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Metagenomic for cultural heritage: techniques for conservation and monitoring.

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Abstract **– This investigation presents the characterization of biodeteriogenic microorganisms in the biological patina of architectural surfaces of the Church of Madonna del Carmine in Melpignano (Lecce, Italy). The aim was to picture the biodiversity of fungal and bacterial consortia inhabiting different part of this monument in order to obtain useful informations for restoration and conservation interventions. Microbial communities were characterized by means high throughput deep sequencing using an Illumina Miseq-based amplicon sequencing of the ribosomal internal transcribed spacer-1 (ITS1) region for fungi and of the V3-V4 hypervariable regions of the 16S ribosomal RNA gene for bacteria.**
Our results showed evidence of important differences among the samples obtained from the different substrates exposed to different environmental conditions and provided useful information to predict the state of deterioration and address future interventions for restoration.

INTRODUCTION

All the materials of the cultural heritage are subject, during their lives and with favourable environmental conditions, to attacks by biological agents that could cause biodeterioration with aesthetic and structural damages. The biodeteriogenic microorganisms can damage the artefact with physical and chemical actions, most of them use the artefact as a nutritional substrate to carry out metabolic reactions and grow in life cycle favourable conditions [1, 2, 3]. Their appropriate identification permits a focused action and a good design of the prevention from future attacks.
The metagenomic method is culture-independent and provides the genetic identification of all microorganisms in a sample by DNA sequencing followed by bioinformatic analysis. [4,5,6].

Fig. 1. The façade of the Madonna del Carmine church.

MELPIGNANO (LE) CASE OF STUDY

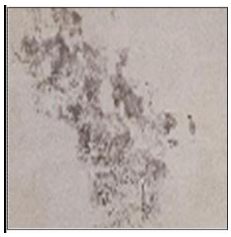
A. Madonna del Carmine church and its patina

The church of Madonna del Carmine is part of the former Augustinian monastery, located in a barycentric position in Salento, southern extremity of the Apulia region [7]. The sixteenth-century church is an excellent example of the architectural style known as Barocco Leccese. The building is entirely made of ashlar of Lecce limestone, a Miocenic calcarenite containing glauconitic minerals with a light yellow coloration.It is characterized by the presence of fossils and bioturbated areas that directly affect the physical and mechanical properties of the material and the type of deterioration.
The nature of the stone and the environmental conditions caused the appearance of deterioration phenomena, such

as the birth of biodeterioration agents.
Inside, the back wall and the walls of the chapels around the high altar are covered with a lime plaster with fine quartz and calcite sand (< 1m).
Northern facades are affected by a wide biological colony of crustose lichens, firmly anchored to the substratum that hide the decorative structure; the parts protected by rain water runoff are subjected to black crusts and dust.
Inside, on the walls of the nave, there are various phenomena wich involve the stone surfaces and lime plasters: water stains, biological patina, weeds, saline efflorescence, etc. Biological patina is the hugest deterioration phenomena therefore it has been started a study for the identification of biodeterioration agents, in order to define an effective restoration project [8].

B. Sampling selection

From the point of view of cultural heritage, metagenomics is a non-destructive technique because it doesn't damage the substratum where the patina grows. In fact a small quantity of sample is required which can be obtained by picking-up a dust sample from the patina, with a brush or with a light pression of a scalpel. During the sampling is is necessary to avoid contaminations by setting up sterile conditions.
Before the sampling phase a preliminary classification of biological patina has been carried out after a visual examination. Inside the church four different types of biological colonization (CB) were visible:



CB_1: Patina with the presence of bacteria and fungi. Poorly anchored to the substratum, it shows grey color and a dusty consistency.

CB_2: Patina with presence of algae (chlorophyta). Discreetly anchored to the substratum, it shows dark green color and a slimy consistency. Filamentary elements are visible.

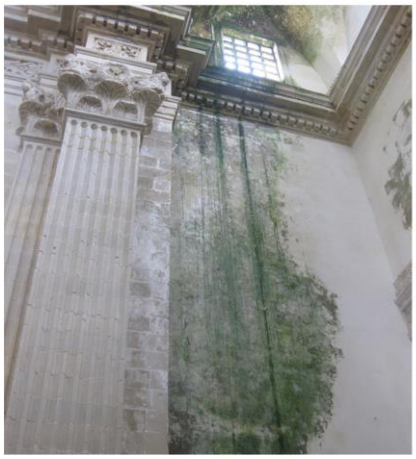
CB_3: Patina with brilliant green color and a sticky consistency, poorly anchored to the substratum.

CB_4: Patina with grey/blue color and a dusty consistency, poorly anchored to the substratum.

On the external façade a thick layer of lichens, predominantly crustose, with the presence of a layer of dust has been recognized.

