



## OPEN Taxonomic and metabolic characterisation of biofilms colonising Roman stuccoes at Baia's thermal baths and restoration strategies

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Stuccoes are very delicate decorative elements of Roman age. Very few of them survived almost intact to present days and, for this reason, they are of great interest to restorers and conservators. In this study, we combined metabarcoding and untargeted metabolomics to characterise the taxonomic and metabolic profiles of the microorganisms forming biofilms on the stuccoes located on the ceiling of the *laconicum*, a small thermal environment in the archaeological park of Baia (southern Italy). We found that some samples were dominated by bacteria while others by eukaryotes. Additionally, we observed high heterogeneity in the type and abundance of bacterial taxa, while the eukaryotic communities, except in one sample (at prevalence of fungi), were dominated by green algae. The metabolic profiles were comparable across samples, with lipids, lipid-like molecules and carbohydrates accounting for roughly the 50% of metabolites. In vitro and in vivo tests to remove biofilms on stuccoes using essential oils blends were successful at a 50% dilution for one hour and half. This integrative study advanced our knowledge on taxonomic and metabolic profiles of biofilms on ancient stuccoes and highlighted the potential impacts of these techniques in the field of cultural heritage conservation.

**Keywords** Ancient stuccoes, Archaeological site, Essential oils, Metabarcoding, Metabolomics, Microbial communities

Stuccoes are traditional decorative elements for ceilings and vaults, but also vertical surfaces, in buildings and villas of the Roman age<sup>1,2</sup>. Before the discovery of Nero's *Domus Aurea* in Rome in the 15th century, the only evidence of these artworks came from written accounts, such as like Pliny the Elder's *Naturalis Historia*<sup>3</sup>. Roman stuccoes are frequently polychrome, but for many years were supposed to be white or monochromatic; however, likewise Greek statues and temples, we now know that the white coloration was generally due to the loss of pigments rather than a lack of them. No single recipe seemed to exist for their preparation<sup>4</sup>. Recent analyses of mineralogical composition of stuccoes from the *Domus Aurea* revealed the same elemental composition of plaster, i.e., the use of calcium hydroxide as binder and calcite as aggregates<sup>5</sup>. Due to the delicate carving techniques, very few stuccoes have survived intact or almost intact to present days and, in rarest cases, have preserved the original colours. Examples can be found in the abovementioned *Domus Aurea* in Rome<sup>6</sup>, in some tombs excavated in the area of Pozzuoli<sup>7,8</sup>, in the area of Baia<sup>9</sup> and Pompeii, Herculaneum and Stabiae<sup>10,11</sup>. Furthermore, after being uncovered by archaeological excavations, as all decorative features, stuccoes are also exposed to deterioration by abiotic and biotic agents, which alter both their structure and aesthetics. For all these reasons, they are of particular conservation interest.

In recent years, the study of deterioration caused by biological agents (biodegradation) has been fostered by the introduction of -omics techniques<sup>12</sup>. These techniques have enabled not only the taxonomic characterisation

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of microbial communities involved in the colonisation of substrata (metabarcoding and metagenomics) but also their metabolic activity (metatranscriptomics, metabolomics, proteomics). Some of these applications like metabarcoding are becoming routinary (e.g.<sup>13–16</sup>), while the others are still at their infancy. Understanding the type and activity of microorganisms is crucial for planning the best strategies for their removal and to prevent future colonisations. To date, no published studies have used -omics tools for the study of biodeterioration of ancient stuccoes.

In this study, we employed a multi-omics approach combining metabarcoding of two molecular markers (16S for bacterial communities and 18S for eukaryotic communities) and untargeted metabolomics to assess the taxonomic and metabolic profiles of the bacterial and eukaryotic community involved in the biodeterioration of Roman stuccoes in a thermal environment called *laconicum* in the archaeological site of Baia (Campania region, Italy). To the best of our knowledge, this is the first attempt to characterise both microorganism and metabolite diversity using -omics approaches in ancient stuccoes. Furthermore, we tested the efficacy of extracts of essential oils at different dilutions to remove the biological patinas from the stuccoes, utilising homemade tiles as test samples.

## Materials and methods

### Study site and sampling

The *laconicum* is located on the terrace of the upper peristyle of the so called “Sosandra sector” in the archaeological park of Baia<sup>17</sup> (Campania region, Italy). The room is characterised by an “L”-shaped plan, with a main rectangular body opening eastward, and a corridor that opens southward into an adjacent room, where the statue of the Venus Sosandra was found in 1953<sup>9</sup>. The room is of small size, with a surface area of approximately 5 m<sup>2</sup>. It is a *balneum*, a private environment with a thermal function, specifically used for steam baths (*laconicum*). The internal wall structure of the *laconicum* is made of *opus reticulatum* flanked by *opus vittatum*, and the ceiling is decorated with stuccoes (Fig. 1A). The narrow corridor showcases a sequence of medallions arranged along the main north-south axis, each connected to its neighbours and to the edges of the ceiling through short, straight bands. The frames are decorated with delicate pearl mouldings, triple for the medallions and double for the connecting strips (Fig. 1B). The medallions have a diameter of 40 cm, while the mouldings are 6 cm wide (triple) and 3 cm wide (double). Among the figures depicted are animals such as lions, swans, and marine creatures and mythological characters like Cupid and Nereids (Fig. 1B). The stuccoes are made of dolomitic lime with calcium and magnesium carbonate as binders. The crystalline structure of the dolomite gives greater compactness and hardness to the lime, making it more resistant. The high quantity of dehydrated calcium sulphate on the surface of some of them can be attributed to the presence of saline efflorescence.

