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Microorganisms and the integrated conservation-intervention process of the renaissance mural paintings from Casas Pintadas in Évora – Know to act, act to preserve

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ABSTRACT

The identification of microorganisms involved in biodeterioration/biodegradation process is determinant to understand their effects on cultural assets, and furthermore, it is a crucial step to define an efficient strategy to protect monuments and artworks from microbiological recolonisation, in order to promote its preservation.

The renaissance mural paintings from the garden of the Inquisition Palace in Évora, classified by UNESCO as World Heritage, were analysed in order to find the microbial population involved in the biodeterioration process, and, to assist and develop an efficient intervention, by biocides application to control the propagation of microbial communities responsible for biodegradation.

The biocides tested revealed good inhibition results against the microbial communities isolated from the paintings, whose action spectrum was noticeably enlarged when the application was carried out combining two different commercial biocides.

In vitro antifungal assays performed with new natural biocompounds revealed efficient inhibition capacity, suggesting the potential of these compounds to be used as a great and safe alternative to the chemical compounds usually used on cultural assets microbial treatments.

1. Introduction

Biodeterioration/Biodegradation of Cultural Heritage is the result of interactions between living organisms, material support and environmental conditions (Nuhoglu et al., 2006; Capodicasa et al., 2010). The biological activity of microorganisms like bacteria, fungi, algae and lichens, contributes to the deterioration of cultural assets, particularly if they are exposed to open air. The interaction of these agents with physico-chemical properties of the materials is considered central to understand the long term deterioration (Ripka et al., 2006; Herrera and Videla, 2009; Wiktor et al., 2009; Mihajlovski et al., 2017), due to the ability to obtain different elements (calcium, aluminium, silicon, iron and potassium) essential for their metabolism, by bio-solubilisation of the materials (Nuhoglu et al., 2006; Karaca et al., 2015).

In mural paintings, the development of diverse organisms is supported by humidity, light, alkaline pH values and the presence of organic and inorganic nutrient sources (Altenburger et al., 1996). In addition, the natural porosity of these paintings makes their surfaces receptive to microbial spores and vegetative cells transported by airborne particles (Saarela et al., 2004; Milanesi et al., 2009), allowing their proliferation.

Fungi are among the most harmful organisms associated to biodeterioration of organic and inorganic substances (Wiktor et al., 2009; De Leo and Urzì, 2015). The destructive potential of these microorganisms is the result of mechanical and chemical

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processes, caused by mycelia penetration inside the plaster of the painting resulting in loss of cohesion and detachment of the paint layer, as well as paint discolouration due to the metabolic products excreted in the surface (Altenburger et al., 1996; Rölleke et al., 1996; Berner et al., 1997; Herrera et al., 2004; Milanesi et al., 2006; Imperi et al., 2007).

Given the several agents that can induce alterations in the paintings, their safeguard is a widespread problem of global relevance (Tomaselli et al., 2000). Therefore, it is crucial to develop efficient approaches to detect potentially harmful or destructive microorganisms, and strategies to conserve and eliminate their contamination. If procedures are taken to prevent microbial growth, biodegradation can be avoided (Gurtner et al., 2000; de los Ríos et al., 2009; Wang et al., 2011).

Thus, for studying deterioration of artistic materials induced by environmental and biological agents, and thereafter proceed to a conservation-restoration, it is necessary the detailed knowledge of the materials originally employed by the artist (Milanesi et al., 2009; Wiktor et al., 2009). On the other hand, biocides application are a very important step to prevent and/or control microbial growth/re-colonisation for one acceptable period of time (Urzì and De Leo, 2007; Fonseca et al., 2010; de los Ríos et al., 2012). However, its application requires attention to chromatic alterations, changes in water absorption capacity, permeability and surface tension (Tretiach et al., 2007).

Treatments with biocides should be tested on a small scale (*in vitro* test), but preferentially on the affected monument to determine their effectiveness against microorganisms, since some studies indicate that the biocides efficacy can be reduced significantly in the case of *in situ* applications compared to the sensitivity of microorganisms observed in laboratory experiments (de los Ríos et al., 2012). Furthermore, artworks exposed to open air, usually, suffer recolonisation after treatment (Nascimbene and Salvadori, 2008), thus development of preservation processes are urgent.

In this work, the biodeterioration of the 16th century renaissance mural paintings from *Casas Pintadas* located in the garden of the Inquisition Palace in Évora (*Portugal*), classified as World Heritage by UNESCO, was investigated and mitigation treatments were asserted.