Six points have been sampled for the analysis, selected for the presence of various types of biological patina and with a massive presence of the phenomenon. All the sampling points show a considerable difference in types of decay (colours and typology of crust) which derive from variability and relative aboundance of the contained species.

- 5 samples were taken in the third chapel (right side) near to Placido Boffelli's altar (1656);
- 1 sample was taken from the external facade (Sample nr. 2).



CB_1 - CB_2 - CB_3

CB_2 - CB_3

Fig. 2-6. Details of the surfaces in the nave with the indications of the patina typologies.

METHODS

Sampling was carried out using sterile conditions and sent to the laboratory to be processing for DNA extraction and amplicon production.

Total DNA was extracted from about 350 mg of samples using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's specifications. The extraction buffer SL1 was used, supplemented with 70 ml of SX enhancer. DNAwas then quantified on a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA). For fungal amplicon production, the ribosomal ITS1 region was targeted, by using primers BITS and B58S3 [9] linked to Illumina adapters as previously described [10].

For bacterial amplicon production, the prokaryote 16S rRNA was targeted using primers designed by Takahashi et al [11]. Illumina sequencing libraries were finally constructed through the link of indexes (Nextera XT Index Kit, Illumina, San Diego, CA), quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific), normalized and pooled. Libraries were subjected to paired-end sequencing (2 _ 250 bp, nano format) on an Illumina MiSeq sequencer at BMR Genomics (Padova, Italy). Sequences obtained underwent to bioinformatics analysis: raw data from sequencing were demultiplexed based on the unique barcode assigned to each sample. Barcodes and primers were then trimmed off. Sequencing quality filters were applied, including a minimum length threshold (100 bp) and removal of singletons. High-quality reads were then clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE and annotated using UNITE fungal ITS reference data set within RDP classifier (http://rdp.cme.msu.edu) and the Worcup ITS reference as training dataset. Bacterial OTUs were clustered using Mothur and annotated using Greengeens bacterial 16S reference data. Finally relative abundances of microbial taxa in each sample were calculated.

RESULTS

A.Analysis of Fungal communities

The analysis of the fungal composition showed the presence of a large variability in the different samples. 34% of the 3317 OTUs identified were not recognized in the databases.Ffor the remaining OTU only those with a prevalence equal to or greater then 9%was considered.

The qualitative and quantitative composition of the fungal communities were in relation with the different ecological conditions: OTU 1 (*Fungi_sp*) 2 (*Hyporeales_sp*) and 5 (*Acremonium*) resulted the most abundant portion of fungal communities in the samples 1, 2, 5 and 6, which differs for the variable presence of other fungal species. Sample 5 appeared clearly different from all the others.

Sample 2 (on facade) contains mainly *Dirina_massiliensis*, wich has not been found in samples 1, 4, 5 and 6 (sampling inside the church) but was identified in sample 3 with only a slight prevalence (0,6%). It is a lichenizing fungus that forms crusty lichens in association with the photo-symbiont of the genus *Trentepohlia*. *Dirina_massiliensis* in Italy is specially present along the coasts on cliffs that receive splashes of salt water, in areas with nothing or little eutrophication; it is found on rocky substrates both silica and calcareous with basic pH; the color of the balloon, is very often gray, however it depends on the amount of calcium oxalate and on the density of the symbiont

cyanobacteria; requires shade or diffused light without direct irradiation of sunlight. The presence of this species in Puglia were previously described by Edwards & al. [12] and by Nimis & Tretiach [13]. Accoding the observations of these researchers, this lichen is able to produce incrustations on different substrates such as gypsum and calcite, on which frescoes are painted, church wall stuccos, glass/glass interface of stained glass windows. The patina can be of different color shades, from grey to white, containing different concentrations of calcium. The ability of this lichen to produce calcium oxydate dihydrate is particularly developed on calcareous substrates that are mainly degraded but has been noted in lesser quantities even on non-calcareous substrates. This seems particularly interesting in terms of biodeterioration of cultural goods. Our current finding agrees with previous studies which described the presence of lichens on the facade of the church. In this analysis we identified this same species also inside the building (sample 3).

Finally dust samples examination allowed the recognition of a notable number of fungi species, some of them typical of the territory (*Omphalotus_olearius*) and of rare species (es. *Rhodosporidium_kratochvilovae*). Species with pathogenic potential have been recognized in every sample, especially inside the church. Among these: *Hypocreales_sp*, *Cryptococcus_diffluens* that was indicated as causative agent of a subcutaneous criptococcosis; *Asperillus_sclerotiorum*, which has been associated with repiratory diseases; *Trichosporon_asahii*, a fungus able to invade the whole organism through blood circulation in subjects with impairment in the innate immune response[14].