Biological patinas were sampled with sterile scalpels by a restoration student (Sara Scamardella) thanks to an agreement between the University of Studies Suor Orsola Benincasa and the archaeological park of Baia. Two samples (S1 and S2) were collected from the inner east side of the *laconicum*, while the other five from the



**Figure 1.** The *laconicum* in the Sector of the Sosandra (Baia archaeological park, southern Italy). (A) Sampling points; (B) details of sampling points.

ceiling decorated with stuccoes (S3-S7). Sample S2 was close to a saline efflorescence. The samples were placed into plastic tubes and stored at -20 °C until later analyses.

### Metabarcoding analyses

The bacterial and eukaryotic components of biological patinas from the stuccoes were characterised through a metabarcoding approach, amplifying the V3-V4 region of 16S rRNA, and the V4 region of 18S rRNA, respectively. Total DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. A qualitative and quantitative analysis of extracted DNA was carried out through visualisation on gel electrophoresis and with a Qubit v4 fluorometer using the dsDNA HS Assay Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Extracted DNA was stored at -20 °C until shipment for amplification and high-throughput sequencing. Metabarcoding analyses were carried out by Integrated Microbiome Resource (IMR; Halifax, Canada) at the condition specified on the company website (<http://imr.bio/protocols>) and using the primer sets ("Bacteria-specific "Illumina" V3-V4, B969F and BA1406R"<sup>18</sup> and "Eukaryote-specific V4, E572F and E1009R"<sup>19</sup>) described in the same webpage. To monitor possible biological contaminations, an extraction blank (sample "ctrl") was processed as negative control alongside the other samples till the sequencing step; in addition, PCR controls were carried out by the company.

Raw data (fastq format) were processed to generate amplicon sequence variants (ASVs) using the dada2 pipeline<sup>20</sup> in R<sup>21</sup>. At the end of the pipeline, singletons were removed and, to account for differences in the number of ASVs across samples, data were normalised to the median value (20,206 for 16S dataset and 138 for 18S dataset) using the "rrarefy" function of the vegan R package<sup>22</sup>. Taxonomic assignment of eukaryotes was carried out using a BLAST approach<sup>23</sup> against the PR2 database v4.14.0<sup>24</sup> (<https://github.com/pr2database/pr2database/releases>) and, for hits not assigned, against the nucleotide (nr) database. Five hits were stored for each query and the final assignment was determined using a lowest common ancestor (lca) approach to identify ASVs at the lowest taxonomic level when different matches occurred at the same similarity percentage. For the latter task, we used the galaxy-tool-lca script<sup>25</sup> (<https://github.com/naturalis/galaxy-tool-lca>), which is partly based on MEGAN's lca method<sup>26</sup>. Taxonomic assignment of bacterial ASVs was carried out using the naive Bayesian classifier method<sup>27</sup> against the Silva reference database v138.1<sup>28</sup> (<https://zenodo.org/record/4587955#.Ylqor9NBw2w>); assignment at species level were determined by exact match (100% identity) between ASVs and sequenced reference strains always within dada2 using the "silva\_species\_assignment\_v138.1" database.

Taxonomic composition of bacterial, eukaryotic, and combined communities was represented as barplots in RStudio<sup>29</sup> using the phyloseq package<sup>30</sup> and plotted with ggplot2<sup>31</sup>. Venn diagrams were built using the file2meco<sup>32</sup> and microeco<sup>33</sup> R packages to assess whether communities with similar taxonomic composition at Domain rank (bacteria vs. eukaryotes) in the barplots were also similar at lower taxonomic levels (phylum and family). A principal component analysis (PCA) was carried out on the ClustVis webserver<sup>34</sup> (<https://biit.cs.ut.ee/clustvis/>) to detect structure in our 16S, 18S, and 16S + 18S data.

### Metabolomic analysis

Approximately 10 mg of pulverized sample material was weighed into a brown vial and exact mass was noted. A quantity of 0.5 mL of acetone: methanol (50:50) was added to each sample. Samples were mixed using a vortex at 1650 rpm for 3 min and then placed in a glass container filled with crushed ice; the container was kept in an ultrasonic bath for 30 min. The extract was filtered into a LC vial using a 0.2 µm filter, and the vial was stored in a freezer (-80 °C) until analysis. The extraction procedure was repeated two times or more for the same sample.