The paintings exhibited in *Casas Pintadas* (Fig. 1) show mythological and exotic scenes decorating a cloister and a small chapel. This space is all that remains of the Noble House belonging to Silveira Henriques family, Masters of the Horse of the kings D. Afonso V and D. João II. However, in the past they have been attributed to Vasco da Gama (Portuguese navigator), fact that has been proved to be a legend. The paintings in the cloister, of a great historical and artistic value, combine exoticism, originality and evocative power, constituting an iconic national and international mural composition (Caetano and de Carvalho, 2014), whose safeguard is imperative.

Thus, the investigation of this iconic mural paintings was performed with the main purposes of: i) characterisation of the materials used, ii) microbial community assessment, iii) mitigation strategies development and iv) investigation of new safe biocides, in order to promote the safeguard of this cultural heritage asset

2. Material and methods

2.1. Sampling process

The sampling process (Fig. 1) was performed on representative areas of the paintings, and, in areas with significant contamination and alterations signs, under the coordination of a Conservator-Restorer, using micro-invasive and non-invasive methods.

Preliminary *in situ* X-ray fluorescence spectrometry and colorimetric analysis were performed in order to select the areas for samples collection. Microsamples (10 samples with less than 1 mm^2) for chromatic layers characterisation were removed using a small chisel, near paint losses or cracks to avoid further damage, to allow a full characterisation of the paintings materials and support mortars.

For microbiological assays, samples were collected under semi-aseptic conditions with sterile swabs and scalpels, placed in a suspension of transport MRD medium (Maximum Recovery Diluent, Merck), until utilisation.

2.2. Material characterisation

Paintings microsamples collected were incorporated in polyester resin (Epofix Fix) and polished to allow cross-section analysis.

Optical microscopy observations were carried out in a Leica DM2500 microscope in reflected light and dark field mode and digitally recorded by a Leica DFC290 HD photo camera, enabling stratigraphy analysis and pigment morphology.

To allow microstructural characterisation of the paint layers and elemental composition (point analysis and 2D mapping), the paint cross-sections were used as such or coated with Au-Pd (Balzers Union SCD 030) and analysed with a HITACHI 3700N variable

Fig. 1. Mural Painting of *Casas Pintadas* located in the garden of the Inquisition Palace (Évora, Portugal) with some sampling process details.

pressure scanning electron microscope (VP-SEM) coupled with a Bruker XFlash 5010 energy dispersive X-ray (EDX) spectrometer with an accelerating voltage of 20 kV.

2.3. Analysis of biological contamination on mortars

Mortar microfragments were coated with gold (Balzers Union SCD030), and analysed by Scanning Electron Microscopy with an accelerating voltage of 10–20 kV in secondary electrons mode, to evaluate the microbial proliferation.

2.4. Microorganisms isolation and characterisation

Samples collected for microbiological studies were mechanically shaken for 1 h, and after serial dilutions (10^{-1} to 10^{-3} mL) were prepared and inoculated (100 μL), under aseptic conditions, in NA (Nutrient Agar), for bacteria isolation, in MEA (Malt Extract Agar) and CRB (Cook Rose Bengal) for filamentous fungi isolation, and, in YPD (Yeast Extract Peptone Dextrose Agar) for yeast growth. The cultures were incubated at 30 °C for 24–48 h, and at 28 °C for 4–5 days, to allow bacterial and fungal development, respectively. After this period, the plates stayed in incubation at the same temperature to detect slow microbial development. The several colonies developed were picked up to obtain pure cultures, and then stored at 4 °C.

The microbial population was characterised based on macroscopic features of the colonies, and, in micro-morphology of the reproductive structures, that were observed in the optical microscope Leica DM 2500P, and the images were acquired with the digital camera Leica DFC290HD. Their identification was also performed, as described on Rosado et al. 2013a,b, sequencing the 16S rDNA or ITS region for bacterial or fungal isolates, by capillary electrophoresis using the ABI PRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDT v1.1 (Applied Biosystems). The nucleotide sequences were aligned with those retrieved from the GenBank (NCBI) databases for the homology analysis using the BLASTN program (Rosado et al., 2013a).

2.5. Antimicrobial activities

The antimicrobial activity of three commercial biocides, namely Preventol PN® (1% and 0.1%) [sodium 2, 3, 4, 5, 6-pentachlorophenplate], Panacide® (1% and 0.1%) [4-chloro-2-[(5-chloro-2-hydroxyphenyl)methyl] phenol] and Linquad® (1% and 0.1%) [Alkylbenzyldimethylammonium chloride], compounds soluble in water, were evaluated against the predominant fungi isolated, under sterile conditions. The commercial compounds were tested at different concentrations against the several fungal isolates (*Aspergillus*, *Cladosporium*, *Penicillium*) from *Casas Pintadas* by agar incorporation method (Caldeira et al., 2011).

