCTTC-SSU1000ArR-3') also synthesized by Eurofins Genomics. Detailed description of the Sequencing-PCR setup, primers, used enzymes and devices can be found in [Tichy et al. \(2023\)](#).

Finally, the barcoded sequences were pooled (bacterial- and archaeal-amplicons separately) in desired ratios to a total molar concentration ranging between 50 and 100 fmol in 10 μ L and ligated to the provided sequencing adapters. The prepared libraries were loaded onto the MinION flow cell FLO-MIN 106D R9 Version (Oxford Nanopore Technologies, Oxford, UK). Sequencing was performed on the MinION Mk1C device for 48 h.

2.5 Bioinformatic analyses

FAST5 files, were basecalled using the Guppy basecalling software (Oxford Nanopore Technologies, Oxford, UK, version 5.0.11 + 2b6dbff) with the high accuracy (HAC) model. FastQC (v0.12.1) ([Andrews et al., 2010](#)) and Nanostat (1.6.0) ([De Coster et al., 2018](#)) were used for quality control. Adapter, barcode, and primer sequences were removed, and chimeric sequences were split using Porechop (0.2.4) ([Wick et al., 2017](#)). A head and tail crop of 40 bases and filtering for a mean quality score of 9 was done with NanoFilt (2.8.0). Additionally, a length filtering was conducted for bacterial and archaeal reads (1,000–1,600 bases for *bacteria*, 700–1,200 bases for *archaea*, according to the expected length of the PCR product).

Emu (v3.4.4) ([Curry et al., 2022](#)) was used to perform metataxonomic classifications with a combination of rrnDB v5.6 ([Stoddard et al., 2015](#)) and NCBI 16S RefSeq from 17 September 2020 ([O'Leary et al., 2016](#)). The resulting database comprises 49,301 sequences from 17,555 unique bacterial and archaeal species.

R version 4.3.2 and the packages phemap (version 1.0.12) ([Kolde, 2010](#)), taxonomizer (version 0.10.7) ([Sherrill-Mix, 2017](#)), phyloseq ([McMurdie and Holmes, 2013](#)), vegan (version 2.7-0) ([Oksanen et al., 2025](#)), microbiome ([Lahti and Shetty, 2017](#)), ggplot2 ([Wickham, 2016](#)), and tidyverse ([Wickham et al., 2019](#)) were used to perform relative abundance analysis, taxonomic clustering and the different diversity indices.

The data for the non-treated samples are derived from NCBI BioProject PRJNA909753 ([Tichy et al., 2023](#)). Data derived from this study are available under the NCBI BioProject accession Number PRJNA1242577.

2.6 Quantitative PCR analyses

To quantify the bacterial and archaeal load in the wall surfaces before and after the treatment, qPCR analyses were performed using Bio-Rad iTaq Universal SYBR Green Supermix. The total reaction volume was 10 μ L. Each reaction mixture consisted of 1 μ L template DNA, 1 μ L primer mix, 5 μ L iTaq Universal SYBR Green Supermix and 3 μ L nuclease free water. The specific primers used for archaeal detection were ARC344F (ACGGGGYGCAGCAGGCGCGA) and ARC744R (CCSGGGTATCTAATCC), while bacterial quantification was performed with the BAC338F (ACTCCTACGGGAGGCAG) and BAC805R (GACTACCAGGTATCTATCC) primers, synthesized by Eurofins Genomics. Each sample was performed in triplicate. Thermal cycling conditions followed the iTaq Universal SYBR Green Supermix

protocol recommendations and Fluorescent Quantitative Detection System (Hangzhou Bioer technology/Shanghai). The PCR parameters included: 4 min denaturation at 95°C, followed by 40 cycles consisting of 5 s. denaturation at 95°C, 30 s. primer annealing and extension at 60°C. For the statistical analysis of the qPCR results between the sampling locations, the statistics program XLSTAT 2023.1.6.1410 was used [Two-sample *t*-test, significance level $\alpha = 0.05$]. The Δ Ct (delta cycle threshold) values were calculated as follows: the mean Ct values of the treated samples (T) minus the mean values of the non-treated samples (NT).

2.7 Salt analysis

For the quantitative analyses of the salt composition High Performance Liquid Chromatography (HPLC) and continuous flow analysis (CFA) were used. The surface of 25 cm² of treated wall surfaces (T) and 25 cm³ of the mineral poultices were analyzed. Non-treated walls (NT) were previously examined ([Tichy et al., 2023](#)). The collected material (poultices and historic surfaces) from the standardized surface area of 25 cm² was weighed before and after drying at 60°C and dissolved in 60 mL demineralised water (according to VDE 0510). The samples were shaken for 3 min. and the non-dissolved particles were allowed to settle for 60 min. Subsequently, 20 mL of supernatant, free of non-dissolved particles, was transferred in a new 50 mL Falcon-Tube and stored at 7°C until analysis. Nitrate detection followed the DIN EN ISO 13395 procedure, involving the reduction of nitrate to nitrite using cadmium, followed by photometric determination after a nitration reaction with 2,6-dimethylphenol in a sulfuric/phosphoric acid solution via CFA.

Sulfate and Chloride ions were detected according DIN EN ISO 10304-1, using HPLC with ion separation and a conductivity detector (IC). Sodium, potassium, calcium, and magnesium ions were quantified in accordance with DIN EN 14911 using HPLC coupled with a conductivity detector. Details of the analytical equipment are provided in the [Supplementary Table S1](#) ([Tichy et al., 2023](#)). To standardize the comparison of salt amounts detected on surfaces (cm²) and those extracted within the mineral poultices (cm³), a conversion factor of 1:1 was applied, and all values are reported in μ g/cm² aligning with the WTA Guideline ([Wissenschaftlich-Technische Arbeitsgemeinschaft für Bauwerkserhaltung und Denkmalpflege, 2019](#)), based on the mass of the detected ionic species. The total retention capacity of ionic species in the mineral poultices was calculated empirically as follows: all cations were normalized to sodium ions (Na⁺ eq.), and all anions to chloride ions (Cl⁻ eq.), based on molar amounts. The total sum of anion and cation equivalents was divided by two (simplified ion balancing through the formation of mean values) and multiplied by the molar mass of sodium chloride (NaCl), providing the empirical total salt retention capacity of the mineral poultice in μ g/cm².

3 Results

3.1 Effectiveness of treatment using different mineral poultices

The effectiveness of the three tested poultices (sepiolite, kaolinite, and vermiculite) was evaluated in terms of their salt storage capacity

and resistance to salt weathering. The salt concentration in the test areas was measured before and after treatment to determine the potential of the poultices to reduce salt content on the surfaces (Figure 2; Supplementary Table S1).

The poultices were tested in two historic buildings, each presenting distinct salt compositions and concentrations (see methods section and Tichy et al., 2023). At the St. Virgil's Chapel, one of the vaults (V2) was examined, while at the Charterhouse of Mauerbach, two rooms (M4 and M6) were investigated (see methods section and Tichy et al., 2023). The three poultices exhibited different behaviors regarding salt retention, extraction efficiency, and resistance to salt weathering at various sampling points.

3.1.1 St. Virgil Chapel

At the beginning of the monitoring campaign the surfaces of the St. Virgil Chapel were predominantly affected by halite (NaCl) salt efflorescence, with sulfates present to lesser extent, as reported previously (Tichy et al., 2023). Results show that all poultice mixtures applied in the chapel of St. Virgil extracted and retained sodium (with a max. in kaolinite after 6 months: 29716.8 $\mu\text{g}/\text{cm}^2$) and chloride ions (max. in vermiculite after 12 months: 76798.2 $\mu\text{g}/\text{cm}^2$) (Supplementary Table S1) during the application period. Additionally, potassium, calcium, magnesium, nitrate, and sulfate accumulated in all poultices (Figure 2a). Notably, all poultices exhibited significant nitrate retention after 1 month, with values of 3336.2 $\mu\text{g}/\text{cm}^2$ (sepiolite), 5099.9 $\mu\text{g}/\text{cm}^2$ (kaolinite), and 3835.6 $\mu\text{g}/\text{cm}^2$ (vermiculite), which subsequently decreased (Figure 2a). Sulfate accumulation peaked after 12 months, with maximum values of 6863.7 $\mu\text{g}/\text{cm}^2$ (sepiolite), 3338.3 $\mu\text{g}/\text{cm}^2$ (kaolinite), and 3296.5 $\mu\text{g}/\text{cm}^2$ (vermiculite).

However, the type of clay mineral exerted a noticeable impact on the ion retention properties, salt resistance, and final extraction efficiency of the main salt, halite. The sepiolite poultice exhibited the lowest performance with signs of salt-weathering after 1 month, manifested as sanding, and also showed the lowest total salt extraction amount (Figure 2a; Supplementary Figure S1). This poultice reached its maximum salt accumulation after 6 months (Supplementary Figure S1). However, the quantity of salts stored was found to be considerably lower in comparison to the other two poultice mixtures (Figure 2a), as demonstrated by the ion chromatography results, which displayed a reduced capacity for the extraction of sodium ions (max. 11008.8 $\mu\text{g}/\text{cm}^2$) and chloride ions (max. 38896.8 $\mu\text{g}/\text{cm}^2$) in comparison with the other two mineral clays.

The kaolinite poultice displayed a salt extraction capacity of max. 111805.9 $\mu\text{g}/\text{cm}^2$ mass eq. NaCl and a higher resilience to salt pressure, although its storage capacity was smaller to that of the vermiculite poultice (Supplementary Figure S1). Furthermore, the results of the analyses conducted after 6 months, showed the highest salt accumulation load (Figure 2a), with the highest amount of retained sodium (29716.8 $\mu\text{g}/\text{cm}^2$) at this time and chloride (71007.8 $\mu\text{g}/\text{cm}^2$) after 12 months of the treatment (Supplementary Table S1).

Finally, the vermiculite poultice demonstrated the most effective extraction and storage capacity (max. 118274.1 $\mu\text{g}/\text{cm}^2$ mass eq. NaCl), as well as the higher resistance to salt-weathering by maintaining an intact surface and structure throughout the entire study period (Supplementary Figure S1). This poultice retained a max. of sodium (27367.2 $\mu\text{g}/\text{cm}^2$) after 6 months and a max. of chloride

(76798.2 $\mu\text{g}/\text{cm}^2$) after 12 months of treatment (Supplementary Table S1).

Regarding the effect of the poultices on the treated surfaces, a reduction in sodium concentration (from 7754.4 $\mu\text{g}/\text{cm}^2$ to 3009.6 $\mu\text{g}/\text{cm}^2$ for sepiolite) and stability in chloride values (from 12550.8 $\mu\text{g}/\text{cm}^2$ to 11803.2 $\mu\text{g}/\text{cm}^2$ for vermiculite) were observed. However, an increase in other ions, particularly potassium, was detected, with concentrations reaching up to 40 times the initial values (Supplementary Table S1).

3.1.2 Charterhouse of Mauerbach

The test areas at the Charterhouse Mauerbach were located in two distinct rooms, labeled M4 and M6, which presented distinct initial salt conditions. M4 had minimal salt contamination, primarily gypsum-related ions (Ca^{2+} and SO_4^{2-}), whereas M6 exhibited extensive salt efflorescence, dominated by sulfates, potassium, and sodium ions (Supplementary Table S1).

3.1.2.1 Test area M4

Since M4 contained mainly gypsum-related salts, it was expected that the poultices would primarily accumulate calcium and sulfate ions (Figure 2b). Indeed, the results of the analyses demonstrated that all poultices primarily extracted sulfate, with maximum values of 763.9 $\mu\text{g}/\text{cm}^2$ (sepiolite), 379.0 $\mu\text{g}/\text{cm}^2$ (kaolinite), and 524.4 $\mu\text{g}/\text{cm}^2$ (vermiculite). Calcium retention was highest in kaolinite after 6 months (444.0 $\mu\text{g}/\text{cm}^2$). However, the accumulation of calcium ions in the poultices was found to be lower than after the treatment directly on the wall.

However, some differences were observed on the retention of other ions depending on the mineral clay used. The sepiolite poultice accumulated the highest concentrations of most ions after 1 month, except for sulfate (with a maximum at 6 months) and chloride (which peaked at 12 months, with 191.0 $\mu\text{g}/\text{cm}^2$) (Supplementary Table S1; Figure 2b).

The kaolinite-based poultice exhibited a distinct behavior. The highest concentration of all total ions was observed after 6 months, with the exception of nitrate ions, which showed their maximum (39.3 $\mu\text{g}/\text{cm}^2$) after 1 month (Supplementary Table S1; Figure 2b). The kaolinite poultice extracted a multiple of the salt ions originally present in the substrate, with the amount of sodium ions extracted reaching up to 170 times the original quantity. However, the amount of extracted sulfates was smaller after 12 months compared to those after 1 and 6 months (Figure 2b).

Concerning the vermiculite poultice, the highest concentrations of sodium (49.2 $\mu\text{g}/\text{cm}^2$), potassium (109.4 $\mu\text{g}/\text{cm}^2$), and magnesium (56.2 $\mu\text{g}/\text{cm}^2$) ions were observed after a period of 1 month (Supplementary Table S1; Figure 2b). Sulfate and calcium showed their maximum retention at 6 months, and the highest quantities of nitrates (17.8 $\mu\text{g}/\text{cm}^2$) and chlorides (20.6 $\mu\text{g}/\text{cm}^2$) were recorded after 12-month period. In comparison to the initial value on the non-treated surface area (NT), the poultice demonstrated the greatest storage capacity for magnesium ions after 1 month, reaching a concentration up to 14.1 times the initial value (Supplementary Table S1).

At the end of treatment, the treated surfaces exhibited increased sulfate (1180.8–2001.6 $\mu\text{g}/\text{cm}^2$), calcium (423.4–837.6 $\mu\text{g}/\text{cm}^2$), and magnesium (50.4–76.8 $\mu\text{g}/\text{cm}^2$) concentrations, regardless of the poultice used (Figure 2b). A slight increase in chloride ions was observed in the area treated with the sepiolite poultice, while the

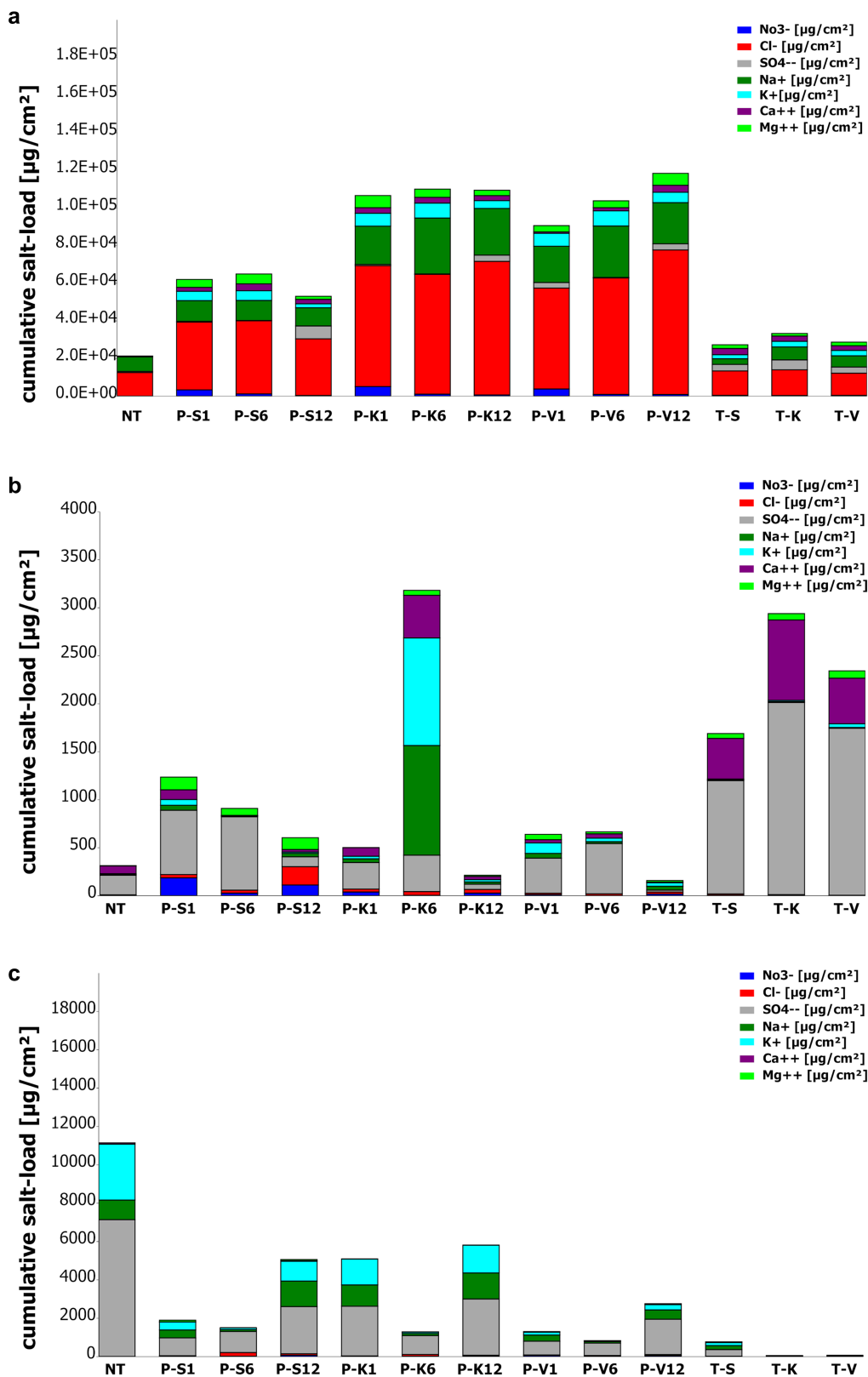


FIGURE 2
Quantitative salt analysis of sampling locations (a) V2; (b) M4, (c) M6 during the application of the poultices (Poultice [P]; Sepiolite [S]; Kaolinite [K]; Vermiculite [V]; non-treated [NT], after the treatment [T + S/K/V]) within the time intervals after 1, 6, and 12 months. Colors correspond to measured ions ($\mu\text{g}/\text{cm}^2$) [sodium (dark-green); potassium (turquoise); magnesium (light-green); calcium (purple); sulfate (gray); nitrate (blue) and chloride (red)].

original value of potassium ions was found to be found approximately fourfold in the area treated with the vermiculite poultice (Supplementary Table S1).

3.1.2.2 Test area M6

In the non-treated M6 test area, the most prevalent ions detected before treatment were sulfate ($7135.2 \mu\text{g}/\text{cm}^2$), potassium ($2918.4 \mu\text{g}/\text{cm}^2$), and sodium ($1022.4 \mu\text{g}/\text{cm}^2$) with a minor presence of calcium ($62.8 \mu\text{g}/\text{cm}^2$) (Supplementary Table S1). These ions were also identified in considerable quantities within all poultices. However, the poultices also demonstrated the accumulation of a greater quantity of nitrate, chloride, and magnesium ions than initially present in the test area (Figure 2c). At the end of the monitoring campaign (12 months) all three poultices exhibited the highest accumulation of the dominant salt ions within their matrix, specifically sulfate ($1842.2\text{--}2937.6 \mu\text{g}/\text{cm}^2$), potassium ($278.4\text{--}1452.0 \mu\text{g}/\text{cm}^2$), and sodium ($487.2\text{--}1356.0 \mu\text{g}/\text{cm}^2$). The maximum concentration of chloride ions in all poultices was detected after 6 months ($51.6\text{--}197.0 \mu\text{g}/\text{cm}^2$).

Regarding salt accumulation in the poultices, the highest quantity of extracted salts was observed in the mineral poultice made of kaolinite after a 12-month period (Figure 2c). The kaolinite-based poultice exhibited superior efficacy in accumulating the dominant species of ions (sulfate, potassium and sodium), present on the non-treated surfaces. In contrast, the sepiolite-based poultice was more effective in accumulating chloride ions. Notably, individual variations were observed among the poultices. The highest concentration of magnesium ions ($85.4 \mu\text{g}/\text{cm}^2$) was detected in the sepiolite poultice after 1 month, whereas in the kaolinite poultice, the highest concentration ($8.4 \mu\text{g}/\text{cm}^2$) was recorded after 6 months. Regarding nitrate extraction, the vermiculite poultice showed the highest retention after 1 month ($63.7 \mu\text{g}/\text{cm}^2$), whereas sepiolite and kaolinite reached a maximum after 12 months (72.1 and $41.9 \mu\text{g}/\text{cm}^2$, respectively; Supplementary Table S1).

At the end of the treatment, compared to the non-treated (NT) areas, the treated (T) areas exhibited a significant decrease in the concentrations of weathering ions. Sulfate decreased from $7135.2 \mu\text{g}/\text{cm}^2$ at the NT surface to $6.2\text{--}350.4 \mu\text{g}/\text{cm}^2$ at the surface, sodium from 1022.4 to $8.6\text{--}205.4 \mu\text{g}/\text{cm}^2$, potassium from 2918.4 to $7.7\text{--}159.6 \mu\text{g}/\text{cm}^2$, and calcium from 62.8 to $16.8\text{--}23.5 \mu\text{g}/\text{cm}^2$, regardless of the poultice used (Figure 2c). Furthermore, a decrease in nitrate and chloride concentrations was observed in the treated areas with kaolinite and vermiculite, while a slight increase in magnesium ions was detected in all treated areas (Supplementary Table S1).

3.2 Effect of the desalination treatment on the surface-associated microbial communities: a metagenomic monitoring

The second objective of this monitoring study was to investigate how the changes in ion concentrations, during and after treatment, affected the salt-associated biofilms on the treated surfaces. For this purpose, the microbial communities present on the walls before and after treatment, as well as on the different mineral poultices after 1, 6, and 12 months of their application, were monitored using a metagenomic approach. Therefore, long-read archaeal- and bacterial-16S rRNA amplicon analysis employing the Nanopore sequencing technology was conducted, as explained in the methods

section. The microbial communities present on the walls before treatment (indicated as non-treated) were previously identified and their detailed composition is shown in Tichy et al. (2023).

3.2.1 Metagenomic monitoring in St. Virgil Chapel

3.2.1.1 Bacterial monitoring

The treatment with mineral poultices showed promising results for the elimination/mitigation of some of the genera that were most abundant on the V2 surface in the Virgil Chapel before treatment. These genera, in particular, *Salinisphaera*, *Methylohalomonas*, *Marinobacter*, *Halomonas*, *Marinimicrobium*, and *Aliifodiniibius*, generally showed a decrease in their relative proportions, or even in some cases, a complete elimination from the wall after treatment (Figure 3a). *Salinisphaera* and *Methylohalomonas*, which formed 40 and 10.5% of the microbial community on the wall before treatment, disappeared completely from the surfaces after treatment, irrespective of the used poultice. During treatment, *Salinisphaera* was detected in the poultices sporadically and in low proportions, i.e., in the sepiolite after 1 month (1.1%), and in the kaolinite and vermiculite after 12 months (1.8 and 0.7%, respectively). *Methylohalomonas* was only detected in the vermiculite poultice, showing low proportions over time (0.6–1.2%). *Marinobacter*, *Halomonas* and *Marinimicrobium* showed a significant decrease in their relative proportion on the treated walls. *Marinobacter* was even eliminated after treatment with vermiculite. However, the proportions of these genera within the poultices throughout the treatment was different. *Marinobacter* was detected inside all three poultices over time with relatively low concentrations in the case of sepiolite (3.9–4.3%), but an enrichment of this genus was seen within the kaolinite (7.0–22.1%) and vermiculite poultices (13.7–78.6%). The same trend was observed with the genus *Halomonas*, which also showed an enrichment within the vermiculite poultice (1.2–10.9%). In contrast, *Marinimicrobium* was not detected in any of the poultices (Figure 3a). The genus *Aliifodiniibius* (19.8% before the treatment) showed a different behavior depending on the poultice used. After treatment with sepiolite- and kaolinite-poultices, there was a decrease in its proportion on the walls (1.8 and 9.7%, respectively), but an increase after treatment with vermiculite (up to 35.4%). Furthermore, this genus was detected inside all the poultices with proportions ranging from 0.7 to 3.3%. Finally, some other genera that showed a low abundance in the untreated surface community (below 1%), such as *Alkalilimnicola*, *Magnetospira* and *Tepidicaulis* also disappeared completely from the treated surfaces and were not detected during treatment in the poultices, with the exception of *Alkalilimnicola*, which was detected in the vermiculite poultice at 6 months of treatment in low proportion (0.6%).

In contrast, an enrichment was observed for other genera, compared to the surfaces prior treatment (Figure 3a). *Bacilli*, including members of the genera *Bacillus*, *Metabacillus*, and *Paenibacillus* showed an increase on treated surfaces, especially on areas treated with sepiolite (up to 38.5, 20.7, and 3.4%, respectively) and kaolinite (up to 21.4, 11.0, and 5.2%, respectively). During the treatment, members of *Metabacillus* and *Paenibacillus* were scarcely detected within the poultices with low relative proportions at different monitoring times, but *Bacillus* showed to be especially enriched in the kaolinite poultice after 12 months (20.0%). Members of *Gracilimonas* and *Persicimonas* showed the same trend, with an increase in their relative proportions in post-treated surfaces with kaolinite (up to 1.7 and 14.5%,

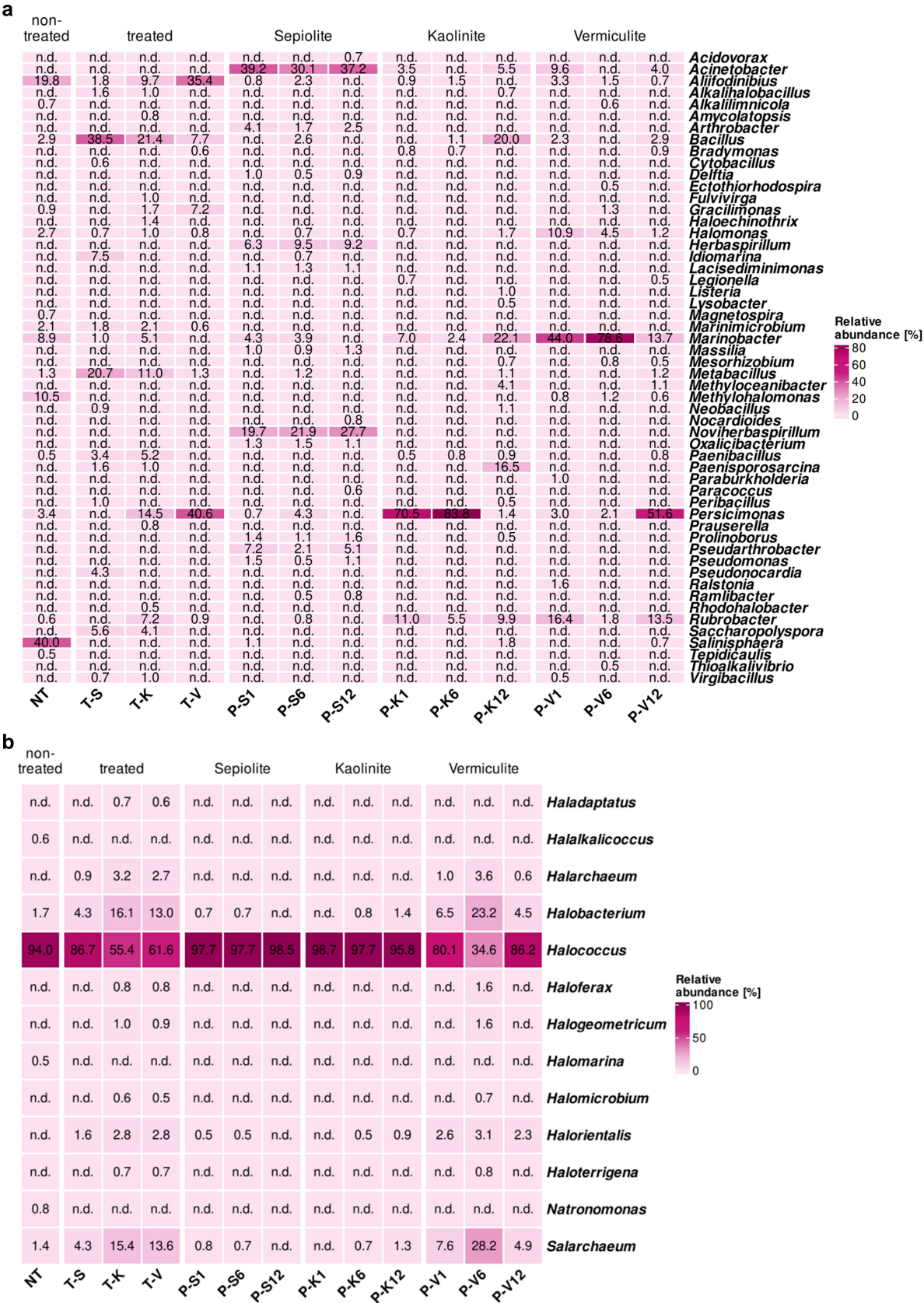


FIGURE 3 Heatmaps showing the relative abundance in percentages of (a) bacteria and (b) archaea, at the genus level (cut-off at 0.5%) for samples taken at the St. Virgil Chapel [V2]. Heatmap colors correspond to the abundance values, the darker the color, the higher the relative abundance. NA denotes (Continued)

FIGURE 3 (Continued)

measurements where the given genus was not detected above the set cut-off value. The samples include following letters for further description: Poultrice [P]; sepiolite [S]; kaolinite [K]; vermiculite [V]; non-treated [NT], after the treatment [T + S/K/V] within the time intervals after 1, 6, and 12 months.

respectively) and even higher with vermiculite (7.2 and 40.6%, respectively), but undetected in sepiolite-treated areas (Figure 3a). However, during the treatment these two genera showed a different trend, *Gracilimonas* was only identified after 6 months within the vermiculite poultrice at a low rate (1.3%), while *Persicimonas* showed in general a strong enrichment in the mineral poultrices, especially in kaolinite (70.5–83.8% in the first half of the treatment) and vermiculite after 12 months (51.6%). Finally, *Rubrobacter* increased its relative abundance on kaolinite-treated surfaces (from 0.6 to 7.2%), and showed an enrichment in the kaolinite and vermiculite poultrices during the treatment (ranging from 1.8 to 16.4%).

In addition, members of 15 other genera, which were either below the detection level or not present on the untreated walls, emerged in the bacterial community of the treated walls, especially in the kaolinite- and/or sepiolite-treated surfaces with low relative proportions (0.5–7.5%). Among them, were mainly members of the *Actinomycetota* phylum, such as *Haloechinothrix*, *Pseudonocardia* and *Saccharopolyspora* in addition of *Alkalihalobacillus*, *Idiomarina* and *Paenisporosarcina*. Of these only *Paenisporosarcina* showed a significant enrichment within the vermiculite poultrice reaching a proportion of 16.5% at the end of the treatment. Some genera were generally detected only sporadically and in very low proportions ($\leq 1\%$), these include members of the *Bacillota* phylum, such as *Cytobacillus*, *Neobacillus*, *Peribacillus*, and *Virgibacillus* in addition to *Amycolatopsis*, *Bradymonas*, *Fulvivirga*, *Prauserella*, and *Rhodohalobacter* (Figure 3a).

Interestingly, some genera were identified exclusively within the mineral poultrices, which were not detected on the wall surfaces either before or after treatment (Figure 3a). In this regard, only the genus *Acinetobacter* was detected within all three mineral clays, with the highest relative abundance in sepiolite (39.2%). However, in general, the genera detected exclusively in the mineral poultrices were different according to the type of clay, indicating their possible origin in their mineral composition. The poultrice with the highest number of bacterial genera was composed of sepiolite and contained the following genera: *Arthrobacter* (1.7–4.1%), *Herbaspirillum* (6.3–9.5%), *Lacisediminimonas* (1.1–1.3%), *Massilia* (1.0–1.3%), *Noviherbaspirillum* (19.7–27.7%), *Oxalicibacterium* (1.1–1.5%), *Pseudoarthrobacter* (2.1–7.2%) and *Pseudomonas* (1.1–1.5%). In addition, the genus *Prolinoborus* (1.1–1.6% within the sepiolite poultrice), was also detected in the kaolinite poultrice after 12 months of treatment, but with a very low proportion (0.5%). The genus *Methyloceanibacter* was detected within the poultrices made of kaolinite and vermiculite (4.1 and 1.1%, respectively, after 12 months) and *Ralstonia* was found only in the vermiculite clay at the beginning of the treatment (1.6%) and was not detectable thereafter.

3.2.1.2 Archaeal monitoring

The archaeal community composition of the non-treated surfaces in area V2 was clearly dominated by the genus *Halococcus* (94.0% of the total archaeal community) (Figure 3b). Interestingly, the relative abundance of this genus was decreased in the treated surfaces after the

application of any mineral poultrice, showing a reduction to 86.7% (sepiolite), 61.6% (vermiculite) and 55.4% (kaolinite). Monitoring of the poultrices during the period of treatment showed a strong enrichment of *Halococcus* within the sepiolite and kaolinite poultrices, with relative values above 95% of the population. In the vermiculite poultrice, a different behavior was observed over time with values ranging from 34.6 to 86.2%. Furthermore, the genera *Natronomonas*, *Halalkalicoccus* and *Halomarina* (0.8, 0.6, and 0.5% in non-treated surfaces, respectively) were completely eliminated from the surfaces treated with the different poultrices and were not detected within any of them along the treatment (Figure 3b).

In contrast, the genera *Halobacterium* and *Salarchaeum*, which showed the second highest abundance on the untreated surface (1.7 and 1.4%, respectively), underwent an increase in relative abundance on the treated walls, with values ranging from 4.3 to 16.1%, depending on the poultrice used (Figure 3b). Their monitoring within the poultrices showed negligible values in the sepiolite and kaolinite clays, but a significant enrichment in the vermiculite poultrice.

Other genera, such as *Haladaptus*, *Halararchaeum*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halorientalis*, and *Haloterrigena* appeared sporadically in some of the treated areas and poultrices, with relatively low proportions (below 4%), indicating a slight increase in diversity in the archaeal community during and after the treatment.

3.2.2 Metagenomic monitoring in Charterhouse Mauerbach

3.2.2.1 Testing location M4

Seven genera that were present on the non-treated M4 test surface, with relatively low proportions (0.6–3.6%), were no longer detected on its surface during and after the desalination treatment, these genera were *Acidothermus*, *Actinoalloteichus*, *Amycolatopsis*, *Goodfellowiella*, *Kutzneria*, *Saccharomonospora*, and *Saccharopolyspora*. In contrast, *Rubrobacter*, the most dominant genus on the surface of M4 before the desalination treatment (86.3%), showed an increase in its relative abundance on the treated surfaces ranging from 92.0 to 99.0%, while within the mineral poultrices, *Rubrobacter* only showed a remarkably high proportion in sepiolite after 1 month (36.4%), and in vermiculite after 12 months (27.9%) (Figure 4).

In addition, members of 6 other genera, not detectable in the non-treated walls, were found in the bacterial community of the vermiculite- and/or kaolinite-treated walls with low relative proportions (0.6–2.2%). These are *Acinetobacter*, which also showed a massive enrichment in all poultrices during treatment (from 19.9 to 64.1%); *Arthrobacter*, also detected within all mineral clays (0.8–2.5%); *Massilia*, only present on the kaolinite-treated surface and enriched in this poultrice (15.0–17.0%), *Pseudarthrobacter*, which showed a significant enrichment in the vermiculite poultrice (11.7–18.6%); *Pseudomonas*, with a higher enrichment in the kaolinite poultrice (5.1–7.0%), and *Yonghaparkia*, associated with the vermiculite poultrice with percentages ranging 8.6–10.7% and transferred to the treated surface (1.8%) (Figure 4).

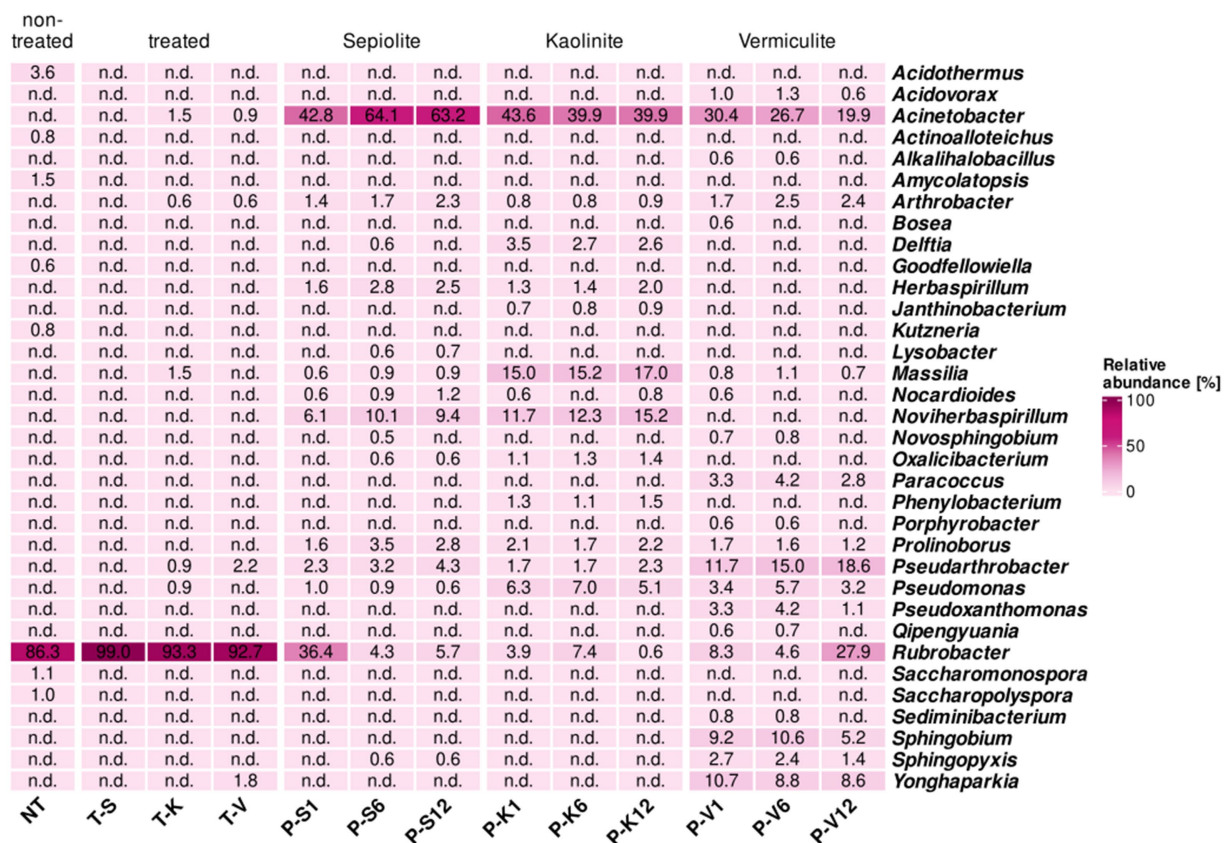


FIGURE 4

Heatmap showing the relative abundance in percentages of bacteria at the genus level (cut-off at 0.5%) for samples taken at the Charterhouse Mauerbach [M4]. Heatmap colors correspond to the abundance values, the darker the color, the higher the relative abundance. NA denotes measurements where the given genus was not detected above the set cut-off value. The samples include following letters for further description: Poultice [P]; sepiolite [S]; kaolinite [K]; vermiculite [V]; non-treated [NT], after the treatment [T + S/K/V] within the time intervals after 1, 6, and 12 months.

Similar to what was observed in the St. Virgil Chapel, some additional genera were detected only within the poultices, which were not being transferred to the walls during the treatment. These genera seem to be specifically related to the clay component of each poultice. Thus, in the sepiolite and kaolinite clays, the following genera were enriched above 1% of the population, respectively: *Delftia*, *Herbaspirillum*, and *Noviherbaspirillum*. In the vermiculite poultice, the genera enriched over 1% of the population within the clay were *Paracoccus*, *Pseudoxanthomonas*, *Sphingobium*, and *Sphingopyxis* (Figure 4).

Concerning the archaeal community at test site M4, members of this community were shown to be at the limit of PCR detection, and therefore, results were not consistent for reliable identification, neither during the desalination treatment on the poultices, nor after treatment on the treated surfaces (see also section 3.3. Quantitative PCR analyses).

3.2.2.2 Testing location M6

The surface before and after the desalination treatment of the testing location M6 was dominated by one highly abundant genus, *Haloechinothrix*. However, this genus faced a reduction from 98.4% toward the range of 67.1–79.3% in the treated surfaces (Figure 5a). Moreover, *Haloechinothrix* showed a significant enrichment in the mineral poultices of sepiolite (up to 48.4% after 6 months), kaolinite

(19.5–27.4%), and partially in vermiculite (4.7–19.9%). In contrast, *Nocardiopsis*, also detected on the non-treated surface (0.6%), increases in proportion (up to 2.8%) on the surfaces treated with kaolinite and vermiculite but disappears from the sepiolite-treated surface.

In addition, members of 12 other genera, which were either below the detection level or not present on the untreated walls in M6, were detected in the bacterial community on the treated walls (Figure 5a). These genera appear sporadically on one or the other treated surfaces with relative abundances below 5%, except *Nesterenkonia*, which was found on all treated walls with proportions between 5.2 and 19.1% and was also detected in all mineral poultices, especially in the one composed of kaolinite (1–6.9%). *Amycolaptosis* and *Saccharomonospora* were also identified on all treated surfaces but with proportions between 0.9–2.5% and 1.1–1.4%, respectively. The former was also detected during treatment within the sepiolite (6 months) and kaolinite poultices (6 and 12 months). *Präuserella* was only identified in the sepiolite-treated surface (0.6%), but not within any poultice. Interestingly, most of the newly detected genera were found on the vermiculite treated surface, such as *Arthrobacter* (1.1%), which was also detected within all of the mineral poultices, with similar proportions ranging from 2.4 to 8.8%. The genera *Paracoccus* (0.6%), *Pseudoarthrobacter* (0.9%), and *Pseudomonas* (2%) also emerged in the vermiculite-treated area, and were also detected within all mineral poultices, but always with a higher

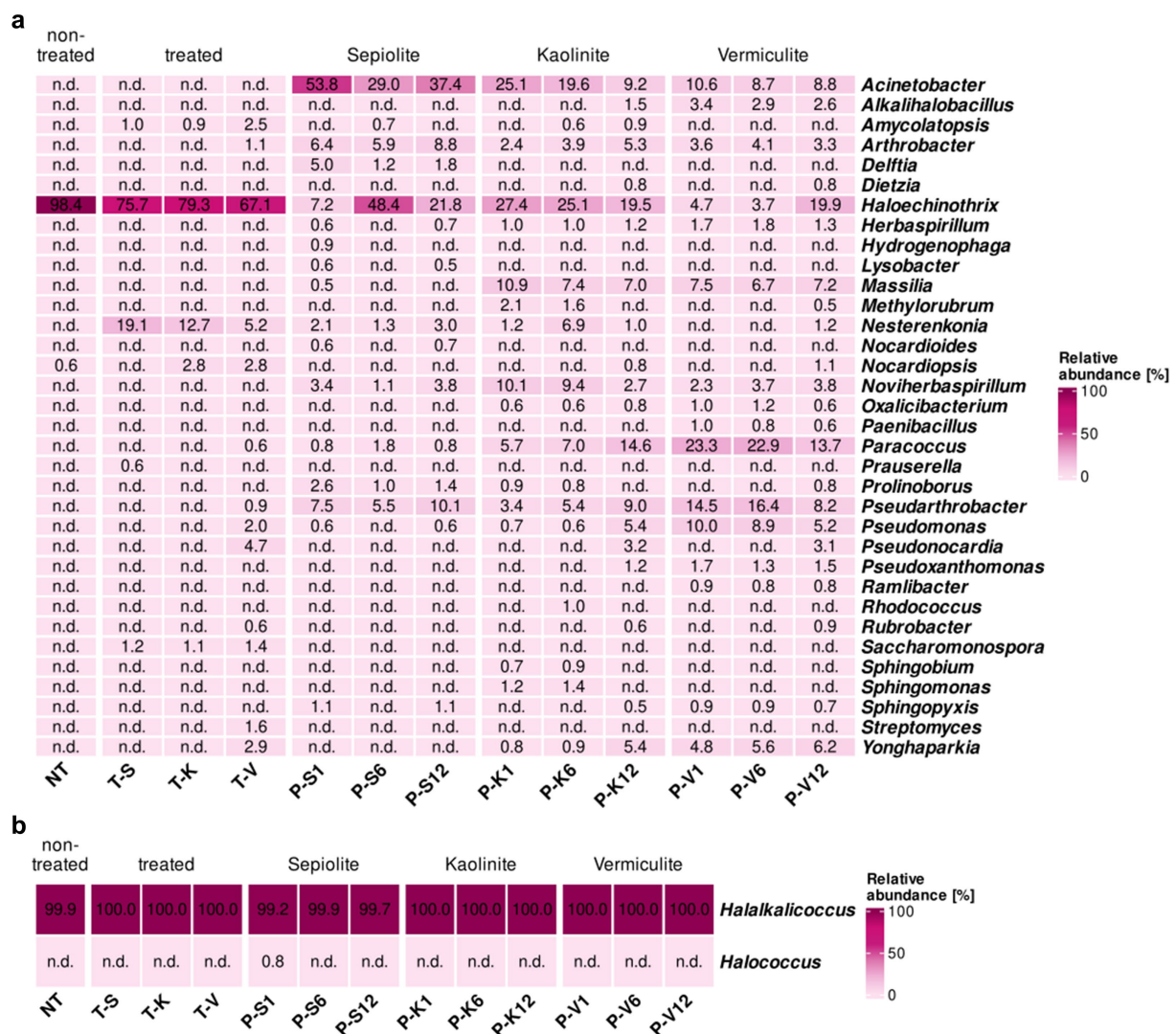


FIGURE 5

Heatmap showing the relative abundance in percentages of (a) bacteria and (b) archaea, at the genus level (cut-off at 0.5%) for samples taken at the Charterhouse Mauerbach [M6]. Heatmap colors correspond to the abundance values, the darker the color, the higher the relative abundance. NA denotes measurements where the given genus was not detected above the set cut-off value. The samples include following letters for further description: Poultice [P]; sepiolite [S]; kaolinite [K]; vermiculite [V]; non-treated [NT], after the treatment [T + S/K/V] within the time intervals after 1, 6, and 12 months.

proportion within the vermiculite clay, reaching significant relative proportions ranging from 5.2 to 23.3%. *Pseudonocardia*, *Rubrobacter*, and *Yonghaparkia* (4.7, 0.6, and 2.9% on the vermiculite-treated surface, respectively) were also detected within the poultices composed of kaolinite and vermiculite, the first two genera only after 12 months of treatment (with proportions ranging 0.6–3.2%) and the latter throughout the treatment, but again showing a higher proportion in the vermiculite clay (4.8–6.2%). Finally, *Streptomyces* was identified at a relative abundance of 1.6% also in the area treated with vermiculite but was not detected in any of the poultices during the desalination treatment (Figure 5a).

Similar to what was observed at the other test sites, some additional genera were only detected inside the poultices, without transferring to the walls during treatment. Six genera were detected inside all poultices, regardless of the clay component, such as *Acinetobacter*, especially enriched in the sepiolite clay (29.0–53.8%) but also with

significant proportional values in the kaolinite (9.2–25.1%) and vermiculite clays (8.7–10.6%); *Herbaspirillum* (from 0.7% in sepiolite up to 1.8% in vermiculite), *Massilia* (from 0.5% in sepiolite up to 10.9% in kaolinite) and *Noviherbaspirillum* (from 1.1% in sepiolite up to 10.1% in kaolinite). In addition, the genera *Prolinoborus* and *Sphingopyxis* were also detected in all mineral clays, but in lower proportions (between 0.5 and 2.6%). Five other genera were detected in the kaolinite and vermiculite poultices in relatively low proportions (between 0.5 and 3.4%) but not in sepiolite, namely *Alkalihalobacillus*, *Dietzia*, *Methylobacterium*, *Oxalicibacterium*, and *Pseudoxanthomonas* (Figure 5a). Finally, additional genera appeared exclusively on one or the other mineral clay at different monitoring times. Thus, in the sepiolite poultice, the genera *Delftia* (1.2–5.0%) emerged in addition to *Hydrogenophaga*, *Lysobacter*, and *Nocardioides* (all three with proportions below 1.0%). In the kaolinite poultice, the genera *Sphingomonas* (1.2–1.4%) together with *Sphingobium* and *Rhodococcus*

(both with proportions $\leq 1.0\%$) were detected and lastly in the vermiculite poultice, the genera *Paenibacillus* and *Ramlibacter* were detected exclusively, but with proportions $\leq 1.0\%$.

The archaeal community on the non-treated surface of test area M6 was absolutely dominated by members of the genus *Halalkalicoccus* (99.9%) and likewise persisted during and after the desalination treatment, both within the desalination poultices as well as on the treated surfaces, irrespective of the mineral clay used (99.2–100.0%) (Figure 5b).

3.2.3 Diversity indices

In the St. Virgil Chapel, *Shannon* and *Simpson's* diversity indices showed a higher diversity for bacteria on the non-treated surface (NT) of area V2, as well as on the surface treated with the sepiolite poultice (TS) compared to the surfaces treated with kaolinite (TK) and vermiculite (TV) (Figure 6a). In contrast, the diversity indices calculated for archaea showed a lower diversity on non-treated surfaces (NT) compared to that on treated surfaces. Only on the surface treated with sepiolite (TS), the diversity was shown to be almost equal (*Shannon's* index) or even lower (*Simpson's* index) after treatment (Figure 6a). The results indicate that, in general, treatment with mineral poultices led to a decrease in bacterial biodiversity and to a slightly increase in archaeal biodiversity, the effect of sepiolite not being as evident as for the other mineral clays.

In the Charterhouse Mauerbach, the diversity indices were calculated for the two rooms tested, M4 and M6. In room M4, a similar behavior to that of the V2 vault of the Virgil Chapel was observed, in terms of bacterial diversity. The highest diversity was observed on the non-treated surface (NT) compared to that of the treated surfaces. After treatment, all diversity values decreased, but interestingly here, and contrary to what was observed in V2, the greatest effect on diversity was exerted by the sepiolite poultice (TS) (Figure 6b). In this test room, diversity analyses could not be performed for archaea, as they were present close to the detection limit.

In room M6, contrary to what was observed in the other two test areas, the lowest *Shannon* and *Simpson's* diversity indices calculated for bacteria were shown on the non-treated surface (NT), which increased significantly after treatment with all mineral clays, especially in the area treated with vermiculite (TV) (Figure 6c). The diversity of the archaeal community did not change drastically after treatment, and remained extremely low, as only one species was detected with a relative abundance above 99% during the whole monitoring period, namely *Halalkalicoccus paucihalophilus* (Supplementary Figure S5).

3.3 Quantitative enumeration of microbial community through qPCR analyses

qPCR analyses were performed before and after the application of the mineral poultices with samples obtained from the different tested areas of both buildings in order to have a (semi)-quantitative monitoring of the possible increase or decrease of the microbial community (bacteria and archaea) due to the treatment.

The samples for the qPCR analyses were taken from the surfaces before (non-treated: NT wall samples) and immediately after removal of the mineral poultice (treated: T wall samples). Results show that in the St. Virgil Chapel, the desalination treatment increased the bacterial Ct (cycle threshold) values on all treated surfaces, irrespective of the mineral poultice used ($[1.55\text{--}4.33]$ ΔCt) (Supplementary Table S2). In contrast, the results of qPCR aimed at archaeal quantification only showed a statistically relevant increase in Ct values after the treatment with kaolinite and sepiolite minerals ($[1.77\text{--}2.07]$ ΔCt). The general increase in the Ct values indicates a decrease in both bacterial and archaeal populations after the treatment with the mineral poultices at the St. Virgil Chapel.

In the Charterhouse Mauerbach the two tested rooms M4 and M6 showed a different performance compared toward the St. Virgil

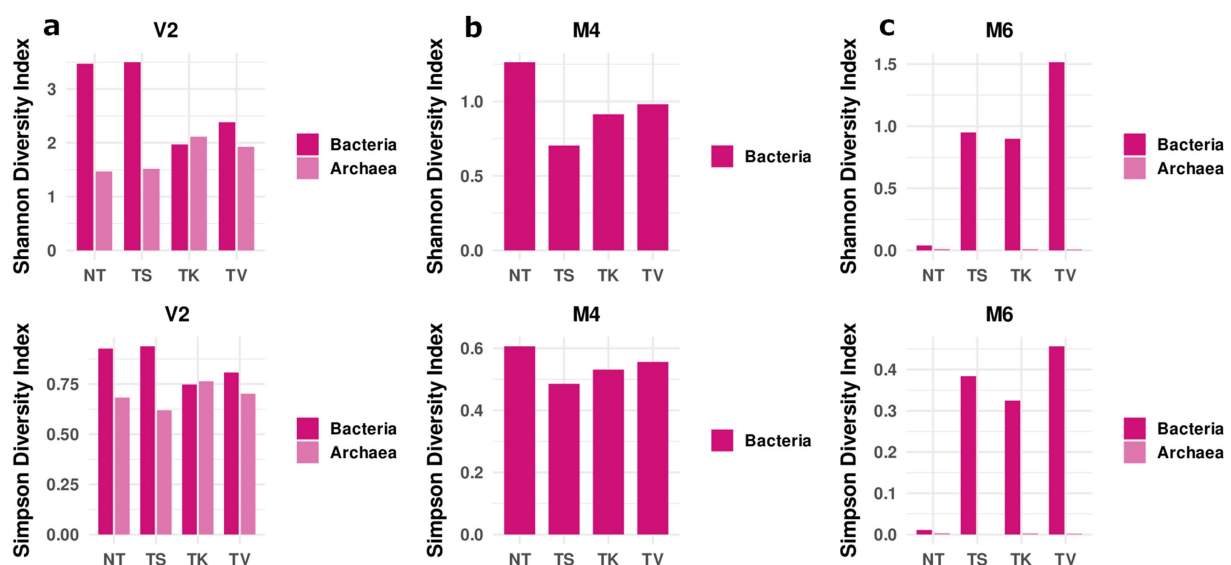


FIGURE 6

Diversity indices. The upper part shows the Shannon diversity index and the lower part shows the Simpson diversity of the sampling locations of (a) the St. Virgil Chapel (V2), and the Charterhouse Mauerbach: (b) of room M4 and (c) of room M6. The samples include following letters for further description: non-treated [NT]; treated [T] with: sepiolite [S]; kaolinite [K]; vermiculite [V].

Chapel. The treated areas in room M4 showed a significant decrease of the Ct values of the bacterial populations for all types of mineral poultices, compared to the values before treatment ($[1.27\text{--}2.76]$ ΔCt , [Supplementary Table S2](#)). The decrease in the Ct-value indicates an enrichment of the bacterial population in M4 after treatment. In contrast, the results for the archaeal population showed steady high Ct values, with non-significant variations before and after treatment, suggesting that the population of archaea in the microbial community of this sample was close to the limit of detection.

The room M6 in the Charterhouse Mauerbach showed the opposite results compared to M4. After the desalination treatment, the bacterial Ct values in areas of M6 raised between $[2.00]$ ΔCt (in T area with kaolinite) and $[2.36]$ ΔCt (in T area with the sepiolite poultice) indicating a reduction in the bacterial population. However, in the area treated with vermiculite, no significant change was observed in the Ct values compared to those observed before treatment, indicating that this clay mineral did not cause any quantitative change in the colonizing microbial population in this area. In contrast, the Ct values of the qPCR targeting the archaeal community decreased considerably on the treated surfaces with respect to the non-treated ones, showing an average decrease of $[6.5]$ ΔCt in the areas treated with the sepiolite and kaolinite poultices and $[5.8]$ ΔCt in the area treated with the vermiculite one, indicating an increase in the archaeal population in all treated areas.

4 Discussion

Long-term monitoring during and after any conservation treatment is essential for assessing their effectiveness and identifying potential secondary effects on both material surfaces and associated microbial communities. Many conservation strategies primarily focus on the immediate removal of salts, often neglecting the long-term impact of ionic redistribution and microbial colonization ([Verges-Belmin and Siedel, 2005](#); [Lubelli et al., 2018](#)). However, changes in salt composition and concentration can significantly alter the physical and chemical properties of treated surfaces, influencing their susceptibility to further deterioration ([Sawdy et al., 2010](#)). Moreover, previous studies have shown that desalination treatments can shift microbial community dynamics, which could potentially favor new biodeterioration risks ([Piñar et al., 2009](#); [Pavlović et al., 2022](#)). Therefore, integrating long-term monitoring into preservation protocols is essential to ensure that treatments provide sustainable and effective protection and to prevent unwanted side effects. This study shows that the long-term performance of different mineral poultices varied depending on the type and initial concentration of salts present on the surfaces and the clay mineral used in the poultices.

4.1 Long-term monitoring at the St. Virgil Chapel

4.1.1 Effectiveness of poultice treatment in test area V2

As briefly mentioned in the methods section, the chapel of St. Sant Virgil is a hypogean environment subject to frequent water ingress. This site presents a unique situation, as water infiltrates from the upper surface after heavy rainfall, carrying de-icing salts from winter applications. This process leads to inhomogeneous salt loads in the

subsurface chapel and continuous formation of visible salt efflorescence. The general condition of the wall surfaces in the chapel has been previously described, as rough, highly porous and structurally weak due to progressive salt weathering ([Tichy et al., 2023](#)). Given the fragile state of the surfaces and the ongoing ingress of water and salts, a long-term treatment was chosen to use mineral poultices as a salt retention surface, aiming to mitigate the formation of salt efflorescence on the wall surfaces. Inorganic mineral poultices were selected, excluding any organic material, due to past incidents in the chapel following desalination treatments with cellulose-based wet poultices, which led to mold growth ([Piñar et al., 2009](#)). For this reason, three mineral poultices (sepiolite, kaolinite, and vermiculite) were applied for 1 year, and their salt retention capacity and resistance to salt weathering were evaluated.

The results of the qualitative and quantitative salt analyses of the three different mineral poultices on test area V2 confirmed their effectiveness in retaining the primary salt, halite (NaCl) ([Figure 2a](#)). However, the type of clay mineral significantly influenced the properties and performance of the poultices. The sepiolite-based poultice demonstrated the lowest performance on a permanently moist substrate with a highly porous substrate. It exhibited signs of salt-weathering after just 1 month of treatment. Maximum salt accumulation occurred at 6 months, but the total retained salt was considerably lower compared to the other poultices. A similar trend was observed in the poultice with kaolinite content, where sodium and chloride were primarily stored during the initial 6 months, and an accumulation of sulfate became evident only after 12 months. Finally, the storage capacity of the vermiculite poultice showed to be comparable to that of the kaolinite poultice, which was previously reported for its extraction efficiency ([Sawdy et al., 2010](#)). However, the vermiculite poultice showed a steady increase in stored ions throughout the 12-month treatment period ([Figure 2a](#)), with nitrate retention peaking after just 1 month.

Regarding resistance to salt-weathering, both vermiculite- and kaolinite-base poultices showed salt efflorescences accumulating on their surfaces after 6 months. However, while the vermiculite poultice maintained structural integrity, the kaolinite poultice exhibited structural damage. In summary, vermiculite showed the best performance in salt retention, likely due to its high ion-exchange capacity ([Verges-Belmin and Siedel, 2005](#)), and demonstrated the highest resistance to salt-induced degradation over the treatment period in this location.

In summary, the application of the poultices at this site proved to be successful in retaining the main weathering salt, namely halite, as well as in displacing the formation of salt efflorescence to the surface of the poultices, thus preventing its formation on the wall surfaces. However, the observed changes in the salt composition in all tested areas of V2 after treatment, regardless of the poultice used, require careful interpretation. While the reduction of sodium chloride on the wall surfaces through accumulation in the poultices is a positive outcome, the increase in magnesium, calcium and potassium ions, along with the rise in nitrate and sulfate ions can lead to new type of salt damage, the long-term effects of which remain uncertain.

These results underline the limitations of mineral poultices as a protective measure. Their effectiveness is strongly dependent on the type and concentration of salts, as well as environmental factors such as moisture ingress and substrate porosity. As shown in area V2, structural degradation of some poultice types began within a few

months, limiting their long-term serviceability. Although vermiculite exhibited promising performance in both salt retention and mechanical stability, its protective capacity is temporary and contingent upon controlled environmental conditions. Consequently, in this location, the application of mineral poultices should be restricted to short- to medium-term interventions, ideally not exceeding 12 months, and should be combined with regular monitoring and timely removal. Their role as a protective system is therefore context-dependent and should not be generalized without a thorough site-specific evaluation.

4.1.2 Effect of mineral poultices on the microbial community V2

As shown in the previous section, and in our previous work (Tichy et al., 2023), the St. Virgil Chapel is dominated by salt efflorescence composed mostly of halite (sodium and chloride ions, Figure 2a). Treatment with the mineral poultices mitigated the weathering damage of halite to the walls, but at the same time altered the ion composition in the walls. On the one hand, anions (nitrates and sulfates) and, on the other hand, cations (potassium, calcium and magnesium) were drastically enriched up to 40 times in the case of potassium, while chlorides showed no significant change and sodium ions were reduced up to 61.2% (Supplementary Table S1). This led to the second objective of this study, which was to monitor how the application of the poultices, and the resulting ionic changes, quantitatively and qualitatively affected the microbial community established in this hypersaline environment.

The first effect observed in terms of the size of the bacterial community, based on the qPCR results (Supplementary Figure S2) was a significant quantitative reduction after the application of all three mineral poultices. At this point it is important to mention that we are aware of the biases that can be introduced in qPCR analyses by dealing with DNA extracts from environmental samples and further amplification using 16S rDNA targeted primers. Therefore, interpretation of the results must be done carefully, considering this approach only as semi-quantitative, due to the problematic of 16S rDNA copy number variation, amplicon length and matrix effects. To similar problematics in environmental metataxonomic approaches we refer to (Whitman et al., 2018). Taking the aforementioned considerations into account, these results show that the mineral poultices were able to reduce the bacterial population size in the associated biofilm in V2.

Diversity indices indicated a reduction in the bacterial biodiversity after treatment, with the effect exerted by the sepiolite poultice being less marked (Figure 6a). The application of the poultices also affected the relative proportions of the different members of the bacterial community. The native community was characterized by four main genera 4, namely *Aliifodiniibius* (*A. roseus*), *Marinobacter* (*M. salarius*), *Methylohalomonas* (*M. lacus*), and *Salinisphaera* (*S. aquimarina*), in addition to other microorganisms well adapted to hypersaline conditions, such as members of the *Halomonas*. After the desalination treatment the genera *Methylohalomonas* and *Salinisphaera* were completely eliminated from the community colonizing the walls (Figure 3a; Supplementary Figure S3A), while *Aliifodiniibius roseus* was able to increase its relative abundance only on the surface treated with vermiculite but decreased its proportion in the areas desalinated with sepiolite and kaolinite. Finally, the genus *Marinobacter*, in addition to *Halomonas*, showed a decrease in their relative proportions, showing

in general that all these dominant genera were affected by ion displacement on the treated walls. Even if the main salt on the walls after treatment was still halite (NaCl), in particular, the K^+ concentration after treatment turned out to be higher (up to 40 times). As described in depth in Tichy et al. (2023), the relative composition of microbial communities in hypersaline environments can differ as a function of the concentration of individual ions, such as the presence/absence of K^+ . The increase in K^+ ions clearly leads to a shift in the structure of the community from species that are able to tolerate high salt concentrations by favoring the synthesis or uptake of compatible solutes, such as *Halomonas* and *Marinobacter*, and hence their decrease, toward those that favor the “salt-in” strategy, i.e., that transport the available K^+ into the cells.

On the contrary, some genera present on the non-treated surfaces with relative low proportions, such as the genera *Bacillus*, *Metabacillus*, and *Paenibacillus* (Figure 3a), in addition to others that were below the detection limit prior treatment, namely *Alkalihalobacillus*, *Cytobacillus*, *Neobacillus*, *Paenisporosarcina*, *Peribacillus*, and *Virgibacillus*, increased in their relative proportion, or emerged, in some of the treated areas, especially in the sepiolite treated one. This group of enriched microorganisms was found to belong to the phylum *Bacillota*. The same was observed for another genus belonging to the phylum *Actinomycetota*, namely *Saccharopolyspora*. All of them share the common capacity of sporulation (Yumoto et al., 2003; Heyrman et al., 2004; Heyrman, 2005; Yoon et al., 2005; Yoon and Oh, 2005; Niederberger et al., 2009; Tang et al., 2009; Shi et al., 2011; Amoozegar et al., 2013; Reddy et al., 2013; Patel and Gupta, 2020). One plausible reason why some of these genera were not detected on the non-treated surface is probably due to the fact that they were present in spore form, which increases the difficulty for DNA extraction significantly (Dineen et al., 2010). The increase in their relative abundance after the treatment may consequently be due to spore germination, following the observed ionic shift. It has been reported that spore germination is possible in hypersaline environments, as in the case within this monitoring, in which highly saline conditions persisted during and after the treatment. *In vitro* experiments have shown germination of *Bacillus* strains even with 4.8 M NaCl, due to a missing environmental salt sensing mechanism (Nagler et al., 2014). The higher relative abundances of spore-forming microorganisms, mainly at the sepiolite-treated surfaces in V2, may be due to the aforementioned ionic shift. Indeed, it has been proven that under higher saline conditions, cations play a more important role than anions in inhibiting germination (Nagler and Moeller, 2015). Moreover, it was observed that the area treated with sepiolite showed lower values of cations (sodium and potassium) than the areas treated with kaolinite and vermiculite (Figure 2a). These cations (sodium and potassium) and their homeostasis play an important role during germination, especially in the intrinsic outflow and following energy dependent reimport of potassium (Swerdlow et al., 1981). Furthermore, during the germination process, osmoprotectants are imported into the spore, also including the de-novo synthesis of osmolytes (Nagler et al., 2016). These osmoprotectants might be provided by the native microbial community present at the tested area in V2, which contains microorganisms capable of synthesizing osmolytes, as shown in Tichy et al. (2023). However, due to the fact that the St. Virgil Chapel is a hypogean environment, osmolyte precursors may also be supplied externally, through the process of degradation of imported biogenic residues (Boch et al., 1994).

An enrichment in spore-forming bacteria, especially of the phylum *Bacillota*, might have long-term implications. In this context, the enormous biodiversity of the genus *Bacillus* comprises members that show great versatility in the production of secondary metabolites. These compounds are known for their natural antagonistic effects on other potential biodeteriogenic invaders, including fungi and bacteria (Caldeira, 2021). In addition, *Bacillus* species are known for their ability to precipitate CaCO_3 (Jroundi et al., 2021). Due to the high salinity conditions at sampling location V2, and the fact that some carbonatogenic *Bacillus* species have been shown to be halotolerant (Jroundi et al., 2017; Saiz-Jimenez and Laiz, 2000), leads to the possible expectation, that *Bacillus* enrichment at this site does not have to be considered as a biodeterioration risk, but even as a positive effect that could contribute to the natural consolidation of the surface, thus being favorable in the restoration treatment.

A reason why the “spore-forming community” did not show to be as dominant in the areas treated with kaolinite and vermiculite might be related to the introduction of a different community dynamic through the presence of a microorganism with predatory behavior, namely *Persicimonas caeni* (Wang et al., 2020), which was already present in a moderate proportion on the V2 surface prior the treatment (3.4%) and enriched in the kaolinite- and vermiculite-treated areas, but not in the sepiolite (Figure 3a). *Persicimonas caeni* shows bacterivory, i.e., the ability to feed on prokaryotic cells, as possible feeding strategy related to the colonization of saline habitats (Mu et al., 2020). Simultaneously *Aliifodinibius roseus* doubled its relative abundance from 19.8 to 35.4% in the presence of this predator. This co-occurrence, on the level of Bradymonadaceae, was also recently reported in hypersaline soil samples (Vera-Gargallo et al., 2023). A possible explanation for this co-occurrence would be either a possible mutualistic halophilic survival strategy or that the prey–predator relationship is in favor of *Aliifodinibius*, as described elsewhere (Gong et al., 2022; Wang et al., 2024). The strong oscillations within the kaolinite and vermiculite mineral poultice, due to the presence of *Persicimonas caeni*, could be related to the “kill-the-winner” model, indicating that predatory fitness is reciprocally linked to the relative abundance of these microorganisms (Cohen et al., 2021). Since prey shape predator behavior (Chen et al., 2011), it has also been reported that intraspecific diversity might increase within communities due to predation (Nair and Velicer, 2021). However, this fact could not be reported in our study, where bacterial diversity indices of kaolinite- and vermiculite-treated surfaces in V2 presented lower numbers compared to untreated areas (Figure 6a). These results suggest that the enrichment of a predator in halophilic/halotolerant communities may present an interesting potential for mitigating certain community members and opens up future potential for shaping diversity indices within these communities on cultural heritage monument surfaces.

Finally, in the wall area treated with kaolinite, sulfate ions reached the highest concentration (Figure 2a). This coincided with a strong increase of *Rubrobacter braccarensis*, from 0.6% in the native community to 7.2% in the treated one. A possible explanation for the enrichment of the treated surface by the genus *Rubrobacter* and co-occurrence of extended amounts of sulfates will be given in detail in the section (4.2.2, sampling location M4).

Concerning the genera that were identified exclusively or mostly within the mineral poultices (Figure 3a), the most noteworthy was the presence of the genus *Acinetobacter* within all three mineral clays, especially in the sepiolite one. In the sepiolite mineral clay, *Acinetobacter*

lwoffii and *johnsonii* showed together a relative abundance of more than 25% during the whole sampling campaign (Supplementary Figure S3A). A wide repertoire of functional characteristics concerning desiccation (Zeidler and Müller, 2019) and osmoregulation (Breisch et al., 2019; Zhao et al., 2023) was reported for this ubiquitous species of *Acinetobacter* (Bouvet and Grimont, 1986). Moreover, *Acinetobacter* has been reported several times in the field of cultural heritage, related to surfaces treatments (Jurado et al., 2010; Jroundi et al., 2017; Skipper et al., 2022). However, throughout the treatment, it was observed that the sepiolite poultice harbored a greater number of genera and species that were only detected specifically within its matrix, such as the case of two organisms associated with the nitrogen cycle, *Herbaspirillum seropedicae* and *Noviherbaspirillum denitrificans*. *H. seropedicae* is capable of N_2 fixation (Baldani and Baldani, 1986) and shows differentiated osmoregulation strategies on the genetic level (Pedrosa et al., 2011), necessary for coping with the high salt concentrations in the mineral poultice (Supplementary Table S1). This species was also reported in other cultural heritage monuments (Haidău et al., 2022). Vice versa *N. denitrificans* shows denitrifying capabilities (Ishii et al., 2017). Other organisms present in lower proportion throughout the monitoring only within the sepiolite poultice were, *Arthrobacter*, *Lacisediminimonas*, *Massilia*, *Oxalicibacterium*, *Pseudomonas*, and *Pseudarthrobacter* (specifically *P. phenanthrenivorans*) (Busse and Schumann, 2019). Also, for that last organism, osmolyte production (Son et al., 2023) and the occurrence in the context of cultural heritage monuments was reported (Skipper et al., 2022).

The kaolinite and vermiculite matrices were mostly inhabited by three genera, namely *Marinobacter*, *Persicimonas* (*P. caeni*), and *Rubrobacter* (*R. braccarensis*) (Figure 3a; Supplementary Figure S3A). In these two poultices an interesting behavior was observed between the relative proportions of the predatory bacteria *P. caeni* and *Marinobacter* species, showing to be inversely related. Within the kaolinite mineral, a strong enrichment of the predatory bacteria was observed in the first 6 months of treatment, but their relative abundance was drastically reduced within the matrix 12 months after application (from 83.3 to 1.4%), related to a possible reduction of available prey or nutrients. This was observed in parallel with an increase in the proportion of *Marinobacter*, which is known to have a higher salt tolerance (Green et al., 2006; Shi et al., 2011; Ng et al., 2014) (Figure 3a; Supplementary Figure S3A). In contrast, in the vermiculite poultice, a strong enrichment of *Marinobacter* species was observed in the first 6 months of treatment (78.6%), along with a relatively low proportion of *P. caeni* (2.1%), which experienced a very significant increase in its proportion (51.6%) combined with a massive decrease in *Marinobacter* species (13.7%) 12 months after application. The indicative susceptibility of the genus *Marinobacter* to bacterivory was already reported elsewhere (Selivanova et al., 2024), in addition to the ability to carry predatory tools at the genetic level itself (Evans et al., 2018). This ambivalence opens an interesting topic for further research on the ability of *Marinobacter* species to act as prey or predator in halophilic environments. Furthermore, the genus *Marinobacter* has already been detected in cultural heritage hypogean environments, linked to the close sea side (Jurado et al., 2022).

In line with what was observed in the bacterial population, the archaeal community also showed a decrease in population after treatment with the mineral poultices in the St. Virgil chapel, especially in the areas treated with the sepiolite and kaolinite mineral clays, based on the qPCR results (Supplementary Figure S2;

Supplementary Table S2). However, the ionic shift appeared to have a minor effect on the archaeal community structure. This community was before and after the desalination treatment highly dominated by the genus *Halococcus* (Figure 3b). This might be linked to the still favorable saline conditions, especially the high concentrations of sodium, potassium and chloride ions persisting after treatment (Supplementary Table S1). The high frequency of different *Halococcus* species within the sampling location V2 could lead to the result of an archaeal sympatric community structure, due to the assumption that these *Halococci* strains prefer similar environmental halophilic conditions. After the treatment with the sepiolite poultice, the detected *Halococcus* species were identical before/after the treatment, but with some variations in their proportions, showing a general increase in the proportion of *H. dombrowskii* and a decrease of *H. salidifoniae* and *H. gingdaonensis*, whereas the areas treated with kaolinite and vermiculite showed more evident community structure shifts (Supplementary Figure S3B). In contrast to what was observed after treatment with the aforementioned poultice, the relative proportion of *H. dombrowskii* decreased after treatment with kaolinite and slightly increased for vermiculite, and the proportions of other three species gained more importance at that area. This was the case of *Halobacterium hubeiense* with the need for NaCl in the range of 15–30% (w/v) and able to utilize many different carbon and energy sources (León et al., 2024), in addition of *Halobacterium* sp. DL1, which is primarily sustained by amino acids (Williams et al., 2014) and *Salarchaeum japonicum*, which similarly does not metabolize sugars (Shimane et al., 2011; Supplementary Figure S3B). Thus, the increase in protein-feeding archaeal species may be related in this treated area to the large increase in the predatory bacterium *Persicimonas caeni*, which could give rise to a new ecological niche colonized by halophilic archaea that feed preferentially on amino acids released after bacterial predation, as has also been described elsewhere (Tschitschko et al., 2016). The fact of these reported protein feeding strategies should be taken as warning, especially concerning the degradation of proteinaceous binders by microorganisms (Sterflinger and Piñar, 2013; Wu et al., 2022). These results highlight the importance of not investigating bacteria and archaea in halophilic environments individually, but simultaneously.

Concerning the genera that were mostly identified within the mineral clay matrices, these were the same genera that were dominant on the non-treated surface (*Halococcus* species), as well as those enriched throughout the treatment, such as *Halobacterium* and *Salarchaeum* in the vermiculite matrix. In addition, other genera, such as *Haladaptus*, *Halarchaeum*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halorientalis*, and *Haloterrigena* sporadically emerged in some of the poultice matrices, with relatively low proportions (below 4%), pointing to a slight increase in diversity in the archaeal community during and after treatment.

4.2 Long-term monitoring at the Charterhouse Mauerbach

4.2.1 Effectiveness of desalination treatment M4

In the test area M4, the primary weathering salt contaminating the surfaces was calcium sulfate, as previously outlined. Although its concentration was low, and salt efflorescences were not evident on the surfaces, it was decided to treat them with the same mineral poultices and for the same duration to allow for comparison. This decision was

also based on the known potential of sulfates to cause significant deterioration of inorganic building materials (Lubelli et al., 2018). The different clay mixtures showed varying preferences in terms of extraction and accumulation of different ions. The sepiolite-based poultice demonstrated the highest ion accumulation after 1 month, with a predominance of nitrate, calcium and magnesium ions, compared to subsequent sampling points. Sulfate ions attained their maximum level after 6 months, while chloride ions required 12 months to reach their peak (Figure 2b). The kaolinite poultice demonstrated the best overall performance, reaching its maximum salt concentration after 6 months. It preferentially accumulated potassium and sodium ions in addition to calcium and sulfate. Nitrate accumulation, as in the sepiolite poultice, was highest after 1 month (Figure 2b). The vermiculite-based poultice exhibited suboptimal outcomes, with overall salt extraction levels lower than those of the other two mixtures (Figure 2b). However, the poultice demonstrated a selective extraction of potassium and magnesium ions.

At the end of the treatment, all treated surfaces in M4 exhibited an increase in calcium and sulfate ions, as well as magnesium, compared to untreated areas (Supplementary Table S1; Figure 2b). However, the area treated with the sepiolite poultice displayed the lowest accumulation of these ions relative to the other two poultices (Figure 2b). Overall, the long-term application of mineral poultices in M4, where no initial salt efflorescence damage was present, resulted in an unfavorable outcome, with higher ion concentrations accumulating on the architectural surfaces after treatment. Consequently, the treatment did not contribute to surface consolidation and raised concerns regarding potential ionic mobilization within the construction material (Verges-Belmin and Siedel, 2005).

These observations highlight important limitations of the material. In environments without visible efflorescence or with low initial salt loads, mineral poultices may not only be ineffective but could potentially redistribute salts into the substrate, as seen in M4. This suggests that their suitability as a protective system is limited to applications where active salt transport and surface crystallization are evident.

4.2.2 Effect of mineral poultices on the microbial community M4

The sampling location M4 in the Charterhouse Mauerbach exhibits a unique attribute, the very low salt concentration (mainly calcium sulfate) prior the desalination treatment and its increase after the treatment with mineral poultices, as mentioned in the section 4.2.1. Therefore, it was interesting to evaluate how the increase in this weathering salt, as well as in other ions, affects the colonizing microbial community in both qualitative and quantitative terms.

The first observation was a significant increase in the size of the bacterial community, based on the qPCR results (Supplementary Figure S2), which showed that the Ct-values decreased significantly for the treatment with sepiolite, kaolinite and vermiculite (Supplementary Figure S2; Supplementary Table S2). Regarding the effects of the increased concentration of ions on the structure of the colonizing community at this site, prior treatment, the surfaces were strictly colonized by mainly one organism of the genus *Rubrobacter*. After the application of the mineral poultices, irrespective of their mineral composition, the relative abundance of this genus was even higher (Figure 4), being correlated with the increase in sulfate,

and possibly magnesium, in all treated surfaces (Figure 2b). This is also in accordance with the diversity indices calculated for this area, which showed to be lower after the treatment (Figure 6b). A plausible explanation for the correlation between the increase in sulfate and the relative proportion of *Rubrobacter* might be the reported genetic capability of members of this genus to mineralize sulfates through the use of extracellular arylsulfatases (ARS) and choline-sulfatases (Miralles et al., 2023). Furthermore, the *in vitro* growth medium for this organism has been described as needing to be supplemented with MgSO₄ (Jurado et al., 2012).

The strong enrichment of *Rubrobacter* in this treated area may also be due to the fact that the mobilized sulfate ions limit the growth potential of other members of the community, even with higher water availability than chloride, as reported for *B. subtilis*, which is able to grow in simple saline brines containing more chloride ions than sulfate ions (Stevens and Cockell, 2020). The even greater presence of members of the genus *Rubrobacter* after treatment implies several negative aspects, such as increased biofilm formation and pink coloration on surfaces, two previously reported capabilities of this bacterium (Tichy et al., 2023), which could represent an increased risk of biodeterioration. Since this organism has been repeatedly detected in previous studies approaching the similar phenomenon of pinkish discoloration of cultural heritage in disparate geographic locations, (Schabereiter-Gurtner et al., 2001; Imperi et al., 2007; Laiz et al., 2009; Piñar et al., 2009; Basile et al., 2025), *Rubrobacter* is still a very interesting target for further scientific research concerning the osmoregulation and desiccation-resistance mechanisms in halophilic environments, especially in those with sulfate dominance.

Regarding the microorganisms detected within all three mineral poultices, and sporadically also on the treated walls, the most abundant genus was *Acinetobacter*, being the most frequently detected species *Acinetobacter lwoffii* and *johnsonii*. Interestingly, it was also within the sepiolite clay where the highest abundance of this genus was recorded, similar to that reported for testing site V2 (Figure 4; Supplementary Figure S4). In addition, another microorganism was detected in all three poultices, but not on the surfaces itself, *Prolinoborus fasciculus*. The detection of that species needs to be carefully taken into account, because the original publication reports no tolerance toward NaCl (Pot et al., 1992). Moreover, there was strong scientific effort and research toward its reclassification, leading to a possible re-introduction as *A. lwoffii* (Glaeser et al., 2020), but ended up as officially rejected (Arahal et al., 2023). The same applies for the genera *Arthrobacter* and *Pseudarthrobacter*, with relative abundance below 2.3% on the treated surfaces of vermiculite and kaolinite, and detected within all mineral clays in different proportions. Both genera have been detected extensively on cultural heritage monuments, (Laiz et al., 2000; Gutarowska et al., 2015; Chimienti et al., 2016; Tescari et al., 2018a; Rosado et al., 2020; Pyzik et al., 2021; Liu et al., 2023). The genus *Arthrobacter* and specifically *Arthrobacter* sp. PGP41, has been reported to have the capability of nitrogen fixation (Xu et al., 2018), osmolyte production based on sugars as substrate (Han et al., 2021), and the possibility of metabolizing sulfurized organic compounds (Kaur et al., 2022), which might be related to the high amount of sulfates through the whole monitoring in the sampling location M4 (Supplementary Table S1). Additional genera detected within all mineral clays were *Massilia*, with the species *Massilia agri*, which tolerates less NaCl (0–2% w/v) (Chaudhary and Kim, 2017), and *Pseudomonas*, with the species

Pseudomonas stutzeri. This last species is well-known not only for its capability to remove nitrate salts, but also for its wide repertoire of survival mechanisms in halophilic environments, extensively reported elsewhere (Bosch-Roig et al., 2016). Furthermore, *P. stutzeri* was also used, inoculated artificially on sepiolite poultices as carrier system, for the nitrate salt removal on cultural heritage monument surfaces (Ranalli and Zanardini, 2021).