Extracted samples were analyzed by mass spectrometry using the Q-Orbitrap EXPLORIS 120 (Thermo Fisher Scientific, Foster City, CA, USA) and by Ultra-Performance Liquid Chromatography–Mass Spectrometry (UPLC-MS) analysis to get the metabolite profile, using a VANQUISH UPLC (Thermo Fisher Scientific, Foster City, CA, USA) coupled to a Q EXACTIVE mass spectrometer (Thermo Fisher Scientific, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source in positive mode. A volume of 5 µL of sample was injected into a Hypersil GOLD™ C18 column (2.1 × 200 mm, 1.9 µm, Thermo Fisher Scientific, Foster City, CA, USA). Mobile phase was A: water + 0.1% TFA and B: Acetonitrile + 0.1% TFA. Gradient settings were: 0 min 5%B, 10 min 70%B, 11 min 70–95%B, isocratic for 1 min. Total flow was 0.35 ml min<sup>-1</sup>, column temperature was 40 °C. Chromatographic data were also recorded using a Photodiode array detector operating with a frequency of 12.5 Hz.

Metabolites were assigned to functional groups using ClassyFire<sup>35</sup> (<http://classyfire.wishartlab.com>) after conversion of chemical names to several formats (ChEBI, KEGG, and InChI codes) in the Chemical Translation Service (CTS; <http://cts.fiehnlab.ucdavis.edu/batch>). Metabolic profiles across samples were showed as barplots of functional groups using the packages phyloseq<sup>30</sup> and ggplot2<sup>31</sup>. A heatmap was plotted to visualise the abundance patterns of selected metabolites across samples; we selected metabolites that occurred at least in two samples and with abundance > 1%. A PCA was also carried out as per metabarcoding data to detect possible structure in our samples. To link the metabolites to specific metabolic pathways, we used the standard KEGG compound names (C codes) previously retrieved in CTS as input for MetaboAnalyst v6.0<sup>36</sup> (<https://www.metaboanalyst.ca/MetaboAnalyst/Secure/Utils/NameMapView.xhtml>), and then we used the KEGG<sup>37,38</sup> mapper search tool (<https://www.genome.jp/kegg/mapper/search.html>).

### Metabarcoding-metabolomics associations

A PerMANOVA analysis with the adonis function of the vegan package<sup>22</sup> was carried out to detect significant associations between abundance of microbial (bacterial and eukaryotic) communities and metabolite concentrations.

## Removal of biological patinas with essential oils

For the removal of biological patinas, we used ESSENZIO (IBIX Biocare, Lugo, Italy), a biodegradable and biocompatible product based on a blend of essential oils (mainly extracts of *Origanum vulgare* and *Thymus vulgaris*). Before in vivo removal of patinas, the product was first tested on homemade tiles of slaked lime and marble powder contaminated with biofilms taken in situ and grown in vitro to simulate the surface of the stuccoes. Based on the results obtained by Cennamo et al.<sup>39</sup>, the mixture was tested at different dilutions in demineralized water (10%, 20% and 50%) and after different application times (30 min, 1 h, 1 h and 30 min, 2 h) on specimens prepared in the laboratory (data not shown). The effectiveness of the treatment was evaluated through a visual comparison with clean specimens. Once the most suitable times and concentrations have been identified, the treatment was extended to the entire portion of the stucco decorations where biological patinas were observed.

## Results

### Metabarcoding analyses

The Illumina sequencing of V3–V4 16S region generated 215,759 raw reads distributed across seven samples. The clean, annotated dataset contained 82,352 sequences corresponding to 265 ASVs in four samples (S1, S2, S3, and S6); after the normalization procedure, 73,039 sequences and 265 ASVs remained (Table S1). The raw eukaryotic dataset based on the 18S–V4 region included 34,150 sequences across seven samples. The clean, annotated dataset contained 23,245 sequences corresponding to 23 ASVs in three samples (S4, S5, and S7); after normalization, 552 sequences and 18 ASVs remained (Table S2). Details regarding the number of sequences discarded in each pre-processing step per sample are provided in Table S3. The negative control (sample “ctrl”) resulted in no sequences for 16S marker and 21 sequences assigned to *Cladosporium* and 24 to humans for 18S marker; since human sequences were discarded as non-target and the fungal ones were not found in any of our samples, we excluded a possible role of contaminants in our diversity estimates.