Cultures of each microorganism were prepared in Malt Extract Agar (MEA) slant and incubated at 28 °C for 7 days. Fungal spore suspensions were prepared by adding a loopful of hyphae and spores in 15 mL of NaCl 0.85% solution. The suspension was filtered by sterilised triple gauze and incorporated (10^5 CFU/mL) in MEA at 45 °C. Sterile filter paper discs with 12.6 mm of diameter (Macherey-Nagel 827 ATD) were placed on agar and impregnated with 20 μL of biocide. The Petri dishes were incubated at 28 °C for 4–5 days. Antimicrobial activity was evaluated accordingly to the inhibition halo developed around the disc. The assays were performed in triplicate.

2.6. In situ biocides application

Preventol PN®, Panacide® and Linquad® were applied in the mural painting of *Casas Pintadas* (combined applications) firstly in slab mortars (3 × 3 cm) to evaluate possible colour alteration. After confirming no chromatic alteration induction, the compounds were applied by spray in small areas (10 × 10 cm) and finally in all affected zones. After these experimental applications, the paintings were subjected to a conservation intervention process, together with a combined application of these biocides to prevent recolonisation.

2.7. Testing green alternative biocides

The use of biocides to control and prevent the monuments to biological damage is widespread (limited by Biocidal Products Directive (BPD) https://echa.europa.eu/), however chemical treatments often involve considerable amounts of potentially dangerous and even toxic compounds (Young et al., 2008; Maxim et al., 2012; Sterflinger and Pinar, 2013; Urzì et al., 2016). To overcome the problem associated to the toxicity of several commercial compounds, green alternative solutions based on biosurfactant lipopeptides produced by *Bacillus* sp. have been performed in our laboratory by Silva et al. (Caldeira et al., 2008; Silva et al., 2015; Silva et al., 2016). Thus, three of these ecological biocompounds (3 mg/mL) denominated BEVOTECH-11, BEVOTECH-14 and BEVOTECH-16, and, a positive control Mycostatin® (Nystatin 4000 UI/mL) known by their antifungal properties, were evaluated against the same fungal isolates used with the chemical compounds, following the same procedures described in the topic 2.5., in order to find effective and ecofriendly solutions that prevent biodeterioration/biodegradation of cultural assets. Relative inhibition percentage was calculated using as reference the positive control Mycostatin®.

3. Results and discussion

3.1. Material characterisation

The material characterisation of mural paintings is an important step to understand an artist technique allowing a deeper knowledge on the pigments and mortars used and providing also crucial parameters for the conservation/restoration process and consequently contributing to its preservation.

In this work, the strategy adopted started with *in situ* analysis which provided a global information about the mural paintings under study, thus allowing the careful selection of the collection points for the analytical and biological analysis.

These paintings showed a simple colour pallet composed by red, yellow, brown, blue and black pigments/shades (Fig. 1). Stratigraphic analysis of the cross sections, by optical microscopy and scanning electron microscopy enabled the characterisation of these pigments.

Microanalysis by SEM-EDX of the red areas showed that red ochre is the pigment responsible for this colouration.

Ochre are composed by clay minerals (aluminosilicates) enriched in iron oxides and hydroxides, goethite (FeO(OH)) and hematite (Fe_2O_3) (Gil et al., 2007). These compounds are easily identified by SEM-EDX by the concomitant presence of aluminium, silicon and potassium in their composition (Fig. 2A). Hematite (Fe_2O_3) is the responsible for the red colour of these pigments although these may also contain other chromophores in their composition, such as goethite (FeO(OH)) and manganese dioxide (MnO_2) that confer orange and brownish nuances (Gil et al., 2009). Both on the upper painting as on the grotesque frieze, red ochres were used alone or in conjunction with bone black, a black pigment obtained from the burning of bones, and identified by the presence of calcium (Ca) and phosphor (P).