Other genera appeared sporadically in one or the other clay, such as *Delftia tsuruhatensis* (Shigematsu et al., 2003), with a reported salinity tolerance based upon NaCl up to 4% (w/v) (Li et al., 2015), and detected on porous limestone on cultural heritage monuments (Jroundi et al., 2010). Furthermore, two genera, *Herbaspirillum* and *Noviherbaspirillum*, which comprise species related to the nitrogen cycle, such as *H. seropedicae* and *N. denitrificans*, were detected within the sepiolite and kaolinite clays (Figure 4; Supplementary Figure S4). These genera were also detected in the V2 testing site, but only within the sepiolite clay.

Interestingly some genera were only detected within the vermiculite clay, two *Paracoccus* species. One of them, *P. marcusii*, is able to tolerate NaCl up to 6% (w/v) (Harker et al., 1998) and described already on inorganic cultural heritage monuments surfaces (Ettenauer et al., 2014). The second, *P. sp. Arc7-R13* (Hollensteiner et al., 2023), was classified as a *P. marcusii* strain, both showing different tools on a genetic level to tolerate high salt loads (Leinberger et al., 2021). Interestingly, it was also reported for *P. marcusii*, that the formation of biofilms by this species on mortar surfaces could be a non-permeable barrier for salt ions (Lv et al., 2015). To our knowledge, this is the first report of this species in the context of a desalination treatment of surfaces affected by salt-weathering in the field of cultural heritage. The fact that this species may contribute to making an impermeable barrier for ions by forming part of a biofilm, opens the unresolved question for future research as to whether microbial biofilms could protect treated surfaces against future salt-weathering.

The genera *Sphingobium*, *Sphingopyxis*, and *Yonghaparkia* were mainly detected within the vermiculite matrix. *Sphingobium yanoikuyae*, a species detected in this study, is able to tolerate 3%, (w/v) of salt (Lee et al., 2019) and it has been reported to feed on sulphonated compounds (Aylward et al., 2013). This might be linked to relatively high amounts of sulfates present (Supplementary Table S1). Members of *Sphingopyxis* have shown the potential for heterotrophic nitrification and aerobic denitrification when using ammonium, nitrate or nitrite as the sole nitrogen source and exhibit a salinity tolerance of up to 5%. Species of this genus are able to regulate osmotic pressure and survive in high salinity environments by increasing metabolic levels of sucrose and D-tagatose. All these features make the members of this genus to be considered as excellent candidates for nitrogen removal treatment in saline environments (Chen et al., 2022). Last but not least *Yonghaparkia alkaliphila* tolerates up to 7% salt (tested as NaCl (w/v)) (Xie et al., 2021), and interestingly, elevated CO₂ levels might be necessary for growth (Ueda et al., 2008).

Considering that the architectural surface under the mineral poultice is made of lime putty (CaCO₃), as previously reported (Tichy et al., 2023), it could be hypothesized that the application of vermiculite on a calcium carbonate surface together with atmospheric CO₂ and water present would at first become acidic (Kaufmann and Dreybrodt, 2007), and then the pH will be buffered by the vermiculite poultice toward neutral end even slightly alkaline, as reported for deionized water with pH 5.95 (Duman and Tunc, 2008; Indrasumunar

and Gresshoff, 2013). This would possibly explain the colonization of *Yonghaparkia alkaliphila* capable of handling an alkaline pH up to 9 (Yoon et al., 2006).

4.2.3 Effectiveness of desalination treatment M6

As mentioned in the results section, test area M6 was characterized by a high concentration of sulfates, in addition to sodium and potassium, and with a minor presence of calcium ions. Salt efflorescences, mainly of thenardite (Na_2SO_4) (Tichy et al., 2023) were visible over the entire surface of this site. Studies have shown that Na_2SO_4 is one of the most destructive salt for sedimentary rocks and granites (Goudie et al., 1970; Kwaad, 1970). Additionally, this was investigated through the usage of saturated solutions of Na_2SO_4 , CaSO_4 , NaCl , and NaNO_3 , including a mixture of sea salts, evaluating the resistance of sandstones and slate (Cooke, 1979; Lubelli et al., 2018).

The results of this monitoring study showed that the kaolinite-based poultice demonstrated the highest efficiency in removing the dominant SO_4^{2-} , Na^+ and K^+ ions, while the vermiculite-based poultice was less effective in retaining these ions (Supplementary Figure S1). The treatment in test area M6 was the most successful in this long-term study, showing that all clay poultices reached maximum salt accumulation after 12 months and achieved an almost complete reduction of weathering salts on the treated surfaces compared to untreated areas. Overall, the kaolinite-based poultice showed the best performance, followed by the vermiculite-based one. However, the possible side effect observed after treatment in M6 was a slight increase of magnesium ions on the treated surface (Supplementary Table S1). Although this could be potentially problematic and warrants further monitoring, the extent of damage associated with magnesium sulfates, as well as potential testing biases, remains under discussion (Lubelli et al., 2018).

These findings highlight both the potential and the limitations of mineral poultices as protective treatments. While the long-term application in M6 successfully reduced the concentration of damaging salts, the duration of effectiveness appears limited to the period of application, which in this study was 12 months. The performance varied notably depending on the specific clay mineral, environmental conditions, and salt composition.

4.2.4 Effect of mineral poultices on the microbial community M6

As mentioned in the previous section 4.2.3, the application of mineral poultices at test site M6 of the Charterhouse Mauerbach showed very promising results in terms of mitigation or even total removal of sulfates. Therefore, it was also interesting to know whether this treatment could also mitigate the biofilm associated with the efflorescence and how the ions shift would affect the community structure colonizing the treated surfaces.

The semi-quantitative qPCR analysis showed that the bacterial community did indeed undergo a reduction in size after 12 months of treatment on the surfaces treated with sepiolite and kaolinite, but no significant reduction on the surface treated with vermiculite (Supplementary Table S2). However, the diversity indices indicated a significant increase in bacterial biodiversity after treatment, irrespective of the mineral clay used (Figure 6c). Concerning the bacterial community structure, prior the desalination treatment the sampling location M6 was strongly dominated by one bacterial genus,

namely *Haloechinothrix* (with over 98% relative proportion) (Figure 5a). Interestingly, after the treatment, the abundance of *Haloechinothrix*, with the single detected species *H. alba* (Supplementary Figure S5A) decreased to levels between 67.1 and 79.3%, depending on the mineral clay applied (see Figure 5a). Conversely, the number of bacterial community members and their associated relative abundance including the genus *Nesterenkonia*, (mainly *N. xinjiangensis*), as well as of the genus *Saccharomonospora* (*S. halophila*) and *Amycolaptosis* (*A. albispora* and *A. xuchangensis*), the last two being under the detection limit in the untreated walls, increased in all treated areas (Figure 5a; Supplementary Figure S5). According to their original description *Haloechinothrix alba* tolerates 9–23% (w/v) salt (tested with NaCl) and a pH range of 4.0–8.0 (Tang et al., 2008), whereas *Nesterenkonia xinjiangensis* present a saline tolerance of 0–25% (w/v) but a tolerance to pH 7.0–12.0 (Li et al., 2004). In addition, the other emerging species such as *S. halophila*, which was isolated from soil, tolerate 10–30% NaCl and a pH range of pH 6.0–11.0 (Al-Zarban et al., 2002) and *A. albispora*, originally isolated from deep-sea sediments, has been reported to tolerate 0–8% (w/v) salinity and pH range of 5.0–11.0 (Zhang et al., 2016). The results show that all emerging species tolerated similar salt levels to the dominant *Haloechinothrix* species, but significantly higher pH thresholds, indicating an adaptation to a more alkaline environment. As mentioned before for the M4 site, through the action of the mineral poultices on a calcium carbonate surface could raise the pH in the poultice and also transiently on the treated surfaces.

Similar to what was observed in site M4, the main genera found within some or all mineral poultices, and sporadically in low proportions in the treated surfaces were, *Acinetobacter*, *Arthrobacter*, *Herbaspirillum*, *Massilia*, *Noviherbaspirillum*, *Paracoccus*, *Pseudarthrobacter*, and *Pseudomonas*, besides the dominant genus *Haloechinothrix* (Figure 5a). The characteristics of these bacteria found predominantly within the mineral clays have already been explained in the paragraph 4.2.2. Consequently, it is observed that the poultices with different mixtures of sepiolite, kaolinite or vermiculite tend to contribute to and enrich a domestic microbial community specific to each clay mineral, considering that the desalination poultices were not sterilized before use, and therefore carried their own microbial community *in situ*.

Regarding the archaeal community, the ion shift produced a significant increase in this population in all treated areas (Supplementary Figure S2), denoted by the marked decrease in Ct values obtained by qPCR analysis after treatment. However, neither the biodiversity (Figure 6c) nor the structure of this community was significantly affected. The archaeal community was shown to be dominated before, during (within the poultices), and after the desalination treatment by a single species, *Halalkalicoccus paucihalophilus*, which is able to survive between 5 and 35% (w/v) NaCl and a pH range of 6.0–8.5 (Liu et al., 2013).

Although the soluble amount of sulfate was successfully removed during treatment with the mineral poultices, there was an additional source of sulfate on the non-treated M6 surfaces, namely syngenite, which are almost insoluble in water [solubility product of -7.2] (Lothenbach and Winnefeld, 2006) (Supplementary Table S3). The formation and occurrence of syngenite on calcareous surfaces of cultural heritage has already been described (Matović et al., 2012). Taking the possible need of *Halalkalicoccus paucihalophilus* for sulfates into account (according to the isolation region of this organism rich in sulfates; Salt

Lake Lop Nur; Liu et al., 2013; Xiyu, 1987), syngenite can function as hidden depot for that required mineral becoming available through biomineralization, but more scientific effort is needed to demonstrate this. In general, the shift after treatment toward microbial community members with higher pH-tolerant capacities in combination with a relatively high amount of free Ca^{2+} ions ($16.8\text{--}23.5\text{ }\mu\text{g}/\text{cm}^2$) indicates a possible increase in alkalinity. In addition, calcium ions have a positive effect on archaeal cell aggregation (Kawakami et al., 2007). To conclude, the sulfate load was removed from the walls and the microbial communities of the biofilm were partially exchanged (bacteria against archaea), resulting in a community possibly more flexibly adapted to high alkalinity and salt fluctuations than the original community.

5 Conclusion

This study shows one of the few existing examples of long-term monitoring of a desalination treatment, evaluating not only salt retention capacities but also the effects of the ionic changes on microbial communities colonizing the treated surfaces. Our results show a clear influence of the type and initial concentration of weathering salts on the performance of the clay mineral used. The three monitored locations exhibited distinct scenarios, highlighting the importance of comprehensive treatment approaches.

In V2, originally dominated by halite, the treatment outcome was gradually positive. The NaCl concentration remained stable after treatment, and its weathering effect on the surfaces was mitigated by its accumulation within and on the poultrices. However, an increase in other ions altered the microbial community composition. qPCR analysis showed a reduction in both bacterial and archaeal biofilms Ct values, while metataxonomic data indicated a partial decline in bacterial biodiversity, particularly in NaCl-adapted genera, alongside an increase in halotolerant and/or spore-forming microorganisms that thrived with the rise in K^+ , Ca^{2+} , and Mg^{2+} or the decrease in Na^+ . Among the tested poultrices, the vermiculite-based one exhibited the best salt retention capacity and the highest resistance to salt weathering over the treatment period.

In M4, the treatment yielded the least favorable outcome. In this area, which initially had a low concentration of CaSO_4 , the poultrices facilitated the mobilization and accumulation of salts on the wall surfaces, leading to increased concentrations of SO_4^{2-} , K^+ , Ca^{2+} , and Mg^{2+} after treatment. This promoted further enrichment of the dominant genus *Rubrobacter*, resulting in reduced microbial biodiversity in the biofilm. The kaolinite-based poultice demonstrated the best performance in terms of salt retention, whereas the sepiolite-based poultice caused the least accumulation of ions on the treated surface.

In M6, the treatment was the most successful. A significant reduction in SO_4^{2-} , K^+ , and Na^+ on the treated surfaces led to an increase in microbial biodiversity. The newly emerging microbial members were possibly better adapted toward a more alkaline environment, likely due to Ca^{2+} solubilization. Overall, the treatment decreased bacterial community size while increasing archaeal populations, which were more suited to the altered conditions. In this case, the kaolinite-based poultice showed the best performance in terms of salt reduction capabilities.

In summary, our findings indicate that no desalination treatment should be applied without prior assessment of the surface properties, salt composition, and colonizing microbial community. Several factors

must be considered before selecting a clay material, including the physical surface conditions and the concentration and chemical composition of the salts present. Furthermore, microbial monitoring should accompany these treatments to assess how changes in the saline environment affect microbial communities, potentially favoring specific microbial groups within biofilms. Additionally, this study underscores the importance of conducting preliminary tests on localized areas before implementing large-scale treatments on historic surfaces. Finally, it is essential to report unsatisfactory results, as these contribute to informed decision-making regarding the use of specific clay materials in future conservation efforts based on empirical data.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/>, PRJNA1242577.

Author contributions

JT: Investigation, Writing – original draft, Methodology, Writing – review & editing. BS: Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing, Methodology. MO: Data curation, Investigation, Writing – review & editing. LF: Data curation, Investigation, Writing – review & editing. MW: Data curation, Writing – review & editing. AG: Data curation, Investigation, Supervision, Writing – review & editing, Methodology. KS: Conceptualization, Supervision, Writing – review & editing, Resources. GP: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing, Project administration.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the Project “Heritage_2020-005_RESTOROMIC” of the Austrian Academy of Sciences (ÖAW).

Acknowledgments

The authors are grateful to Astrid Huber and Peter Hunger of the Charterhouse Mauerbach (BDA) and to Elisabeth Graff of the Wien Museum for the permissions granted to investigate these historic buildings and for their availability and support throughout this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations,

or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1603289/full#supplementary-material>

References

- Adamiak, J., Otlewska, A., and Gutarowska, B. (2015). Halophilic microbial communities in deteriorated buildings. *World J Microbiol Biotechnol* 31, 1489–1499. doi: 10.1007/s11274-015-1913-3
- Al-Zarban, S. S., Al-Musallam, A. A., Abbas, I., Stackebrandt, E., and Kroppenstedt, R. M. (2002). *Saccharomonospora halophila* sp. nov., a novel halophilic actinomycete isolated from marsh soil in Kuwait. *Int. J. Syst. Evol. Microbiol.* 52, 555–558. doi: 10.1099/00207713-52-2-555
- Amoozegar, M. A., Didari, M., Bagheri, M., Fazeli, S. A. S., Schumann, P., Spröer, C., et al. (2013). *Bacillus salsus* sp. nov., a halophilic bacterium from a hypersaline lake. *Int. J. Syst. Evol. Microbiol.* 63, 3324–3329. doi: 10.1099/ijso.0.050120-0
- Andrews, S. *FastQC: a quality control tool for high throughput sequence data*. (2010). Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- Arahall, D. R., Bull, C. T., Busse, H.-J., Christensen, H., Chuvochina, M., Dedysh, S. N., et al. (2023). Judicial Opinions 123–127. *Int. J. Syst. Evol. Microbiol.* 72. doi: 10.1099/ijsem.0.005708
- Arnold, A., and Zehnder, K. (1991). "Monitoring wall paintings affected by soluble salts" in *The Conservation of Wall Paintings*, 103–136.
- Aylward, F. O., McDonald, B. R., Adams, S. M., Valenzuela, A., Schmidt, R. A., Goodwin, L. A., et al. (2013). Comparison of 26 Sphingomonad genomes reveals diverse environmental adaptations and biodegradative capabilities. *Appl. Environ. Microbiol.* 79, 3724–3733. doi: 10.1128/AEM.00518-13
- Baldani, I., and Baldani, V. L. D. (1986). Characterization of *Herbaspirillum seropedicae* gen. nov. sp. nov. a Root-Associated Nitrogen-Fixing Bacterium. *Int. J. Syst. Bacteriol.* 36, 86–93. doi: 10.1099/00207713-36-1-86
- Basile, A., Riggio, F. P., Tescari, M., Chebbi, A., Sodo, A., Bartoli, F., et al. (2025). Metagenome-resolved functional traits of *Rubrobacter* species implicated in rosy discoloration of ancient frescoes in two Georgian cathedrals. *Sci. Total Environ.* 958:178135. doi: 10.1016/j.scitotenv.2024.178135
- Bergaya, F., and Lagaly, G. (Eds.) (2013). *Developments in clay science*. 2nd Edn. Amsterdam: Elsevier.
- Boch, J., Kempf, B., and Bremer, E. (1994). Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J. Bacteriol.* 176, 5364–5371. doi: 10.1128/jb.176.17.5364-5371.1994
- Bosch-Roig, P., Decorosi, F., Giovannetti, L., Ranalli, G., and Viti, C. (2016). Connecting phenotype to genome in *Pseudomonas stutzeri* 5190: an artwork biocleaning bacterium. *Res. Microbiol.* 167, 757–765. doi: 10.1016/j.resmic.2016.09.003
- Bosch-Roig, P., Lustrato, G., Zanardini, E., and Ranalli, G. (2015). Biocleaning of cultural Heritage stone surfaces and frescoes: which delivery system can be the most appropriate? *Ann. Microbiol.* 65, 1227–1241. doi: 10.1007/s13213-014-0938-4
- Bouvet, P. J. M., and Grimont, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov. *Acinetobacter haemolyticus* sp. nov. *Acinetobacter johnsonii* sp. nov. and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwofii*. *Int. J. Syst. Bacteriol.* 36, 228–240. doi: 10.1099/00207713-36-2-228
- Breisch, J., Waclawska, I., and Averhoff, B. (2019). Identification and characterization of a carnitine transporter in *Acinetobacter baumannii*. *MicrobiologyOpen* 8:e00752. doi: 10.1002/mbo3.752
- Busse, H.-J., and Schumann, P. (2019). Reclassification of *Arthrobacter enclensis* as *Pseudarthrobacter enclensis* comb. nov., and emended descriptions of the genus *Pseudarthrobacter*, and the species *Pseudarthrobacter phenanthrenivorans* and *Pseudarthrobacter scleromae*. *Int. J. Syst. Evol. Microbiol.* 69, 3508–3511. doi: 10.1099/ijsem.0.003652
- Caldeira, A. T. (2021). "Green mitigation strategy for cultural Heritage using bacterial biocides" in *Microorganisms in the deterioration and preservation of cultural Heritage*. ed. E. Joseph (Cham: Springer International Publishing), 137–154.
- Charola, A. E. (2000). Salts in the deterioration of porous materials: an overview. *J. Am. Inst. Conserv.* 39, 327–343. doi: 10.1179/019713600806113176
- Charola, A. E., and Bläuer, C. (2015). Salts in masonry: an overview of the problem. *Restoration Build. Monuments* 21, 119–135. doi: 10.1515/rbm-2015-1005
- Chaudhary, D. K., and Kim, J. (2017). *Massilia Agri* sp. nov., isolated from reclaimed grassland soil. *Int. J. Syst. Evol. Microbiol.* 67, 2696–2703. doi: 10.1099/ijsem.0.002002
- Chen, H., Athar, R., Zheng, G., and Williams, H. N. (2011). Prey bacteria shape the community structure of their predators. *ISME J.* 5, 1314–1322. doi: 10.1038/ismej.2011.4
- Chen, P., Zhang, F., Zhang, L., Liu, H., Zhang, Q., Xing, Z., et al. (2022). Characterization of a novel salt-tolerant strain *Sphingopyxis* sp. CY-10 capable of heterotrophic nitrification and aerobic denitrification. *Bioresour. Technol.* 358:127353. doi: 10.1016/j.biortech.2022.127353
- Chimienti, G., Piredda, R., Pepe, G., van der Werf, I. D., Sabbatini, L., Crecchio, C., et al. (2016). Profile of microbial communities on carbonate stones of the medieval church of san Leonardo di Siponto (Italy) by Illumina-based deep sequencing. *Appl. Microbiol. Biotechnol.* 100, 8537–8548. doi: 10.1007/s00253-016-7656-8
- Cohen, Y., Pasternak, Z., Müller, S., Hübschmann, T., Schattenberg, F., Sivakala, K. K., et al. (2021). Community and single cell analyses reveal complex predatory interactions between bacteria in high diversity systems. *Nat. Commun.* 12:5481. doi: 10.1038/s41467-021-25824-9
- Cojoc, L. R., Enache, M. I., Neagu, S. E., Lungulescu, M., Setnescu, R., Ruginescu, R., et al. (2019). Carotenoids produced by halophilic bacterial strains on mural paintings and laboratory conditions. *FEMS Microbiol. Lett.* 366:fnz243. doi: 10.1093/femsle/fnz243
- Cooke, R. U. (1979). Laboratory simulation of salt weathering processes in arid environments. *Earth Surf. Process.* 4, 347–359. doi: 10.1002/esp.3290040405
- Curry, K. D., Wang, Q., Nute, M. G., Tyshayeva, A., Reeves, E., Soriano, S., et al. (2022). Emu: species-level microbial community profiling of full-length 16S rRNA Oxford Nanopore sequencing data. *Nat. Methods* 19, 845–853. doi: 10.1038/s41592-022-01520-4
- De Coster, W., D'Hert, S., Schultz, D. T., Cruts, M., and Van Broeckhoven, C. (2018). NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics* 34, 2666–2669. doi: 10.1093/bioinformatics/bty149
- Dineen, S. M., Aranda, R., Anders, D. L., and Robertson, J. M. (2010). An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil: soil DNA extraction kit evaluation. *J. Appl. Microbiol.* 109, 1886–1896. doi: 10.1111/j.1365-2672.2010.04816.x
- Duman, O., and Tunç, S. (2008). Electrokinetic properties of vermiculite and expanded vermiculite: effects of pH, clay concentration and mono- and multivalent electrolytes. *Sep. Sci. Technol.* 43, 3755–3776. doi: 10.1080/01496390802219109
- Ettenauer, J. D., Jurado, V., Piñar, G., Miller, A. Z., Santner, M., Saiz-Jimenez, C., et al. (2014). Halophilic microorganisms are responsible for the rosy Discolouration of saline environments in three historical buildings with mural paintings. *PLoS One* 9:e103844. doi: 10.1371/journal.pone.0103844
- Ettenauer, J., Sterflinger, K., and Piñar, G. (2010). Cultivation and molecular monitoring of halophilic microorganisms inhabiting an extreme environment presented by a salt-attacked monument. *Int. J. Astrobiol.* 9, 59–72. doi: 10.1017/S1473550409990383
- Evans, M. V., Panescu, J., Hanson, A. J., Welch, S. A., Sheets, J. M., Nastasi, N., et al. (2018). Members of *Marinobacter* and *Arcobacter* influence system biogeochemistry during early production of hydraulically fractured natural gas Wells in the Appalachian Basin. *Front. Microbiol.* 9:2646. doi: 10.3389/fmicb.2018.02646
- Gallo, C., Rizzo, P., and Guerra, G. (2020). Antimicrobial release from cleaning poultices for the conservation and disinfection of stone surfaces. *Appl. Clay Sci.* 193:105667. doi: 10.1016/j.clay.2020.105667
- Glaeser, S. P., Pulami, D., Blom, J., Eisenberg, T., Goesmann, A., Bender, J., et al. (2020). The status of the genus *Prolinoborus* (Pot et al. 1992) and the species *Prolinoborus fasciculus* (Pot et al. 1992). *Int. J. Syst. Evol. Microbiol.* 70, 5165–5171. doi: 10.1099/ijsem.0.004404
- Gong, Y., Ping, X.-Y., Zeng, C.-H., Wang, S.-X., Zhou, Y., Wang, M.-Y., et al. (2022). Predation capacity of *Bradymonabacteria*, a recently discovered group in the order *Bradymonadales*, isolated from marine sediments. *Arch. Microbiol.* 204:695. doi: 10.1007/s00203-022-03303-z

- Goudie, A., Cooke, R., and Evans, I. (1970). Experimental investigation of rock weathering by salts. *Area* 2, 42–48. Available online at: <https://www.jstor.org/stable/20000488>.
- Green, D. H., Bowman, J. P., Smith, E. A., Gutierrez, T., and Bolch, C. J. S. (2006). *Marinobacter algicola* sp. nov., isolated from laboratory cultures of paralytic shellfish toxin-producing dinoflagellates. *IJSEM* 56, 523–527. doi: 10.1099/ijms.0.63447-0
- Gutarowska, B., Celikkol-Aydin, S., Bonifay, V., Otlewska, A., Aydin, E., Oldham, A. L., et al. (2015). Metabolomic and high-throughput sequencing analysis—modern approach for the assessment of biodeterioration of materials from historic buildings. *Front. Microbiol.* 6:979. doi: 10.3389/fmicb.2015.00979
- Haidău, C., Năstase-Bucur, R., Bulzu, P., Levei, E., Cadar, O., Mirea, I. C., et al. (2022). A 16S rRNA gene-based Metabarcoding of phosphate-rich deposits in Muierilor cave, South-Western Carpathians. *Front. Microbiol.* 13:877481. doi: 10.3389/fmicb.2022.877481
- Han, S.-R., Kim, B., Jang, J. H., Park, H., and Oh, T.-J. (2021). Complete genome sequence of *Arthrobacter* sp. PAMC25564 and its comparative genome analysis for elucidating the role of CAZymes in cold adaptation. *BMC Genomics* 22:403. doi: 10.1186/s12864-021-07734-8
- Harker, M., Hirschberg, J., and Oren, A. (1998). *Paracoccus marcusii* sp. nov., an orange gram-negative coccus. *Int. J. Syst. Bacteriol.* 48, 543–548. doi: 10.1099/00207713-48-2-543
- Heritage, A., Heritage, A., Funke, F., Vergès-Belmin, V., and Bourges, A. (2013). “Current use of poultries in the conservation of monuments” in *Desalination of historic buildings* (London: Stone and Wall Paintings. Archetype Publications), 8–13.
- Heyrman, J. (2005). *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. *Int. J. Syst. Evol. Microbiol.* 55, 111–117. doi: 10.1099/ijms.0.63240-0
- Heyrman, J., Vanparys, B., Logan, N. A., Balcaen, A., Rodriguez-Díaz, M., Felske, A., et al. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse a grasslands. *Int. J. Syst. Evol. Microbiol.* 54, 47–57. doi: 10.1099/ijms.0.02723-0
- Hollensteiner, J., Schneider, D., Poehlein, A., Brinkhoff, T., and Daniel, R. (2023). Pan-genome analysis of six *Paracoccus* type strain genomes reveal lifestyle traits. *PLoS One* 18:e0287947. doi: 10.1371/journal.pone.0287947
- Imperi, F., Caneva, G., Cancellieri, L., Ricci, M. A., Sodo, A., and Visca, P. (2007). The bacterial aetiology of rosy discoloration of ancient wall paintings. *Environ. Microbiol.* 9, 2894–2902. doi: 10.1111/j.1462-2920.2007.01393.x
- Indrasumunar, A., and Gresshoff, P. M. (2013). Vermiculite's strong buffer capacity renders it unsuitable for studies of acidity on soybean (*Glycine max* L.) nodulation and growth. *BMC. Res. Notes* 6:465. doi: 10.1186/1756-0500-6-465
- Ishii, S., Ashida, N., Ohno, H., Segawa, T., Yabe, S., Otsuka, S., et al. (2017). *Noviherbaspirillum denitrificans* sp. nov., a denitrifying bacterium isolated from rice paddy soil and *Noviherbaspirillum autotrophicum* sp. nov., a denitrifying, facultatively autotrophic bacterium isolated from rice paddy soil and proposal to reclassify *Herbaspirillum massiliense* as *Noviherbaspirillum massiliense* comb. nov. *Int. J. Syst. Evol. Microbiol.* 67, 1841–1848. doi: 10.1099/ijsem.0.001875
- Jroundi, F., Fernández-Vivas, A., Rodríguez-Navarro, C., Bedmar, E. J., and González-Muñoz, M. T. (2010). Bioconservation of deteriorated monumental Calcarene stone and identification of Bacteria with Carbonatogenic activity. *Microb. Ecol.* 60, 39–54. doi: 10.1007/s00248-010-9665-y
- Jroundi, F., Gonzalez-Muñoz, M. T., and Rodríguez-Navarro, C. (2021). “Protection and consolidation of stone Heritage by bacterial Carbonatogenesis” in *Microorganisms in the deterioration and preservation of cultural Heritage*. ed. E. Joseph (Cham: Springer International Publishing), 281–299.
- Jroundi, F., Schiro, M., Ruiz-Agudo, E., Elert, K., Martín-Sánchez, I., González-Muñoz, M. T., et al. (2017). Protection and consolidation of stone heritage by self-inoculation with indigenous carbonatogenic bacterial communities. *Nat. Commun.* 8:279. doi: 10.1038/s41467-017-00372-3
- Jurado, V., Del Rosal, Y., Jimenez De Cisneros, C., Liñan, C., Martín-Pozas, T., Gonzalez-Pimentel, J. L., et al. (2022). Microbial communities in carbonate precipitates from drip waters in Nerja cave, Spain. *PeerJ* 10:e13399. doi: 10.7717/peerj.13399
- Jurado, V., Miller, A. Z., Alias-Villegas, C., Laiz, L., and Saiz-Jimenez, C. (2012). *Rubrobacter bracarensis* sp. nov., a novel member of the genus *Rubrobacter* isolated from a biodeteriorated monument. *Syst. Appl. Microbiol.* 35, 306–309. doi: 10.1016/j.syapm.2012.04.007
- Jurado, V., Porca, E., Pastrana, M. P., Cuezva, S., Fernandez-Cortes, A., and Saiz-Jimenez, C. (2010). Microbiological study of bulls of indulgence of the 15th–16th centuries. *Sci. Total Environ.* 408, 3711–3715. doi: 10.1016/j.scitotenv.2010.05.008
- Kaufmann, G., and Dreybrodt, W. (2007). Calcite dissolution kinetics in the system $\text{CaCO}_3\text{--H}_2\text{O--CO}_2$ at high undersaturation. *Geochim. Cosmochim. Acta* 71, 1398–1410. doi: 10.1016/j.gca.2006.10.024
- Kaur, A., Van Der Peet, P. L., Mui, J. W.-Y., Herisse, M., Pidot, S., and Williams, S. J. (2022). Genome sequences of *Arthrobacter* spp. that use a modified sulfoglycolytic Embden–Meyerhof–Parnas pathway. *Arch. Microbiol.* 204:193. doi: 10.1007/s00203-022-02803-2
- Kawakami, Y., Hayashi, N., Ema, M., and Nakayama, M. (2007). Effects of divalent cations on *Halobacterium salinarum* cell aggregation. *J. Biosci. Bioeng.* 104, 42–46. doi: 10.1263/jbb.104.42
- Kolde, R. (2010). Pheatmap: Pretty Heatmaps. doi: 10.32614/CRAN.package.pheatmap
- Kwaad, F. (1970). Experiments on the granular disintegration of granite by salt action. Publication 16 Fysisch Geografisch materials and structures Bodemkundig Laboratorium from field to laboratory, 67–80.
- Lahti, L., and Shetty, S. (2017). microbiome. doi: 10.18129/B9.BIOC.MICROBIOME
- Laiz, L., Miller, A. Z., Jurado, V., Akatova, E., Sanchez-Moral, S., Gonzalez, J. M., et al. (2009). Isolation of five *Rubrobacter* strains from biodeteriorated monuments. *Naturwissenschaften* 96, 71–79. doi: 10.1007/s00114-008-0452-2
- Laiz, L., Recio, D., Hermosin, B., and Saiz-Jimenez, C. (2000). “Microbial communities in salt Efflorescences” in *Of microbes and art*. eds. O. Ciferri, P. Tiano and G. Mastromei (Boston, MA: Springer US), 77–88.
- Laue, S., Schaab, C., Drese, D., Krauthäuser, D., Helfmeier, G., and Vogt, J. (2021). “20 years long-term monitoring of the salt loaded crypt of St. Maria im Kapitol, Cologne” in *Salt weathering of buildings and stone sculptures*, 307.
- Lee, J.-C., Song, J.-S., and Whang, K.-S. (2019). *Sphingobium pinisoli* sp. nov., isolated from the rhizosphere soil of a Korean native pine tree. *Antonie Van Leeuwenhoek* 112, 815–825. doi: 10.1007/s10482-018-01215-x
- Leinberger, J., Holste, J., Bunk, B., Freese, H. M., Spröer, C., Dlugosch, L., et al. (2021). High potential for secondary metabolite production of *Paracoccus marcusii* CP157, isolated from the crustacean *Cancer pagurus*. *Front. Microbiol.* 12:688754. doi: 10.3389/fmicb.2021.688754
- Leissner, J., Kilian, R., Kotova, L., Jacob, D., Mikolajewicz, U., Broström, T., et al. (2015). Climate for culture: assessing the impact of climate change on the future indoor climate in historic buildings using simulations. *Herit. Sci.* 3:38. doi: 10.1186/s40494-015-0067-9
- Lenz, R. (2017). “Systemoptimierte Salzinderungskompressen (Material – Wirkung)” in *Retrospektive Und Perspektive: Methoden Und Techniken in Der Wandmalereirestauration. Presented at the Werkstattgespräch des Bayerischen Landesamtes für Denkmalpflege im Literaturhaus München, 9. und 10. Mai 2106* (Volk Verlag), 123–133. doi: 10.5165/hawk-hhg/368
- León, M. J., Sánchez-Porro, C., de la Haba, R. R., Pfeiffer, F., Dyall-Smith, M., Oksanen, H. M., et al. (2024). *Halobacterium hubeiense* sp. nov., a haloarchaeal species isolated from a bore core drilled in Hubei Province, PR China. *Int. J. Syst. Evol. Microbiol.* 74:006296. doi: 10.1099/ijsem.0.006296
- Li, C.-T., Yan, Z.-F., Chu, X., Hussain, F., Xian, W.-D., Yunus, Z., et al. (2015). *Delftia deserti* sp. nov., isolated from a desert soil sample. *Antonie Van Leeuwenhoek* 107, 1445–1450. doi: 10.1007/s10482-015-0440-4
- Liu, B.-B., Tang, S.-K., Zhang, Y.-G., Lu, X.-H., Li, L., Cheng, J., et al. (2013). *Halalkalicoccus paucihalophilus* sp. nov., a halophilic archaeon from Lop Nur region in Xinjiang, northwest of China. *Antonie Van Leeuwenhoek* 103, 1007–1014. doi: 10.1007/s10482-013-9880-x
- Liu, J., Wu, F., Xiang, T., Ma, W., He, D., Zhang, Q., et al. (2023). Differences of airborne and mural microorganisms in a 1,500-year-old Xu Xianxiu's tomb, Taiyuan, China. *Front. Microbiol.* 14:1253461. doi: 10.3389/fmicb.2023.1253461
- Li, W.-J., Chen, H.-H., Zhang, Y.-Q., Schumann, P., Stackebrandt, E., Xu, L.-H., et al. (2004). *Nesterenkonia halotolerans* sp. nov. and *Nesterenkonia xinjiangensis* sp. nov., actinobacteria from saline soils in the west of China. *Int. J. Syst. Evol. Microbiol.* 54, 837–841. doi: 10.1099/ijms.0.02935-0
- Londono, S. C., Hartnett, H. E., and Williams, L. B. (2017). Antibacterial activity of aluminum in clay from the Colombian Amazon. *Environ. Sci. Technol.* 51, 2401–2408. doi: 10.1021/acs.est.6b04670
- Lothenbach, B., and Winnefeld, F. (2006). Thermodynamic modelling of the hydration of Portland cement. *Cem. Concr. Res.* 36, 209–226. doi: 10.1016/j.cemconres.2005.03.001
- Lubelli, B., Cnudde, V., Diaz-Goncalves, T., Franzoni, E., van Hees, R. P. J., Ioannou, I., et al. (2018). Towards a more effective and reliable salt crystallization test for porous building materials: state of the art. *Mater. Struct.* 51:55. doi: 10.1617/s11527-018-1180-5
- Lubelli, B., and Van Hees, R. P. J. (2010). Desalination of masonry structures: fine tuning of pore size distribution of poultries to substrate properties. *J. Cult. Herit.* 11, 10–18. doi: 10.1016/j.culher.2009.03.005
- Lv, J., Mao, J., and Ba, H. (2015). Influence of marine microorganisms on the permeability and microstructure of mortar. *Constr. Build. Mater.* 77, 33–40. doi: 10.1016/j.conbuildmat.2014.11.072
- Matović, V., Erić, S., Kremenović, A., Colomban, P., Srećković-Batočanin, D., and Matović, N. (2012). The origin of syngenite in black crusts on the limestone monument King's gate (Belgrade fortress, Serbia) – the role of agriculture fertiliser. *J. Cult. Herit.* 13, 175–186. doi: 10.1016/j.culher.2011.09.003
- McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217
- Miralles, I., Ortega, R., and Montero-Calasanz, M. D. C. (2023). Functional and biotechnological potential of microbiome associated with soils colonised by cyanobacteria in drylands. *Appl. Soil Ecol.* 192:105076. doi: 10.1016/j.apsoil.2023.105076
- Mu, D.-S., Wang, S., Liang, Q.-Y., Du, Z.-Z., Tian, R., Ouyang, Y., et al. (2020). *Bradyonabacter*, a novel bacterial predator group with versatile survival strategies in saline environments. *Microbiome* 8:126. doi: 10.1186/s40168-020-00902-0

- Nagler, K., Krawczyk, A. O., De Jong, A., Madela, K., Hoffmann, T., Laue, M., et al. (2016). Identification of differentially expressed genes during *Bacillus subtilis* spore outgrowth in high-salinity environments using RNA sequencing. *Front. Microbiol.* 7:1564. doi: 10.3389/fmicb.2016.01564
- Nagler, K., and Moeller, R. (2015). Systematic investigation of germination responses of *Bacillus subtilis* spores in different high-salinity environments. *FEMS Microbiol. Ecol.* 91:fiv023. doi: 10.1093/femsec/fiv023
- Nagler, K., Setlow, P., Li, Y.-Q., and Moeller, R. (2014). High salinity alters the germination behavior of *Bacillus subtilis* spores with nutrient and nonnutrient Germinants. *Appl. Environ. Microbiol.* 80, 1314–1321. doi: 10.1128/AEM.03293-13
- Nair, R. R., and Velicer, G. J. (2021). Predatory Bacteria select for sustained prey diversity. *Microorganisms* 9:2079. doi: 10.3390/microorganisms9102079
- Ng, H. J., López-Pérez, M., Webb, H. K., Gomez, D., Sawabe, T., Ryan, J., et al. (2014). *Marinobacter salarius* sp. nov. and *Marinobacter similis* sp. nov., Isolated from Sea Water. *PLoS ONE* 9, e106514. doi: 10.1371/journal.pone.0106514
- Niederberger, T. D., Steven, B., Charvet, S., Barbier, B., and Whyte, L. G. (2009). *Virgibacillus arcticus* sp. nov., a moderately halophilic, endospore-forming bacterium from permafrost in the Canadian high Arctic. *Int. J. Syst. Evol. Microbiol.* 59, 2219–2225. doi: 10.1099/ijs.0.002931-0
- O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufu, S., Haddad, D., McVeigh, R., et al. (2016). Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res.* 44, D733–D745. doi: 10.1093/nar/gkv1189
- Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., et al. (2025). Vegan: Community ecology package. doi: 10.32614/CRAN.package.vegan
- Pastor, A., Gámiz, B., Cruz-Yusta, M., Sánchez, L., and Pavlovic, I. (2020). Carbendazim-clay complexes for its potential use as antimicrobial additives in mortars. *Build. Environ.* 183:107214. doi: 10.1016/j.buildenv.2020.107214
- Patel, S., and Gupta, R. S. (2020). A phylogenomic and comparative genomic framework for resolving the polyphyly of the genus *Bacillus*: proposal for six new genera of *Bacillus* species, *Peribacillus* gen. Nov., *Cytobacillus* gen. Nov., *Mesobacillus* gen. Nov., *Neobacillus* gen. Nov., *Metabacillus* gen. Nov. and *Alkalihalobacillus* gen. Nov. *Int. J. Syst. Evol. Microbiol.* 70, 406–438. doi: 10.1099/ijsem.0.003775
- Pavlović, J., Bosch-Roig, P., Rusková, M., Planý, M., Pangallo, D., and Sanmartín, P. (2022). Long-amplicon MinION-based sequencing study in a salt-contaminated twelfth century granite-built chapel. *Appl. Microbiol. Biotechnol.* 106, 4297–4314. doi: 10.1007/s00253-022-11961-8
- Pedrosa, F. O., Monteiro, R. A., Wassem, R., Cruz, L. M., Ayub, R. A., Colauto, N. B., et al. (2011). Genome of *Herbaspirillum seropedicae* strain SmR1, a specialized Diazotrophic endophyte of tropical grasses. *PLoS Genet.* 7:e1002064. doi: 10.1371/journal.pgen.1002064
- Pel, L., Sawdy, A., and Voronina, V. (2010). Physical principles and efficiency of salt extraction by poulticing. *J. Cult. Herit.* 11, 59–67. doi: 10.1016/j.culher.2009.03.007
- Piñar, G., Ettenauer, J., and Sterflinger, K. (2014). “La vie en rose”: a review of the rosy discoloration of subsurface monuments” in *The conservation of subterranean cultural Heritage*. ed. C. Saiz-Jimenez (London: CRC Press), 113–124.
- Piñar, G., Ripka, K., Weber, J., and Sterflinger, K. (2009). The micro-biota of a sub-surface monument the medieval chapel of St. Virgil (Vienna, Austria). *Int. Biodeterior. Biodegradation* 63, 851–859. doi: 10.1016/j.ibiod.2009.02.004
- Piñar, G., Saiz-Jimenez, C., Schabereiter-Gurtner, C., Blanco-Varela, M. T., Lubitz, W., and Rölleke, S. (2001). Archaeal communities in two disparate deteriorated ancient wall paintings: detection, identification and temporal monitoring by denaturing gradient gel electrophoresis. *FEMS Microbiol. Ecol.* 37, 45–54. doi: 10.1111/j.1574-6941.2001.tb00852.x
- Pinna, D., Galeotti, M., Perito, B., Daly, G., and Salvadori, B. (2018). In situ long-term monitoring of recolonization by fungi and lichens after innovative and traditional conservative treatments of archaeological stones in Fiesole (Italy). *Int. Biodeterior. Biodegradation* 132, 49–58. doi: 10.1016/j.ibiod.2018.05.003
- Pot, B., Willems, A., Gillis, M., and De Ley, J. (1992). Intra- and intergeneric relationships of the genus *Aquaspirillum*: *Prolinoborus*, a new genus for *Aquaspirillum fasciculus*, with the species *Prolinoborus fasciculus* comb. nov. *Int. J. Syst. Bacteriol.* 42, 44–57. doi: 10.1099/00207713-42-1-44
- Pyzik, A., Ciuchcinski, K., Dziurzynski, M., and Dziejew, L. (2021). The bad and the good—microorganisms in cultural Heritage environments—an update on biodeterioration and biotreatment approaches. *Materials* 14:177. doi: 10.3390/ma14010177
- Ranalli, G., and Zanardini, E. (2021). Biocleaning on cultural Heritage: new frontiers of microbial biotechnologies. *J. Appl. Microbiol.* 131, 583–603. doi: 10.1111/jam.14993
- Reddy, G. S. N., Manasa, B. P., Singh, S. K., and Shivaji, S. (2013). *Paenisporsarcina indica* sp. nov., a psychrophilic bacterium from a glacier, and reclassification of *Sporosarcina antarctica* Yu et al., 2008 as *Paenisporsarcina Antarctica* comb. nov. and emended description of the genus *Paenisporsarcina*. *Int. J. Syst. Evol. Microbiol.* 63, 2927–2933. doi: 10.1099/ijs.0.047514-0
- Ripka, K., Denner, E. B. M., Michaelsen, A., Lubitz, W., and Piñar, G. (2006). Molecular characterisation of *Halobacillus* strains isolated from different medieval wall paintings and building materials in Austria. *Int. Biodeterior. Biodegradation* 58, 124–132. doi: 10.1016/j.ibiod.2006.05.004
- Rölleke, S., Witte, A., Wanner, G., and Lubitz, W. (1998). Medieval wall paintings—a habitat for archaea: identification of archaea by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified gene fragments coding for 16S rRNA in a medieval wall painting. *Int. Biodeterior. Biodegradation* 41, 85–92. doi: 10.1016/S0964-8305(98)80011-5
- Rosado, T., Dias, L., Lança, M., Nogueira, C., Santos, R., Martins, M. R., et al. (2020). Assessment of microbiota present on a Portuguese historical stone convent using high-throughput sequencing approaches. *MicrobiologyOpen* 9, 1067–1084. doi: 10.1002/mbo3.1030
- Saiz-Jimenez, C., and Laiz, L. (2000). Occurrence of halotolerant/halophilic bacterial communities in deteriorated monuments. *Int. Biodeterior. Biodegradation* 46, 319–326. doi: 10.1016/S0964-8305(00)00104-9
- Sawdy, A., Lubelli, B., Voronina, V., and Pel, L. (2010). Optimizing the extraction of soluble salts from porous materials by poultices. *Stud. Conserv.* 55, 26–40. doi: 10.1179/sic.2010.55.1.26
- Schabereiter-Gurtner, C., Piñar, G., Vybiral, D., Lubitz, W., and Rölleke, S. (2001). *Rubrobacter*-related bacteria associated with rosy discoloration of masonry and lime wall paintings. *Arch. Microbiol.* 176, 347–354. doi: 10.1007/s002030100333
- Schröer, L., Boon, N., De Kock, T., and Cnudde, V. (2021). The capabilities of bacteria and archaea to alter natural building stones – a review. *Int. Biodeterior. Biodegradation* 165:105329. doi: 10.1016/j.ibiod.2021.105329
- Selivanova, E. A., Yakimov, M. M., Kataev, V. Y., Khlopko, Y. A., Balkin, A. S., and Plotnikov, A. O. (2024). The cultivation of halophilic microalgae shapes the structure of their prokaryotic assemblages. *Microorganisms* 12:1947. doi: 10.3390/microorganisms12101947
- Sherrill-Mix, S. (2017). Taxonomizr: Functions to work with NCBI accessions and taxonomy. doi: 10.32614/CRAN.package.taxonizr
- Shigematsu, T., Yumihara, K., Ueda, Y., Numaguchi, M., Morimura, S., and Kida, K. (2003). *Delfia tsuruhatensis* sp. nov., a terephthalate-assimilating bacterium isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* 53, 1479–1483. doi: 10.1099/ijs.0.02285-0
- Shimane, Y., Hatada, Y., Minegishi, H., Echigo, A., Nagaoka, S., Miyazaki, M., et al. (2011). *Salarchaeum japonicum* gen. Nov., sp. nov., an aerobic, extremely halophilic member of the Archaea isolated from commercial salt. *Int. J. Syst. Evol. Microbiol.* 61, 2266–2270. doi: 10.1099/ijs.0.025064-0
- Shi, R., Yin, M., Tang, S.-K., Lee, J.-C., Park, D.-J., Zhang, Y.-J., et al. (2011). *Bacillus luteolus* sp. nov., a halotolerant bacterium isolated from a salt field. *Int. J. Syst. Evol. Microbiol.* 61, 1344–1349. doi: 10.1099/ijs.0.021683-0
- Skipper, P. J. A., Skipper, L. K., and Dixon, R. A. (2022). A metagenomic analysis of the bacterial microbiome of limestone, and the role of associated biofilms in the biodeterioration of heritage stone surfaces. *Sci. Rep.* 12:4877. doi: 10.1038/s41598-022-08851-4
- Son, Y., Min, J., Shin, Y., and Park, W. (2023). Morphological and physiological adaptations of psychrophilic *Pseudarthrobacter psychrotolerans* YJ56 under temperature stress. *Sci. Rep.* 13:14970. doi: 10.1038/s41598-023-42179-x
- Steiger, M., Charola, A. E., and Sterflinger, K. (2011). Weathering and Deterioration. In *Stone in Architecture*. eds. S. Siegesmund and R. Snethlage (Berlin, Heidelberg: Springer Berlin Heidelberg), 227–316.
- Sterflinger, K., and Piñar, G. (2013). Microbial deterioration of cultural heritage and works of art — tilting at windmills? *Appl. Microbiol. Biotechnol.* 97, 9637–9646. doi: 10.1007/s00253-013-5283-1
- Stevens, A. H., and Cockell, C. S. (2020). A systematic study of the limits of life in mixed ion solutions: physicochemical parameters do not predict habitability. *Front. Microbiol.* 11:1478. doi: 10.3389/fmicb.2020.01478
- Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K., and Schmidt, T. M. (2015). rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res.* 43, D593–D598. doi: 10.1093/nar/gku1201
- Swerdlow, B. M., Setlow, B., and Setlow, P. (1981). Levels of H⁺ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. *J. Bacteriol.* 148, 20–29. doi: 10.1128/jb.148.1.20-29.1981
- Tang, S.-K., Tian, X.-P., Zhi, X.-Y., Cai, M., Wu, J.-Y., Yang, L.-L., et al. (2008). *Halactinospora alba* gen. Nov., sp. nov., a halophilic filamentous actinomycete of the family *Nocardiopsaceae*. *Int. J. Syst. Evol. Microbiol.* 58, 2075–2080. doi: 10.1099/ijs.0.065531-0
- Tang, S.-K., Wang, Y., Cai, M., Zhi, X.-Y., Lou, K., Xu, L.-H., et al. (2009). *Saccharopolyspora halophila* sp. nov., a novel halophilic actinomycete isolated from a saline lake in China. *Int. J. Syst. Evol. Microbiol.* 59, 555–558. doi: 10.1099/ijs.0.65705-0
- Tescari, M., Frangipani, E., Caneva, G., Casanova Municchia, A., Sodo, A., and Visca, P. (2018a). *Arthrobacter agilis* and rosy discoloration in “Terme del Foro” (Pompeii, Italy). *Int. Biodeterior. Biodegradation* 130, 48–54. doi: 10.1016/j.ibiod.2018.03.015
- Tescari, M., Visca, P., Frangipani, E., Bartoli, F., Rainer, L., and Caneva, G. (2018b). Celebrating centuries: pink-pigmented bacteria from rosy patinas in the house of bicentenary (Herculaneum, Italy). *J. Cult. Herit.* 34, 43–52. doi: 10.1016/j.culher.2018.02.015

- Tichy, J., Waldherr, M., Ortbauer, M., Graf, A., Sipek, B., Jembrih-Simbuerger, D., et al. (2023). Pretty in pink? Complementary strategies for analysing pink biofilms on historical buildings. *Sci. Total Environ.* 904:166737. doi: 10.1016/j.scitotenv.2023.166737
- Tschischko, B., Williams, T. J., Allen, M. A., Zhong, L., Raftery, M. J., and Cavicchioli, R. (2016). Ecophysiological distinctions of Haloarchaea from a hypersaline Antarctic Lake as determined by Metaproteomics. *Appl. Environ. Microbiol.* 82, 3165–3173. doi: 10.1128/AEM.00473-16
- Ueda, K., Tagami, Y., Kamihara, Y., Shiratori, H., Takano, H., and Beppu, T. (2008). Isolation of Bacteria whose growth is dependent on high levels of CO₂ and implications of their potential diversity. *Appl. Environ. Microbiol.* 74, 4535–4538. doi: 10.1128/AEM.00491-08
- Vera-Gargallo, B., Hernández, M., Dumont, M. G., and Ventosa, A. (2023). Thrive or survive: prokaryotic life in hypersaline soils. *Environ. Microbiome* 18:17. doi: 10.1186/s40793-023-00475-z
- Verges-Belmin, V., and Siedel, H. (2005). Desalination of masonries and monumental sculptures by Poulting: a review / Entsalzen von Mauerwerk und Steinfiguren mit Hilfe von Kompressen: Ein Überblick. *Restoration Build. Monuments* 11, 391–408. doi: 10.1515/rbm-2005-6000
- Wang, S., Gong, Y., Chen, G.-J., and Du, Z.-J. (2024). The predatory properties of *Bradymonabacteria*, the representative of facultative prey-dependent predators. *Microorganisms* 12:2008. doi: 10.3390/microorganisms12102008
- Wang, S., Mu, D., and Du, Z.-J. (2020). *Persicomonas caeni* gen. Nov., sp. nov., the representative of a novel wide-ranging predatory taxon in *Bradymonadales*. *Front. Microbiol.* 11:698. doi: 10.3389/fmicb.2020.00698
- Whitman, T., Neurath, R., Perera, A., Chu-Jacoby, I., Ning, D., Zhou, J., et al. (2018). Microbial community assembly differs across minerals in a rhizosphere microcosm. *Environ. Microbiol.* 20, 4444–4460. doi: 10.1111/1462-2920.14366
- Wickham, H. (2016). ggplot2: Elegant graphics for data analysis, 2. Springer International Publishing, Cham.
- Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., et al. (2019). Welcome to the Tidyverse. *JOSS* 4:1686. doi: 10.21105/joss.01686
- Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Completing bacterial genome assemblies with multiplex MinION sequencing. *Microbial Genom.* 3:e000132. doi: 10.1099/mgen.0.000132
- Williams, L. B., and Haydel, S. E. (2010). Evaluation of the medicinal use of clay minerals as antibacterial agents. *Int. Geol. Rev.* 52, 745–770. doi: 10.1080/00206811003679737
- Williams, T. J., Allen, M. A., DeMaere, M. Z., Kyrpides, N. C., Tringe, S. G., Woyke, T., et al. (2014). Microbial ecology of an Antarctic hypersaline lake: genomic assessment of ecophysiology among dominant haloarchaea. *ISME J.* 8, 1645–1658. doi: 10.1038/ismej.2014.18
- Wissenschaftlich-Technische Arbeitsgemeinschaft für Bauwerkserhaltung und Denkmalpflege. (2019). Desalination of porous building material by means of poultices, Deutsche Fassung vom Dezember 2019, Ausgabe 12/2019/D. ed, WTA Guideline 3-13 12.2019/D, 2019. Fraunhofer IRB Verl, Stuttgart.
- Wu, F., Gu, J.-D., Li, J., Feng, H., and Wang, W. (2022). “Microbial colonization and protective management of wall paintings” in *Cultural Heritage Microbiology: Recent Developments*. eds. R. Mitchell, J. Clifford and A. Vasanthakumar (London: Archetype Publications), 57–84.
- Xie, F., Pei, S., Huang, X., Wang, L., Kou, J., and Zhang, G. (2021). *Microcella flavibacter* sp. nov., isolated from marine sediment, and reclassification of *Chryseoglobus frigidaquae*, *Chryseoglobus indicus*, and *Yonghaparkia alkaliphila* as *Microcella frigidaquae* comb. nov., *Microcella indica* nom. Nov., and *Microcella alkalica* nom. Nov. *Antonie Van Leeuwenhoek* 114, 2133–2145. doi: 10.1007/s10482-021-01668-7
- Xiyu, Z. (1987). Salt lakes and their origins in Xinjiang, China. *Chin. J. Ocean. Limnol.* 5, 172–185. doi: 10.1007/BF02845001
- Xu, X., Xu, M., Zhao, Q., Xia, Y., Chen, C., and Shen, Z. (2018). Complete genome sequence of cd(II)-resistant *Arthrobacter* sp. PGP41, a plant growth-promoting bacterium with potential in microbe-assisted phytoremediation. *Curr. Microbiol.* 75, 1231–1239. doi: 10.1007/s00284-018-1515-z
- Yoon, J.-H., Kang, S.-J., Schumann, P., and Oh, T.-K. (2006). *Yonghaparkia alkaliphila* gen. Nov., sp. nov., a novel member of the family *Microbacteriaceae* isolated from an alkaline soil. *Int. J. Syst. Evol. Microbiol.* 56, 2415–2420. doi: 10.1099/ijss.0.64258-0
- Yoon, J.-H., Kang, S.-J., Yeo, S.-H., and Oh, T.-K. (2005). *Paenibacillus alkaliterrae* sp. nov., isolated from an alkaline soil in Korea. *Int. J. Syst. Evol. Microbiol.* 55, 2339–2344. doi: 10.1099/ijss.0.63771-0
- Yoon, J.-H., and Oh, T.-K. (2005). *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int. J. Syst. Evol. Microbiol.* 55, 1945–1948. doi: 10.1099/ijss.0.63332-0
- Yumoto, I., Yamaga, S., Sogabe, Y., Nodasaka, Y., Matsuyama, H., Nakajima, K., et al. (2003). *Bacillus krulwichiae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and m-hydroxybenzoate. *Int. J. Syst. Evol. Microbiol.* 53, 1531–1536. doi: 10.1099/ijss.0.02596-0
- Zehnder, K. (2007). Long-term monitoring of wall paintings affected by soluble salts. *Environ. Geol.* 52, 353–367. doi: 10.1007/s00254-006-0463-2
- Zeidler, S., and Müller, V. (2019). Coping with low water activities and osmotic stress in *Acinetobacter baumannii*: significance, current status and perspectives. *Environ. Microbiol.* 21, 2212–2230. doi: 10.1111/1462-2920.14565
- Zhang, G., Wang, L., Li, J., and Zhou, Y. (2016). *Amycolatopsis albispora* sp. nov., isolated from deep-sea sediment. *IJSEM* 66, 3860–3864. doi: 10.1099/ijsem.0.001277
- Zhao, Y., Wei, H.-M., Yuan, J.-L., Xu, L., and Sun, J.-Q. (2023). A comprehensive genomic analysis provides insights on the high environmental adaptability of *Acinetobacter* strains. *Front. Microbiol.* 14:1177951. doi: 10.3389/fmicb.2023.1177951



OPEN ACCESS

EDITED BY

Erasmus Gámez-Espinosa,
National University of La Plata, Argentina

REVIEWED BY

Benjamín Otto Ortega,
Autonomous University of Campeche, Mexico
Alan Santiago Tarda,
CONICET Instituto de Limnología
Dr. RA Ringuet (ILPLA), Argentina

*CORRESPONDENCE

Xiaobo Liu
✉ xbliu@njust.edu.cn

[†]These authors have contributed equally to
this work

RECEIVED 27 March 2025

ACCEPTED 14 May 2025

PUBLISHED 27 May 2025

CITATION

Ma C, Zhang X, Wu F and Liu X (2025)
Identifying keystone taxa and metabolisms of
epilithic biofilms is crucial to the conservation
of stone heritage from biodeterioration.
Front. Microbiol. 16:1600865.
doi: 10.3389/fmicb.2025.1600865

COPYRIGHT

© 2025 Ma, Zhang, Wu and Liu. This is an
open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Identifying keystone taxa and metabolisms of epilithic biofilms is crucial to the conservation of stone heritage from biodeterioration

Chenchen Ma^{1†}, Xiaoying Zhang^{1†}, Fasi Wu² and Xiaobo Liu^{1*}

¹School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, China, ²Department of Conservation Research, National Research Center for Conservation of Ancient Wall Paintings and Earthen Sites, Dunhuang Academy, Dunhuang, China

Background: Outdoor stone heritage accounts for a large portion of UNESCO World Heritage Sites and is an essential carrier of the ancient civilization of our society. Unfortunately, they usually suffer from serious biodeterioration by diverse microbial colonizers, especially when the environment is available. As microbial communities evolve with the environment, it is important to link the bio-deteriogens to biodeterioration processes accurately.

Methods: We used an integrative high-throughput sequencing and comparative metabolomic approach to unravel the biodeterioration of the Leizhou Stone Dog monuments.

Results: The divergence and similarity of the composition of microbial biofilms colonizing the monuments indicated that photoautotrophic bacteria (e.g., *Leptolyngbya*, *Chroococcidiopsis*, and *Chloroplast*) and nitrifying archaea (e.g., the family *Nitrososphaeraceae*) and/or bacteria (e.g., *Massilia* and *Bacillus*) are the keystone taxa governing the biodeterioration processes. Further, the correlation between the keystone taxa and physicochemical properties confirmed the consistency of the observations of the keystone metabolisms involved in the biodeterioration processes.

Conclusion: Our study highlights the necessity of a case-by-case diagnosis of the keystone taxa and metabolisms before any therapy, advancing the conservation science of cultural heritage.

KEYWORDS

biodeterioration, epilithic biofilms, stone heritage, keystone taxa, heritage conservation

1 Introduction

As early as prehistoric times, rocks and carved stones have been used for making daily tools, designing totems, and building shelters worldwide. For this reason, stone monuments are considered the most crucial evidence of the past civilization of our society, as indexed by the UNESCO World Heritage List.¹ Unfortunately, most stone monuments have been exposed to an outdoor environment, leaving them at long-term risk from frequent environmental fluctuation,

¹ <https://whc.unesco.org/en/list/>

increasing atmospheric pollution, and climate change. Further, the changing environment has dramatically exacerbated both abiotic and biotic deterioration processes of outdoor stone monuments. Notably, geomicrobiological activity and impact often evolve with changing environmental factors, increasing the difficulty of conserving stone monuments.

In most cases, outdoor stone monuments are substantially colonized by colorful biofilms (Villa et al., 2022). The epilithic biofilms are composed of archaea, bacteria, fungi, lichens, and microalgae with different metabolic functions and trophic manners (Liu et al., 2020; Wu et al., 2021). For example, phototrophic microorganisms, usually predominated by cyanobacteria, have been widely observed to grow on stone monuments (Rossi et al., 2012; Zhang et al., 2024). These microorganisms can grow on outdoor stone monuments only relying on the availability of water (Wang et al., 2025), and they are commonly reported as the pioneers of microbial colonizers (Bellinzoni et al., 2003). Together with phototrophs, some chemolithotrophs (e.g., ammonia-oxidizing bacteria or archaea) can convert CO₂ into organic carbons to support the growth of subsequent heterotrophs (e.g., most bacteria and fungi) in the biofilms. Collectively, the members inside the biofilms drive the geochemical cycles of carbon, nitrogen, and sulfur that are mainly from atmospheric deposition (Mitchell and Gu, 2000), resulting in the continual deterioration of stone by biogenic acids (Liu et al., 2020; Qian et al., 2024). For example, our previous work showed that ammonia-oxidizing archaea and bacteria cooperatively contribute to the production of nitric acid responsible for the biodeterioration of stone monuments at the Bayon Temple of Angkor Thom in Cambodia (Meng et al., 2016). We also found

that sulfur-oxidizing bacteria (e.g., *Sulfurovum* and *Acidiphilium*) release sulfuric acid, resulting in the blackening of basalt sculptures of the Leizhou Stone Dog monuments (Wang and Liu, 2021).

Thanks to the multi-omics techniques, we can obtain much microbial information on deteriorated monuments using minimal samples. Although these culture-independent approaches allow us to rapidly construct a public database of microbial information on the biodeterioration of stone monuments, we need a case-by-case analysis of the monuments as they are exposed to different environments. However, we proposed the research priority of the keystone taxa in the biofilms, which act as drivers of microbiome structure and functioning (Banerjee et al., 2018) and as good indicators of pending community shifts (Herren and McMahon, 2018).

Our focus is on the Stone Dog monuments located at the Leizhou Peninsula in tropical South China, where a humid hot climate prevails because of the influence of continental and maritime monsoons as well as typhoons. Thus, microbial biofilms thrive on the stone monuments due to the preferable temperature and humidity over the year (Wang et al., 2021). Our previous work showed that most of them suffer from severe biodeterioration caused by diverse microorganisms, including fungi, cyanobacteria, and sulfur-oxidizing bacteria (Wang and Liu, 2021; Wang et al., 2021), resulting in a considerable loss of appearance (Figure 1). However, our further work indicated that micro-environments significantly affect community structures and biodeterioration processes of epilithic biofilms on the monuments (Meng et al., 2023). Contrary to our initial hypothesis of congruence between the



FIGURE 1

Sculptures of the Leizhou Stone Dog. (a) An overview of the sculptures displayed in a courtyard; (b–f) are the appearances of the sculptures M0137, M0211, M0251, M0295, and M0307, respectively.

biodeterioration of the monuments in the same yard, we observed divergent physicochemical features of the soils at the bottom of the monuments, highlighting the need for a nuanced framework capturing the keystone microorganism-metabolite interplay. Therefore, identifying the keystone taxa and metabolisms involved in microbial biodeterioration of the monuments is essential to bridge the link between microbial biofilm and biodeterioration in cultural heritage microbiology research (Luo and Gu, 2025).

Here, we used an integrative high-throughput sequencing and comparative metabolomic approach to unravel the biodeterioration of the Leizhou Stone Dog monuments. To avoid the divergence of abiotic deterioration, we selected the monuments in the same yard as the subject. We tracked the divergent physicochemical features of the soils at the bottom of the monuments and systematically compared the divergence and similarity of the microbial composition of the biofilms colonizing the monuments to identify the keystone taxa governing the biodeterioration of each stone monument. Furthermore, the correlation between the keystone taxa and physicochemical properties was evaluated to check the consistency of the observations of the species-based metabolisms of the keystone taxa, leading to the biodeterioration of the monuments.

2 Materials and methods

2.1 Sample collection

Samples were collected in January 2024 in Leizhou (110°5'57"E, 20°54'56"N), Guangdong Province, China. The local climatic conditions during the period were featured as follow: a typical monsoon prevailing, an average temperature of 23°C, and an average precipitation of 1,000 mm (relative humidity of about 70%), but no precipitation in 1 week prior to sampling. Five sets of stone dog sculptures suffering from deterioration in an open-air courtyard were selected and coded M0137, M0211, M0252, M0295, and M0307, respectively (Figure 1). Both the biofilm samples and soil samples were collected for each sculpture. The epilithic biofilms colonizing each sculpture were sampled in 5 cm × 5 cm squares via the non-destructive method. Meanwhile, about 10 g of the soils at the bottom of the sculpture were collected for chemical assays. All the samples were packaged and stored at 4°C before laboratory-based analyses.

2.2 Ion chromatography analysis

The base soil was thoroughly mixed before ion chromatography analysis (Dick and Tabatabai, 1979). Briefly, 1 g of the soil sample was added to 5 mL of distilled water. After 15 min of mixing on the vortex followed by 5 min centrifugation at 1,000g, the supernatant was filtered using the microporous membrane (0.22 µm in diameter) for subsequent determination of anion and cation concentrations. Upon the detection limit of ion chromatography, the initial concentration would be diluted if necessary. Then, the prepared supernatant was injected into an ion chromatograph to detect anion and cation concentrations. The Dionex™ Aquion™ ICS 2100 (Thermo Fisher Scientific, MA, United States) system determined the anion concentration with the column IonPac™ AS19 (4 × 250 mm). The cation concentration was examined using the Dionex™ ICS 6000 (Thermo Fisher Scientific, MA,

United States) system, with the column IonPac™ CS12A (4 × 250 mm). The entire detection was carried out at 25°C.

2.3 DNA extraction and sequencing

Following the manufacturer's introduction, the ALFA-SEQ Soil DNA Kit (50) (FINDROP, CN) was used to extract the genomic DNA of biofilm samples. The concentration and purity were detected by Thermo NanoDrop One (Thermo Fisher Scientific, MA, United States).

The V4-V5 regions of 16S rRNA genes were amplified using specific primers (Bacteria: 515F-GTGCCAGCMGCCGCGGTAA, 907R-CCGTCAATTCMTTTRAGTT; Archaea: 524F10extF-TGYCAGCCGCCGCGGTAA, Arch958RmodR-YCCGGCGTTGAV-TCCAATT), in which a 12-bp barcode was appended (Tamaki et al., 2011; Pires et al., 2012). Primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, United States).

The length and concentration of the PCR products were detected by 1% agarose gel electrophoresis. Samples with bright main strips between 400–450 bp could be used for further assays. PCR products were mixed in equidensity ratios according to the GeneTools Analysis Software (Version 4.03.05.0, SynGene). Then, a mixture of PCR products was purified with E.Z.N.A. Gel Extraction Kit (Omega, USA).

Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, United States) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, MA, United States). Finally, the library was sequenced on an Illumina Nova6000 platform, and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China). The high-throughput sequencing data supporting this study's findings were available in the National Center for Biotechnology Information (NCBI) under accession no. PRJNA1170002.

2.4 Bioinformatics and taxonomy analyses

Downstream analysis of high-throughput sequencing data was carried out by QIIME2 (Quantitative Insights Into Microbial Ecology 2) (Bolyen et al., 2019). The primers were removed to obtain the paired-end Clean Reads using Cutadapt software (Martin, 2011), according to the primer information at the beginning and end of the sequence. The paired ends sequencing data were denoised and spliced, and chimeras were removed using the DADA2 algorithm (Callahan et al., 2016). The sequence quality was controlled by specifying the truncation length of the front and back ends. The quality statistics of the spliced sequences were carried out, and the lowest value was leveled. The operational taxonomic units (OTUs) were extracted at a threshold of 97% of identity among the reads. The OTUs were classified by the feature-classifier module in QIIME2 to obtain the taxonomy of bacteria and archaea, referring to the Sliva 16S rRNA (v.138.2).

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was performed to assess the differences between chemical properties of the soils. Assuming the soils

were mostly derived from underlying stone from biological activity, their chemical properties were then analyzed against microbial metabolism from biofilms using Waller-Duncan's *post hoc* test in IBM SPSS v.25.0 (Chicago, IL, United States). Stacked columns were performed to display bacterial and archaeal community structures using the “ggplot2” package in R v.4.4.1.² The heatmap showing the differences in genus abundance among different samples was standardized using the Z-Score method, and the calculation formula was as follows:

$$value = \frac{x - \mu}{\sigma}$$

where μ was the mean and σ was the standard deviation of the data (Singh and Singh, 2020).

Taxonomic trees of the shared OTUs of bacteria and archaea were generated by Cytoscape software with the node and edge files calculated by R according to the shared OTU table. To better explain the relationship between keystone taxa and biodeterioration, we employed Mantel's test to compare different phenotypes with physicochemical properties of the base soils and identify the correlation of consistent biological information from functional results of metabolomic pathway analysis (Ten-Doménech et al., 2021). Mantel's test was carried out to examine the relationships between the chemical properties and the structure of the core taxa using the “linkET” and “ggplot2” packages (Huang, 2021). To mine the metabolism information of the microbial community, the algorithms PICRUST2 and FAPROTAX were applied to predict the functional potential of the bacterial and archaeal community by 16S rRNA gene sequencing profiles. The PICRUST2 predictions were mapped to the annotated genes catalog of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Douglas et al., 2020). The FAPROTAX algorithm extrapolated taxonomic profiles of microbial communities into putative functional profiles based on a database of cultured microorganisms (Louca et al., 2016).

3 Results

3.1 Physicochemical properties of the soils under the bio-deteriorated monuments

Four anions (i.e., NO_2^- , Cl^- , SO_4^{2-} and NO_3^-) and five cations (i.e., Mg^{2+} , NH_4^+ , Na^+ , K^+ and Ca^{2+}) were detected among the five samples (Figure 2). Generally, the contents of anions in each sample ranked as $\text{NO}_3^- > \text{SO}_4^{2-} > \text{Cl}^- > \text{NO}_2^-$, with NO_3^- and SO_4^{2-} being almost 5 and 4 times the content of NO_2^- , respectively (Figure 2a). Moreover, the contents of cations in each sample (except M0307) were ordered as $\text{Ca}^{2+} > \text{K}^+ > \text{Na}^+ > \text{NH}_4^+ > \text{Mg}^{2+}$, with the contents of Ca^{2+} and K^+ higher than the other three cations ($p < 0.05$, Figure 2b).

Despite the monuments located in the same yard, the content of each cation or anion exhibited significant differences among the samples ($p < 0.05$). For example, the contents of NO_3^- , SO_4^{2-} , and Cl^- in M0251 and M0307 were the highest and the lowest among the

samples, respectively. Similarly, the contents of cations in M0307 were the lowest compared with those in the other samples. Also, the content of Ca^{2+} in M0211 doubled that of Ca^{2+} in the other four samples, whereas K^+ , Na^+ , and NH_4^+ exhibited the highest content in M0251.

3.2 Diversity and composition of epilithic biofilms on the monuments

We investigated the biodiversity and composition of epilithic biofilms colonizing the monuments to unravel microbial communities relevant to the biodeterioration of the monuments (Wang and Liu, 2021; Meng et al., 2023). Generally, the bacterial diversity was ranked as $\text{M0295} > \text{M0211} \approx \text{M0251} > \text{M0307} \approx \text{M0137}$, whereas archaeal diversity was ordered as $\text{M0251} \approx \text{M0137} > \text{M0295} \approx \text{M0307} > \text{M0211}$ ($p < 0.05$, Supplementary Figure S1).

Bacteria in all the biofilms mainly included eight phyla, such as Proteobacteria, Cyanobacteria, Firmicutes, and Chloroflexi (Figure 3a). Consistently, Proteobacteria were the predominant community, followed by two phototrophic phyla, Cyanobacteria and Chloroflexi, accounting for 35–50% of bacterial populations in the biofilms. Differentially, Proteobacteria and Firmicutes co-predominated and accounted for more than 90% of bacterial communities on the monument M0307, which might be a consequence of the distinctive physicochemical properties of the soil under M0307. Moreover, the most abundant genera listed in order were *Massilia*, *Leptolyngbya_PCC-6,306*, *Exiguobacterium*, *Bacillus*, *Acinetobacter*, *Chloroplast*, *Chthonomonas*, *Spirosoma*, *Chroococcidiopsis_SAG_2023*, *Bryobacter* and *C0119* (Figure 3b). Commonly, *Massilia* spp. were the predominant bacteria in all the biofilms. Differently, *Exiguobacterium*, *Bacillus*, and *Acinetobacter* predominated in bacterial communities on the monument M0307, *Leptolyngbya_PCC-6,306* predominated on M0137, M0211 and M0295, *Chloroplast* on M0251 and *Bacillus* on M0137.

Archaeal communities in the biofilms mainly included four phyla Crenarchaeota, Halobacterota, Nanoarchaeota, and Euryarchaeota, with Crenarchaeota accounting for more than 95% of the populations (Figure 3c). Interestingly, abundances of Halobacterota (~3%) and Euryarchaeota (~2%) in the biofilm on M0307 were significantly higher than the others. Moreover, archaeal populations were mainly affiliated to seven genera, including two unclassified genera of *Nitrososphaeraceae*, *Ca. Nitrocosmicus*, *Halomarina*, *Nitrososphaera*, *Methanobacterium* and *Nitrosotaleaceae* (Figure 3d). Notably, archaeal populations of M0295, M0211, M0251, and M0137 were purely predominated by an unclassified genus of *Nitrososphaeraceae*, which shared 90% of archaeal populations of M0307 with the other unclassified genus of *Nitrososphaeraceae*. Besides, *Ca. Nitrocosmicus* accounted for 4% of archaeal members of M0307 and 2% of archaeal members of M0295 and M0211. Members in the family *Nitrososphaeraceae* are typical ammonia-oxidizing archaea that convert ammonia to nitrite (Bei et al., 2024), while the latter could be further oxidized to nitrate by aerobic nitrite-oxidizing bacteria (e.g., *Bacillus* spp.) (Abeliovich, 2006; Daims et al., 2016). Thus, the predominance of the families *Nitrososphaeraceae* and *Bacillus* explains the observation that the content of nitrate is significantly higher than that of nitrite and/or ammonia in all the soils.

Although we had an overview of the microbial compositions of the epilithic biofilms on the bio-deteriorated monuments, the core taxa involved in the biodeterioration processes were still unclear. Thus,

² <https://www.r-project.org/>

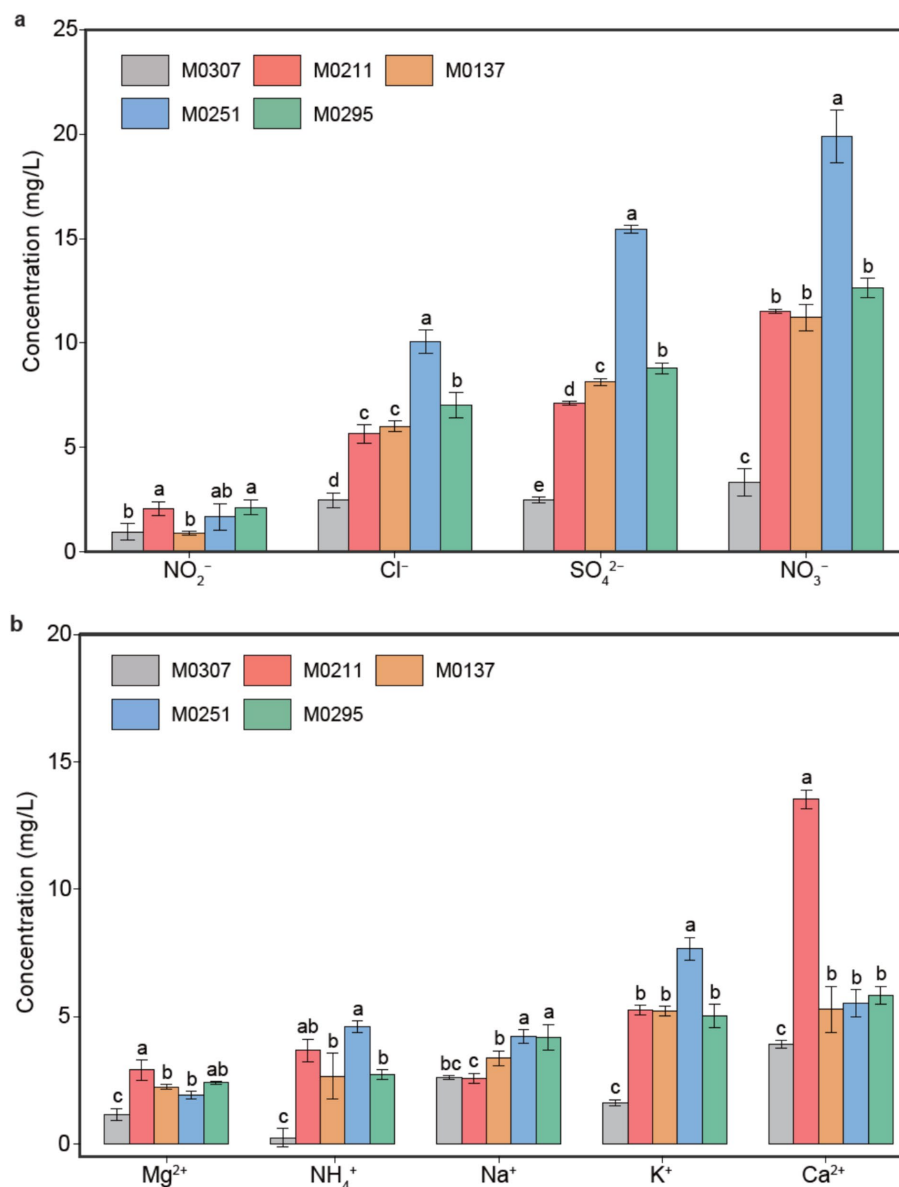


FIGURE 2
Physicochemical properties of the soils at the bottom of each sculpture. Contents of anions (a) and cations (b) in the soils.

further exploration of the populations of the epilithic biofilms colonizing the monuments is required to unravel the keystone taxa that might lead to biodeterioration.

3.3 Keystone taxa of the epilithic biofilms

To figure out the keystone taxa relevant to the biodeterioration of the monuments, we further extracted and compared the predominant species across the biofilm communities. The distribution of the 15 most abundant OTU showed that members of the genera *Massilia*, *Exiguobacterium*, *Leptolyngbya*, *Acinetobacter*, *Bacillus*, *Chroococcidiopsis* and *Chloroplast* were the predominant bacterial species of all the biofilm communities (Figure 4a). Specifically, the predominant species in the biofilm M0137 were mainly affiliated to the genera *Massilia*, *Leptolyngbya*, *Bacillus*, and two unclassified

genera of the families *Ktedonobacteraceae* and *Nostocaceae*. Bacterial communities of the biofilms M0211, M0251, and M0295 were mainly predominated by *Massilia* spp., followed by *Leptolyngbya* spp., *Chloroplast* spp., and *Leptolyngbya* spp., corresponding to the biofilm. Unlike the other biofilms, bacterial species in the biofilm M0307 mainly belonged to the two genera *Exiguobacterium* and *Acinetobacter*, followed by *Massilia* and *Bacillus*. The observations of the most abundant species were generally consistent with the composition of bacterial genera, which further confirmed the potential keystone taxa of these genera involved in the biodeterioration of the monuments.