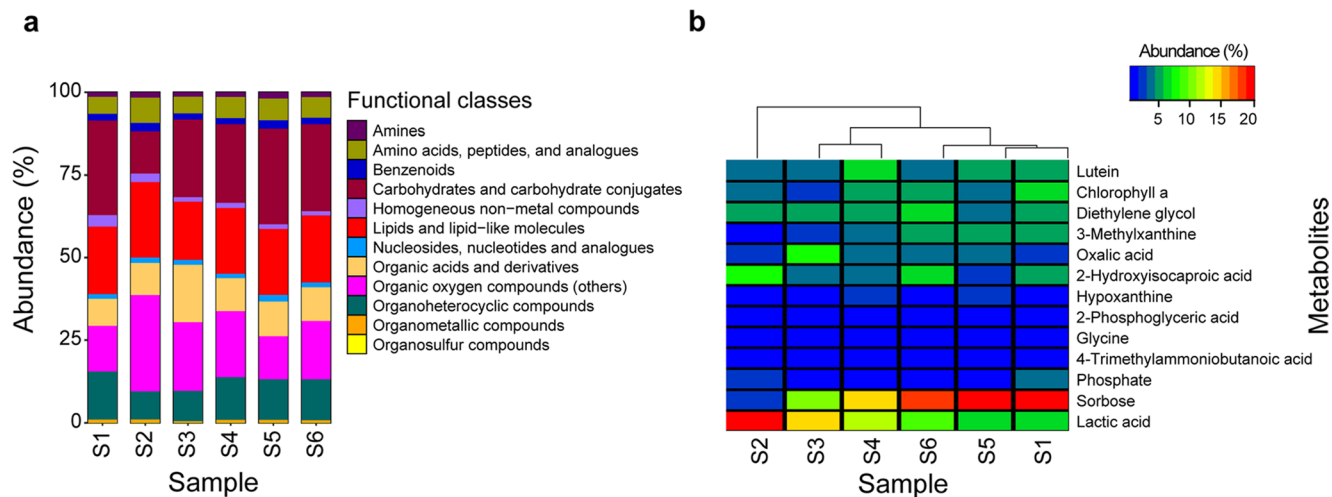
In four out of seven samples, microbial community was constituted almost exclusively by bacteria, while in the remaining three samples, by eukaryotes (Fig. 2A); regarding samples from stuccoes, three out of five (S4, S5, and S7) were dominated by eukaryotes. Bacterial taxa were absent in samples S4, S5, and S7, while eukaryotic taxa occurred in all but S2 sample. The 95.1% of bacterial ASVs assigned to phylum level were shared by all samples (Fig. 2B), while only the 59.6% of ASVs at family level was shared (Fig. 2C). Regarding eukaryotes, only the 33.8% of ASVs attributed to phylum level was shared, and the remaining 66.2% of ASVs were exclusive to sample S7 (Fig. 2D). At family rank, no ASVs were shared, with the 67.6% of them exclusive to sample S7, the 29.9% to sample S5 and only 2.5% to sample S4 (Fig. 2E). A total of 14 bacterial families with abundance > 5% of total sequences were identified (Fig. 2F). *Euzebyaceae* was the only family present in all taxa and at notable abundance; members of *Nitrliruptoraceae* were present only in three samples (S1, S3, and S6). Not considering unclassified bacteria and families collapsed at abundance < 5%, bacterial community of most samples was represented by 6–8 families; the only exception was sample S2, with only three families (*Euzebyaceae*, *Termosynechocaceae*, and *Trueperaceae*), of which *Termosynechocaceae* included roughly the half of ASVs. With the only exception of *Euzebyaceae*, *Pseudonocardiaceae*, and *Rhizobiaceae*, all the other families were represented by single genera or unclassifiable sequences at lower taxonomic levels (Table S1). Most of the latter were in the family *Nitrliruptoraceae*, while among cyanobacteria we observed some taxa particularly abundant for which assignment was not achieved even at family level (Table S1). Only five assignments at species level were obtained (*Aliihoeflea aestuarii*, *Halomonas chromatireducens*, *Nocardiopsis exhalans*, *Pelagibacterium lentulum*, and *Streptomyces sodiophilus*), plus two ambiguities (*Brevundimonas bacteroides*/*B. variabilis* and *Nocardiopsis exhalans*/*N. valliformis*).

The eukaryotic component was dominant in samples S4, S5 and S7 (Fig. 2A) but overall, taxonomically limited, accounting almost exclusively for chlorophytes and fungi (Fig. 2G). Among the former ones, we identified *Picocystis salinarum* R.A.Lewin, and *Ctenocladus circinnatus* Borzi, and assigned other ASVs to the genera *Picochlorum* W.J.Henley & al., and *Pseudostichococcus* L.Moewus (Table S2); for fungi, the only attribution at genus level was for *Cyphellophora* de Vries (*Eurotiomycetes*), while all the other ASVs belonged to the classes *Dothideomycetes* and *Sordariomycetes*. Such taxa were not equally distributed across samples, with *Picocystis salinarum* occurring only in samples S1 and S3, *Ctenocladus* in samples S1 and S6, *Picochlorum* in sample S7, and *Pseudostichococcus* in samples S5 and S6. Similarly, ASVs assigned to fungi, despite particularly abundant in sample S7, were exclusive of each sample and never shared (Table S2). The PCA analysis based on the bacterial dataset (Supplementary Figure S1a) separated along the first axis (46.5% of variance) sample S6 from S2, S3 and S1, the latter further separated from the others on the second axis by 33.7% of variance. Similarly, in the PCA analysis based on eukaryotic data (Supplementary Figure S1b), sample S6 was separated by all the others on PC1 (43.3% of variance), and S1 by the remnant samples by PC2 (32%); eukaryotic communities of samples S3, S4, and S5 were closely related. The same pattern of distinctiveness of samples S6 and S1 along PC1 and PC2, respectively was observed in the combined dataset (Supplementary Figure S1c). The PCA based on metabolomic data (Supplementary Figure S1d) showed a different pattern, with samples S1, S4, and S6 separated from the others on the first axis (57.3% of variance), and S1 and S2 from the others on the second axis (17.3% of variance).