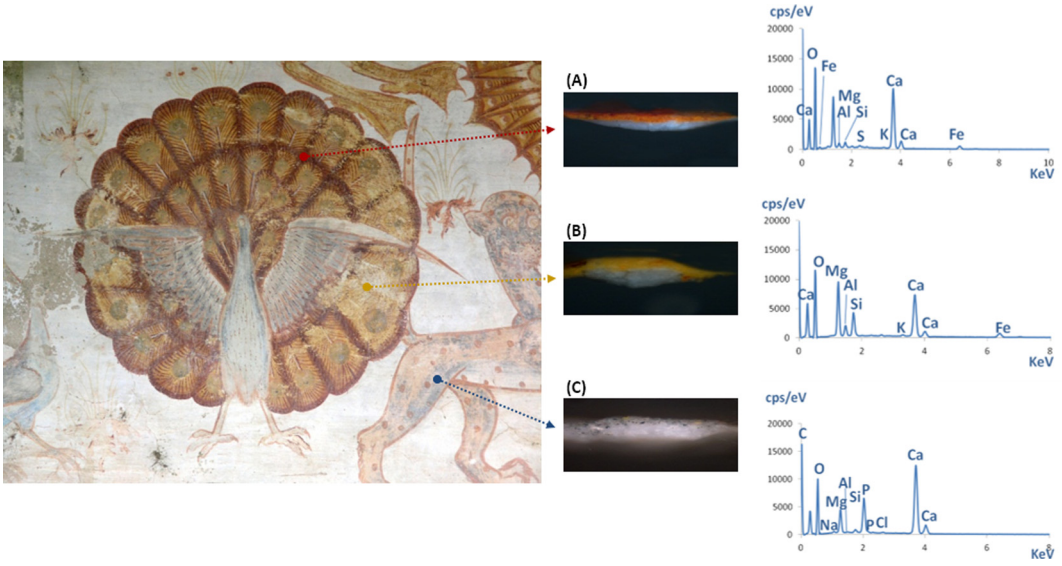


Fig. 2. SEM micrograph in back-scattered mode and EDX 2D elemental maps of a cross-section from red (A), yellow (B) and blue (C) areas from *Casas Pintadas*.

Like the red areas, yellow and brown colours were obtained with clay-based earth pigments, whose composition is similar to the red ochre, however the type and proportion of the iron compound present is different. In the yellow ochre, the chromophore responsible for the colour is mainly goethite, an iron oxohydroxide (FeO(OH)) compound (Fig. 2B). On the other hand, the brownish shades can be explained by the presence of manganese oxides, organic matter or others clay minerals (e.g. smectite).

On the background of the grotesque frieze, currently dark bluish grey is observed, however, no chromophore was identified. The absence of blue pigment particles in the samples analysed by optical microscopy and scanning electron microscopy, the detection of animal black and yet the presence of red and yellow ochres in the adjacent areas seem to indicate the possible use of an optical blue (Ashok, 1993).

In the black areas of the painting, the presence or absence of phosphor revealed the application of bone black or charcoal, respectively. Both pigments are produced by calcination of organic matter (bones and wood).

3.2. Microbiological study

To assess the biodegradation of *Casas Pintadas*, samples from areas with noticeable signs of alteration were analysed. This approach involved a detailed study in order to characterise the biological agents that promote degradation of the murals and to understand their propagation in the deteriorated areas. Thereby, mortar microfragments were observed by SEM (Fig. 3) which confirmed microbiological contamination, showing the capacity of microorganisms to thrive in the paintings. It was possible to observe the proliferation of fungal hyphae on the surface and by the microstructure of the mortars, fact that may explain the detachment and cracking observed in some areas of the painting (Fig.3A–D).

In Fig. 3B-D is evident the micellar structures of the filamentous fungi, forming a biofilm on the surface of the mortar, covering some areas of the paint, which can induce alterations due to the metabolic activity of the microorganisms or colour acquisition from the development of the microorganisms in the surface of the walls. Biofilms are biological deposits of a highly hydrated gel of extracellular polymeric substances containing microbial cells and inorganic detritus that can drastically change the physico-chemical characteristics of the environment in contact with the structural material and generally increase its aggressiveness (Herrera et al., 2004; Harding et al., 2009; Zucconi et al., 2012), being dramatic for the structure and aesthetical aspect of the artworks.

Once detected microbial proliferation in the mural paintings of *Casas Pintadas* it was necessary to characterise this population, in order to identify the harmful microorganisms in the degradation process and the areas with the greatest contamination levels.

The microbiological study allowed the isolation and characterisation of several bacterial strains such as cocci and bacilli Gram-positive and *Actinomycetes* sp., yeast strains and filamentous fungi of the genera *Aspergillus*, *Cladosporium* and *Penicillium*; microorganisms frequently associated to mural paintings alteration (Garg et al., 1995; Gorbushina et al., 2004; Sterflinger, 2010; Zucconi et al., 2012; Rosado et al., 2013b, 2014). This characterisation (Table 1) was based on macro- and micromorphological features of each microbial isolates, being the identification of the fungal strains complemented with ITS region sequencing. These results showed high microbial contamination in the paintings surface, fact that can be correlated with the damages observed, whose dissemination affects the visual appearance of the paintings. Furthermore, highly contaminated areas showed higher degradation levels, due to fungal proliferation, as it was possible to detect by SEM analysis. Indeed, the biodeterioration of these paintings were due to the presence both of heterotrophic and phototrophic microorganisms but the latter were not considered in this study, but are being monitored actually.

Thus, the high biological contamination together with the outdoor location of these paintings, exposed to variations of temperature, humidity and luminosity during the day and over the seasons of the year, seems to be related with the chromatic alterations, cracking and detachment of some areas of the paintings (Garg et al., 1995; Ciferri, 1999; Gorbushina and Petersen, 2000; Milanesi et al., 2006; Guiamet et al., 2011). Since the paintings are in an outdoor environment and subject to extreme humidity due to the harsh winters in this location and taking into consideration that the biological attack is strongly influenced by water availability (Guiamet et al., 2013), it is determinant to take into account these important factors on a conservation strategy.