B.Analysis of Bacteria communities

Like fungi composition the bacterial composition allows us to depict a distinctive picture for each sample. The 5 most representative taxon/class was considered for the comparison. The most abundant taxons in sample 1 were *Actinobacteria* (33%) and *Cyanobacteria* (24%); *Deinococcus Thermus* showed an high prevalence (53%) only in sample 2 (façade of the church); in sample 4, 92% of bacteria were represented by Cyanobacteria (*Chloroplast class*). *Actinobacteria* were observed in almost all samples. These bacteria are Gram positive and can be found both in marine and terrestrial environments. *Actinobacteria* are all aerobic and, some species, pathogens. From the literature they are mainly mesophilic, with an optimum growth at temperatures between 25 and 30 °C. Some of therm are ophilic. *Actinobacteria* can grow at temperatures ranging from 50 to 60 ° C. *Deinococcus* is a genus belonging to the *Deinococcales* group of *Deinococcus-Thermus*, a bacterial phylum highly resistant to environmental hazards. This organism is able to survive under extreme conditions: cold, dehydration, vacuum and acidity: for this reason, it is also considered a polyestremophilic bacterium. *Deinococcus* is aerobic and colonizes habitats rich in organic matter. This organism is also extremely resistant to ionizing radiation, ultraviolet light, dehydration, oxidation and exposure to electrophilic agents, for its ability to repair the functional structure of its chromosomes after damaging..

Gammaproteobacteria (found in sample 1, 3, 5, 6) are a class of Gram negative bacteria belonging to the *Proteobacteria* phylum, some of which are pathogens. They usually colonize light environments with anoxia conditions. Some of these are photosynthetic and produce sulfur as a waste product.

Chloroplast (in sample 3) are Gram negative bacteria belonging to the phylum of *Cyanobacteria*. They are nitrogen-fixing photosynthetic organisms and mainly colonize aquatic environments but are also capable of growing on solid substrates by forming patinas. High temperature and the alkalinity of water are conditions favouring the diffusion of Cyanobacteria.

CONCLUSIONS

The analysis of the characteristics of the most represented microorganisms in a sample, which are also likely to have a greater role in degradation, provides useful informations about their interactions with the substrates.

Already in the 1980s, due to a spread of lichen colonies, various studies were carried out in order to identify them on various monuments in the center of Italy: *Caloplaca citrina (Flavoplaca citrina)*, a yellow lichen, *Verrucaria nigrescens*, looking blackcurrant on limestone rocks; *Verrucaria sp.*, grayish, present in the marine environment; *Xantoria parietina*, with partially crocheted tallo, partly yellowish foliage; *Lecanora pruinosa* (*Myriolecis pruinosa*) common crostous lichen on limestone, pale yellow to orange [15].

Our present analysis aimed at the identification of microorganisms (fungi and bacteria) on dust samples from masonry and stone materials of the Carrmine church of Melpignano (Lecce, Italy). The study was carried out with a metagenomic approach and allowed us to identify in a short time and with a high degree of resolution, a considerable number of fungal species, some of which are typical of the area where the monument stands (eg *Omphalotus_olearius*). We can detectedalso very rare species (eg *Rhodosporidium_kratochvilovae*), present only in traces, thus confirming the sensitivity of the analytical method. Species with pathogenic potential such as *Cryptococcus_diffluens*, *Aspergillus sclerotiorum*, *Trichosporon_asahii*) have also been identified. These last species were found in all the sampled parts but their prevalence was higher within the building. The quantitative composition of the fungal communities resulted significantly different in the different samples, reflecting the different ecological conditions. The same results was obtained analyzing the bacterial composition.

Information on contamination by both fungi and bacteria allows us to have a very complete picture of biological colonization since it is known that there are microbial associations for which different microorganisms can act in synergy or competition. The evaluation of these associations is essential to plan a correct intervention for restoration minimizing the adverse effects.

After the microorganisms' identification, appropriate conservation intervention must be set up, with particular attention to create unfavourable environmental conditions, in order to better control the microorganism regrowth. The results of our type analysis could also help in the choose of specific biocides that can be proved against microorganisms of the same type.

It is necessary to underline that the use of biocidal products is not decisive so as a consequence, in order to control biodeterioration, it is necessary to repeat the treatments over time. Furthermore, since the biocides could be harmful, the use of non-aggressive products with the implementation of other strategies minimizing harmful substances must be applied. The monitoring of environmental conditions, making them unfavorable to the decomposition and the monitoring of microbial contamination using metagenomics could be very helpful even when the biodeteriogen attack is not macroscopically evident allows for planning of intervention ante factum. Lastly, it has been emphasized that this type of analysis requires minimal sample traces, which involves benefiting a considerable reduction in invasivity of the withdrawals combined with a high sensitivity.

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