### Metabolomic analyses and metabarcoding-metabolomics associations

Employing mass spectrometry, we identified and annotated 162 metabolites across six powdered material samples (Table S4). Sample S7 was excluded from the analysis due to insufficient source material, which led to a failed extraction. Almost a quarter of metabolites belonged to lipids and lipid-like (fatty acids) molecules (23.5%) and carbohydrates (21.6%), followed by organic acids and derivatives (13.6%) and amino acids, peptides, and analogues (12.3%) (Fig. 3A); a list of the other classes of compounds is available in Table S4. According to the heatmap based on the most abundant metabolites (Fig. 3B), the sample S2 showed a different metabolic





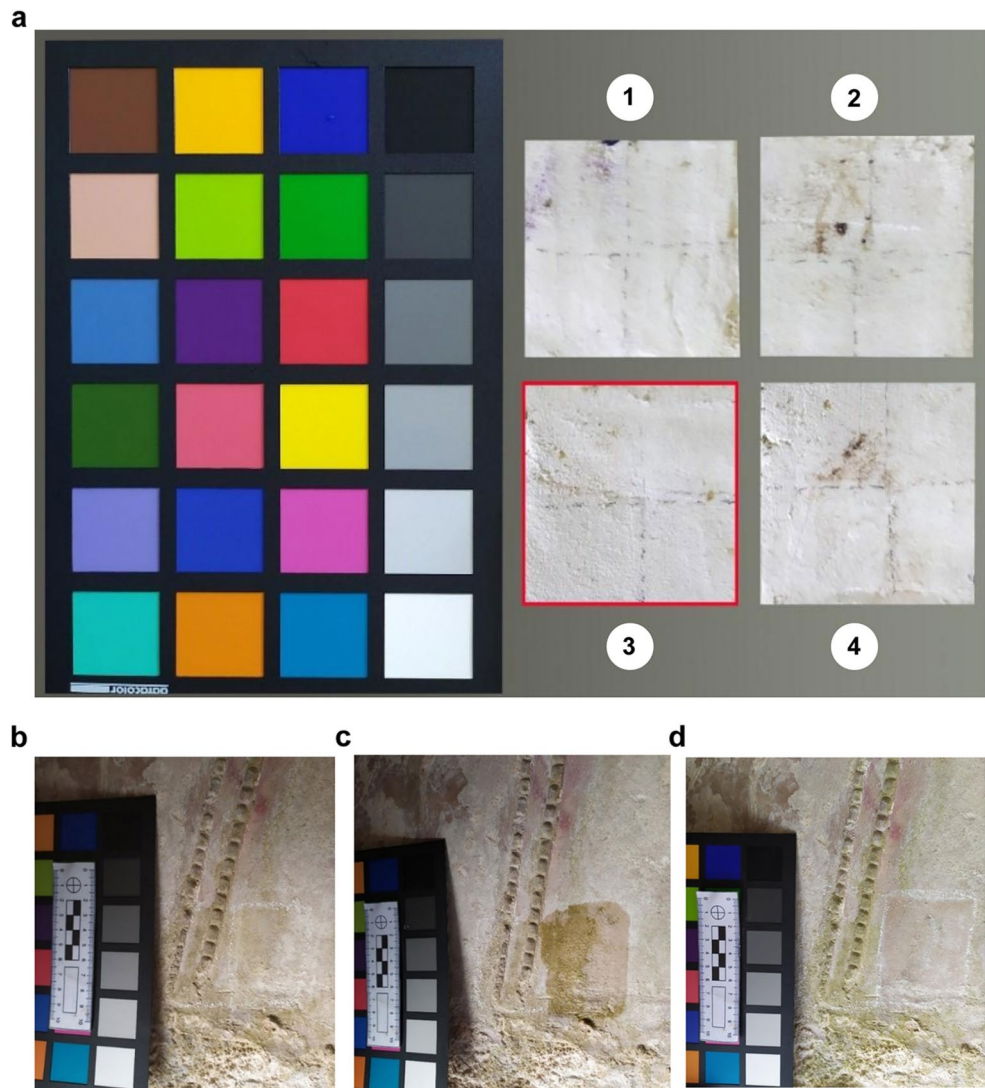
**Figure 3.** Metabolic patterns of samples. **(A)** Barplots of relative abundance of functional groups of metabolites; **(B)** metabolic profile of the most abundant metabolites (see Material and methods for further details) across samples.

### Removal of biofilms

The conditions that were found to be optimal for removing the biological patinas on the mortar test specimens were dilution of ESSENZIO at 50% in demineralized water and application time of an hour and a half (Fig. 4A, point 3). In all the other tiles, dark spots are visible as indicative of the presence of microorganisms. After this trial, the above-mentioned treatment was applied in-situ on all the stuccoes under restoration; an example of treatment is provided in Fig. 4B and C, and 4D. Since the biofilm layers were rather thin and not uniformly distributed, it was not necessary to use a support that would allow for a longer exposure time. At last, the surface was rinsed and cleaned with scalpel and swab. After 11 months from the treatment, no biofilms have been observed yet. An example of stucco decoration before and after removal of biofilms with essential oils is provided in Supplementary Figure S2.