Furthermore, we extracted the shared species across the bacterial communities (Figure 4b). As a result, it showed that the five biofilm samples shared only 81 species with most of the species owned by individuals, especially M0295, M0211, and M0251, suggesting the differentiation of bacterial communities among the biofilm samples. To identify which shared 81 species represented

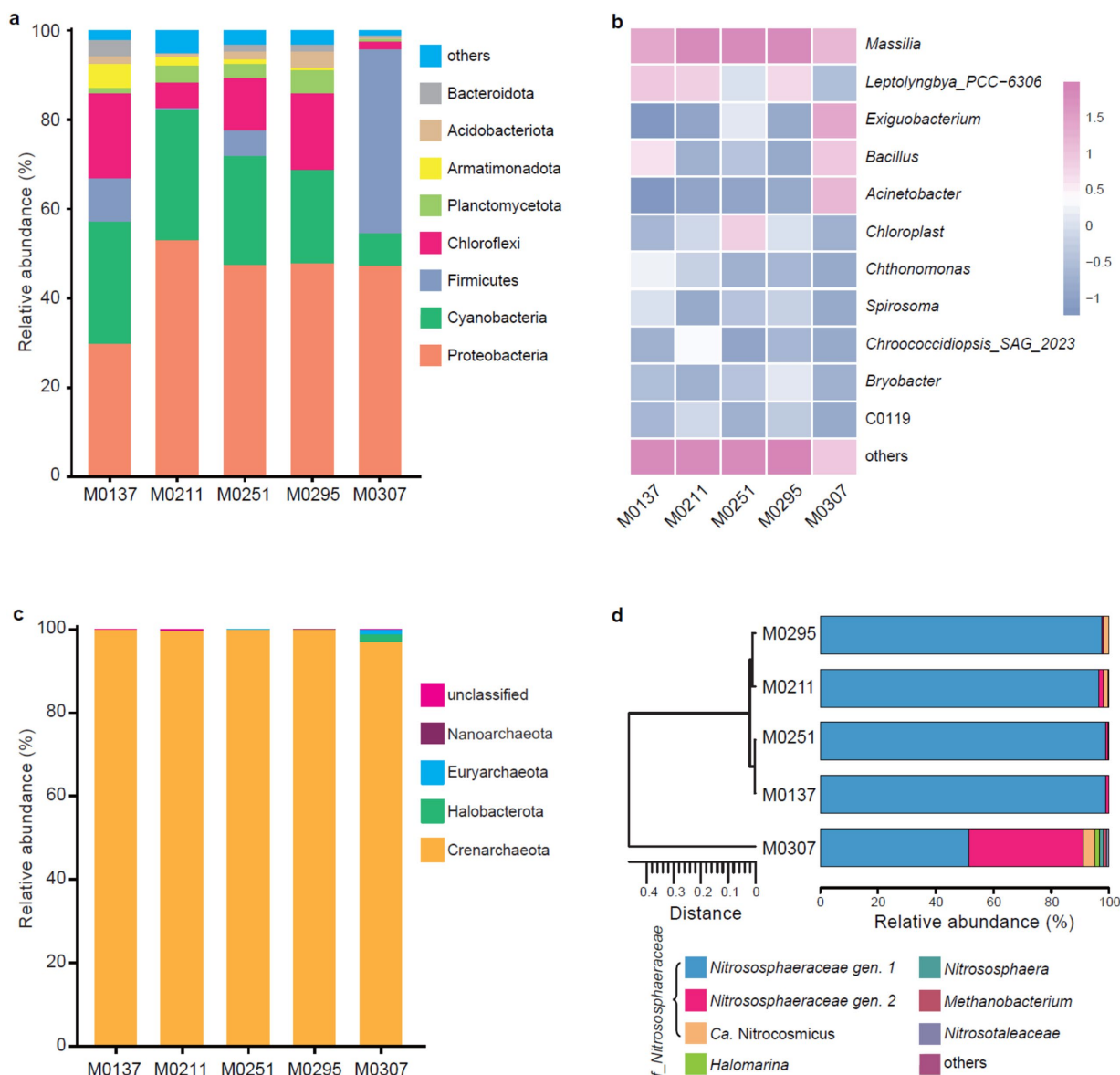


FIGURE 3

Bacterial and archaeal compositions of the epilithic biofilms colonizing the sculptures. **(a)** Bacterial populations at the phylum level. Others represent the phyla with the relative abundance less than 1%. **(b)** Bacterial populations at the genus level. Others include the unclassified genera and the genera with the relative abundance less than 1%. **(c)** Archaeal populations at the phylum level. **(d)** Composition of the archaeal genera. Others include the unclassified genera or the genera with the relative abundance less than 1%. On the left is a cluster tree drawn by similarity clustering based on different sample distance matrices.

each sample, we investigated their abundance distribution among the biofilm samples. We found that these shared species mainly belonged to the genera *Massilia*, *Exiguobacterium*, *Bacillus*, *Leptolyngbya*, *Acinetobacter*, *Chloroplast*, *Chroococcidiopsis*, *Spirosoma* and *C0119* (Figure 4c). Together with the unassigned members, they represented the most bacterial populations of each sample, especially M0307 and M0137, indicating their possibility of keystone taxa. Specifically, the shared *Massilia* spp. represented more than 30% of bacterial populations of M0295, M0211, and M0251, which was consistent with the distribution of the most abundant species (Figure 4a). Besides the unassigned members, the shared *Massilia* spp., *Leptolyngbya* spp., and *Bacillus* spp. represented 20, 10, and 5% of the populations of M0137, respectively. In particular,

75% of the populations of M0307 were represented by the shared *Exiguobacterium* spp. (25%), *Acinetobacter* spp. (20%), *Massilia* spp. (18%) and *Bacillus* spp. (12%). These results outlined the keystone taxa of the bacterial populations in each sample. Moreover, the taxonomic tree of the shared 81 OTUs provided an overview of their affiliation and abundances in all the biofilm samples (Figure 4d). Based on the taxonomic tree, the shared *Massilia* spp. represented 28.3% of the total bacterial populations, followed by *Exiguobacterium* (6.1%), *Leptolyngbya* (4.8%), *Bacillus* (4.8%), *Acinetobacter* (4.1%), and *Chloroplast* (1.9%), which confirmed that they were the keystone taxa involved in the biodeterioration of the monuments.

By contrast, the archaeal populations were relatively simple. The most abundant 10 OTUs indicated that they were affiliated to the two

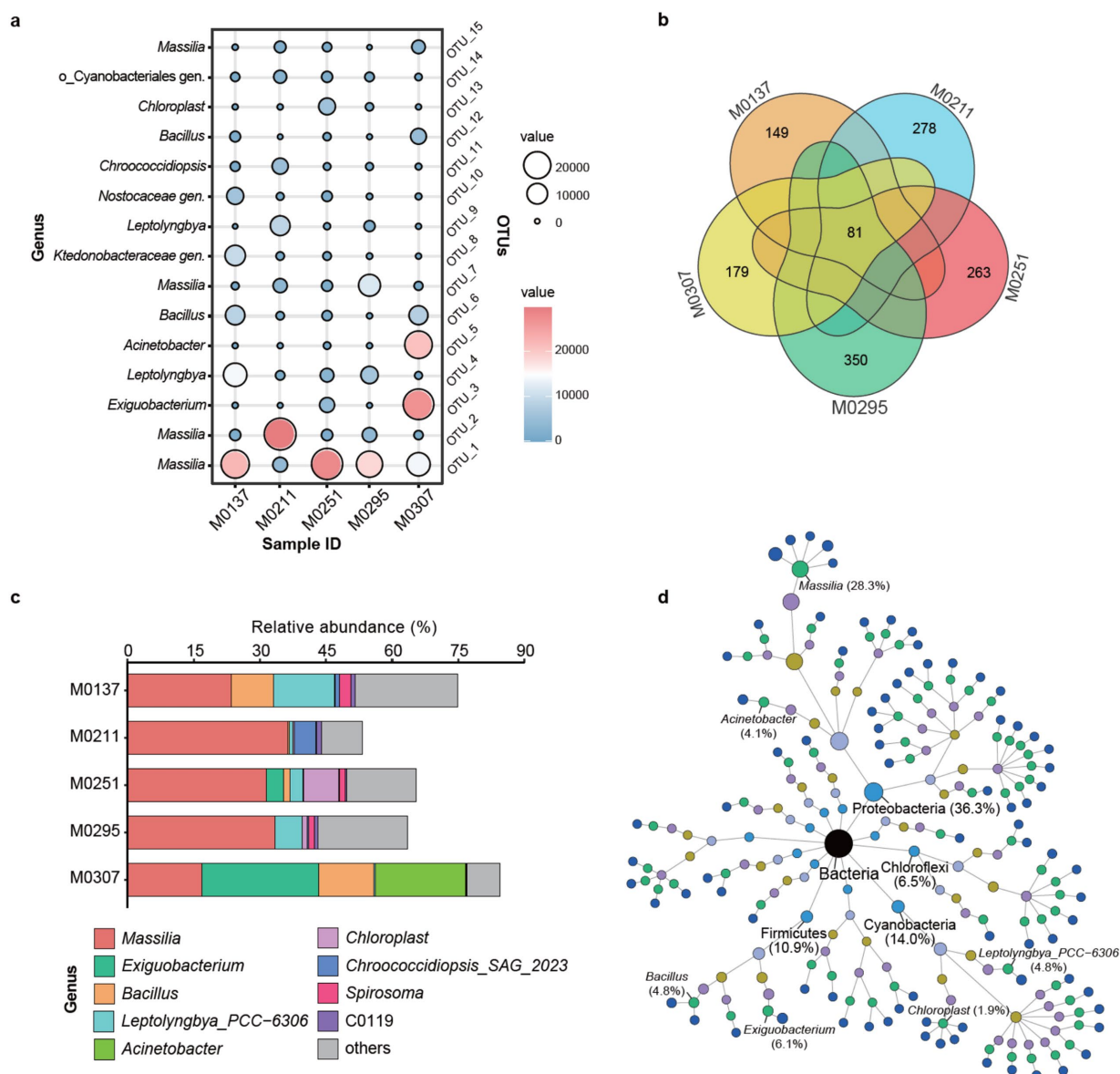


FIGURE 4

Distribution of the keystone bacteria in the epilithic biofilms. **(a)** Distribution of the keystone species across the biofilms. Bubble color and size represent the abundance distribution of each species. **(b)** Venn analysis of common and private bacterial populations among the biofilms. **(c)** Relative abundance distribution of the shared 81 species across the biofilms. Others indicate the unclassified members. **(d)** Taxonomic trees of the shared species. Color of the nodes represents the different taxonomic levels of domain, phylum, class, order, family, genus, and species. Size of the nodes reflects the relative abundance.

genera of the family *Nitrososphaeraceae* (Figure 5a), especially the *Nitrososphaeraceae* gen. 1, suggesting their keystone taxa of the archaeal populations. The archaeal species (OTU-1) predominated the archaeal populations of all the biofilms, especially M0137, M0211, and M0295. Another archaeal species (OTU-2) was the second predominant member of the archaeal populations of M0251. Significantly, the archaeal populations of M0307 were not only predominated by the species (OTU-1) but also by other four species (i.e., OTU-2, OTU-3, OTU-4, and OTU-5), indicating the differentiation of M0307 to the other four biofilms. Moreover, the number of the shared species was four, with M0307 owning the unique species (Figure 5b), further suggesting its differentiation in the archaeal community. Unexpectedly, the common species were all affiliated to *Nitrososphaeraceae* gen. 1 and

represented more than 90% of the archaeal populations of the biofilm samples, except M0307 (Figure 5c). In M0307, the archaeal populations were represented by *Nitrososphaeraceae* gen. 1 (48%) and *Nitrososphaeraceae* gen. 1 (4%). Further, the taxonomic tree of the shared archaeal OTUs confirmed *Nitrososphaeraceae* gen.1 as the keystone taxa in all the biofilm samples (Figure 5d).

3.4 Correlations of keystone taxa with biodeterioration

The Mantel's test showed that the contents of Cl^- , SO_4^{2-} and NO_3^- were significantly correlated with the contents of NH_4^+ ($p < 0.05$) and

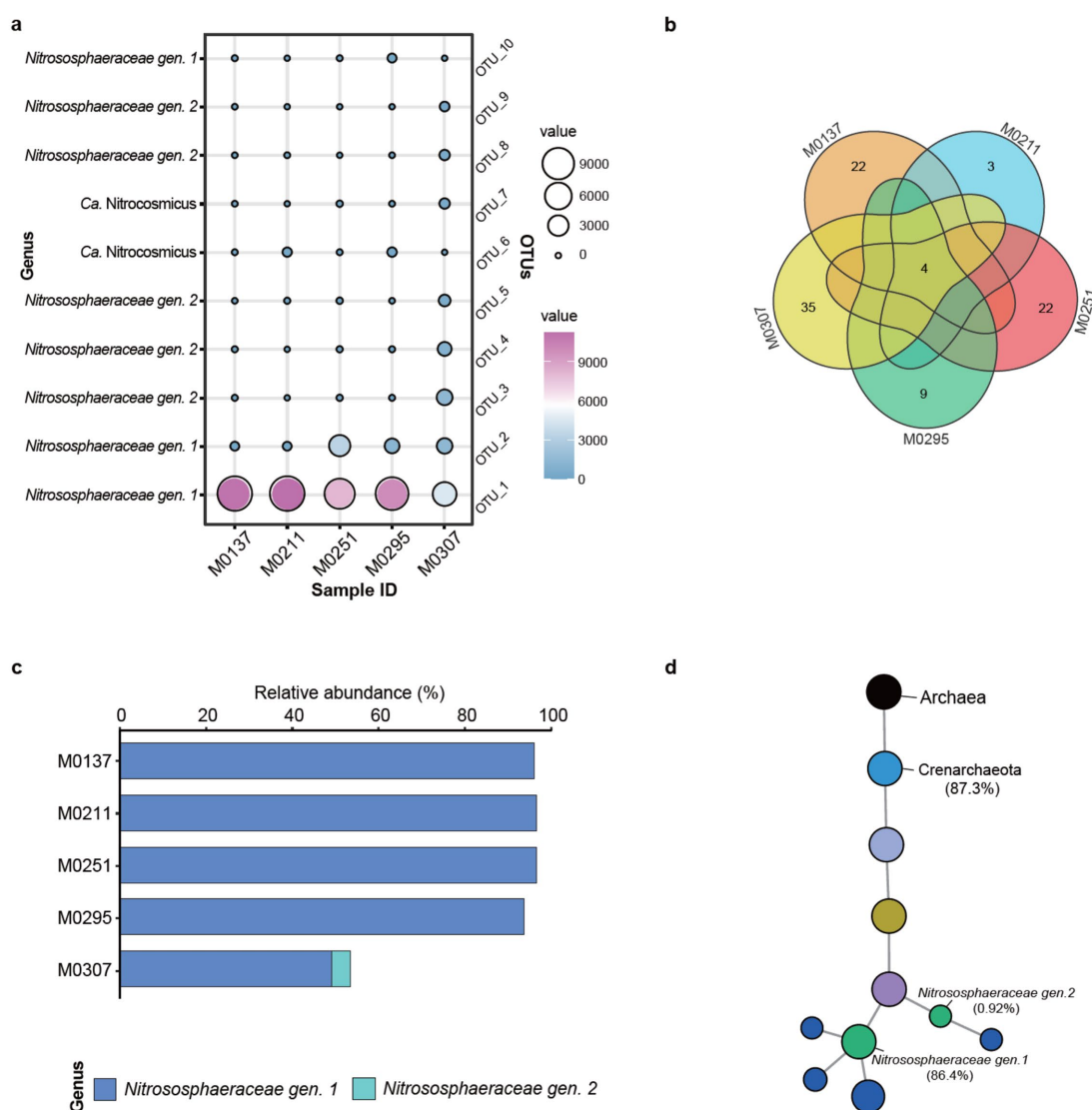


FIGURE 5

Distribution of the keystone archaea in the epilithic biofilms. **(a)** Distribution of the most abundant 10 archaeal species across the biofilms. Bubble color and size represent the abundance distribution of each species. **(b)** Venn analysis of common and private archaeal populations among the biofilms. **(c)** Relative abundance distribution of the shared species across the biofilms. **(d)** Taxonomic trees of the shared species. Color of the nodes represents the different taxonomic levels of domain, phylum, class, order, family, genus, and species. Size of the nodes reflects the relative abundance.

K^+ ($p < 0.01$), instead of Ca^{2+} or Mg^{2+} (Figure 6). Moreover, the content of Cl^- was extremely significantly correlated with the contents of SO_4^{2-} and NO_3^- ($p < 0.001$), while the content of K^+ was significantly correlated with that of NH_4^+ ($p < 0.01$).

In the bacterial keystone taxa (Figure 6a), the genera *Spirosoma*, *Chroococcidiopsis*, and *C0119* were highly significantly correlated with Na^+ , Ca^{2+} , and Mg^{2+} ($p < 0.01$), respectively, indicating they might be the keystone taxa leading to mineral solutions of stone monuments (Keshari and Adhikary, 2014; Yu et al., 2024). Importantly, the two genera *Massilia* and *Bacillus* were solely significantly correlated with NO_2^- , whereas NO_3^- was significantly correlated with *Leptolyngbya*, *Acinetobacter*, *Chloroplast*, and *C0119*, with *Acinetobacter* and *Chloroplast* correlated with NH_4^+ as well, suggesting they were potential keystone bacteria involved in nitrification and/or denitrification (Qiao et al., 2020). Moreover, the most abundant *Nitrososphaeraceae* gen 1 was significantly correlated with NO_3^- , but

we found no potential keystone taxa correlated with SO_4^{2-} (Figure 6b).

3.5 Potential metabolic processes involved in biodeterioration

Generally, there were no significant differences in the total metabolisms in bacterial populations among the samples (Figure 7a), where amino acid metabolism, carbohydrate metabolism, and metabolisms of cofactors and vitamins were the predominant processes. Specifically, the differences were mainly found in C metabolisms rather than N and S metabolisms (Figure 7b). Bacterial populations of the biofilm M0307 were characterized by chemoheterotrophy and aromatic compound degradation. In contrast, bacterial populations of the other four biofilms consistently relied on

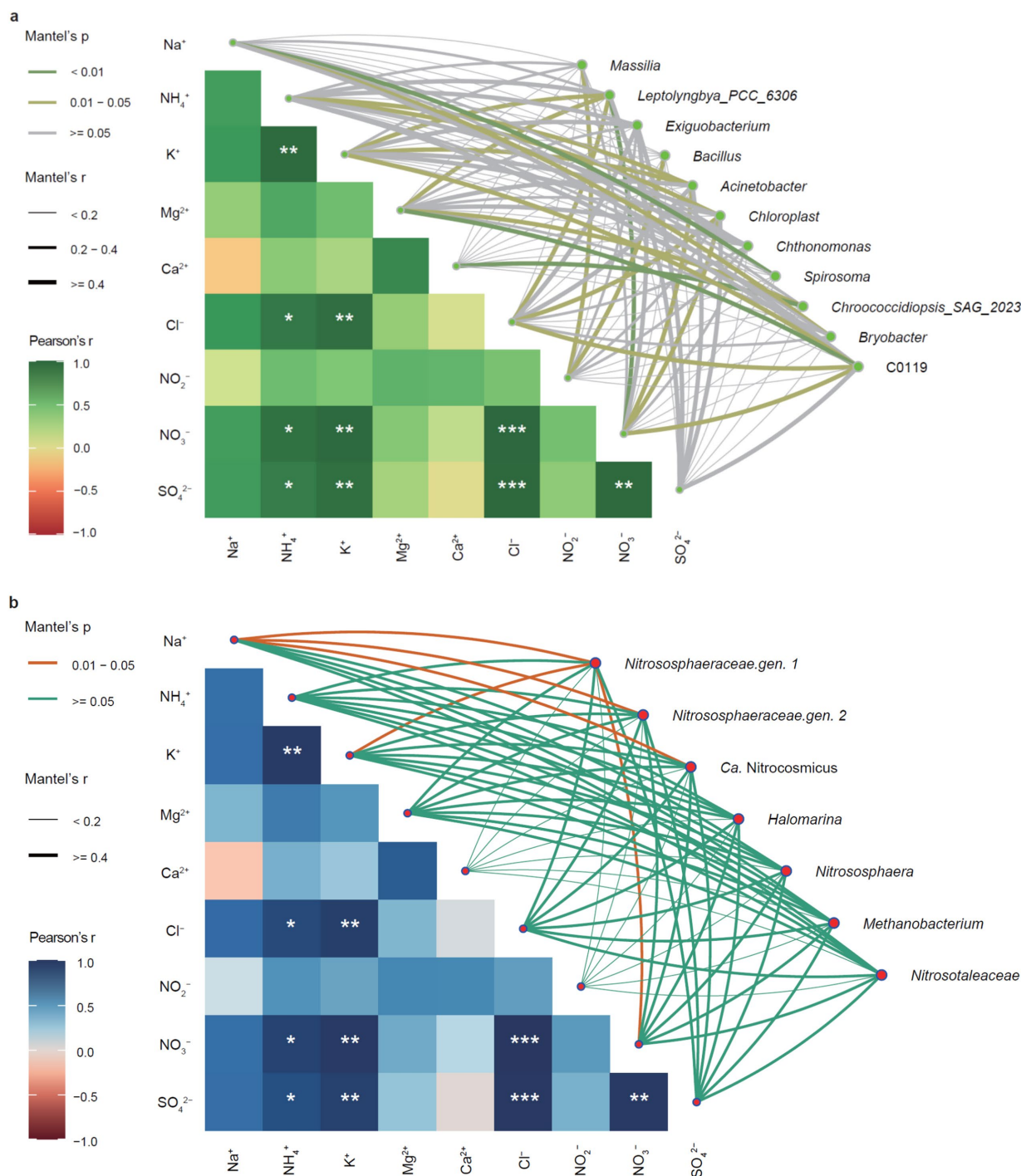


FIGURE 6

Correlation networks between keystone taxa and physicochemical properties. **(a)** Correlations between keystone bacteria and physicochemical properties. **(b)** Correlations between keystone archaea and physicochemical properties. Pearson's coefficient indicates the correlation between different physicochemical properties, whereas the correlation of Mantel's test shows the effect of physicochemical properties on the keystone bacterial and archaeal communities. Line thickness indicates the degree of correlation and p -value represents the degrees of significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

phototrophic processes, corresponding to the dominant phototrophic taxa in epilithic biofilms (Méndez et al., 2024), such as *Leptolyngbya* and *Chroococcidiopsis*.

Similarly, the general metabolisms of archaeal populations among the samples also exhibited no differences, with amino acid

metabolism, carbohydrate metabolism, and metabolisms of cofactors and vitamins predominated over the general metabolisms (Figure 7c). Interestingly, aerobic ammonia oxidation and nitrification were consistent characteristics of archaeal populations of all the samples (Figure 7d), which further

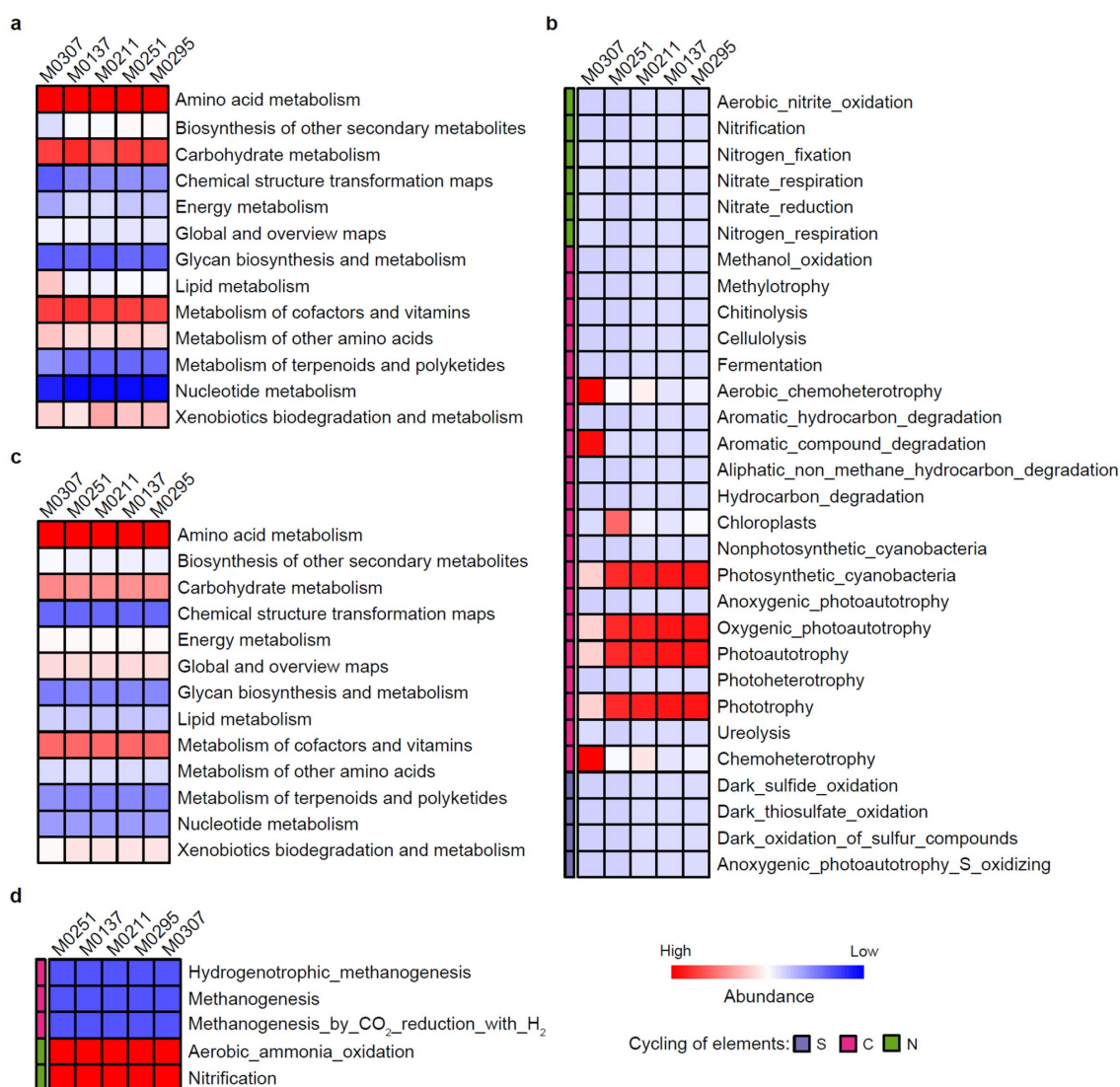


FIGURE 7

Keystone metabolisms of the epilithic biofilms. **(a)** General metabolisms of bacterial populations of the epilithic biofilms. **(b)** Bacterial metabolic processes involved in biogeochemical cycling of C, N, and S. **(c)** General metabolisms of archaeal populations of the epilithic biofilms. **(d)** Archaeal metabolic processes involved in biogeochemical cycling of C, N, and S. The relative abundance of the general metabolisms was predicted by the PICRUST2 analysis, whereas that of the metabolic processes responsible for biogeochemical cycling of C, N, and S was predicted by the FAPROTAX analysis.

confirmed our observations of the keystone taxa of the family *Nitrososphaeraceae* (Figure 6b).

4 Discussion

We used an integrative high-throughput sequencing and comparative metabolomic approach to unravel the biodeterioration of the Leizhou Stone Dog monuments. The divergence and similarity of the microbial composition of the biofilms colonizing the monuments were compared to identify the keystone taxa governing the biodeterioration of each stone monument. The correlation between the keystone taxa and physicochemical properties confirmed the consistency of the observations. In addition, the species-based functional information of the keystone taxa supported

the potential metabolisms leading to the biodeterioration of the monuments.

We examined the contents of soluble salts in the soils deposited at the bottom of the bio-deteriorated monuments to track the potential chemical processes involved in biodeterioration. Collectively, the observations indicate that abundant nitrates and sulfates are accumulated in the soils. The Mantel's test showed that excessive sulfuric and nitric acids might be needed to maintain the residual Ca²⁺ or Mg²⁺ in solution in the soils. Meanwhile, the significantly higher contents of SO₄²⁻ and NO₃⁻ than those of Ca²⁺ and Mg²⁺ could also support this inference (Figure 2). These observations suggest significant differences in the contents of soluble minerals in the soils, indicating that different intensities of deterioration occur on the monuments. Such deterioration could be attributed to both abiotic and biotic processes depending on the environment the monuments

were exposed to (Liu et al., 2022b). The monuments were all in the same yard and suffered the same abiotic impacts (Figure 1). Thus, biodeterioration caused by epilithic biofilms on the monuments would be the most likely reason to explain the differences in these observations.

While physicochemical features elucidated the potential deterioration processes, the divergence of microbial communities highlighted the contributions of biotic deterioration rather than abiotic deterioration because the monuments were exposed to the same environment. Notably, the community compositions across the biofilm samples indicated common and divergent populations for each sample, consistent with our previous observation that the micro-environment determines community structures and biodeterioration processes of epilithic biofilms (Meng et al., 2023). For example, the most abundant *Nitrososphaeraceae* *gen 1* was significantly correlated with NO_3^- (Figure 6b), confirming that they were the keystone taxa responsible for nitrification that have been reported to be responsible for biodeterioration of stone monuments (Meng et al., 2016; Ding et al., 2021). Consistently, the two genera of *Nitrososphaeraceae* and *Ca. Nitrocosmicus* were significantly relevant to Na^+ and K^+ , suggesting they are the keystone taxa responsible for the salt efflorescences of the monuments (Mihajlovski et al., 2019). This highlights the importance of the micro-environment in shaping the biofilm community colonizing stone monuments and their biodeterioration, suggesting the necessity of a case-by-case treatment before any solution is used to protect from microbial attacks (Liu et al., 2022a).

We also predicted the potential metabolisms of the keystone taxa to further identify the C, N, and S metabolic pathways that might be relevant to the biodeterioration. Collectively, the observations of the potential metabolisms were consistent with the keystone taxa in the biofilms colonizing the deteriorated monuments. Thus, we claimed that photoautotrophy and/or chemoheterotrophy of bacterial populations and nitrification of archaeal populations would be the keystone metabolic processes leading to the biodeterioration of the

Leizhou Stone Dog monuments (Figure 8). Future work aims to elucidate causal mechanisms through complementary methods such as mapping multi-omics data into metabolic pathways using chromatographic techniques, stable notable labeling, and microbial isolation studies, especially the keystone taxa. We acknowledge the limitations of relying solely on direct species-based functional prediction for metabolisms involved in biodeterioration, including the inability to differentiate active microbes or determine metabolites, as well as issues with sampling errors. Moving forward, implementing this culture-independent technique alongside culture-dependent and chemical techniques could provide a better understanding of the complex biodeterioration processes of stone monuments (Liu et al., 2022a).

Furthermore, incorporating longer timescales and expanded climate representation will further refine our understanding of the succession of the most relevant keystone taxa and metabolisms involved in the biodeterioration of the stone monuments. For example, continuous *in-situ* detection and comparison of the incident radiation on the monument surfaces between different seasons could greatly benefit a full explanation why the abundance or photosynthetic yields of photoautotrophic microorganisms evolves in the biofilms (Figueroa et al., 2017). Moreover, we observed some cyanobacterial inhabitants of low light (Hauer et al., 2015), such as *Leptolyngbya* and *Chroococcidiopsis*, which were also detected on other cultural heritage stone buildings in tropical and subtropical climates (Keshari and Adhikary, 2014; Ortega-Morales et al., 2019), indicating that an upper-to-lower layer analysis of microbial composition is required to confirm the divergence of spatial distribution in the epilithic biofilms (Czerw-Marcinkowska and Massalski, 2018). Interestingly, despite having some abundant genera in common with other observations (Silva et al., 2022), such as *Spirosoma* and *Massilia*, their contribution to biodeterioration of stone monuments is still unclear. However, the genus *Massilia* are typical soil bacteria of the *Oxalobacteraceae*, but their members are often pigmented bacteria (Yang et al., 2019), indicating their

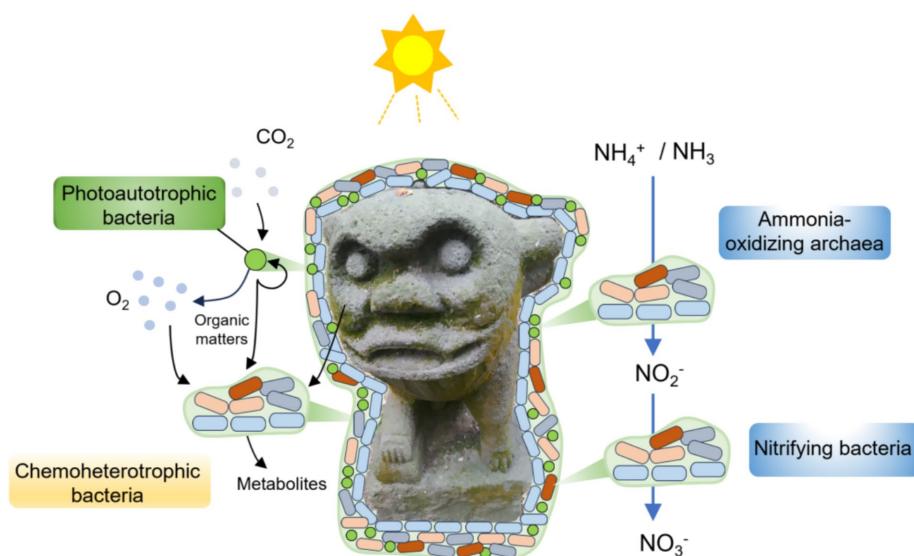


FIGURE 8

Keystone taxa and metabolisms of epilithic biofilms involved in the biodeterioration of the Leizhou Stone Dog monuments.

involving in discoloration of stone monuments. By integrating keystone taxa and metabolism formulas into future mechanistic models of biodeterioration diagnosis and therapy, we can gain a focused picture of measures for the sustainable conservation of stone monuments from microbial damage.

In conclusion, we have successfully identified the keystone taxa and metabolisms involved in microbial biodeterioration of the Leizhou Stone Dog monuments. We propose an integrative culture-independent and culture-dependent approach to the necessity of a case-by-case diagnosis of the keystone taxa and metabolisms before any therapy, shedding light on the complex biodeterioration processes of stone monuments and advancing the conservation science of cultural heritage.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: <https://www.ncbi.nlm.nih.gov/>, accession number: PRJNA1170002.

Author contributions

CM: Writing – original draft, Investigation, Software, Formal analysis, Data curation, Methodology. XZ: Writing – original draft, Software, Investigation, Methodology, Conceptualization. FW: Conceptualization, Validation, Writing – review & editing. XL: Validation, Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This study was supported

by the National Natural Science Foundation of China (Grant Nos. 32100101 and 32370105) and the Fundamental Research Funds for the Central Universities (Grant Nos. 30922010305 and 1225011021289).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1600865/full#supplementary-material>

References

- Abeliovich, A. (2006). The nitrite oxidizing Bacteria. *Prokaryotes* 5, 861–872. doi: 10.1007/0-387-30745-1_41
- Banerjee, S., Schlaeppi, K., and Van Der Heijden, M. G. A. (2018). Keystone taxa as drivers of microbiome structure and functioning. *Nat. Rev. Microbiol.* 16, 567–576. doi: 10.1038/s41579-018-0024-1
- Bei, Q., Reitz, T., Schädler, M., Hodgskiss, L. H., Peng, J., Schnabel, B., et al. (2024). Metabolic potential of *Nitrososphaera*-associated clades. *ISME J.* 18:wrae086. doi: 10.1093/ismejo/wrae086
- Bellinzoni, A. M., Caneva, G., and Ricci, S. (2003). Ecological trends in travertine colonisation by pioneer algae and plant communities. *Int. Biodeterior. Biodegradation* 51, 203–210. doi: 10.1016/S0964-8305(02)00172-5
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghali, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9
- Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869
- Czerwik-Marcinkowska, J., and Massalski, A. (2018). "Diversity of cyanobacteria on limestone caves" in *Cyanobacteria*. ed. A. Tiwari (Rijeka: IntechOpen).
- Daims, H., Lückner, S., and Wagner, M. (2016). A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends Microbiol.* 24, 699–712. doi: 10.1016/j.tim.2016.05.004
- Dick, W. A., and Tabatabai, M. A. (1979). Ion chromatographic determination of sulfate and nitrate in soils. *Soil Sci. Soc. Am. J.* 43, 899–904. doi: 10.2136/sssaj1979.03615995004300050016x
- Ding, X., Lan, W., Li, Y., Yan, A., Katayama, Y., Koba, K., et al. (2021). An internal recycling mechanism between ammonia/ammonium and nitrate driven by ammonia-oxidizing archaea and bacteria (AOA, AOB, and Comammox) and DNRA on Angkor sandstone monuments. *Int. Biodeterior. Biodegradation* 165:105328. doi: 10.1016/j.ibiod.2021.105328
- Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6
- Figuerola, F. L., Álvarez-Gómez, F., Del Rosal, Y., Celis-Plá, P. S. M., González, G., Hernández, M., et al. (2017). In situ photosynthetic yields of cave photoautotrophic biofilms using two different pulse amplitude modulated fluorometers. *Algal Res.* 22, 104–115. doi: 10.1016/j.algal.2016.12.012
- Hauer, T., Mülhsteinová, R., Bohunická, M., Kaštovský, J., and Mareš, J. (2015). Diversity of cyanobacteria on rock surfaces. *Biodivers. Conserv.* 24, 759–779. doi: 10.1007/s10531-015-0890-z
- Herren, C. M., and McMahon, K. D. (2018). Keystone taxa predict compositional change in microbial communities. *Environ. Microbiol.* 20, 2207–2217. doi: 10.1111/1462-2920.14257
- Huang, H. (2021). *linkET: Everything is linkable. R package version 0.0.3*. Available online at: <https://github.com/Hy4m/linkET> (Accessed June 06, 2021).

- Keshari, N., and Adhikary, S. P. (2014). Diversity of cyanobacteria on stone monuments and building facades of India and their phylogenetic analysis. *Int. Biodeterior. Biodegradation* 90, 45–51. doi: 10.1016/j.ibiod.2014.01.014
- Liu, X., Koestler, R. J., Warscheid, T., Katayama, Y., and Gu, J.-D. (2020). Microbial deterioration and sustainable conservation of stone monuments and buildings. *Nat. Sustain.* 3, 991–1004. doi: 10.1038/s41893-020-00602-5
- Liu, X., Qian, Y., Wang, Y., Wu, F., Wang, W., and Gu, J.-D. (2022a). Innovative approaches for the processes involved in microbial biodeterioration of cultural heritage materials. *Curr. Opin. Biotechnol.* 75:102716. doi: 10.1016/j.copbio.2022.102716
- Liu, X., Qian, Y., Wu, F., Wang, Y., Wang, W., and Gu, J.-D. (2022b). Biofilms on stone monuments: biodeterioration or bioprotection? *Trends Microbiol.* 30, 816–819. doi: 10.1016/j.tim.2022.05.012
- Louca, S., Jacques, S. M. S., Pires, A. P. F., Leal, J. S., Srivastava, D. S., Parfrey, L. W., et al. (2016). High taxonomic variability despite stable functional structure across microbial communities. *Nat. Ecol. Evol.* 1:0015. doi: 10.1038/s41559-016-0015
- Luo, L., and Gu, J.-D. (2025). Bridging the link between microbial biofilm and biodeterioration in cultural heritage research. *Int. Biodeterior. Biodegradation* 198:106001. doi: 10.1016/j.ibiod.2025.106001
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200
- Méndez, A., Sanmartín, P., Balboa, S., and Trueba-Santiso, A. (2024). Environmental proteomics elucidates phototrophic biofilm responses to ornamental lighting on stone-built heritage. *Microb. Ecol.* 87:147. doi: 10.1007/s00248-024-02465-1
- Meng, H., Luo, L., Chan, H. W., Katayama, Y., and Gu, J.-D. (2016). Higher diversity and abundance of ammonia-oxidizing archaea than bacteria detected at the Bayon Temple of Angkor Thom in Cambodia. *Int. Biodeterior. Biodegradation* 115, 234–243. doi: 10.1016/j.ibiod.2016.08.021
- Meng, S., Qian, Y., Liu, X., Wang, Y., Wu, F., Wang, W., et al. (2023). Community structures and biodeterioration processes of epilithic biofilms imply the significance of micro-environments. *Sci. Total Environ.* 876:162665. doi: 10.1016/j.scitotenv.2023.162665
- Mihajlovski, A., Lepinay, C., Mirval, A. L., Tournon, S., Boust, F., and Di Martino, P. (2019). Characterization of the archaeal and fungal diversity associated with gypsum efflorescences on the walls of the decorated Sorcerer's prehistoric cave. *Ann. Microbiol.* 69, 1071–1078. doi: 10.1007/s13213-019-01506-2
- Mitchell, R., and Gu, J.-D. (2000). Changes in the biofilm microflora of limestone caused by atmospheric pollutants. *Int. Biodeterior. Biodegradation* 46, 299–303. doi: 10.1016/S0964-8305(00)00105-0
- Ortega-Morales, O., Montero-Muñoz, J. L., Baptista Neto, J. A., Beech, I. B., Sunner, J., and Gaylarde, C. (2019). Deterioration and microbial colonization of cultural heritage stone buildings in polluted and unpolluted tropical and subtropical climates: a meta-analysis. *Int. Biodeterior. Biodegradation* 143:104734. doi: 10.1016/j.ibiod.2019.104734
- Pires, A. C. C., Cleary, D. F. R., Almeida, A., Cunha, A., Dealtry, S., Mendonça-Hagler, L. C. S., et al. (2012). Denaturing gradient gel electrophoresis and barcoded pyrosequencing reveal unprecedented archaeal diversity in mangrove sediment and rhizosphere samples. *Appl. Environ. Microbiol.* 78, 5520–5528. doi: 10.1128/AEM.00386-12
- Qian, Y., Liu, X., Hu, P., Gao, L., and Gu, J.-D. (2024). Identifying the major metabolic potentials of microbial-driven carbon, nitrogen and sulfur cycling on stone cultural heritage worldwide. *Sci. Total Environ.* 954:176757. doi: 10.1016/j.scitotenv.2024.176757
- Qiao, Z., Sun, R., Wu, Y., Hu, S., Liu, X., and Chan, J. (2020). Microbial heterotrophic nitrification-aerobic denitrification dominates simultaneous removal of aniline and ammonium in aquatic ecosystems. *Water Air Soil Pollut.* 231:112. doi: 10.1007/s11270-020-04476-3
- Rossi, F., Micheletti, E., Bruno, L., Adhikary, S. P., Albertano, P., and De Philippis, R. (2012). Characteristics and role of the exocellular polysaccharides produced by five cyanobacteria isolated from phototrophic biofilms growing on stone monuments. *Biofouling* 28, 215–224. doi: 10.1080/08927014.2012.663751
- Silva, N. C., Madureira, A. R., Pintado, M., and Moreira, P. R. (2022). Biocontamination and diversity of epilithic bacteria and fungi colonising outdoor stone and mortar sculptures. *Appl. Microbiol. Biotechnol.* 106, 3811–3828. doi: 10.1007/s00253-022-11957-4
- Singh, D., and Singh, B. (2020). Investigating the impact of data normalization on classification performance. *Appl. Soft Comput.* 97:105524. doi: 10.1016/j.asoc.2019.105524
- Tamaki, H., Wright, C. L., Li, X., Lin, Q., Hwang, C., Wang, S., et al. (2011). Analysis of 16S rRNA amplicon sequencing options on the Roche/454 next-generation titanium sequencing platform. *PLoS One* 6:e25263. doi: 10.1371/journal.pone.0025263
- Ten-Domènech, I., Moreno-Torres, M., Castell, J. V., Quintas, G., and Kuligowski, J. (2021). Extracting consistent biological information from functional results of metabolomic pathway analysis using the Mantel's test. *Anal. Chim. Acta* 1187:339173. doi: 10.1016/j.aca.2021.339173
- Villa, F., Wu, Y.-L., Zerbini, A., and Cappitelli, F. (2022). In living color: pigment-based microbial ecology at the mineral-air interface. *Bioscience* 72, 1156–1175. doi: 10.1093/biosci/biac091
- Wang, L., Huang, J., Sanmartín, P., Martino, P. D., Wu, F., Urzì, C. E., et al. (2025). Water determines geomicrobiological impact on stone heritage. *Nat. Geosci.* 18, 108–111. doi: 10.1038/s41561-024-01631-x
- Wang, Y., and Liu, X. (2021). Sulfur-oxidizing bacteria involved in the blackening of basalt sculptures of the Leizhou Stone Dog. *Int. Biodeterior. Biodegradation* 159:105207. doi: 10.1016/j.ibiod.2021.105207
- Wang, Y., Zhang, H., Liu, X., Liu, X., and Song, W. (2021). Fungal communities in the biofilms colonizing the basalt sculptures of the Leizhou Stone Dogs and assessment of a conservation measure. *Herit. Sci.* 9:36. doi: 10.1186/s40494-021-00508-1
- Wu, F., Zhang, Y., He, D., Gu, J.-D., Guo, Q., Liu, X., et al. (2021). Community structures of bacteria and archaea associated with the biodeterioration of sandstone sculptures at the Beishiku Temple. *Int. Biodeterior. Biodegradation* 164:105290. doi: 10.1016/j.ibiod.2021.105290
- Yang, E., Zhao, M., Li, S., Wang, Y., Sun, L., Liu, J., et al. (2019). *Massilia atriviolacea* sp. nov., a dark purple-pigmented bacterium isolated from soil. *Int. J. Syst. Evol. Microbiol.* 69, 2135–2141. doi: 10.1099/ijsem.0.003449
- Yu, C. X., Wang, P., Ding, H. Y., Hu, Y., Wang, F., Chen, H. P., et al. (2024). Effects of the epiphytic patterns on endophytes and metabolites of *Dendrobium nobile* Lindl. *Front. Plant Sci.* 15:1326998. doi: 10.3389/fpls.2024.1326998
- Zhang, Y., Wu, F., Gu, J.-D., He, K., Fang, Z., Liu, X., et al. (2024). Dominance by cyanobacteria in the newly formed biofilms on stone monuments under a protective shade at the Beishiku Temple in China. *Environ. Res.* 251:118576. doi: 10.1016/j.envres.2024.118576



OPEN ACCESS

EDITED BY

Virginia Helena Albarracín,
CONICET Center for Electron Microscopy
(CIME), Argentina

REVIEWED BY

Fasi Wu,
Dunhuang Research Academy, China
Graciela Pucci,
National University of Patagonia San Juan
Bosco, Argentina

*CORRESPONDENCE

Jiao Pan
✉ jiaopan@ustb.edu.cn

[†]These authors have contributed equally to
this work

RECEIVED 14 April 2025

ACCEPTED 19 May 2025

PUBLISHED 03 June 2025

CITATION

Wang Y, Wang Y, Guan Z, Wang Z, Duan Y,
Min C, Zhong Y, Hou L and Pan J (2025)
Analysis and prevention of microbial
degradation of shadow puppetry artifacts
preserved in the National Shadow Puppetry
Museum in Chengdu.
Front. Microbiol. 16:1611451.
doi: 10.3389/fmicb.2025.1611451

COPYRIGHT

© 2025 Wang, Wang, Guan, Wang, Duan, Min,
Zhong, Hou and Pan. This is an open-access
article distributed under the terms of the
[Creative Commons Attribution License
\(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction
in other forums is permitted, provided the
original author(s) and the copyright owner(s)
are credited and that the original publication
in this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Analysis and prevention of microbial degradation of shadow puppetry artifacts preserved in the National Shadow Puppetry Museum in Chengdu

Yu Wang^{1†}, Yuanyuan Wang^{1†}, Zhiqian Guan¹, Zeao Wang¹,
Yangbo Duan^{2,3}, Chen Min³, Yuhan Zhong³, Lilong Hou⁴ and
Jiao Pan^{1*}

¹Key Laboratory of Archaeomaterials and Conservation, Ministry of Education, Institute of Cultural Heritage and History of Science and Technology, University of Science and Technology Beijing, Beijing, China, ²School of Archaeology and Museology, Sichuan University, Chengdu, China, ³Chengdu Museum (National Shadow Puppetry Museum in Chengdu), Chengdu, China, ⁴Department of Microbiology, College of Life Sciences, Nankai University, Tianjin, China

Shadow puppetry, an integral and crucial component of China's intangible cultural heritage, currently faces the significant threat of microbial degradation. This is primarily due to the organic materials used in its artifacts. This study centers on the shadow puppets housed in the National Shadow Puppetry Museum in Chengdu. By employing Scanning Electron Microscopy (SEM) and high-throughput sequencing techniques, it has revealed a diverse array of co-existing microorganisms on the surfaces of these puppets. These include species from genera such as *Aspergillus*, *Streptomyces*, *Nocardiopsis*, *Pseudomonas*, and *Saccharopolyspora*, among others. Eleven microbial species were successfully isolated, wherein four were identified as predominant: *Pseudomonas* sp. WH. S-B1, *Streptomyces* sp. WH. S-B2, *Nocardiopsis* sp. WH. S-B6, and *Aspergillus fumigatus* WH. S-F2. Notably, these four strains demonstrated the ability to degrade collagen. The antimicrobial experiment results indicated that 0.3% isothiazolinone-based antimicrobial agents BC01 and 50 mg/mL carvacrol exhibited a certain degree of antimicrobial activity against these predominant strains. Overall, this research provides a robust foundation for the conservation of shadow puppet artifacts. It does so by thoroughly analyzing the mechanisms of microbial degradation and screening effective antimicrobial agents.

KEYWORDS

shadow puppetry artifacts, microbial degradation, microbial community, antimicrobial agents, cultural relics preservation

1 Introduction

Shadow puppetry, a quintessential medium of China's intangible cultural heritage, is a multifaceted art form that seamlessly integrates painting, carving, opera, and the esthetics of light and shadow (Liang et al., 2023; Huang et al., 2015; Yang et al., 2024). The artifacts are primarily crafted from animal hides, such as cowhide and sheepskin, through traditional processes encompassing tanning, carving, and dyeing (Duan et al., 2024). Abundant in collagen and organic dyes, these artifacts act as "living fossils," meticulously documenting the evolution of ancient Chinese social culture, folk beliefs, and artisanal techniques (Lei et al., 2023; Li and Cao, 2021; Liu et al., 2019). The existing

shadow puppetry artifacts from the Ming and Qing dynasties not only boast high artistic and esthetic value but also serve as irreplaceable physical evidence for the study of ancient social and cultural life (Prahmana and Istiandaru, 2021; Padovani, 2023; Coar and Usta, 2009). Nevertheless, due to the organic composition of shadow puppetry artifacts, they are highly vulnerable to damage caused by temperature and humidity fluctuations, pollutant erosion, and microbial activity during long-term preservation (Zhang et al., 2024). Among these threats, microbial-induced material degradation has become a critical concern endangering the preservation of these cultural relics. Conducting systematic research not only overcomes the empirical limitations of traditional cultural relic preservation but also provides theoretical frameworks for elucidating the degradation mechanisms of organic cultural heritage (Sun et al., 2024; Sterflinger and Piñar, 2013).

Recent investigations have demonstrated that microbial communities inhabiting the surface of shadow puppet artifacts can gradually deteriorate the collagen fiber network of leather. This occurs through the secretion of metabolic byproducts such as proteases and lipases, ultimately resulting in a loss of mechanical integrity and the detachment of pigment layers (Perini et al., 2019; Zugno et al., 2011; Strzelczyk et al., 1987). International research efforts have identified key degrading microbial groups. This includes fungi from the *Aspergillus* and *Penicillium* genera, as well as *Bacillus* bacteria. Their metabolic activities exhibit significant correlations with the pH and moisture levels of the leather (Sterflinger, 2010). The most recent research, utilizing metagenomic sequencing technology, has unveiled the spatial heterogeneity of microbial communities. It has revealed that microenvironments within the crevices of artifacts are particularly susceptible to the formation of synergistic degradation systems involving actinobacteria and fungi (Shi et al., 2020; Gadd et al., 2024; Wang et al., 2023).

Nevertheless, the current systematic research on the microbial deterioration of shadow puppet relics remains fraught with several limitations. Firstly, the dynamic fluctuations of microbial communities under varying environmental conditions have not been comprehensively explored. Secondly, existing preventive and control measures predominantly draw on the experiences from the conservation of other cultural relic types. As a result, their specificity and efficacy demand further in-depth investigation (Zhang et al., 2022). Conducting a holistic analysis of microbial deterioration in shadow puppet relics and formulating scientifically robust prevention and control strategies are of critical significance for the long-term conservation and cultural heritage succession of these invaluable artifacts.

This research is centered on the shadow puppets housed in the National Shadow Puppetry Museum in Chengdu. The majority of these shadow puppet cultural relics were donated by individuals from diverse backgrounds. Consequently, their preservation conditions vary significantly, some of these artifacts are afflicted with microbial contamination. Through painstaking investigation and sampling, a systematic examination was carried out on the microbial degradation processes occurring on the surfaces of these shadow puppet artifacts. Subsequently, appropriate antimicrobial agents were screened, thereby providing a foundation for the conservation and protection of shadow puppet artifacts.

2 Materials and methods

2.1 Sample collection and microbial investigation

In October 2022 and March 2023, a microbiological survey was conducted on shadow puppet artifacts stored in the repository of the National Shadow Puppetry Museum in Chengdu. Crafted entirely from cowhide, these shadow puppet samples were sourced from various rural areas, primarily including Shaanxi, Sichuan, and Hebei. The majority of the shadow puppets date back to the period spanning from the Republic of China era to modern and contemporary times. The artifacts were housed in a climate-controlled warehouse. The temperature was maintained at $20 \pm 2^\circ\text{C}$, with a daily variation of less than 2°C , while the humidity was kept at $50 \pm 5\%$, with a daily fluctuation of less than 5%. The study detected microbial colonization on the surfaces of certain cultural relics (Figure 1). To evaluate the severity of microbial degradation, a grading system was established based on the colony coverage area. Samples S1–S5 were classified as having mild contamination, S6–S10 as moderate, and S11–S16 as severe. Microbial plaques were collected from the shadow puppet surface using sterile cotton swabs and then streaked onto Potato Dextrose Agar (PDA), Luria-Bertani (LB), and Gauze's No. 1 media plates. These plates were transported to the laboratory for subsequent microbial isolation and purification. Meanwhile, surface plaques were carefully scraped with a sterile scalpel and placed into sterile EP tubes. These samples were transported on dry ice to the laboratory and stored at -80°C for subsequent amplicon sequencing and metagenomic analysis. During the survey, the ambient temperature in the repository was 18°C with a relative humidity of 56%.

2.2 Scanning electron microscope observation

Microbial samples were collected from the surfaces of shadow puppet artifacts and adhered to a substrate using carbon conductive adhesive. The samples were then dried in a desiccator. Once dried, each sample was mounted onto an SEM specimen stage. Gold sputtering was performed at a current of 24 mA for 300 s. Subsequently, the samples were examined using a SEM, and images were captured. The measurement parameters were set as follows: electron high tension (EHT) at 15.0 kV, working distance (WD) ranging from 9.5 to 10.7 mm, and magnification (Mag) between 2,000 $\times$ and 10,000 $\times$.

2.3 Isolation and identification of culturable microorganisms

For the isolation and cultivation of fungi, PDA medium (1.2% potato extract, 2% glucose, 2% agar) was employed. LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 2% agar, pH7.2) was utilized for bacterial isolation and cultivation. Gauze's No.1 Medium (1% soluble starch, 0.2% KNO_3 , 0.05% $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2% agar, pH7.5) served as the growth medium for actinomycetes. These microorganisms were incubated under specific conditions: fungi were cultured at 28°C for 5 days, bacteria at



FIGURE 1

Microbial plaque on shadow puppet relics. S1–S5 were categorized as having mild contamination, S6–S10 exhibited moderate contamination, and S11–S16 showed severe contamination.

37°C for 2 days, and actinomycetes at 28°C for 10 days. After the incubation period, all microbial colonies that developed on the media were systematically isolated and purified. Genomic DNA was then extracted from these pure cultures and amplified using polymerase chain reaction (PCR) to generate PCR products. For fungal amplification, the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') were used, while the bacterial amplification employed primers 341F (5'-CCTACGGGAGG CAGCAG-3') and 907R (5'-CCCCGTCAATTCATTTGAGTTT-3'). The PCR reaction conditions remained uniform across all samples, with the following reaction program: an initial denaturation step at 98°C for 3 min, followed by 33 cycles of 98°C for 10 s, 57°C for 10 s, and 72°C for 50 s. A final extension step was conducted at 72°C for 5 min, and the samples were held at 4°C. The resulting PCR products were sent to GENEWIZ (Beijing, China) for sequencing. Subsequently, the sequence homology of the amplified fragments was analyzed using tools available on the National Center for Biotechnology Information (NCBI) platform. The obtained sequences have been deposited in GenBank for public access and further research.

2.4 Amplicon sequencing and metagenomic sequencing analysis

Samples S1–S10 exhibited relatively mild microbial contamination, and their microbial compositions were analyzed using amplicon sequencing. Conversely, samples S11–S16 presented more severe

microbial challenges. To comprehensively examine both their microbial communities and the leather degradation processes mediated by these microorganisms, metagenomic sequencing was employed. Total genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, Germany; Cat. No. 47014), strictly following the manufacturer's protocols. The extracted DNA samples were then submitted to NovoMagic Technology Co., Ltd. (Beijing, China) for sequencing. The raw sequencing data are publicly available in the NCBI Sequence Read Archive (SRA) under the study accession number PRJNA1231699 and PRJNA1230850.

2.5 Detection of microbial collagen degradation ability

When microorganisms were inoculated into a medium where gelatin served as the sole nitrogen source (comprising 1% gelatin, 1% glucose, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% NaCl, 2% agar), their growth on this medium indicated their capacity to degrade collagen (Osathanunkul et al., 2013; Fuente et al., 2024).

2.6 Antimicrobial experiment

Building on previous experimental findings and relevant literature (Wang et al., 2024; Li et al., 2025), the antimicrobial agents selected for this study were the isothiazolinone-based agent BC01 and carvacrol,

TABLE 1 Antimicrobial agents used in the study.

Antimicrobial agents	Main components	Concentration	Solvent	Manufacturer
BC01	3% Isothiazolinone	0.3% (m/v)	H ₂ O	Beijing Baicheng Wangda Biotechnology Co., Ltd.
Carvacrol	Carvacrol	50 mg/mL	DMSO	Macklin Biochemical Technology Co., Ltd.

a principal component of a specific plant essential oil (Table 1). The experimental procedure was as follows: First, microorganisms were uniformly spread across the appropriate culture medium. Subsequently, four sterile filter paper disks, each with a diameter of 7 mm, were placed on the medium's surface. Then, 5 μ L of each antimicrobial agent, along with the negative control solution, were separately dispensed onto the filter paper disks. Finally, the inoculated culture medium was transferred to the corresponding incubator for cultivation. The presence and size of inhibition zones were observed to assess the antimicrobial efficacy of the tested agents.

3 Results

3.1 SEM results of samples from the surface of shadow puppet cultural relics

SEM analysis provided conclusive evidence for the presence of microbial hyphae and spores (Figure 2). Significantly, both fungal and actinomycete species were simultaneously detected in multiple samples, including S1, S2, S7, and S14. This finding indicated that diverse microbial community inhabited the surface of shadow puppet cultural relics. These results strongly suggested that the visible microbial colonies on the artifacts are the result of complex synergistic interactions among multiple microbial species, rather than the growth of a single organism. In addition to microbial structures, SEM imaging also revealed the presence of insects on the surfaces of the shadow puppets (Supplementary Figure S1). These insects, when crawling on the puppets, can inadvertently transport microbial hyphae and spores, thereby facilitating the spread and exacerbating the contamination of these cultural artifacts by microorganisms. This biotic interaction represents a significant threat to the long-term preservation of shadow puppet cultural relics.

3.2 Microbial composition on the surface of the shadow puppet cultural relics

High-throughput sequencing analysis of samples S1–S16 (Supplementary Figures S2–S4) revealed the presence of diverse microbial communities. Fungi predominantly belonging to the phylum Ascomycota and bacteria primarily classified under Actinobacteria were the dominant taxonomic groups on the surfaces of these cultural relics (Supplementary Figures S2–S4). Specifically, Firmicutes accounted for 14.22% of the prokaryotic community in samples S1–S5 (Supplementary Figure S2B), Proteobacteria constituted 30.68% of prokaryotes in samples S6–S10 (Supplementary Figure S3B), and Pseudomonadota represented 5.05% of the total microbial population in samples S11–S16

(Supplementary Figure S4). In-depth taxonomic profiling demonstrated significant variations in the dominant microbial communities across different shadow puppet artifacts. Samples S1–S5 shared similar microbial profiles, with *Aspergillus* (fungi) and *Streptomyces* (bacteria) as the predominant genera (Figures 3A,C). In contrast, samples S6–S10 exhibited notable heterogeneity: *Aspergillus* and *Pseudomonas* were enriched in S6; *Pseudomonas* was the dominant genus in S7; S8 was characterized by the co-occurrence of *Aspergillus*, *Microascus*, *Pseudomonas*, and *Nocardia*, S9 featured elevated abundances of *Aspergillus*, *Streptomyces*, and *Pseudomonas*; and S10 was dominated by *Aspergillus*, *Arachnomyces*, and *Streptomyces* (Figures 3B,D). Metagenomic sequencing of samples S11–S16 further identified prokaryote-dominated communities, with *Nocardiopsis*, *Streptomyces*, *Saccharopolyspora*, *Brevibacterium*, and *Brachybacterium* as the key taxonomic representatives (Figure 4). These findings highlight the complex and diverse microbial ecosystems associated with shadow puppet cultural relics, which may have implications for their conservation and preservation.

3.3 Database annotation

To elucidate the potential degradation mechanisms of microorganisms on shadow puppets, the sequencing results were annotated across multiple databases. Analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database revealed that metabolic pathways represented a significant proportion in each sample, ranking among the primary functional categories. This finding underscores the critical role of metabolic functions within these microbial communities (Figure 5A). Functional annotation based on the eggNOG database demonstrated that, while there was a degree of similarity in the overall composition of functional categories across samples, notable variations in specific proportions were observed. Transcription, amino acid transport, metabolism, and related categories were consistently highly represented in multiple samples. For example, in sample S11, the category of unknown function was the most abundant, followed by transcription, amino acid transport and metabolism. In contrast, sample S12 showed elevated proportions of carbohydrate transport and metabolism, inorganic ion transport and metabolism, among others (Figure 5B). Examination of the Comprehensive Antibiotic Resistance Database (CARD) revealed significant differences in the relative abundance and composition of antibiotic-resistance genes. Sample S13 exhibited the highest relative abundance, primarily attributable to genes such as *vanW* gene in *vanI* cluster (Drug Class: glycopeptide antibiotic), *vanX* gene in *vanO* cluster (Drug Class: glycopeptide antibiotic), and *vanY* gene in *vanA* cluster (Drug Class: glycopeptide antibiotic). Sample S15 also had a relatively high relative abundance with a complex gene composition, including

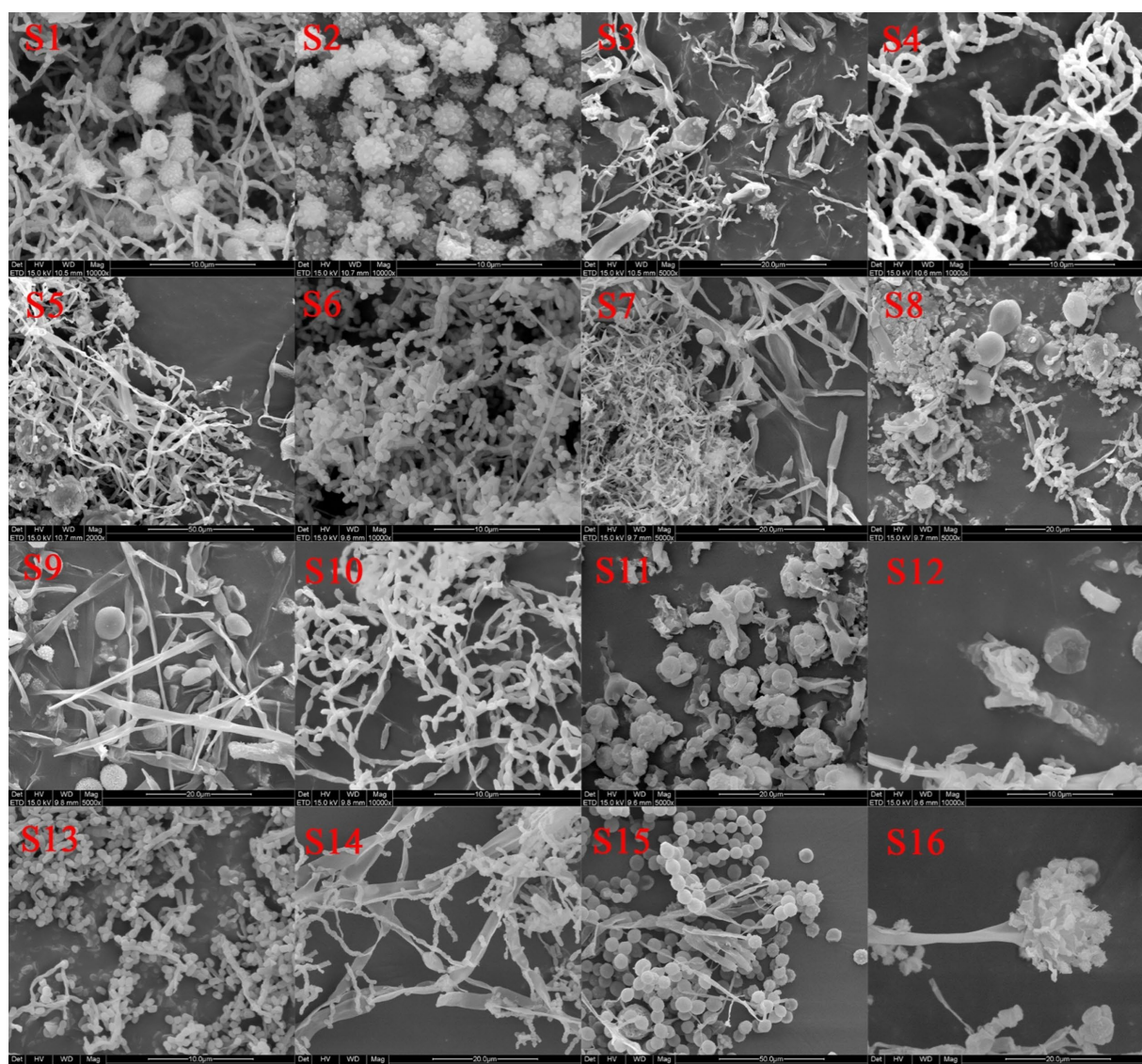


FIGURE 2

SEM results of samples from the surface of shadow puppet cultural relics.

genes such as *aadA5* (Drug Class: aminoglycoside antibiotic). In contrast, samples S11, S12, and S16 showed lower relative abundances, characterized by dispersed gene components and minor proportions. Sample S14 displayed a moderate relative abundance, predominantly composed of genes like the *vanY* gene in *vanG* cluster (Drug Class: glycopeptide antibiotic). These results illustrated the distinct distribution patterns of antibiotic-resistance genes across samples, providing essential data for understanding the dissemination and evolution of these genes in different environmental contexts (Figure 5C).

3.4 Isolation and identification of dominant microorganisms

Eleven distinct microbial species were successfully isolated and purified using standard microbiological culture techniques,

including four bacterial strains, two actinomycete strains, and five fungal isolates (Table 2). Among these, four strains—*Pseudomonas* sp. (WH. S-B1), *Streptomyces* sp. (WH. S-B2), *Nocardiopsis* sp. (WH. S-B6), and *Aspergillus fumigatus* (WH. S-F2)—corroborated the findings from high-throughput sequencing data, confirming their status as predominant microorganisms on the shadow puppet artifacts (Figure 6). These four strains were thus selected for further in-depth investigation. Growth assays on media supplemented with gelatin as the sole nitrogen source demonstrated that *Pseudomonas* sp., *Streptomyces* sp., *Nocardiopsis* sp., and *Aspergillus fumigatus* were capable of utilizing gelatin for growth (Figure 7). This phenotypic characteristic strongly suggests that these four strains possess the metabolic potential to degrade collagen, a major structural protein component of shadow puppets. These results provide crucial insights into the biochemical mechanisms underlying the deterioration of shadow puppet cultural relics.

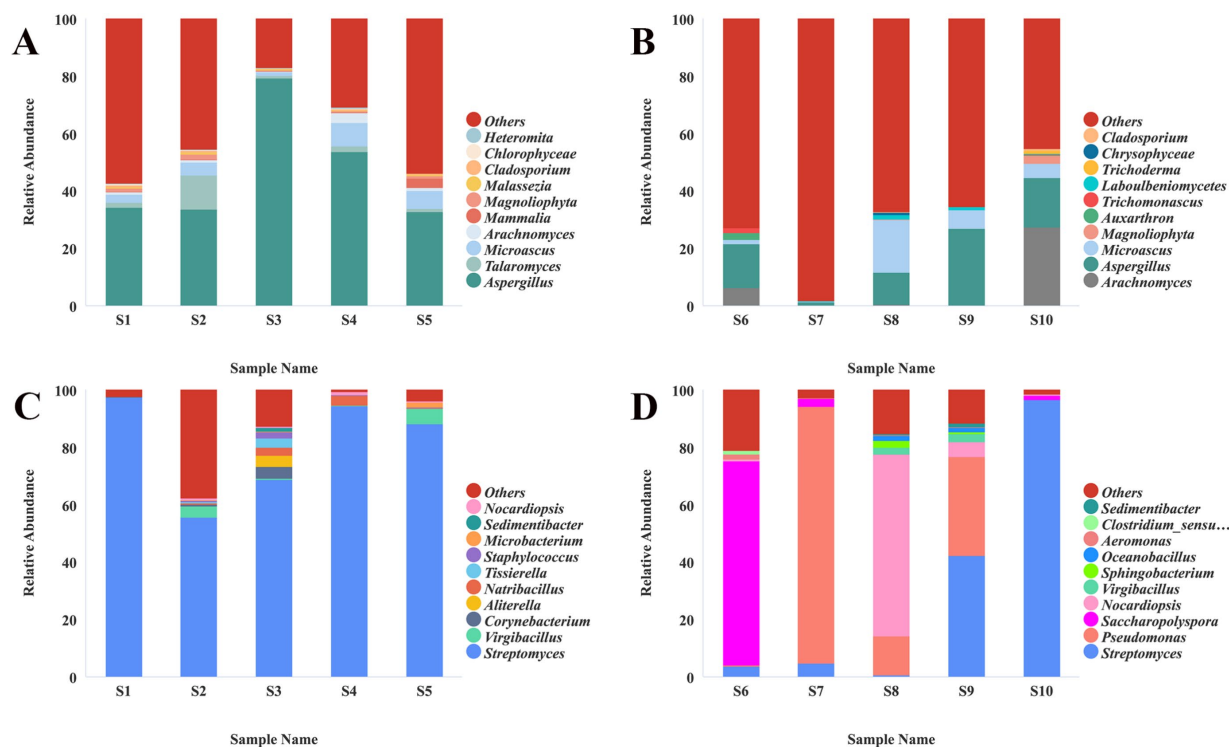


FIGURE 3

The relative abundance of microorganisms on the surface of shadow puppet cultural relics. (A) Eukaryotes, sample S1–S5; (B) eukaryotes, sample S6–S10; (C) prokaryotes, sample S1–S5; (D) prokaryotes, sample S6–S10.

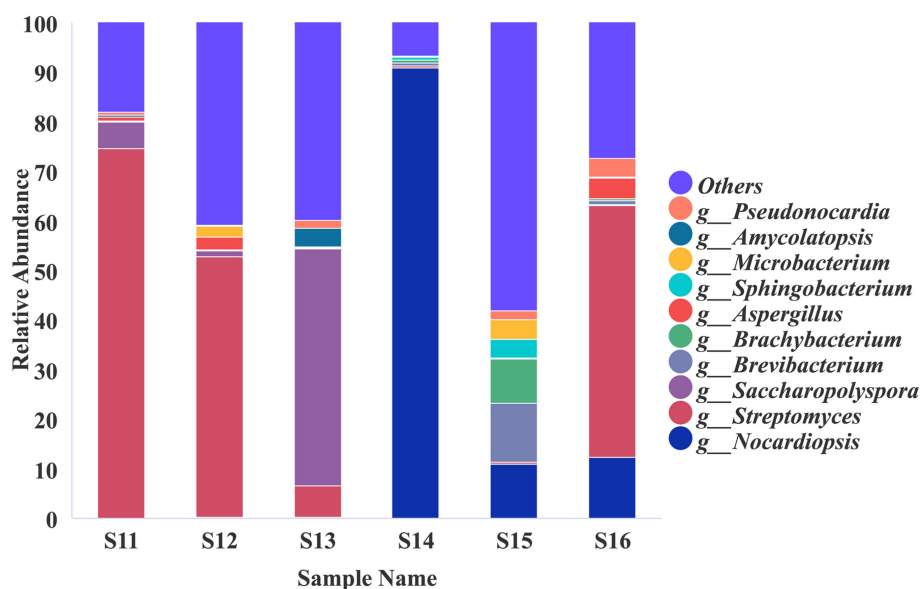


FIGURE 4

Relative abundance of microorganisms at the genus level on the surface of the Sample S11–S16.

3.5 Sensitivity of dominant microorganisms to different antimicrobial agents

To identify effective antimicrobial agents with minimal adverse effects on cultural relics, two candidates were selected for laboratory

testing: BC01, an isothiazolinone-based antimicrobial agent currently utilized in cultural heritage conservation, and carvacrol, a main component of certain plant essential oils. The antimicrobial assay results indicated that 0.3% BC01 and 50 mg/mL carvacrol exhibited limited inhibitory activity against *Pseudomonas* sp. (WH. S-B1).

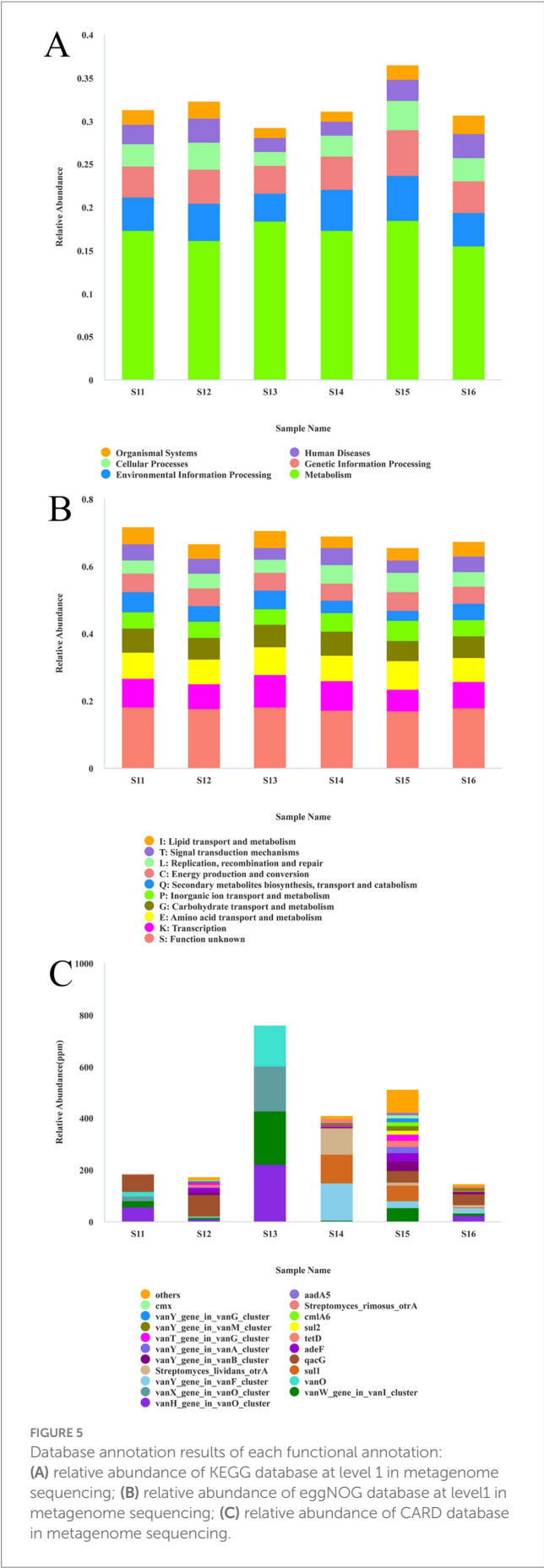


TABLE 2 Molecular identification of strains isolated from the shadow puppet artifacts.

Microorganisms	Genus	Genbank number
WH. S-B1	<i>Pseudomonas</i> sp.	PV569711
WH. S-B2	<i>Streptomyces</i> sp.	PV569712
WH. S-B3	<i>Lysinibacillus fusiformis</i>	PV569713
WH. S-B4	<i>Brachybacterium</i> sp.	PV569714
WH. S-B5	<i>Bacillus subtilis</i>	PV569715
WH. S-B6	<i>Nocardiopsis</i> sp.	PV569716
WH. S-F1	<i>Penicillium aethiopicum</i>	PV576011
WH. S-F2	<i>Aspergillus fumigatus</i>	PV576012
WH. S-F3	<i>Cladosporium cladosporioides</i>	PV576013
WH. S-F4	<i>Penicillium oxalicum</i>	PV576014
WH. S-F5	<i>Gymnoascus</i> sp.	PV576015

Conversely, BC01 demonstrated notable antibacterial and antifungal effects against *Streptomyces* sp. (WH. S-B2), *Nocardiopsis* sp. (WH. S-B6), and *Aspergillus fumigatus* (WH. S-F2), with the most pronounced effect observed against *Nocardiopsis* sp. Carvacrol showed significant inhibitory efficacy against *Streptomyces* sp. and *Nocardiopsis* sp. (Figure 8). These findings suggest that BC01 and carvacrol hold potential for application in the conservation of shadow puppet cultural relics, warranting further investigation into their long-term efficacy and compatibility with artifact materials.

4 Discussion

This study represents the first investigation into the microbial degradation of shadow puppet cultural relics. The SEM analysis provided conclusive evidence for the co-existence of diverse microbial species, including fungi and actinomycetes, on the surface of these artifacts. This finding aligns with previous research (Zhang et al., 2022), which indicates that the degradation of cultural heritage is often the result of synergistic interactions among multiple microorganisms. High-throughput sequencing further elucidated the complex microbial communities associated with the shadow puppets. The prevalence of Ascomycota among the fungal taxa and Actinobacteria within the bacterial groups across all samples (S1–S16) highlights their potential significance in the degradation process. Distinct patterns of microbial dominance were observed: Firmicutes were relatively abundant in S1–S5, Proteobacteria dominated in S6–S10, and Pseudomonadota were prevalent in S11–S16. These variations likely reflect differences in the microenvironmental conditions of individual artifacts, such as variations in preservation history, exposure to pollutants, and the chemical composition of the leather resulting from diverse tanning and dyeing processes. The identification of dominant genera, including *Aspergillus*, *Streptomyces*, *Pseudomonas*, and *Nocardiopsis*, is consistent with international research on the microbial degradation of organic materials (Creangă, 2010; Piñar et al., 2020; Bicchieri et al., 2019; Pangallo et al., 2010). The ability of four selected strains (*Pseudomonas* sp., *Streptomyces* sp., *Nocardiopsis* sp., and *Aspergillus fumigatus*) to grow on media containing gelatin as the sole nitrogen source strongly

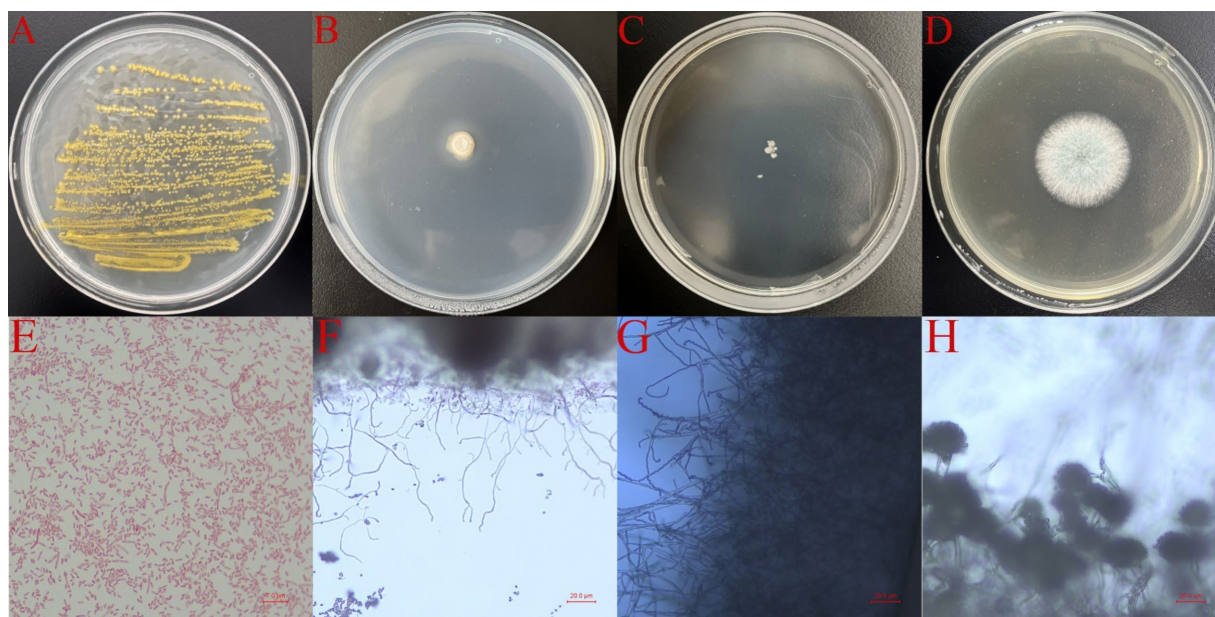


FIGURE 6

The morphology of four microorganisms on the culture medium and under the microscope. (A) *Pseudomonas* sp., WH. S-B1, LB medium; (B) *Streptomyces* sp., WH. S-B2, Gauze's No.1 medium; (C) *Nocardiosis* sp., WH. S-B6, Gauze's No.1 medium; (D) *Aspergillus fumigatus*, WH. S-F2, PDA medium; (E) WH. S-B1; (F) WH. S-B2; (G) WH. S-B6; (H) WH. S-F2.

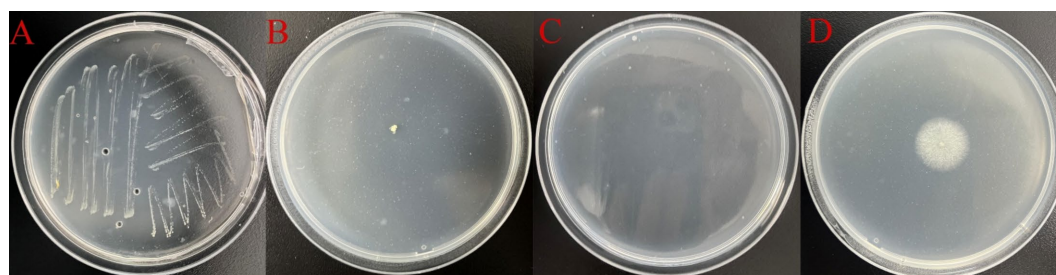


FIGURE 7

The four microorganisms on the gelatin medium. (A) *Pseudomonas* sp., WH. S-B1; (B) *Streptomyces* sp., WH. S-B2; (C) *Nocardiosis* sp., WH. S-B6; (D) *Aspergillus fumigatus*, WH. S-F2.