In this way, to ensure the longevity of the intervention and to avoid the fast recolonisation, remediation strategies were envis-

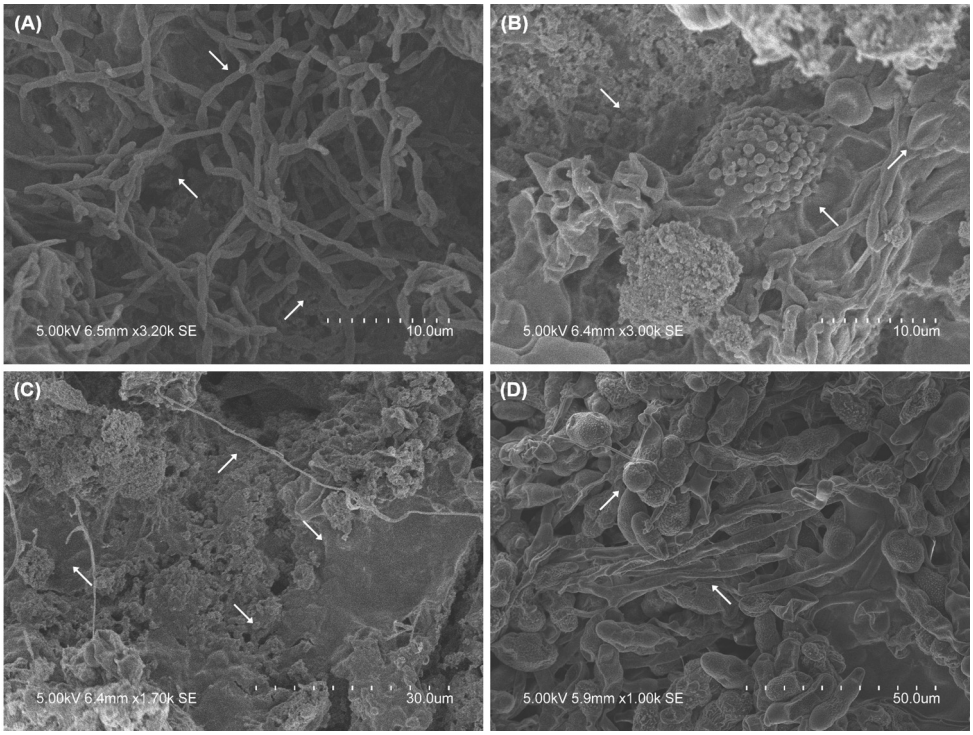


Fig. 3. SEM micrograph of mortar microfragments, evidencing biofilms formation (A-D), Actinomycete filaments (A), filamentous fungi and hyphae proliferation (B-D) in the surface of the mortar and the penetration of these microorganisms in depth.

aged. Therefore, in this way biocide tests were performed to control efficiently the microbial proliferation.

3.3. Biocides application

The use of biocides to treat mural paintings is very important to prevent the proliferation of microorganisms as already reported by other authors (Blazquez et al., 2000; Warscheid and Braams, 2000; Ascaso et al., 2002; Domenech-Carbo et al., 2006; Urzì and De Leo, 2007; Moreau et al., 2008; Fonseca et al., 2010; Gaylarde et al., 2011; de los Ríos et al., 2012; Maxim et al., 2012; Pinna et al., 2012; Speranza et al., 2012).

To determine the efficacy of different biocides to inhibit the growth of the microorganisms which are developing on the paintings, laboratory tests, using commercial compounds and natural biocompounds, were carried out against high cell concentration of fungal strains, previously isolated from the mural paintings of the *Casas Pintadas*. The results of the antifungal activity are summarised in Table 2 and analysed according to the inhibition halo formed in the solid cultures.

Among the commercial compounds tested, Preventol PN® 1% was the most efficient agent for inhibiting the development of all the fungal isolates, promoting almost total inhibition for majority of these microrganisms. This compound has hydrophilic and hydrophobic chemical groups, able to disrupt the cell membrane structure of the microorganism, causing leakage of intracellular materials (Ascaso et al., 2002) and consequently their death.

In the case of Panacide® compound, the inhibition induced was satisfactory for all the microorganisms tested, being less effective for fungi of the genus *Cladosporium*, however inhibits considerably their growth. The compound Linquad® produced satisfactory results, however showed lower inhibition capacity than the other biocides tested. The majority of the fungal isolates were inhibited with more efficiency by Preventol PN® 1%, followed by Panacide® and then Linquad®. At the concentration 0.1% the biocides Panacide® and Linquad® did not present inhibition capability.