### Discussion

The mechanisms of biodeterioration of cultural heritage have been studied for many years. From the first, culture-based approaches, which often favour the detection of few, fast-growing opportunistic species over other, less abundant ones<sup>40–42</sup>, the introduction of culture-independent methods has revealed an astonishing taxonomic diversity of microorganisms colonising monuments and historical manufactures<sup>43–45</sup>. However, such pioneer techniques as DGGE (Denaturing Gradient Gel Electrophoresis) and ARISA (Automated Ribosomal Intergenic Spacer Analysis), to cite just a few, did not provided a taxonomic information about the members of a given microbial community, and were often coupled to culture-based approaches<sup>46,47</sup>. The introduction of Next Generation Sequencing (NGS) approaches and their affordability of in terms of data processing, and reduced costs over years, has empowered the knowledge of microorganisms causing biodeterioration of cultural heritage in terms of taxonomic, physiological and metabolic diversity<sup>48–50</sup>. Among the various techniques, DNA metabarcoding has attracted great interest in the last few years, by allowing the simultaneous amplification and sequencing of short fragments of previously established markers as 16S, 18S, 23S, and ITS2 for the characterization of prokaryotes, eukaryotes, algae and fungi (e.g.,<sup>14,51,52</sup>). These studies have contributed to enrich the “taxonomic library” of microorganisms associated to different cultural heritage items as common stone monuments<sup>53,54</sup>, man-made artefacts<sup>55</sup>, and wall- and traditional paintings<sup>56</sup> to peculiar materials as ceramics<sup>57</sup>. Stuccoes fall within the latter group, being very fragile materials that rarely survive intact or almost intact to present days. Available literature on the taxonomic diversity of microorganisms forming biofilms on such materials is from the vault of a XVIII century Italian church<sup>58</sup>, and Mayan stucco masks from Guatemala<sup>59</sup> or buildings from Mexico<sup>60</sup>. In all of these studies, no similarities in microbial communities of stuccoes were observed: fungi (*Aspergillus*, *Chaetomium*, *Sarocladium*, and *Stachybotrys*) were dominant in the Italian church, while cyanobacteria (especially *Gloeocapsopsis*, *Pseudoanabaena*, and *Rhabdoderma* in the former, and *Gloeocapsa*, *Synechocystis*-like, and *Xenococcus* in the latter) were the dominant taxa. In both Mayan samples, as also reported by Garcia de Miguel et al.<sup>61</sup>, eukaryotic algae except for *Chlorella* were absent from most samples, and this trend was explained with their exposition to direct sunlight, which was likely responsible for desiccation. The *laconicum* object of this study is, on the contrary, a small, semi-confined environment, with rather uniform values of temperature, light and humidity across the year. High humidity (75–95%) is also favoured by its exposition close to a hill and phenomena of capillary rise of water from the ground and infiltration from several sides. This environment is also constantly protected from direct sunlight and excessive ventilation. This could be the reason why we found several eukaryotic algae like *Ctenocladus*, *Picochlorum*, *Picocystis salinarum*, and *Pseudostichococcus*, in addition to different genera of cyanobacteria (*Leptolyngbya*, *Loriellopsis*, *Nodosilinea*, and *Nodularia*,) and fungi (*Cyphellophora*). In addition to climatic factors, the diversity of taxa found on stuccoes



**Figure 4.** Removal of biological patinas with ESSENZIO. (A) Test treatment with contact times of half an hour (1), one hour (2), one hour and half, and two hours (4); (B) stucco's area before treatment; (C) stucco's area during the application of the biocidal product, and before being covered with film; (D) result after treatment.

could be explained with the porosity and water retention capacity of this material stuccoes, which is known to present high bioreceptivity<sup>62</sup>. It should be taken into consideration that most of the biological patina here found developed both in direct contact with the surface of the stuccoes and on the thin limestone encrustations that were present above them. Regarding bacterial colonisation of stuccoes data are even more limited, but Agarossi et al.<sup>63</sup>, found *Nocardia* and *Streptomyces* as the most abundant genera in the subterranean Neo-Phytagorean basilica of Porta Maggiore in Rome (1st century AD). We found several bacterial genera (excluding cyanobacteria) particularly abundant like *Chelativorans*, *Longispora*, *Nitrolancea*, *Phytoactinopolyspora*, and *Pseudonocardia*, but none of them was typical of stuccoes, but also found on adjacent, non-stucco samples. Nonetheless, microbial colonization is also driven by the spatially different micro-environments, including both exposure and physical-chemical characteristic of stuccoes. For instance, *Halomonas chromatireducens*, a species of halophilic bacteria, was only found in sample S1, which is in proximity to saline incrustations, while *Chelativorans*, a genus of Gram-negative, strictly aerobic bacteria generally isolated from nutrient-poor environments<sup>64</sup> was abundant in all samples. In sample S1, the one close to saline incrustations, we also found bacteria typical of marine or tidal environments as *Oceanicaulis*, *Aliihoeflea aestuarii*, and *Pelagibacterium lentulum*: their occurrence could be due to the influence of nearby sea sprays (the distance of the *laconicum* from the sea is of just 200 m), as also explained for other samples collected in Baia<sup>15</sup> and for some eukaryotic species mentioned above, typical of saline environments. Salt-tolerant bacteria and archaea have been reported as colonisers of stone monuments, especially in porous building materials subjected to rainwater and rising damp that contain soluble salts<sup>65–67</sup>. We did not find any archaeal sequences in our dataset, and we cannot exclude, beside a real absence, a bias due to primer choice. According to the company's protocol (<https://imr.bio/protocols.html>), the primer pair for the V3-V4 region<sup>18</sup> of 16S should have a moderate coverage (0–90%) at amplifying archaea. Further studies using