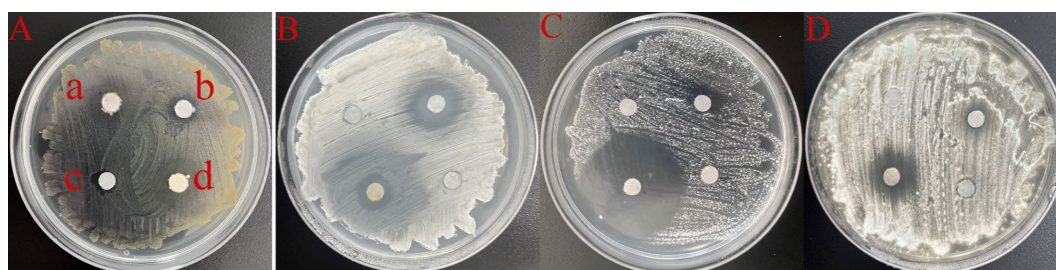


FIGURE 8

The antimicrobial effects of 0.3% BC01 and 50 mg/mL carvacrol. (A) DMSO; (B) 50 mg/mL carvacrol; (C) 0.3% BC01; (D) H₂O. Among them, DMSO and H₂O served as the negative controls. (A) *Pseudomonas* sp., WH. S-B1; (B) *Streptomyces* sp., WH. S-B2; (C) *Nocardiosis* sp., WH. S-B6; (D) *Aspergillus fumigatus*, WH. S-F2.

suggests their potential to degrade collagen, a major structural component of leather artifacts. Collagen degradation compromises the mechanical strength and structural integrity of shadow puppets, a

critical factor in their deterioration. Previous studies have shown that microorganisms secrete proteases and lipases, which can break down collagen fiber network and pigment layers (Zugno et al., 2011;

Israelroming et al., 2012). In summary, the identification of these degradation-associated microorganisms and their functional capabilities provides fundamental insights into the mechanisms underlying the deterioration of shadow puppet cultural relics.

Previous international studies have consistently identified *Aspergillus* and *Penicillium* as dominant fungal genera and *Bacillus* as a key bacterial genus involved in the degradation of various organic cultural relics (Yu et al., 2022; Du et al., 2024; Zhang et al., 2021; Lao et al., 2024; Shao et al., 2023; Zhao et al., 2023; Zhang et al., 2023; Huang et al., 2021). In contrast, while *Aspergillus* was also frequently detected in this study, other genera including *Pseudomonas*, *Streptomyces*, and *Nocardiopsis* emerged as significant contributors to the degradation of shadow puppet relics. This divergence in microbial composition can be attributed to the unique material properties of shadow puppet, which are predominantly crafted from animal hides subjected to specialized tanning and dyeing processes. These manufacturing techniques create a distinct ecological niche that selects for specific microbial taxa, differentiating the microbial communities associated with shadow puppets from those found on other organic artifacts such as wooden objects or paper documents. Recent advancements in metagenomic sequencing have highlighted the spatial heterogeneity of microbial communities with cultural relics (Gadd et al., 2024; Wang et al., 2023). Our study supports this emerging paradigm, as significant variations in microbial composition were observed across different shadow puppet samples (S1–S16). Although the spatial heterogeneity of microenvironments within shadow puppets, particularly in creases and folds where actinobacteria and fungi often form synergistic degradation systems, was not explicitly investigated in this study, it represents a promising avenue for future research. By elucidating these similarities and differences with previous research, this study contributes to a more comprehensive understanding of microbial-mediated degradation processes in organic cultural heritage, thereby facilitating the development of targeted conservation strategies. In this study, the antibacterial agents 0.3% BC01 and 50 mg/mL carvacrol demonstrated promising *in-vitro* bacteriostatic activity against the four selected microbial strains. However, their long-term efficacy and safety in actual cultural heritage conservation scenarios remain uncertain. Several critical aspects require further investigation. Firstly, potential chemical interactions between these agents and the leather materials of shadow puppets may occur. Such interactions could lead to material degradation, color change, or other physical alterations over time, potentially compromising the structural integrity of the artifacts. Secondly, the impact of these agents on the esthetic and historical value of shadow puppets needs to be carefully evaluated. Any treatment should preserve the unique characteristics and historical significance of these cultural relics without causing unintended damage. Furthermore, the optimal application methods for ensuring uniform distribution and sustained protection of these antibacterial agents have not been explored in this study. Previous research (Chen et al., 2024; Xu et al., 2020) has emphasized that the application techniques play a crucial role in the effectiveness of conservation treatments. Inadequate application could result in uneven protection, leaving certain areas of the artifacts vulnerable to microbial attack. In conclusion, while the initial findings on the antibacterial properties of BC01 and carvacrol are encouraging, comprehensive studies on their long-term performance, material compatibility, and appropriate application methods are essential before they can be safely and effectively implemented in the conservation of shadow puppet cultural relics.

5 Conclusion

This study investigated shadow puppets in the National Shadow Puppetry Museum in Chengdu, identifying diverse co-existing microorganisms on their surfaces and isolating four predominant collagen-degrading strains. Antimicrobial experiments showed that 0.3% isothiazolinone-based BC01 and 50 mg/mL carvacrol had antimicrobial effects to a certain extent. This research provides a basis for shadow puppet artifact preservation against microbial degradation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary material](#).

Author contributions

YuW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. YuaW: Conceptualization, Investigation, Methodology, Writing – original draft. ZG: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. ZW: Formal analysis, Investigation, Writing – original draft. YD: Investigation, Resources, Writing – original draft. CM: Formal analysis, Investigation, Methodology, Resources, Writing – original draft. YZ: Formal analysis, Investigation, Methodology, Resources, Writing – original draft. LH: Investigation, Methodology, Writing – original draft. JP: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the National Key R&D Program of China (2022YFF0904100) and the Fundamental Research Funds for the Central Universities from the University of Science and Technology Beijing.

Acknowledgments

We gratefully acknowledge the technical assistance of Yang Zhou and Hailiang Yang of the China National Silk Museum, Dongsheng Wei of Nankai University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations,

or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1611451/full#supplementary-material>

References

- Bicchieri, M., Biocca, P., Colaizzi, P., and Flavia, P. (2019). Microscopic observations of paper and parchment: the archaeology of small objects. *Herit. Sci.* 7, 47–59. doi: 10.1186/s40494-019-0291-9
- Chen, Z., Liu, X., Chen, H., Li, J., Wang, X., and Zhu, J. (2024). Application of epoxy resin in cultural relics protection. *Chin. Chem. Lett.* 35, 109194–109142. doi: 10.1016/j.ccllet.2023.109194
- Coar, A. M., and Usta, C. (2009). Main types and satire in language in the traditional Turkish shadow puppet play. *Bilig-Türk Dunyası Sosyal Bilimler Dergisi* 51, 13–32.
- Creangă, D. (2010). The inventory and classification of types of damage to objects from ethnographic collections. *Codrul Cosmin.* 16, 21–30. doi: 10.1161/01.STR.12.6.775
- Du, B., Pan, L., and Zheng, M. (2024). Preparation of AgNPs/oregano essential oil composite film and its antibacterial application in the conservation of paper relics. *Inorg. Chem. Commun.* 160:112008. doi: 10.1016/j.inoche.2023.112008
- Duan, Y., Zhang, M., Min, C., Lin, Y., and Li, L. (2024). Proteomic analysis of collagen: a mass spectrometry approach to material identification of shadow puppet cultural relics. *Appl. Biochem. Biotechnol.* 196, 5903–5919. doi: 10.1007/s12010-023-04822-1
- Fuente, M., Delgado, D., Beitia, M., Barreda-Gómez, G., Acera, A., Sanchez, M., et al. (2024). Validation of a rapid collagenase activity detection technique based on fluorescent quenched gelatin with synovial fluid samples. *BMC Biotechnol.* 24, 1–12. doi: 10.1186/s12896-024-00869-y
- Gadd, G. M., Fomina, M., and Pinzari, F. (2024). Fungal biodeterioration and preservation of cultural heritage, artwork, and historical artifacts: extremophily and adaptation. *Microbiol. Mol. Biol. Rev.* 88:e0020022. doi: 10.1128/mmb.00200-22
- Huang, X., Han, Y., Du, J., Guo, P., Wang, Y., Ma, K., et al. (2021). Inhibitory effect of cinnamaldehyde on main destructive microorganisms of Nanhai no. 1 shipwreck. *Appl. Sci. (Basel)* 11:5262. doi: 10.3390/app11115262
- Huang, X., Sun, S., Zhang, K., Xu, T., Wu, J., and Zhu, B. (2015). A method of shadow puppet figure modeling and animation. *Front. Inf. Technol. Electron. Eng.* 16, 367–379. doi: 10.1631/FITEE.1400351
- Israelroming, F., Gherghina, E., Luță, G., Bălan, D., Popa, A., and Ștefan, R. (2012). Studies on biodegradation of tanned leather. *Sci. Bull.* 16, 119–123.
- Lao, G., Zhou, Z., Wu, R., Wang, C., Wu, W., Lv, S., et al. (2024). Exploring the key deteriorative microorganisms on ancient ivories unearthed from the Sanxingdui ruins site during temporary cold storage. *Front. Microbiol.* 15:1400157. doi: 10.3389/fmicb.2024.1400157
- Lei, X., Li, L., Liu, X., and Duan, Z. (2023). Preservation evaluation method of Chinese traditional shadow puppet relics based on pixel lattice and membership function. *Herit. Sci.* 11:166. doi: 10.1186/s40494-023-01006-2
- Li, T., and Cao, W. (2021). Research on a method of creating digital shadow puppets based on parameterized templates. *Multimed. Tools Appl.* 80, 1–20. doi: 10.1007/s11042-021-10726-1
- Li, T., Zhang, H., Tan, X., Zhang, R., Wu, F., Yu, Z., et al. (2025). Antifungal activity evaluation of three traditional Chinese herbs against biodeterioration of cultural heritage. *Int. Biodeterior. Biodegrad.* 202:106093. doi: 10.1016/j.ibiod.2025.106093
- Liang, H., Dong, X., Pan, J., and Zheng, X. (2023). Virtual scene generation promotes shadow puppet art conservation. *Comput. Anim. Virtual Worlds* 34:e2148. doi: 10.1002/cav.2148
- Liu, S., Hua, G., and Li, Y. (2019). 2.5D human pose estimation for shadow puppet animation. *KSII Trans. Internet Inf. Syst.* 13:18. doi: 10.3837/tiis.2019.04.017
- Osathanunkul, M., Buddhachatt, K., and Chomdej, S. (2013). A modified colorimetric method of gelatinolytic assay using bacterial collagenase type II as a model. *Anal. Biochem.* 433, 168–170. doi: 10.1016/j.ab.2012.09.036
- Padovani, F. (2023). Varying discourse and use of intangible cultural heritage in Shaanxi Huaxian shadow puppets. *China Perspect.* 132, 39–48. doi: 10.4000/chinaperspectives.14601
- Pangallo, D., Chovanova, K., and Makova, A. (2010). Identification of animal skin of historical parchments by polymerase chain reaction (PCR)-based methods. *J. Archaeol. Sci.* 37, 1202–1206. doi: 10.1016/j.jas.2009.12.018
- Perini, N., Mercuri, F., Thaller, M. C., Orlanducci, S., Castiello, D., Talarico, V., et al. (2019). The stain of the original salt: red heats on chrome tanned leathers and purple spots on ancient parchments are two sides of the same ecological coin. *Front. Microbiol.* 10:2459. doi: 10.3389/fmicb.2019.02459
- Piñar, G., Tafer, H., Schreiner, M., Miklas, H., and Sterflinger, K. (2020). Decoding the biological information contained in two ancient Slavonic parchment codices: an added historical value. *Environ. Microbiol.* 22, 3218–3233. doi: 10.1111/1462-2920.15064
- Prahmana, R. C. I., and Istiandaru, A. (2021). Learning sets theory using shadow puppet: a study of Javanese ethnomathematics. *Mathematics* 9:2938. doi: 10.3390/math9222938
- Shao, Y., Luan, Y., Hao, C., Song, J., Li, L., and Song, F. (2023). Antimicrobial protection of two controlled release silver nanoparticles on simulated silk cultural relic. *J. Colloid Interface Sci.* 652, 901–911. doi: 10.1016/j.jcis.2023.08.116
- Shi, Z., Han, C., Zhang, X., Tian, L., and Wang, L. (2020). Novel synergistic mechanism for lignocellulose degradation by a thermophilic filamentous fungus and a thermophilic actinobacterium based on functional proteomics. *Front. Microbiol.* 11:539438. doi: 10.3389/fmicb.2020.539438
- Sterflinger, K. (2010). Fungi: their role in deterioration of cultural heritage. *Fungal Biol. Rev.* 24, 47–55. doi: 10.1016/j.fbr.2010.03.003
- Sterflinger, K., and Piñar, G. (2013). Microbial deterioration of cultural heritage and works of art—tilting at windmills? *Appl. Microbiol. Biotechnol.* 97, 9637–9646. doi: 10.1007/s00253-013-5283-1
- Strzelczyk, A. B., Kuroczkin, J., and Krumbein, W. E. (1987). Studies on the microbial degradation of ancient leather bookbindings: part I. *Int. Biodeterior.* 23, 3–27. doi: 10.1016/0265-3036(87)90039-X
- Sun, S., Xu, Z., Ren, M., Li, S., Luo, Y., and Tian, Y. (2024). Identification of microbial diversity in buried ivory soil at the Sanxingdui site in Guanghan City, China, using high-throughput sequencing. *Front. Microbiol.* 15:1384650. doi: 10.3389/fmicb.2024.1384650
- Wang, Y., Wang, C., Hou, L., Yang, X., Li, C., Cui, S., et al. (2024). Microbial diversity and biodegradation mechanism of microorganisms in the Dingtao M2 tomb. *Int. J. Mol. Sci.* 25:12270. doi: 10.3390/ijms252212270
- Wang, B., Zhu, C., Hu, Y., Zhang, B., and Wang, J. (2023). Dynamics of microbial community composition during degradation of silks in burial environment. *Sci. Total Environ.* 883:163694. doi: 10.1016/j.scitotenv.2023.163694
- Xu, J., Zhang, T., Jiang, Y., Yang, D., Qiu, F., Chen, Q., et al. (2020). Preparation of self-healing acrylic copolymer composite coatings for application in protection of paper cultural relics. *Polym. Eng. Sci.* 60, 288–296. doi: 10.1002/pen.25282
- Yang, C., Lou, M., Chen, X., and Ren, Z. (2024). An interactive collaborative creation system for shadow puppets based on smooth generative adversarial networks. *Comput. Mater. Contin.* 79, 4107–4126. doi: 10.32604/cmc.2024.049183
- Yu, Q., Xue, Z., Hu, R., Zhong, N., Zeng, T., Tang, H., et al. (2022). Reflective fiber-optic sensor for on-line nondestructive monitoring of *aspergillus* on the surface of cultural paper relics. *Biomed. Opt. Express* 13, 3324–3338. doi: 10.1364/BOE.457037
- Zhang, M., Hu, Y., Liu, J., Pei, Y., Tang, K., and Lei, Y. (2022). Biodeterioration of collagen-based cultural relics: a review. *Fungal Biol. Rev.* 39, 46–59. doi: 10.1016/j.fbr.2021.12.005
- Zhang, G., Liu, S., Li, Y., Zhou, Z., Cheng, Q., and Wang, Q. (2023). Microbial pollution assessment of storage environment for the ancient Buddhist relics in Tibetan palace museum. *Aerobiologia* 39, 275–283. doi: 10.1007/s10453-023-09791-w
- Zhang, X., Min, C., Zhang, M., and Li, L. (2024). Investigation of photoinduced deterioration in shadow puppet artifacts collected from the National Shadow Puppetry Museum in Chengdu, China. *Heliyon* 10:e39186. doi: 10.1016/j.heliyon.2024.e39186

Zhang, D., Pan, Q., Jia, M., Zhang, X., and Wang, Y. (2021). Three birds with one stone: preventive protection of paper materials by ZnO-PHMB and UV-531 composite systems. *Langmuir* 37, 8445–8454. doi: 10.1021/acs.langmuir.1c00770

Zhao, H., Liu, P., Huang, Y., and Zhang, H. (2023). Nanocomposites composed of modified natural polymer and inorganic nanomaterial for safe, high-efficiency,

multifunctional protection of paper-based relics. *Sci. China Technol. Sci.* 66, 2225–2236. doi: 10.1007/s11431-022-2391-6

Zugno, L., Hurlow, E., and Oppong, D. (2011). Fungal growth on wetblue: methods to measure impact on leather quality. *J. Am. Leather Chem. Assoc.* 106, 1–7.



OPEN ACCESS

EDITED BY

Ines Morais Caldas,
Faculdade de Medicina Dentária da
Universidade do Porto, Portugal

REVIEWED BY

Adam Pyzik,
University of Warsaw, Poland
Marina Fomina,
National Academy of Sciences of Ukraine,
Ukraine

*CORRESPONDENCE

Virginia Helena Albarracín
✉ cime@conicet.gov.ar

RECEIVED 14 April 2025

ACCEPTED 26 June 2025

PUBLISHED 10 July 2025

CITATION

Alonso-Reyes DG, Galván FS, Alvarado NN,
D'Arpino MC, Martinez LJ, Esquivel HJ,
Gallardo CA, Silva Manco MJ and
Albarracín VH (2025) Echoes of 1816:
microbial footprints in heritage artifacts from
Argentina's museum of independence.
Front. Microbiol. 16:1611832.
doi: 10.3389/fmicb.2025.1611832

COPYRIGHT

© 2025 Alonso-Reyes, Galván, Alvarado,
D'Arpino, Martinez, Esquivel, Gallardo, Silva
Manco and Albarracín. This is an open-access
article distributed under the terms of the
[Creative Commons Attribution License
\(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction
in other forums is permitted, provided the
original author(s) and the copyright owner(s)
are credited and that the original publication
in this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Echoes of 1816: microbial footprints in heritage artifacts from Argentina's museum of independence

Daniel Gonzalo Alonso-Reyes^{1,2}, Fátima Silvina Galván^{1,2},
Natalia Noelia Alvarado^{1,2}, María Cecilia D'Arpino^{1,3},
Luciano José Martínez^{1,3}, Hernán José Esquivel¹,
Cecilia Aymara Gallardo¹, María Julia Silva Manco^{1,2} and
Virginia Helena Albarracín^{1,2,3,4*}

¹Laboratorio de Microbiología Ultraestructural y Molecular, Centro Integral de Microscopía Electrónica (CIME), Universidad Nacional de Tucumán, CONICET NOA SUR, CONICET, Tucumán, Argentina, ²Facultad de Agronomía, Zootecnia y Veterinaria, Universidad Nacional de Tucumán, Tucumán, Argentina, ³Instituto de Salud y Calidad de Vida, Universidad de San Pablo, Tucumán, Argentina, ⁴Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, Tucumán, Argentina

Introduction: Historical artifacts preserved in museums are invaluable cultural treasures but are often susceptible to biodeterioration driven by microbial colonization. Despite increasing awareness of microbial impacts on heritage conservation, systematic microbiological studies of such objects remain limited, particularly in Latin America.

Methods: This study presents the first comprehensive investigation of bacteria inhabiting heritage artifacts from Casa Histórica de la Independencia, Argentina's Museum of Independence. Samples were collected from a range of materials, including wood, textiles, architectural elements, and exterior walls. Microbial colonization was assessed using scanning electron microscopy (SEM), and bacterial isolates were phenotypically and taxonomically characterized via VITEK MALDI-TOF mass spectrometry. Selected isolates were further subjected to genomic analysis.

Results: SEM imaging revealed diverse and well-structured biofilms with intricate three-dimensional architectures embedded in extracellular polymeric substances. A total of 49 bacterial strains were isolated, predominantly Gram-positive genera such as *Bacillus*, *Micrococcus*, and *Kocuria*. The 19th-century albumen print photograph emerged as the most biodiverse artifact, yielding 21 distinct strains, including extremophilic genera such as *Streptomyces*, *Oceanobacillus*, and *Caldibacillus thermoamylovorans*. The albumen layer's protein-rich and halophilic properties likely promoted microbial colonization. Notably, *Pseudomonas* species were found exclusively on this photographic material. Human-associated taxa, such as *Staphylococcus epidermidis* and *Staphylococcus equorum*, were primarily detected in high-contact zones, while exterior surfaces exhibited unique microbial profiles, including opportunistic pathogens.

Discussion: The findings highlight a complex and substrate-specific microbial landscape across the museum's collection. The presence of halophiles and human-associated bacteria points to both intrinsic material properties and external contamination sources. This study shows the importance of incorporating microbiological data into conservation protocols. By characterizing the artifact-associated microbiota, we contribute to the emerging field of heritage microbiology and future bio-informed preservation strategies.

KEYWORDS

biodiversity, cultural heritage, museum, extremophiles, electron microscopy, Tucuman, microbiomes, biodeterioration

1 Introduction

Museums serve a fundamental role in the conservation of cultural heritage (CH), safeguarding irreplaceable artifacts that embody humanity's identity, memory, and history. Preventive conservation is vital because cultural assets are inherently vulnerable to deterioration processes that threaten their integrity (Gutarowska et al., 2014; Grabek-Lejko et al., 2017; Negi and Sarethy, 2019; Baldan et al., 2021; Liu et al., 2022). Microbial contamination is a common issue in heritage collections, as both organic and inorganic materials can be colonized by bacteria and fungi depending on their bioreceptivity and environmental exposure. These microorganisms persist in museum environments by adapting their versatile metabolisms to low-nutrient conditions and limited light, and by tolerating subtle microclimatic fluctuations that may occur despite environmental controls. Indeed, microbes may have been present in the original piece since its first production, as invisible part of the materials used to craft the artifact. Microbes settle into specific ecological niches, often remaining undetected until visible biofilm formation, discoloration, or structural weakening became apparent. Over time, their activity can cause significant damage to textiles, wood, paper, and other organic materials, manifesting as discoloration, structural weakening, odor, and cracking (Skóra et al., 2015; Grabek-Lejko et al., 2017; Negi and Sarethy, 2019). The consequences of microbial growth extend beyond artifact damage, potentially diminishing monetary value, and bringing about costly decontamination procedures (Sterflinger and Piñar, 2013). Despite these challenges, the cultural and historical value of many CH objects is immeasurable and cannot be quantified solely in monetary terms.

Biodeterioration is defined as “any undesirable change in a material brought about by the vital activities of organisms” (Perito and Cavalieri, 2018). While biodeterioration cannot be entirely halted, advances in microbial ecology and technological interventions offer strategies to mitigate its effects. A deeper understanding of microbial communities on CH objects is critical for identifying their deteriorative mechanisms and implementing effective control measures (Perito and Cavalieri, 2018; Baldan et al., 2021). These aspects have become an important research field for both, conservators and microbiologists (Adamiak et al., 2018). This issue also raises occupational health concerns, as museum staff may be exposed to harmful biological agents because of the quality of the indoor air (Baldan et al., 2021), or during the handling of contaminated materials, such as paper, wood, and textiles (Borrego et al., 2010; Gutarowska et al., 2014; Stratigaki et al., 2024). Indeed, many studies indicated that conservators, librarians, and archivists face risks related to microbial exposure (Zielińska-Jankiewicz et al., 2008; Wiszniewska et al., 2009; Karbowska-Berent et al., 2011; Baldan et al., 2021).

Traditional methods of investigating microorganisms on CH objects have primarily been culture-dependent, offering insights into their deteriorative roles. Recently, amplicon sequencing has emerged as a powerful yet resource-intensive tool for microbial identification (Kraková et al., 2012a, 2018). An alternative approach could be the combination of the automated VITEK system for rapid biochemical identification (Książczyk et al., 2016), with whole-genome sequencing

(WGS) for functional profiling of key strains. Scanning electron microscopy (SEM) has also proven effective in biodeterioration studies, providing visual insights into microbial activity on CH surfaces (Díaz-Herráiz, 2015; Puškárová et al., 2016; Elhagrassy, 2018; Liu et al., 2018b; Stratigaki et al., 2024).

The National Museum “Casa Histórica de la Independencia” (CHM), located in San Miguel de Tucumán, Argentina, stands as a vivid example of how heritage and identity intersect. Renowned as the site where the independence of the United Provinces of the Río de la Plata was declared on July 9, 1816—a date later established as Argentine Independence Day—the CHM is among the most visited historical museums in the country (Chatruc, 2021). In addition to its architectural significance, the museum preserves an extensive collection of historical furniture, documents, a large library, and a photographic archive. Among its most valuable items is *Provincia de Tucumán* by Arsenio Granillo, a 213-page volume commissioned by Governor Federico Helguera. This rare publication (Granillo, 1872), which includes original albumen prints depicting the city and its sugar mills of photographer Angel Paganelli, features the only known photograph of the museum's original facade — a crucial visual reference during the building's restoration in 1943.

The objective of this study is to provide the first comprehensive microbiological characterization of the CHM collections, using them as models to investigate the role of cultivable bacteria in the biodeterioration of heritage materials. We aim to identify and classify viable bacterial strains colonizing historical artifacts and architectural elements, determine the prevalence of specific taxa in relation to substrate types and microenvironmental conditions, and assess the functional potential of these microorganisms—particularly their capabilities for biodegradation and resistance to environmental stressors. Additionally, this study seeks to evaluate the potential biohazards posed by microbial communities in museum environments, contributing to the development of more effective preventive conservation strategies. To achieve these goals, we employed an integrative approach combining scanning electron microscopy (SEM) for visualizing microbial colonization, culture-dependent methods for bacterial isolation, MALDI-TOF mass spectrometry for rapid taxonomic identification, and whole-genome sequencing (WGS) for functional profiling.

2 Materials and methods

2.1 Brief description of the Casa Histórica museum

The CHM is located in San Miguel de Tucumán, the capital of Tucumán Province, Argentina at a pedestrian zone with significant foot traffic.¹ The Casa Histórica is a preserved colonial-style building and

1 <https://sketchfab.com/3d-models/museo-nacional-casa-historica-5b37f88491c0456bbd15fe87f4b8b171>

functions as a museum, offering guided tours and exhibitions related to Argentina's independence and the revolutionary era. The museum features four courtyards, a cistern, and native trees from the region. It currently holds over 700 items in its collection including the original Act of the Declaration of Independence and various other pieces such as period furniture, weapons, personal items, dishware, religious artifacts, paintings, portraits, coins, and commemorative plaques and medals from the 18th and 19th centuries. The museum's library contains classic texts, recent historiographical debates, museum studies, conservation, restoration, literature, journals and early provincial newspapers. Moreover, the Museum conserves an extensive book library and a photographic archive. Among the highlights is *Provincia de Tucumán* by Arsenio Granillo, a 213-page book (Granillo, 1872) commissioned by Governor Federico Helguera, features descriptive articles and news about Tucumán, accompanied by original albumen print photographs by Angel Paganelli, depicting the city and its sugar mills.

2.2 Selection of the artifacts for the sampling

Before sampling, an extensive survey and photographic documentation were conducted to capture the condition of cultural heritage items affected by different types of deterioration. This preliminary assessment informed the selection of specific sampling locations within the building and on key artifacts of cultural significance.

The initial phase of sampling took place in August 2019. Samples were taken from the museum's exterior, including facade elements like the front door and painted walls (Figure 1A), which are subjected to annual repainting, weathering, and urban pollution. Within the museum, samples were taken from the window and furniture in the "Salón de la Jura" (Figure 1B). Additional historical artifacts that required preservation evaluations were also sampled (Table 1 and Figure 1).

The Salón de la Jura or Hall of the Oath is a central room within the CHM (Figure 1B). Samples for our study were collected from one chair where congressmen sat and from the table where the Declaration of Independence was signed; this furniture remains in its original state, dating back to 1810. The wooden frame from the armrest and backrest and the velvet-covered seat of one of the chairs were sampled to assess the bacteria present on these distinct materials (Figures 1C,D). The table's wooden surface was swabbed in multiple locations, especially in the areas less exposed to regular cleaning (Figure 1E). Additionally, we took samples from biodeteriorated zones of one of the windows of the room, subjected to infestation by woodworm eggs (Figures 1F,G).

Juan Bautista Alberdi (1810–1884) was a distinguished statesman, diplomat, and writer who played a pivotal role in shaping Argentina's political landscape. The Museum holds a captivating collection of artifacts attributed to Alberdi, including a childhood suit made from natural red silk which was sampled (Figures 1H,I).

The second phase of sampling in December 2021 targeted a key artifact from the CHM: the 1872 book *Provincia de Tucumán* authored by Dr. Arsenio Granillo (Figures 1J–L). Stored in a metallic safe case to preserve its condition, this book has survived with minimal light exposure, thermal fluctuations, and limited handling. Samples were taken from both the albumen print and the paper support.

2.3 Sampling procedures for direct material observation and bacterial isolation

To conduct the sampling, a non-invasive approach was employed, using precision tools to extract material samples without compromising the structural integrity of the pieces. Micro-sampling techniques, supported by visual documentation and environmental controls, were used to assess the physical condition of the albumen prints and the rest of the elements identify potential areas of degradation, such as yellowing, flaking, or damage from previous handling.

For the direct observation of microorganisms on heritage objects, freshly-open 3 M™ adhesive tape was used (Michaelsen et al., 2009). Strips of 2–3 cm were cut in advance using sterile scissors under aseptic conditions and sterilized by exposure to germicidal UV-C radiation for 15 min. Subsequently, the strips were gently adhered to the different surfaces and then transferred to 2 mL tubes containing Karnovsky's fixative solution (2.66% w/v paraformaldehyde and 1.66% w/v glutaraldehyde in 0.1 M phosphate buffer, pH 7.2), for transportation to the lab. The tape sampling method was not used for the book of Granillo, to avoid any undesired consequences over its conservation.

For isolation of individual bacterial strains, sample collection was performed with dry and sterilized cotton swabs. The swab was passed over the entire area of visibly damaged material; each swab covered approximately a 10 cm² area to standardize sample collection. Immediately after sampling, the swabs were used to inoculate plates with Luria-Bertani (LB) agar media. In most cases, selective media were used: parallel LB agar plates were supplemented with nalidixic acid (10 µg mL⁻¹) and cycloheximide (10 µg mL⁻¹) to inhibit the growth of Gram-negative bacteria and fungi, respectively (Rasuk et al., 2017). However, in the case of the albumen book, only non-selective LB medium was used to favor the recovery of a broader spectrum of microbial taxa, including both Gram-positive and Gram-negative bacteria. Parallel agar plates were supplemented with nalidixic acid (10 µg mL⁻¹) and cycloheximide (10 µg mL⁻¹) to inhibit the growth of Gram-negative bacteria and fungi, respectively (Rasuk et al., 2017). This non-invasive method is considered essential for the study of collections in museum environments (Michaelsen et al., 2009; Piñar et al., 2015). Once the colonies appeared, they were collected with a sterile loop for purification and subsequent streaking allows generating axenic cultures. We stored the cells in glycerol 20% at –20°C. Subcultures were performed in LB broth for morphological and molecular identification analysis. From the pure cultures of the bacterial isolates, macroscopic practices were carried out to determine the structure of the colonies and their pigmentation, and microscopic practices (optical and electronic) of individual cells to establish shapes, sizes, and spatial arrangement. Additionally, the Gram reactions of all isolates were recorded.

All isolated strains in this work are kept and curated in the CeBAC collection (Cepario de Bacterias Ambientales del CIME), Catalogue Urban Microbiome Strains, under the corresponding codes indicated in Table 1.

2.4 Scanning electron microscopy (SEM)

For SEM studies, the adhesive tapes or pure axenic cultures of isolated strains were fixed in a mixture of aldehydes (Karnovsky fixative) overnight at 4°C, and were prepared following the processing method

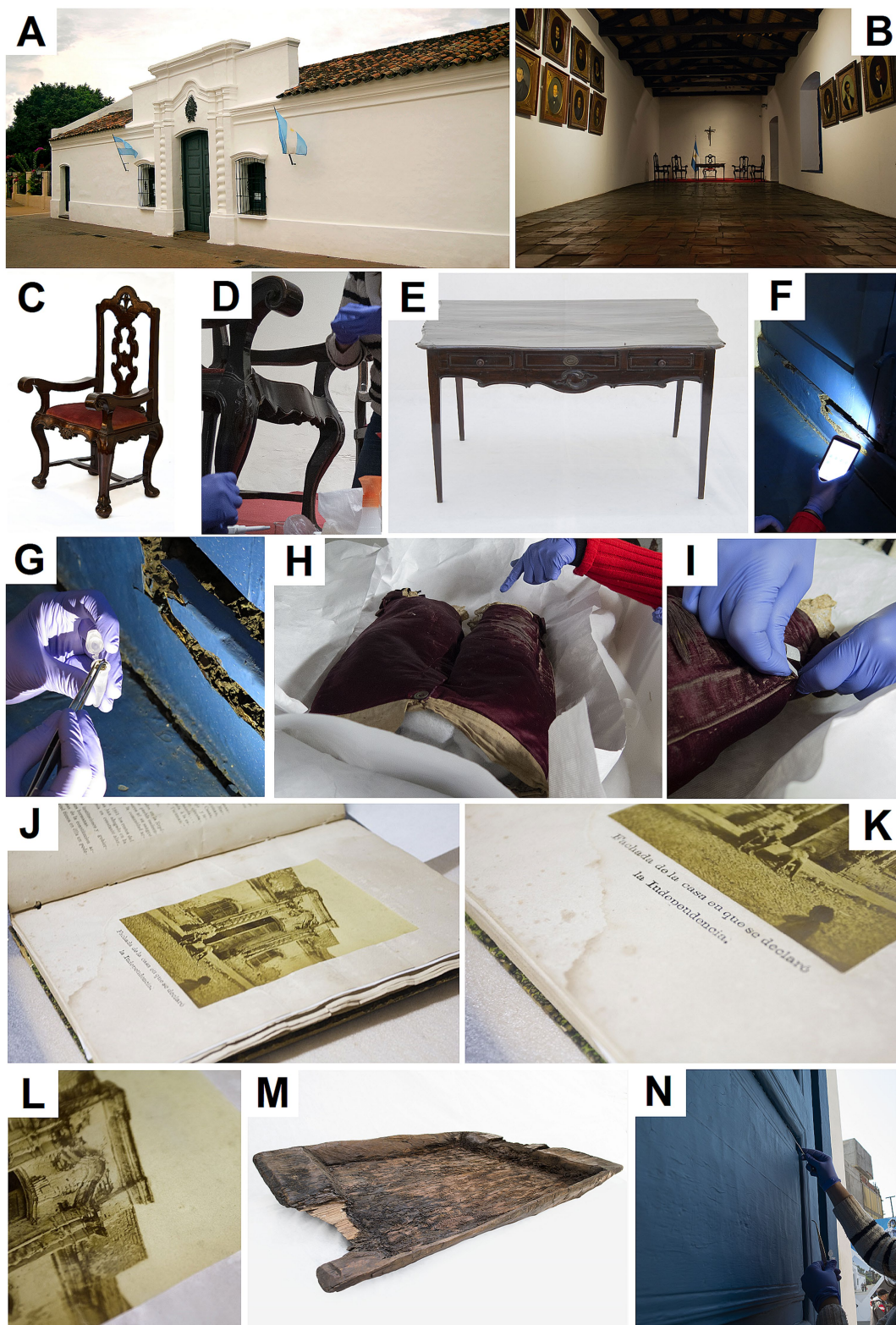


FIGURE 1

(A) Exterior facade of the museum. (B) interior of the "Salón de la Jura," (B) a historic room central to the declaration of Argentine independence. (C–E) Furniture from the "Salón de la Jura": (C) colonial chair; (D) detail of chair leg and surface during sampling; (E) table sampled for microbial analysis. (F,G) window from the "Salón de la Jura": (F) overview of the window structure; (G) close-up of the deteriorated frame during sampling. (H,I) Garment belonging to Juan Bautista Alberdi: (H) general view of preserved trousers; (I) detail of fiber structure showing signs of microbial alteration. (J–L) Nineteenth-century albumen print photo-book: (J) open view of interior; (K) detail showing biological staining and foxing; (L) close-up of albumen photograph. (M) Historic wooden washing trough recovered from the museum's collection. (N) Main entrance door of the museum, sampled at the lower panel. Ph: Cecilia Gallardo y Alejandro Torres.

TABLE 1 Taxonomic classification, morphological description, and microscopy-based traits of bacterial strains isolated from the Casa Histórica de la Independencia Museum (CHM).

ID	Taxonomical identification by VITEK	Source	Macroscopical characterization		Microscopical characterization	
			Colour	Surface and texture	Morphology	Gram
CH - 001	<i>Micrococcus luteus</i>	SJ Chair (textile)	Intense-yellow	Smooth and creamy	Coccoid	(+)
CH - 002	<i>Staphylococcus equorum</i>	SJ Chair (textile)	Pale-yellow	Smooth and creamy	Coccoid	(+)
CH - 003	<i>Bacillus simplex</i>	SJ Chair (textile)	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 004	<i>Bacillus licheniformis</i>	SJ Chair (textile)	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 005	<i>Micrococcus</i> sp.	SJ Chair (wood)	Orange	Smooth and creamy	Coccoid	(+)
CH - 006	<i>Kocuria rosea</i>	SJ Chair (wood)	Orange	Smooth and creamy	Coccoid	(+)
CH - 007	Unidentified	SJ Chair (wood)	Pale-yellow	Smooth and creamy		
CH - 008	<i>Kocuria rosea</i>	SJ Table (wood)	Orange	Smooth and creamy	Coccoid	(+)
CH - 009	<i>Micrococcus luteus</i>	SJ Table (wood)	Intense-yellow	Smooth and creamy	Coccoid	(+)
CH - 010	<i>Bacillus altitudinis/pumilus</i>	SJ Table (wood)	Cream-colored	Rough and mucoid	Bacillus	(+)
CH - 011	<i>Staphylococcus equorum</i>	Washing trough (wood)	Pale-yellow	Smooth and creamy	Coccoid	(+)
CH - 012	<i>Bacillus</i> sp.	Washing trough (wood)	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 013	<i>Actinomyces odontolyticus</i>	Alberdi's Suit (silk and lace)	White	Dry and hard	Mycelium	(+)
CH - 014	<i>Micrococcus luteus</i>	SJ Window (blue painted wood)	Intense-yellow	Smooth and creamy	Coccoid	(+)
CH - 015	<i>Microbacterium aurum</i>	SJ Window (blue painted wood)	Yellow	Smooth and creamy	Bacillus	(+)
CH - 016	Unidentified	SJ Window (blue painted wood)	Yellow	Smooth and creamy		
CH - 017	<i>Microbacterium aurum</i>	Entrance door (blue painted wood)	Yellow	Smooth and creamy	Bacillus	(+)
CH - 018	<i>Bacillus altitudinis/pumilus</i>	Entrance door (blue painted wood)	Pale-yellow	Smooth and creamy	Bacillus	(+)
CH - 019	<i>Clostridium subterminale</i>	Entrance door (blue painted wood)	Yellow	Smooth and creamy	Bacillus	(+)
CH - 020	<i>Micrococcus luteus</i>	Entrance door (blue painted wood)	Intense-yellow	Smooth and creamy	Coccoid	(+)
CH - 021	<i>Corynebacterium pseudodiphtheriticum</i>	Entrance door (blue painted wood)	Orange	Smooth and creamy	Bacillus	(+)
CH - 022	Unidentified	Entrance door (blue painted wood)	Yellow	Smooth and creamy		
CH - 023	<i>Kocuria rosea</i>	Entrance door (blue painted wood)	Orange	Smooth and creamy	Coccoid	(+)
CH - 024	<i>Corynebacterium pseudodiphtheriticum</i>	Exterior walls (white painted bricks)	Intense-orange	Smooth and creamy	Bacillus	(+)
CH - 025	<i>Kocuria rosea</i>	Exterior walls (white painted bricks)	Orange	Smooth and creamy	Coccoid	(+)
CH - 026	<i>Turicella otitidis</i>	Exterior walls (white painted bricks)	Yellow	Smooth and creamy	Bacillus	(+)
CH - 027	<i>Bacillus megaterium</i>	Exterior walls (white painted bricks)	Cream-colored	Smooth and hard	Bacillus	(+)
CH - 028	<i>Kocuria rosea</i>	Exterior walls (white painted bricks)	Orange	Smooth and creamy	Coccoid	(+)

(Continued)

TABLE 1 (Continued)

ID	Taxonomical identification by VITEK	Source	Macroscopical characterization		Microscopical characterization	
			Colour	Surface and texture	Morphology	Gram
CH - 029	<i>Bacillus altitudinis/pumilus</i>	GB Albumen print	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 030	Unidentified	GB Albumen print	Intense-orange	Smooth and mucoid	Cocoid	(+)
CH - 031	<i>Bacillus galactosidilyticus</i>	GB Albumen print	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 032	<i>Pseudomonas</i> sp.	GB Albumen print	Cream-colored	Smooth and creamy	Rod-shaped	(−)
CH - 033	<i>Pseudomonas</i> sp.	GB Albumen print	Cream-colored	Smooth and creamy	Rod-shaped	(−)
CH - 034	<i>Pseudomonas</i> sp.	GB Albumen print	Cream-colored	Smooth and creamy	Rod-shaped	(−)
CH - 035	<i>Kocuria rosea</i>	GB Albumen print	Pale-yellow	Smooth and creamy	Cocoid	(+)
CH - 036	<i>Streptomyces griseus</i>	GB Albumen print	White	Dry and hard	Mycelium	(+)
CH - 037	Unidentified	GB Albumen print	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 038	<i>Kocuria rosea</i>	GB Albumen print	Intense-orange	Smooth and creamy	Cocoid	(+)
CH - 039	<i>Bacillus galactosidilyticus</i>	GB Albumen print	Cream-colored	Smooth and mucoid	Bacillus	(+)
CH - 040	<i>Pseudomonas</i> sp.	GB Albumen print	Pale-yellow	Smooth and mucoid	Bacillus	(+)
CH - 041	<i>Bacillus thermoamylovorans</i>	GB Albumen print	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 042	<i>Streptomyces</i> sp.	GB Albumen print	Pale-cream	Smooth and hard	Mycelium	(+)
CH - 043	<i>Micrococcus luteus</i>	GB Albumen print	Intense-yellow	Smooth and creamy	Cocoid	(+)
CH - 044	Unidentified	GB Albumen print	Pale-yellow	Smooth and mucoid		(+)
CH - 045	<i>Bacillus cereus</i> group	GB Albumen print	Cream-colored	Smooth and creamy	Bacillus	(+)
CH - 046	<i>Staphylococcus epidermis</i>	GB Albumen print	Cream-colored	Smooth and dry	Cocoid	(+)
CH - 047	<i>Micrococcus luteus</i>	GB Albumen print	Intense-yellow	Smooth and creamy	Cocoid	(+)
CH - 048	Unidentified	GB Albumen print	Orange	Smooth and mucoid	Cocoid	(+)
CH - 049	<i>Pseudomonas</i> sp.	GB paper sheet	Cream-colored	Smooth and creamy	Rod-shaped	(−)

This table presents 49 cultivable bacterial isolates recovered from heritage artifacts and architectural elements of the CHM. For each strain, the VITEK-based taxonomic assignment is listed. The source column specifies the sampled object (e.g., photograph, textile, wood, and wall). Macromorphological traits, including colony color, surface appearance, and texture, were recorded after 24–48 h growth on LB agar. Micromorphological observations, including Gram reaction and cell shape, were determined via light microscopy. Strains are curated in the CeBAC collection under the specified isolate codes.

already previously described in [Alonso-Reyes et al. \(2021\)](#). During post-fixation, samples were washed with 0.1 M phosphate buffer twice and dehydrated in a graded concentration series of ethanol (50, 70, 90, and 100%), following acetone 100%. This was followed by critical point drying (Denton Vacuum model DCP-1). The samples were then mounted on aluminum supports and subjected to gold sputtering (JEOL model JFC-1100) and examined using a Zeiss Supra 55VP scanning electron microscope (Carl Zeiss NTS GmbH, Germany), in the Electron Microscopy Core Facility (CIME-CONICET-UNT).

2.5 Identification of microbial strains by MALDI-TOF MS (VITEK)

MALDI-TOF analysis was performed with a bioMérieux VITEK MALDI-TOF mass spectrometer (bioMérieux, France), and the spectra were compared to the VITEK MS SARAMIS database for research use only (RUO), version 4.09, using the SuperSpectra algorithm (referred to here as MALDI-TOF MS). For MALDI-TOF MS analysis, a colony from a pure plate with 24 h growth was applied to a slide and covered with 1 µL of matrix solution (α -cyano-4-hydroxycinnamic acid) and dried completely before analysis. The identification was considered

definitive when the probability provided was >70%. This service was provided by Laboratorios CIBIC in Rosario, Argentina.

2.6 DNA extraction

Eight representative bacterial genomes were selected for DNA sequencing ([Table 2](#)) to represent the ecological and substrate diversity of the CHM samples, including three isolates from outdoor surfaces, two from the indoor furniture surfaces of the well-preserved Salón de la Jura, and three strains from a uniquely preserved photographic artifact. The selection was guided by phenotypic variability, distinct cultural origin, and presumed relevance to biodeterioration processes, as determined by preliminary MALDI-TOF MS identification and morphological traits. The DNA was obtained using a commercial extraction kit (DNA Puriprep B-kit for bacteria, Inbio Highway, Argentina); following the manufacturer's instructions. For Gram-positive bacteria, the pellet was suspended in 162 µL of BRB buffer and 18 µL of lysozyme, and then incubated for 30 min at 37°C. For gram-negative bacteria, the use of lysozyme was omitted. Next, 200 µL of BT buffer and 20 ul of proteinase K were added to each sample. It was incubated for 30 min at 56°C. After, RNaseA (10 mg/mL), was added and incubated for 30 min at 37°C. Then the lysis buffer (200 µL)

was added, vortex pulsed for 10 s and the homogenate was incubated for 10 min at 56°C. Then, 200 µL of 100% ethanol was added to each sample, shaken by inversion, and transferred to a microcolumn. It was centrifuged for 1 min at 12,000 g, and the filtrate was separated. Then, two column washes were performed each with 500 µL of Blav1 and Blav2 buffer, respectively. Finally, the column was dried by centrifuging for 3 min at 12,000 g. DNA elution was carried out with 60 µL of pH 9 elution buffer, equilibrated at 70°C. DNA concentration and quality were measured using a µDrop plate (Thermo Scientific).

2.7 Genome sequencing, assembly, and analysis

Whole genome sequencing service was performed by Novogene UK services² using the Illumina Novaseq platform with pair-end 150 strategy. Quality control of the reads was also performed by Novogene. The Illumina reads were assembled using SPAdes assembler v. 3.15.4 (Bankevich et al., 2012), with—careful and—cov-cutoff auto options. The resulting eight assemblies were uploaded to the NCBI database under the Bioproject accession number PRJNA1233209, and are also available at Zenodo.³ The sequencing process and quality control yielded approximately 100 million reads and 15 Gb of data, with an average of 12 million reads and 1.88 Gb per genome. Assembly resulted in fewer than 115 contigs for all strains, except for the CH-041 strain, which had 200 contigs. The functional annotation was performed using Arche v. 1.0 (Alonso-Reyes and Albarracín, 2025) pipeline with—kegg option, and the resultant KEGG identifiers (KO) were mapped through the KEGG mapper reconstruct tool.⁴ The Arche pipeline, employing GeneMarkS-2 for gene prediction, achieved high annotation rates for protein-coding sequences (CDS). The lowest annotation rate was observed in CH-026, with ~85% of CDS annotated, while CH-036 reached approximately 97% annotation. The genomic potential for the synthesis of secondary metabolites was elucidated through antiSMASH v. 7.1.0 (Blin et al., 2023). To estimate the taxonomic identity of each strain at genus level, 16 s-rRNA and DNA gyrase B genes were retrieved from the genomes using barnap and blast tools, and then blasted against the NCBI NR database. For complete taxa identification, average nucleotide identity (ANI) analysis was performed using pyani (Pritchard et al., 2016), against genus-specific genomes deposited in NCBI database. No statistical analyses were applied in this study, as the focus was on descriptive microbial characterization using microscopy, culture-based isolation, MALDI-TOF identification, and genomic analyses.

3 Results

3.1 SEM analysis of microbial communities on heritage objects and facade surfaces

Biofilms on heritage surfaces including furniture, wooden objects (chair, table, window and washing trough) and textile garments were analyzed using scanning electron microscopy (Figure 2). In all cases,

the adhesive tape sampling method proved to be a useful, simple, and non-destructive technique for collecting microbial communities for immediate imaging analysis. The surfaces of objects within and outside the museum were highly colonized with microbes forming well-structured biofilms.

Most observed biofilms exhibited three-dimensional cellular organization and were surrounded by substantial amounts of extracellular material. For instance, the wooden chair showed spindle or hazelnut-shaped bacterial cells embedded in an abundant exopolysaccharide (EPS) matrix (Figure 2A). The wooden table displayed a homogeneous bacterial biofilm with coccoid units connected by thin, short filaments (Figure 2B). In the wooden washing trough, compact coconut-shaped biofilms were observed attached to wooden rests (Figure 2C), while forming an interconnected by extracellular filaments or vesicles. Other areas revealed a filamentous bacterial mantle (Figure 2F) likely belonging to the genus *Streptomyces* or related Actinobacteria. On garments, microbial filaments were much scarce (Figures 2G,H); they were adhered tightly to deteriorated fibers of the clothing, with fibrous materials and deposits visible at higher magnifications. In this sample, diverse fungal spores were also found. In the window sample, we observed a biofilm formed by heterogeneous microbial community dominated by large cocci and compacted bacilli, with minimal extracellular material facilitating cell interactions (Figure 2A).

The facade also showed high colonization of the surfaces by biofilms; the entrance door exhibited a thick biofilm structure composed of cocci, dividing bacilli, larger rough-surfaced bacilli, and filamentous bacteria, all forming strongly interconnected aggregates (Figure 2J). The exterior walls demonstrated consolidated coccoid biofilms held together by EPS, likely offering protection against environmental factors, while other regions showed sparse adherence of bacilli in simple distributions.

3.2 Isolation and VITEK-MS-based identification

A total of 49 bacterial strains were isolated from various samples (Table 1 and Figures 3, 4), of which 12 were associated with the museum's exterior surfaces and 37 with interior environments. Each sampled niche exhibited a distinct profile of bacterial taxa, reflecting the contrasting conditions of outdoor and indoor environments. Moreover, each artifact sampled from within the Casa Histórica de la Independencia can be regarded as an isolated microbial island, shaped by its unique environmental conditions, material composition, age, usage and historical context. The microbial communities inhabiting these artifacts form a complex and dynamic microecosystem, where each niche harbors a distinctive collection of bacteria specifically adapted to its substrate (Figure 3). Gram-positive bacteria dominated the isolates, as expected given the use of selective isolation media supplemented with antibiotics to exclude fungi and gram-negative bacteria. The most prolific source of isolates was the albumen photograph, where no inhibitors were used. Here 21 distinct strains were recovered, predominantly from the genera *Bacillus*, *Oceanobacillus*, and *Streptomyces*. In contrast, the Alberdi's garment gave a unique isolate: *Actinomyces odontolyticus*. Morphological characteristics of each strain (texture, color, spore production) were recorded to differentiate isolates (Table 1).

Four strains were isolated from the textile upholstery of a chair (*Micrococcus luteus*, *Staphylococcus equorum*, *Bacillus licheniformis*

² <https://www.novogene.com/amea-en/>

³ <https://doi.org/10.5281/zenodo.15169295>

⁴ <https://www.genome.jp/kegg/mapper/reconstruct.html>

TABLE 2 Genomic sequencing, assembly statistics, and taxonomic identification of selected bacterial strains isolated from the CHM.

Strain code	CH-041	CH-031	CH-036	CH-007	CH-015	CH-021	CH-019	CH-026
Isolation place	Albumen picture	Albumen picture	Albumen picture	SJ Chair (wood)	SJ Window	Main door	Main door	Outside wall
Raw data (Gb)	1.4	1.5	1.6	2.3	2.1	2.2	1.8	2.2
Length after assembly (bp)	4,277,215	3,925,509	7,608,646	3,319,459	3,250,763	3,907,984	3,187,239	3,685,277
Contigs (≥200 nt)	113	33	79	20	42	80	28	56
CDS	4,067	3,925	6,773	3,143	3,063	3,584	3,223	3,416
Annotated genes	3,664	3,737	6,577	2,887	2,766	3,208	2,988	2,909
Taxonomy with Vitek	<i>Bacillus thermoamylovorans</i>	<i>Bacillus galactosidilyticus</i>	<i>Streptomyces griseus</i>	Unidentified	<i>Microbacterium aurum</i>	<i>Corynebacterium pseudodiphtheriticum</i>	<i>Clostridium subterminale</i>	<i>Turicella otitidis</i>
Taxonomy with 16S/gyrB	<i>Caldibacillus thermoamylovorans</i> (gyrB)	<i>Oceanobacillus</i> sp. (gyrB)	<i>Streptomyces globisporus</i> (16S)	<i>Microbacterium imperiale</i> (16S)	<i>Microbacterium aurum</i> (16S)	<i>Kocuria rosea</i> (16S)	<i>Bhargavaea ginsengi</i> (16S)	<i>Pseudoclavibacter</i> sp. (16S)
Taxonomy with ANI	<i>Caldibacillus thermoamylovorans</i>	<i>Oceanobacillus kimchii</i>	<i>Streptomyces rubiginosohelvolus</i>	<i>Microbacterium</i> sp.	<i>Microbacterium aurum</i>	<i>Kocuria</i> sp.	<i>Bhargavaea massiliensis</i>	<i>Pseudoclavibacter</i> sp.
Closest match in NCBI genomes	<i>Caldibacillus thermoamylovorans</i> MSL 185.1 <i>Spacecraft cleanroom</i> ASM2371276v1 (97.93%)	<i>Oceanobacillus kimchii</i> p3-SID1558 <i>Human back skin</i> ASM2514966v1 (99.47%)	<i>Streptomyces</i> sp. f150 <i>Soil</i> ASM255143v1 (98.48%)	<i>Microbacterium arborescens</i> <i>Leaves of Ricinus comunis</i> ASM333966v1 (87.82%)	<i>Microbacterium</i> sp. DE14.009 <i>Geothermal springs</i> ASM1476380v1 (97.98%)	<i>Kocuria</i> sp. <i>Duodenal mucosa of celiac disease patient</i> ASM148375v1 (98.71%)	<i>Bhargavaea massiliensis</i> Marseille-Q1000 human urine GCA_902825275.1 (99.32%)	<i>Pseudoclavibacter helvolus</i> PS02339 <i>Danish slaughterhouse wall</i> ASM3001191v1 (87.11%)

This table summarizes key genomic features of eight representative bacterial isolates selected from distinct environments within the Casa Histórica de la Independencia Museum (CHM), including the albumen print photograph, museum furniture, and exterior surfaces. For each strain, the table includes: (i) the isolation source (artifact or location), (ii) total amount of raw sequencing data generated (in gigabases), (iii) genome assembly length (in base pairs), (iv) number of assembled contigs ≥200 nt, and (v) predicted number of protein-coding sequences (CDS). Whole genome sequencing was performed using Illumina NovaSeq PE150 technology. Assemblies were generated with SPAdes v3.15.4, and functional annotation was conducted using the Arche pipeline. Taxonomic identifications were refined using VITEK-MS, 16S and gyrB gene markers, and ANI-based comparisons against reference genomes in NCBI.

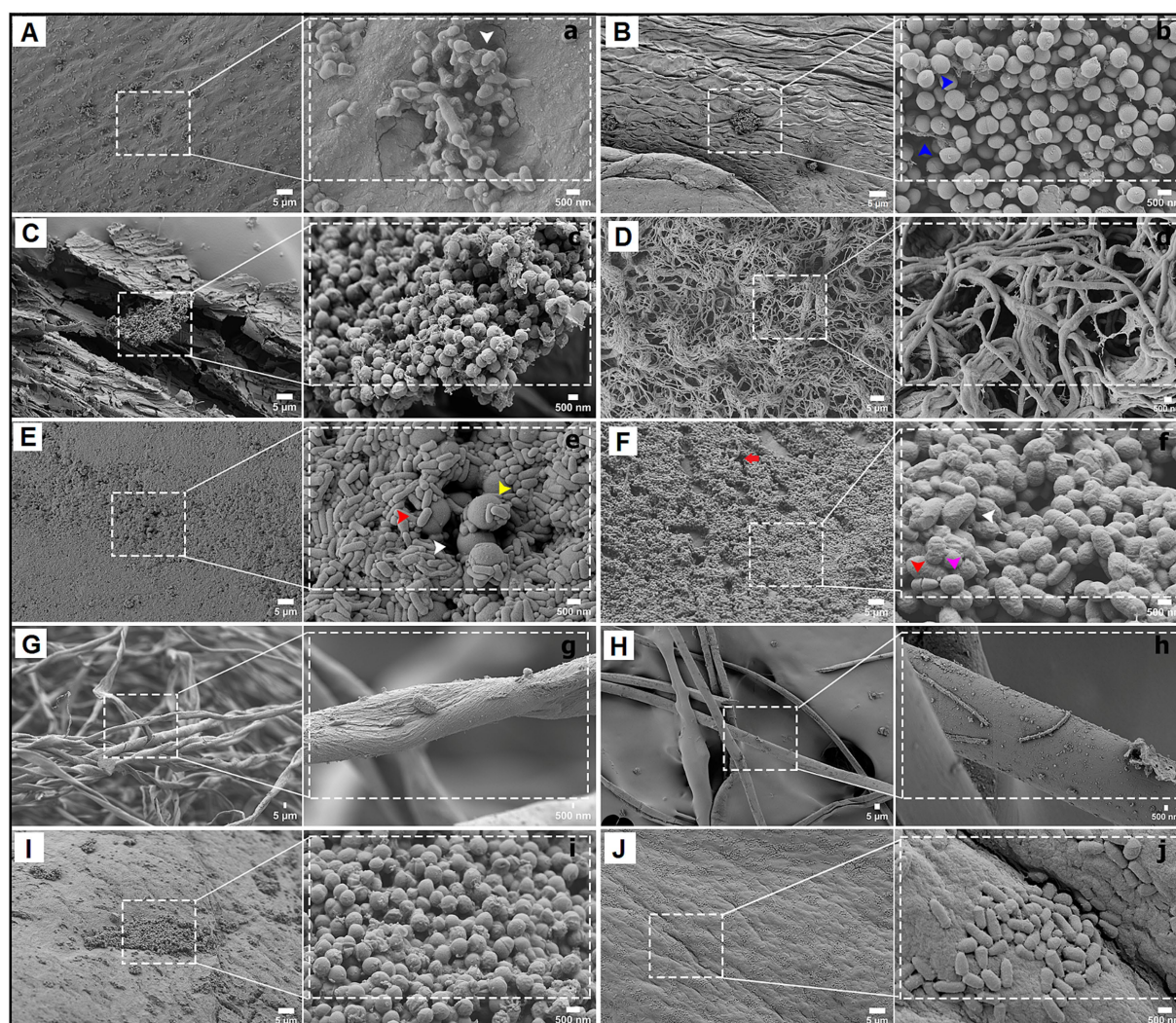


FIGURE 2

Scanning electron microscopy (SEM) of biofilms detected on heritage surfaces from the Casa Histórica de la Independencia Museum (CHM). Each panel displays colonized materials sampled from key museum artifacts. Left subpanels show wide field images (5 μ m scale), while right subpanels present higher magnifications of microbial structures (500 nm scale). (A,a) Chair armrest showing spindle-shaped and hazelnut-like bacterial cells embedded in an extracellular polymeric substance (EPS) matrix. (B,b) Table surface colonized by coccoid bacterial cells interconnected by thin filaments (blue arrowheads). (C,c) Washing trough showing clusters of coconut-shaped biofilm-forming bacteria within cracks of the wood. (D,d) Filamentous network likely corresponding to Actinobacteria (e.g., *Streptomyces*). (E,e) Homogeneous mat of short bacilli (red arrowhead), with sporulated forms (yellow arrowhead) embedded in EPS. (F,f) Multispecies biofilm on wooden surface containing cocci (pink arrowhead), bacilli (red arrowhead), and irregular morphologies (white arrowheads), with clear spatial organization. (G,g) Fibrous textile structure from Alberdi's garment with bacterial filaments entangled with fabric strands. (H,h) Sparse colonization of textile fibers showing limited microbial structures and occasional debris. (I,i) Surface of a window plank with compact coccoid cells embedded in minimal EPS. (J,j) Main entrance door showing a consolidated biofilm of cocci and bacilli within surface depressions.

and *Bacillus simplex*) while three strains were found on the wooden frame of the same chair (*Micrococcus* sp., *Kocuria* sp. and one unidentified strain). Additional 15 isolates were recovered from other wooden elements, including the table (*Kocuria rosea*, *Micrococcus luteus*, *Bacillus altitudinis/pumilus*), the window frame (*Micrococcus luteus*, *Microbacterium aurum* and one unidentified strain), the museum's entrance door (*Microbacterium aurum*, *Bacillus altitudinis/pumilus*, *Clostridium subterminale*, *Micrococcus luteus*, *Corynebacterium pseudodiphtheriticum*, *Kocuria rosea* and one unidentified strain) and a washing trough (*Staphylococcus equorum* and *Bacillus* sp.) where meat of slaughter animals was prepared. Finally, five strains were isolated from the front wall of the building

made of white painted bricks (*Corynebacterium pseudodiphtheriticum*, *Kocuria rosea*, *Turicella otitidis*, *Bacillus megaterium*).

3.3 Genome-based taxonomical identification

Although VITEK MALDI-TOF MS was used for preliminary identification, higher-resolution taxonomic resolution was achieved using 16S rRNA and *gyrB* gene markers and refined through ANI analyses, which provided species-level assignment where reference genomes were available. The *gyrB* gene was used when the 16S-rRNA

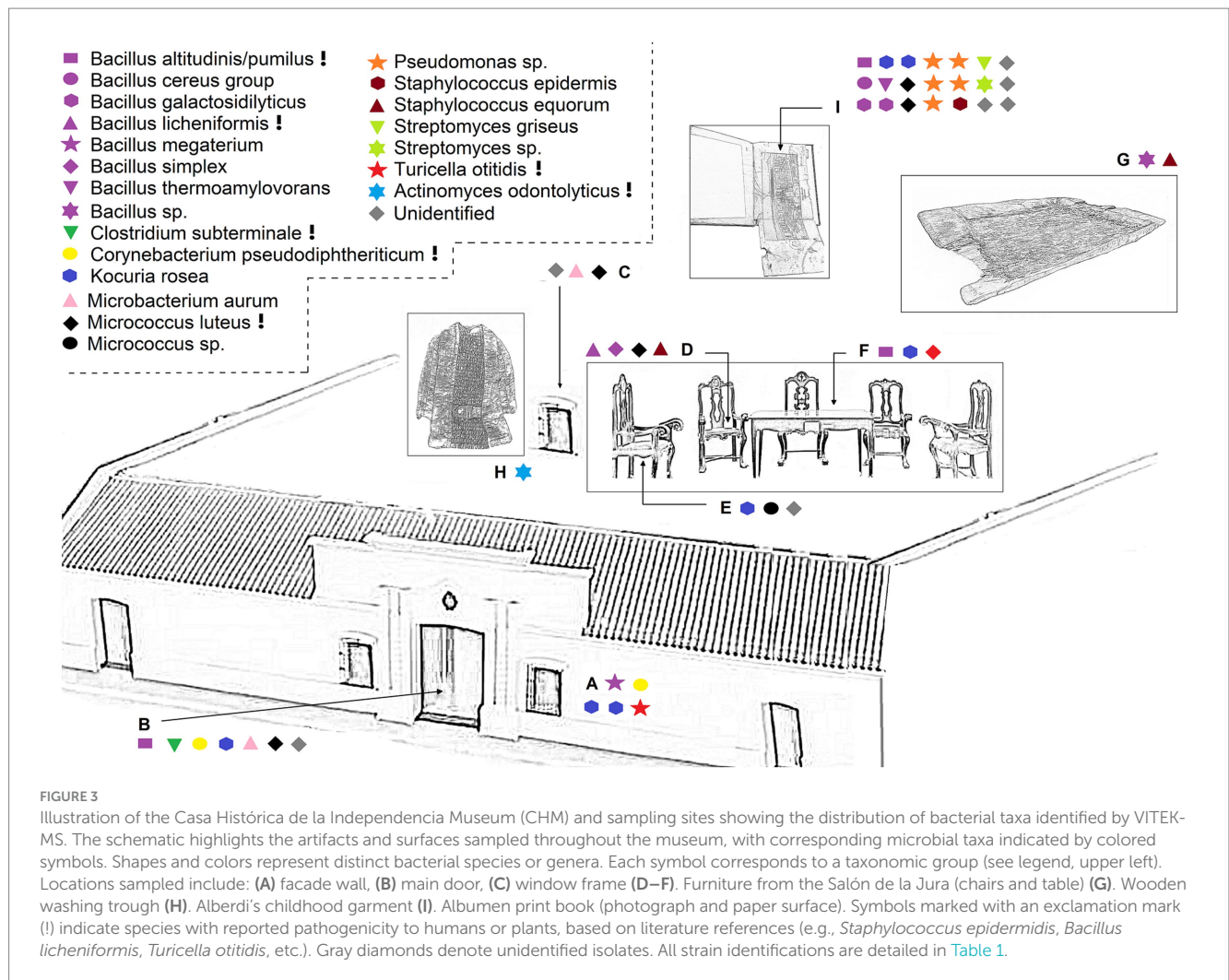


FIGURE 3

Illustration of the Casa Histórica de la Independencia Museum (CHM) and sampling sites showing the distribution of bacterial taxa identified by VITEK-MS. The schematic highlights the artifacts and surfaces sampled throughout the museum, with corresponding microbial taxa indicated by colored symbols. Shapes and colors represent distinct bacterial species or genera. Each symbol corresponds to a taxonomic group (see legend, upper left). Locations sampled include: (A) facade wall, (B) main door, (C) window frame (D–F). Furniture from the Salón de la Jura (chairs and table) (G). Wooden washing trough (H). Alberdi's childhood garment (I). Albumen print book (photograph and paper surface). Symbols marked with an exclamation mark (!) indicate species with reported pathogenicity to humans or plants, based on literature references (e.g., *Staphylococcus epidermidis*, *Bacillus licheniformis*, *Turicella otitidis*, etc.). Gray diamonds denote unidentified isolates. All strain identifications are detailed in Table 1.

gene was incomplete or truncated. ANI analyses were performed using genus-specific genomes from the NCBI database for more precise identification (Table 2).

VITEK identification results for most strains differed from those obtained through MGs. Only strains CH-041 and CH-015 maintained the same species classification by both methods. CH-036 was identified as belonging to the genus *Streptomyces* by VITEK, MGs, and ANI, but the species differed between methods. VITEK accurately classified CH-031 at the family level (Bacillaceae), but MGs identified it at the genus level as *Bacillus*, whereas ANI suggested *Oceanobacillus*. CH-021 and CH-026 only matched at the class level (Actinomycetes) by both methods, while CH-019 matched at the phylum level (Bacillota). CH-007 could not be classified by VITEK but was identified as *Microbacterium* by MGs and ANI. Significant differences were evident in species-level classifications; MGs and ANI agreed on the species for CH-041 (*Caldibacillus thermoamylovorans*) and CH-015 (*Microbacterium aurum*). In CH-031, where MGs did not yield a species classification, ANI clustered it with *Oceanobacillus kimchi* due to >95% similarity to a reference genome in the NCBI database (Richter and Rosselló-Móra, 2009). Notably, CH-007, and CH-026 did not align with any reference genome above the 95% ANI threshold, suggesting they might belong to novel species.

Pairwise comparisons between the CHM genomes and publicly available genomes revealed their closest phylogenetic matches (Table 2). Most strains exhibited >97% sequence similarity; however, CH-026 and CH-007 showed lower similarities of 87.11 and 87.82%, respectively. Notably, three strains — *Oceanobacillus kimchi* CH-031, *Kocuria* sp. CH-021, and *Bhargavaea massiliensis* CH-019 — showed high-confidence matches to species commonly associated with the human microbiome (Table 2). The G + C content varied largely between genomes, ranging from ~35% in CH-031 to ~72% in CH-021.

3.4 Functional annotation of genomes: insights into biodeterioration and environmental resilience

The functional annotation carried out using Arche led to useful information regarding the genomic potential of the strains (Figures 5A–E and Supplementary Tables S1–S15). Genes involved in resistances to antibiotics, osmotic stress, and toxic metals were reported from the annotation. Furthermore, relevant genes related to the biodeterioration of the cultural heritage were found, such as those required for the degradation of wood and paper, and the production of pigments. *Streptomyces* sp. CH-036 revealed the highest numbers

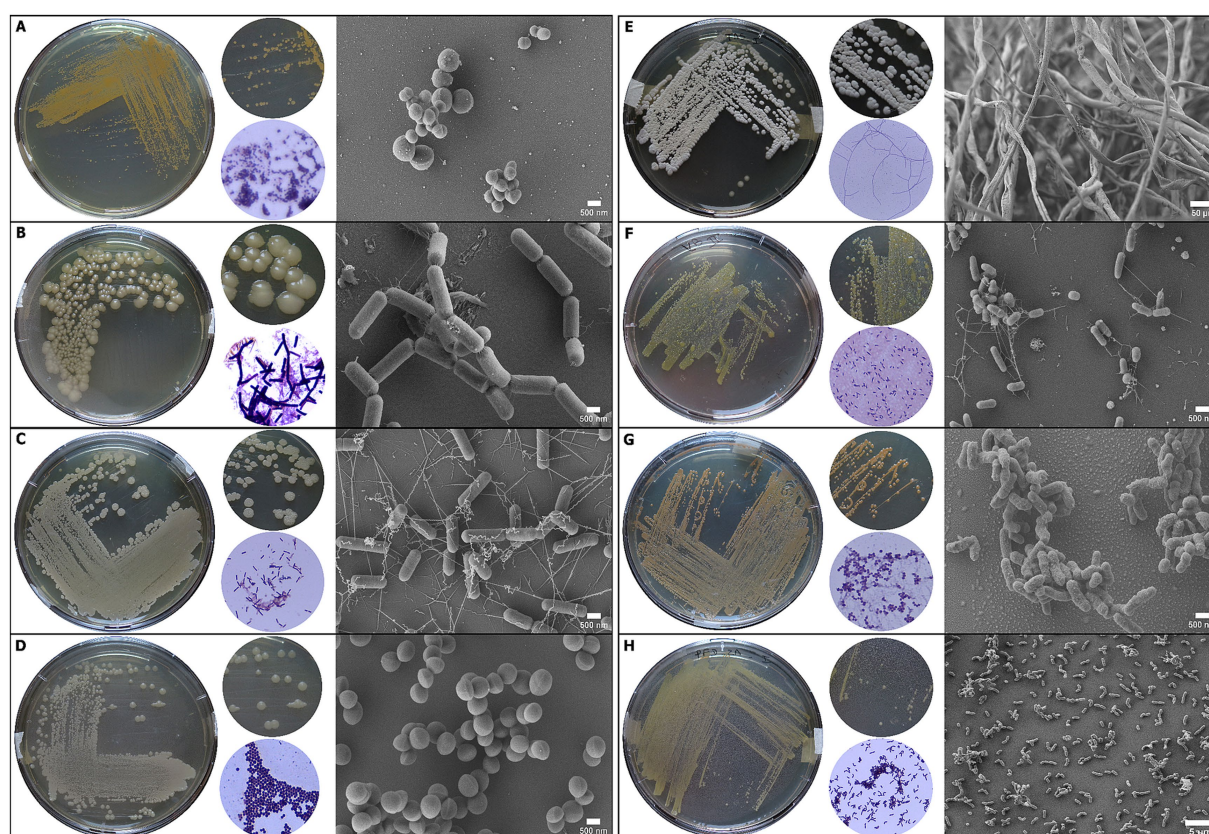


FIGURE 4

Morphological characterization of selected bacterial strains isolated from the CHM using colony morphology, Gram staining, and scanning electron microscopy (SEM). Each row (A–H) corresponds to an individual bacterial strain: (A) CH-005 (*Micrococcus* sp.), (B) CH-003 (*Bacillus* sp.), (C) CH-010 (*Bacillus* sp.), (D) CH-011 (*Staphylococcus* sp.), (E) CH-013 (*Streptomyces* sp.), (F) CH-015 (*Microbacterium aurum*), (G) CH-021 (*Kocuria* sp.), (H) CH-026 (*Turicella otitidis*). From left to right, each panel shows: (i) culture plate image on LB agar with colony pigmentation and texture; (ii) enlarged view of colony morphology; (iii) Gram-stained smear observed under light microscopy, indicating cell wall characteristics and arrangements; (iv) SEM micrograph showing fine-scale cell morphology and surface features. All SEM images were acquired at 500 nm or 5 μ m scale depending on cellular dimensions. The combination of macroscopic, microscopic, and ultrastructural views allows accurate differentiation of morphotypes, aiding taxonomic confirmation and highlighting structural diversity among strains colonizing museum artifacts.

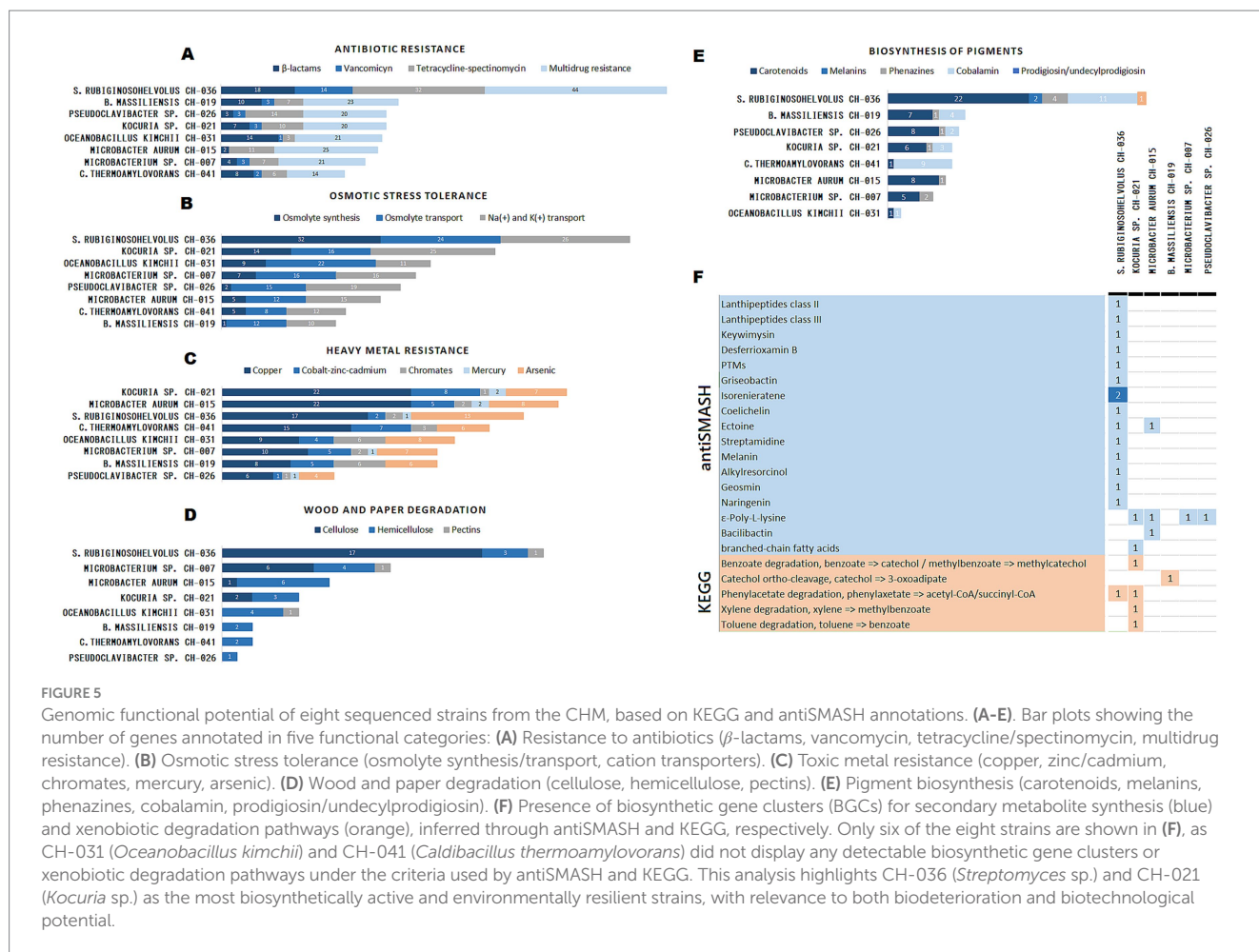
of genes in all the systems mentioned before; except for toxic metal resistance (the numbers are still relatively high). Moreover, CH-036 reported more than twice as many genes related to biodeterioration (Figures 5D,E) as the rest of the strains studied. Particularly, those genes related to the degradation of cellulose and production of pigments, which are potentially harmful to paper documents, were highly abundant in CH-036. *Kocuria* sp. CH-021 strain displayed a high potential to resist both the osmotic and toxic metal stresses. A relatively elevated number of cation transporters were annotated in CH-021's genome, and genes related to copper and chromate homeostasis were also noticeable. Genes for the degradation of cellulose and hemicellulose are also present, together with the capability to produce some pigments. On the other hand, both *Oceanobacillus* sp. CH-031 and *Caldibacillus* sp. CH-041 had a reduced biodeteriorative potential; a tiny group of genes devoted to pigment production and degradation of wood and paper were found with none of the latter related to cellulose degradation. Finally, CH-041 showed a clear toxic metal resistance profile considering the coding-sequences found in the genome.

Further functional capabilities of relevance were studied, such as the potential for the synthesis of secondary metabolites, and

degradation of xenobiotics (Figure 5F). The results of the screening with antiSMASH revealed *Streptomyces* sp. CH-036 as the most diverse of the strains regarding to the hosting of biosynthetic gene clusters (BGCs) devoted to the production of secondary metabolites. Among the putative products of these BGCs are lanthipeptides, and ϵ -poly-L-lysines, both compounds with reported antimicrobial properties (Repka et al., 2017; Wang et al., 2021). Other compounds reported from CH-036 genome were desferrioxamin (Holden and Nair, 2019), and isorenieratene, which is a light-induced yellow pigment (Takano et al., 2005). On the other hand, KEGG identifiers retrieved from the annotation of *Kocuria* sp. CH-021 genome revealed several complete pathways for xenobiotic degradation, including those for the degradation of benzoate, phenylacetate, xylene, and toluene.

4 Discussion

This study revealed significant microbial colonization on historical artifacts and architectural surfaces, highlighting both the conservation challenges and the potential scientific value of these microbial communities. SEM analysis of historical artifacts showed extensive



biofilm development in most samples. In some cases, like the Alberdi's garment, it was observed tightly adhered microbial filaments to altered original material of the pieces, providing direct evidence of biodeterioration processes that threaten cultural heritage.

Microbial colonization in the CHM is shaped by distinct ecological niches, with differences observed between the outdoor urban microbiome, the indoor museum microbiome, and the albumen print book which remains stored in a metallic case. The exteriors, primarily represented by microbial communities on the museum's facade, entrance door, and exterior walls, is continuously exposed to fluctuating environmental conditions such as temperature variations, UV radiation, precipitation, and air pollutants. In contrast, the interiors comprise microbial communities inhabiting historical furniture, textiles, and architectural surfaces within the controlled indoor environment of the museum. These niches are characterized by relatively stable temperature and humidity conditions, lower UV exposure, and reduced air circulation. Finally, the book endures minimal light exposure, thermal fluctuations, and limited handling which could favor specialized communities.

It is important to acknowledge that the use of LB medium in the isolation procedures likely introduced a bias toward fast-growing, copiotrophic bacteria. While this approach successfully recovered strains with potential relevance to biodeterioration processes, it inevitably captures only a fraction of the microbial diversity present in the sampled environments. The rich nutrient content of LB does not

reflect the oligotrophic and substrate-specific conditions typically found on cultural heritage materials. Consequently, slow-growing and metabolically specialized microorganisms may have been underrepresented in our isolate collection. Future efforts could benefit from using selective or minimal media supplemented with heritage-relevant substrates, such as albumin, cellulose, or lignin, to recover a broader spectrum of metabolically active and ecologically relevant taxa. Nonetheless, the genomic and phenotypic insights derived from the isolates obtained here provide valuable initial data for understanding microbial contributions to biodeterioration in museum contexts. Finally, as such isolates may not fully represent the actively growing microbial community responsible for ongoing biodeterioration, their ecological relevance should be interpreted with caution and complemented by future culture-independent analyses (Liu et al., 2022).

Worth to note is that the use of multiple complementary methods for bacterial identification allowed us to assess the strengths and limitations of each approach. While VITEK MALDI-TOF MS offered rapid initial classification, its performance was constrained by the scope of the clinical-focused database, potentially leading to misclassification or low-resolution results for environmental isolates. In contrast, genome-resolved methods based on 16S rRNA and *gyrB* sequences, along with average nucleotide identity (ANI) comparisons, provided superior accuracy and confidence in taxonomic assignment, particularly for uncommon or novel strains. Therefore, for

species-level resolution, ANI proved to be the most reliable method in this study. This multi-method approach also allowed us to identify potential novel species and refine our understanding of microbial diversity associated with heritage materials.