Due to the toxicity of Preventol PN®, less concentrated solutions were tested, however with Preventol PN® 0.1% their inhibitory capacity decreases considerably.

According to these results, mixtures of biocides, composed by the most effective compounds, should be employed in artworks treatment, to allow an effective microbiological elimination and avoid a quick recolonisation (Gaylarde et al., 2011; Bruno et al., 2014).

This strategy was applied in this work and the results showed that the biocides have the ability to inhibit the growth of all isolated fungi, promoting good inhibition results particularly with a combined application of Preventol PN® and Panacide® in the mural paintings of *Casas Pintadas* (Fig. 4).

The information obtained on the material characterisation and microbiological identification were integrated on the intervention plan, executed to rehabilitate the mural paintings of *Casas Pintadas*. During the conservation-restoration process, painting layers were fixed, holes were filled and some areas were retouched. One has to take into consideration that after the conservation-restoration process it is important to control and prevent possible recolonistion. Therefore, preventive conservation measures were taken and presently a long term *in situ* monitoring is ongoing that encompasses weekly measures of temperature and relative humidity in the paintings area, and monthly photogrametry/photographic assessment and collection of possible neoformation products (salts) and microorganisms.

The biocides considered in this work were able to eliminate and control microorganisms development. *In situ* application of biocides in these paintings did not promote chromatic alterations neither mortar damages (data not shown).

Despite the inhibitory capacity of these chemical compounds, it is necessary to find alternative solutions due to their toxicity to humans and environment. To solve this problem, several studies

Table 1
Macro- and micromorphological features of the predominant microbial isolates, being the identification of the fungal strains complemented with ITS region sequencing.

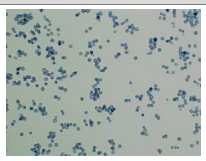
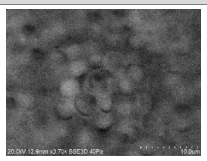
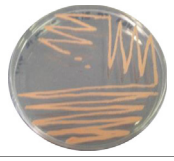
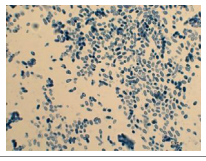
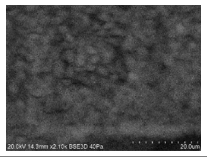
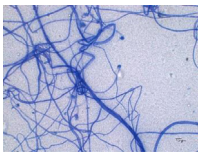
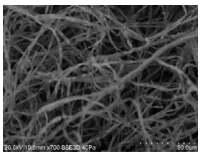
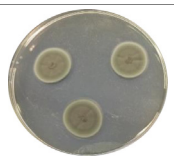
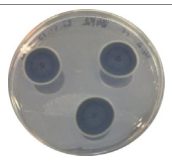
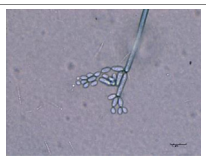
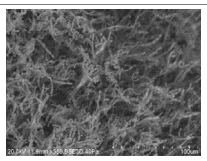
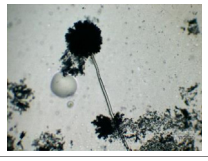
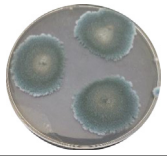
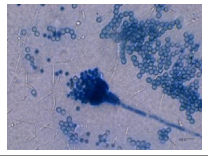
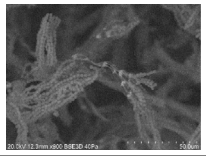
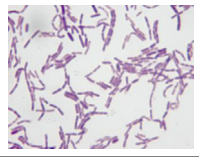
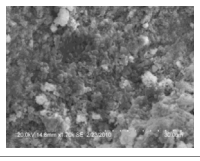
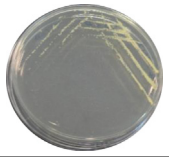
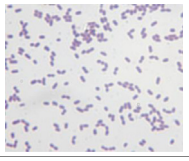
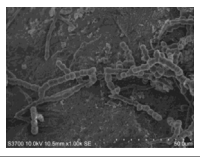
Code	Macroscopic features		Microscopic features		Identif.
	Front	Back	Optical microscopy	SEM	
Z1LA					*Cryptococcus* sp.
Z1LB					Yeast
Z1F2					Mycellium
Z4F2					*Cladosporium* sp.
Z4F4					*Aspergillus* sp.
Z5F1					*Penicillium* sp.
Z1A					Bacillus
Z3C					Coccus

Table 2
Effect of commercial compounds against different fungal strains isolated from *Casas Pintadas* (Z1LA – *Cryptococcus* sp.; Z1LB – Unidentified yeast; Z1F2 – Unidentified fungus; Z3F2 – *Aspergillus* sp.; Z4F2 – *Cladosporium* sp.; Z4F4 – *Aspergillus niger*; Z5F1 – *Penicillium* sp.).