archaeal-specific primers are needed to assess their contribution in the biodeterioration of cultural heritage sites, especially the ones interested by saline incrustations.

Regarding the biodeterioration potential of the microorganisms here detected, some of them were already known to be involved in aesthetic (e.g., discolouration) and structural (e.g., corrosion, deterioration and decay) damages to cultural heritage items. Among the most abundant bacterial genera identified, we report *Actinomycetospora*<sup>68</sup>, *Egibacter*<sup>69</sup>, *Loriellopsis*<sup>70</sup>, *Pseudonocardia*<sup>68,71,72</sup>, *Rubrobacter*<sup>68,70,73</sup>, *Streptomyces*<sup>71</sup>, and *Truepera*<sup>74</sup>. Although none of these studies focused on the biodeterioration of stuccoes, it is likely that these microorganisms could have a similar impact on this substrate. In addition, other abundant bacteria, such as *Chelativorans*, have been indirectly associated with wood decay<sup>75</sup>, or biopolymers degradation in marine environments, as in the case of *Oceanocaulis*<sup>76</sup>. For eukaryotes, the available literature on the biodeterioration is mostly focused on algae and fungi, especially from stone monuments<sup>77–79</sup>. Regarding stuccoes, most of studies have investigated the role of fungi, reporting *Aspergillus*<sup>80</sup> and *Chaetomium*, *Penicillium*, *Sarcocladium*, and *Stachybotrys*<sup>8</sup>. Among these, in our study we only found *Sarcocladium* in the stucco sample S6. Other studies (see above) reported only on the presence of microorganisms, therefore allowing no inferences on their role in biodegradative phenomena. About algae, the genus *Ctenocladus* here found was already reported<sup>81</sup> for stuccoes, despite the direct role in biodeterioration was not assessed. For the genera *Picochlorum*, *Picocystis*, and *Pseudostichococcus*, we report their occurrence for the first time on stuccoes and we did not find any study implying their involvement in the deterioration of cultural heritage.

Metabolomic studies are still at their infancy in the field of cultural heritage (e.g.<sup>82–84</sup>), but are gaining increasing importance due to their capabilities at providing qualitative and quantitative data on small molecules that are part of the metabolic pathways. Specifically, untargeted metabolomics is particularly useful to search for biomarkers likely involved in the biodeterioration of CH, by collecting data from hundreds to thousands of metabolites that belong to various classes of chemicals in a single analysis<sup>12</sup>. According to the studies reviewed in<sup>12</sup>, pathways like biosynthesis and degradation of aminoacids, ubiquinone and other terpenoid-quinone biosynthesis, pigments biosynthesis and degradation were shared among different CH objects. In our study, most of metabolites belonged to biosynthesis of secondary metabolites, microbial metabolism, biosynthesis of aminoacids, and carbon and protein metabolism, indicating active metabolic processes. In some cases, it has been possible to link the presence of specific metabolites to particular taxa, e.g. chlorophyll a to photosynthetic eukaryotes and cyanobacteria, and diatoxanthin to *Picocystis salinarum*. Diatoxanthin is a pigment typical of heterokonts (especially diatoms) and, outside this group, to date it has been only found in this species; because we found no diatom sequences in our samples and *P. salinarum* was particularly abundant in some of them, we are confident in the inferred attribution of such metabolite to this species. However, our correlation analysis between abundance of taxa and metabolites provided weak signals. Finding statistically significant correlations could be difficult in CH studies for several reasons. One factor responsible for this could be the bias in the sampling strategy of metabolomics but also metagenomics studies. Indeed, historical objects are sampled in non-invasive ways, which result in collecting very small amounts of superficial material from small portions that could not be representative of the entire surface<sup>12</sup>. For instance, there is the risk that the communities living in the deeper layers of the biofilm, which are often at contact with the CH, are not sampled to avoid damages to the object. In addition, it should be considered that a statistically robust sampling could not be often achieved because some manufactures are unique (impossibility to compare biofilms from similar substrata) or, on the same manufacture, the number of biofilms that can be collected is limited (different degree of biodeterioration). Another affecting factor could be the choice of the barcoding marker, with broad-spectrum markers unable of capturing fine taxonomic resolution that could be, instead, relevant to link some organisms to specific metabolites. For instance, the universal 18S-V4 region could have underestimated the diversity of some fungi groups, which are better detected with ITS region marker, by amplifying other eukaryotic taxa<sup>85</sup>. However, the nature of detected metabolites could be responsible for such weak associations. Indeed, most of metabolites found here and in other studies on different substrata (e.g.<sup>86</sup>) belong to generic pathways and cannot be associated to specific communities.