The albumen prints in the book *Provincia de Tucumán* represent a unique microenvironment, as even subtle microclimatic differences can shape distinct microbial communities and deterioration patterns (Meng et al., 2023). Composed primarily of denatured egg white proteins, cellulose and salts they can be a suitable substrate for microbial colonization and biodeterioration. Several strains isolated from the CHM's photo book were classified by VITEK, MGs, and ANI as belonging to the genera *Bacillus*, *Oceanobacillus*, *Micrococcus*, *Streptomyces*, *Staphylococcus* and *Kocuria* (Table 1 and Figure 3). These taxa were consistently reported as microbial contamination in historical archives and audiovisual materials (Villalba et al., 2004; Puškárová et al., 2016; Mazzoli et al., 2018; Branysova et al., 2023). They have proven amylolytic, cellulolytic, proteolytic, and/or lipolytic capacities related to biodeterioration of paper, photographs, and/or other visual material which were mainly determined by culture-dependent methods (Villalba et al., 2004; Branysova et al., 2023). The amylolytic capacities can be used for the consumption of starch from paper (used as a glue in its manufacturing) while the proteolytic activity presents a threat especially to visual materials with a gelatinous binder. Some species with cellulolytic properties pose a risk to collodion visual materials (as the main component of collodion is cellulose nitrate) while others have the ability to produce alkaline serine proteases which are harmful to albumin materials (Villalba et al., 2004; Branysova et al., 2023).

Notably, *Pseudomonas* strains were exclusively isolated from the photograph, aligning with its role as a frequent contaminant in photographic manufacturing due to its ability to metabolize gelatin and resist silver nitrate (Gadd et al., 1989; Borrego et al., 2010; Kraková et al., 2012b; Sterflinger and Piñar, 2013; Madigan et al., 2014; Grabek-Lejko et al., 2017; Mazzoli et al., 2018; Warner Marien, 2022). *Pseudomonas* spp. and *Streptomyces* spp. poses a risk not only because of its potential catalytic properties, but also because of the ability of some members to produce deteriorative pigments. For example, some *Streptomyces* spp. ruined graphic art inside some Etruscan and Roman tombs due to the production of violet pigments, while members of *Pseudomonas* produced both green (in an alkaline environment) and red (in acidic conditions) pigments during wool degradation of historical textiles (Díaz-Herráiz, 2015; Gutarowska et al., 2016; Mazzoli et al., 2018). In accordance with culture-dependent methods, genomic analysis over the strain *Streptomyces* sp. CH-036 (Table 2) revealed strong capabilities to degrade components of paper (Figure 5D). Moreover, a high number of genes for the biosynthesis of several pigments were reported (Figure 5E) including isorenieratene (Figure 5F), a light-induced pigment (Takano et al., 2005). A similar pigment-related genes were found in *Rubrobacter* strains colonizing the pink biofilms on frescoes from Georgian cathedrals, where their persistence was linked to both light exposure and metabolic adaptability (Basile et al., 2025). These findings should encourage CHM authorities to implement precautionary measures that prevent the exposure of the albumen print book to light, humidity, or warm temperatures—conditions that could facilitate microbial proliferation. In particular, humidity and water availability are widely recognized as the primary drivers of microbial growth and subsequent biodeterioration on cultural heritage surfaces. Recent evidence

highlights that even subtle moisture levels can dramatically shape microbial colonization and biochemical activity, especially in porous materials and confined microenvironments in cultural heritage (Liu et al., 2018a; Wang et al., 2025). Therefore, managing humidity is critical not only for prevention, but also for potentially shifting microbial activity toward less harmful states. Finally, preservation strategies, including illumination control and environmental stabilization, have proven effective in mitigating biodeterioration in mural paintings at the Positano Roman Archaeological Museum (Antonelli et al., 2024).

Resistance genes, including multidrug efflux systems and detoxification enzymes, were reported in pigment-producing bacteria from fresco biofilms, where they contributed to the resilience of microbial communities against biocidal treatments (Basile et al., 2025). Interestingly, the genome of *Streptomyces* CH-036 also showed a strong antibiotic resistance profile of genes (Figure 5A). This finding is noteworthy, as certain *Streptomyces* species present in museum environments have been proposed to pose potential risks to the human respiratory system (Borrego et al., 2010). Furthermore, it was pointed out the importance of regular disinfection routines, after several *Bacillus* spp., *Staphylococcus* spp., and *Streptomyces* spp. with potentially pathogenic characteristics were reported in museum collections (Gutarowska et al., 2014; Kraśnicki et al., 2025). It is thus important keep away threats to health from the CHM's staff and visitors, especially those who are immune-compromised. Protection elements should be used in the manipulation of museum artifacts, and the implementation of disinfection protocols must carefully balance microbial control with material preservation. For instance, ethanol mist has been validated as a biocidal method that significantly reduces microbial load without damaging collagen structures, making it a promising option for delicate heritage materials (Kraśnicki et al., 2025). Also, we encourage the adoption of appropriate measures to avoid new infectious agents coming from the outside of the CHM's. Proof of the unprotected handling of the book of albumen print is the presence of the commensal species *Staphylococcus epidermis*, related to the human skin microbiome (Villalba et al., 2004). Also, a 99.47% of similarity was found between the genomes of *Oceanobacillus kimchii* CH-031 and *Oceanobacillus kimchii* p3-SID1558 isolated from the human back skin (Table 2).

Some microbes having their optimal growth during the different manufacturing processes of the photo book could have been preserved via spores once the manufacturing conditions changed. Indeed, 7 of the 17 strains from the book identified taxonomically by the VITEK method are producers of endospores (*Bacillus* spp.) or resistance spores (*Streptomyces* spp.). Both endospores and resistance spores are metabolically inactive structures, extremely resistant to adverse environmental conditions, such as high concentrations of salts, absence of nutrients or low levels of humidity. The adaptive advantage conferred on bacteria is to allow them to remain as spores for as long as necessary until the conditions are suitable (Cano and Borucki, 1995; Nicholson et al., 2000; Madigan et al., 2014). Apparently, this could explain the isolation of the thermophilic strain *Caldibacillus thermoamylovorans* CH-041 which grows optimally at 50°C, a similar finding previously reported for thermophilic *Geobacillus* spp. in albumen supports (Puškárová et al., 2016). The most likely hypothesis is that the thermophile proliferated during the initial manufacturing (Puškárová et al., 2016). Different methods were used to harden the albumen layers, including steaming and storing the albumen-coated

papers inside a warm hayloft for half a year. The temperature of a hayloft can sometimes reach 50°C or more, providing an optimal environment for the growth of CH-041 and other thermophiles (Puškárová et al., 2016). Considering that CH-041 survived for 160 years under environmental conditions far cooler than the ~50°C required for its optimal growth its successful reactivation today suggests a level of genomic stability and preservation over time (Schuenemann et al., 2018; Bonczarowska et al., 2022). A deeper look in to the genomics of CH-041 revealed a scarce amount of genes for antibiotic resistance (Figure 5A), and biodeterioration (Figures 5D,E), making it the most environment friendly strain reported for the CHM.

A particularly intriguing case was observed in Alberdi's suit, a 19th-century silk and lace garment, where we identified a unique bacterial strain, classified by VITEK as *Actinomyces odontolyticus*. This filamentous bacterium is typically found in human microbiomes, particularly in the oral cavity, and profusely isolated from dental caries (Batty, 1958). Manifestations of infection by *A. odontolyticus* include thoracic, abdominal, pelvic and central nervous system disease (Razok et al., 2022). Recently, it was proposed as driver of Colorectal Cancer (Breau, 2024). Its presence on the textile suggests potential contamination from human handling or storage conditions, a closed cartoon box that avoids contamination and humidity changes. Notably, the possibility that the original owner of the suit, Juan Bautista Alberdi, introduced this bacterium as a child cannot be ruled out, given the high prevalence of dental infections in toddlers and young children. The genus *Actinomyces* sp. was previously cited in a list of the most common bacteria responsible for biodeterioration of cultural heritage (Grabek-Lejko et al., 2017). A more detailed study of the strain, including genome sequencing should reveal with certainty its taxonomic identity and toxicity, considering the hypothesis that it could have originated from an infected person.

The museum's wooden furniture is affected by frequent procedures of cleaning and disinfection, as well as repairs and preventive actions against wood decay. Frequently, the wooden material in the museum is sensible to other pests, such as insects. For this reason, it is not expected to these wooden items to suffer biodeterioration driven primarily by bacteria. In spite of this, the isolates from the wooden parts of the chair, table, window, washing trough, and main access door could have cellulolytic or lignocellulolytic capabilities required for the degradation of wood. The most commonly reported bacteria associated with wood-decay environments are genera such as *Clostridium*, and cosmopolitan taxa such as *Bacillus* (Liu et al., 2018b; Pyzik et al., 2021), both isolated from the aforementioned elements (Table 1). A notable high number of genes related to the breakup of cellulose and hemicellulose were found in the genomes of *Microbacterium* sp. CH-007, *Kocuria* sp. CH-021, and *Microbacterium* sp. CH-015 (Figure 5D), which were isolated from the chair wood, the entrance door and the window, respectively, (Table 1 and Figure 3). This could be supporting some kind metabolic activity over the wooden compounds of the furniture. Moreover, the strongest profiles of metal resistances belong to CH-021 and CH-015, and are probably the reflection of the chemical treatments used over the years to preserve the wood from decay. Most of these wood preservatives recruit toxic metals in their formulas, like copper chromate arsenate (CCA) which was widely used in the past but now restricted due its toxicity (Woźniak, 2022). Finally, SEM micrographs of the washing trough showed the presence of filamentous bacteria probably from the genus *Streptomyces* or related (Figure 2D). Previous studies showed that filamentous actinomycetes are associated with wood decay (Antai

and Crawford, 1981; Jayasinghe and Parkinson, 2008), while other claim that their presence in wood could prevent worst scenarios of biodeterioration like those caused by fungi (Mortabit et al., 2015; Cai and Kuo, 2022).

One of the strains we isolated from the chair textile was classified as *Bacillus licheniformis*, a species which is in the list of pathogens infecting humans (Bartlett et al., 2022). *B. licheniformis* was reported in several cases of infection, even in a patient with no history of any immune deficiency (Haydushka et al., 2012). Another intriguing species is *Staphylococcus equorum*, isolated from both the textile chair and the washing trough. *S. equorum* is a commensal and obligate aerobe usually found on the skin of various farm animals, such as horses, dairy cattle and goats (Vermeersch et al., 2023; Reydam et al., 2024; Senoussi et al., 2024). This microorganism probably has colonized the seat and meat washing trough during the nineteenth century, when people of the city interacted routinely with farm animals and used horses as their primary vehicle or cattle as meat source.

Regarding the outdoor environment, which includes the main entrance door and the outside wall, the microorganisms found there are related to the street environment and human activity. For example, the species identified by VITEK as *Bacillus pumilus* CH-018, *Clostridium subterminale* CH-019, *Corynebacterium pseudodiphtheriticum* CH-024, *Turicella otitidis* CH-026, and *Micrococcus luteus* CH-020 are either known human pathogens, or related to the human microbiome (Stackebrandt et al., 1995; Bartlett et al., 2022; Zhou et al., 2022; Ahamad et al., 2023). Furthermore, when ANI comparisons are used the genome of the strain CH-019 shares a 99.32% of similarity with *Bhargavaea massiliensis* Marseille-Q1000 isolated from a urine sample in patients with urinary tract infections (Table 2, Section 3.3). Likewise, ANI showed a 98.71% of similarity between the genomes of CH-021 and *Kocuria* sp. CD08_4, an isolate from the duodenal mucosa of a celiac disease patient (Munish et al., 2017). Finally, CH-017 and CH-027 were both classified by Vitek as *B. pumilus* and *B. megaterium* which have been reported as plant pathogens in other work (Bull et al., 2012; Yuan and Gao, 2015).

The genomic profiles of some of the isolates reveal a promising biotechnological potential beyond their role in biodeterioration. Several *Bacillus* and *Pseudomonas* strains identified in this study have previously been proposed as candidates for bioprecipitation of calcium carbonate crystals, a process with practical applications in bioremediation and consolidation of stone and plaster monuments. Notably, *Bacillus megaterium* and *Bacillus simplex*, both detected among the isolates, are among the most effective carbonate bioprecipitators and are used in protective treatments for historical architecture (Paramo-Aguilera, 2012; Pyzik et al., 2021). In addition, members of the *Bacillus* genus have been suggested for use in biotreatments of textiles and paper artifacts, particularly for their enzymatic capabilities in cleaning and stabilizing cellulosic and proteinaceous materials. Similarly, *Micrococcus* spp. are known for their capacity to produce bioactive molecules and enzymes of interest to the pharmaceutical and bioremediation industries, including antimicrobial compounds and pollutant-degrading enzymes (Díaz-Herráiz, 2015).

Importantly, our genomic analyses revealed that *Streptomyces* sp. CH-036 and *Kocuria* sp. CH-021 harbor genes involved in the synthesis of valuable secondary metabolites and the degradation of xenobiotic compounds (see Figure 5F and section 3.4). These findings position these strains as promising sources of novel biotechnological resources and reinforce the concept of cultural heritage environments as overlooked reservoirs of useful microorganisms. Further exploration of these isolates

could yield new applications in conservation, biotechnology, and environmental remediation.

While our investigation prioritized the culturable fraction of the bacterial microbiome, primarily due to its accessibility and relevance for subsequent genomic and phenotypic analyses, it is important to recognize that fungal organisms also play a pivotal role in the biodeterioration of cultural heritage biological origin (Zhgun et al., 2020; Antonelli et al., 2024; Stratigaki et al., 2024; Kraśnicki et al., 2025). Fungi are well-documented agents of degradation, particularly in cellulose- and protein-rich substrates, where their enzymatic capabilities enable persistent and often aggressive colonization. However, characterizing fungal communities typically requires distinct culturing conditions, longer incubation times, and complementary approaches such as ITS-based metabarcoding or cultivation on specific mycological media. Considering these methodological divergences, we prioritized a focused bacterial analysis in this first stage. Future studies could expand the scope by integrating fungal community profiling, thereby offering a more comprehensive understanding of the microbial consortia contributing to heritage deterioration.

5 Concluding remarks and prospect

This study provides the first comprehensive microbiological survey of the Casa Histórica de la Independencia Museum, revealing a rich and complex microbial landscape shaped by historical, material, and environmental factors. Through the integration of scanning electron microscopy, microbial isolation, MALDI-TOF MS, and genome sequencing, we uncovered distinct bacteria inhabiting both outdoor and indoor surfaces of cultural heritage significance. This work advances the field of heritage microbiology and supports efforts to safeguard culturally significant objects through a deeper understanding of their microbial ecosystems. Future work should expand to metagenomic and metatranscriptomic approaches, allowing for a deeper exploration of microbial dynamics, functional activity, and long-term succession on heritage substrates.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/>, bioproject accession number PRJNA1233209.

Author contributions

DA-R: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. FG: Investigation, Methodology, Writing – original draft, Writing – review & editing. NA: Investigation, Methodology, Writing – review & editing. MDA: Investigation, Methodology, Writing – review & editing. LM: Methodology, Writing – review & editing. HE: Methodology, Writing – review & editing. CG: Methodology, Writing – review & editing. MS: Methodology, Writing – review & editing. VA: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing, Formal analysis, Supervision.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. All authors are members of the National Research Council (CONICET) in Argentina. VA was a recipient of a Georg Foster Scholarship for Experienced Researchers, Alexander von Humboldt Foundation (2021–2023) and of a grant of the Williams Foundation (Impacto en el Territorio Argentino 2024). This work was funded by National University of Tucuman with Projects PIUNT G603 (2017–2022) and G703 (2023–2027). Funding was also available through research Project in Thematic Areas of Museums and National Institutes 2021–2022 (CONICET and Ministry of Culture of Argentina) and Universidad San Pablo-Tucumán (Project ISYCAV-842 2019–2021).

Acknowledgments

The authors sincerely thank the Museo Casa Histórica de la Independencia staff, especially former Director Cecilia Guerra Orozco and Cecilia Barrionuevo and Ana Oliva from the restoration, conservation and museology department, for kindly granting access to the museum's collections. Electron micrographs used in this study were taken at the Center for Electron Microscopy (CIME) belonging to UNT and CONICET, in Tucumán, Argentina.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of *Frontiers*, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1611832/full#supplementary-material>

References

- Adamiak, J., Otlewska, A., Tafer, H., Lopandic, K., Gutarowska, B., Sterflinger, K., et al. (2018). First evaluation of the microbiome of built cultural heritage by using the ion torrent next generation sequencing platform. *Int. Biodeterior. Biodegrad.* 131, 11–18. doi: 10.1016/j.ibiod.2017.01.040
- Ahamad, A., Yuan, C., Chung, C., Blair, B., Tran, A., and Tehreem, B. (2023). Metabolism and gene sequence variation in *Turicella otitidis* implies its adaptability and pathogenicity in extra-otic infection: a systematic review. *BMC Infect. Dis.* 23:735. doi: 10.1186/s12879-023-08721-y
- Alonso-Reyes, D. G., and Albarracín, V. H. (2025). Arche: An advanced flexible tool for high-throughput annotation of functions on microbial contigs. *IEEE Transactions on Computational Biology and Bioinformatics*.
- Alonso-Reyes, D. G., Galván, F. S., Portero, L. R., Alvarado, N. N., Farías, M. E., Vazquez, M. P., et al. (2021). Genomic insights into an andean multiresistant soil actinobacterium of biotechnological interest. *World J. Microbiol. Biotechnol.* 37:166. doi: 10.1007/s11274-021-03129-9
- Antai, S. P., and Crawford, D. L. (1981). Degradation of softwood, hardwood, and grass lignocelluloses by two *Streptomyces* strains. *Appl. Environ. Microbiol.* 42, 378–380. doi: 10.1128/aem.42.2.378-380.1981
- Antonelli, F., Iafrate, S., Tescari, M., Giandomenico, M., Kumbaric, A., and Bartolini, M. (2024). The Hypogean Roman archaeological Museum of Positano: study of the evolution of biological threats and development of adequate control protocols. *Microorganisms* 12:520. doi: 10.3390/microorganisms12081520
- Baldan, M., Manente, S., and Izzo, F. C. (2021). The role of bio-pollutants in the indoor air quality of old museum buildings: artworks biodeterioration as preview of human diseases. *Environ. Monit. Assess.* 193:787. doi: 10.1007/s10661-021-09568-z
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021
- Bartlett, A., Padfield, D., Lear, L., Bendall, R., and Vos, M. (2022). A comprehensive list of bacterial pathogens infecting humans. *Microbiology* 168:1269. doi: 10.1099/mic.0.001269
- Basile, A., Riggio, F. P., Tescari, M., Chebbi, A., Sodo, A., Bartoli, F., et al. (2025). Metagenome-resolved functional traits of *Rubrobacter* species implicated in rosy discoloration of ancient frescoes in two Georgian cathedrals. *Sci. Total Environ.* 958:178135. doi: 10.1016/j.scitotenv.2024.178135
- Batty, I. (1958). *Actinomyces odontolyticus*, a new species of actinomycete regularly isolated from deep carious dentine. *J. Pathol. Bacteriol.* 75, 455–459. doi: 10.1002/path.1700750225
- Blin, K., Shaw, S., Augustijn, H. E., Reitz, Z. L., Biermann, F., Alanjary, M., et al. (2023). antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic Acids Res.* 51, W46–W50. doi: 10.1093/nar/gkad344
- Bonczarowska, J. H., Susat, J., Mühlemann, B., Jasch-Boley, I., Brather, S., Höke, B., et al. (2022). Pathogen genomics study of an early medieval community in Germany reveals extensive co-infections. *Genome Biol.* 23:250. doi: 10.1186/s13059-022-02806-8
- Borrego, S., Guiamet, P., Gómez de Saravia, S., Batistini, P., Garcia, M., Lavin, P., et al. (2010). The quality of air at archives and the biodeterioration of photographs. *Int. Biodeterior. Biodegrad.* 64, 139–145. doi: 10.1016/j.ibiod.2009.12.005
- Branysova, T., Limpouch, O., Durovic, M., Demnerova, K., and Stiborova, H. (2023). Bacterial diversity on historical audio-visual materials and in the atmosphere of Czech depositories. *Microbiol. Spectr.* 11:1176. doi: 10.1128/spectrum.01176-23
- Breau, K. A. (2024). *Actinomyces odontolyticus*: from carries to colorectal Cancer. *Cell. Mol. Gastroenterol. Hepatol.* 17, 879–880. doi: 10.1016/j.jcmgh.2024.02.009
- Bull, C. T., de Boer, S. H., Denny, T. P., Firrao, G., Fischer-Le Saux, M., Saddler, G. S., et al. (2012). List of new names of plant pathogenic bacteria (2008–2010) prepared by the international society of plant pathology committee on the taxonomy of plant pathogenic bacteria (ISPP-CTPPB). *J. Plant Pathol.* 94, 21–27.
- Cai, L., and Kuo, C.-J. (2022). Epsilon poly-L-lysine as a novel antifungal agent for sustainable wood protection. *Front. Microbiol.* 13:908541. doi: 10.3389/fmicb.2022.908541
- Cano, R. J., and Borucki, M. K. (1995). Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican Amber. *Science* 268, 1060–1064. doi: 10.1126/science.7538699
- Chatruc, C. (2021). Los diez museos más visitados de la Argentina La Nación. Available online at: <https://www.lanacion.com.ar/cultura/los-diez-museos-mas-visitados-de-la-argentina-nid20092021/> (Available December 10, 2024).
- Díaz-Herráiz, M. (2015). Caracterización de Comunidades Microbianas en Tumbas Etruscas y Romanas. Sevilla: Universidad de Sevilla.
- Elhagrassey, A. F. (2018). Isolation and characterization of actinomycetes from mural paintings of Snu- Sert-anhk tomb, their antimicrobial activity, and their biodeterioration. *Microbiol. Res.* 216, 47–55. doi: 10.1016/j.micres.2018.08.005
- Gadd, G. M., Laurence, O. S., Briscoe, P. A., and Trevors, J. T. (1989). Silver accumulation in *Pseudomonas stutzeri* AG259. *Biol. Met.* 2, 168–173. doi: 10.1007/BF01142556
- Grabek-Lejko, D., Tekiel, A., and Kasprzyk, I. (2017). Risk of biodeterioration of cultural heritage objects, stored in the historical and modern repositories in the regional Museum in Rzeszów (Poland). A case study. *Int. Biodeterior. Biodegrad.* 123, 46–55. doi: 10.1016/j.ibiod.2017.05.028
- Granillo, A. (1872). Provincia de Tucumán. San Miguel de Tucumán: Razon.
- Gutarowska, B., Pietrzak, K., Machnowski, W., and Milczarek, J. M. (2016). Historical textiles – a review of microbial deterioration analysis and disinfection methods. *Text. Res. J.* 87, 2388–2406. doi: 10.1177/0040517516669076
- Gutarowska, B., Pietrzak, K., and Skóra, J. (2014). Disinfection as a factor reducing microbial threat at workposts in museum and library - a comparison of the effectiveness of photocatalytic ionization, UV irradiation and chemical misting. *Int. J. Curr. Microbiol. Appl. Sci.* 3, 945–959.
- Haydushka, I. A., Markova, N., Kirina, V., and Atanassova, M. (2012). Recurrent sepsis due to *Bacillus licheniformis*. *J. Glob. Infect. Dis.* 4, 82–3. doi: 10.4103/0974-777X.93768
- Holden, P., and Nair, L. S. (2019). Deferoxamine: an Angiogenic and antioxidant molecule for tissue regeneration. *Tissue Eng. Part B Rev.* 25, 461–470. doi: 10.1089/ten.teb.2019.0111
- Jayasinghe, B. A. T. D., and Parkinson, D. (2008). Actinomycetes as antagonists of litter decomposer fungi. *Appl. Soil Ecol.* 38, 109–118. doi: 10.1016/j.apsoil.2007.09.005
- Karbowska-Berent, J., Górny, R. L., Strzelczyk, A. B., and Wlazło, A. (2011). Airborne and dust borne microorganisms in selected polish libraries and archives. *Build. Environ.* 46, 1872–1879. doi: 10.1016/j.buildenv.2011.03.007
- Kraková, L., Chovanová, K., Selim, S. A., Šimonovičová, A., Puškarová, A., Maková, A., et al. (2012). A multiphasic approach for investigation of the microbial diversity and its biodegradative abilities in historical paper and parchment documents. *Int. Biodeterior. Biodegrad.* 70, 117–125. doi: 10.1016/j.ibiod.2012.01.011
- Kraková, L., Šoltys, K., Otlewska, A., Pietrzak, K., Purkrtová, S., Savická, D., et al. (2018). Comparison of methods for identification of microbial communities in book collections: culture-dependent (sequencing and MALDI-TOF MS) and culture-independent (Illumina MiSeq). *Int. Biodeterior. Biodegrad.* 131, 51–59. doi: 10.1016/j.ibiod.2017.02.015
- Kraśnicki, K., Pydyn, N., Papis, A., Guzińska, K., Kaźmierczak, D., Maciolek, U., et al. (2025). Reducing microbial contamination on historical leather artifacts at the Auschwitz-Birkenau state museum using ethanol in the form of mist. *Front. Microbiol.* 16:6114. doi: 10.3389/fmicb.2025.1576114
- Książczyk, M., Kuczkowski, M., Dudek, B., Korzekwa, K., Tobiasz, A., Korzeniowska-Kowal, A., et al. (2016). Application of routine diagnostic procedure, VITEK 2 compact, MALDI-TOF MS, and PCR assays in identification procedure of bacterial strain with ambiguous phenotype. *Curr. Microbiol.* 72, 570–582. doi: 10.1007/s00284-016-0993-0
- Liu, Z., Fu, T., Hu, C., Shen, D., Macchioni, N., Sozzi, L., et al. (2018b). Microbial community analysis and biodeterioration of waterlogged archaeological wood from the Nanhai no. 1 shipwreck during storage. *Sci. Rep.* 8, 7170–7111. doi: 10.1038/s41598-018-25484-8
- Liu, X., Meng, H., Wang, Y., Katayama, Y., and Gu, J.-D. (2018a). Water is a critical factor in evaluating and assessing microbial colonization and destruction of Angkor sandstone monuments. *Int. Biodeterior. Biodegrad.* 133, 9–16. doi: 10.1016/j.ibiod.2018.05.011
- Liu, X., Qian, Y., Wang, Y., Wu, F., Wang, W., and Gu, J.-D. (2022). Innovative approaches for the processes involved in microbial biodeterioration of cultural heritage materials. *Curr. Opin. Biotechnol.* 75:102716. doi: 10.1016/j.copbio.2022.102716
- Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., and Stahl, D. A. (2014). Brock biology of microorganisms. 14th Edn. San Francisco, CA: Benjamin Cummings.
- Mazzoli, R., Giuffrida, M. G., and Pessione, E. (2018). Back to the past: “find the guilty bug—microorganisms involved in the biodeterioration of archeological and historical artifacts.” *Appl. Microbiol. Biotechnol.* 102, 6393–6407. doi: 10.1007/s00253-018-9113-3
- Meng, S., Qian, Y., Liu, X., Wang, Y., Wu, F., Wang, W., et al. (2023). Community structures and biodeterioration processes of epilithic biofilms imply the significance of micro-environments. *Sci. Total Environ.* 876:162665. doi: 10.1016/j.scitotenv.2023.162665
- Michaelsen, A., Piñar, G., Montanari, M., and Pinzari, F. (2009). Biodeterioration and restoration of a 16th-century book using a combination of conventional and molecular techniques: a case study. *Int. Biodeterior. Biodegrad.* 63, 161–168. doi: 10.1016/j.ibiod.2008.08.007
- Mortabit, D., Zyani, M., and Koraichi, S. I. (2015). Biological control of wood decay fungi by *Streptomyces* strains's extract. *Int. J. Innov. Sci. Res.* 14, 100–103.
- Munish, C. A., Munesh, K., Rakesh, K., Kumar, D. D., Kumar, B. S., and Shanmugam, M. (2017). Genome sequence of *Kocuria polaris* strain CD08_4, an isolate from the duodenal mucosa of a celiac disease patient. *Genome Announc.* 5:1158. doi: 10.1128/genomea.01158-17
- Negi, A., and Sarethy, I. P. (2019). Microbial biodeterioration of cultural heritage: events, colonization, and analyses. *Microb. Ecol.* 78, 1014–1029. doi: 10.1007/s00248-019-01366-y

- Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., and Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev.* 64, 548–572. doi: 10.1128/mmbr.64.3.548-572.2000
- Paramo-Aguilera, L. A. (2012). Caracterización de Comunidades Microbianas con Potencial Biotecnológico Para la Prevención del Deterioro Estructural. Mexico: Instituto Politécnico Nacional.
- Perito, B., and Cavalieri, D. (2018). Innovative metagenomic approaches for detection of microbial communities involved in biodeterioration of cultural heritage. *IOP Conf. Ser. Mater. Sci. Eng.* 364:2074. doi: 10.1088/1757-899X/364/1/012074
- Piñar, G., Tafer, H., Sterflinger, K., and Pinzari, F. (2015). Amid the possible causes of a very famous foxing: molecular and microscopic insight into Leonardo da Vinci's self-portrait. *Environ. Microbiol. Rep.* 7, 849–859. doi: 10.1111/1758-2229.12313
- Pritchard, L., Glover, R. H., Humphris, S., Elphinstone, J. G., and Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal. Methods* 8, 12–24. doi: 10.1039/C5AY02550H
- Puškárová, A., Bučková, M., Habalová, B., Kraková, L., Maková, A., and Pangallo, D. (2016). Microbial communities affecting albumen photography heritage: a methodological survey. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep20810
- Pyzik, A., Ciuchcinski, K., Dziurzynski, M., and Dziewit, L. (2021). The bad and the good-microorganisms in cultural heritage environments-an update on biodeterioration and biotreatment approaches. *Materials* 14, 1–15. doi: 10.3390/ma14010177
- Rasuk, M. C., Ferrer, G. M., Kurth, D., Portero, L. R., Farias, M. E., and Albarracín, V. H. (2017). UV-resistant Actinobacteria from high-altitude Andean lakes: isolation, characterization and antagonistic activities. *Photochem. Photobiol.* 93, 865–880. doi: 10.1111/php.12759
- Razok, A., Ali, M., Aker, L., and Ziglam, H. (2022). *Actinomyces odontolyticus* bacteraemia associated with cervical and mediastinal abscesses in an immunocompetent patient: first reported case in Qatar. *New Microbes New Infect.* 45:100956. doi: 10.1016/j.nmni.2022.100956
- Repka, L. M., Chekan, J. R., Nair, S. K., and van der Donk, W. A. (2017). Mechanistic understanding of Lanthipeptide biosynthetic enzymes. *Chem. Rev.* 117, 5457–5520. doi: 10.1021/acs.chemrev.6b00591
- Reydams, H., Toledo-Silva, B., Mertens, K., Piepers, S., Vereecke, N., Souza, F. N., et al. (2024). Phenotypic and genotypic assessment of iron acquisition in diverse bovine-associated non-aureus staphylococcal strains. *Vet. Res.* 55:6. doi: 10.1186/s13567-023-01260-z
- Richter, M., and Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. USA* 106, 19126–19131. doi: 10.1073/pnas.0906412106
- Schuenemann, V. J., Avanzi, C., Krause-Kyora, B., Seitz, A., Herbig, A., Inskip, S., et al. (2018). Ancient genomes reveal a high diversity of *Mycobacterium leprae* in medieval Europe. *PLoS Pathog.* 14:e1006917. doi: 10.1371/journal.ppat.1006997
- Senoussi, A., Aissaoui-Zitoun, O., Chenchouni, H., Senoussi, S., Saoudi, Z., Pediliggieri, C., et al. (2024). Microbial screening of animal skin bags used in traditional cheesemaking. *Int. J. Food Microbiol.* 411:110549. doi: 10.1016/j.ijfoodmicro.2023.110549
- Skóra, J., Gutarowska, B., Pielech-Przybylska, K., Stępień, Ł., Pietrzak, K., Piotrowska, M., et al. (2015). Assessment of microbiological contamination in the work environments of museums, archives and libraries. *Aerobiologia* 31, 389–401. doi: 10.1007/s10453-015-9372-8
- Stackebrandt, E., Koch, C., Gvozdiak, O., and Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. Nov., *Nesterenkonia* gen. Nov., *Kytococcus* gen. Nov., *Dermacoccus* gen. Nov., and *Micrococcus* cohn 1872 gen. Emend. *Int. J. Syst. Bacteriol.* 45, 682–692. doi: 10.1099/00207713-45-4-682
- Sterflinger, K., and Piñar, G. (2013). Microbial deterioration of cultural heritage and works of art - tilting at windmills? *Appl. Microbiol. Biotechnol.* 97, 9637–9646. doi: 10.1007/s00253-013-5283-1
- Stratigaki, M., Armirotti, A., Ottonello, G., Manente, S., and Traviglia, A. (2024). Fungal and bacterial species richness in biodeteriorated seventeenth century venetian manuscripts. *Sci. Rep.* 14, 7003–7018. doi: 10.1038/s41598-024-57228-2
- Takano, H., Obitsu, S., Beppu, T., and Ueda, K. (2005). Light-induced carotenogenesis in *Streptomyces coelicolor* A3(2): identification of an extracytoplasmic function sigma factor that directs photodependent transcription of the carotenoid biosynthesis gene cluster. *J. Bacteriol.* 187, 1825–1832. doi: 10.1128/JB.187.5.1825-1832.2005
- Vermeersch, A. S., Ali, M., Gansemans, Y., Van Nieuwerburgh, F., Ducatelle, R., Geldhof, P., et al. (2023). An in-depth investigation of the microbiota and its virulence factors associated with severe udder cleft dermatitis lesions. *J. Dairy Sci.* 107, 3219–3234. doi: 10.3168/jds.2023-24180
- Villalba, L. S., Mikán, J. F., and Sánchez, J. (2004). Actividades hidrolíticas y caracterización isoenzimática de poblaciones microbianas aisladas del patrimonio documental del Archivo General de Colombia. *Ther. Nova* 2, 50–58. doi: 10.22490/24629448.7
- Wang, L., Huang, J., Sanmartín, P., Martino, P., Wu, F., Urzì, C. E., et al. (2025). Water determines geomicrobiological impact on stone heritage. *Nat. Geosci.* 18, 108–111. doi: 10.1038/s41561-024-01631-x
- Wang, L., Zhang, C., Zhang, J., Rao, Z., Xu, X., Mao, Z., et al. (2021). Epsilon-poly-L-lysine: recent advances in biomanufacturing and applications. *Front. Bioeng. Biotechnol.* 9:748976. doi: 10.3389/fbioe.2021.748976
- Warner Marien, M. (2022). Photography: A cultural history. 5th Edn. London: Laurence King Publishing.
- Wiszniewska, M., Walusiak-Skorupa, J., Pannenko, I., Draniak, M., and Palczynski, C. (2009). Occupational exposure and sensitization to fungi among museum workers. *Occup. Med.* 59, 237–242. doi: 10.1093/occmed/kqp043
- Woźniak, M. (2022). Antifungal agents in wood protection—a review. *Molecules* 27:392. doi: 10.3390/molecules27196392
- Yuan, Y., and Gao, M. (2015). Genomic analysis of a ginger pathogen *Bacillus pumilus* providing the understanding to the pathogenesis and the novel control strategy. *Sci. Rep.* 5:10259. doi: 10.1038/srep10259
- Zhgun, A., Avdanina, D., Shumikhin, K., Simonenko, N., Lyubavskaya, E., Volkov, I., et al. (2020). Detection of potential biodeterioration risks for tempera painting in 16th century exhibits from state Tretyakov gallery. *PLoS One* 15:e0230591. doi: 10.1371/journal.pone.0230591
- Zhou, Y., Wang, S., and Weng, X. B. (2022). *Clostridium subterminale* infection in a patient with diffuse large B-cell lymphoma and haemophagocytic syndrome: a case report and literature review. *J. Int. Med. Res.* 50:558. doi: 10.1177/03000605221129558
- Zielińska-Jankiewicz, K., Kozajda, A., Piotrowska, M., and Szadkowska-Stańczyk, I. (2008). Microbiological contamination with moulds in work environment in libraries and archive storage facilities. *Ann. Agric. Environ. Med.* 15, 71–78.



OPEN ACCESS

EDITED BY

Duraipandiyar Veeramuthu,
Loyola College, India

REVIEWED BY

T. P. Ajeesh Krishna,
Rajagiri College of Social Sciences, India
Joseph Devadass Balthazar,
Entomology Research Institute, India

*CORRESPONDENCE

Ioana Cristina Marinaș
✉ ioanacristinamarinas@gmail.com
Irina Gheorghe-Barbu
✉ irina.gheorghe@bio.unibuc.ro

[†]These authors have contributed equally to this work and share first authorship

[‡]These authors have contributed equally to this work

[§]These authors have contributed equally to this work and share last authorship

RECEIVED 11 June 2025

ACCEPTED 11 August 2025

PUBLISHED 22 August 2025

CITATION

Dumbravă AȘ, Corbu VM, Marinaș IC, Pericleanu R, Marinescu L, Motelica L, Trusca DR, Ianovici N, Șesan TE, Gheorghe-Barbu I, Ficăi D, Oprea OC, Ficăi A and Chifiriuc MC (2025) Multifunctional protective strategies for wooden cultural heritage: antimicrobial efficacy of polyacrylic resins, siloxane coupling agents, and silver nanoparticles. *Front. Microbiol.* 16:1642335. doi: 10.3389/fmicb.2025.1642335

COPYRIGHT

© 2025 Dumbravă, Corbu, Marinaș, Pericleanu, Marinescu, Motelica, Trusca, Ianovici, Șesan, Gheorghe-Barbu, Ficăi, Oprea, Ficăi and Chifiriuc. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Multifunctional protective strategies for wooden cultural heritage: antimicrobial efficacy of polyacrylic resins, siloxane coupling agents, and silver nanoparticles

Andreea Ștefania Dumbravă^{1,2†}, Viorica Maria Corbu^{3,4†}, Ioana Cristina Marinaș^{3*‡}, Radu Pericleanu¹, Liliana Marinescu⁵, Ludmila Motelica^{6,7,8}, Doina Roxana Trusca⁸, Nicoleta Ianovici⁹, Tatiana Eugenia Șesan^{1,10}, Irina Gheorghe-Barbu^{1,3*‡}, Denisa Ficăi^{5,6,7,8§}, Ovidiu Cristian Oprea^{5,6,7,8}, Anton Ficăi^{5,6,7,8} and Mariana Carmen Chifiriuc^{1,3,7§}

¹Department of Microbiology and Botany, Faculty of Biology, University of Bucharest, Bucharest, Romania, ²Department of Technological Irradiation (IRASM), Horia Hulubei National Institute of Physics and Nuclear Engineering–IFIN-HH, Magurele, Romania, ³Research Institute of the University of Bucharest, University of Bucharest, Bucharest, Romania, ⁴Department of Genetics, Faculty of Biology, University of Bucharest, Bucharest, Romania, ⁵Faculty of Chemical Engineering and Biotechnologies, National University of Science and Technology Politehnica Bucharest, Bucharest, Romania, ⁶Research Center for Advanced Materials, Products and Processes, National University of Science and Technology Politehnica Bucharest, Bucharest, Romania, ⁷Academy of Romanian Scientist, Bucharest, Romania, ⁸National Center for Micro and Nanomaterials, National University of Science and Technology Politehnica Bucharest, Bucharest, Romania, ⁹Department of Biology, Faculty of Chemistry, Biology, Geography, Environmental Biology and Biomonitoring Research Center, West University of Timisoara, Timisoara, Romania, ¹⁰Romanian Academy of Agricultural and Forestry Sciences, Bucharest, Romania

Introduction: This study evaluates two innovative protective treatments for wooden cultural heritage objects vulnerable to biodeterioration. The first involves polyacrylic resin solutions embedded with silver nanoparticles (AgNPs), while the second uses the siloxane-based coupling agent 3-mercaptopropyltrimethoxysilane (3-MPTMS) to enhance AgNP adhesion to wood surfaces.

Methods: Antimicrobial, anti-biofilm, and anti-metabolic activities were assessed using both qualitative and quantitative assays against biodeteriogenic strains (*Penicillium chrysogenum*, *Bacillus cereus*, and *Bacillus megaterium*). Additional analyses included extracellular nitric oxide (NO) production, phytotoxicity testing on *Allium cepa*, and correlations with biochemical parameters.

Results: Polyacrylic resins incorporating AgNPs exhibited significant antimicrobial properties, with stronger effects against bacteria. Treatments combining AgNPs with MPTMS or acrylic resins demonstrated enhanced inhibitory effect on microbial viability, adhesion, and degradative enzymes secretion. Sub-inhibitory concentrations of resin solutions modulated the extracellular NO levels, correlated with metabolic stress responses. Notably, ecotoxicity testing confirmed minimal phytotoxic impact, supporting the safety of these materials for cultural heritage applications.

Conclusion: The findings support the use of AgNP-enhanced polyacrylic resins and silanized coatings as effective, non-destructive conservation strategies for wooden heritage artifacts, offering durable protection against microbial deterioration while maintaining environmental safety.

KEYWORDS

wood heritage objects, siloxane-based coupling agent, silver nanoparticles, polyacrylic resins, antimicrobial, anti-biofilm, anti-metabolic

1 Introduction

Preserving wood heritage objects presents a formidable challenge for conservators and researchers, given their high susceptibility to biodeterioration, a process predominantly driven by microbial activity. As an organic material, wood is particularly prone to colonization by fungi, bacteria, and other microorganisms, leading to significant structural damage, discoloration, and a gradual loss of cultural and historical value (Cappitelli et al., 2020). Microbial deterioration compromises the physical integrity of the artifacts and erodes the cultural narratives they represent. The microbial community involved in wood artifacts degradation comprises a variety of microbial species, each of them presenting specific degradation mechanisms (Schrader et al., 2025). For instance, species such as *Bjerkandera adusta*, *Ganoderma lucidum*, *Irpex lacteus*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* classified as white rot fungi compromise the structural integrity of wooden artifacts through the secretion of lignocellulolytic enzymes, including endoglucanases, cellobiohydrolases, and β -glucosidases. In contrast, brown rot fungi such as *Antrodia vaillantii*, *Coniophora puteana*, *Gloeophyllum trabeum*, and *Serpula lacrymans* produce non-enzymatic compounds such as oxalic acid which diffuses in the plant cell wall and then generates reactive oxygen species such as hydroxyl radicals that depolymerize cellulose and hemicellulose (Martínez et al., 2005; Eastwood et al., 2011; Alfieri et al., 2021; Schrader et al., 2025). In addition to fungi, bacteria contribute notably to the decay of wooden artifacts, especially in environments with limited oxygen availability—such as waterlogged, buried, or highly humid conditions—where their metabolic activity becomes more pronounced. Depending on their mechanisms of wood tissue invasion, biodeteriorating bacteria are classified into several functional groups, including cavitation bacteria, tunneling bacteria, and erosion bacteria—each exhibiting distinct metabolic adaptations that enable their survival under specific environmental conditions (Chu et al., 2025; Wang et al., 2025). Regardless of their classification, the growing demand for effective conservation measures underscores the urgent need for innovative strategies that can provide long-lasting protection against microbial degradation while preserving the structural and esthetic integrity of the wood (Cirone et al., 2023; Pournou, 2020).

Polyacrylic resins have been widely used in the conservation of wooden artifacts due to their adhesive properties and ability to form protective coatings as well as stability (Kyei et al., 2022). Overall, polyacrylic resins are used in the treatment of the wood heritage because of its capability to bind to the wood structure and even to consolidate the fragile wood structure, to protect against moisture, UV or even to assure a higher scratch-resistance (especially as composite with silica, glass or wood flour) and these effects persist for long time. They are reversible and insoluble in water but dissolve readily in

organic solvents, which are commonly employed in conserving wooden artworks. While highly resistant to extremes temperature, acids, and alkali, acrylic resins can undergo degradation over time from factors such as microbial activity, UV radiation, and mechanical wear, leading to alterations in their physical and mechanical characteristics (Cataldi et al., 2014; Cataldi et al., 2015). Nonetheless, the use of these treatments alone frequently fails to provide comprehensive protection against microbial colonization and the ensuing biodeterioration processes (Campana et al., 2022; Ion et al., 2018). Ongoing researches are now underway to pass from the traditionally petrochemical based acrylic resins to bio-based feedstock (Wen et al., 2023).

Recent advancements in improving the protective performance of polyacrylic resins have emphasized the integration of antimicrobial agents, including nanoparticles such as silver (AgNPs), titanium dioxide (TiO₂), and zinc oxide (ZnO) (Arya et al., 2019; De Caro et al., 2024). For instance, TiO₂ and SiO₂ nanoparticles integrated into acrylic resins significantly suppressed *Streptococcus mutans* and *Lactobacillus acidophilus*, obtaining reductions of 98–99% in microbial viability under UV exposure (Sodagar et al., 2016). Similarly, ZnO NPs exhibit significant antimicrobial properties, effectively inhibiting bacterial and fungal colonization when incorporated into thin-film coatings or applied to heritage materials like paper and stone (Cinteză and Tănase, 2021). Recent investigations have shown that the incorporation of AgNPs into acrylic or silane-modified resin systems considerably improves antibacterial protection on cultural heritage substrates. In a 2024 study, AgNPs were biosynthesised *in situ* during the synthesis of an acrylic polymer coating (by plant extract and AgNO₃ mixing). The resulting nanocomposite decreased microbial load on stone heritage models by 2–4 log units compared to acrylic alone (Lak et al., 2024). Furthermore, a treatment combining a polyvinyl chloride-abietic acid (PVC-AA) copolymer with AgNPs was developed to improve the characteristics of pine wood, with a focus on dimensional stability, water repellence, and decay resistance. The results showed that while the impregnation procedure did not improve the wood's physical qualities, the PVC-AA-AgNPs treatment did greatly increase resistance to *T. versicolor* and *C. puteana*, demonstrating its potential as a protective treatment for wooden historical objects (Can and Hazer, 2022).

AgNPs are recognized for their high antimicrobial activity and broad-spectrum efficacy, being effective against bacteria, fungi, and viruses. These features make AgNPs an ideal additive for conservation materials, including resins, specifically designed to protect wood from microbial colonization and subsequent damage and deterioration (Baglioni et al., 2021). The antimicrobial properties of AgNPs are attributed to their ability to release silver ions, which can interact with microbial cell membranes, proteins, and DNA, leading to cell damage and death (Rai et al., 2009). Despite their efficacy, the integration of

AgNPs into conservation materials presents significant challenges. One primary concern is represented by NPs cytotoxicity, which could adversely impact both the artifacts and the surrounding environment. Consequently, their use in conservation requires careful assessment and rigorous evaluation to balance their protective benefits against any possible risks (Silva et al., 2017). One significant concern is the potential for phytotoxic effects, which could arise from the release of silver ions or other chemical interactions involving the siloxane-based coupling agents. Phytotoxicity could lead to unintended damage to the wood or surrounding organic materials, compromising the long-term safety and integrity of the conserved artifacts. Therefore, a comprehensive evaluation of these materials must include not only the assessment of their antimicrobial efficacy but also of their potential impact on the artifacts and the environment (Ameen et al., 2021). Plant-based (green synthesis) AgNPs (PB-AgNPs) offers a biocompatible and environmentally sustainable alternative to conventional chemical methods. This process utilizes phytochemicals such as polyphenols, flavonoids, and terpenoids as natural reducing and capping agents, enabling NP formation under mild conditions while significantly reducing ecological and human health risks (Iravani et al., 2014). Biologically produced AgNPs are often regarded as eco-friendly alternatives because they minimize the use of hazardous chemicals. However, they can still present ecotoxicological concerns, including alterations to soil microbial communities, accumulation in plant tissues, and disruption of key ecological processes such as nitrogen cycling and organic matter breakdown (Colman et al., 2014; Ottoni et al., 2020). Among the biological agents used for green synthesis, are particularly advantageous due to their rapid growth, ability to secrete bioactive enzymes, and natural resistance to heavy metals. Various fungal species, such as *Fusarium*, *Aspergillus*, *Trichoderma*, *Cladosporium*, *Ganoderma*, and the wood-degrading *Gloeophyllum striatum* have been effectively utilized in the biosynthesis of AgNPs with antimicrobial properties (Cui et al., 2022; Wrońska et al., 2023; Al-Zaban et al., 2021; Osorio-Echavarría et al., 2021; Santos et al., 2021; Zawadzka et al., 2021; Tończyk et al., 2025).

In terms of biological safety, NPs exhibit dose-dependent and cell-type-specific cytotoxicity. Cytotoxicity mechanisms often involve oxidative stress, DNA damage, and cell cycle arrest through interactions with proteins and nucleic acids (Ribeiro et al., 2018). Factors such as particle size, crystallinity, surface functionalization, and aging-related silver ion release further modulate toxicity (Wang et al., 2014; Kittler et al., 2010; Siddiqi et al., 2018). AgNPs synthesized using *Pseudomonas putida* showed no cytotoxicity on Hep-2 cells at concentrations up to 25 µg/mL, but caused 70% cell death at 100 µg/mL (Gopinath et al., 2017). Similarly, Khorrami et al. (2018) found that biosynthesized AgNPs induced 70% cell death in MCF-7 breast cancer cells at 60 µg/mL, while exhibiting much lower toxicity toward noncancerous L-929 cells. The lower toxicity compared to other commercial NPs could be possibly due to better encapsulation and biocompatible surface coatings (Khan et al., 2020). The minimal toxicity to normal cells at low doses reinforces the potential of green AgNPs for selective anticancer applications (Ghabban et al., 2022).

Siloxane-based coupling agents are organosilane compounds (OS) that exhibit very good properties like fire retardancy, resistance to microbial degradation, and increased weathering stability (Liu et al., 2023). Wood substrates treated with OS demonstrate improved functional performance, since these agents can enhance the surface hydrophobicity, thereby reducing moisture

absorption—a critical factor in microbial growth and biodeterioration (Aziz et al., 2021; Alfieri et al., 2021). OS are derived from silane (SiH₄) by substituting one or more hydrogen atoms with organic or functional groups, yielding the general formula R_nSiH_{4-n} (e.g., trimethylsilane, tetraethylsilane, trimethylsilylamine, chlorosilane, trimethoxysilane) (Liu et al., 2023; Broda et al., 2019; Panov and Terziev, 2009). These R groups—such as alkyl, amino, sulfhydryl, methoxy, glycidyloxy, or vinyl—provide compatibility with organic materials, making OS compounds effective coupling agents between organic and inorganic phases. Given their bi-functional molecular structure, upon hydrolysis, one end of the silane molecule reacts with inorganic substrates or hydroxyl-rich wood surfaces, while the other end may engage in covalent bonding with polymer matrices. This amphiphilic nature enhances interfacial compatibility between nanoparticles and polymeric phases (Jamil et al., 2018). Recent studies have highlighted the synergistic potential of OS compounds, especially when combined with nanoparticles or other bioactive agents, in enhancing wood durability. OS-treated wood is increasingly utilized across diverse sectors, including structural engineering, architectural finishing, furniture manufacturing, electronic components, and aerospace materials (Liu et al., 2023).

By combining AgNPs with silanization agents, it is possible to create a dual-functioning resin that not only inhibits microbial colonization but also strengthens the physical barrier against environmental factors that contribute to wood degradation (Ferreira et al., 2023; Nayaki et al., 2023). Similarly, OS treatments might enhance the biological resistance of thermally modified wood. Although thermal treatment reduces moisture content and increases initial resistance to biological agents, this effect diminishes over time. The addition of silane treatments post-thermal modification was found to extend and reinforce the wood's resistance to microbial attack (Kamperidou, 2019). Oscillating techniques are used to improve OS treated wood durability, water resistance and to assure biological protection.

In this context, the present study aims to evaluate the efficacy of two distinct protective formulations for wood heritage conservation. The first formulation is based on polyacrylic resins incorporating AgNPs, while the second involves surface modification using the siloxane-based coupling agents 3-mercaptopropyltrimethoxysilane (3-MPTMS), to facilitate enhanced binding of AgNPs. Both formulations are intended to protect wooden heritage objects from biodeterioration. The study focuses on investigating their antimicrobial, anti-biofilm, and anti-metabolic activities and their correlation with microbial and biochemical parameters, using treated wood model systems. Additionally, the formulations are evaluated for their preservation efficacy and potential phytotoxicity, with the overarching goal of assessing their suitability as integrated solutions for extending the longevity of wooden artifacts susceptible to microbial degradation.

2 Materials and methods

2.1 Synthesis of AgNPs

Synthesis of AgNPs using *Classical method*, at room temperature (AgNPs_c), *Turkevich method* (by reduction in the presence of appropriate agents – AgNPs_t), and *hydrothermal method* (AgNPs_h) was described in our previous work (Corbu et al., 2023).

Silver nitrate (AgNO_3) was purchased from Sigma Aldrich, polyvinylpyrrolidone (PVP), from Roth, and trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) from Alfa Aesar. All reagents and solvents were used without further purification.

2.1.1 Synthesis of AgNPs by the classical method, at room temperature (AgNPc)

A volume of 500 mL of 0.0001 M AgNO_3 solution was introduced into a Berzelius beaker, then 30 mL of 0.02999 M trisodium citrate was added. The solution thus obtained was left under continuous agitation (600–700 rpm) for 12 min, at room temperature. After 12 min, 30 mL of 0.00007 M PVP was added. The solution was stirred for 1 min (200 rpm), after which 3 or 5 mL of 0.1 M sodium borohydride solution was added. The final mixture was stirred for 5–7 min, after which 1.2 mL of 30% hydrogen peroxide was added. After adding H_2O_2 , the obtained solution was stirred until a blue or emerald blue color appeared. The concentration of the obtained NPs was 10 ppm.

2.1.2 Synthesis of AgNPs by the Turkevich method (AgNPt)

An amount of 0.01 g AgNO_3 was dissolved in 500 mL H_2O . The obtained solution was kept at 70–75° C under magnetic stirring for 75 min. To this solution was added dropwise a solution containing 0.30 g sodium salt of citric acid as reducing agent. When the solution became slightly yellowish, 10 mL of PVP were added. The concentration of the obtained NPs was 100 ppm.

2.1.3 Synthesis of AgNPs by the solvothermal method (AgNPsol)

An amount of 0.888 g of PVP K 30 was added to 80 mL of PEG 400. The mixture was stirred and heated to 80° C until the obtained solution became transparent. When the temperature reached 80° C, 2 mL of AgNO_3 with a concentration of 0.5 M was quickly added to the mixture, under stirring. A dark yellow color was observed, and the mixing continued at 80° C until the appearance of a dark brown color. The mixture was poured into a Teflon vat, and the temperature increased to 220° C while maintaining a constant pressure of 1 bar for 2 h. The reddish-brown mixture was cooled and then removed from the vat. The concentration of the obtained NPs was 1,000 ppm.

2.2 Treatment of wooden materials

2.2.1 Treatment with 3-MPTMS of wooden materials

In the case of treatment with 3-MPTMS (silane with –SH side group), the wood samples were introduced in 35 mL silanization solution (3-MPTMS), in a Berzelius flask and left under continuous stirring (600–700 rpm) 1 h, at room temperature. Subsequently, the wood samples were put in contact with the solution with Ag solution of concentration 100 ppm (Ag100) for 1 h under continuous stirring (600–700 rpm). In the final the wooden samples were removed from AgNPs solution and dried at room temperature.

2.2.2 Treatment with based acrylic resin of wooden materials

To be silanized, the beech wood materials were washed and dried at 115° C for 2 h. After washing and drying, the surfaces of

standardized wood samples (1*1 cm²) were impregnated using a painting brush with a commercial water-based acrylic resin, Adicril ARD-795, containing 78% acrylic binder from Bear Química, with or without AgNPs.

The samples were processed using a commercial water-based acrylic emulsion, Adicril ARD-795, containing 78% acrylic binder from Bear Química. The obtained emulsion was then mixed for 20 min using a Dispermat VMA mixer equipped with a 60 mm diameter blade, operating at 1,000 rpm, with AgNPs at a concentration of 10 ppm and 1,000 ppm. Four samples were obtained according to [Supplementary Table S1](#). Both AgNPc samples, designated as AgNPc 3 mL (D1) and AgNPc 5 mL (D1), were synthesized through the reduction of silver nitrate using sodium borohydride (NaBH_4) and sodium citrate. The primary difference between these samples lies in the varying sodium borohydride content used during the synthesis of AgNP ([Marinescu et al., 2022](#)).

All samples contain acrylic binder and three of them contain different percentage of AgNPs characterized by variation of concentration as indicated in [Supplementary Table S1](#).

2.2.3 Characterization of wooden surfaces and treated materials

Morphological and structural characterization of model surfaces and treated materials were achieved using specific physico-chemical methods: IR microscopy, Dynamic Light Scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). IR spectroscopy measurements were performed using a Thermo Nicolet iS50 FTIR spectrometer equipped with a diamond crystal-based ATR module capable of recording spectra over the range of 400–4,000 cm⁻¹. To obtain a better signal-to-noise ratio, the spectra were obtained by summing 16 scans recorded at a resolution of 8 cm⁻¹. The acquisition time was set to 3 scans/s using a DTGS type detector and automatic beam splitter for the ability to record the spectrum, especially in transmission mode, in the range 10–25,000 cm⁻¹, i.e., from far IR to near IR. The DLS measurements were done using a DELSA Max Pro, light scattering analyzer reusable PEEK flow cells, BCI-3216-DMP, DLS detector angle (degree) 163.5°. The acquisition parameters for this technique were: 5 s acquisition time, 1 s read interval, with 15 s collection period for 3 acquisitions, at 10 Hz electric field frequency, with auto-attenuation set at 0% level, normal laser mode, with set temp at 20° C. SEM images were recorded using a Quanta Inspect F – FEI microscope equipped with an EDX spectrometer. Morphological and structural characterization of AgNPs was published previously by our team ([Corbu et al., 2023](#)).

2.2.4 Evaluation of antimicrobial activity against biodeteriogenic strains

2.2.4.1 Microbial strains

Biodeteriogenic strains were previously isolated and identified using classical methods and MALDI TOF MS from Romanian cultural heritage churches ([Corbu et al., 2023](#)). For this study we selected two bacterial (i.e., *Bacillus cereus* and *Bacillus megaterium* encoded C16156 and NS5-R) and two filamentous fungi strains (*Penicillium chrysogenum* encoded NS4-2B and NS11-C) previously isolated from wood cultural heritage objects.

2.2.4.2 Qualitative antimicrobial activity of industrial resins

A volume of 10 µL of the four aqueous solutions prepared from the industrial acrylic styrene resin – ADICRIL ARD-795 (1% D1 3 mL AgNPsol, 1% D1 5 mL AgNPc, 0.1% S3 AgNPc and the base coat acrylic resin) was distributed on Sabouraud Dextrose Agar (SDA) in the case of filamentous fungi strains and, respectively, on Mueller-Hinton agar (MHA) for the bacterial strains. After incubation at 37° C for 24 h in the case of bacterial strains, and at room temperature for 6 days in the case of fungal strains, the growth inhibition zone around the disk was measured (mm) (Gheorghe et al., 2021).

2.2.4.3 Quantitative antimicrobial evaluation of industrial resins

The quantitative antimicrobial evaluation of industrial resins and determination of minimum inhibitory concentration (MIC) values were performed as previously described using twofold microdilutions of the screened resins on RPMI (Roswell Park Memorial Institute) 1,640 medium in the case of filamentous fungi strains and Mueller-Hinton Broth (MHB) for bacterial strains in 96 multi-well plates (Corbu et al., 2023).

2.2.4.4 Microbial viability determination in the presence of support materials treated with solutions containing NPs and resins

For wood materials, quantitative evaluation of the antimicrobial activity was evaluated by determining microbial viability in the presence of wood materials treated with siloxane-based coupling agents 3-MPTMS (L-MPTMS) and, respectively, silanization agent + silver nanoparticles (L-MPTMS-Ag100), adapted from previously described methods (Suflet et al., 2024; Adetunji and Odetokun, 2012). Thus, wood fragments with a total area of 1 cm² treated with siloxane-based coupling agents and, respectively, siloxane-based coupling agents + AgNPs were immersed in culture medium (RPMI 1640 for filamentous fungi and MHB for bacterial strains) in a final volume of 2 mL, over which 1/0.5 McFarland suspension of the filamentous fungi/bacterial strains in saline solution, was added. After incubation (6 days at room temperature in the case of filamentous fungi and 24 h at 37° C in the case of bacterial strains) serial decimal dilutions (10⁻¹⁰) were inoculated on SDA culture medium (100 µL/dilution) and, respectively, on MH agar (10 µL), followed by incubation (48 h at room temperature and, respectively, 24 h at 37° C). At the end of the incubation period, cell viability was determined as the percentage ratio between the number of colony-forming units (CFU/mL) in each sample (in the presence of functionalized materials) and the CFU/mL obtained in the control (untreated microbial culture).

2.2.4.5 The influence of resins solutions and treated wooden materials on the microbial adherence capacity to the inert substratum

To evaluate the influence of resins solutions on the microbial adherence capacity, the crystal violet microtiter method was used,

followed by determination of microbial adherence capacity (Corbu et al., 2024):

$$PICA\% = \frac{As}{Ac} \times 100,$$

where As = the absorbance at 490 nm of the biofilm formed and treated with sub-inhibitory concentration of resins solutions,

and Ac = the absorbance at 490 nm of the formed biofilm untreated.

The same protocol was applied for the colonized wood materials after washing three times with saline solution and fixation with methanol. The percentage of adherence capacity inhibition (PICA%) was calculated according to the previous formula, where:

As = absorbance value of the adherence capacity to the treated wooden material (AgNPs and resins and, respectively, wood materials treated with L-MPTMS and L-MPTMS-Ag100);

Ac = absorbance value of the adherence capacity to the untreated material.

To demonstrate the release of antimicrobial compounds into the environment, the effect on the adherence capacity to the surface of the inert substrate was determined in both cases.

2.2.4.6 The influence of wood materials and coatings on extracellular nitric oxide

The extracellular nitric oxide (NO) was quantified using previously reported methods with certain changes (Quinteros et al., 2016). The NO release was evaluated by making a total nitrite spectrophotometric analysis using the Griess reagent. After 24 h of incubation for bacteria and 8 days for fungi suspension, 100 µL of the supernatant was collected and centrifuged at 10,000 rpm for 10 min to remove cells. Then, 50 µL of 2% sulphanilamide in 5% (v/v) H₃PO₄ and 50 µL of 0.1% N-(1-naphthyl)-ethylenediamine aqueous solution were added to 50 µL of supernatant. The mixture was incubated at room temperature for 30 min in the dark. The azo dye was quantified at λ = 540 nm. All measurements were performed in triplicate and results are expressed as mean ± SD. To measure the amount of nitric oxide, a calibration curve with NaNO₂ values from 1 to 100 µM was created (R² = 0.9991).

2.2.4.7 The influence of resins solutions on enzymatic and organic acid production

Specific culture media supplemented with cellulose, Tween 20, Tween 80, starch, milk and organic acids were inoculated with treated and untreated microbial suspension followed by the determination of the ratio of the colony diameter (C) to the diameter of the specific inhibition zone occurring around the colony (D) (Corbu et al., 2021):

$$Inhibition(\%) = \frac{D2 - C2}{D1 - C1} \times 100,$$

where C1—colony diameter of control strain, D1—inhibitory effect zone diameter of control strain, C2—colony diameter of treated strain, and D2—inhibitory effect zone diameter of treated strain.

Microbial strains cultivated in the presence of wooden materials treated with AgNPs and resins and, respectively, wood materials treated with MPTMS and MPTMS-Ag100 were evaluated also regarding the influence against the production capacity to secrete

organic acids and enzymes involved in the biodegradation process (cellulase, phenoloxidase, esterase, caseinase and amylase) by cultivation on specific culture media (Corbu et al., 2023). The microbial culture grown in the presence of treated materials was centrifuged 10 min, 10,000 rpm and the cell pellets were re-suspended in saline water to an optical density equivalent to the 1 McFarland standard. The obtained microbial suspensions were seeded spot (10 μ L) on the specific agar media. The seeded plates were then incubated for 5 days/24 h at 22/37° C. The influence on the enzymatic capacity and on the production of organic acids was evaluated according to the same relationship used in the case of silanization solution against the enzymatic or organic acid production.

2.2.5 Ecotoxicity of treated wood materials

To evaluate the ecotoxic effects of the wood material functionalized with AgNPs and resins and, respectively, wood materials treated with MPTMS and MPTMS-Ag100, the *Allium* assay was employed (Corbu et al., 2023; Datcu et al., 2020). *Allium cepa* var. *rubra* bulbs were carefully chosen based on their external morphology and physical soundness. Prior to treatment, all viable bulbs were kept in tap water at ambient temperature for 6 days to encourage root growth. For the control group, bulbs were placed in distilled water, while the experimental groups were exposed to the wood material functionalized with AgNPs and resins and, respectively, wood materials treated with MPTMS and MPTMS-Ag100. After a 24-h exposure period, the bulbs were assessed measuring the fresh biomass by weighing the bulbs on an analytical balance. The data were subjected to statistical analysis using one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance, with significance considered at $p < 0.05$.

2.2.6 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) and the statistical analyses of antibacterial and anti-adherence activities, nitric oxide (NO) content, enzymatic and organic acid production, and ecotoxicity were performed using GraphPad Prism version 9 (GraphPad

Software, San Diego, CA, United States). Dunnett's and Tukey's multiple comparisons tests were used to correct for multiple comparisons between control and test groups. The significance level was set at $p < 0.05$.

3 Results

3.1 Synthesis of AgNPs

The AgNPs were obtained using three different synthesis methods: the classical one, performed at room temperature (AgNPc), Turkevich method, involving reduction in the presence of appropriate agents – AgNPt and the hydrothermal method (AgNPsol); the results of the structural characterization by X-ray diffraction (XRD), FTIR, DLS, SEM and UV-Vis spectroscopy has been published previously (Corbu et al., 2023). AgNPs were in the nanometric range, while they provide the prerequisites and had good stability because the predicted zeta potential is between $[-19.60$ and -35.45 mV] which leads to a sufficiently high repulsion to keep the suspension stable. In the TEM images (Figure 1) recorded on the three types of AgNPs, it can easily be observed that the size of NPs varies between 10 and 20 nm. By corroborating these data with the DLS, it can be concluded that the particles remain independent, without concerning coalescence.

3.2 Characterization of wooden surfaces/ materials treated

The morphological and structural characterization of the treated surfaces was performed using appropriate physicochemical techniques, specifically FTIR spectroscopy and SEM microscopy. For the wood model materials (L), the surface exhibited a high degree of homogeneity, with identical spectral maps observed across all four monitored wavenumbers. Comparable results were obtained for the samples treated with MPTMS and AgNPs, further confirming that the wood surface was uniformly coated (Figure 2).

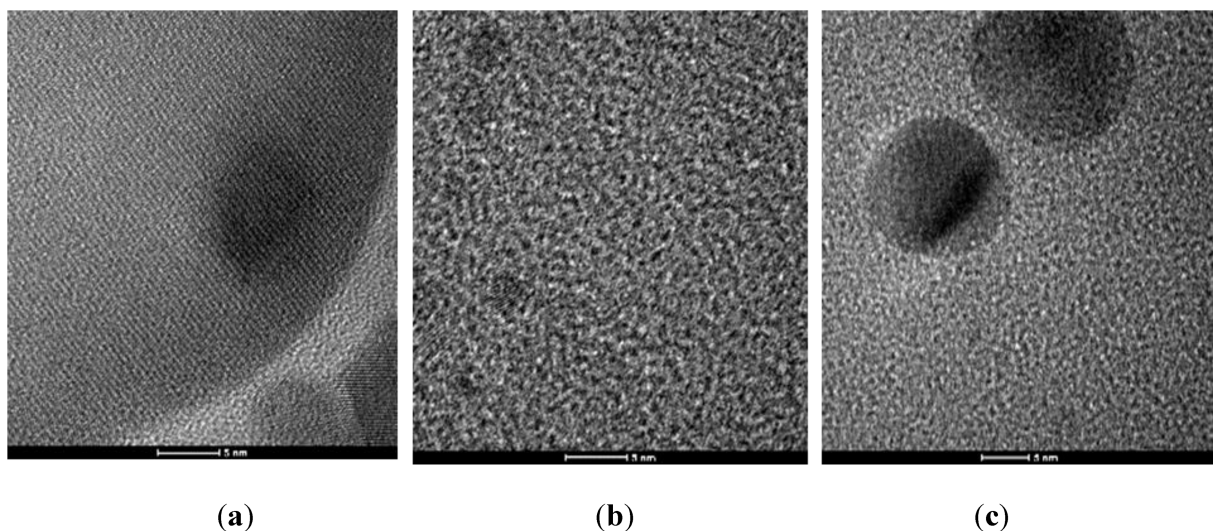


FIGURE 1
TEM images recorded on AgNPc (a), AgNPt (b), and AgNPsol (c).

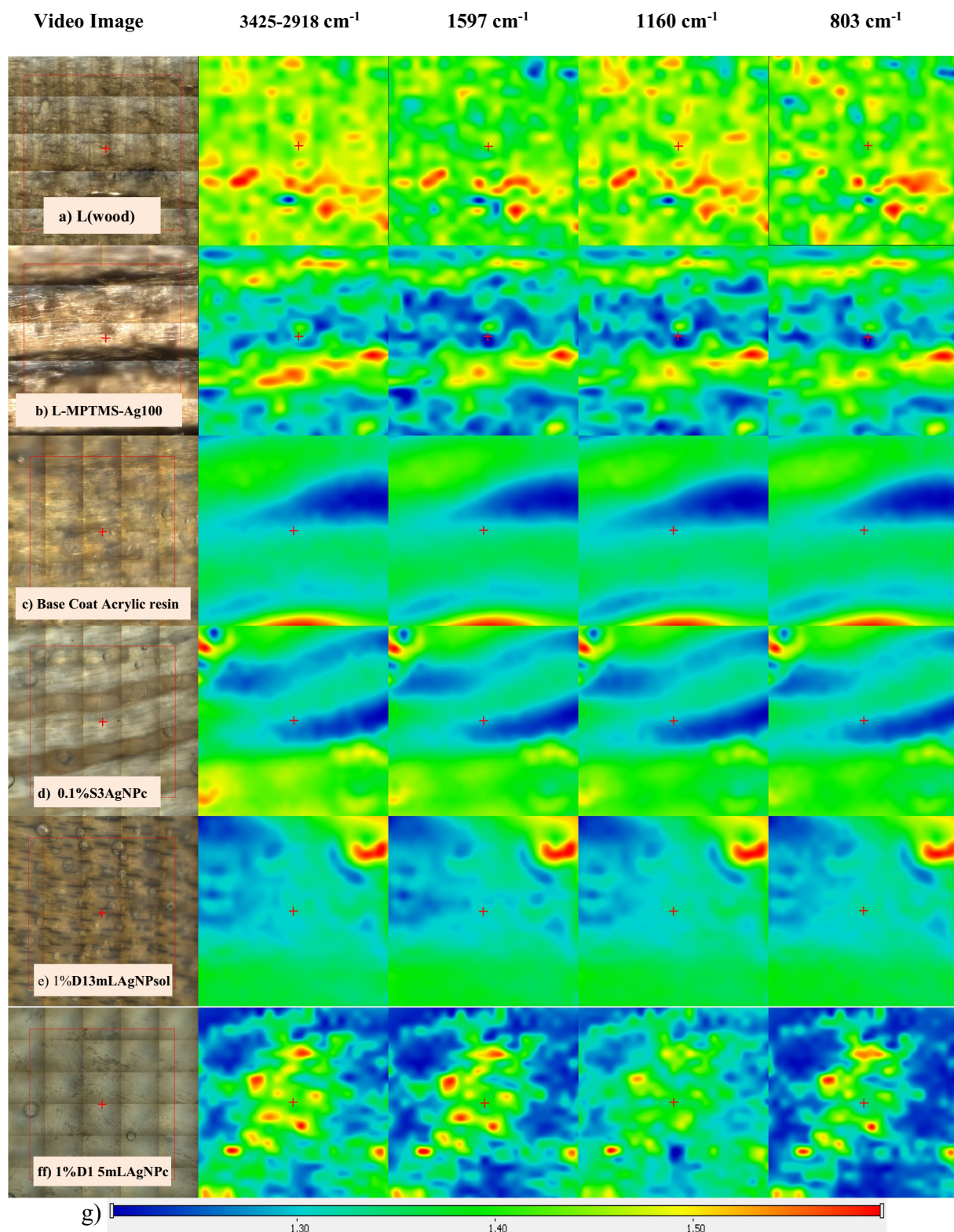


FIGURE 2

(a) FTIR microscopy recorded on (a) wooden model material (L), (b) L-MPTMS-Ag100 solutions, (c) L-Base coat acrylic resin, (d) L-0.1%S3 AgNPc, (e) L-1%D1 3mL AgNPsol, (f) L-0.1%S3 AgNPc; (g) color scale bar to highlight the red to blue differences of the absorbances; for all the reference wood surface and treated surfaces the video image as well as the four FTIR maps are indicated (3425–2,918; 1,597; 1,160 and 803 cm^{-1}).

The film morphology and depth penetration were investigated by **SEM microscopy**, at higher magnification. SEM images of untreated and treated samples provided information on the surface appearance.

In the case of wood surfaces, SEM images show uniform surface coverage but also an impregnation in the material structure. This coating effect is barely visible in cross-section, but the tangential

longitudinal (TL) plane in [Figures 3a–c](#) clearly shows transverse pits under the siloxane layer.

SEM images of wood surfaces treated with commercial water-based epoxy resin show smooth resin deposits ([Figure 4](#)), indicating that the cured resin forms a uniform coating. Both the surface deposits and the coating on the cell walls act as physical barriers against moisture and wood-degrading organisms. While these barriers may initially inhibit the escape of moisture and the intrusion of fungi, they are not entirely impermeable; over time, both moisture and fungal agents may eventually penetrate the protective layers.

EDX spectra ([Figure 5](#)) confirm the silanization of the surfaces by the presence of the Si, but also that of AgNP. In both cases, these peaks

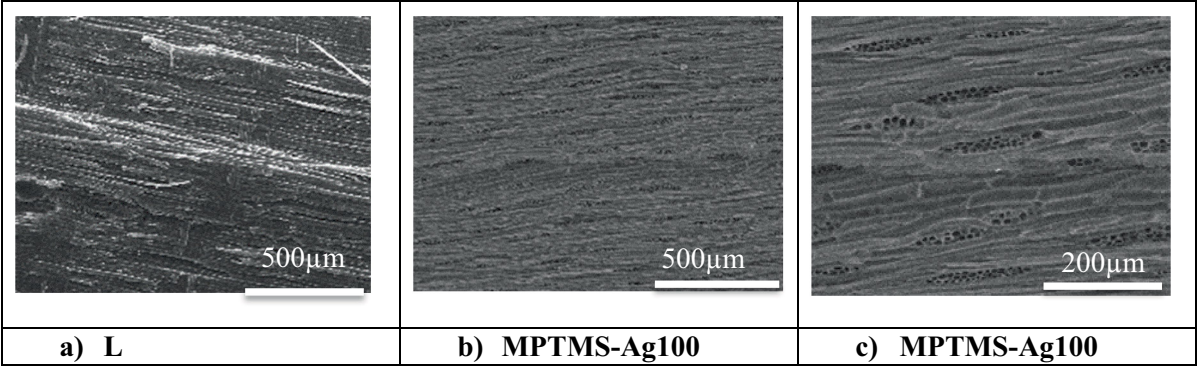


FIGURE 3
SEM images recorded on wooden model material treated with MPTMS solution containing AgNPs, at different magnifications. **(a)** L. **(b)** MPTMS-Ag100. **(c)** MPTMS-Ag100.

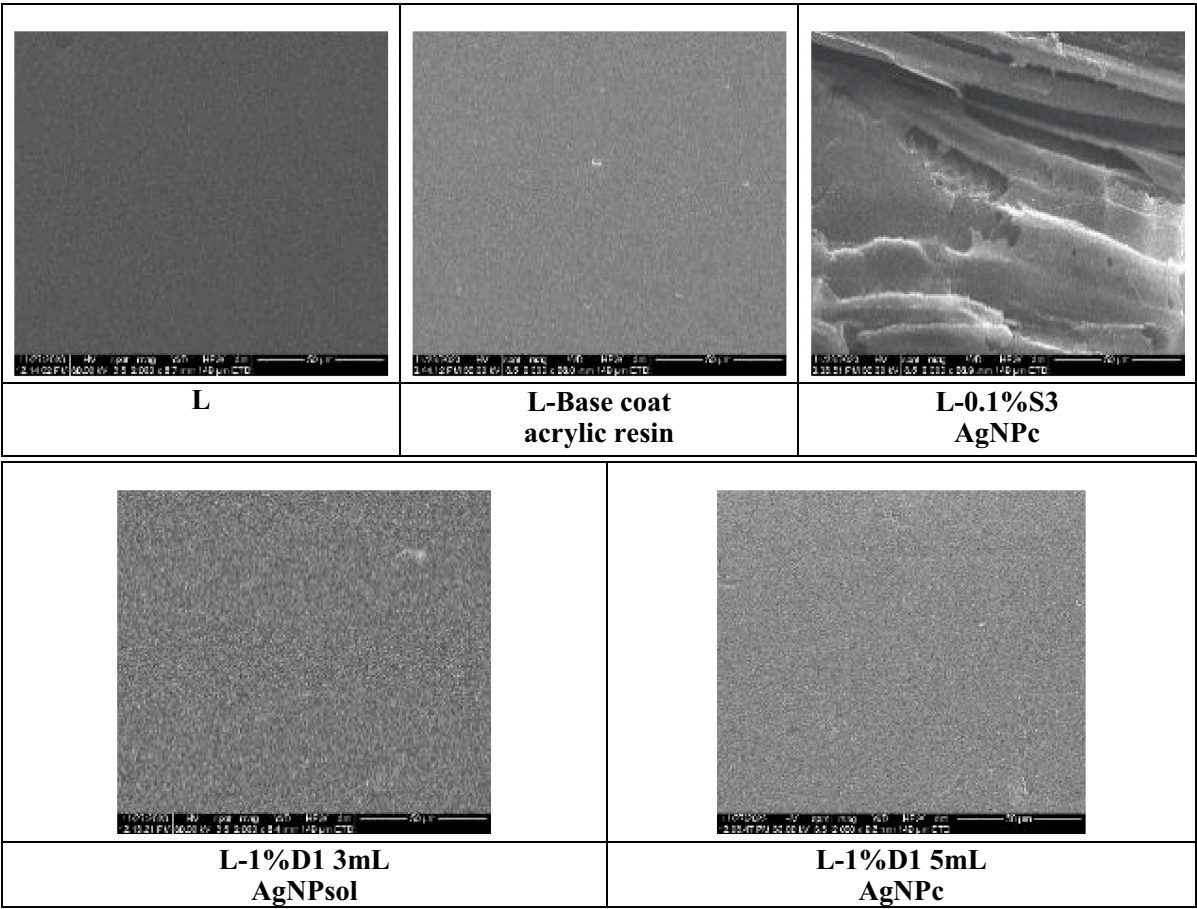


FIGURE 4
SEM images recorded on wooden model material treated with commercial water-based acrylic resin, Adicril ARD-795, with and without AgNPs.

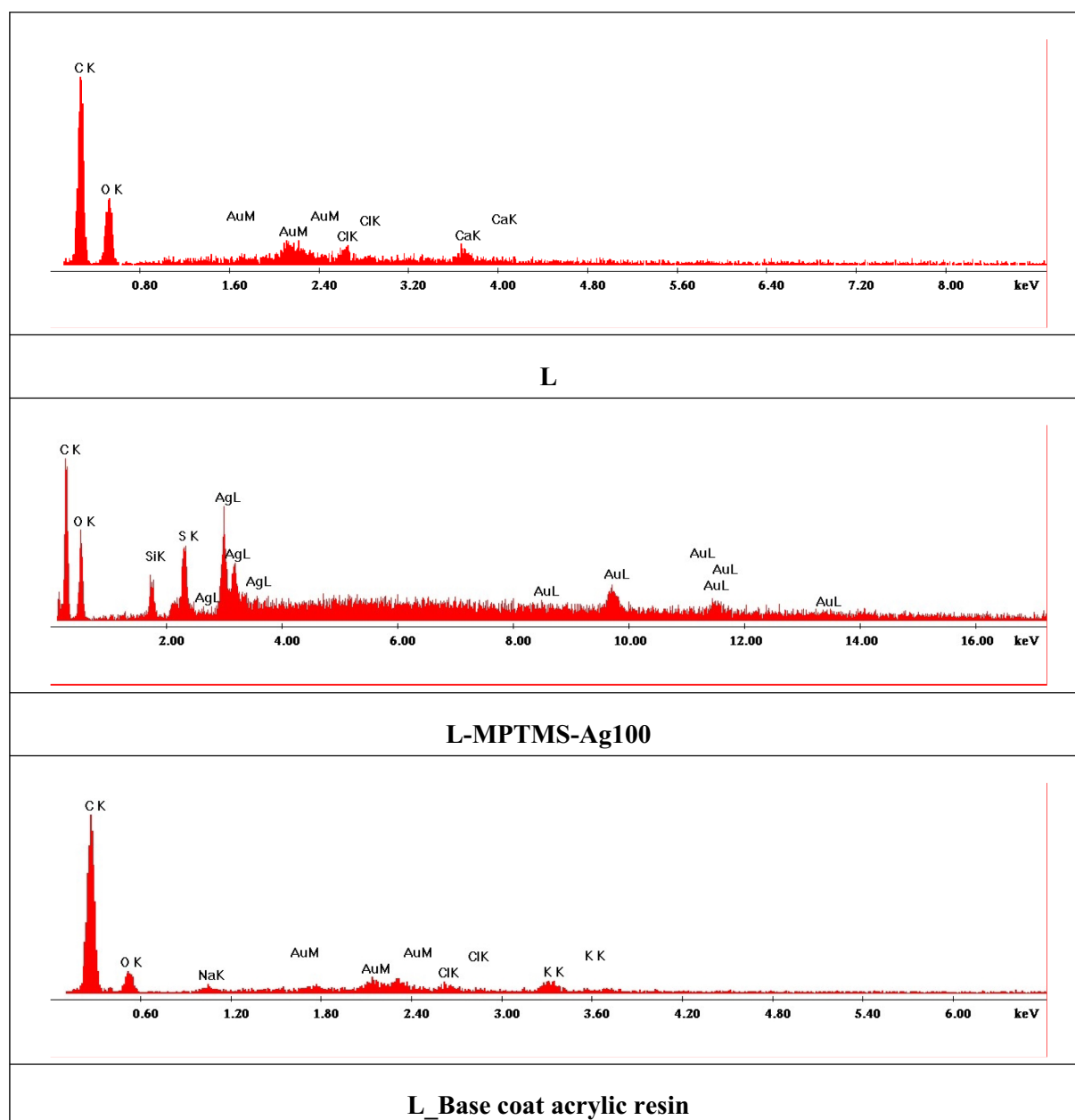


FIGURE 5

EDX spectra recorded on wooden model material treated with MPTMS or epoxy resin solutions with and without AgNPs solutions.

are clearly identifiable, and these elements are not present in the wood reference materials neither in the base coat acrylic resin which means that first, the silanization process was efficient and second, the AgNPs retained well. Considering that MPTMS treatment means a simultaneous surface modification with siloxane and thiol moieties, certainly sulfur is also present, in a relative high amount, the ratio between Si and S being proportional with their atomic weight. These results can be explained considering that MPTMS possess a sensitive hydrolytic center that reacts with organic and inorganic substrates to form stable siloxanic covalent bonds and possess an additional thiolic group with high affinity for silver. In the case of the EDX spectrum recorded on the material treated only with the base coat acrylic resin, a decrease in the intensity of the specific oxygen peak is observed as a

result of the coating with acrylic resin while C and O are the major elements and Na as minor element. Au should be ignored being used to metalize the surface for getting better resolution during SEM analyses.