	Inhibition halo (mm)						
	Z1LA	Z1LB	Z1F2	Z3F2	Z4F2	Z4F4	Z5F1
Preventol 0.1%	27.8 ± 1.7	25.7 ± 3.9	18.7 ± 3.1	22.9 ± 2.5	21.2 ± 1.4	27.2 ± 1.5	20.2 ± 5.0
Preventol 1%	50.3 ± 0.6	–	35.9 ± 1.2	43.3 ± 2.9	44.7 ± 0.8	45.7 ± 1.2	40.6 ± 4.0
Panacide	24.7 ± 0.6	27.5 ± 1.5	30.3 ± 1.5	40.3 ± 0.6	37.3 ± 3.8	22.7 ± 1.5	22.0 ± 0.0
Linquad	31.7 ± 2.9	33.0 ± 2.0	26.3 ± 1.5	34.3 ± 0.6	31.3 ± 1.5	16.0 ± 1.0	19.7 ± 0.6

are being performed in the development of green solutions which are eco-friendly and without negative effects on the environment or human beings. Biotechnological based compounds, obtained by bacterial cells of the genera *Bacillus* are being used on growth inhibition studies, which can be a great alternative for cultural heritage safeguard mitigation strategies, due to the capacity of these

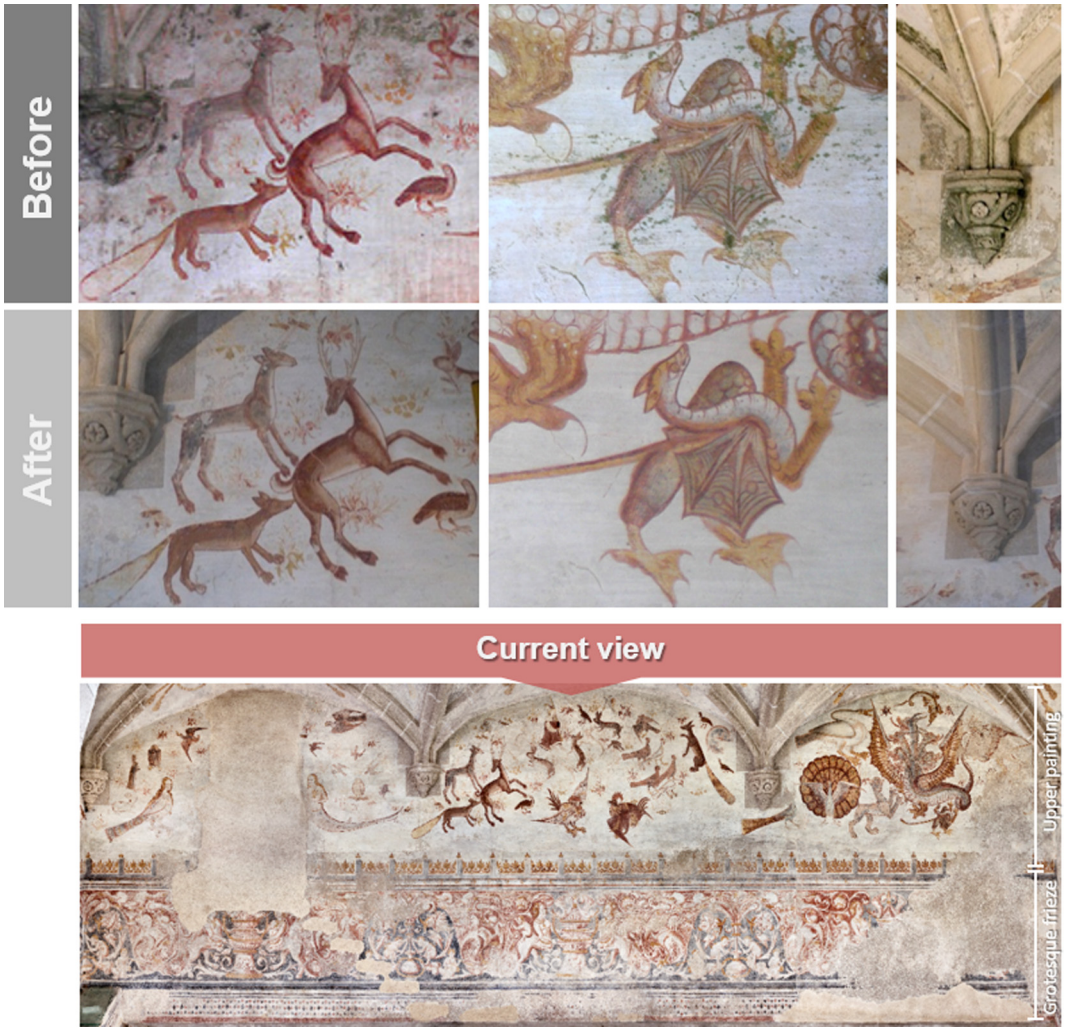


Fig. 4. Details of the mural paintings of *Casas Pintadas*, before and after treatment with biocide and conservation-intervention process, and the current aspect of the paintings.

microorganisms to produce a great diversity of secondary metabolites with antagonistic action against many fungal strains (Silva et al., 2015).