The use of green biocides such as essential oil extracts represents a valid alternative to synthetic biocides, allowing a restoration to be carried out using products with low toxicity, easy to handle and environmentally sustainable. Indeed, in the last few years there was an increase in the use of phyto-derivatives like liquorice leaf extract and essential oils as safer and eco-friendly alternative to chemicals to be used as natural biocides for the restoration of cultural heritage<sup>87–89</sup>. Despite the wide spectrum of efficacy of essential oils across different taxa and domains, it was found that Gram negative bacteria were generally more resistant than Gram positive bacteria, and that some had major effects on fungi than others<sup>90</sup>. In the field of cultural heritage, attention has been paid on extract oils from the family Lamiaceae, especially from *Thymus* and *Origanum*, because they have proven to be effective on different microorganisms and for their availability in commerce as ready-to-use blends<sup>91–93</sup>. An example of the application of these essential oils concerns the restoration of some artworks from the Catholic cemetery for foreigners in Rome<sup>94</sup>, where up to three applications were repeated into a hydroalcoholic solution (70% ethanol and 30% water), with a concentration of 5%. In other cases, as the removal of biofilms under the tiles of the floor mosaic of Leda's House in the archaeological park of Solunto in Sicily<sup>95</sup> and on the sculpture "The Silvano" in the archaeological museum of Florence<sup>96</sup>, applications of a thymus blend at 15% and a bush application in 2% demineralized water respectively, were proven to be effective. Regarding the specific application of ESSENZIO by IBIX Biocare, successful restoration interventions were achieved for the mosaics located in the room XIX of the Insula of the Muses in Ostia Antica<sup>97</sup> and a mosaic fountain in Ravenna<sup>98</sup>. From the in-depth analysis of the different application methods tested in the studies described above, we identified the most important parameters to take into consideration for the application of essential oils and we were able to remove the biofilms on the surfaces of the *laconicum*. The treatment with ESSENZIO at 50%



in demineralized water with an application exposure of one hour and half was successful, and no biofilms were observed after eleven months from the restoration.

## Conclusions

Genetic and biochemical high-throughput techniques (-omic tools) have opened new paths toward the study of microorganisms forming biofilms on cultural heritage and their metabolic activity that is responsible of biodeterioration phenomena. Despite the potential of such -omic tools, which reflects in increasing literature on metabarcoding studies of CH objects, the number of studies using metabolomics and integrating both approaches is still small. In this study, we reported on the taxonomic and metabolite diversity of microorganisms forming biofilms on ancient stuccoes. At best of our knowledge, this is the first wide-spectrum intervention on ancient Roman stuccoes (besides the work by Bruno et al.<sup>87</sup> on catacombs in Rome), as well as the first study integrating -omics approaches as metabarcoding and metabolomics on such fragile cultural heritage object. We confirmed that metabarcoding is a powerful technique to quickly and thoroughly characterise the taxonomic diversity of microorganisms in complex matrices as biofilms in respect to classical, culture-based approaches, also in fragile and delicate materials as stuccoes. In addition, we demonstrated that it is possible to extract and isolate numerous metabolites from very low amount of material and that such untargeted-metabolomics analyses are indicative of metabolic pathways active in the biofilms. However, we also argued that the paucity of biological material collected from stuccoes or cultural heritage items in general, as well as the scattered distribution of biofilms in the study system could affect the detection of statistically significant correlations between abundance of taxa and metabolites. Last, we have proven that a treatment based on essential oils from thyme and oregano effectively removes both bacterial and eukaryotic biofilms from stuccoes, thus confirming its utility in restoration of cultural heritage. To promote the adoption of such integrative approaches, future efforts should focus on the establishment of standardised strategies for sampling, data pre-processing, and statistical analyses of cultural heritage objects. Additionally, it is important not to overlook the value of culture-based methods in enriching taxonomic and metabolomics reference libraries, which are essential for any -omic study.

## Data availability

Raw Illumina reads are available in fastq format in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA1135078. All other information not included in the main text is provided as Supplementary Information alongside this manuscript.

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## Author contributions

P.C. conceived and designed the experiments; P.C. obtained the funding for the study; P.C. and S.S. were involved in the sampling activity; D.D.L. and R.P. carried out the experiments on metabarcoding; J.T. and M.L. produced the metabolomics data; M.M.C. and S.S. were involved in the restoration of stuccoes; D.D.L. and R.P. analysed the data; D.D.L. prepared the original draft of the manuscript; D.D.L., J.T., M.M.C., O.D.C., P.C., and R.P. were involved in review and editing of the manuscript. All authors have read and agreed to the final version of the manuscript.

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## Declarations

### Ethical approval

This article does not include any studies of human participants or animals by the authors of this investigation.

### Competing interests

The authors declare no competing interests.

### Additional information

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