The liquid resin uptake (LU) showed a strong dependency on the type of modification applied. In the case of beech wood, water absorption remained significantly high, with the final mass after immersion increasing by 100–160%. This indicates that treatments with AgNPs and silanization using MPTMS do not effectively reduce water uptake. Nevertheless, these treatments retain important benefits, such as imparting antimicrobial properties, which may contribute to the long-term resistance against microbial attack.

As shown in [Supplementary Table S2](#), a noticeable reduction in water absorption can be achieved through resin treatments. However,

such measures are primarily necessary under conditions of prolonged exposure to high humidity or direct immersion in water.

Water absorption after 4 days of immersion did not result in any visible degradation of the experimental wood models. In all cases, the solutions obtained after 96 h showed no detectable reaction to chloride testing for Ag^+ ions, indicating that ion release was minimal and remained below the technical detection limit.

3.3 Antimicrobial activity of the resin coatings and treated wood materials against biodeteriogenic microorganisms

3.3.1 The effect of synthetic resin solutions on the biodeteriogenic microorganism's growth and metabolic activity

Representative strains, previously characterized as highly relevant as biodeteriogenic microbial models, were selected for determining the impact of synthetic resin solutions on microbial growth and metabolism.

3.3.1.1 Growth inhibition assessment

The qualitative evaluation of the antimicrobial activity of polyacrylic resin solutions revealed that all three tested solutions exhibit an inhibitory effect against the tested bacterial and fungal biodeteriogenic strains after incubation. The antibacterial activity against *B. megaterium* C16156 exhibited a decreasing trend in the following order: 1% D1 3 mL AgNPsol > 1% D1 5 mL AgNPc > 0.1% S3 AgNPc > base coat acrylic resin, indicating the superior efficacy of AgNPs-based formulations, particularly those incorporating AgNPsol.

In contrast, for *B. cereus* C16156, the most pronounced antibacterial effect was observed with the 1% D1 5 mL AgNPc, followed by 1% D1 3 mL AgNPsol, and the base coat acrylic resin, with an arbitrary inhibition unit of 2 (see [Supplementary Table S3](#); [Supplementary Figure S1](#)). In the case of fungal strains, the inhibitory areas were no longer detectable after 5 days of incubation.

Following the quantitative assessment of antimicrobial activity, the MIC values against the bacterial strains ranged from 1.56 to 4.08%, indicating moderate to strong antimicrobial effects depending on the formulation. The highest antibacterial efficiency was observed in the case of 1% D1 3 mL AgNPsol (MIC = 1.56%) against *B. megaterium* NS5-R strain, and 1% D1 5 mL AgNPc (MIC = 1.56%) against *B. cereus* C16156 strain. These findings underscore the superior efficiency of AgNP-enhanced coatings compared to both the base acrylic resin and lower-concentration AgNP formulations.

In the case of the fungal strains, the MIC values exhibited a notable variation between *P. chrysogenum* NS4-2B and NS11C strains. For *P. chrysogenum* NS4-2B strain, all formulations including the base coat and AgNP-containing variants demonstrate high MIC values ranging from 20.83 to 25%, suggesting lower susceptibility of this strain. Interestingly, 1% D1 5 mL AgNPc, although the most concentrated, did not enhance antifungal activity and showed the highest MIC (25%), indicating potential strain tolerance. Conversely, *P. chrysogenum* NS11C strain was more sensitive to the AgNP-based formulations, with MIC values ranging from 2.34 to 12.5%. The lowest MIC (2.34%) was recorded for the 1% D1 5 mL AgNPc formulation, followed by 0.1% S3 AgNPc (6.25%) and 1% D1 3 mL AgNPsol (8.33%). The base coat alone exhibited an MIC of 12.5%, further

highlighting the enhanced antifungal efficacy of biogenic AgNPs against this fungal isolate. These findings confirm that the antimicrobial efficacy of AgNPs is both formulation and strain-dependent, with higher NP concentrations generally providing improved inhibitory effects, especially against susceptible bacterial and fungal strains (see [Supplementary Table S4](#)).

3.3.1.2 Influence on microbial adherence to the inert surface

The anti-adherence potential (PICA%) of the tested resin solutions was evaluated at sub-inhibitory concentrations (MIC/2 and MIC/4) against selected bacterial and fungal strains. Among the bacterial isolates, a notable reduction of the adherence capacity to the inert substratum was observed. For *B. cereus* C16156, the 1% D1 5 mL AgNPc demonstrated the highest anti-adherence effect, with PICA values of 11.92% at MIC/2 and 13.92% at MIC/4 ($p < 0.0001$), indicating a strong inhibitory response. In contrast, the base coat resin showed a moderate effect (58.29% at MIC/2), while the 0.1% S3 AgNPc formulation exhibited no inhibitory activity and even exceeded the control (PICA > 111%), suggesting a potential strain-specific stimulatory response. For *B. megaterium* NS5-R, similar inhibitory trends were obtained. The 1% D1 5 mL AgNPc again achieved the most significant reduction in adherence, with PICA values of 11.96% (MIC/2) and 20.79% (MIC/4) ($p < 0.0001$), while the base resin and 0.1% S3 AgNPc displayed variable and less effective results.

In contrast, the fungal strains exhibited divergent responses. For *P. chrysogenum* NS4-2B, all tested formulations including those with AgNPs enhanced adherence capacity relative to the untreated control. Notably, 1% D1 5 mL AgNPc resulted in PICA values of 221.72% (MIC/2) and 277.15% (MIC/4), indicating an undesirable stimulatory effect on adherence and biofilm formation. Conversely, *P. chrysogenum* NS11C responded favorably to the same formulation, with marked inhibition of adherence capacity. The 1% D1 5 mL AgNPc reduced PICA to 18.36% (MIC/2) and 10.65% (MIC/4) ($p < 0.0001$), significantly outperforming both the base resin and the 0.1% S3 AgNPc treatments. In this case, the resin's anti-adherence efficiency appears to be strain-dependent, highlighting the variable biofilm response mechanisms between fungal isolates ([Figure 6](#)) (see [Supplementary Table S5](#)).

3.3.1.3 Influence on biodeteriogenic metabolic activity

To evaluate the potential protective role of the tested resin solutions against microbial biodeterioration of cultural heritage objects, was assessed their influence on the production of key extracellular enzymes (esterases, proteases, amylases) and organic acids. These biochemical markers are known contributors to the degradation of organic and inorganic substrates, including historical surfaces. The analysis was conducted at sub-inhibitory concentrations (MIC/2 and MIC/4), and metabolic activity was quantified relative to untreated controls.

In *B. megaterium* NS5-R, exposure to 0.1% S3 AgNPc significantly stimulated organic acid production, reaching 142.86% at MIC/2 and 150% at MIC/4. In contrast, treatment with 1% D1 3 mL AgNPsol and 1% D1 5 mL AgNPc resulted in notable inhibition of acid production, with MIC/2 values of 57.14 and 92.86%, respectively. Esterase activity (targeting ester bonds in long-chain fatty acids) was most effectively suppressed by the 1% D1 3 mL AgNPsol solution, which reduced esterase TW20 production to 66.67% (MIC/2) and 88.89% (MIC/4),

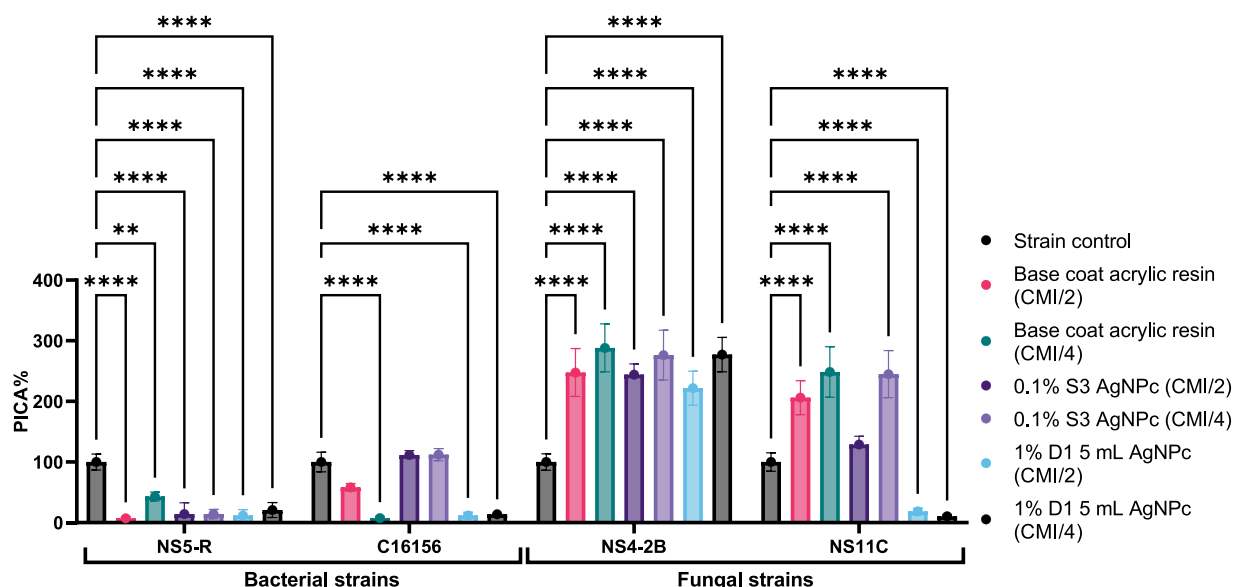


FIGURE 6

The anti-adherence activity of resin solutions tested against bacterial and microfungi strains (Dunnett's multiple comparisons test, ** $p < 0.01$; **** $p < 0.0001$).

and TW80 to 95% (MIC/2) and 115% (MIC/4). The base coat resin showed modest modulation of enzyme levels, while 0.1% S3 AgNPc moderately increased esterase TW20 production to 188.89% (MIC/2), suggesting a possible stress-induced response. Amylase activity, a critical enzyme in polysaccharide degradation, was variably affected, with the 1% D1 3 mL AgNPc increasing secretion to 123.08% at MIC/2 and 130.77% at MIC/4. In *B. cereus* C16156, the production of all evaluated enzymes and acids demonstrated limited response, with most values approximating 100%, regardless of formulation or concentration (Figure 7) (see Supplementary Table 6). These findings suggest an increased tolerance of this strain to the metabolic modulation effects of the tested coatings.

In *P. chrysogenum* NS4-2B, a clear inhibitory effect was observed in caseinase activity, with 0.1% S3 AgNPc reducing activity to 28.57% at MIC/2, and the base coat acrylic resin and 1% D1 5 mL AgNPc, both exhibiting comparable inhibitory effects at MIC/4. Notably, esterase TW20 and TW80 production was completely suppressed at MIC/2 by the 0.1% S3 AgNPc treatment. Additionally, organic acid production was moderately reduced (to ~63–97%), while amylase activity was strongly inhibited (0% at MIC/2 and MIC/4) by 1% D1 3 mL AgNPc. In *P. chrysogenum* NS11C, the enzyme production profile differed significantly. The most pronounced reduction in amylase activity was again observed in the case of 0.1% S3 AgNPc (MIC/2–33.33%; MIC/4–41.67%) and 1% D1 5 mL AgNPc solutions (MIC/4–58.33%). Caseinase inhibition was also evident, particularly at MIC/2 in the case of 0.1% S3 AgNPc (28%) and 1% D1 3 mL AgNPc solutions (75%). Interestingly, this strain demonstrated higher tolerance to esterase inhibition, with values remaining above 40% for all formulations (Figure 7) (see Supplementary Table S7).

These findings indicate that the tested resin solutions, especially 0.1% S3 AgNPc and 1% D1 3 mL AgNPc, can significantly inhibit the secretion of enzymes and acids involved in microbial biodeterioration, though the magnitude of inhibition is strain- and enzyme-specific. This suggests a promising application of such

coatings in the preventive conservation of heritage objects, particularly when tailored to the microbial profile of the treated environment.

3.3.1.4 Extracellular nitric oxide release and correlation with biodeteriogenic features

Considering that one of the ways by which AgNPs might inhibit microbial adherence is the increased NO concentration in the extracellular environment, was studied the effect of a sub-inhibitory AgNPs concentration (MIC/4) on the extracellular release of NO for all tested strains (Figure 8). In our study, it can be observed that extracellular NO for fungal strains was significantly higher than for bacteria, where concentrations of NO were below the limit of detection. The treatment of *P. chrysogenum* NS4-2B strain with MIC/4 of 1% D1 5 mL AgNPc exhibited a significantly higher concentration of extracellular NO compared to untreated strain ($p < 0.0001$) as well as to the resin base ($p < 0.0001$) and 0.1% S3 AgNPc ($p < 0.0001$) treatments. The same trend was observed for the *P. chrysogenum* NS11C fungal strain (Figure 8), but with lower values compared to *P. chrysogenum* NS4-2B strain.

In the case of the *P. chrysogenum* NS11C strain, treatment with resin base or AgNPs 0.1% S3 produces moderate growth. The highest values were for the samples 1% D1 3 mL AgNPc, which showed a significant increase compared to the control and the other treatments ($p < 0.001$), and AgNPs 1% D1 5 mL, which had the highest level of NO for this strain ($p < 0.0001$ compared to control). AgNPs induce the release of NO depending on the type of AgNPs, suggesting an effect dependent on the method of nanoparticle synthesis and dosage.

In the case of *P. chrysogenum* NS4-2B fungal strain, the results were similar to those of the strain NS11C, but the released NO values were higher for all treatments. The treatment with 1% D1 5 mL AgNPc leads to the production of the highest concentration of NO ($1.87 \pm 0.15 \mu\text{M NaNO}_2$), significantly higher than all other conditions ($p < 0.0001$). All treatments with 0.1% S3 and resin lead to increased levels compared to the untreated control ($p < 0.0001$). Therefore,

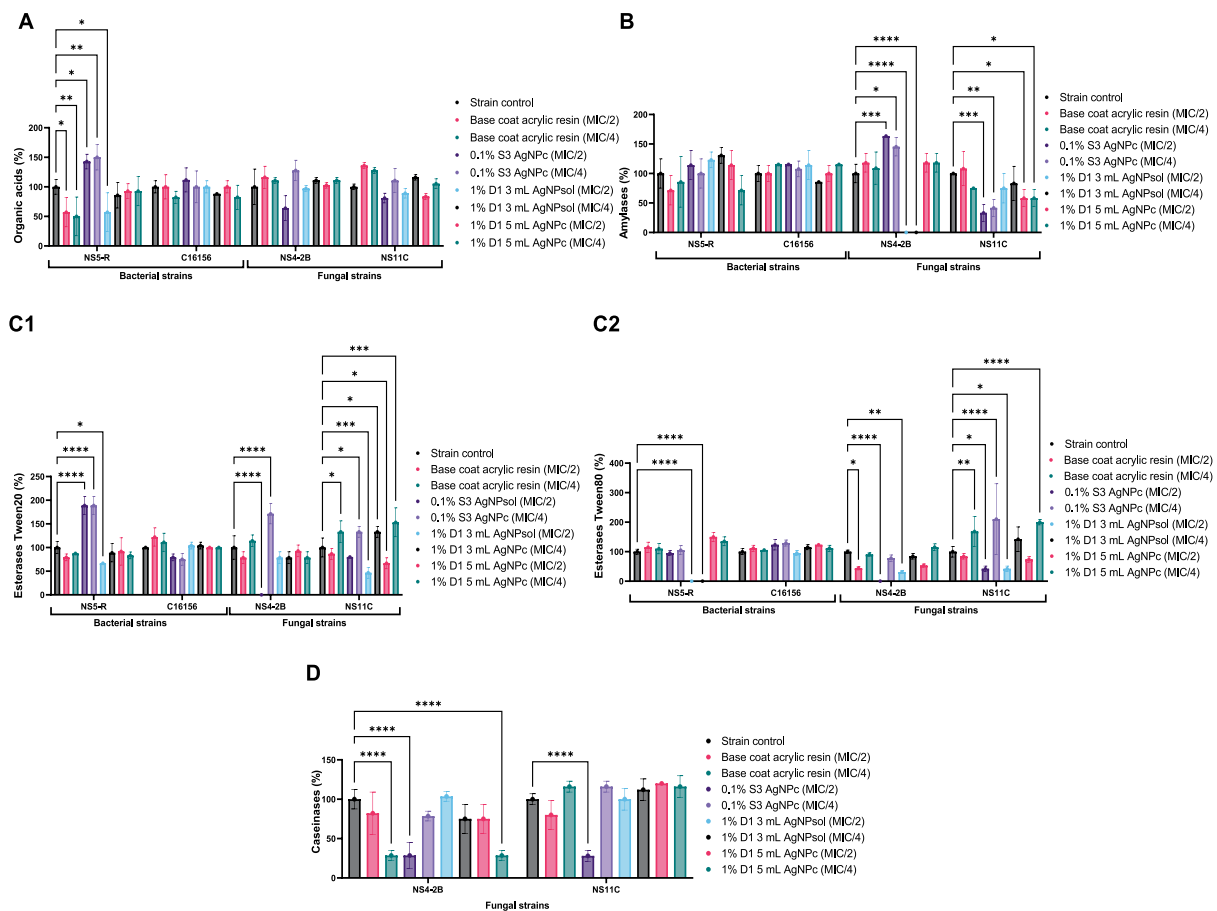


FIGURE 7

The effect of resin solutions at subinhibitory concentrations on the ability of microbial strains to secrete (A) organic acids; (B) amylases; (C) esterases (1-enzyme substrate Tween 20; 2-enzyme substrate Tween 80), (D) caseinases (Dunnett's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$;

*** $p < 0.001$, **** $p < 0.0001$).

P. chrysogenum NS4-2B strain exhibits the highest capacity for NO release in the presence of AgNPs.

The Pearson correlation was applied for each fungal strain with the following parameters: PICA (%), caseinase, esterase T80, esterase T20, organic acid and amylase. The relationship between extracellular concentration of NO produced at levels of MIC/4 and microbial adherence showed that the higher the NO content, the stronger the adherence inhibition of *P. chrysogenum* NS11C strain ($r = -0.30$) (Figure 8b). In the case of *P. chrysogenum* NS4-2B strain, the correlation was better but with the reverse trend, i.e., the higher the concentration of NO, the better the adhesion ($r = 0.87$) (Figure 8c). In the case of *P. chrysogenum* NS11C strain, direct correlations between NO vs. caseinase activity ($r = 0.81$) and NO vs. esterase's activity (T20, $r = 0.98$ and T80, $r = 0.82$) have been obtained, i.e., the higher the extracellular content of NO, the better the enzymatic activity. Esterase and protease activity stimulation may be an adaptive mechanism for substrate degradation and the release of nutrients needed for survival in stressful environments. In response to NO, the inhibition of amylase activity could indicate a selective control of enzymatic pathways or a transfer of metabolic resources.

In the case of *P. chrysogenum* NS4-2B strain (Figure 8c), the following correlations can be highlighted: NO content vs. caseinase

activity ($r = -0.95$), NO content vs. esterase activity (T20, $r = 0.97$; T80, $r = 0.46$), and NO content vs. PICA ($r = 0.87$). Nitric oxide is a crucial signaling molecule in eukaryotic organisms that controls metabolism and cellular behavior in addition to mediating the stress response. Compared to other fungi, *P. chrysogenum* NS4-2B strain exhibits a different impact on biological processes due to increase NO release, which is most likely brought on by AgNPs. The study found that NO and adhesion capability had a strong positive correlation, suggesting that NO may promote the formation of biofilms or surface contact. NO may encourage ester breakdown, which is essential for fungal metabolism and biodeterioration processes, as it is also strongly linked to T20 esterase activity. Nonetheless, there was a significant inverse relationship between NO and caseinase activity, suggesting a metabolic regulatory mechanism whereby the strain reduces its proteolytic activity in reaction to AgNPs.

3.3.2 Microbial viability and biodeteriogenic activity on treated wood samples

3.3.2.1 Microbial growth

The first step of the evaluation involved assessing the viability of microbial strains in the presence of treated materials by quantifying

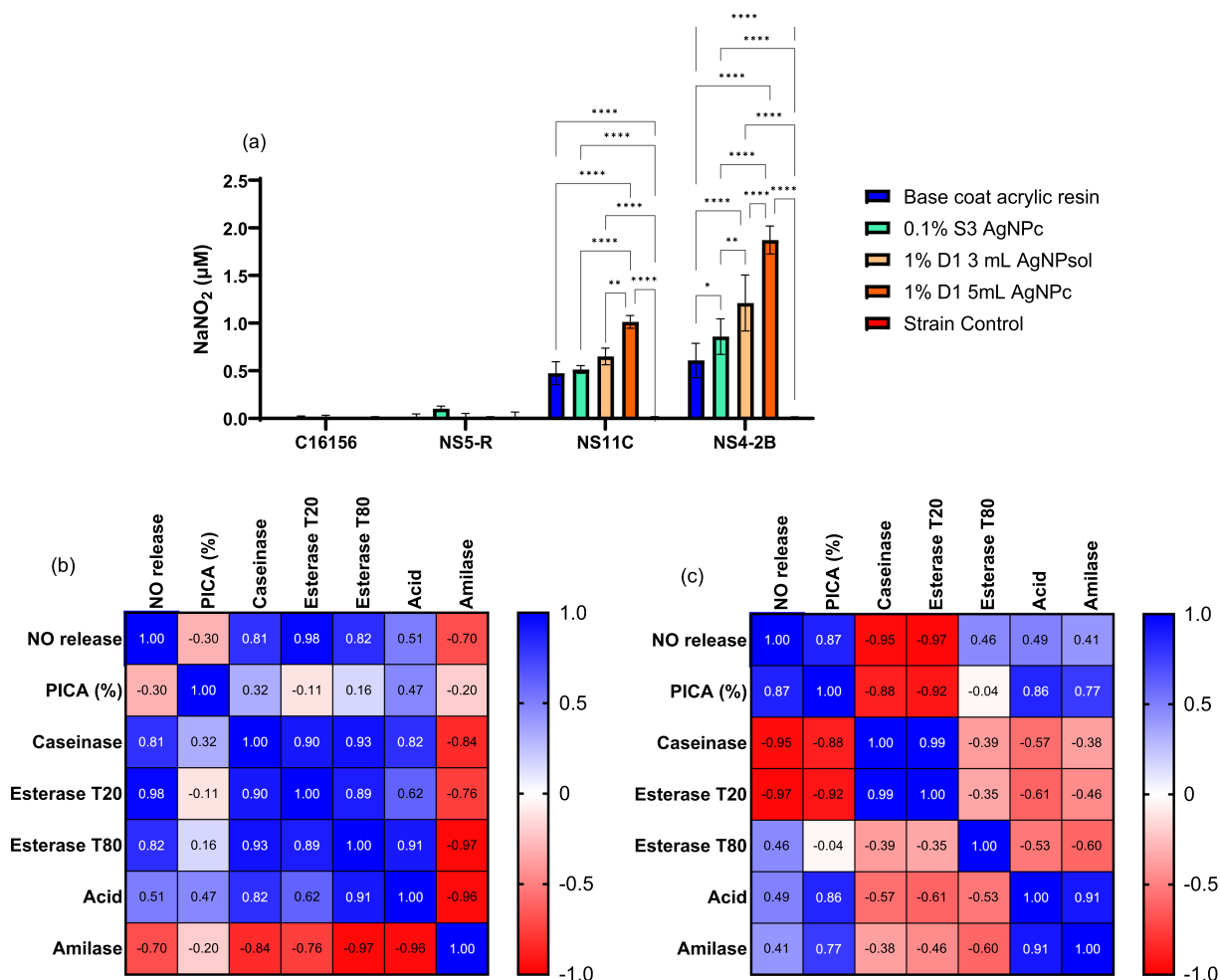


FIGURE 8

Extracellular NO content determined by Griess's reaction for AgNPs in presence of biodeteriogenic strains (a) (Tukey's method, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$); and Pearson correlation between extracellular NO vs. anti-adherence and enzymatic activities for NS11C strain (b) and NS4-2B strain (c).

colony-forming units per milliliter (CFU/mL) after 24 h of contact for bacterial strains, and after 6 days for microfungi. As illustrated in Figures 9a,b, the percentage of microbial viability varied depending on both the type of antimicrobial agent applied and the microbial strain tested.

As shown in Figure 9a, treatment of wooden substrate with the silane-functionalized formulation containing AgNPs (L-MPTMS-Ag100), led to a marked and statistically significant reduction in bacterial viability compared to both the untreated control and the growth control. Specifically, the viability percentage of *B. megaterium* NS-5R decreased to 30.66% while for *B. cereus* C16156 the viability was reduced to 36.93% reflecting a robust antimicrobial effect ($p < 0.0001$). This contrasts with the L-MPTMS without AgNPs treatment which exhibited only partial inhibition reducing bacterial viability to 78.78% for *B. megaterium* NS-5R respectively, 76.06% for *B. cereus* C16156 ($p < 0.05$ – 0.01 depending on the strain). However, the overall antibacterial effect of the treatment is consistent, with differences recorded for the strains analyzed possibly reflecting strain-specific differences in cell wall composition or subtle variations in membrane lipid content, etc.

The fungal strains exhibited a more complex response. For the fungal strain *P. chrysogenum* NS4-2B, exposure to untreated wood led

to an increase in viability up to 131.60% likely due to its ability to metabolize lignocellulosic compounds present in wood as carbon source.

Yet, treatment with L-MPTMS alone showed moderate inhibitory effect by reducing with the fungal viability to 91.08% compared to the untreated material (Figure 9a; see Supplementary Tables S8, S9) ($p < 0.01$). The inhibitory effect is likely not due to direct antifungal activity but rather to reduced accessibility of lignocellulosic substrate resulting from increased surface hydrophobicity and chemical modification of the wood surface following L-MPTMS application. Such surface alteration may hinder fungal adhesion and enzymatic degradation processes, thereby limiting fungal proliferation. In case of *P. chrysogenum* NS11C strain no significant alteration of fungal viability were recorded proving once again that the impact of L-MPTMS alone or supplemented with AgNPs is strain dependent.

As shown in Figure 9b the base coat acrylic resin failed to reduce microbial viability appreciably across the strains tested. However, adding AgNPs improved the outcomes. For *B. cereus* C16156 both 1% D1 AgNP solutions formulated with 3 or 5 mL of AgNPs led to significant reductions in viability to 86.19% and 88.43% ($p < 0.01$). Fungal strains demonstrated greater resistance to the applied

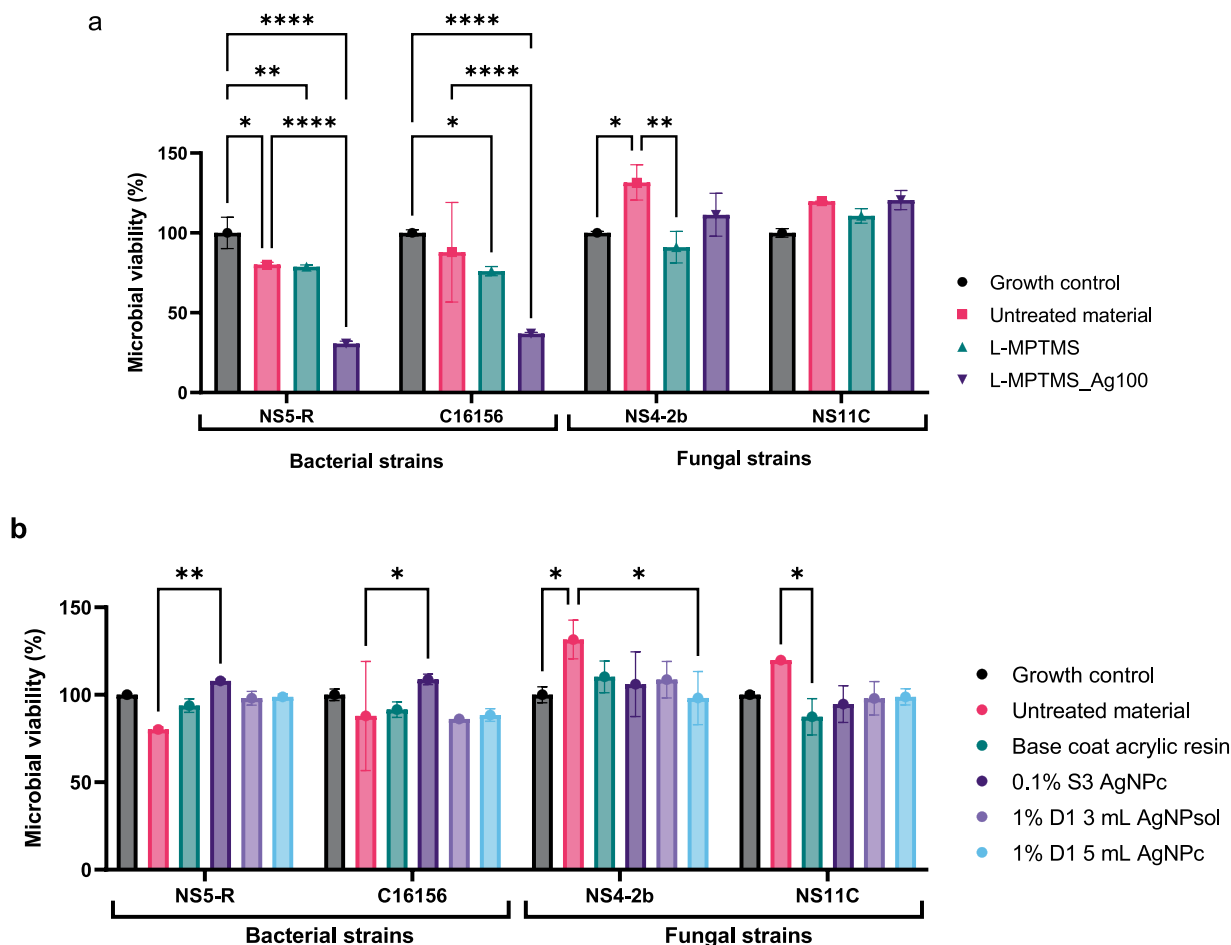


FIGURE 9

Microbial viability in the presence of wooden material treated with L-MPTMS and L-MPTMS-Ag100 (a) and of wooden material treated with synthetic resins (b) (Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

treatments. For the fungal strain, all tested acrylic resin formulations led to a decrease in microbial viability relative to the untreated wood (131.60 and 119.78%), though the reductions were not statistically significant when compared with the growth control (see [Supplementary Tables S8, S9](#)). Notably, the 1% D1 5mL AgNPc formulation showed the greatest antifungal effect among resin treatments, though the overall viability remained above 80% for both fungal strains. Interestingly, a reduction of NS11C viability compared to the untreated substrate was observed when base coat acrylic resin treatment was applied probably due to the inaccessibility of nutrients from the wooden material. Overall, the results indicate that AgNPs enhance the antimicrobial properties of both silane- and resin- based wood treatments, with bacterial strains being more susceptible than fungal strains.

3.3.2.2 Adherence capacity of the biodeteriogenic strains

Based on the results obtained for microbial viability, the study continued with determination of the impact of the treatments on the microbial adherence capacity. As shown in [Figure 10](#), treatment of wood with L-MPTMS and the AgNPs enhanced L-MPTMS significantly reduced microbial adhesion of both bacterial and fungal strains (compared to the untreated material ($p < 0.0001$ for

most comparisons). The most substantial reductions were recorded in case of *B. cereus* C16156 and *P. chrysogenum* NS4-2B with PICA% values decreasing to 61.78 and 56.93%, respectively, following L-MPTMS treatment, and further down to 75.24 and 38.43% when treated with L-MPTMS-Ag100 (see [Supplementary Table S8](#)). These results confirmed the above-mentioned statement regarding the fact that the treatment with L-MPTMS alters the physiochemical properties of wood surfaces in terms of hydrophobicity or surface roughness thereby impairing microbial attachment. Adding AgNPs in the L-MPTMS-Ag100 formulation appears to amplify this anti-adhesive effect, possibly through a combination of surface modification and the well-documented antimicrobial activity of AgNPs which can inhibit microbial surface colonization at early stages. Notably, the fungal strain *P. chrysogenum* NS4-2B exhibited a strong response to the AgNPs enhanced treatment, with PICA% reduced nearly by half.

The evaluation of microbial adherence capacity to resin-treated wooden materials revealed that the base coat acrylic resin treatment exhibited the most pronounced anti-adhesive effect among the tested industrial resins. Specifically, a significant reduction in surface colonization was observed for *B. megaterium* NS5-R, with PICA% decreasing up to 50.13%. However, adding

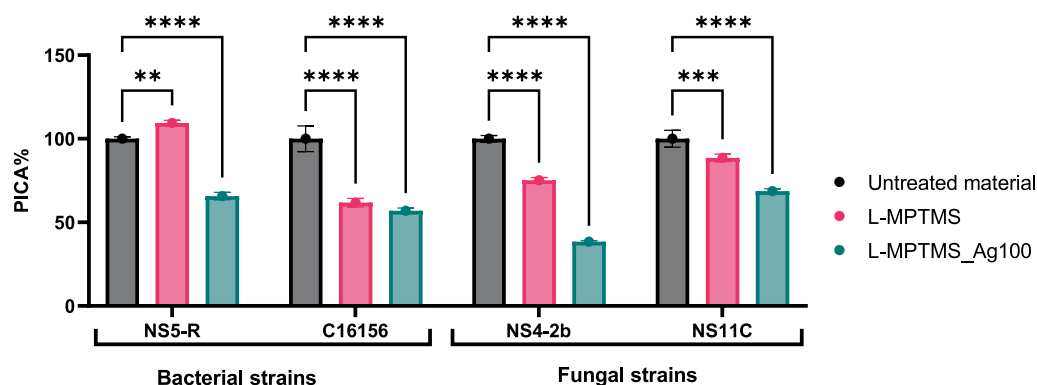


FIGURE 10

The inhibitory effect of treated wood material on the adhesion capacity of biodeteriogenic bacterial and fungal strains (Dunnett's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

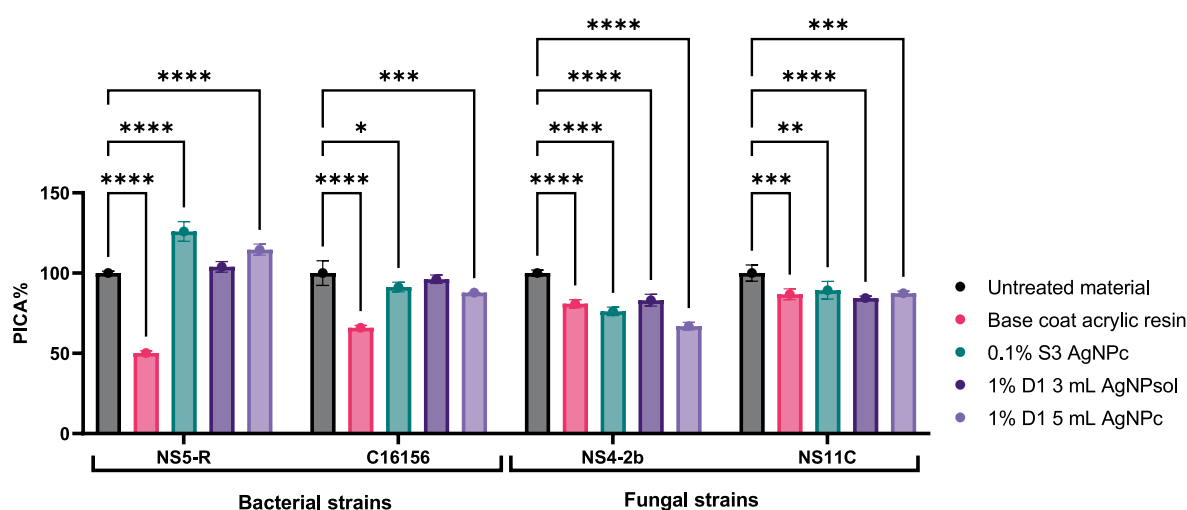


FIGURE 11

The inhibitory effect of wood material treated with industrial resins on the adhesion capacity of bacterial strains and filamentous fungal strains (Dunnett's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

AgNPs significantly reduced the adhesive capacity for 3 out of 4 strains, while for the fourth one, *B. megaterium* NS5-R, an increase of the PICA% value was observed (114.58 in case of 1% D1 5mL AgNPc respectively, 125.97% in case of 0.1% S3 AgNPc) (Figure 11, see Supplementary Table S9).

Since large quantities of active antimicrobial compounds can be released from the substrate in the presence of high humidity, the study continued for determining the impact of these released compounds on the adherence capacity to other substrates. According to Figure 12, no significant changes in the adherence capacity of bacterial strains to the inert substratum as a result of exposure to the antimicrobial compounds released have been noticed. In the case of the fungal strains, a decrease in the adherence capacity compared to the untreated material presence has been observed. Thus, it can be concluded that some of the antimicrobial compounds used to treat surfaces reach the culture medium and interfere with the metabolism of non-adherent cells, inhibiting their ability to further colonize the surface. In case of wood material treated with MPTMS the adhesion

of *B. megaterium* NS5-R to the inert substratum was moderately inhibited by the L-MPTMS_Ag100 formulation, resulting in a PICA% of 77.62%. Furthermore, *B. cereus* strain C16156B adhesion to the inert substratum was enhanced, with PICA% values ranging from 109.86 to 130.96%. Fungal adhesion capacity was not significantly inhibited, with only a slight reduction observed for the L-MPTMS material, showing PICA% values around 83% (Figure 12a, see Supplementary Table S8).

In contrast, regarding the wood material treated with acrylic resins in the presence of NS5-R *B. megaterium*, compared to the untreated material, all treatments incorporating 0.1% S3 AgNPc, 1% D1 3 mL AgNPc, 1% D1 5 mL AgNPc acrylic solutions slightly inhibited the capacity to adhere to the inert substratum with PICA% values around 91%. Meanwhile, for the *B. cereus* C16156 strain, surface adhesion was stimulated in the presence of all the tested materials, with PICA% values surpassing 116%, suggesting limited inhibitory effects. For *P. chrysogenum* NS4-2b and NS11C fungal strains, the adhesion capacity remained generally unaffected by most

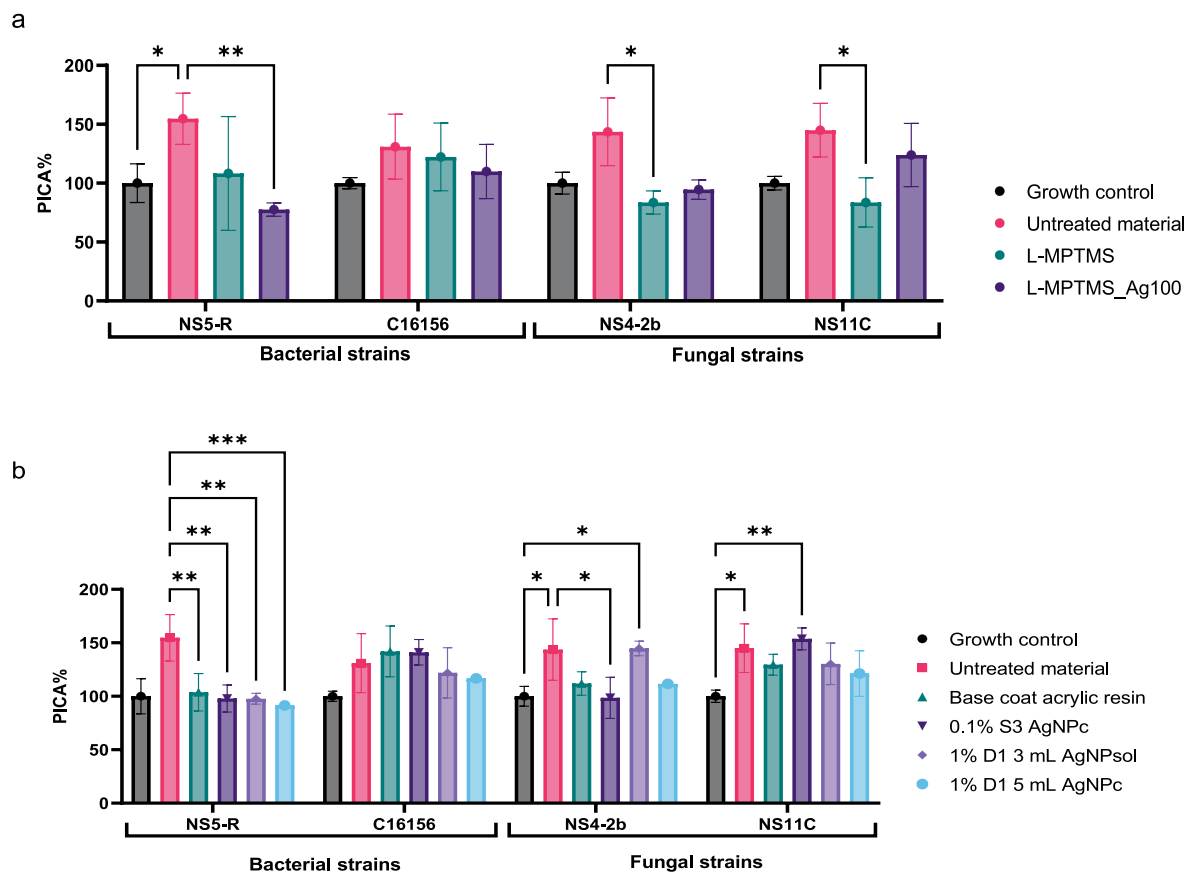


FIGURE 12

The inhibitory effect of compounds released from wood materials type (a) and wood materials type treated with acrylic resins (b) on the adhesion capacity of bacterial and filamentous fungal strains to the inert substrate (Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

treatments. However, a modest inhibitory effect was observed for NS4-2b strain, particularly after treatment with 0.1% S3 AgNPc when PICA% values decreasing to approximately 98% (Figure 12b, see Supplementary Table S9).

3.3.2.3 Biodeteriogenic metabolic features

When exposed to the treated materials it was observed that the AgNPs solution retained its functional bioactivity by significantly reducing the secretion of organic acids by *P. chrysogenum* NS4-2B, with up to 30% while for the *P. chrysogenum* NS11C a similar effect was observed in case of esterase activity (up to 60% inhibition). More precisely in the case of bacterial strains exposed to the AgNPs coating solution completely inhibited the esterase activity of *B. megaterium* NS 5-R strain and up to 30% for the *B. cereus* C16156 strain (see Supplementary Table S10). These results suggest that AgNPs maintain their impact on key metabolic pathways even after being applied on the wooden surface as functionalized coatings. Beyond esterase and organic acid production, further enzymatic assays demonstrated that AgNP exposure also impaired the activity of other extracellular enzymes critical for microbial survival and competitiveness. Specifically, cellulase production was completely inhibited in the presence of L-MPTMS in case of *B. megaterium* NS5-R and *B. cereus* C16156 but highly simulated when AgNPs were added (550% respectively, 180%). Overall, the

results indicate that proteolytic and cellulolytic functions are influenced by the treatment based on L-MPTMS with or without AgNPs mediated stress. Although amylase activity showed variable susceptibility, certain isolates exhibited significant reductions, suggesting strain-specific sensitivity possibly linked to differences in cell wall structure or enzyme regulation.

Treating the surface of the wood material with the resin solution has a minor impact on the ability of microbial strains to secrete enzymes or organic acids. However, a pronounced inhibitory effect was observed in the case of the strain *P. chrysogenum* NS11C whose ability to secrete caseinase and organic acids, respectively, was inhibited in the presence of material treated with 0.1 S3 2020 solution (71.42 and 70.49%). Similarly, the ability of *B. cereus* strain C16156 to secrete esterase when cultured in the presence of 1%D1 5mL AgNPs treated material was reduced to 54.55% (see Supplementary Table S11). Beyond these prominent examples, other strains exhibited varying degrees of sensitivity across enzymatic functions. Moderate reductions were observed in amylase activity in case of bacterial isolates, highlighting that while the inhibition is not universal, multiple enzymatic pathways can be impacted depending on the treatment composition, concentration and microbial target. The amylase production was severely reduced in presence of 1% D1 5mL AgNPc (to 39.39%) for *B. megaterium* NS5-R strain but no significant impact was recorded for the *B. cereus* C16156 strain.

3.3.2.4 Extracellular NO release and correlation with biodegradative features

According to the data in Figure 13a, the addition of silver ions (L-MPTMS_Ag100) decreases this response ($p < 0.05$), possibly due to an antimicrobial effect; in contrast, *P. chrysogenum* NS4-2B and NS11C fungal strains showed very low levels of NO, with no significant

differences between treatments. The wood material, particularly the silane-functionalized one (L-MPTMS), significantly stimulates the extracellular release of NO in the case of bacterial strains (*B. cereus* C16156 and *B. megaterium* NS5-R) when compared to the untreated material ($p < 0.0001$). The NO release demonstrated how treatments applied to wood surfaces impact microbial physiology, and the

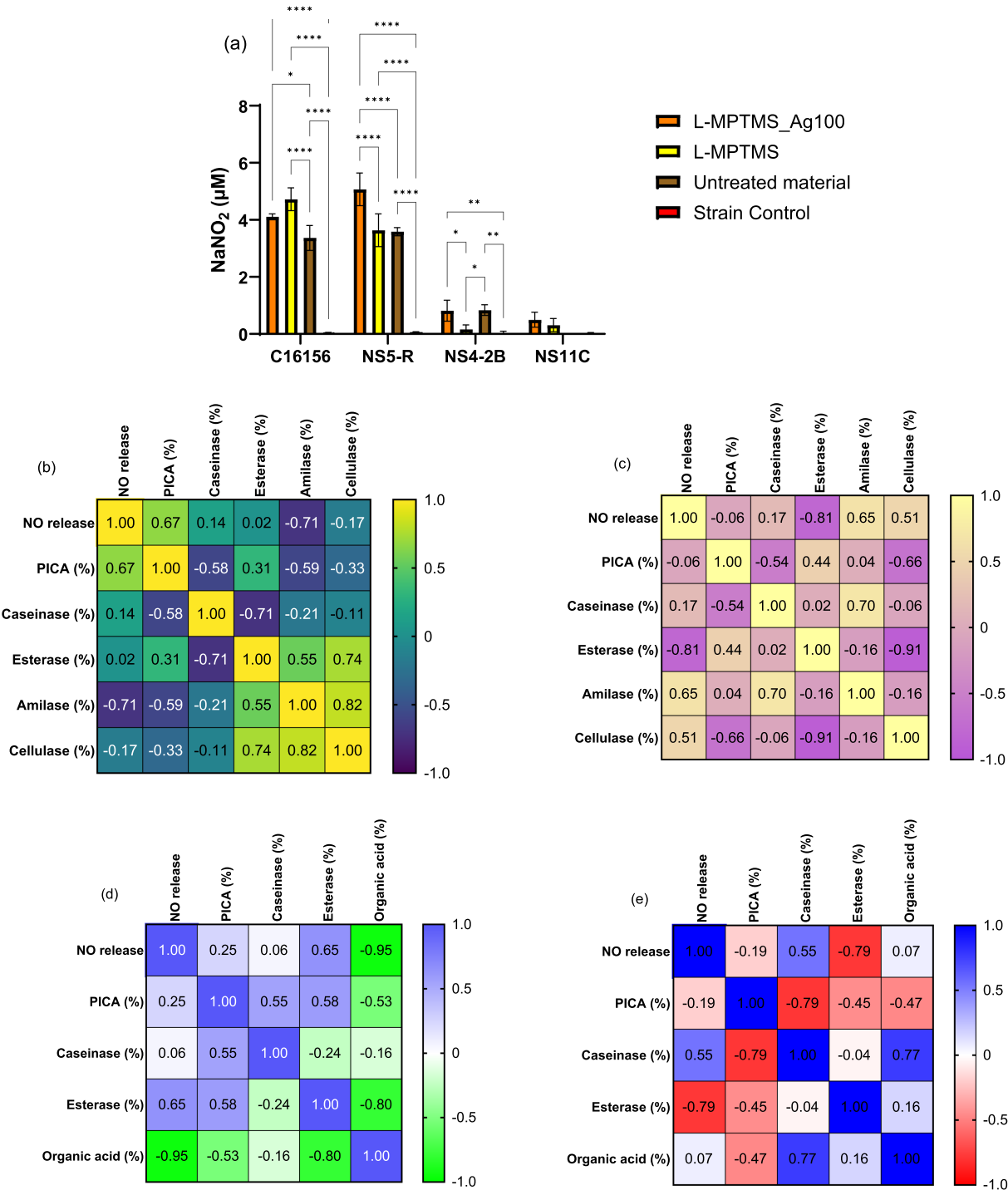


FIGURE 13 Extracellular NO content determined by Griess's reaction for the biodeteriogenic strains in presence of silanized wood materials (a) (Tukey's method, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$); and Pearson correlation between extracellular NO vs. anti-adherence and enzymatic activities for C16156 (b), NS5-R (c), NS4-2B (d) and NS11C (e) strains.

differences between bacteria and fungi highlight how specific the response is depending on the type of microbe and the properties of the surface. Therefore, keeping an eye on NO can be a helpful way to assess how well antimicrobial treatments work on porous materials like wood.

In the case of *B. cereus* C16156 strain, the correlations with esterase and caseinase activities were not significant (Figure 13b). Instead, the activity of cellulase and amylase decreases, and the inhibition of adhesion increases with NO content increase. It is noticeable that, in the case of *B. megaterium* NS5-R bacterial strain, high concentrations of NO inhibit only esterase activity (Figure 13c). Regarding *P. chrysogenum* NS4-2B strain, the acidity decreased with increasing the NO content, which may indicate a decrease in the degree of wood material degradation caused by acid hydrolysis (Figure 13d). Enzyme inactivation such as esterase activity ($r = -0.80$) and enhanced PICA ($r = -0.53$) might also result from the acid pH. The variations in enzymatic activity and microbial adhesion capacity recorded for *B. megaterium* NS5-R strain were different (Figure 13e) indicating that these correlations are highly dependent on the microbial strains under investigation. NO can serve as either a stimulant or an inhibitor, influencing processes such as substrate adherence, polymer breakdown, and organic acid secretion. Increased NO release in bacterial strains such as *B. cereus* C16156 may limit carbohydrate breakdown and surface adherence, potentially through oxidative stress defense mechanisms. *B. megaterium* NS5-R responds differently, blocking esterases while boosting amylase activity, indicating a distinct metabolic response to NO. A negative association between NO and extracellular acidity in filamentous fungi may imply a barrier in organic acid secretion, which reduces the effectiveness of wood material decomposition. The diversity in *P. chrysogenum* NS11C response indicates its high metabolic complexity and versatility.

In the case of resins, it can be observed that the treatment base can significantly change the behavior of biodeteriogenic microorganisms. Thus, in the case of bacterial strains a significant decrease in NO has been observed compared to untreated material, practically the treatment leading to the protection of cells from nitrosative stress. In the case of fungi, it has been observed that the 1%D1 5 mL AgNPc treatment leads to a significant increase in the extracellular NO content (Figure 14a). The Pearson correlation showed that the higher the NO content, the lower the enzymatic activity for amylase, esterase and caseinase. For the bacterial strain *B. cereus* C16156 (Figure 14b), an increase in extracellular NO content was associated with a strong inhibition of caseinase activity ($r = -0.79$), whereas amylase activity correlated positively with microbial adhesion ($r = 0.82$), implying that this enzyme may play a functional role in the colonization process. Furthermore, the acidity significantly influences the activity of these enzymes, even though it is not directly correlated with the NO content. In the case of *B. megaterium* NS5-R strain (Figure 14c), the Pearson correlation showed that the activity of amylase was decreased as the concentration of NO increases. In the case of this strain, a very good correlation with PICA (%) can be observed, in the sense that the higher the NO content, the greater the microbial adhesion. In the instance of *B. megaterium* NS5-R strain (Figure 14c), high doses of NO greatly favored adhesion (PICA, $r = 0.92$), but strongly hindered amylase activity ($r = -0.93$), indicating an adaptive response in which the cell prioritizes attachment over degradation activities.

For *P. chrysogenum* NS4-2B strain, it can be observed that the acidity is inversely proportional to the extracellular content of NO and

PICA, having the ability to inactivate caseinase (Figure 14d). A negative correlation between NO and extracellular acidity ($r = -0.58$) indicates that nitric oxide reduces the acidic environment, which favors proteolytic activity (caseinase), given that higher acidity correlates negatively with it ($r = -0.77$). In the case of *P. chrysogenum* NS11C strain, the increase in acidity can lead to a reduction in microbial adhesion (Figure 14e). *P. chrysogenum* NS11C fungal strain had lesser correlations, but there was a very significant negative association between microbial adherence and caseinase activity ($r = -0.98$), indicating a possible metabolic trade-off between colonization and extracellular enzyme synthesis.

Overall, the findings showed that NO release affects microbial responses differently depending on the strain, inhibiting hydrolytic enzymatic activity while also influencing adhesion and extracellular acidity. NO interferes with biofilm development and can disperse established biofilms through cell signaling pathways at low levels (10^{-9} – 10^{-6} mM). High NO concentrations (0.001–1 mM) remove biofilms by directly killing embedded bacteria (Yang et al., 2018).

3.4 Ecotoxicity

The main objective of the ecotoxicity test in this study consists in assessing the short-term impact on the bulbs of *A. cepa*, i.e., for 24 h, after direct contact. In terms of fresh biomass, the weight of *A. cepa* bulbs increased across all variants. Thus, Figures 15a,b reveal that none of the coating solutions were significantly harmful related to the control. Coating with resin 1%D1 5 mL AgNPc resulted in the lowest biomass growth value when compared to the control, although this was not statistically significant ($p > 0.05$) (Figure 15b). In the case of silane coatings, the L-MPTMS-Ag100 variation resulted in a modest decrease in *A. cepa* bulb biomass, although the p -value was higher than 0.05 which is beneficial and can be attributed to the high affinity of the thiol groups to the AgNPs surface. It may be stated that the solutions provided do not exhibit phytotoxicity.

The increase in fresh biomass across all tested samples suggests that the applied materials, whether acrylic resins with AgNPs or silane-treated materials, did not cause a significant stress response on plant metabolism throughout the 24 h exposure period. Surface interactions, such as the affinity of thiols in silanes for AgNPs, can influence the degree of Ag⁺ ion release, as seen in L-MPTMS-Ag100 and 1% D1 AgNPc. However, the lack of significant decreases in fresh biomass supports the notion that these materials do not exhibit acute phytotoxicity and are thus compatible with plant biological media under similar testing conditions.

4 Discussion

This study explored the antimicrobial and protective efficacy of treatments combining commercial polyacrylic resins, silver nanoparticles (AgNPs), and siloxane-based coupling agents (3-MPTMS) for the conservation of wooden heritage materials. Polyacrylic resins are widely used as surface coatings due to their advantageous properties, including rapid drying time, optical clarity, favorable mechanical strength, strong adhesion, and excellent chemical stability (Cocca et al., 2004). Protective treatments employing commercially available acrylic resins are not only easy to apply and

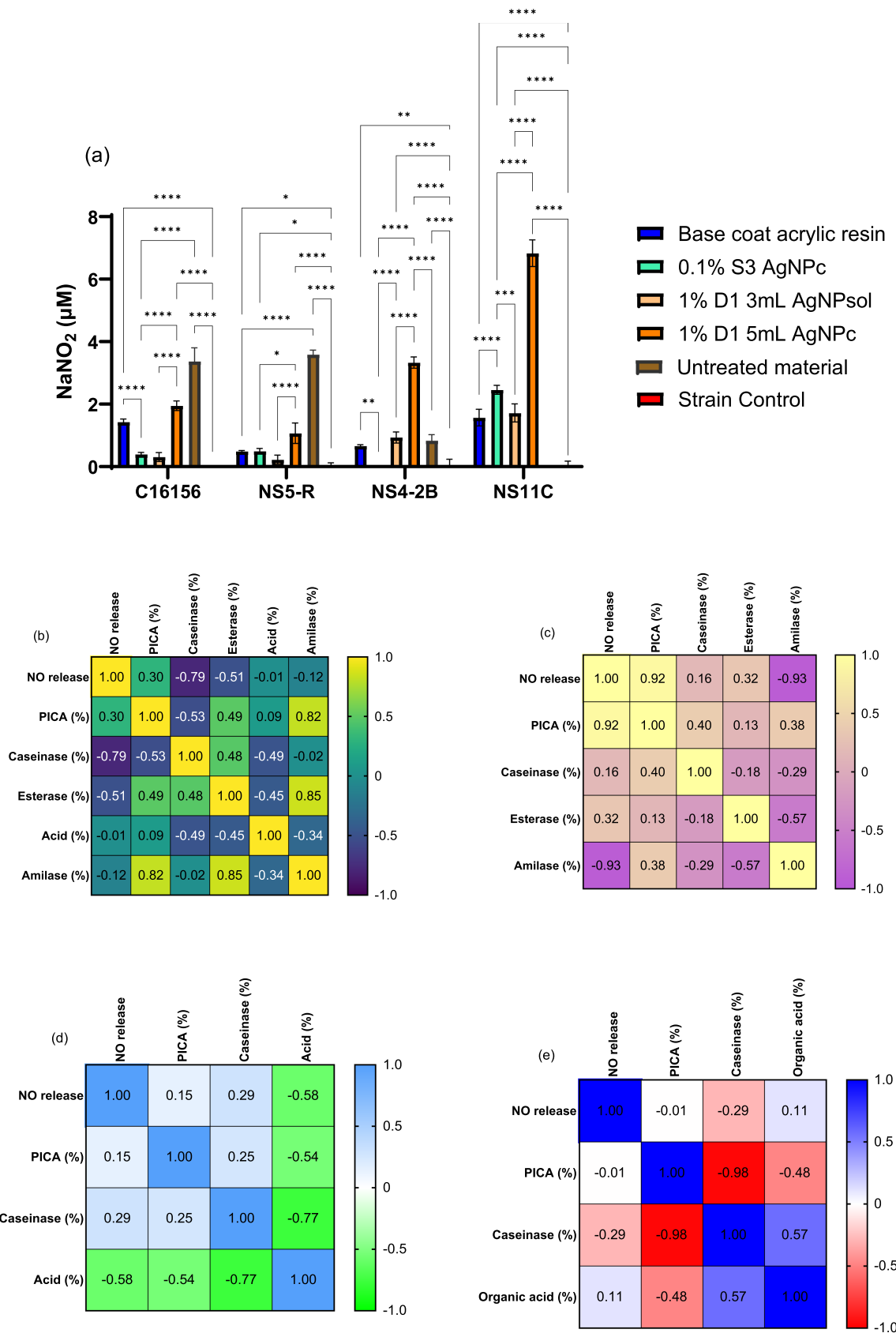


FIGURE 14
Extracellular NO content determined by Griess's reaction for the biodeteriogenic strains in presence of wood materials coated with resins (a) (Tukey's method, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$); and Pearson correlation between extracellular NO vs. anti-adherence and enzymatic activities for C16156 (b), NS5-R (c), NS4-2B (d) and NS11C (e) strains.

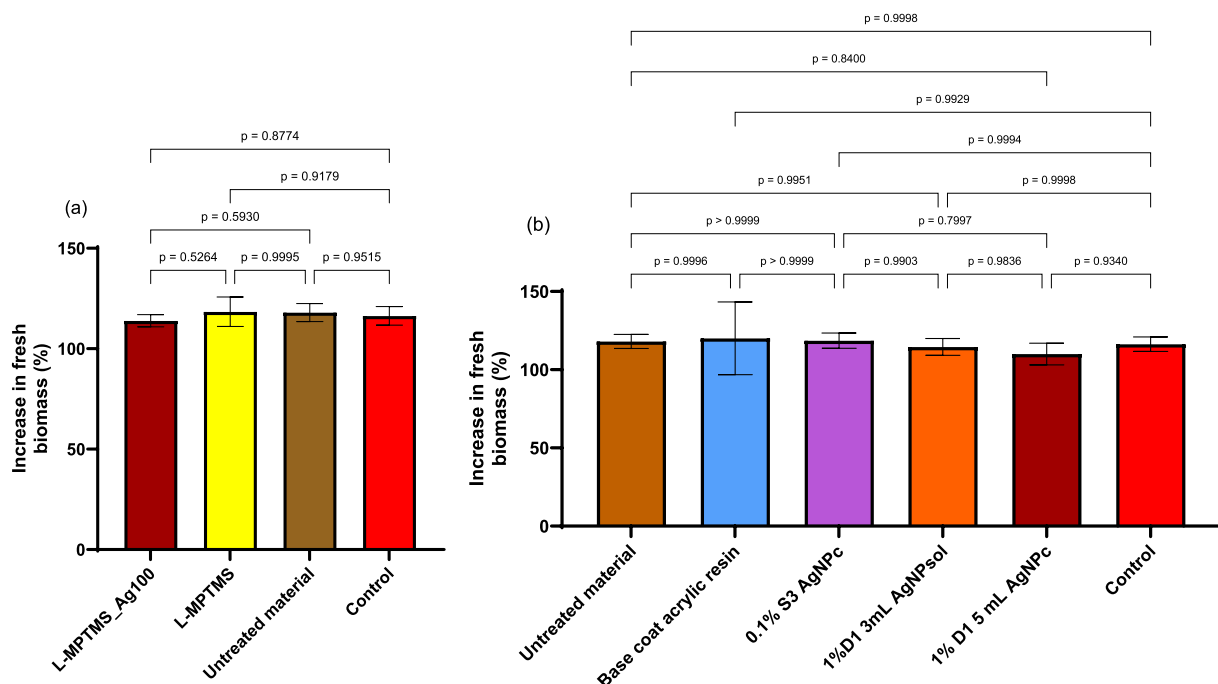


FIGURE 15

Comparative results for the increase in fresh biomass of *A. cepa* bulbs after 24 h: (a) wood-based materials coated with silane-based coatings, and (b) wood-based materials coated with acrylic resins. Statistical analysis method used was one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance (significant relevance was set $*p < 0.05$).

cost-effective, but also non-destructive to the treated surfaces. These resins are capable of penetrating the substrate, providing structural reinforcement and enabling deep impregnation, particularly in microcracks and fissures, rather than forming a superficial coating alone. The consolidation of wooden elements using resins is well documented, for instance in the repair of wooden elements in Nanzen Temple in Shanxi (Wang et al., 2023), as well as in the restoration of ancient architectural heritage in Brazil and Spain (Da Silva Bertolini et al., 2013; Vilches Casals et al., 2013).

Other polymer-based systems such as FEVE resins modified with Ag₂O/OH-MWCNTs have also shown enhanced viscosity, thermal stability, and antimicrobial activity against *Trichoderma* spp., *Aspergillus niger*, and white rot fungi (Teri et al., 2024). Similarly, the use of polyacrylic resins enhanced with AgNPs and siloxane-based coupling agent 3-MPTMS represents a potent strategy for combating microbial contamination and preserving materials susceptible to biodeterioration.

The role of the coupling agent is to create a bond between the silicon in the siloxane groups (Si-MetOH) and the -OH hydroxyl groups on the structure of the substrates. The siloxanes used possess a hydrolytic center that reacts with inorganic substrates to form stable covalent bonds and an organic group that modifies the physical interactions of the treated substrates. Through hydrolysis and condensation reactions, alkoxy groups of siloxanes eliminate water or alcohol to form a crosslinked network, partially shrinking the pores of the treated surface through the pore-lining effect (Marinescu et al., 2023). Terminal -SH moieties on the siloxane molecules covalently bind to AgNPs, promoting self-assembly and enhancing penetration into wood structure.

Compared to other coating systems, surface silanization methods are easy to apply, cost-effective, non-destructive, and compatible with

solvent systems (especially alcohol-based ones) that facilitate faster evaporation and deeper substrate penetration. Siloxane-based coupling agents are proposed as alternatives to benzotriazole due to their hydrophobicity, transparency, and corrosion-inhibiting properties, making them suitable for cultural heritage conservation (Artesani et al., 2020). Silane coatings interact covalently with metal surfaces when hydrolyzed, though achieving uniform films requires multiple layers depending on substrate properties (Schreiber, 2000).

The combined application of AgNPs with silanization agents enhances antimicrobial efficacy and mechanical reinforcement. Silane-modified epoxy resins, for example, improve tensile strength and reduce water absorption in viscose fabric composites (Rajan et al., 2018). Similarly, advanced mortar coatings treated with silanization agents have demonstrated exceptional durability under UV exposure, offering long-term protection for historical buildings (Jelincic and Glivetić, 2020). Nonetheless, high NP concentrations may alter the visual appearance of treated substrates, requiring optimized formulations (Ntelia and Karapanagiotis, 2020).

In our study, treatments combining AgNPs with MPTMS or acrylic resins significantly reduced microbial viability, particularly for bacterial strains such as *Bacillus cereus* and *Bacillus megaterium*. This finding aligns with prior research showing that AgNPs disrupt microbial membranes, produce reactive oxygen species, and damage DNA (Rai et al., 2009; Seth et al., 2012). Notably, untreated wood enhanced the growth of *P. chrysogenum*, emphasizing the biodeteriogenic potential of fungal colonization.

Sub-inhibitory concentrations of resin solutions inhibited bacterial adherence to inert substrata, with PICA% values significantly lower than controls. This anti-adherence activity was accompanied by increased oxidative stress, as evidenced by

elevated extracellular nitric oxide (NO) levels, particularly in fungal strains. These findings support the hypothesis that NO plays a role in microbial stress responses and biofilm inhibition (Bowman et al., 2011; Yang et al., 2018). In contrast, bacterial strains produced lower extracellular NO, and AgNPs decreased NO production while MPTMS increased it, suggesting distinct nitrosative stress responses (Stern and Zhu, 2014).

Enzymatic activity was also influenced by treatment type and microbial strain. The strongest inhibition of esterase and amylase secretion was observed for fungal strains treated with 0.1% S3 and 1% D1 AgNPs. Bacterial esterase activity was completely inhibited in *B. megaterium* and significantly reduced in *B. cereus*. These data support the dose-dependent, species-specific effects of AgNPs on microbial enzymatic systems (Quinteros et al., 2016).

The potential ecological impact of these treatments was assessed using the *Allium cepa* test system. No significant phytotoxic effects were observed, as all treated samples supported normal biomass growth. This confirms the environmental safety of these formulations, addressing concerns about the release of AgNPs from treated surfaces (Ameen et al., 2021; Souza et al., 2020).

Regarding NO, its multifunctional role in microbial physiology was confirmed. While some microbial strains increase NO production as a defensive response, others reduce it under AgNP exposure. This differential response influences not only microbial adhesion but also enzymatic activity and degradation potential. Studies have shown that NO interacts with microbial enzymes, potentially inactivating them via S-nitrosylation and impairing pathogenicity (Brandes et al., 2007; Stern and Zhu, 2014).

The study was further extended to evaluate the inhibitory efficacy of resin solutions applied to model materials, specifically 1 cm² wood fragments. Additionally, the analysis included a wood model pre-treated with MPTMS and subsequently functionalized with AgNPs synthesized via the Turkevich method (Ag100), which demonstrated high levels of antimicrobial activity in a previous study (Marinescu et al., 2022). The wood model materials treated with L-MPTMS and L-MPTMS-Ag100 decreased the microbial adhesion and enzymatic degradation. Our results are in agreement with a previous study showing that organosilicon compounds can preserve degraded wood by reinforcing structure and protecting against further biodeterioration (Broda et al., 2023; Broda and Plaza, 2023). The same effect was reported for 3-MPTMS and AgNPs deposited on other substrates like cotton fabric, enhancing its antimicrobial activity (Kim et al., 2011).

Similarly, the most effective resin formulation, 1% D1 5 mL AgNPs, strongly inhibited microbial adherence and enzymatic activity, supporting its suitability for conservation use. This aligns with findings on AgNP-embedded catechol-formaldehyde resin microspheres and other antimicrobial coatings that inhibit bacterial and fungal growth with low toxicity (Liu et al., 2024).

Finally, it is essential to consider the long-term fate of nanoparticles. While AgNPs show great promise in conservation, their ecological and toxicological profiles must be closely monitored. Existing literature highlights the potential for AgNPs to affect soil and plant microbiomes, but our findings show minimal short-term phytotoxicity and support their use in responsible, controlled conservation applications (Cvijetko et al., 2017; Sajid et al., 2015).

5 Conclusion

This study demonstrated that polyacrylic resins incorporating AgNPs possess substantial antimicrobial properties, with notably stronger effects against bacterial strains. The incorporation of AgNPs, particularly in combination with MPTMS or polyacrylic matrices, significantly enhanced the inhibition of microbial viability, adhesion, and the secretion of degradative enzymes. Additionally, sub-inhibitory concentrations of resin-based treatments modulated extracellular NO levels, indicating activation of microbial stress responses. Importantly, ecotoxicological evaluations confirmed that these functionalized materials exhibit minimal phytotoxicity, supporting their environmental safety and suitability for use in the conservation of cultural heritage. Furthermore, treatments applied to wood model substrates, especially L-MPTMS and L-MPTMS-Ag100, effectively reduced microbial adhesion and enzymatic degradation, reinforcing their protective potential for organic heritage materials. These findings support the application of AgNP-functionalized polyacrylic resins as safe and effective antimicrobial coatings for the preservation of cultural heritage objects, with both immediate and long-term implications for biodeterioration control strategies.