Therefore, in this work and to envisage a future alternative application, three different natural biocompounds, produced in our laboratory (Silva et al., 2015, 2016), were tested *in vitro* against the fungal population present on the mural paintings of *Casas Pintadas* and encouraging results were obtained.

All the new natural biocompounds tested revealed inhibition capacity of the different fungal strains (Fig. 5) present in the paintings of *Casas Pintadas*. The biocompound BEVOTECH-11 was the most efficient inhibitory agent, forming in some cases higher inhibition levels than Preventol 0.1% (Z1F2), Panacide (Z1LA) and Linquad (Z4F4) (Table 2). However, the biocompounds BEVOTECH-14 and BEVOTECH-16 promoted also good results, and BEVOTECH-14 seems to be more efficient for Z1LB than BEVOTECH-11 and BEVOTECH-16 (Fig. 5). These biocompounds showed large spectra of fungal inhibition, displaying inhibition levels and evidencing that they can be a natural alternative to the commercial chemical compounds. Furthermore, these novel biocompounds are harmless and handling safe, acting by nontoxic mechanisms and hence could represent a new approach in this field (Silva et al., 2016).

The present promising results prompt the use of this novel biocides in future monitoring research programs for microbiological growth control, due to their inhibition efficiency, non-harmful and ecofriendly characteristics (Silva et al., 2016), to evaluate their effectiveness for the control of microbial population on Cultural Heritage assets.

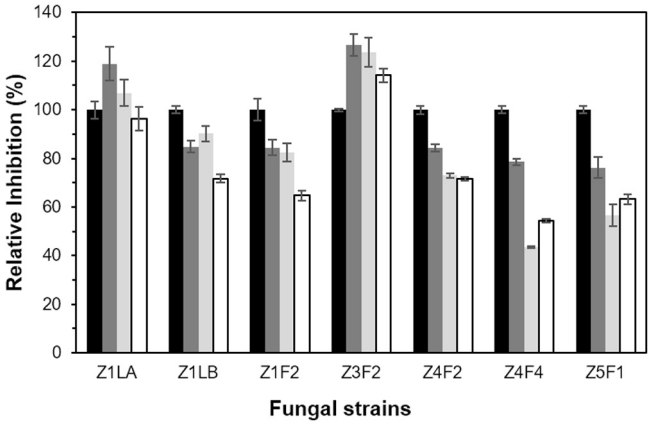


Fig. 5. Antifungal activity of the biocompounds (■- Mycostatin®; ■ - BEVOTECH-11; ■ - BEVOTECH-14; □- BEVOTECH-16) against different fungal strains isolated from *Casas Pintadas* (Z1LA – *Cryptococcus* sp.; Z1LB – Unidentified yeast; Z1F2 – Unidentified fungus; Z3F2 - *Aspergillus* sp.; Z4F2 – *Cladosporium* sp.; Z4F4 – *Aspergillus niger*; Z5F1 – *Penicillium* sp.). Mycostatin® was used as positive control.

4. Conclusions

Several microorganisms like bacteria, yeast and fungi were identified on the frescoes of *Casas Pintadas*, however the main contamination agents isolated were filamentous fungi of the genera *Aspergillus*, *Cladosporium* and *Penicillium*.

The microbial population detected is responsible for the biodegradation of the mural paintings in *Casas Pintadas* and may have an important role on the overall degradation process. Due to a wide microbial diversity present in these paintings it was necessary to develop combined application of biocides to prevent efficiently their proliferation. The greatest efficacy against fungi was obtained for formulations with Preventol PN® and Panacide® which were used by the conservation team on the treatment of these paintings.

Alternative safer biocides were investigated and novel biocompounds isolated from bacteria's cultures showed effective inhibition levels, representing a groundbreaking and environmentally safe alternative to the commercial toxic compounds, encouraging their future application on biocontaminated cultural assets to prevent their recolonisation.

In conclusion, to efficiently eliminate and control the development of the microorganisms actively involved in the biodegradation process it is crucial to have a deep knowledge of the decay processes and remediation solutions before the conservation-restoration intervention, and, on the other hand to develop preventive monitoring programs to ensure the longevity of the intervention and the safeguard of the artwork.

Conflict of interest

The authors have no conflict of interest to declare.

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