Future studies should focus on assessing the efficacy of these materials against a broader range of biodeteriogenic taxa, including white rot fungi and erosion bacteria, within more complex microbial communities. In addition, omics-based analyses and molecular-level investigations (e.g., transcriptomics, proteomics, and metabolomics) could provide deeper insight into antimicrobial mechanisms and stress pathways. Lastly, long-term performance and environmental stability of these treatments should be evaluated under simulated and real environmental conditions, including fluctuations in temperature, humidity, UV exposure, and microbial succession. Monitoring aging behavior, leaching potential, and re-treatability will be essential for validating the materials' safety, durability, and compatibility with conservation ethics and standards.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

AD: Writing – original draft, Formal analysis, Investigation, Data curation, Writing – review & editing. VC: Conceptualization, Data curation, Methodology, Formal analysis, Investigation, Writing – original draft. IM: Formal analysis, Data curation, Software, Investigation, Writing – original draft, Visualization. RP: Formal analysis, Writing – original draft, Data curation, Investigation. LiM: Writing – original draft, Formal analysis, Methodology, Data curation, Investigation. LuM: Writing – original draft, Formal analysis, Data curation, Investigation. DT: Formal analysis, Data curation, Investigation, Writing – original draft. NI: Data curation, Writing – original draft, Investigation, Visualization. TŞ: Visualization, Supervision, Writing – review & editing, Validation. IG-B: Investigation, Conceptualization, Supervision, Validation, Funding acquisition, Resources, Writing – original draft, Methodology. DF: Methodology,

Conceptualization, Validation, Investigation, Supervision, Funding acquisition, Resources, Writing – original draft. OO: Formal analysis, Writing – original draft, Software, Data curation, Investigation, Methodology. AF: Validation, Supervision, Writing – original draft, Conceptualization, Methodology, Investigation. MC: Resources, Validation, Visualization, Writing – review & editing, Supervision.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The authors acknowledge the financial support of the Research Projects PN-III-P2-2.1-PED-2021-2526 (736 PED/2022), PN-IV-P7-7.1-PED-2024-2249 (9 PED/2025) funded by Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI) and by contract no.760238/28.12.2023, code CF 162/31.07.2023, funded by the EU's Next Generation EU instrument through the National Recovery and Resilience Plan of Romania - Pillar III-C9-I8.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Adetunji, V. O., and Odetokun, I. A. (2012). Assessment of biofilm in *E. coli* O157:H7 and *Salmonella* strains: influence of cultural conditions. *Am. J. Food Technol.* 7, 582–595. doi: 10.3923/ajft.2012.582.595
- Alfieri, P. V., Mohamed, C., and Canosa, G. (2021). Nano/silane impregnation system for wood protection from biotic and abiotic factors. *Int. J. Conserv. Sci.* 12, 849–868.
- Al-Zaban, M. I., Mahmoud, M. A., and Alharbi, M. A. (2021). A polyphasic approach to the characterization of potential silver-nanoparticles-producing and non-producing isolates of *Alternaria* species and antifungal activity against mycotoxigenic fungi. *Biotechnol. Biotechnol. Equip.* 35, 290–302. doi: 10.1080/13102818.2021.1875876
- Ameen, F., Alsamhary, K., Alabdullatif, J. A., and AlNadhari, S. (2021). A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. *Ecotoxicol. Environ. Saf.* 213:112027. doi: 10.1016/j.ecoenv.2021.112027
- Artesani, A., Di Turo, F., Zuchelli, M., and Travaglia, A. (2020). Recent advances in protective coatings for cultural heritage—an overview. *Coatings* 10:3. doi: 10.3390/coatings10030217
- Arya, G., Sharma, N., Mankamna, R., and Nimesh, S. (2019). “Antimicrobial silver nanoparticles: future of nanomaterials” in *Microbial Nanobionics*, ed. R. Prasad, Nanotechnology in the Life Sciences (Cham: Springer International Publishing).
- Aziz, T., Ullah, A., Fan, H., Jamil, M. I., Khan, F. U., Ullah, R., et al. (2021). Recent progress in silane coupling agent with its emerging applications. *J. Polym. Environ.* 29, 3427–3443. doi: 10.1007/s10924-021-02142-1
- Baglioni, M., Poggi, G., Chelazzi, D., and Baglioni, P. (2021). Advanced materials in cultural heritage conservation. *Molecules* 26:3967. doi: 10.3390/molecules26133967
- Bowman, L. A. H., McLean, S., Poole, R. K., and Fukuto, J. M. (2011). The diversity of microbial responses to nitric oxide and agents of Nitrosative stress. In *Advances in microbial physiology*, 59. Amsterdam: Elsevier.
- Brandes, N., Rinck, A., Leichert, L. I., and Jakob, U. (2007). Nitrosative stress treatment of *E. coli* targets distinct set of thiol-containing proteins. *Mol. Microbiol.* 66, 901–914. doi: 10.1111/j.1365-2958.2007.05964.x
- Broda, M., Jakes, J. E., Li, L., and Antipova, O. A. (2023). Archeological wood conservation with selected organosilicon compounds studied by XFM and nanoindentation. *Wood Sci. Technol.* 57, 1277–1298. doi: 10.1007/s00226-023-01503-4
- Broda, M., Mazela, B., and Dutkiewicz, A. (2019). Organosilicon compounds with various active groups as Consolidants for the preservation of waterlogged archaeological wood. *J. Cult. Herit.* 35, 123–128. doi: 10.1016/j.culher.2018.06.006
- Broda, M., and Plaza, N. Z. (2023). Durability of model degraded wood treated with organosilicon compounds against fungal decay. *Int. Biodeterior. Biodegrad.* 178:105562. doi: 10.1016/j.ibiod.2022.105562
- Campana, R., Sabatini, L., Giorgi, L., Pettinari, G., Valentini, L., and Gobbi, P. (2022). A multidisciplinary approach in examining the susceptibility to microbial attack of Polyacrylic and polyurethane resins used in art restoration. *Int. J. Mol. Sci.* 23:11725. doi: 10.3390/ijms231911725
- Can, A., and Hazer, B. (2022). ‘Improvement of dimensional stability, water resistance, and decay resistance of pine wood by the incorporation of polyvinyl chloride-Abietic acid copolymer with AgNPs’. The 3rd International Electronic Conference on Forests—Exploring New Discoveries and New Directions in Forests October 15, 22.
- Cappitelli, F., Cattò, C., and Villa, F. (2020). The control of cultural heritage microbial deterioration. *Microorganisms* 8:10. doi: 10.3390/microorganisms8101542
- Cataldi, A., Deflorian, F., and Pegoretti, A. (2015). Microcrystalline cellulose filled composites for wooden artwork consolidation: application and physic-mechanical characterization. *Mater. Des.* 83, 611–619. doi: 10.1016/j.matdes.2015.06.037
- Cataldi, A., Dorigato, A., Deflorian, F., and Pegoretti, A. (2014). Thermo-mechanical properties of innovative microcrystalline cellulose filled composites for art protection and restoration. *J. Mater. Sci.* 49, 2035–2044. doi: 10.1007/s10853-013-7892-6
- Chu, S., Li, Y., Wang, X., Li, N., Song, J., and Lin, L. (2025). Degradation condition and microbial analysis of waterlogged archaeological wood from the second shipwreck site on the northwestern continental slope of the South China Sea. *NPJ Herit. Sci.* 13:10. doi: 10.1038/s40494-025-01628-8
- Cinteză, L. O., and Tănase, M. A. (2021). ‘Multifunctional ZnO Nanoparticle: Based Coatings for Cultural Heritage Preventive Conservation’. In *Thin films*, edited by A. E. Ares. London: IntechOpen.
- Cirone, M., Figoli, A., Galiano, F., La Russa, M. F., Macchia, A., Mancuso, R., et al. (2023). Innovative methodologies for the conservation of cultural heritage against biodeterioration: a review. *Coatings* 13:1986. doi: 10.3390/coatings13121986
- Cocca, M., D’Arienzo, L., D’Orazio, L., Gentile, G., and Martuscelli, E. (2004). Polyacrylates for conservation: chemico-physical properties and durability of different commercial products. *Polym. Test.* 23, 333–342. doi: 10.1016/S0142-9418(03)00105-3
- Colman, B. P., Espinasse, B., Richardson, C. J., Matson, C. W., Lowry, G. V., Hunt, D. E., et al. (2014). Emerging contaminant or an old toxin in disguise? Silver nanoparticle impacts on ecosystems. *Environ. Sci. Technol.* 48, 5229–5236. doi: 10.1021/es405454v
- Corbu, V. M., Dumbravă, A. Ș., Marinescu, L., Motelica, L., Chircov, C., Surdu, A. V., et al. (2023). Alternative mitigating solutions based on inorganic nanoparticles for the

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1642335/full#supplementary-material>

- preservation of cultural heritage. *Frontiers in Materials* 10:1272869. doi: 10.3389/fmats.2023.1272869
- Corbu, V. M., Georgescu, A.-M., Marinas, I. C., Pericleanu, R., Mogos, D. V., Dumbravă, A. Ș., et al. (2024). Phenotypic and genotypic characterization of resistance and virulence markers in *Candida* Spp. isolated from community-acquired infections in Bucharest, and the impact of AgNPs on the highly resistant isolates. *Journal of Fungi* 10:563. doi: 10.3390/jof10080563
- Corbu, V. M., Gheorghe, I., Marinas, I. C., Geană, E. I., Moza, M. I., Csutak, O., et al. (2021). Demonstration of *Allium Sativum* extract inhibitory effect on Biodeteriogenic microbial strain growth, biofilm development, and enzymatic and organic acid production. *Molecules* 26:7195. doi: 10.3390/molecules26237195
- Cui, X., Zhong, Z., Xia, R., Liu, X., and Qin, L. (2022). Biosynthesis optimization of silver nanoparticles (AgNPs) using *Trichoderma longibrachiatum* and biosafety assessment with silkworm (*Bombyx mori*). *Arab. J. Chem.* 15:104142. doi: 10.1016/j.arabjc.2022.104142
- Cvijetko, P., Milošić, A., Domijan, A.-M., Vinković Vrček, I., Tolić, S., Peharec Štefanić, P., et al. (2017). Toxicity of silver ions and differently coated silver nanoparticles in *Allium Cepa* roots. *Ecotoxicol. Environ. Saf.* 137, 18–28. doi: 10.1016/j.ecoenv.2016.11.009
- Da Silva Bertolini, M., De Borges Macedo, L., De Almeida, D. H., Icimoto, F. H., and Rocco Lahr, F. A. (2013). Restoration of structural timber elements using epoxy resin: analysis of mechanical properties. *Adv. Mater. Res.* 778, 582–587. doi: 10.4028/www.scientific.net/AMR.778.582
- Datu, A.-D., Ciobanu, D.-G., Boros, B.-V., Ostafe, V., and Ianovici, N. (2020). A new approach for phytotoxicity testing using *Allium cepa* bulbs. *Rom. Biotechnol. Lett.* 25, 1488–1494. doi: 10.25083/rbl/25.2/1488.1494
- De Caro, T., Toro, R. G., Cassone, L., Barbaccia, F. I., Zaratti, C., Colasanti, I. A., et al. (2024). Functionalization of artwork packaging materials utilizing ag-doped TiO₂ and ZnO nanoparticles. *Molecules* 29:3712. doi: 10.3390/molecules29153712
- Eastwood, D. C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., et al. (2011). The plant Cell Wall-decomposing machinery underlies the functional diversity of Forest Fungi. *Science* 333, 762–765. doi: 10.1126/science.1205411
- Ferreira, M. T., Soldado, E., Borsoi, G., Mendes, M. P., and Flores-Colen, I. (2023). Nanomaterials applied in the construction sector: environmental, human health, and economic indicators. *Appl. Sci.* 13:12896. doi: 10.3390/app132312896
- Ghabban, H., Alnomasy, S. F., Almohammed, H., Al Idriss, O. M., Rabea, S., and Eltahir, Y. (2022). Antibacterial, cytotoxic, and cellular mechanisms of green synthesized silver nanoparticles against some cariogenic bacteria (*Streptococcus mutans* and *Actinomyces viscosus*). *J. Nanomater.* 2022, 1–8. doi: 10.1155/2022/9721736
- Gheorghe, I., Avram, I., Corbu, V. M., Popa, M., Balotescu, I., Blăjan, I., et al. (2021). *In vitro* evaluation of MgB₂ powders as novel tools to fight fungal biodeterioration of heritage buildings and objects. *Front. Mater.* 7:601059. doi: 10.3389/fmats.2020.601059
- Gopinath, V., Priyadarshini, S., Loke, M. F., Arunkumar, J., Marsili, E., MubarakAli, D., et al. (2017). Biogenic synthesis, characterization of antibacterial silver nanoparticles and its cell cytotoxicity. *Arab. J. Chem.* 10, 1107–1117. doi: 10.1016/j.arabjc.2015.11.011
- Ion, R.-M., Grigorescu, R.-M., Iancu, L., Ghioca, P., and Radu, N. (2018). “Polymeric Micro- and Nanosystems for wood artifacts preservation” in New uses of Micro and nanomaterials. eds. M. R. Pagnola, J. Useche Vivero and A. G. Marrugo (London: Intech).
- Iravani, S., Korbekandi, H., Mirmohammadi, S. V., and Zolfaghari, B. (2014). Synthesis of silver nanoparticles: chemical, physical and biological methods. *Res. Pharm. Sci.* 9, 385–406.
- Jamil, M. I., Ali, A., Haq, F., Zhang, Q., Zhan, X., and Chen, F. (2018). Icephobic strategies and materials with Superwettability: design principles and mechanism. *Langmuir* 34, 15425–15444. doi: 10.1021/acs.langmuir.8b03276
- Jelincic, D., and Glivetić, D. (2020). Cultural heritage and sustainability: practical guide. Interreg Europe programme.
- Kamperidou, V. (2019). The biological durability of thermally- and chemically-modified black pine and poplar wood against Basidiomycetes and Mold action. *Forests* 10:1111. doi: 10.3390/f10121111
- Khan, M. R., Hoque, S. M., Hossain, K. F. B., Siddique, M. A. B., Uddin, M. K., and Rahman, M. M. (2020). Green synthesis of silver nanoparticles using *Ipomoea aquatica* leaf extract and its cytotoxicity and antibacterial activity assay. *Green Chem. Lett. Rev.* 13, 303–315. doi: 10.1080/17518253.2020.1839573
- Khorrami, S., Zarrabi, A., Khaleghi, M., Danaei, M., and Mozafari, M. (2018). Selective cytotoxicity of green synthesized silver nanoparticles against the MCF-7 tumor cell line and their enhanced antioxidant and antimicrobial properties. *Int. J. Nanomedicine* 13, 8013–8024. doi: 10.2147/ijn.s189295
- Kim, S. S., Park, J. E., and Lee, J. (2011). Properties and antimicrobial efficacy of cellulose fiber coated with silver nanoparticles and 3-mercaptopropyltrimethoxysilane (3-MPTMS). *J. Appl. Polym. Sci.* 119, 2261–2267. doi: 10.1002/app.32975
- Kittler, S., Greulich, C., Diendorf, J., Köller, M., and Eppel, M. (2010). Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. *Chem. Mater.* 22, 4548–4554. doi: 10.1021/cm100023p
- Kyei, S. K., Eke, W. I., Darko, G., and Akaranta, O. (2022). Natural polyhydroxy resins in surface coatings: a review. *J. Coat. Technol. Res.* 19, 775–794. doi: 10.1007/s11998-021-00604-8
- Lak, M., Mohammadi, P., and Ghadam, P. (2024). The engineered in situ silver nanocomposite as a surface protective coating with antimicrobial activity used in stony cultural heritage. *Polym. Bull.* 81, 10277–10292. doi: 10.1007/s00289-024-05156-6
- Liu, J.-L., Qu, L.-Y., Shi, Y.-L., Yang, A.-L., Zhang, L., and Xu, Y.-J. (2024). Fabrication of antimicrobial viscose fibers containing silver nanoparticle@catechol formaldehyde resin microspheres. *Cellulose* 31, 703–715. doi: 10.1007/s10570-023-05647-1
- Liu, H., Zhou, T., Sun, X., and Zong, G. (2023). Organosilane-modified wood materials: a review of research and applications. *Bioresour.* 18, 6561–6582. doi: 10.15376/biores.18.3.Liu
- Marinescu, L., Fica, D., Fica, A., Oprea, O., Nicoara, A. I., Vasile, B. S., et al. (2022). Comparative antimicrobial activity of silver nanoparticles obtained by wet chemical reduction and Solvothermal methods. *Int. J. Mol. Sci.* 23:5982. doi: 10.3390/ijms23115982
- Marinescu, L., Motelica, L., Fica, D., Fica, A., Oprea, O. C., Andronescu, E., et al. (2023). A two-step surface modification methodology for the advanced protection of a stone surface. *Nano* 14:68. doi: 10.3390/nano14010068
- Martínez, A. T., Speranza, M., Ruiz-Dueñas, F. J., Ferreira, P., Camarero, S., Guillén, F., et al. (2005). Biodegradation of lignocelluloses: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int. Microbiol.* 8, 195–204.
- Nayaki, V. T., Karthigeyan, S., Ali, S. A., Kalarani, G., Ranganathan, K., and Ranganathan, A. (2023). Chemical characterization of silanized silver nanoparticles impregnated in poly (methyl methacrylate) resin: an *in vitro* study. *J. Indian Prosthodont. Soc.* 23, 45–49. doi: 10.4103/jips.jips_203_22
- Ntelia, E., and Karapanagiotis, I. (2020). Superhydrophobic Paraloid B72. *Prog. Org. Coat.* 139:105224. doi: 10.1016/j.porgcoat.2019.105224
- Osorio-Echavarría, J., Osorio-Echavarría, J., Ossa-Orozco, C. P., and Gómez-Vanegas, N. A. (2021). Synthesis of silver nanoparticles using white-rot fungus anamorphous Bjerkandera Sp. R1: influence of silver nitrate concentration and fungus growth time. *Sci. Rep.* 11:3842. doi: 10.1038/s41598-021-82514-8
- Ottoni, C. A., Lima Neto, M. C., Léo, P., Ortolan, B. D., Barbieri, E., and De Souza, A. O. (2020). Environmental impact of biogenic silver nanoparticles in soil and aquatic organisms. *Chemosphere* 239:124698. doi: 10.1016/j.chemosphere.2019.124698
- Panov, D., and Terziev, N. (2009). Study on some alkoxysilanes used for hydrophobation and protection of wood against decay. *Int. Biodeterior. Biodegrad.* 63, 456–461. doi: 10.1016/j.ibiod.2008.12.003
- Pournou, A. (2020). ‘Biodeterioration of Wooden Cultural Heritage: Organisms and Decay Mechanisms in Aquatic and Terrestrial Ecosystems’ | SpringerLink. Available online at: <https://link.springer.com/book/10.1007/978-3-030-46504-9>.
- Quinteros, M. A., Cano Aristizábal, V., Dalmasso, P. R., Paraje, M. G., and Páez, P. L. (2016). Oxidative stress generation of silver nanoparticles in three bacterial genera and its relationship with the antimicrobial activity. *Toxicol. In Vitro* 36, 216–223. doi: 10.1016/j.tiv.2016.08.007
- Rai, M., Yadav, A., and Gade, A. (2009). Silver nanoparticles as a new generation of antimicrobials. *Biotechnol. Adv.* 27, 76–83. doi: 10.1016/j.biotechadv.2008.09.002
- Rajan, R., Rainosalo, E., Thomas, S. P., Ramamoorthy, S. K., Zavašnik, J., Vuorinen, J., et al. (2018). Modification of epoxy resin by silane-coupling agent to improve tensile properties of viscose fabric composites. *Polym. Bull.* 75, 167–195. doi: 10.1007/s00289-017-2022-2
- Ribeiro, A. P. C., Anbu, S., Alegria, E. C. B. A., Fernandes, A. R., Baptista, P. V., Mendes, R., et al. (2018). Evaluation of cell toxicity and DNA and protein binding of green synthesized silver nanoparticles. *Biomed. Pharmacother.* 101, 137–144. doi: 10.1016/j.biopha.2018.02.069
- Sajid, M., Ilyas, M., Basheer, C., Tariq, M., Daud, M., Baig, N., et al. (2015). Impact of nanoparticles on human and environment: review of toxicity factors, exposures, control strategies, and future prospects. *Environ. Sci. Pollut. Res.* 22, 4122–4143. doi: 10.1007/s11356-014-3994-1
- Santos, T. S., Silva, T. M., Cardoso, J. C., Albuquerque-Júnior, R. L. C. d., Zielinska, A., Souto, E. B., et al. (2021). Biosynthesis of silver nanoparticles mediated by Entomopathogenic Fungi: antimicrobial resistance, Nanopesticides, and toxicity. *Antibiotics* 10:852. doi: 10.3390/antibiotics10070852
- Schrader, L., Brischke, C., Trautner, J., and Tebbe, C. C. (2025). Microbial decay of wooden structures: actors, activities and means of protection. *Appl. Microbiol. Biotechnol.* 109:59. doi: 10.1007/s00253-025-13443-z
- Schreiber, F. (2000). Structure and growth of self-assembling monolayers. *Prog. Surf. Sci.* 65, 151–257. doi: 10.1016/S0079-6816(00)00024-1
- Seth, D., Hausladen, A., Wang, Y.-J., and Stamler, J. S. (2012). Endogenous protein S-Nitrosylation in *E. coli*: regulation by OxyR. *Science* 336, 470–473. doi: 10.1126/science.1215643
- Siddiqi, K. S., Husen, A., and Rao, R. A. K. (2018). A review on biosynthesis of silver nanoparticles and their biocidal properties. *J. Nanobiotechnol.* 16:14. doi: 10.1186/s12951-018-0334-5
- Silva, L. P., Silveira, A. P., Bonatto, C. C., Reis, I. G., and Milreu, P. V. (2017). “Silver nanoparticles as antimicrobial agents” in Nanostructures for antimicrobial therapy (Amsterdam: Elsevier).
- Sodagar, A., Khalil, S., Kassae, M. Z., Shahrudi, A. S., Pourakbari, B., and Bahador, A. (2016). Antimicrobial properties of poly (methyl methacrylate) acrylic resins incorporated with silicon dioxide and titanium dioxide nanoparticles on

- cariogenic Bacteria. *Journal of Orthodontic Science* 5, 7–13. doi: 10.4103/2278-0203.176652
- Souza, I. R., Silva, L. R., Fernandes, L. S. P., Salgado, L. D., Silva de Assis, H. C., Firak, D. S., et al. (2020). Visible-light reduced silver nanoparticles' toxicity in *Allium Cepa* test system. *Environ. Pollut.* 257:113551. doi: 10.1016/j.envpol.2019.113551
- Stern, A. M., and Zhu, J. (2014). "An introduction to nitric oxide sensing and response in Bacteria" in *Advances in applied microbiology*, vol. 87 (Amsterdam: Elsevier).
- Suflet, D. M., Constantin, M., Pelin, I. M., Popescu, I., Rumbu, C. M., Horhoge, C. E., et al. (2024). Chitosan-oxidized pullulan hydrogels loaded with essential clove oil: synthesis, characterization, antioxidant and antimicrobial properties. *Gels* 10:227. doi: 10.3390/gels10040227
- Teri, G., Cheng, C., Han, K., Huang, D., Li, J., Luo, Y., et al. (2024). Study on the properties of FEVE modified with Ag₂O/OH-MWCNTS nanocomposites for use as adhesives for wooden heritage objects. *Molecules* 29:1365. doi: 10.3390/molecules29061365
- Tończyk, A., Niedziałkowska, K., and Lisowska, K. (2025). Ecotoxic effect of Mycogenic silver nanoparticles in water and soil environment. *Sci. Rep.* 15:10815. doi: 10.1038/s41598-025-95485-x
- Vilches Casals, M., Rodríguez Trujillo, V., and Labèrnia Badia, C. (2013). Timber structure repair of an emblematic Catalan industrial building with wood grafts and epoxy resins. *Adv. Mater. Res.* 778, 998–1005. doi: 10.4028/www.scientific.net/AMR.778.998
- Wang, Y., Chen, Q., Lei, Y., Albu Kaya, M. G., Goh, K. L., and Tang, K. (2025). Identification, deterioration, and protection of organic cultural heritages from a modern perspective. *NPJ Herit. Sci.* 13:71. doi: 10.1038/s40494-025-01601-5
- Wang, Q., Feng, X., and Liu, X. (2023). Advances in historical wood consolidation and conservation materials. *Bioresources* 18, 6680–6723. doi: 10.15376/biores.18.3.Wang
- Wang, X., Ji, Z., Chang, C. H., Zhang, H., Wang, M., Liao, Y.-P., et al. (2014). Use of coated silver nanoparticles to understand the relationship of particle dissolution and bioavailability to cell and lung toxicological potential. *Small* 10, 385–398. doi: 10.1002/sml.201301597
- Wen, Y., Mo, H., Tan, B., Lu, X., Wang, B., and Liu, N. (2023). Progress in synthesis and properties of oxetane-based energetic polymers. *Eur. Polym. J.* 194:112161. doi: 10.1016/j.eurpolymj.2023.112161
- Wrońska, N., Placzowska, S., Niedziałkowska, K., and Lisowska, K. (2023). The synergistic effect of biosynthesized silver nanoparticles and Phyto compound as a novel approach to the elimination of pathogens. *Molecules* 28:7921. doi: 10.3390/molecules28237921
- Yang, L., Feura, E. S., Ahonen, M. J. R., and Schoenfisch, M. H. (2018). Nitric oxide-releasing macromolecular scaffolds for antibacterial applications. *Adv. Healthc. Mater.* 7:1800155. doi: 10.1002/adhm.201800155
- Zawadzka, K., Felczak, A., Nowak, M., Kowalczyk, A., Piwoński, I., and Lisowska, K. (2021). Antimicrobial activity and toxicological risk assessment of silver nanoparticles synthesized using an eco-friendly method with *Gloeophyllum striatum*. *J. Hazard. Mater.* 418:126316. doi: 10.1016/j.jhazmat.2021.126316



OPEN ACCESS

EDITED BY

Mai Bingjie,
Shanghai University, China

REVIEWED BY

Biao Wang,
Zhejiang University, China
Xingyue Li,
Chongqing University, China

*CORRESPONDENCE

Fasi Wu

✉ wufs@dha.ac.cn

Huyuan Feng

✉ fenghy@lzu.edu.cn

RECEIVED 30 June 2025

ACCEPTED 01 September 2025

PUBLISHED 24 September 2025

CITATION

Ma W, Chen Q, Wu F, He D, Duan Y, Yue Y, Gu J-D, Yang X and Feng H (2025) Difference and environmental drivers of bacterial communities on wall paintings of the Maijishan and Mogao Grottoes, China. *Front. Microbiol.* 16:1657118. doi: 10.3389/fmicb.2025.1657118

COPYRIGHT

© 2025 Ma, Chen, Wu, He, Duan, Yue, Gu, Yang and Feng. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Difference and environmental drivers of bacterial communities on wall paintings of the Maijishan and Mogao Grottoes, China

Wenxia Ma^{1,2}, Qiqi Chen², Fasi Wu^{3*}, Dongpeng He^{2,3}, Yulong Duan⁴, Yongqiang Yue⁵, Ji-Dong Gu^{6,7}, Xiaoyan Yang¹ and Huyuan Feng^{2*}

¹Key Laboratory of Western China's Environmental Systems (Ministry of Education), Key Scientific Research Base of Bioarchaeology in Cold and Arid Regions (National Cultural Heritage Administration), College of Earth and Environmental Sciences, Lanzhou University, Lanzhou, Gansu, China, ²MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou, Gansu, China, ³National Research Center for Conservation of Ancient Wall Paintings and Earthen Sites, Conservation Institute, Dunhuang Academy, Dunhuang, Gansu, China, ⁴Key Laboratory of Extreme Environmental Microbial Resources and Engineering, Gansu Province, Northwest Institute of Eco-Environment and Resources, Chinese Academy of Sciences, Lanzhou, Gansu, China, ⁵Institute of Maijishan Grottoes Art, Dunhuang Academy, Tianshui, Gansu, China, ⁶Environmental Science and Engineering Group, Guangdong Technion-Israel Institute of Technology, Shantou, Guangdong, China, ⁷Guangdong Provincial Key Laboratory of Materials and Technologies for Energy Conversion, Guangdong Technion-Israel Institute of Technology, Shantou, Guangdong, China

The Maijishan and Mogao Grottoes, both UNESCO World Heritage Sites along the Silk Road, are increasingly threatened by microbial biodeterioration. To characterize bacterial communities of different microbial damages on wall paintings and identify environmental drivers, we combined high-throughput DNA/RNA sequencing with microenvironmental monitoring and conducted a cross-site comparison. At Maijishan, bacterial communities associated with black and white mycelia showed no significant compositional differences within the same cave but varied markedly between caves, indicating site-specific community assembly. Actinobacteria (>50%), particularly *Pseudonocardia* and *Actinomyces*, predominated, while RNA-based analysis revealed active populations of *Escherichia* and *Stenotrophomonas*, likely introduced via exogenous contamination from animal activities. In contrast, black spots from the Mogao Grottoes were dominated by Actinobacteria, Firmicutes, and Proteobacteria, with *Rhodococcus* as a core genus. No core bacterial OTUs were shared between the sites, suggesting strong microenvironmental filtering. Multivariate analysis identified substrate properties (total organic carbon, total nitrogen, pH) and microclimatic fluctuations (diurnal temperature/humidity ranges) as critical drivers. Maijishan's persistently humid conditions (RH > 70% for over 180 days/year) favored Actinobacteria proliferation, whereas Mogao's arid climate (RH < 70% for over 240 days/year) selected for xerotolerant Firmicutes. These results reveal distinct site-specific microbial colonization patterns and provide a scientific basis for targeted conservation strategies to mitigate microbial damage and preserve these invaluable wall paintings.

KEYWORDS

microbial damages, DNA-RNA high-throughput sequencing, relative humidity, environmental monitoring, sustainable preservation

1 Introduction

Wall paintings represent one of the most ancient and enduring expressive forms of expression human creativity, bearing exceptional artistic, scientific, and historical significance on a global scale. As prominent components of grotto architecture, they provide rich insights into the evolution of societies and civilizations (Garg et al., 1995; Sansupa et al., 2023; Wu et al., 2022). However, these cultural assets face increasing threats by biodeterioration, a process exacerbated by global climate change characterized by rising temperature variability and intensified hydrological extremes (Leissner et al., 2015; Liu et al., 2020; Ding et al., 2022; Hu and Hewitt, 2024). Among the various degradation agents, microbial activity plays a central role, driving damage through both physicochemical and biochemical mechanisms. Bacteria, in particular, are often the pioneering colonizers on wall-painting surfaces (Cuezva et al., 2012; Ma et al., 2015; Meng et al., 2017; Mihajlovski et al., 2017; Sugiyama et al., 2017; Ma C. et al., 2023). Their metabolites, such as organic acids, pigments and extracellular polymeric substances (EPS), which accelerate the aesthetic value and structural integrity reducing (Abdel-Halim et al., 2013; Cojoc et al., 2019; Dominguez-Moñino et al., 2017; Elhagrassy, 2018). Through photoautotrophic or chemoautotrophic metabolism, bacterial communities fix carbon and generate organic substrates, facilitating the succession of heterotrophic fungi or archaea (Lan et al., 2010; Li et al., 2021; Liu et al., 2020; Zhang et al., 2019). This trophic cascade fundamentally reshapes microhabitat conditions, promoting secondary colonization by other microorganisms (Xu et al., 2018).

Biodeterioration poses major challenges to wall painting preservation, understanding these microbial communities is thus essential for heritage conservation (Ciferri, 1999; Martin-Sanchez et al., 2012; Ma et al., 2020; Pei et al., 2023). While previous research has relied heavily on culture-dependent methods or DNA-based high-throughput sequencing to characterize microbial diversity, these approaches have limitations. DNA sequencing captures the total community composition, including dormant or dead cells, but does not directly reveal which members are metabolically active. RNA enables the identification of taxa actively participating in biodeterioration at the sampling moment, the integration of DNA and RNA sequencing provides a more complete and dynamic view of microbial communities by resolving both the taxonomic structure (DNA) and the metabolically active fraction (RNA). Applying such an integrated method significantly enhances our ability to investigate the viability of these microbial communities and allows more accurate assessment of microbial threats, thereby improving the scientific basis for preventive conservation (Sanmartín et al., 2018; Meng et al., 2020; Liu W. et al., 2022).

Microbial communities colonizing cultural heritage materials are shaped by a complex interplay of environmental conditions, biological and physicochemical factors (Dornieden et al., 2000; Wu et al., 2021; Zhang et al., 2021). Previous studies have demonstrated that regional climate type and microclimatic fluctuations are key determinants of microbial diversity in heritage sites (Ding et al., 2022), with temperature and precipitation patterns identified as key drivers of microbial diversity (Biagioli et al., 2024; Chen and Gu, 2022; Liu et al., 2018). Bacterial communities, in particular, tend to respond more rapidly and sensitively to environmental variability than fungi, which often display greater adaptability and resilience (Barnard et al., 2013; Chen et al., 2023). This sensitivity means that climate-induced

environmental shifts can trigger rapid changes in bacterial assemblages, potentially leading to irreversible deterioration trajectories (Viles and Cutler, 2012; Li et al., 2021; Ding et al., 2022).

This study investigates microbial biodeterioration at two UNESCO World Heritage Sites along the Ancient Silk Road: the Maijishan Grottoes and the Mogao Grottoes. In recent years, microbial proliferations have been observed in Caves No. 28 and No. 30 at Maijishan, appearing as black and white mycelial growths (Ma et al., 2025). While black spot formations have previously been documented in Cave No. 256 at Mogao (Ma W. et al., 2023). By comparing the bacterial communities and microenvironmental conditions across these geographically distinct sites (separated by over 1,400 km), this study aims to: (1) elucidate taxonomic divergences between black and white mycelia in Maijishan Grottoes; (2) identify site-specific bacterial community signatures distinguishing the two sites; and (3) evaluate the relative influence of geographic isolation versus environmental filtering on bacterial community assembly. These findings aim to advance our understanding of microbial biogeography in wall painting environments and provide a scientific foundation for developing site-specific conservation strategies.

2 Materials and methods

2.1 Description of study sites

The Maijishan Grottoes (34°05′–35°10′N, 104°35′–106°44′E), a UNESCO World Cultural Heritage Site, are located in the Tianshui Maiji Mountain, a part of Qinling Mountain range. This cultural site comprises 221 caves, featuring 10,632 clay sculptures and approximately 1,000 m² of wall paintings. Situated in a transitional zone between subtropical and warm temperate climates, the region is characterized by dense forest coverage, high humidity and stable temperatures year-round. In contrast, the Mogao Grottoes (39°53′–41°35′N, 92°13′–95°30′E), also designated as a UNESCO World Cultural Heritage Site, are positioned along the eastern cliff of Mingsha Mountain, approximately 25 km southeast of Dunhuang. This site includes 735 caves, with over 45,000 m² of wall paintings and 2,400 sculptures, and is located in a cold desert climate zone marked by low humidity, minimal precipitation, and high potential evaporation (Figures 1A–C).

Both grotto complexes were constructed during the 4th century CE (Maijishan: 384 CE; Mogao: 366 CE), with similar wall painting techniques and materials. These include mineral pigments, natural sediments, original binders (e.g., casein, animal glue, gelatin), and plant fibers (e.g., hemp, wheat straw, cotton) used for structural reinforcement. Microbial colonization and biodeterioration have been observed to varying degrees in both the Maijishan Grottoes and the Mogao Grottoes. This study focused on Cave No. 256 at the Mogao Grottoes, where black spot deterioration is present (Figures 1D,E,G), and Caves No. 28 and No. 30 at the Maijishan Grottoes, which exhibit black and white mycelial growth on wall paintings (Figures 1E,H,I).

2.2 Sampling

Caves No. 28 and No. 30, situated within the middle-lower sections of the Maijishan Grottoes (Supplementary Figures S1B,C,F),

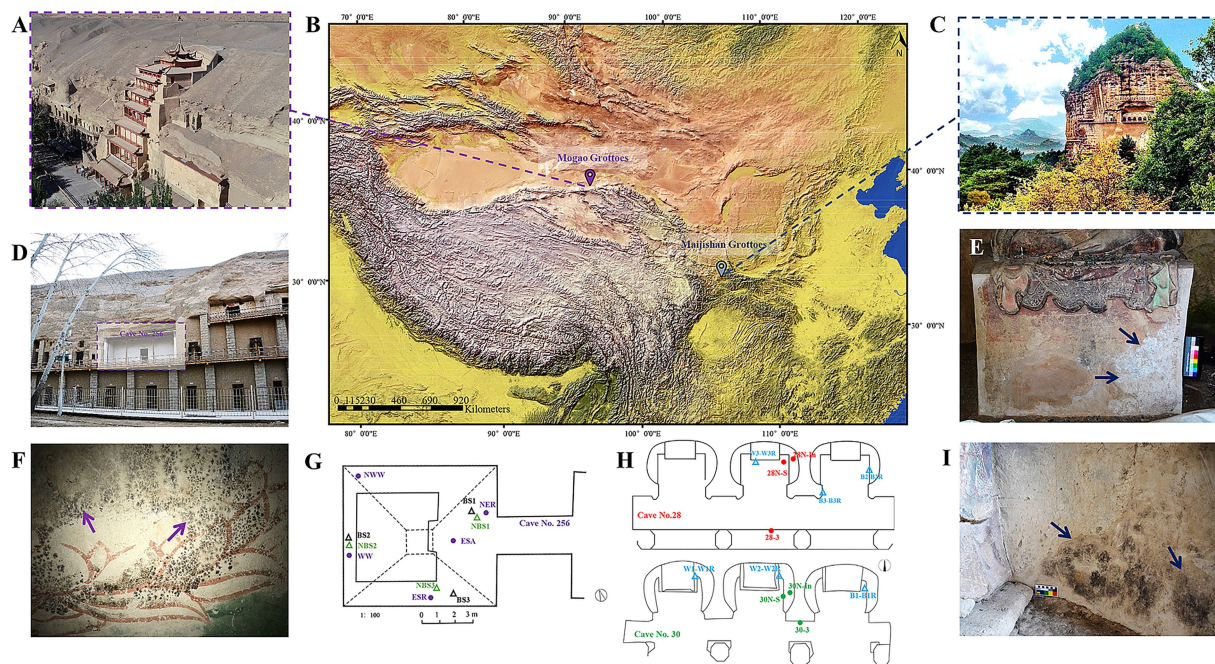


FIGURE 1

Locations of the study site and typical microbial damages of the Maijishan Grottoes and the Mogao Grottoes in China. (A–C) The geographical locations and exterior illustration of the Maijishan and Mogao Grottoes. (D,F) The appearance of Cave No. 256 and black spots on wall paintings at the Mogao Grottoes. (G) Schematic diagram of sampling and environmental monitoring sites in Cave No. 256, the black and green triangles represent the sampling sites for black-spots samples (BS) and no-spots samples (NBS), respectively, while the purple dots represent the temperature and relative humidity monitoring sites. (E,I) White and black mycelium on the wall paintings of Caves No. 28 and No. 30 at the Maijishan Grottoes. (H) Schematic diagram of sampling and environmental monitoring sites, the blue triangles represent the sampling sites, while the red and green dots represent the temperature and relative humidity monitoring sites.

with black (Supplementary Figures S1A,D,I) and white (Supplementary Figures S1E,G,H) mycelia colonizing wall paintings. In August 2021, a total of 12 microbial samples (each pair consisting of two samples) were collected from colonized wall painting surfaces. Each sample was collected using sterile forceps within a targeting 10×10 cm area (Supplementary Figures S1A,D,E,G–I). Each collected mycelia sample was thoroughly mixed and equally divided: one portion for DNA extraction (designated W1–W3 for white and B1–B3 for black mycelia), and the other preserved in RNeasy Lysis Buffer (Qiagen, Germany) for RNA analysis (designated W1R–W3R and B1R–B3R) to assess viable metabolism. Sampling involved carefully removing only mycelia without damage to paintings or restored materials, with each weighed approximately 50 mg. Additionally, fragments of non-colonized wall paintings (totaling 10–15 g) were collected from both caves for physicochemical characterization. All samples were stored in sterile Eppendorf tubes and transported to the laboratory at Lanzhou University for further analysis. The sampling was conducted under the authorization and supervision of professionals from the Maijishan Grottoes Art Research Institute.

For comparative analysis, previously collected and sequenced samples from Cave No. 256 at the Mogao Grottoes (Ma W. et al., 2023) were re-analyzed, including black-spot samples (BS) and non-spot (NBS) samples. These samples collected from the wall painting surfaces with the same sampling method, with specific sample information and detailed data provided in the previous publication (Ma W. et al., 2023).

2.3 DNA extraction, RNA extraction, and MiSeq high-throughput sequencing analysis

Total genomic DNA and rRNA from each sample were extracted using the PowerSoil® DNA Isolation Kit and PowerSoil® RNA Kit (MO BIO Laboratories, United States), following the manufacturer's protocols. RNA was subsequently reverse-transcribed into complementary DNA (cDNA) with the cDNA Synthesis Kit (Sigma-Aldrich, United States). Nucleic acid concentrations (DNA/cDNA) were quantified using a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, United States), and integrity was verified by 1% agarose gel electrophoresis. The bacterial V3–V4 regions of the 16S rRNA gene were amplified using primers 338F/806R (Peiffer et al., 2013). PCR conditions consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min (detailed reaction mixtures in Supplementary Table S1). Triplicate amplifications per sample were pooled, purified with a Gel Extraction Kit (AXYGEN, China), and quantified via QuantiFluor™-ST Fluorimeter (Promega, United States). Libraries were prepared using the NEXTflex® Rapid DNA-Seq Kit (Bioo Scientific, United States) and sequenced on an Illumina MiSeq PE300 platform (Majorbio Bio-Pharm Technology Co., China).

Raw FASTQ data were processed with Trimmomatic (Bolger et al., 2014) and FLASH (Magoč and Salzberg, 2011), with reads <50 bp discarded and no assembly performed. High-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity

via UPARSE (Edgar, 2013). Taxonomic classification of bacterial OTUs was performed using the RDP Classifier against the SILVA and UNITE v7.0 databases with a 70% confidence threshold.

2.4 Environmental data collection

Approximately 3 g of homogenized wall painting substrate from the Majijishan Grottoes was oven-dried at 105°C until constant weight to determine gravimetric moisture content (MC). pH and electrical conductivity (EC) were measured using a Sartorius PB-10 pH meter (1:5 w/v in deionized water) and Leici DDSJ-318 conductivity meter (1:5 w/v in 1 M KCl), respectively. Total organic carbon (TOC) and total nitrogen (N) were quantified by high-temperature combustion (450°C and 1,250°C) using a Vario EL cube CHNS analyzer.

Continuous environmental monitoring was conducted from 2021 to 2022 in Caves No. 28 and No. 30 (Figure 1H). HOBO® U23-001 loggers recorded air (28-3, 30-3) and wall surface (28 N-S, 30 N-S) temperature (T) and relative humidity (RH) at hourly intervals. iButton® DS1923 sensors embedded in wall paintings measured internal T and RH every 2 h (28 N-IN, 30 N-IN). The environmental monitoring protocol in Cave No. 256 at the Mogao Grottoes followed the same design as detailed in our previous study (Ma W. et al., 2023), with monitoring locations shown in Figure 1G.

2.5 Statistical analysis

All statistical analyses were performed using SPSS 13.0. The α -diversity indices (Shannon, Simpson, Ace, and Chao) were calculated with MOTHUR (version 1.30.1) (Schloss et al., 2009). Redundancy analysis (RDA) was conducted in R (v4.0.3) using the vegan package to explore relationships between microbial communities and environmental

variables. Sankey diagrams were generated with the ggalluvial package within the R environment. Differences between sample groups were tested with the Wilcoxon rank-sum test ($p < 0.05$). Environmental parameter visualizations were created using Origin 8.0 software.

3 Results and analysis

3.1 Bacterial community diversity and taxonomic composition in Majijishan Grottoes

High-throughput sequencing of the bacterial V3–V4 regions from both DNA and cDNA libraries yielded 782,304 raw sequences, clustered into 526 OTUs, with the specific sequence counts, OTU numbers, and diversity indices for each sample presented in the Supplementary Table S2. Alpha-diversity metrics revealed minimal differences among the four sampling types (Black-DNA, Black-RNA, White-DNA, White-RNA, $p < 0.05$, Wilcoxon rank-sum test), with the Black-RNA group exhibiting the highest Shannon index, followed by Black-DNA, White-DNA, and White-RNA (Supplementary Table S2). Taxonomic classification identified 21 bacterial phyla, 182 families, and 302 genera. The dominant phyla were Actinobacteria, Proteobacteria, Cyanobacteria, and Firmicutes, with Actinobacteria exceeding 50% relative abundance in all but one RNA sample (B1R). RNA samples showed higher proportions of Proteobacteria, suggesting elevated microbial viability (Figure 2A).

At the genus level, *Pseudonocardia* and *Actinomycetospora* dominated, accounting for >60% relative abundance in most samples (excluding W3R and B1R, Figure 2B). *Pseudonocardia* was predominant in black mycelial and some white mycelial samples (B1, B2, B2R, B3, B3R, W3, W3R), while *Actinomycetospora* was more abundant in other white mycelial groups (W1, W1R, W2, W2R).

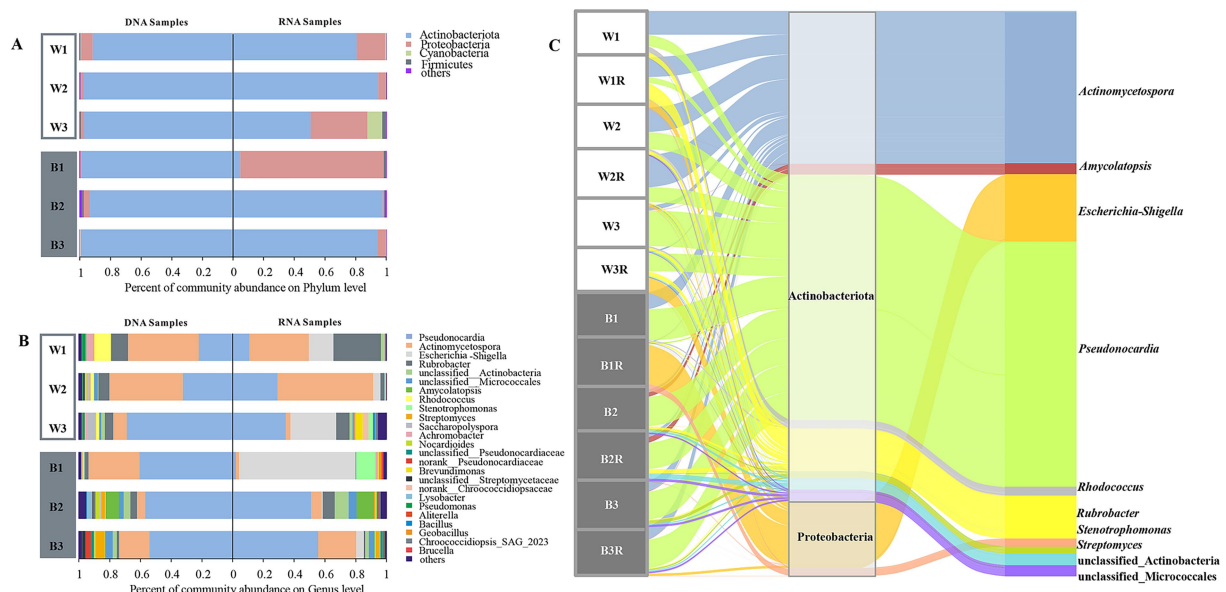


FIGURE 2
Community structures of bacteria detected at DNA and RNA levels. (A,B) Bacterial phyla and genera. (C) The Sankey diagram of bacterial microbial community structures based on the 16S rRNA gene at DNA and RNA levels.

(Figure 2C). Kruskal-Wallis H test revealed no significant taxonomic differences among the four groups. Among the top ten most abundant bacterial genera, *Pseudonocardia* and *Actinomycetospora* exhibited similar content in four groups. Notably, RNA samples consistently showed higher levels of *Escherichia* and *Stenotrophomonas* in bacterial taxa, indicating higher microbial viability (Supplementary Figure S2).

3.2 Influence of environmental factors and painting materials on bacterial communities in Maijishan Grottoes

The wall painting substrates in Caves No. 28 and No. 30 exhibited weak alkalinity (pH 8–9). Cave No. 28 showed lower moisture content and electrical conductivity than Cave No. 30 but had higher total organic carbon (TOC) and total nitrogen (N) levels (Table 1).

To identify key environmental drivers, variance inflation factor (VIF) analysis employed to eliminate inappropriate factors (e.g., moisture content, MC) with a threshold of 10. Redundancy Analysis (RDA) was then utilized to ascertain the relationships among microbial samples, basic physicochemical characteristics, and environmental factors (Figure 3A). While black and white mycelia showed no significant compositional differences, samples clustered strongly by cave origin regardless of mycelium color. In Cave No. 28, bacterial community composition was primarily shaped by temperature and pH, whereas in Cave No. 30, total nitrogen and electrical conductivity were the dominant factors. To rigorously control the false discovery rate (FDR) in high-dimensional correlation testing, we applied Benjamini-Hochberg correction to all pairwise associations. Heatmap visualization (Figure 3B) revealed that temperature (T) and relative humidity (RH) exerted the most contrasting effects on community structure. Six Actinobacterial genera, including unclassified_c_Actinobacteria, *Streptomyces*, and norank_f_Pseudonocardiaceae taxa displayed significant positive correlations with T and negative correlations with RH ($q < 0.0001$, FDR-corrected), while *Streptomonospora*, unclassified_f_Nocardioidaceae, *Amycolatopsis*, and unclassified_o_Micrococcales taxa showed positive correlations with T ($q < 0.05$, FDR-corrected). Furthermore, total nitrogen (N) exhibited significant negative correlations with unclassified_o_Micrococcales, *Kribbella* and *Actinophytocola* ($q < 0.05$, FDR-corrected), whereas total organic carbon (TOC) demonstrated pronounced positive association with *Actinophytocola* ($q < 0.05$, FDR-corrected).

3.3 Comparison of bacterial composition in samples from the Maijishan and Mogao Grottoes

Comparative analysis of alpha-diversity revealed statistical differences in bacterial diversity within black microbial samples

between the Maijishan Grottoes and the Mogao Grottoes ($p < 0.05$, Wilcoxon rank-sum test, Figures 4A–D). Both sites were dominated by Actinobacteria, Proteobacteria and Firmicutes, but their relative abundances exhibited site-specific patterns: Actinobacteria were more abundant in Maijishan samples, whereas Proteobacteria and Firmicutes showed higher abundance in Mogao samples ($p < 0.05$, Wilcoxon rank-sum test, Figure 4E). Upset analysis revealed that Maijishan groups (B-DNA, B-RNA, W-RNA, W-DNA) obtained 163, 175, 187, and 303 OTUs respectively, significantly fewer than the OTU numbers in Mogao samples (467 and 508 OTUs). Among these, 575 OTUs were detected only in the Mogao Grottoes samples, including 289 OTUs detected in both black spots and non-spots samples. Additionally, 209 OTUs were detected only in the Maijishan Grottoes samples, including 65 OTUs detected in both black mycelia DNA/RNA and white mycelia DNA/RNA samples (Figure 5). Only 13 OTUs were shared among the six types of samples from the two sites, none of which could be resolved at the species level, and all had low read counts.

Hierarchical clustering of the top 50 bacterial genera revealed site-specific community composition (Figure 6). Maijishan samples clustered by cave origin, one was for 5 samples collected from Cave No. 30, the other group included 6 samples collected from Cave No. 28 (Figure 6A). Genera were organized into three main clusters, Cluster 1, which included *Rubrobacter*, *Pseudonocardia*, *Actinomycetospora*, *Escherichia-Shigella*, and *Stenotrophomonas*, was predominantly present across nearly all samples. Cluster 2, comprising *Nocardioides*, *Lysobacter*, *Amycolatopsis*, etc., was primarily concentrated in samples derived from Cave No. 28. In contrast, Cluster 3, which included *Saccharopolyspora*, *Rhodococcus*, *Achromobacter*, and *Pseudomonas*, exhibited no discernible distribution pattern. In contrast, Mogao Cave No. 256 samples were divided into two main groups based on sampling locations, the samples located proximal to the tunnel passageway formed one group (BS1, BS3, and NBS1), whereas interior-positioned samples formed another (BS2, NBS2 and NBS3). Dominant genera formed two key clusters: Cluster 1 included *Escherichia-Shigella* and *Achromobacter*, Cluster 2 included *Rhodococcus*, *Ralstonia*, and *Bacillus*, distributed across various samples.

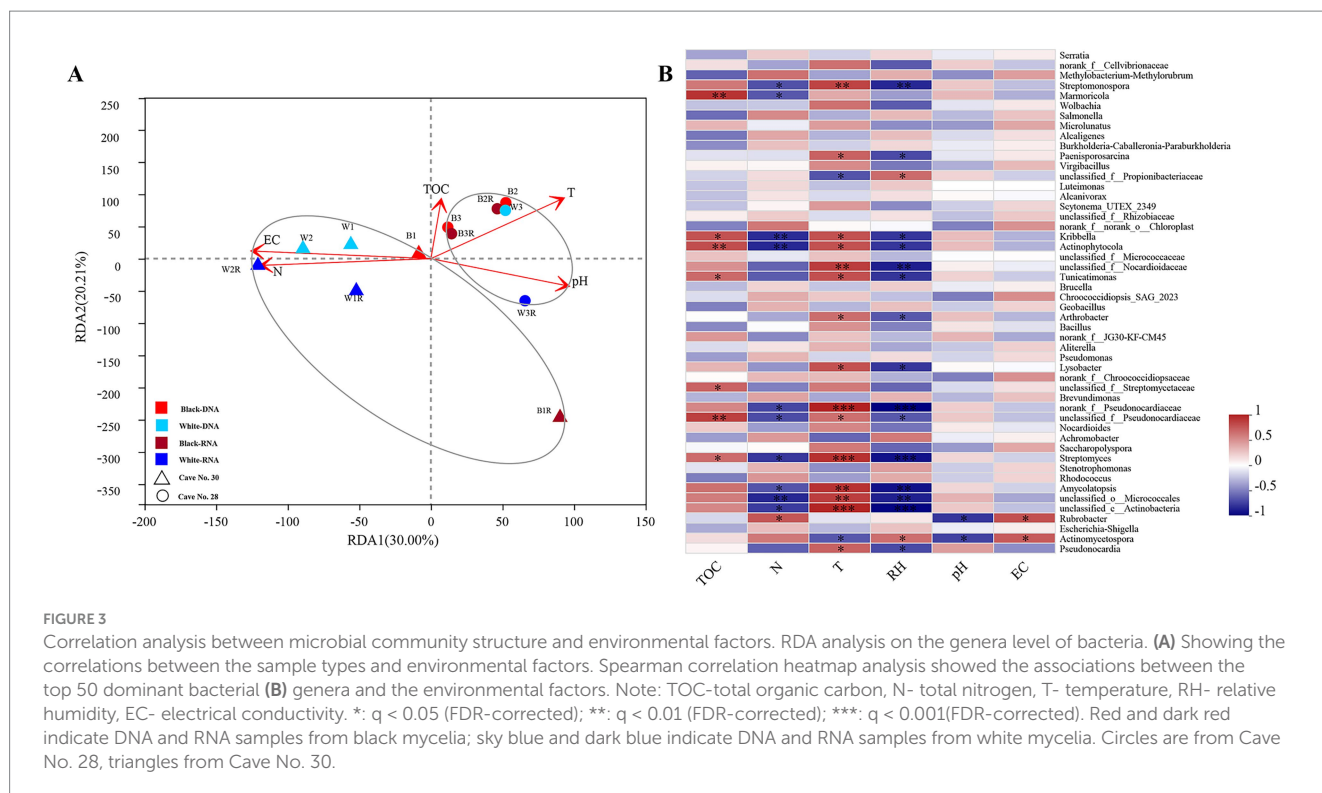
3.4 Comparison of diurnal variation of temperature in the Maijishan and Mogao Grottoes

In Maijishan Grottoes, mean diurnal range of air temperature (28-3, 30-3) were significantly higher than those of wall painting surfaces (28 N-S, 30 N-S) and plaster layers (28 N-IN, 30 N-IN). Within each cave system, the mean diurnal range of temperature of wall painting surfaces exceeded plaster layers, indicating that air temperature was more susceptible to external environmental

TABLE 1 Comparative analysis of physicochemical parameters of two caves at the Maijishan Grottoes.

Sampling cave	pH	MC (%)	EC	TOC	TN
Cave No. 28	8.380 ± 0.221	3.133	1.470 ± 0.116	1.556 ± 0.104	0.255 ± 0.017
Cave No. 30	8.166 ± 0.022	3.657	2.876 ± 0.122	1.156 ± 0.089	0.107 ± 0.032

The pH, moisture content (MC), electrical conductivity (EC), total organic carbon (TOC) and total nitrogen (TN) of Cave No. 28 and Cave No. 30 in the Maijishan Grottoes.



disturbances. Cave No. 30 experienced the most pronounced thermal variability, with the mean diurnal range of air temperature exceeding 5°C annually and peaking at 12°C during November–December, markedly higher than equivalent periods in Cave No. 28. Both caves exhibited minimal diurnal temperature fluctuations (mean range < 2°C) across wall painting surfaces and plaster substrates. However, Cave No. 30 displayed consistently elevated temperature ranges at corresponding monitoring points relative to Cave No. 28 (Supplementary Figure S3A). Contrastingly, the Cave No. 256 of Mogao Grottoes demonstrated smaller mean diurnal range of temperature across five monitoring points. Excluding cave air (ESA), all measurement positions maintained sub-0.5°C fluctuation thresholds, the west wall (WW) exhibited the highest mean diurnal range of temperature in plaster layers for nearly half the year (January, February, August, November, and December, Supplementary Figure S3B).

3.5 Comparison of diurnal variation of relative humidity in Maijishan and Mogao Grottoes

RH data showed that the mean diurnal RH range at all plaster layers monitoring points in both caves of Maijishan Grottoes (2% ~ 10%) exceeded those in Mogao Grottoes Cave No. 256 of (1% ~ 5%). In Maijishan, Cave No. 30 exhibited the highest RH fluctuation in cave air (30-3), peaking at 23.32% in December, while its plaster layer (30 N-IN) remained relatively stable humidity (Figure 7A). Both caves showed higher daily RH variations on wall painting surfaces than in plaster support layers, suggesting that surface RH is more sensitive to ambient changes. At Cave No. 256 of Mogao Grottoes, RH in cave air showed strong seasonal variation associated

with precipitation (Figure 7B), with diurnal fluctuations ranging from 2.11% (February) to 9.80% (July). From February to October, RH fluctuations in cave air exceeded those in plaster layers. The west wall (WW) plaster layer displayed exceptional RH stability, with fluctuations remaining below 2%, likely due to its location far from the cave entrance and limited air exchange (Figure 7B).

Daily maximum RH frequency analysis showed that in Maijishan Grottoes (Caves No. 28 and 30), RH most often fell within 70–80% at all monitoring points (Table 2). In Cave No. 28, RH > 70% occurred on 214 days at the plaster layer (28 N-IN), 207 days at the wall painting surface (28 N-S), and 185 days in cave air (28-3). In Cave No. 30, corresponding values were 209 days (30 N-IN), 202 days (30 N-S), and 146 days (30-3), respectively. Extreme RH events (>90%) were more common in Cave 28 and more frequent in plaster layers than on wall painting surfaces. In contrast, at Mogao Cave No. 256, daily maximum RH at most points fell within 20–30%, with RH > 70% occurring only on a few days, and never exceeding 80% (Table 2). Notably, the west wall (WW), showing visible water damage, had 73 days with RH > 60%, much higher than other plaster sites. The northeast roof (NER) near the entrance remained RH < 60% year-round.

4 Discussion

4.1 The core taxa and their biodeterioration potential in Maijishan Grottoes

In this study, combined DNA-based and RNA-based Illumina MiSeq sequencing was employed to comprehensively characterize both total and metabolically active bacterial communities associated with black and white mycelial biofilms in the Maijishan Grottoes. RNA-level

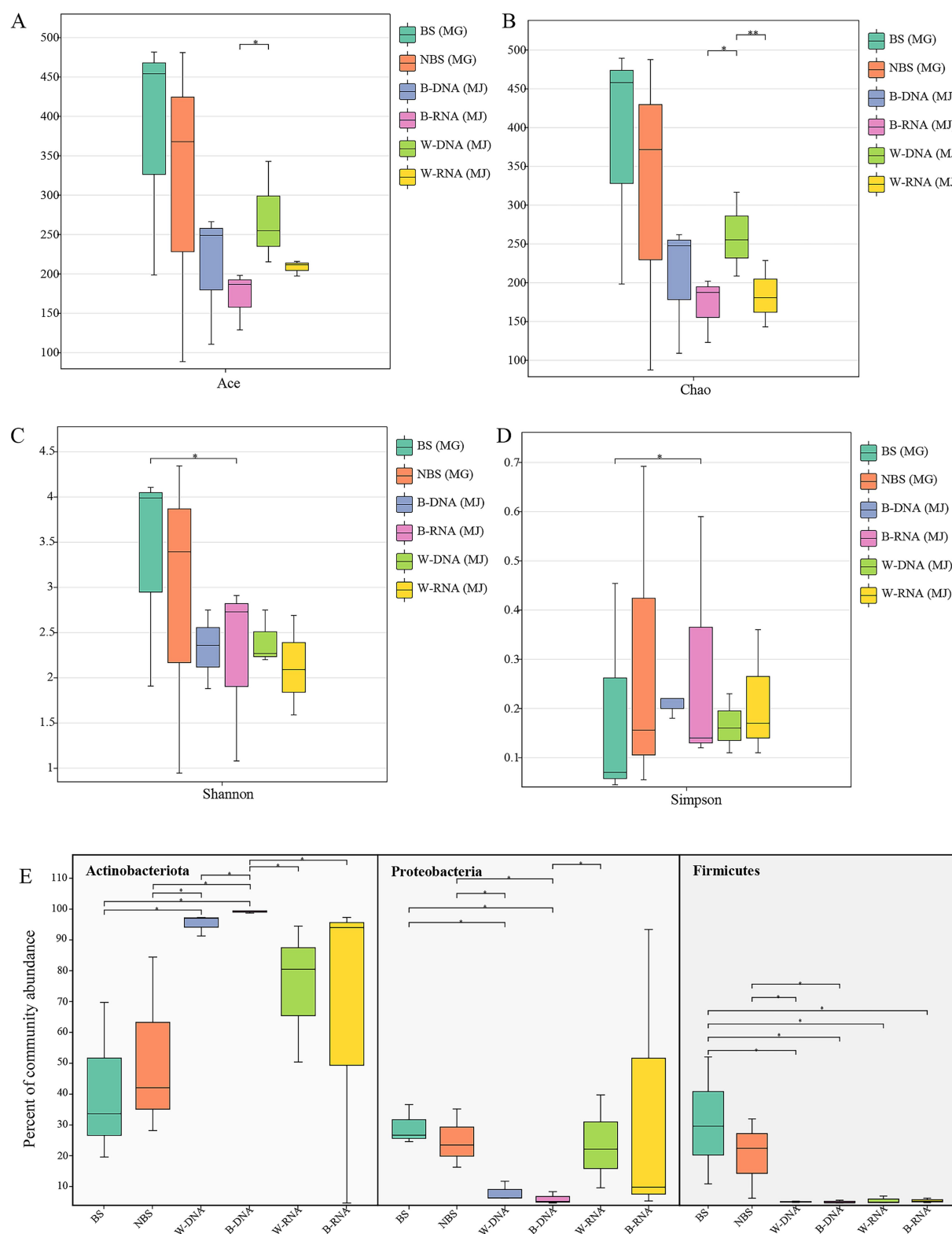
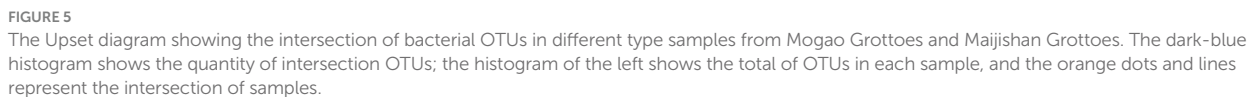


FIGURE 4

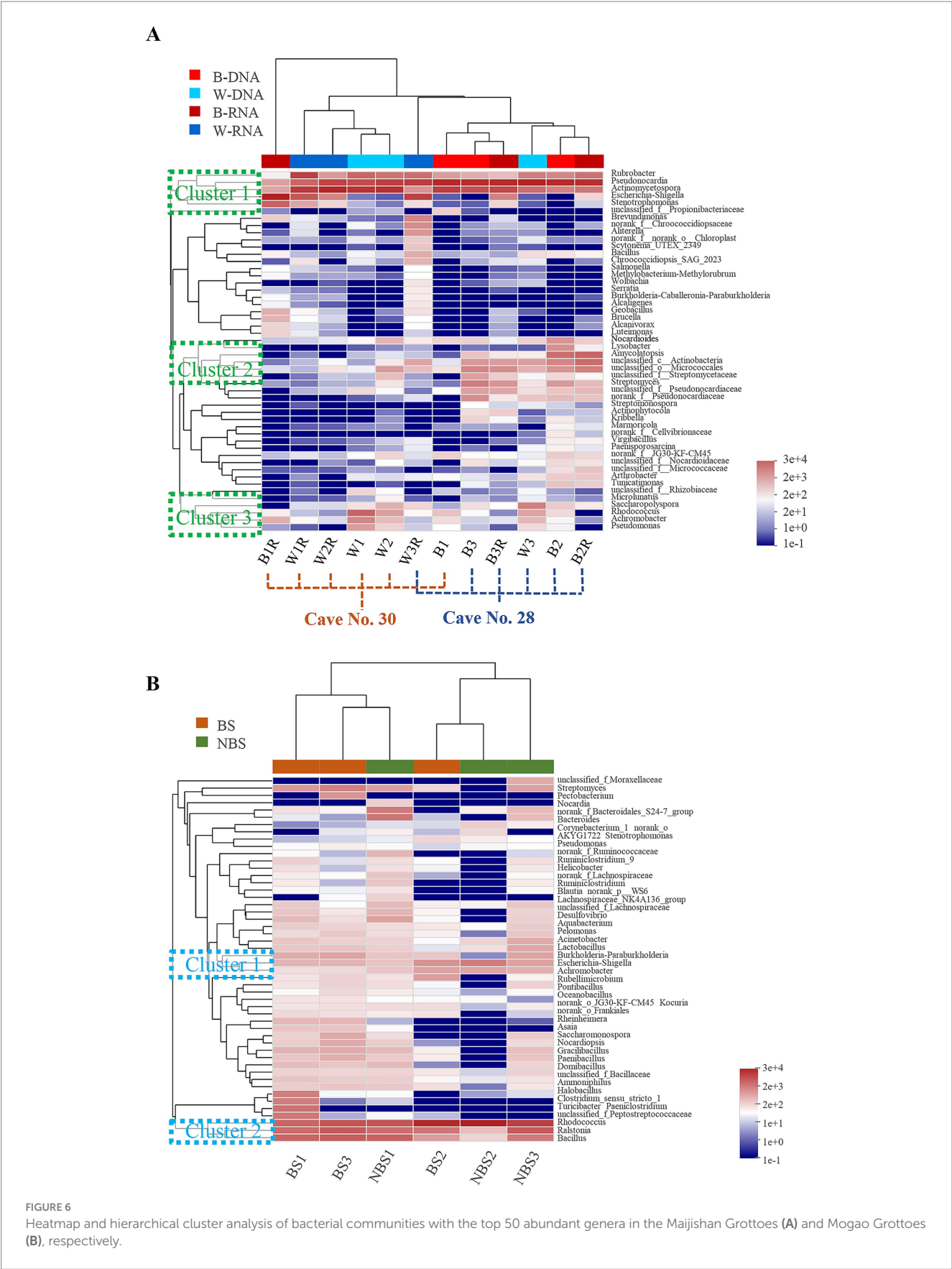
Comparison of bacterial alpha diversity and dominant bacterial phyla between the Majijishan Grottoes and Mogao Grottoes by Wilcoxon rank-sum test. (A,B) Species richness estimators (ACE and Chao indices), (C,D) alpha diversity indices (Shannon and Simpson), and dominant bacterial phyla (E).

sequencing enabled insight into microbial activity, complementing DNA-based community assessments and offering a more dynamic perspective on active biodegradation risks (Meng et al., 2020; Li et al.,

2021). Bacterial α -diversity and overall taxonomic profiles showed no significant differences between black and white mycelia within Majijishan Grottoes, suggesting that community composition alone



Microbial biofilms across diverse cultural heritage sites often shared microbial phyla (Hathaway et al., 2014; Duan et al., 2021). Regarding bacterial community structures, Actinobacteria dominated most samples in both DNA and RNA samples (except for sample B1R). Actinobacteria and Proteobacteria are typical phyla have been widely documented in heritage environments such as caves or subterranean sites (Jurado et al., 2005, 2008; Li et al., 2024). As prolific producers of secondary metabolite producers, Actinobacteria contribute to wall painting deterioration by releasing pigments, acids, and antimicrobial compounds, thereby promoting chromatic biofilm formation (Lepinay et al., 2018; Oliveira et al., 2017; Wang et al., 2012).



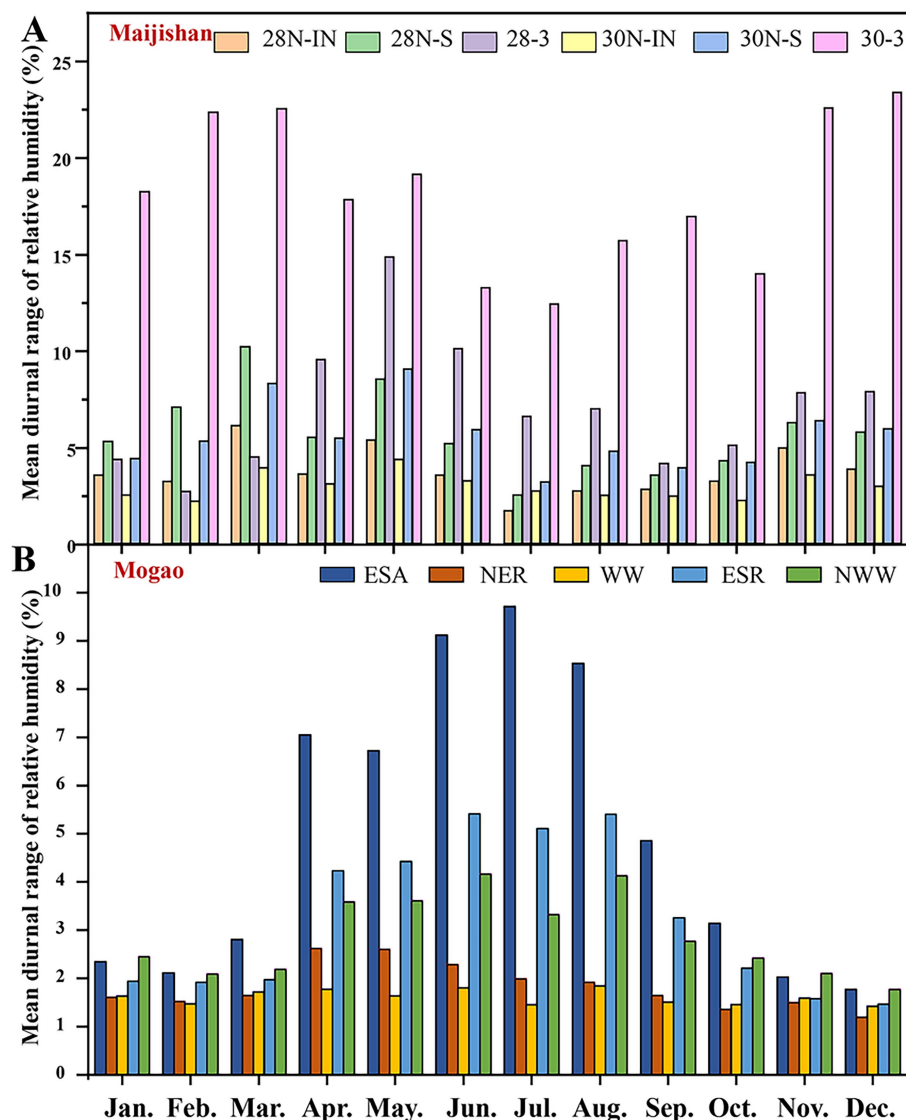


FIGURE 7

Mean diurnal range of relative humidity within Caves No. 28 and No. 30 of the Maijishan Grottoes (A) and Cave No. 256 of the Mogao Grottoes (B) for entire year. Six monitoring sites of the Maijishan Grottoes comprising wall painting plaster layer of Cave No. 28 and Cave No. 30 (28N-IN, 30N-IN), wall painting surface of Cave No. 28 and Cave No. 30 (28N-S, 30N-S), air of Cave No. 28 and Cave No. 30 (28-3, 30N-3); Five monitoring sites of the Mogao Grottoes comprising one for air at the eastern part of the southern slope (ESA) and four for murals at the north part of the northeast roof (NER), the west wall (WW), the eastern part of southern roof (ESR), and the northwest wall (NWW).

Proteobacteria serve as keystone chemoorganotrophic degraders, functioning as sensitive biological indicators of active biodeterioration (Ai et al., 2022; Khadka et al., 2017).

At the genus level, *Pseudonocardia* was particularly dominant in samples from Cave No. 30, corroborating its established role as an important taxon in wall paintings at the Maijishan Grottoes (Duan et al., 2017). This genus exhibited acidogenic metabolism capable of modulating local pH to favor oligotrophic proliferation, a critical adaptation enabling dominance in xeric wall painting substrates (Duan et al., 2017; Stomeo et al., 2008). *Pseudonocardia* thrived in subterranean heritage sites under harsh conditions, such as low light, scarce organic matter, high salinity, elevated humidity and temperature (Laiz et al., 1999), their rapid growth could lead to the formation of white spots on wall paintings (Duan et al., 2021). Another dominant genus, *Actinomycetospora*, was implicated in microbial outbreaks

across multiple caves of the Maijishan Grottoes (He et al., 2021). Importantly, RNA sequencing revealed that *Escherichia-Shigella* and *Stenotrophomonas* were substantially more abundant at the RNA-level than in DNA profiles, indicating high metabolic activity despite their relatively lower overall abundance. Thereinto, *Escherichia-Shigella* (Proteobacteria) often associated with intestinal microbiota (Stoddard et al., 1998; Wang et al., 2013), likely originate from animal vectors such as rodents, birds, or arthropods inhabiting the forested surroundings of the Maijishan Grottoes. These animals are likely to act as vectors for microbial dispersal, introducing exogenous microorganisms (e.g., intestinal microbiota via excreta) into the cave ecosystem. Their activity may exacerbate biodeterioration risks by altering microhabitat conditions or facilitating organic matter accumulation, thereby fostering microbial proliferation on wall paintings (Liu W. et al., 2022; Bastian et al., 2010). Additionally, *Rubrobacter* was identified in

TABLE 2 Daily maximum relative humidity distribution frequency of monitoring sites in investigative caves of two heritage sites.

Relative humidity (%)	Monitoring sites										
	Cave No. 256 of the Mogao Grottoes					Cave No. 28 and 30 of the Maijishan Grottoes					
	ESA	NER	WW	ESR	NWW	28 N-IN	28 N-S	28-3	30 N-IN	30 N-S	30-3
0–10	0	0	0	0	0	0	0	0	0	0	0
10–20	75	1	0	87	65	0	0	0	0	0	0
20–30	119	189	130	97	105	0	0	1	0	0	2
30–40	64	90	70	69	68	13	23	26	8	23	25
40–50	45	55	62	45	42	33	28	28	45	32	34
50–60	32	30	30	34	45	56	48	71	72	61	66
60–70	25	0	65	32	33	49	59	54	31	47	92
70–80	5	0	8	1	7	84	78	81	83	84	102
80–90	0	0	0	0	0	73	74	65	65	69	43
90–100	0	0	0	0	0	57	55	39	61	49	1

Six monitoring sites of Maijishan Grottoes comprising wall painting plaster layer of Cave No. 28 and Cave No. 30 (28 N-IN, 30 N-IN), wall painting surface of Cave No. 28 and Cave No. 30 (28 N-S, 30 N-S), air of Cave No. 28 and Cave No. 30 (28-3, 30 N-3); Five monitoring sites of the Mogao Grottoes comprising one for air at the eastern part of the southern slope (ESA) and four for wall painting at the north part of the eastern slope (NER), the west wall (WW), the eastern part of southern slope (ESR), and the northwest wall (NWW).

samples, aligning with findings from the Angkor Thom Bayon Temple (Zhang et al., 2018) and other caves of the Maijishan Grottoes (Duan et al., 2017; He et al., 2021), which was recognized as a core genus associated with rosy discoloration on wall paintings, sandstone, and masonry (Schabereiter-Gurtner et al., 2001; Imperi et al., 2007; Nugari et al., 2009). Together, the RNA results demonstrate that a subset of the bacterial community is actively engaged in processes with direct biodeterioration potential, highlighting the importance of integrating RNA data into conservation-oriented microbial risk assessments.

Under favorable growth conditions, microorganisms can colonize on wall paintings by utilizing carbon sources, nitrogen sources, and mineral nutrients derived from original painting materials and restoration plasters (Meng et al., 2020). In this study, several differences were observed in total organic carbon, total nitrogen, and electrical conductivity between Cave No. 28 and Cave No. 30. These differences might alter microenvironmental conditions (Li and Gu, 2022) and provide distinct nutrient substrates for microbial proliferation (Imperi et al., 2007; Sterflinger and Pinzari, 2012). Combining with cave-specific temperature and humidity, microenvironmental conditions might lead the differences in bacterial community composition between the two caves.

4.2 Difference of bacterial community and their adaptations

Across both sites, Actinobacteria, Proteobacteria, and Firmicutes were the core bacterial phyla associated with biodeterioration, consistent with their recognized roles as pioneer colonizers across diverse lithic heritage substrates (Li et al., 2017; Liu et al., 2019; Xing et al., 2023). Actinobacteria was the dominant bacterial phylum at two sites of this study. However, the relative abundance of Actinobacteria in Mogao samples was markedly lower in the Maijishan Grottoes samples. The Maijishan Grottoes exhibited sustained high humidity levels year-round, with extreme humidity events exceeding 90%, which are mainly attributed to their unique geographical setting characterized by dense forest coverage, frequent precipitation, and limited cave

ventilation (Yao et al., 2022). The observed variation in Actinobacteria abundance may originate from their hydrophilic properties, the persistently humid microenvironment promoted actinobacterial spore germination and growth (Zvyagintsev et al., 2007). Conversely, samples from the Mogao Grottoes exhibited a higher relative abundance of Firmicutes. Certain members of the phylum Firmicutes, particularly within the classes Bacilli (e.g., *Bacillus*) and Clostridia (e.g., *Clostridium*), are capable of forming endospores, which serve as a key mechanism for surviving extreme environments such as drought. These endospores possess a multilayered protective structure that resists desiccation, high temperatures, and UV radiation, significantly enhancing their survival rates under arid conditions (Setlow, 2007; Mckenney et al., 2013). The arid climate of the Dunhuang region selected for the desiccation-resistant metabolic strategies of Firmicutes, enabling their ecological dominance in the Mogao Grottoes' ecosystems (Ma et al., 2015). Proteobacteria emerged as the dominant taxa driving the biodeterioration processes on cultural heritage substrates, particularly Alpha-proteobacteria, played a pivotal role in heritage biodeterioration through their metabolic versatility and adaptive strategies. Part of Proteobacteria exhibited oligotrophic capabilities, enabling colonization of nutrient-poor stone surfaces via the utilization of diverse organic compounds and UV-resistant pigment production (Pinhassi and Berman, 2003; Yu et al., 2024). At the genus level, *Rhodococcus* was dominant in samples of the Mogao Grottoes, they were also frequently documented in global heritage biodeterioration cases. Conversely, *Pseudonocardia* dominated the samples of Maijishan Grottoes, a genus commonly associated with wall paintings in caves and tombs (Stomeo et al., 2008; Portillo et al., 2009; Liu W. et al., 2022). Several studies have confirmed that Actinobacteria (e.g., *Pseudonocardia*) exhibit strong adaptability to oligotrophic conditions with limited organic nutrients. As pioneering colonizers in natural caves, they initiate early growth and establish material foundations for other microorganisms (Ciferri, 1999). Moreover, *Pseudonocardia* actively modifies environmental pH through metabolic processes, creating localized slightly alkaline microenvironments that promote rapid proliferation, then contributed to biodeterioration (Portillo and Gonzalez, 2011).

Only 13 OTUs belonged to few overlapping bacterial genera were identified between the two sites, revealing the possible role of site-specific environmental conditions (e.g., humidity, temperature, and substrate composition) in shaping bacterial community composition on wall paintings, the microenvironment exerting selective pressure on bacterial communities (Allen et al., 2020). Microbial damages to wall paintings were typically shaped by the metabolic activities and successional dynamics of diverse microbial communities, comprising bacteria, fungi, and extracellular polymeric substances (EPS). Studies indicated that bacteria play critical roles in microbial biofilms as ecological modulators, dynamically reshaping the composition of other biofilm-associated microorganisms (e.g., fungi, algae) in response to microenvironmental fluctuations (Liu et al., 2020). In our study, bacteria likely serve analogous functions: though not dominant in white/black mycelia, they might respond to microenvironmental variations and influence these mycelia on wall paintings.

4.3 Interplay between environments and bacterial communities

The Maijishan Grottoes, situated in a forested mountainous region with persistent high humidity, the temperature and RH fluctuations might be predominantly influenced by seasonal changes and heat exchange of cliff rock. The Cave No. 28 and Cave No. 30 maintained RH > 70% for over half the year, creating ideal conditions for microbial outbreaks and biodeterioration (He et al., 2022). Rainfall infiltration through cliff fractures and monsoon-driven precipitation (July–September) elevate RH to near 90% for >50 days annually, triggering efflorescence, cracking, pigment layer exfoliation and plaster layers destabilization via thermal expansion-contraction cycles (Bertolin et al., 2020; Li and Gu, 2022; Wang et al., 2025; Rivera et al., 2006). Despite high microbial risk in the Maijishan Grottoes, the bacterial and fungal concentrations (mean: 754 CFU/m³ and 645 CFU/m³, respectively) maintained at a lower level (Duan et al., 2018). Air microbial concentrations between 500 and 1,000 CFU/m³ indicated moderate visitor impact, belonging to balanced level, necessitating moderate crowd control (Porca et al., 2011).

The Mogao Grottoes, located in a desert region with distinct seasons, experienced extreme aridity and significant diurnal temperature fluctuations. Cave No. 256 maintained a relatively lower humidity, with RH consistently below 70%. This stable microenvironment can inhibit microbial colonization, favoring heritage preservation (Loli and Bertolin, 2018). In addition, this cave exhibited minimal daily temperature variation (diurnal range <1°C), which typically supports microbial proliferation, but the arid conditions (RH < 70% for over two-thirds of the year) inhibited numerous microbial growth (Spagnuolo et al., 2019; Sterflinger, 2010). Low precipitation, intense solar radiation, and high evaporation accelerated salt efflorescence on wall paintings via the migration of capillary water, leading efflorescence and then induced painting layers detachment. Microbial communities undergo niche selection on the wall paintings with efflorescence progression, the drought-adapted taxa potentially accelerating growth and deterioration (Ma et al., 2020).

In the Mogao Grottoes samples, more than half of the OTUs were exclusively detected in the two types (BS, NBS) of samples from this location, while at the Maijishan Grottoes, over one-third of the OTUs were shared across all four sample types (W-DNA, B-DNA, W-RNA,

B-RNA). The bacterial communities demonstrated significant divergence in both diversity and structural composition at two sites, with community characteristics showing strong spatial dependency on sampling locations. The composition of microbial communities on cultural heritage surfaces are also shaped by dynamic interactions between substrate physicochemical properties (e.g., organic carbon content, nitrogen content, electric conductivity) and site-specific environmental stressors within the microenvironment such as pH, temperature and humidity (Chen and Gu, 2022; Dias et al., 2023; Ding et al., 2021; Duan et al., 2018, 2021; Zhang et al., 2018). Among these variables, environmental conditions emerged as an important determinant, critically modulating both microbial taxonomic dominance and their metabolic functionality across heritage ecosystems (Biagioli et al., 2024). Concurrently, increasing global tourism compounds risks through organic contamination and microclimatic destabilization (Ai et al., 2022; Li et al., 2022). In these semi-enclosed ecosystems, niche filtering overrides dispersal limitations (Saarela et al., 2004; Vellend, 2010). Climatic parameters critically modulate the ecological dominance of microbial taxa in heritage sites (Biagioli et al., 2024). Bacterial communities exhibited remarkable adaptability and resilience under different environmental stress, with their composition dynamically adjusting to maintain stability in response to fluctuant microenvironmental conditions (Chen et al., 2023).

5 Conclusion

This study revealed significant differences in bacterial communities and drivers of microbes on wall paintings in the Maijishan and Mogao Grottoes. Persistent high humidity and cliff seepage in the Maijishan Grottoes might promote Actinobacteria dominance (e.g., *Pseudonocardia*), their metabolic byproducts (organic acids, pigments) might exacerbate painting layer degradation. In contrast, the arid conditions of the Mogao Grottoes selected for desiccation-tolerant Firmicutes (e.g., *Bacillus*) and halophilic Proteobacteria (e.g., *Halomonas*), with salt efflorescence posing a primary threat to integrity of wall paintings. Therefore, site-specific microbial risks call for tailored conservation interventions. For the Maijishan Grottoes, strategies should prioritize humidity regulation (e.g., cyclic dehumidification) and control of Actinobacterial spore. In addition, RNA-level analysis identified metabolically active enteric bacteria (e.g., *Escherichia*) in the Maijishan Grottoes, potentially introduced by wildlife, thus more attention is necessary for the future preventative conservation. The lack of shared dominant genera between two heritage sites emphasizes the importance of localized microbial surveillance and environment-specific preservation approaches. These findings may advance our understanding of microbial ecology in cultural heritage environments and potentially support the development of evidence-based, sustainable conservation strategies for ancient wall paintings.

Data availability statement

The datasets presented in this study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/>, accession number PRJNA950462.

Author contributions

WM: Funding acquisition, Data curation, Visualization, Writing – review & editing, Methodology, Writing – original draft, Conceptualization. QC: Validation, Writing – review & editing, Investigation. FW: Writing – review & editing, Methodology, Funding acquisition, Supervision, Resources, Conceptualization. DH: Project administration, Funding acquisition, Supervision, Writing – review & editing, Formal analysis. YD: Software, Funding acquisition, Writing – review & editing. YY: Investigation, Writing – review & editing, Formal analysis. J-DG: Writing – review & editing. XY: Writing – review & editing, Formal analysis. HF: Conceptualization, Funding acquisition, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This study was supported by the National Natural Science Foundation of China (No. 32400082; 32460278); National Youth Top Talent Program of China; The Science and Technology Plan of Gansu Province, China (No. 23JRRA644); The Open Project of Gansu Provincial Research Center for Conservation of Dunhuang Cultural Heritage (No. GDW2021ZD08); and The Project of Gansu Provincial Bureau of Cultural Heritage (No. GSWW202229).

Acknowledgments

We would like to give our sincere gratitude to Junjian Hu, Ruibo Pei and Peng Xu from the Institute of Maijishan Grottoes Art for their help during the fieldwork.

Reference

- Abdel-Halim, M. E. F., Sakr, A. A., Ali, M. F., Ghaly, M. F., and Sohlenkamp, C. (2013). Characterization of Streptomyces isolates causing colour changes of mural paintings in ancient Egyptian tombs. *Microbiol. Res.* 168, 428–437. doi: 10.1016/j.micres.2013.02.004
- Ai, J., Guo, J., Li, Y., Zhong, X., Lv, Y., Li, J., et al. (2022). The diversity of microbes and prediction of their functions in karst caves under the influence of human tourism activities—a case study of Zhijin cave in Southwest China. *Environ. Sci. Pollut. Res.* 29, 25858–25868. doi: 10.1007/s11356-021-17783-x
- Allen, R., Hoffmann, L. J., Larcombe, M. J., Louisson, Z., and Summerfield, T. C. (2020). Homogeneous environmental selection dominates microbial community assembly in the oligotrophic South Pacific gyre. *Mol. Ecol.* 29, 4680–4691. doi: 10.1111/mec.15651
- Barnard, R. L., Osborne, C. A., and Firestone, M. K. (2013). Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *ISME J.* 7, 2229–2241. doi: 10.1038/ismej.2013.104
- Bastian, F., Jurado, V., Nováková, A., Alabouvette, C., and Saiz-Jimenez, C. (2010). The microbiology of Lascaux cave. *Microbiology* 156, 644–652. doi: 10.1099/mic.0.036160-0
- Bertolin, C., De Ferri, L., Grottesi, G., and Strojceki, M. (2020). Study on the conservation state of wooden historical structures by means of acoustic attenuation and vacuum microbalance. *Wood Sci. Technol.* 54, 203–226.
- Biagioli, F., Coleine, C., Delgado-Baquerizo, M., Feng, Y., Saiz-Jimenez, C., and Selbmann, L. (2024). Outdoor climate drives diversity patterns of dominant microbial taxa in caves worldwide. *Sci. Total Environ.* 906:167674. doi: 10.1016/j.scitotenv.2023.167674
- Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170
- Chen, J., and Gu, J. (2022). The environmental factors used in correlation analysis with microbial community of environmental and cultural heritage samples. *Int. Biodeterior. Biodegrad.* 173:105460. doi: 10.1016/j.ibiod.2022.105460
- Chen, J., Zhao, Q., Li, F., Zhao, X., Wang, Y., Zhang, L., et al. (2023). Nutrient availability and acid erosion determine the early colonization of limestone by lithobiontic microorganisms. *Front. Microbiol.* 14:1194871. doi: 10.3389/fmicb.2023.1194871
- Ciferri, O. (1999). Microbial degradation of paintings. *Appl. Environ. Microbiol.* 65, 879–885. doi: 10.1128/AEM.65.3.879-885.1999
- Cojoc, L. R., Enache, M. I., Neagu, S. E., Lungulescu, M., Setnescu, R., Ruginescu, R., et al. (2019). Carotenoids produced by halophilic bacterial strains on mural paintings and laboratory conditions. *FEMS Microbiol. Lett.* 366:fnz243. doi: 10.1093/femsle/fnz243
- Cuezva, S., Fernandez-Cortes, A., Porca, E., Pašić, L., Jurado, V., Hernandez-Marine, M., et al. (2012). The biogeochemical role of Actinobacteria in Altamira cave, Spain. *FEMS Microbiol. Ecol.* 81, 281–290. doi: 10.1111/j.1574-6941.2012.01391.x
- Dias, L., Pires, V., Sitzia, F., Lisci, C., Candeias, A., Caldeira, A. T., et al. (2023). Evaluating the biosusceptibility of natural stone as an supporting tool to prevent cultural heritage biodeterioration. *Eur. Phys. J. Plus* 138:570. doi: 10.1140/epjp/s13360-023-04185-w
- Ding, X., Lan, W., Li, Y., Yan, A., Katayama, Y., Koba, K., et al. (2021). An internal recycling mechanism between ammonia/ammonium and nitrate driven by ammonia-oxidizing archaea and bacteria (AOA, AOB, and Comammox) and DNRA on Angkor sandstone monuments. *Int. Biodeterior. Biodegrad.* 165:105328. doi: 10.1016/j.ibiod.2021.105328
- Ding, X., Lan, W., Yan, A., Li, Y., Katayama, Y., and Gu, J.-D. (2022). Microbiome characteristics and the key biochemical reactions identified on stone world cultural heritage under different climate conditions. *J. Environ. Manag.* 302:114041. doi: 10.1016/j.jenvman.2021.114041
- Dominguez-Moñino, I., Diaz-Herrera, M., Jurado, V., Laiz, L., Miller, A. Z., Santos, J. L., et al. (2017). Nature and origin of the violet stains on the walls of a Roman tomb. *Sci. Total Environ.* 598, 889–899. doi: 10.1016/j.scitotenv.2017.04.017

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1657118/full#supplementary-material>

- Dornieden, T., Gorbushina, A. A., and Krumbein, W. E. (2000). Biodecay of cultural heritage as a space/time-related ecological situation—an evaluation of a series of studies. *Int. Biodeterior. Biodegrad.* 46, 261–270. doi: 10.1016/S0964-8305(00)00107-4
- Duan, Y., Wu, F., He, D., Gu, J.-D., Feng, H., Chen, T., et al. (2021). Bacterial and fungal communities in the sandstone biofilms of two famous Buddhist grottoes in China. *Int. Biodeterior. Biodegrad.* 163:105267. doi: 10.1016/j.ibiod.2021.105267
- Duan, Y., Wu, F., Wang, W., Gu, J.-D., Li, Y., Feng, H., et al. (2018). Differences of microbial community on the wall paintings preserved *in situ* and *ex situ* of the Tiantishan grottoes, China. *Int. Biodeterior. Biodegrad.* 132, 102–113. doi: 10.1016/j.ibiod.2018.02.013
- Duan, Y., Wu, F., Wang, W., He, D., Gu, J.-D., Feng, H., et al. (2017). The microbial community characteristics of ancient painted sculptures in Majishan grottoes, China. *PLoS One* 12:e0179718. doi: 10.1371/journal.pone.0179718
- Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604
- Elhagrassy, A. F. (2018). Isolation and characterization of actinomycetes from mural paintings of Snu- Sert-anh tomb, their antimicrobial activity, and their biodeterioration. *Microbiol. Res.* 216, 47–55. doi: 10.1016/j.micres.2018.08.005
- Garg, K. L., Jain, K. K., and Mishra, A. K. (1995). Role of fungi in the deterioration of wall paintings. *Sci. Total Environ.* 167, 255–271. doi: 10.1016/0048-9697(95)04587-Q
- Hathaway, J. J. M., Garcia, M. G., Balasch, M. M., Spilde, M. N., Stone, F. D., Dapkevicius, M. D. L. N. E., et al. (2014). Comparison of bacterial diversity in Azorean and Hawaiian lava cave microbial mats. *Geomicrobiol. J.* 31, 205–220. doi: 10.1080/01490451.2013.777491
- He, D., Wu, F., Ma, W., Gu, J.-D., Xu, R., Hu, J., et al. (2022). Assessment of cleaning techniques and its effectiveness for controlling biodeterioration fungi on wall paintings of Majishan grottoes. *Int. Biodeterior. Biodegrad.* 171:105406. doi: 10.1016/j.ibiod.2022.105406
- He, D., Wu, F., Ma, W., Zhang, Y., Gu, J.-D., Duan, Y., et al. (2021). Insights into the bacterial and fungal communities and microbiome that causes a microbe outbreak on ancient wall paintings in the Majishan grottoes. *Int. Biodeterior. Biodegrad.* 163:105250. doi: 10.1016/j.ibiod.2021.105250
- Hu, H., and Hewitt, R. J. (2024). Understanding climate risks to world cultural heritage: a systematic analysis and assessment framework for the case of Spain. *Herit. Sci.* 12:194. doi: 10.1186/s40494-024-01299-x
- Imperi, F., Caneva, G., Cancellieri, L., Ricci, M. A., Sodo, A., and Visca, P. (2007). The bacterial aetiology of rosy discoloration of ancient wall paintings. *Environ. Microbiol.* 9, 2894–2902. doi: 10.1111/j.1462-2920.2007.01393.x
- Jurado, V., Boiron, P., Kroppenstedt, R. M., Laurent, F., Couble, A., Laiz, L., et al. (2008). *Nocardia altamirensis* sp. nov., isolated from Altamira cave, Cantabria, Spain. *Int. J. Syst. Evol. Microbiol.* 58, 2210–2214. doi: 10.1099/ijs.0.65482-0
- Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L., and Saiz-Jimenez, C. (2005). *Agromyces subbeticus* sp. nov., isolated from a cave in southern Spain. *Int. J. Syst. Evol. Microbiol.* 55, 1897–1901. doi: 10.1099/ijs.0.63637-0
- Khadka, B., Adeolu, M., Blankenship, R. E., and Gupta, R. S. (2017). Novel insights into the origin and diversification of photosynthesis based on analyses of conserved indels in the core reaction center proteins. *Photosynth. Res.* 131, 159–171. doi: 10.1007/s1120-016-0307-1
- Lan, W., Li, H., Wang, W.-D., Katayama, Y., and Gu, J.-D. (2010). Microbial community analysis of fresh and old microbial biofilms on Bayon Temple sandstone of Angkor Thom, Cambodia. *Microb. Ecol.* 60, 105–115. doi: 10.1007/s00248-010-9707-5
- Laiz, L., Groth, I., Gonzalez, I., and Saiz-Jimenez, C. (1999). Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). *J. Microbiol. Meth.* 36:129.
- Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676
- Lavoie, K. H., Winter, A. S., Read, K. J. H., Hughes, E. M., Spilde, M. N., and Northup, D. E. (2017). Comparison of bacterial communities from lava cave microbial mats to overlying surface soils from lava beds National Monument, USA. *PLoS One* 12:e0169339. doi: 10.1371/journal.pone.0169339
- Leissner, J., Kilian, R., Kotova, L., Jacob, D., Mikolajewicz, U., Broström, T., et al. (2015). Climate for culture: assessing the impact of climate change on the future indoor climate in historic buildings using simulations. *Herit. Sci.* 3:38. doi: 10.1186/s40494-015-0067-9
- Lepinay, C., Mihajlovski, A., Touron, S., Seyer, D., Boust, F., and Di Martino, P. (2018). Bacterial diversity associated with saline efflorescences damaging the walls of a French decorated prehistoric cave registered as a world cultural heritage site. *Int. Biodeterior. Biodegrad.* 130, 55–64. doi: 10.1016/j.ibiod.2018.03.016
- Li, J., Deng, M., Gao, L., Yen, S., Katayama, Y., and Gu, J.-D. (2021). The active microbes and biochemical processes contributing to deterioration of Angkor sandstone monuments under the tropical climate in Cambodia – a review. *J. Cult. Herit.* 47, 218–226. doi: 10.1016/j.culher.2020.10.010
- Li, J., He, Y., He, C., Xiao, L., Wang, N., Jiang, L., et al. (2024). Diversity and composition of microbial communities in Jinsha earthen site under different degree of deterioration. *Environ. Res.* 242:117675. doi: 10.1016/j.envres.2023.117675
- Li, Q., Zhang, B., Wang, L., and Ge, Q. (2017). Distribution and diversity of bacteria and fungi colonizing ancient Buddhist statues analyzed by high-throughput sequencing. *Int. Biodeterior. Biodegrad.* 117, 245–254. doi: 10.1016/j.ibiod.2017.01.018
- Liu, S.-J., Zhu, H.-Z., Zhang, Z.-F., Zhou, N., Jiang, C.-Y., Wang, B.-J., et al. (2019). Diversity, distribution and co-occurrence patterns of bacterial communities in a karst cave system. *Front. Microbiol.* 10:1726. doi: 10.3389/fmicb.2019.01726
- Liu, W., Zhou, X., Jin, T., Li, Y., Wu, B., Yu, D., et al. (2022). Multikingdom interactions govern the microbiome in subterranean cultural heritage sites. *Proc. Natl. Acad. Sci. USA* 119:e2121141119. doi: 10.1073/pnas.2121141119
- Liu, X., Koestler, R. J., Warscheid, T., Katayama, Y., and Gu, J.-D. (2020). Microbial deterioration and sustainable conservation of stone monuments and buildings. *Nat. Sustain.* 3, 991–1004. doi: 10.1038/s41893-020-00602-5
- Liu, X., Meng, H., Wang, Y., Katayama, Y., and Gu, J.-D. (2018). Water is a critical factor in evaluating and assessing microbial colonization and destruction of Angkor sandstone monuments. *Int. Biodeterior. Biodegrad.* 133, 9–16. doi: 10.1016/j.ibiod.2018.05.011
- Liu, X., Qian, Y., Wang, Y., Wu, F., Wang, W., and Gu, J.-D. (2022). Innovative approaches for the processes involved in microbial biodeterioration of cultural heritage materials. *Curr. Opin. Biotechnol.* 75:102716. doi: 10.1016/j.copbio.2022.102716
- Li, X., Zhou, X., Wu, C., Petropoulos, E., Yu, Y., and Feng, Y. (2022). Temperature and moisture gradients drive the shifts of the bacterial microbiomes in 1000-year-old mausoleums. *Atmos.* 14:14. doi: 10.3390/atmos14010014
- Li, Y.-H., and Gu, J.-D. (2022). A more accurate definition of water characteristics in stone materials for an improved understanding and effective protection of cultural heritage from biodeterioration. *Int. Biodeterior. Biodegrad.* 166:105338. doi: 10.1016/j.ibiod.2021.105338
- Loli, A., and Bertolin, C. (2018). Indoor multi-risk scenarios of climate change effects on building materials in Scandinavian countries. *Geosciences* 8:347. doi: 10.3390/geosciences8090347
- Ma, C., Fang, Z., Li, X., and Liu, X. (2023). Identification of bacterial communities involved in bioweathering crusts on limestone sculptures of the Longmen grottoes. *Coatings* 13:1506. doi: 10.3390/coatings13091506
- Ma, W., Wu, F., He, D., Gu, J.-D., Chen, Y., Yue, Y., et al. (2025). Fungal community structure and viability in biofilms on wall paintings of the Majishan grottoes. *NPJ Herit. Sci.* 13:386. doi: 10.1038/s40494-025-01962-x
- Ma, W., Wu, F., He, D., Li, J., Zhang, Q., Yang, X., et al. (2023). The biodeterioration outbreak in Dunhuang Mogao grottoes analyzed for the microbial communities and the occurrence time by C-14 dating. *Int. Biodeterior. Biodegrad.* 178:105533. doi: 10.1016/j.ibiod.2022.105533
- Ma, W., Wu, F., Tian, T., He, D., Zhang, Q., Gu, J.-D., et al. (2020). Fungal diversity and its contribution to the biodeterioration of mural paintings in two 1700-year-old tombs of China. *Int. Biodeterior. Biodegrad.* 152:104972. doi: 10.1016/j.ibiod.2020.104972
- Ma, Y., Zhang, H., Du, Y., Tian, T., Xiang, T., Liu, X., et al. (2015). The community distribution of bacteria and fungi on ancient wall paintings of the Mogao grottoes. *Sci. Rep.* 5:7752. doi: 10.1038/srep07752
- Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507
- Martin-Sanchez, P. M., Nováková, A., Bastian, F., Alabouvette, C., and Saiz-Jimenez, C. (2012). Two new species of the genus *Ochroconis*, *O. lascauxensis* and *O. anomala* isolated from black stains in Lascaux cave, France. *Fungal Biol.* 116, 574–589. doi: 10.1016/j.funbio.2012.02.006
- Mckenney, P. T., Driks, A., and Eichenberger, P. (2013). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.* 11, 33–44. doi: 10.1038/nrmicro2921
- Meng, H., Katayama, Y., and Gu, J.-D. (2017). More wide occurrence and dominance of ammonia-oxidizing archaea than bacteria at three Angkor sandstone temples of Bayon, Phnom Krom and wat Athvea in Cambodia. *Int. Biodeterior. Biodegrad.* 117, 78–88. doi: 10.1016/j.ibiod.2016.11.012
- Meng, H., Zhang, X., Katayama, Y., Ge, Q., and Gu, J.-D. (2020). Microbial diversity and composition of the Preah Vihear temple in Cambodia by high-throughput sequencing based on genomic DNA and RNA. *Int. Biodeterior. Biodegrad.* 149:104936. doi: 10.1016/j.ibiod.2020.104936
- Mihajlovski, A., Gabarre, A., Seyer, D., Boust, F., and Di Martino, P. (2017). Bacterial diversity on rock surface of the ruined part of a French historic monument: the Chaalis abbey. *Int. Biodeterior. Biodegrad.* 120, 161–169. doi: 10.1016/j.ibiod.2017.02.019
- Nugari, M. P., Pietrini, A. M., Caneva, G., Imperi, F., and Visca, P. (2009). Biodeterioration of mural paintings in a rocky habitat: the crypt of the original sin (Matera, Italy). *Int. Biodeterior. Biodegrad.* 63, 705–711. doi: 10.1016/j.ibiod.2009.03.013
- Oliveira, C., Gunderman, L., Coles, C., Lochmann, J., Parks, M., Ballard, E., et al. (2017). 16S rRNA gene-based metagenomic analysis of Ozark cave bacteria. *Diversity* 9:31. doi: 10.3390/d9030031
- Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. USA* 110, 6548–6553. doi: 10.1073/pnas.1302837110

- Pei, S., Wu, F., Chen, Y., Ma, W., He, D., Zhang, Q., et al. (2023). Mechanisms of lead-containing pigment discoloration caused by *Naumannella cuiyingiana* AFT2T isolated from 1500 years tomb wall painting of China. *Int. Biodeterior. Biodegrad.* 185:105689. doi: 10.1016/j.ibiod.2023.105689
- Pinhassi, J., and Berman, T. (2003). Differential growth response of colony-forming α - and γ -proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Appl. Environ. Microbiol.* 69, 199–211. doi: 10.1128/AEM.69.1.199-211.2003
- Porca, E., Jurado, V., Martín-Sánchez, P. M., Hermosin, B., Bastian, F., Alabouvette, C., et al. (2011). Aerobiology: an ecological indicator for early detection and control of fungal outbreaks in caves. *Ecol. Indic.* 11, 1594–1598. doi: 10.1016/j.ecolind.2011.04.003
- Portillo, M. C., and Gonzalez, J. M. (2011). Moonmilk deposits originate from specific bacterial communities in Altamira cave (Spain). *Microb. Ecol.* 61, 182–189. doi: 10.1007/s00248-010-9731-5
- Portillo, M. C., Saiz-Jimenez, C., and Gonzalez, J. M. (2009). Molecular characterization of total and metabolically active bacterial communities of “white colonizations” in the Altamira cave, Spain. *Res. Microbiol.* 160, 41–47. doi: 10.1016/j.resmic.2008.10.002
- Qin, Y., and Xia, Y. (2024). Melanin in fungi: advances in structure, biosynthesis, regulation, and metabolic engineering. *Microb. Cell Factories* 23:334. doi: 10.1186/s12934-024-02614-8
- Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nat. Biotechnol.* 35, 833–844. doi: 10.1038/nbt.3935
- Rivera, A. B., Rainer, L., and Vagts, L. (2006). “Out of their native earth: the history of excavation and conversation of ancient hopi murals from Awatovi and Kawaika-a” in *The conservation of decorated surfaces on earthen architecture*. eds. L. Rainer and A. B. Rivera (Los Angeles, CA: Getty Conservation Institute), 53–65.
- Saarela, M., Alakomi, H.-L., Suihko, M.-L., Maunukela, L., Raaska, L., and Mattila-Sandholm, T. (2004). Heterotrophic microorganisms in air and biofilm samples from Roman catacombs, with special emphasis on actinobacteria and fungi. *Int. Biodeterior. Biodegrad.* 54, 27–37. doi: 10.1016/j.ibiod.2003.12.003
- Sanmartín, P., DeAraujo, A., and Vasanthakumar, A. (2018). Melding the old with the new: trends in methods used to identify, monitor, and control microorganisms on cultural heritage materials. *Microb. Ecol.* 76, 64–80. doi: 10.1007/s00248-016-0770-4
- Sansupa, C., Suphaphimol, N., Nonthijun, P., Ronsuek, T., Yimklan, S., Semakul, N., et al. (2023). Life on the wall: the diversity and activity of microbes on 13th – century AD. Lan Na mural painting. *Front. Microbiol.* 14:1220901. doi: 10.3389/fmicb.2023.1220901
- Schabereiter-Gurtner, C., Piñar, G., Vybiral, D., Lubitz, W., and Rölleke, S. (2001). Rubrobacter-related bacteria associated with rosy discoloration of masonry and lime wall paintings. *Arch. Microbiol.* 176, 347–354. doi: 10.1007/s002030100333
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09
- Setlow, P. (2007). I will survive: DNA protection in bacterial spores. *Trends Microbiol.* 15, 172–180. doi: 10.1016/j.tim.2007.02.004
- Spagnuolo, A., Vetromile, C., Masiello, A., Alberghina, M. F., Schiavone, S., and Lubritto, C. (2019). Climate and cultural heritage: the case study of “real Sito di Carditello”. *Heritage* 2, 2053–2066. doi: 10.3390/heritage2030124
- Sterflinger, K. (2010). Fungi: their role in deterioration of cultural heritage. *Fungal Biol. Rev.* 24, 47–55. doi: 10.1016/j.fbr.2010.03.003
- Sterflinger, K., and Pinzari, F. (2012). The revenge of time: fungal deterioration of cultural heritage with particular reference to books, paper and parchment. *Environ. Microbiol.* 14, 559–566. doi: 10.1111/j.1462-2920.2011.02584.x
- Stoddard, C. S., Coyne, M. S., and Grove, J. H. (1998). Fecal bacteria survival and infiltration through a shallow agricultural soil: timing and tillage effects. *J. Environ. Qual.* 27, 1516–1523. doi: 10.2134/jeq1998.00472425002700060031x
- Stomeo, F., Portillo, M. C., Gonzalez, J. M., Laiz, L., and Saiz-Jimenez, C. (2008). *Pseudonocardia* in white colonizations in two caves with Paleolithic paintings. *Int. Biodeterior. Biodegrad.* 62, 483–486. doi: 10.1016/j.ibiod.2007.12.011
- Sugiyama, J., Kiyuna, T., Nishijima, M., An, K.-D., Nagatsuka, Y., Tazato, N., et al. (2017). Polyphasic insights into the microbiomes of the Takamatsuzuka tumulus and Kitora tumulus. *J. Gen. Appl. Microbiol.* 63, 63–113. doi: 10.2323/jgam.2017.01.007
- Vellend, M. (2010). Conceptual synthesis in community ecology. *Q. Rev. Biol.* 85, 183–206. doi: 10.1086/652373
- Viles, H. A., and Cutler, N. A. (2012). Global environmental change and the biology of heritage structures. *Glob. Change Biol.* 18, 2406–2418. doi: 10.1111/j.1365-2486.2012.02713.x
- Wang, L., Huang, J., Sanmartín, P., Martino, P. D., Wu, F., Urzì, C. E., et al. (2025). Water determines geomicrobiological impact on stone heritage. *Nat. Geosci.* 18, 108–111. doi: 10.1038/s41561-024-01631-x
- Wang, W. F., Wu, F. S., Ji, A. H., and Feng, H. Y. (2013). Advancement and prospect of bionic techniques in the conservation of the cultural heritage. *Appl. Mech. Mater.* 461, 469–475. doi: 10.4028/www.scientific.net/AMM.461.469
- Wang, W., Ma, Y., Ma, X., Wu, F., Ma, X., An, L., et al. (2012). Diversity and seasonal dynamics of airborne bacteria in the Mogao grottoes, Dunhuang, China. *Aerobiologia* 28, 27–38. doi: 10.1007/s10453-011-9208-0
- Wu, F., Gu, J.-D., Li, J., Feng, H., and Wang, W. (2022). “Microbial colonization and protective management of wall paintings” in *Cultural heritage microbiology: Recent developments*. eds. R. Mitchell, J. Clifford and A. Vasanthakumar (London: Archetype Publications), 57–84.
- Wu, F., Zhang, Y., He, D., Gu, J.-D., Guo, Q., Liu, X., et al. (2021). Community structures of bacteria and archaea associated with the biodeterioration of sandstone sculptures at the Beishiku Temple. *Int. Biodeterior. Biodegrad.* 164:105290. doi: 10.1016/j.ibiod.2021.105290
- Xing, W., Qi, B., Chen, R., Ding, W., and Zhang, F. (2023). Metagenomic analysis reveals taxonomic and functional diversity of microbial communities on the deteriorated wall paintings of Qinling tomb in the southern tang dynasty, China. *BMC Microbiol.* 23:140. doi: 10.1186/s12866-023-02887-w
- Xu, H.-B., Tsukuda, M., Takahara, Y., Sato, T., Gu, J.-D., and Katayama, Y. (2018). Lithoautotrophic oxidation of elemental sulfur by fungi including *Fusarium solani* isolated from sandstone Angkor temples. *Int. Biodeterior. Biodegrad.* 126, 95–102. doi: 10.1016/j.ibiod.2017.10.005
- Yao, S., Yan, Z., Ma, Q., Xu, B., Zhang, Z., Bi, W., et al. (2022). Analysis of the annual hygrothermal environment in the Majiashan grottoes by field measurements and numerical simulations. *Build. Sci.* 22:109229. doi: 10.1016/j.buildenv.2022.109229
- Yu, Y., Zhang, J., Chen, R., Coleine, C., Liu, W., Delgado-Baquerizo, M., et al. (2024). Unearthing the global patterns of cultural heritage microbiome for conservation. *Int. Biodeterior. Biodegrad.* 190:105784. doi: 10.1016/j.ibiod.2024.105784
- Zhang, G., Gong, C., Gu, J., Katayama, Y., Someya, T., and Gu, J.-D. (2019). Biochemical reactions and mechanisms involved in the biodeterioration of stone world cultural heritage under the tropical climate conditions. *Int. Biodeterior. Biodegrad.* 143:104723. doi: 10.1016/j.ibiod.2019.104723
- Zhang, X., Ge, Q., Zhu, Z., Deng, Y., and Gu, J.-D. (2018). Microbiological community of the Royal Palace in Angkor Thom and Beng Mealea of Cambodia by Illumina sequencing based on 16S rRNA gene. *Int. Biodeterior. Biodegrad.* 134, 127–135. doi: 10.1016/j.ibiod.2018.06.018
- Zhang, Y., Wu, F., Su, M., He, D., Gu, J.-D., Guo, Q., et al. (2021). Spatial and temporal distributions of microbial diversity under natural conditions on the sandstone stelae of the Beishiku Temple in China. *Int. Biodeterior. Biodegrad.* 163:105279. doi: 10.1016/j.ibiod.2021.105279
- Zomer, A., Ingham, C. J., Von Meijenfeldt, F. A. B., Escobar Doncel, Á., Van De Kerkhof, G. T., Hamidjaja, R., et al. (2024). Structural color in the bacterial domain: the ecogenomics of a 2-dimensional optical phenotype. *Proc. Natl. Acad. Sci. USA* 121:e2309757121. doi: 10.1073/pnas.2309757121
- Zvyagintsev, D. G., Zenova, G. M., Doroshenko, E. A., Gryadunova, A. A., Gracheva, T. A., and Sudnitsyn, I. I. (2007). Actinomycete growth in conditions of low moisture. *Biol. Bull. Russ. Acad. Sci.* 34, 242–247. doi: 10.1134/S1062359007030053



OPEN ACCESS

EDITED BY

Fadwa Jroundi,
University of Granada, Spain

REVIEWED BY

Cristina Cattò,
University of Milan, Italy
Xiaobo Liu,
Nanjing University of Science
and Technology, China
Hairong Wang,
Carnegie Mellon University Stores,
United States

*CORRESPONDENCE

Irit Nir
✉ iritnir1@gmail.com
Ariel Kushmaro
✉ arielkus@bgu.ac.il

RECEIVED 11 July 2025

REVISED 23 November 2025

ACCEPTED 02 December 2025

PUBLISHED 21 January 2026

CORRECTED 09 February 2026

CITATION

Nir I, Sharaby A, Barak H, Pavan MJ,
Friedlander LR, Multanen V and Kushmaro A
(2026) Extensive biofilm covering on
sgraffito wall art: a call for proactive
monitoring.
Front. Microbiol. 16:1664404.
doi: 10.3389/fmicb.2025.1664404

COPYRIGHT

© 2026 Nir, Sharaby, Barak, Pavan,
Friedlander, Multanen and Kushmaro. This is
an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](#). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Extensive biofilm covering on sgraffito wall art: a call for proactive monitoring

Irit Nir^{1*}, Anath Sharaby¹, Hana Barak¹, Mariela J. Pavan²,
Lonia R. Friedlander², Victor Multanen¹ and Ariel Kushmaro^{1,2,3*}

¹Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer Sheva, Israel, ²Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer Sheva, Israel, ³The Goldman Sonnenfeldt School of Sustainability and Climate Change, Ben-Gurion University of the Negev, Beer Sheva, Israel

Background: The study focuses on a black and white sgraffito decoration attached to a cement exterior wall in Kibbutz Yiftach, Israel. Since its creation in 1971, the artwork has experienced weathering processes, resulting in peeling, flaking, and the development of a microbial layer on the wall art decoration. Before its restoration in 2022, this study was initiated, aiming to address three primary questions: What is the composition of the microbial communities? What is the distribution of these microbial communities throughout the wall, and how do they interact with the substrate materials?

Methods: Complementary methods, including mineral analysis, microscopic observations, and molecular techniques, were implemented to answer the study questions.

Results: Five main groups of bacteria (e.g., Cyanobacteria, Actinobacteria, Proteobacteria, Bacteroidota, and Chloroflexi), as well as various types of fungi, were revealed. Nevertheless, although the same phyla were detected across samples, each displayed distinct diversity at the order level. Microscopic observations revealed the attachment of microbial components to both the porous plaster and the rough cement.

Discussion: The study shows a well-developed microbial coating with a localized colonization pattern, underscoring the need for continued long-term monitoring of both the wall-art materials and their environmental conditions to support data-driven conservation.

KEYWORDS

sgraffito, wall art, lime mortar, bio-weathering, next-generation sequencing (NGS)

1 Introduction

Sgraffito is a wall art technique commonly used in murals and architectural decoration by applying layers of colored plaster or paint to a surface and then scratching or carving away to reveal the layers underneath (Jagiello, 2022). The term “sgraffito” comes from the Italian word “sgraffire,” meaning “to scratch” (Aldersey-Williams, 2013). This technique can create a wide range of designs, from simple geometric patterns to more complex, detailed images, and is notable for its ability to add depth and texture to a surface through carved lines and shapes (Baker, 2007). The plaster components are mostly composed of lime mortar, a traditional building material made by mixing lime, sand,



FIGURE 1

(A) The original sgraffito bichromate wall painting titled "Bo-Shalom" (meaning "Come, Peace," expressing a desire for peace), from its creation period in 1971. (B) Deterioration patterns of the wall include dark coating and surface flaking. (C) The sgraffito wall art after the cleaning and restoration project (2022).

and water (Elert et al., 2002). The way lime mortars are made involves heating limestone (CaCO_3) to turn it into quicklime (CaO), then mixing it with water to make slaked lime, and finally combining it with materials like sand to form the mortar (Wojcieszak et al., 2024). Over time, lime mortar also absorbs carbon dioxide from the air, contributing to a natural hardening process known as carbonation (Campo et al., 2021). For centuries, masonry construction has relied on its flexibility, breathability, and durability (Ashurst, 1983; Carran et al., 2011; Mundy, 2013). Initially, artists created sgraffito designs in monochrome black and white, utilizing soot to create the dark black tone. The sgraffito technique was popular in Italy during the Renaissance to modernize medieval buildings and express religious views, with examples such as the works of Andrea del Sarto and the Palazzo Pitti in Florence (Ferguson, 2010; Jagiełło, 2022). Throughout the 1970s, Israel's wall decoration artists predominantly employed this approach. Halperin, a local artist knowledgeable in the European sgraffito technique, devised a regional plaster combination that included sand sourced from Nubian sandstone he gathered in the Negev Desert, Israel. To evaluate the quality of the material he created, he asked local artists to experiment with its application in decorating building walls nationwide. These decorations featured local symbolic motifs derived from the natural environment and biblical narratives. The number of locations using the technology decreased over time, and weathering processes began, causing the structures to disintegrate. Simultaneously, a substantial initiative to document and preserve the works has commenced (Farkash and Tal, 2017).

Sgraffito, like many forms of wall art, is vulnerable to deterioration processes, including biodeterioration. In this process, living organisms such as fungi, bacteria, and algae colonize the surfaces and pores of lime mortar, causing changes to the

materials (Cassar, 2002). Microbial growth can lead to staining and discoloration, negatively affecting the aesthetic value of historical and heritage buildings (Gauri and Holdren, 1981; Gu, 2003; Liu et al., 2020; Warscheid and Braams, 2000). In regions with high moisture levels, the plaster and paint layers may absorb water, encouraging the growth of microorganisms. Consequently, sgraffito artworks can experience surface erosion and loss of image details over time (Caron, 2015; Liu et al., 2022). Understanding the mechanisms of microbial colonization and its impact is important when designing conservation strategies for lime mortar structures (McNamara et al., 2010). This study focuses on a sgraffito wall artwork adorning the concrete wall of a shelter in a northern Israeli village called "Kibbutz Yiftach." Created by a local artist, the piece depicts children playing and dancing in the fields, images that evoke a sense of peace and innocence. To emphasize this contrast and express a desire for peace, the artist inscribed the Hebrew words "Bo Shalom" ("Come Peace") into the artwork. The artist carved the figurative images through a layer of white plaster, revealing a layer of black plaster underneath (Figure 1A). Since its creation in 1971, the artwork has undergone a weathering process that resulted in a black or dark green cover, loss of aesthetic value, and degradation of the plaster material (Figure 1B). Notwithstanding its symbolic, local communal importance and artistic distinctiveness, the opportunity for cleaning and restoration of the piece did not emerge until 2022 (Figure 1C). Before starting the restoration, this study was initiated, aiming to address three primary questions: What is the composition of the microbial communities? What is their distribution along the wall, and what are their interactions with the substrate materials? The data obtained will expand our knowledge regarding the microbial colonization of sgraffito wall decoration and will serve as a basis for further long-term monitoring and preservation of this unique wall art technique.

2 Materials and methods

2.1 Study site and sampling

The sgraffito wall decoration entitled “Bo-shalom” is situated on an external concrete wall, which is a part of a shelter complex adjacent to school buildings in the “Kibbutz Yiftach” village, located in northern Israel (Figure 1). Climatically, the area is classified as a Mediterranean climate zone characterized by an annual precipitation of approximately 530 mm, which primarily occurs during the winter months. July is the hottest month in the region, with an average temperature of about 27°C (81°F), while January is the coldest at about 11°C (52°F). The climatic data were sourced from the Kibbutz Yiftach meteorological station, which is located about 500 m from the study site (33.1259°N, 35.5521°E), and published on the Israel Meteorology Services (IMS) website¹ (accessed 15.11.2025) (Israel Meteorological Service, 2025) and are presented in Supplementary Table 1. Samples were collected at four points along the wall decoration (Y1–Y4) and at four points along the adjoining concrete wall (Y5–Y8), oriented in the same direction, which were covered by spots of dark green and black crust layer (Figure 2). The Samples were collected about a week before the conservation project was conducted in August 2022. A hammer and chisel, surface-sterilized with 70% ethanol were used to collect slabs of about 4 cm in size. Each of the samples was divided into two fragments. One fragment was used for molecular analysis, and the other for microscopic and chemical study. The samples were transferred to the environmental microbiology lab at Ben Gurion University, Israel, for further analysis. Samples targeted for microscopic and chemical examination were preserved at room temperature, whereas those intended for molecular analysis were kept cold at 4°C until processing (The testing methodologies employed for each sample are specified in Supplementary Table 2).

¹ <https://ims.gov.il/en/node/310>

2.2 Wall-art materials characterization

To determine the materials that were used for the creation of the Sgraffito, including the pigments that were used, X-ray powder diffraction (XRD), X-ray fluorescence (XRF) analysis, and Raman spectroscopy were implemented.

2.2.1 X-ray powder diffraction analysis

Phase analysis of the mural samples (Y1–Y4) was performed using the XRD method. The samples were hand-ground using an agate mortar and pestle to the consistency of fine silt as described by Bish and Reynolds (1989). Samples were placed on a bias-cut quartz plate, which gives no diffraction in the normal range for cement or geologic materials, for analysis. The data were gathered using an Empyrean II X-ray powder diffractometer (Malvern Panalytical, Almelo, Netherlands) ($K\alpha$ radiation, $\lambda = 1.541\text{\AA}$) set to 40 kV and 30 mA and with an attached X'Celerator linear detector. Data was collected in Bragg-Brentano reflectance geometry from 10 to 80° 2 θ , with a step size of 0.0334° and a counting time of 50 s per step. Phase identification and Rietveld refinement for phase quantification were both performed using HighScore Plus version 5.1 software, in combination with the ICDD Powder Diffraction File database (PDF-5 +) (version 2024). The average error on the quantitative analysis is ± 0.5 wt%.

2.2.2 X-ray fluorescence measurements

The XRF measurements were performed using an Axios XRF Spectrometer (Malvern Panalytical, Ltd., Almelo, Netherlands). Powder samples were mounted directly into the instrument, and analysis of the emitted elemental fluorescence lines was performed on the data as it was collected using the native Panalytical-provided Omnian XRF analysis software.

2.2.3 Raman spectroscopy analysis

Micro-Raman spectroscopy was used to study the pigments and mineral composition of representative samples (Y2 and Y4

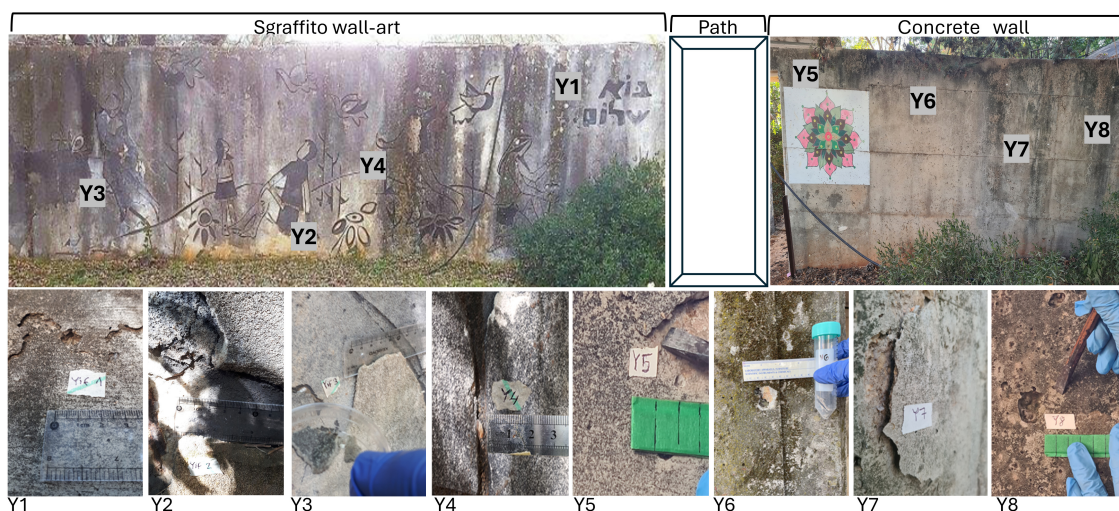


FIGURE 2

Sampling site: the “Sgraffito” wall art and the adjacent concrete wall, indicating the positions of sampling points Y1–Y8 (above), together with close-up photographs of the samples collected in this study (at the lower portion of the figure).

from the mural and Y5–Y8 from the exposed concrete wall). The measurements were taken using a LabRAM HR Evolution Confocal Raman Microscope (Horiba Ltd., Kyoto, Japan), which has a Sincerity CCD detector that is cooled to -60°C and has a resolution of $1,024 \times 256$ pixels. 785, 532, and 325 nm lasers were used as excitation sources, with power on the sample ranging from 4 to 80 mW for the 785 nm laser, 1–3.4 mW for the 532 nm laser, and 0.3–0.15 mW for the UV laser. The $10\times$ and $50\times$ LWD objectives were used to focus on the Vis and NIR lasers, and the $40\times$ for the UV laser. The confocal hole was set to 100 and 200 μm , using 600 and 1,800 gr/mm gratings for the visible and UV ranges, respectively. Acquisition time ranged from 1 to 60 s. LabSpec 6, version 6.5.1.24, was implemented to acquire baseline-correct, denoise, and average the spectra using. Identification of the compounds was performed either by comparing their spectra with those obtained from Raman spectral databases (KnowItAll Informatics Systems, 2025, John Wiley and Sons Inc., United States) or by comparing them with Raman spectra in the literature.

2.3 Microscopic observations of the biofilm structure

The samples collected from the wall decoration site were initially examined under a light stereomicroscope (SMZ1500, Nikon, Japan) at magnifications of up to $40\times$, without any specific preparation (Supplementary File 3). This allowed us to gather an overview of the morphological characteristics of the biofilm. Images were captured using a digital camera (Nikon 1J5) connected to the microscope for this purpose. To distinguish between inorganic (e.g., substrate materials) and organic materials (e.g., microbial cells) in the biofilm, a light polarized microscope with polarized light (PPL) and cross-polarized light (XPL) was used (DM1750, Leica Microsystems, Germany). Images were taken using a Canon M6 MaII II digital camera. The Thermo Fisher Verios 460L field-emission scanning electron microscope (FESEM) coupled with Energy-Dispersive X-ray Spectroscopy (EDS) was used to characterize the microbial cells-substrate interface. The samples were prepared as follows: after fixation with 2.5% buffered glutaraldehyde, the samples were subsequently dehydrated using an ascending serial ethanol gradient and immersed in a hexamethyldisilazane (HMDS)/ethanol gradient solution at concentrations of 25, 50, 75, 90, 95, and 100%. The treated specimens were air-dried and then sputter-coated with a 20-nm layer of gold using an EMITECH K575x sputtering device (Emitech Ltd., United Kingdom).

2.4 Taxonomic analysis of the biofilm components using molecular methods

2.4.1 DNA extraction

Substrate samples (Y1, Y2, Y3, and Y4 related to lime mortar; Y5, Y6, Y7, and Y8 related to concrete samples) were crushed in a laboratory hood to obtain small aggregates using sterile pliers. Aggregates (0.5 g) from each sample were transferred into separate bead tubes for DNA extraction. DNA extraction was performed using a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories,

Inc., Carlsbad, CA, United States) following the protocol supplied by the manufacturer. The purity and concentration of DNA samples were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The extracted DNA was submitted to the Genomics and Microbiome Core Facility at Rush University, United States.

2.4.2 DNA sequencing

For bacterial community analysis, the V3–V4 hypervariable region of the 16S rRNA gene was amplified using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GACTACHVGGGTATCTAATCC-3') (Caporaso et al., 2011; Klindworth et al., 2013). To study the fungal community, the internal transcribed spacer (ITS) region was copied using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') (Gardes and Bruns, 1993; White et al., 1990). Amplicon sequencing was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) with paired-end (2×300 bp) sequencing at the Genomics and Microbiome Core Facility at Rush University, United States.

2.4.3 Bioinformatic analysis

The DADA2 pipeline (Callahan et al., 2016) was implemented through QIIME 2 for quality filtering, denoising, chimera removal, and amplicon sequence variant (ASV) identification. Based on quality score profiles, we truncated the forward and reverse reads at positions 250 and 225 bp, respectively. No bases were trimmed from the start of the sequences ($-p\text{-trim-left-f } 0$, $-p\text{-trim-left-r } 0$). The minimum overlap between paired-end reads was set to 15 bp, and pseudo-pooling was employed for ASV inference. Chimeric sequences were identified and removed using the pooled method with a minimum fold parent abundance of 4. The maximum expected error (maxEE) filter threshold was set to 4 for both forward and reverse reads. After denoising, the feature table and representative sequences were summarized. Taxonomy was assigned to ASVs using a naive Bayes classifier trained on the Silva 99% reference database (Bokulich et al., 2018; Quast et al., 2013; Yilmaz et al., 2014). Sequences classified as mitochondria or chloroplasts were filtered out from the final feature table and representative sequences. For the fungal ITS sequences, the DADA2 pipeline was implemented with modified parameters to accommodate the higher variability and quality characteristics of the ITS regions. Forward and reverse reads were truncated at 200 bp, with no bases trimmed from the start of the sequences. The maximum expected error filter threshold was set to 5.0 for both forward and reverse reads, and the minimum quality score truncation threshold ($-p\text{-trunc-q}$) was disabled by setting it to 0. The minimum overlap between paired-end reads was reduced to 10 bp to account for the variable length of the ITS region. ASVs were inferred using the independent pooling method, and the chimera checking step was disabled due to the high variability of the ITS regions. Taxonomy was assigned to the resulting ASVs using a naive Bayes classifier trained on the UNITE database version 10 (99% threshold, dated February 19, 2025) (Nilsson et al., 2019).

2.4.4 Statistical analysis

All the statistical analyses were conducted using QIIME 2 version 2021.4 (Bolyen et al., 2019). Raw sequence data were

imported and demultiplexed using QIIME 2 standard procedures. To quantify the differences between the studied microbial groups colonizing the mural (Y1, Y2, and Y4) and the concrete (Y5, Y6, and Y7) substrate samples, Bray-Curtis Principal Coordinate Analysis (PCoA) was implemented along with the PERMANOVA statistical test. Additionally, alpha diversity statistical measures were implemented to describe the diversity within samples.

3 Results

3.1 Wall-art materials analysis

3.1.1 XRD analysis

Figure 3 illustrates the XRD analysis results of samples Y1, Y2, Y3, and Y4 that were obtained from different points of the mural materials. All the samples contain the predominant phases of quartz (SiO_2) and calcite (CaCO_3). Samples Y1 and Y3 have dolomite [$\text{CaMg}(\text{CO}_3)_2$], but Y4 is devoid of it. The remaining trace phases consist of silicates, specifically the pyroxene mineral augite [$\text{CaMg}(\text{SiAl})_2\text{O}_6$] or a solid-solution mixed phase from the albite-anorthite feldspar series [$(\text{NaCa})\text{Al}(\text{SiAl})_3\text{O}_8$]. The present phases are consistent with the minerals of lime mortar mixed with sand. Other phases may be present that are amorphous and therefore undetectable by XRD.

3.1.2 XRF analysis

Supplementary Table 4 displays the XRF analysis results, encompassing components detected at concentrations over 0.1%. Calcium and Silica are the predominant ingredients, comprising 40–50% of the mural materials. These results correspond with the knowledge of lime mortar constituents (Manoharan and Umarani, 2022).

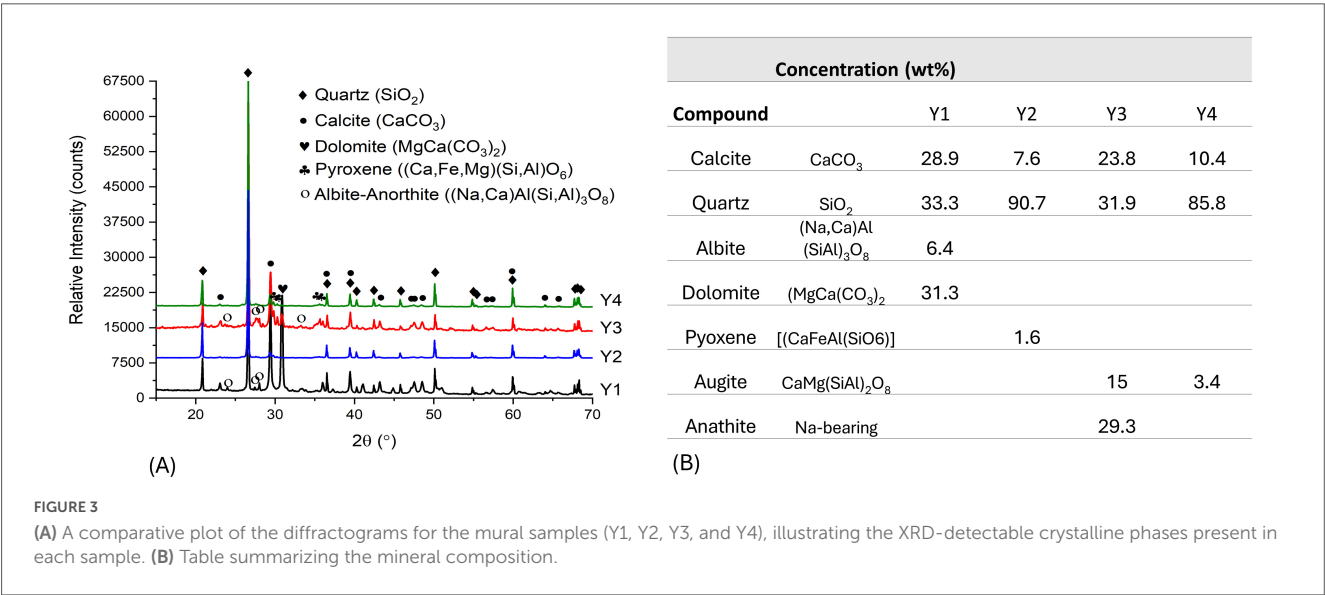
3.1.3 Raman spectroscopy analysis

Figure 4 and Supplementary Table 5 present results from the Raman spectroscopy analysis of samples Y2 and Y4 that were obtained from the mural and samples Y5, Y6, Y7, and Y8

obtained from the adjacent concrete wall. The analysis identified calcite (CaCO_3), dolomite [$\text{CaMg}(\text{CO}_3)_2$], and quartz (SiO_2), which are the principal components of the lime mortar mixture. Additionally, the hornblende ($(\text{CaNa})_2(\text{MgFeAl})_5(\text{AlSi})_8\text{O}_{22}(\text{OH})_2$) silicates were determined. Other pigments and mineral phases included amorphous carbon (a main constituent in coal used as black pigment), hematite ($\alpha\text{-Fe}_2\text{O}_3$), and goethite ($\alpha\text{-FeOOH}$), typical red and yellow-brown iron oxides, as well as anatase and rutile, which are naturally occurring forms of crystalline titanium oxide (TiO_2) commonly used in white paints. Among organic compounds, carotene, melanin, and chlorophyll were detected in samples Y2, Y4, Y5, and Y6, suggesting potential microbial colonization. In contrast, samples Y7 and Y8 showed only mineral phases, likely due to strong fluorescence interference.

3.2 Microscopic observations of the coating

Samples of the coated building materials (e.g., mortar and cement) were examined using a range of microscopic techniques. Stereoscopic light microscope enabled observation of black round spots of fungi, lichen green-yellow thalli, and dark-green moss leaves on the surfaces of samples Y2 and Y4, respectively (Figures 5A,D). These observations indicate the presence of a well-developed microbial community where early-stage photosynthetic colonizers create conditions that allow subsequent vascular plant growth. Scanning electron microscopy provided detailed insight into the structure of the microbial coating. Figure 5C indicates filaments attached to the substrate materials. Figure 5F shows amorphous matrix binding to the substrate aggregates, which was not chemically identified in his study. Polarized light microscopy (Figures 5B,E) further distinguished organic structures (cells) from inorganic matrix components under plain and cross-polarized light, confirming microorganism attachment to the substrate materials, which in turn may cause the disaggregation of the mortar materials.



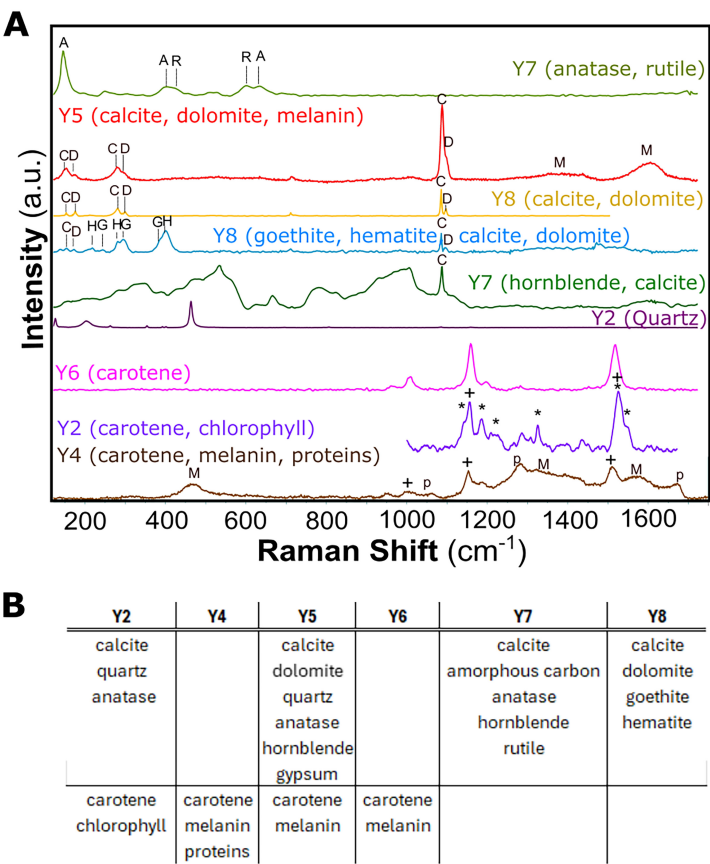


FIGURE 4
(A) Raman spectroscopy scattering analysis obtained from different points at Y2, Y4, Y5, Y6, Y7, and Y8 samples. The band position for every constituent is marked as follows: A, anatase; R, rutile; C, calcite; AC, amorphous carbon; G, goethite; H, hematite; D, dolomite; *, chlorophyll and +, carotene M, melanin, and p, proteins (B) Table of the minerals and pigments detected in each of the samples Y2, Y4, Y5, and Y8)

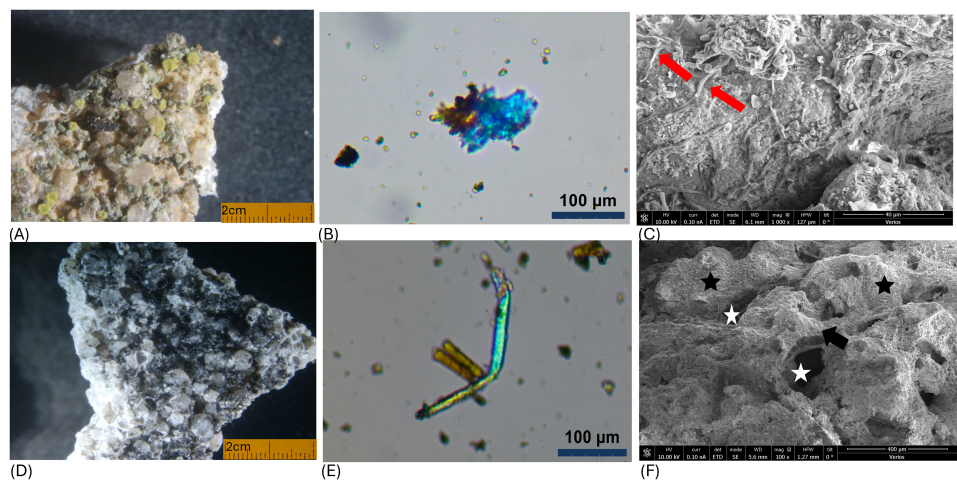


FIGURE 5
Microscopic observations of samples obtained from the wall surface: (A,D) Light stereomicroscope images of the biofilm with x10 magnification. (B,E) Images taken with cross-polarized light (XPL) demonstrate the phototrophic cells of the biofilm attached to the mortar aggregates (in light blue) and to the cement fibers. (C) Scanning Electron Microscope (SEM) micrographs of microbial filaments (fungi) attached to the surface of a concrete sample. (F) Scanning Electron Microscope image of microbial cells embedded in an amorphous matrix (black arrow) on the plaster aggregates (black asterisks). White asterisks indicate the presence of microcavities between the aggregates.

3.3 Characterization of the biofilm components using DNA sequencing

DNA was extracted from all the samples collected. Samples Y3 and Y8, however, did not produce DNA of sufficient quality for sequencing. Consequently, libraries were acquired from six samples: Y1, Y2, Y4 (mural), and Y5, Y6, and Y7 (concrete). A total of 182,545 high-quality sequences were obtained from the 16S rRNA gene libraries after quality filtering, denoising, and chimera removal, with read counts ranging from 20,824 to 48,251 per sample (mean = 30,424). For the ITS region, a total of 99,724 high-quality sequences were retained after processing, with read counts ranging from 13,748 to 23,317 per sample (mean = 16,621). Statistical analysis revealed that each sample contained a variety of microbes, and there were no specific groupings based on the types of materials used (e.g., lime mortar and concrete) (Figures 6C, 7C).

3.3.1 Bacterial taxa diversity

The study revealed 20 bacterial phyla and 181 bacterial genera in six of the eight samples: Y1, Y2, Y4, Y5, Y6, and Y7. The analysis of the bacterial communities found four main groups: Cyanobacteria, Actinobacteria, Proteobacteria, and Chloroflexi, which together made up about 88–92% of all the bacteria, along with a few other groups that each made up less than 5% of the total. About 1–2% of the ASVs obtained were not assigned to any specific taxa and are marked as “others” (Figure 6). The Phylum Cyanobacteria dominates samples Y1, Y4, and Y5, accounting

for 40–58%, while Actinobacteria dominates samples, Y2, Y6, and Y7, accounting for 35–45%. The phylum Proteobacteria made up about 20–38% of the total, and the Chloroflexi phylum accounted for 3–10% of the total bacterial types (Figure 6A). All the samples revealed Cyanobacteriales as the dominant order within the Cyanobacteria (Figure 6B). The family-level identification revealed two main groups that were found in all the samples. Among these groups, the Chroococcidiopsaceae family was found in amounts ranging from 8% in sample Y4 to 45% in sample Y2, while the Nostocaceae family was found in smaller amounts, from 1% in sample Y7 to 30% in sample Y6. In the Actinobacteria group, five families were the most common: Geodermatophilaceae, Micromonosporaceae, Nocardioidaceae, Pseudonocardiaceae, and Micrococcaceae, which constitute about 72–84% of the Actinobacteria community. The dominant class in samples Y1, Y4, and Y7 was Geodermatophilaceae (35–48%), while in samples Y6 and Y4, the Pseudonocardiaceae class was the most dominant (40–50%) (Figure 6B and Supplementary Table 6).

3.3.2 Fungal taxa diversity

About 2–50% of the total ASVs retrieved were unidentified. All the samples included the Ascomycota phylum among the identified taxa. In samples Y1, Y2, Y4, and Y6, the Ascomycota phylum was dominant and present in more than 80% of the fungal taxa. (In samples Y1 and Y6, it is present in more than 95% of the taxa). All samples exhibited a relatively low abundance of the phylum Mortierellomycota, ranging from 2 to 18%. Approximately

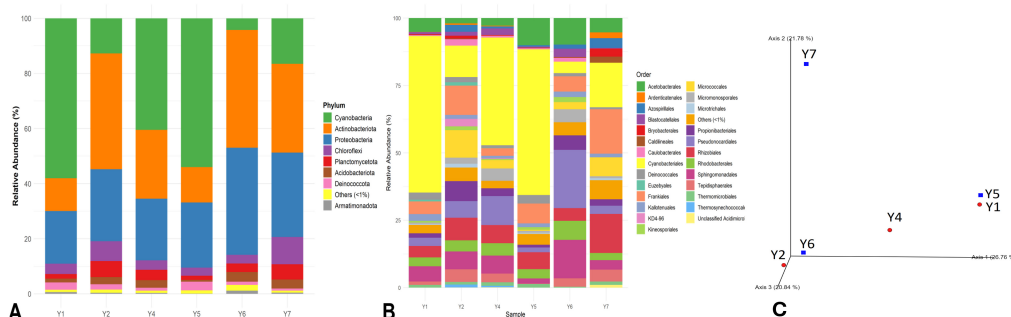


FIGURE 6

Relative abundance of bacterial communities at the phylum level (A) and order level (B). (C) Bray-Curtis dissimilarity PCoA plot of the bacterial communities colonizing the wall art samples (Y1, Y2, and Y4) and the exposed concrete samples (Y5, Y6, and Y7).

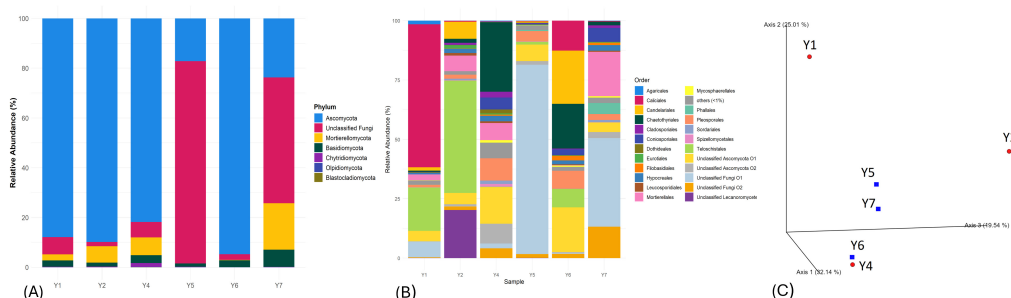


FIGURE 7

Relative abundance of fungi communities at the phylum level (A) and at the order level (B). (C) Bray-Curtis dissimilarity PCoA plot of the fungi communities colonizing the wall art samples (Y1, Y2, and Y4) and the concrete samples (Y5, Y6, and Y7).

1–7% of all the samples contained representatives from the Basidiomycota. The phyla Chytridiomycota, Olpidiomyces, and Blastocladiomycota were present in less than 1% of all the samples (Figure 7A). At the order level, ASVs' distribution varied among sites. All the samples tested showed lichenized fungi in the Lecanoromycetes group (Ascomycota), especially in samples Y1 (90%) and Y2 (80%). The distribution of the Candelariomycetes, Eurotiomycetes, and Dothideomycetes was detected mostly in the samples Y4, Y6, and Y7 (Figure 7B and Supplementary Table 7).

3.3.3 Comparison between microbial colonization on the different substrates

Principal Coordinate Analysis (PCoA) was performed on the six samples to explore differences in microbial community composition. Originally, eight samples were collected, but two failed to yield DNA of sufficient quality for sequencing, resulting in a reduced sample size. The six samples present two groups regarding the substrate material, mortar or cement. The analysis revealed no clear separation between the samples in the ordination space (Figures 6C, 7C). Additionally, PERMANOVA analysis using Bray–Curtis dissimilarity indicated no significant compositional differences between substrate types for either bacterial ($p = 0.518$) or fungal ($p = 0.743$) communities (Supplementary Table 8). Consistently, Kruskal–Wallis tests of alpha diversity showed no significant differences between cement and rock samples for observed features (bacteria: $H = 0.048$, $p = 0.827$; fungi: $H = 1.19$, $p = 0.275$) or Shannon entropy (bacteria: $H = 0.048$, $p = 0.827$; fungi: $H = 0.43$, $p = 0.513$). Although individual samples varied in richness and diversity, no substrate-specific patterns were detected. Together, these results indicate that microbial diversity is not structured by substrate type and is more likely influenced by local microenvironmental heterogeneity.

4 Discussion

Since 1971, the “Come, Peace” sgraffito has undergone progressive weathering. Close examination showed a mature microbial layer incorporating lichens and mosses, reaching ~0.5 cm thickness in several areas consistent with conditions favoring biofilm development (e.g., humidity, substrate porosity) (Liu et al., 2022; Sanmartín et al., 2021b). Moreover, the SEM study pointed to microbial cells, filaments, and hyphae adhering to construction particles. This may result in the removal of material grains from the substrate, thereby weakening its integrity over time. The chemical tests on the samples from the damaged sgraffito showed that they mainly contained two substances: silica oxide (quartz) and calcium carbonate (calcite). Silica oxide and calcium carbonate are known as the major components comprising lime mortars and cement concrete. Interestingly, the analytical examination's findings point to elevated concentrations of iron oxides in the art materials. This finding may be explained by the assumption that the sand used for the lime mortar mixture originated from Nubian sandstone sourced from the Negev Desert (Farkash and Tal, 2017; Weissbrod and Sneh, 1997).

Among the bacterial domain, Cyanobacteria, Actinobacteria, and Proteobacteria were detected as the principal bacterial taxa. This discovery agrees with established research concerning the

bacterial communities surviving in building materials (Dyda et al., 2019; Kukletová and Chromková, 2018). The Cyanobacteria group is crucial in biofilm formation as the primary energy producer in this nutrient-deficient environment. Interestingly, *Literella*, a cyanobacterium genus that was recently described within the fissures and cracks of granite stones and rocks were detected in all the samples (Jung et al., 2020). Four of the samples contained members of the filamentous cyanobacterium genus *Scytonema* which is already known as a colonizer of black crusts on building materials (De Miguel et al., 1995; Gaylarde et al., 2004; Gaylarde et al., 2007). *Nostoc* is a genus of filamentous cyanobacteria that can survive on stone monuments due to their ability to remain desiccated for months or years (Ramírez et al., 2011). All the samples collected included members of the Actinobacteria group, such as *Blastococcus*, *Nocardioideis*, *Micromonosporaceae*, and *Pseudonocardia*. Furthermore, we identified representatives from *Modestobacter*, *Micrococcaceae*, and *Geodermatophilus* in five of the samples. Actinobacteria, a prevalent soil bacterium, are recognized as a significant group residing in building materials (Abdulla et al., 2008; Saadouli et al., 2022; Urzi et al., 2004).

Concerning the fungi, the most represented phylum on the examined walls is Ascomycota. Within the Ascomycota phylum, the most prevalent groups were Lecanoromycetes, Dothideomycetes, and Eurotiomycetes. Lecanoromycetes are lichen-forming fungi with a worldwide range, predominantly found in cryptoendolithic colonies that inhabit stones and rocks in both natural environments and man-made structures (Cozzolino et al., 2021). In our study, we found the *Teloschistaceae*, a substantial family of lichen-forming fungi, colonizing both the lime mortar and concrete samples. Their ability to cause damage to building materials has been recorded in marble structures, where they may infiltrate up to 10 mm into the stone and disintegrate the marble surface (Fuentes et al., 2021). The results revealed other important taxa of Ascomycetes: Dothideomycetes and Eurotiomycetes, which are part of the black meristematic fungal communities (MCF) (De Leo et al., 2019; Selbmann et al., 2015; Sert et al., 2007). Members of this fungal group, such as *Knufia*, *Coniosporium*, and *Cladosporium*, were detected in all samples and are already known for their ability to cause damage by penetrating, degrading, and discoloring the stone surface. The production of protective pigments, such as melanin and carotenoids, revealed in various samples (Figure 4), further enhances their resilience (Erdmann et al., 2022; Isola et al., 2015). Most rock-inhabiting fungi can infiltrate between crystal structures (Figure 5E; Knabe and Gorbushina, 2018; Rabbachin et al., 2024). Interestingly, we identified taxa commonly referred to as sooty-mold fungi, such as representatives from the Trichomeriaceae and Capnodiaceae families (Chomnunti et al., 2014). Their presence may indicate an alternative pathway for biofilm formation on the wall decoration materials (Harding et al., 2009). We link this phenomenon to a large tree that covered much of the wall surface before conservation work began. The tree harbors a species of leaf aphids that feed on its sap and secrete a sugary substance known as honeydew (Meng et al., 2023).

Despite the disparity in material composition, which might have resulted in variations in microbial populations, no significant difference was found between the groups. This may be because over the years, the biofilm was well developed, containing microorganisms from all domains. Another reason could be

attributed to the differentiation of microclimatic conditions along the wall. In regions shaded by the adjacent *Pistacia palaestina*, the coating appeared dark to black, while in locations with slightly greater sun exposure, it also exhibited green patches (Figure 2). Interestingly, a recent study of epilithic biofilms on Stone Dog sculptures on the Leizhou Peninsula, China, found that despite identical exposure within a small courtyard, the biofilms exhibited high biodiversity and distinct community compositions (Sanmartín et al., 2021a). This underscores the critical influence of microenvironmental conditions and the need for precise microclimatic measurements, including radiation and humidity (Del Mondo et al., 2021; Sanmartín et al., 2021a).

5 Conclusion

The sgraffito wall decoration has experienced substantial deterioration and weathering since its creation, significantly diminishing the visibility of the artwork. Analytical results revealed a well-established, diverse microbial community, including fungi, bacteria, lichens, and mosses attached to the substrate. This process may reduce the structural integrity of the wall-art materials. Notably, variations in microbial composition appear to be more closely linked to localized microclimatic conditions than to the substrate materials themselves. Thus, future studies should address microclimatic measures, physicochemical characteristics, and metabolic functions of the microbial communities. Due to its climatic exposure and porous composition, the artwork remains vulnerable to microbial re-colonization. Therefore, sustained biological monitoring combined with conservation expertise and community engagement is essential for its long-term preservation.

Data availability statement

The data presented in this study are publicly available. The data can be found here: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1265466/>, accession number PRJNA1265466.

Author contributions

IN: Conceptualization, Data curation, Writing – original draft, Visualization. AS: Data curation, Investigation, Writing – review & editing. HB: Data curation, Formal analysis, Software, Visualization, Writing – review & editing. MP: Data curation, Visualization, Investigation, Writing – review & editing. LF: Data curation, Investigation, Visualization, Writing – review & editing. VM: Data curation, Visualization, Writing – review & editing. AK: Conceptualization, Writing – original draft, Visualization, Data curation, Investigation.

Funding

The author(s) declared that financial support was not received for this work and/or its publication.

Acknowledgments

We thank the Avram and Stella Goldstein-Goren Fund and the John A. Ungar Chair in Biotechnology for partial support and Esti Kramarsky-Winter for her scientific comments and editing. We wish to acknowledge S. Farkash, the manager of Studio Tchelet, and the professional restoration team for his cooperation. Special thanks to the wall décor artist N. Ben-Zvi, affiliated with Kibbutz Yiftach, whose work has enriched both the local community and the public realm.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was used in the creation of this manuscript. During the preparation of this work, the author(s) used ChatGPT with caution to improve language and readability. After using this tool or service, the author(s) reviewed and edited the content as needed and took full responsibility for the publication.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Correction note

A correction has been made to this article. Details can be found at: [10.3389/fmicb.2026.1795378](https://doi.org/10.3389/fmicb.2026.1795378).

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1664404/full#supplementary-material>

References

- Abdulla, H., May, E., Bahgat, M., and Dewedar, A. (2008). Characterization of actinomycetes isolated from ancient stone and their potential for deterioration. *Polish J. Microbiol.* 57, 213–220. doi: 10.31086/pm.2008.23
- Aldersey-Williams, H. (2013). *The art of making: techniques and traditions of traditional crafts*. London: Thames and Hudson.
- Ashurst, J. (1983). *Mortars, plasters and renders in conservation*. London: Ecclesiastical Architects' and Surveyors' Association.
- Baker, L. (2007). *Exploring decorative art techniques*. Oxford: Oxford University Press.
- Bish, D. L., and Reynolds, R. C. Jr. (1989). "Sample preparation for X-Ray diffraction," in *Modern powder diffraction. Reviews in mineralogy*, vol. 20, eds D. L. Bish and J. E. Post (Washington, DC: Mineralogical Society of America), 73–99. doi: 10.1515/9781501509018-007
- Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6:90. doi: 10.1186/s40168-018-0470-z
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N., Abnet, C., Al-Ghalith, G., et al. (2019). Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869
- Campo, F. P., Tua, C., Biganzoli, L., Pantini, S., and Grosso, M. (2021). Natural and enhanced carbonation of lime in its different applications: A review. *Environ. Technol. Rev.* 10, 224–237. doi: 10.1080/21622515.2021.1982023
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U S A* 15:4516. doi: 10.1073/pnas.100080107
- Caron, R. (2015). *Biodeterioration of decorative art: Impacts and prevention strategies*. Berlin: Springer.
- Carran, D., Hughes, J., Leslie, A., and Kennedy, C. (2011). A short history of the use of lime as a building material beyond Europe and North America. *Int. J. Architectural Heritage* 6, 117–146. doi: 10.1080/15583058.2010.511694
- Cassar, J. (2002). The conservation of architectural heritage: A case study of the biodeterioration of lime mortar in Mediterranean climates. *Int. J. Architectural Heritage* 1, 119–136. doi: 10.1080/15583050210146176
- Chomnunti, P., Hongsanan, S., Aguirre-Hudson, B., Tian, Q., Peršoh, D., Dharni, K. M., et al. (2014). The sooty molds. *Fungal Divers.* 66, 1–36. doi: 10.1007/s13225-014-0278-5
- Cozzolino, A., Adamo, P., Bonanomi, G., and Motti, R. (2021). The role of lichens, mosses, and vascular plants in the biodeterioration of historic buildings: A review. *Plants* 11:3429. doi: 10.3390/plants11243429
- De Leo, F., Antonelli, F., Pietrini, A. M., Ricci, S., and Urzi, C. (2019). Study of the endolithic activity of black meristematic fungi isolated from a marble statue in the Quirinale Palace's Gardens in Rome, Italy. *Facies* 65, 1–10. doi: 10.1007/s10347-019-0564-5
- De Miguel, J. M. G., Sanchez-Castillo, L., Ortega-Calvo, J. J., Gil, J. A., and Saiz-Jimenez, C. (1995). Deterioration of building materials from the Great Jaguar Pyramid at Tikal, Guatemala. *Building Environ.* 30, 591–598. doi: 10.1016/0360-1323(94)00055
- Del Mondo, A., Zuccaro, G., Petraretti, M., Pollio, A., and De Natale, A. (2021). Water absorption coefficient drives Nostoc sp. colonization on mineral substrates. *Int. Biodeterioration Biodegradation* 164:105291. doi: 10.1016/j.ibiod.2021.105291
- Dyda, M., Pyzik, A., Wilkojc, E., Kwiatkowska-Kopka, B., and Sklodowska, A. (2019). Bacterial and fungal diversity inside the medieval building constructed with sandstone plates and lime mortar as an example of the microbial colonization of a nutrient-limited extreme environment (Wawel Royal Castle, Krakow, Poland). *Microorganisms* 7:416. doi: 10.3390/microorganisms7100416
- Elert, K., Rodríguez-Navarro, C., Pardo, E. S., Hansen, E., and Cazalla, O. (2002). Lime mortars for the conservation of historic buildings. *Stud. Conserv.* 47, 62–75. doi: 10.1179/sic.2002.47.1.62
- Erdmann, E. A., Nitsche, S., Gorbushina, A. A., and Schumacher, J. (2022). Genetic engineering of the rock inhabitant *Knufia petricola* provides insight into the biology of extremotolerant black fungi. *Front. Fungal Biol.* 8:862429. doi: 10.3389/ffunb.2022.862429
- Farkash, S., and Tal, M. (2017). "Sgraffito in Israel, presentation in Sgraffito change—materials, techniques, topics, and preservation," in *Proceedings of the International Conference of the HAWK University of Applied Sciences and Arts*, (Germany).
- Ferguson, W. (2010). *Renaissance art and architecture*. Milton Park: Routledge.
- Fuentes, E., Carballeira, R., and Prieto, B. (2021). Role of exposure on the microbial consortia on historical rural granite buildings. *Appl. Sci.* 11:3786. doi: 10.3390/app11093786
- Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi: 10.1111/j.1365-294x.1993.tb00005.x
- Gauri, K. L., and Holdren, G. C. (1981). "Biodeterioration of stone surfaces," in *Stone: damage and conservation*, ed. G. C. Holdren (West Conshohocken: ASTM International), 115–122.
- Gaylarde, C. C., Ortega-Morales, B. O., and Bartolo-Pérez, P. (2007). Biogenic black crusts on buildings in unpolluted environments. *Curr. Microbiol.* 54, 162–166. doi: 10.1007/s00284-006-0432-8
- Gaylarde, C., Gaylarde, P., Copp, J., and Neilan, B. (2004). Polyphasic detection of cyanobacteria in terrestrial biofilms. *Biofouling* 20, 71–79. doi: 10.1080/08927010410001681237
- Gu, J. D. (2003). Microbiological deterioration and degradation of synthetic polymeric materials: Recent research advances. *Int. Biodeterioration Biodegradation* 52, 69–91. doi: 10.1016/S0964-8305(02)00177-4
- Harding, M. W., Marques, L. L., Howard, R. J., and Olson, M. E. (2009). Can filamentous fungi form biofilms? *Trends Microbiol.* 17, 475–480. doi: 10.1016/j.tim.2009.08.007
- Isola, D., Zucconi, L., Onofri, S., Caneva, G., de Hoog, G. S., and Selbmann, L. (2015). Extremotolerant rock-inhabiting black fungi from Italian monumental sites. *Fungal Divers.* 76, 75–96. doi: 10.1007/s13225-015-0342-9
- Israel Meteorological Service. (2025). Available online at: <https://ims.gov.il> (accessed 15.11.2025)
- Jagiello, M. (2022). Sgraffito as a method of wall decoration in the renaissance and mannerist silesia. *Arts* 11:25. doi: 10.3390/arts11010025
- Jung, P., Mikhailyuk, T., Emrich, D., Dultz, K., Dultz, S., and Büdel, B. (2020). Shifting boundaries: Ecological and geographical range extension based on three new species in the cyanobacterial genera *Cyanocohniella*, *Oculatella*, and *Literella*. *J. Psychol.* 56, 1216–1231. doi: 10.1111/jpy.13025
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808
- Knabe, N., and Gorbushina, A. A. (2018). Territories of rock-inhabiting fungi: Survival on and alteration of solid air-exposed surfaces. *Methods Microbiol.* 45, 145–169. doi: 10.1016/bs.mim.2018.06.001
- Kukletová, I., and Chromková, I. (2018). Testing of algae colonization growth risk on building materials. *Materials Sci. Eng.* 379:012041. doi: 10.1088/1757-899X/379/1/012041
- Liu, X., Koestler, R. J., Warscheid, T., Katayama, Y., and Gu, J. D. (2020). Microbial deterioration and sustainable conservation of stone monuments and buildings. *Nat. Sustainability* 3, 991–1004. doi: 10.1038/s41893-020-00602-5
- Liu, X., Qian, Y., Wu, F., Wang, Y., Wang, W., and Gu, J. D. (2022). Biofilms on stone monuments: Biodeterioration or bioprotection? *Trends Microbiol.* 30, 816–819. doi: 10.1016/j.tim.2022.05.012
- Manoharan, A., and Umarani, C. (2022). Lime mortar, a boon to the environment: Characterization case study and overview. *Sustainability* 14:6481. doi: 10.3390/su14116481
- McNamara, C. J., Perry, T. D., Bearce, K. A., Hernandez-Duque, G., and Mitchell, R. (2010). "Epilithic and endolithic bacterial communities in limestone from a maya archaeological site," in *Cultural heritage microbiology: Fundamental studies in conservation science*, eds C. J. McNamara and R. Mitchell (Washington, DC: American Society for Microbiology (ASM Press)).
- Meng, S., Qian, Y., Liu, X., Wang, Y., Wu, F., Wang, W., et al. (2023). Community structures and biodeterioration processes of epilithic biofilms imply the significance of micro-environments. *Sci. Total Environ.* 876:162665. doi: 10.1016/j.scitotenv.2023.162665
- Mundy, P. (2013). *Preserving the past: a technical guide to historic building conservation*. Milton Park: Routledge.
- Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., et al. (2019). The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47. doi: 10.1093/nar/gky1022
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219
- Rabbachin, L., Nir, I., Waldherr, M., Vassallo, Y., Piñar, G., Graf, A., et al. (2024). Diversity of fungi associated with petroglyph sites in the Negev Desert, Israel, and their

potential role in bioweathering. *Front. Fungal Biol.* 5:1400380. doi: 10.3389/ffunb.2024.1400380

Ramírez, M., Hernández-Maríné, M., Mateo, P., Berrendero, E., and Roldán, M. (2011). Polyphasic approach and adaptive strategies of *Nostoc cf. commune* (Nostocales, Nostocaceae) growing on Mayan monuments. *Fottea* 11, 73–86. doi: 10.5507/fot.2011.008

Saadouli, I., Marasco, R., Mejri, L., Hamden, H., Guerfali, M. M., Stathopoulou, P., et al. (2022). Diversity and adaptation properties of actinobacteria associated with Tunisian stone ruins. *Front. Microbiol.* 13:997832. doi: 10.3389/fmicb.2022.997832

Sanmartín, P., Méndez, A., Carballeira, R., and Elena López, E. (2021a). New insights into the growth and diversity of subaerial biofilms colonizing granite-built heritage exposed to UV-A or UV-B radiation plus red LED light. *Int. Biodeterioration Biodegradation* 161:105225. doi: 10.1016/j.ibiod.2021.105225

Sanmartín, P., Miller, A. Z., Prieto, B., and Viles, H. A. (2021b). Revisiting and reanalyzing the concept of bio receptivity 25 years on. *Sci. Total Environ.* 20:145314. doi: 10.1016/j.scitotenv.2021.145314

Selbmann, L., Zucconi, L., Isola, D., and Onofri, S. (2015). Rock black fungi: Excellence in the extremes, from the Antarctic to space. *Curr. Genet.* 61, 335–345. doi: 10.1007/s00294-014-0457-7

Sert, H. B., Sümbül, H., and Sterflinger, K. (2007). Microcolonial fungi from antique marbles in Perge Side Termessos (Antalya/Turkey). *Antonie Van Leeuwenhoek* 91, 217–227. doi: 10.1007/s10482-006-9111-9

Urzi, C., Salamone, P., Schumann, P., Rohde, M., and Stackebrandt, E. (2004). *Blastococcus saxosidens* sp. nov., and emended descriptions of the genus *Blastococcus* Ahrens and Moll 1970. *Int. J. Syst. Evol. Microbiol.* 54, 253–259. doi: 10.1099/ijls.0.02745-0

Warscheid, T., and Braams, J. (2000). Biodeterioration of stone: A review. *Int. Biodeterioration Biodegradation* 46, 343–368. doi: 10.1016/S0964-8305(00)00109-8

Weissbrod, T., and Sneh, A. (1997). Stratigraphy and sedimentology of the Lower cretaceous amir formation in Israel and Sinai: A marine clastic deposit with tidal channels. *Israel J. Earth Sci.* 46, 79–93.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. (1990). “Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics,” in *PCR protocols: A Guide to methods and applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, (New York, NY: Academic Press), 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1

Wojcieszak, M., Fontaine, L., Elsen, J., Hayen, R., Lehouck, A., and Boudin, M. (2024). Historic lime mortar composition and terminology for radiocarbon dating—case studies based on thin-section petrography and cathodoluminescence. *Radiocarbon* 66, 1814–1834. doi: 10.1017/RDC.2024.14

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and “All-species Living tree project (LTP)” taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209



OPEN ACCESS

APPROVED BY
Frontiers Editorial Office,
Frontiers Media SA, Switzerland

*CORRESPONDENCE

Irit Nir
✉ iritnir1@gmail.com
Ariel Kushmaro
✉ arielkus@bgu.ac.il

RECEIVED 24 January 2026
REVISED 24 January 2026
ACCEPTED 26 January 2026
PUBLISHED 09 February 2026

CITATION

Nir I, Sharaby A, Barak H, Pavan MJ,
Friedlander LR, Multanen V and
Kushmaro A (2026) Correction: Extensive
biofilm covering on sgraffito wall art: a
call for proactive monitoring.
Front. Microbiol. 17:1795378.
doi: 10.3389/fmicb.2026.1795378

COPYRIGHT

© 2026 Nir, Sharaby, Barak, Pavan,
Friedlander, Multanen and Kushmaro.
This is an open-access article distributed
under the terms of the [Creative
Commons Attribution License \(CC BY\)](#).
The use, distribution or reproduction in
other forums is permitted, provided the
original author(s) and the copyright
owner(s) are credited and that the
original publication in this journal is
cited, in accordance with accepted
academic practice. No use, distribution
or reproduction is permitted which does
not comply with these terms.

Correction: Extensive biofilm covering on sgraffito wall art: a call for proactive monitoring

Irit Nir^{1*}, Anath Sharaby¹, Hana Barak¹, Mariela J. Pavan²,
Lonia R. Friedlander², Victor Multanen¹ and Ariel Kushmaro^{1,2,3*}

¹Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer Sheva, Israel, ²Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer Sheva, Israel, ³The Goldman Sonnenfeldt School of Sustainability and Climate Change, Ben-Gurion University of the Negev, Beer Sheva, Israel

KEYWORDS

sgraffito, wall art, lime mortar, bio-weathering, next-generation sequencing (NGS)

A Correction on

[Extensive biofilm covering on sgraffito wall art: a call for proactive monitoring](#)

by Nir, I., Sharaby, A., Barak, H., Pavan, M. J., Friedlander, L. R., Multanen, V., and Kushmaro, A. (2026). *Front. Microbiol.* 16:1664404. doi: 10.3389/fmicb.2025.1664404

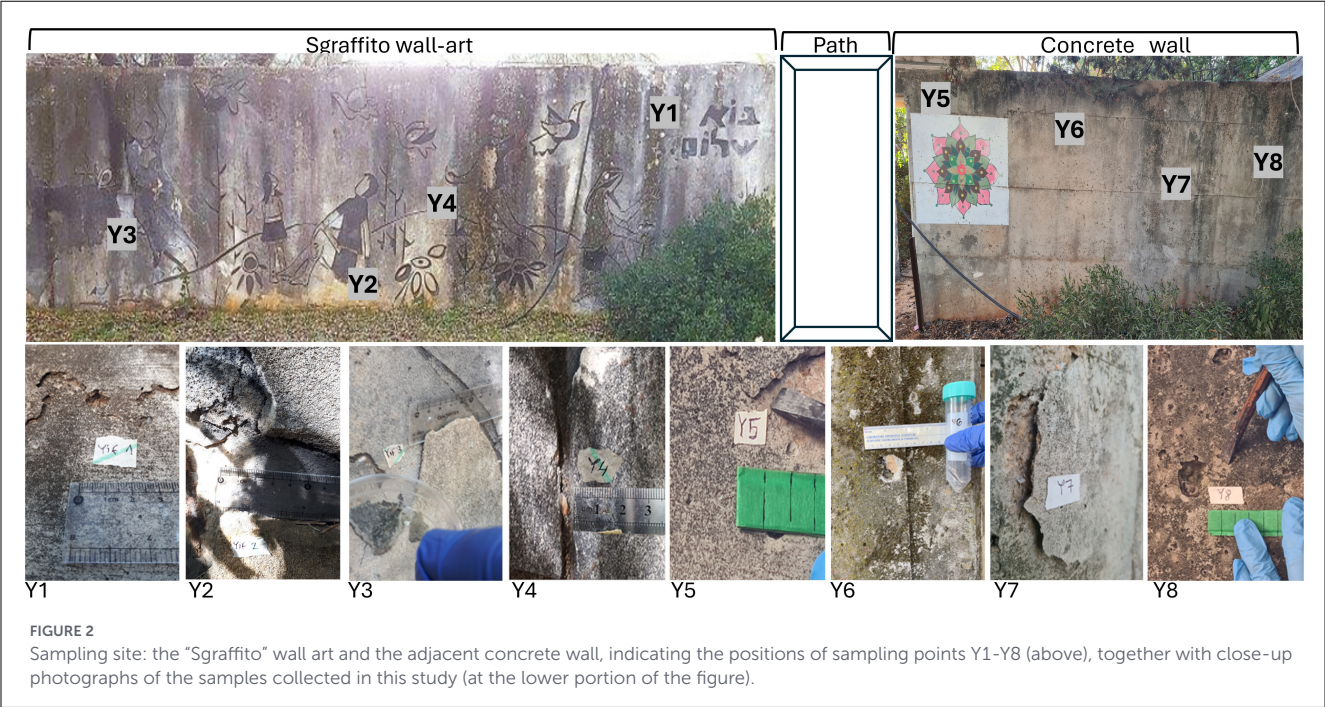
Error in Figure 2

There was a mistake in [Figure 2](#) as published. The sample numbers/location Y1, Y2, Y3, and Y4 were missing from the upper section of [Figure 2](#). The corrected [Figure 2](#), with the sample numbers Y1, Y2, Y3, and Y4, appears below.

The original version of this article has been updated.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.



Frontiers in Microbiology

Explores the habitable world and the potential of microbial life

The largest and most cited microbiology journal which advances our understanding of the role microbes play in addressing global challenges such as healthcare, food security, and climate change.

Discover the latest Research Topics

[See more →](#)

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact

