METHODS

Application of Molecular Techniques to the Elucidation of the Microbial Community Structure of Antique Paintings

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Abstract This paper uses molecular techniques to describe the microstructure and microbiological communities of sixteenth century artwork and their relationships. The microbiological populations, analysed by denaturing gradient gel electrophoresis (DGGE), were highly influenced by the chemical composition of the pictorial layers detected by energy-dispersive X-ray analysis. DGGE revealed that the diversity of microbial communities was lower in pictorial layers composed of pigments with metals, such as Pb, Cu and Hg, than in those found in pictorial layers without such compounds. The number of cultivable microorganisms, mainly fungi and bacteria, was very low in comparison to those found by DGGE, revealing the presence of both cultivable and as-yet-uncultivated (or not viable) species in the samples analysed. Both fungi and bacteria were present in a non-random spatial distribution. Environmental scanning electron microscopy and fluorescent in situ hybridisation analyses revealed that bacterial populations were usually found in close contact with the surface of the pictorial layers, and fungal populations were located on the bacterial biofilm. This work shows, for the first time, the correlation between the diversity of the microbial populations and the chemical composition of the pictorial layers of an artwork.

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Introduction

Paintings are composed of a wide range of organic and inorganic constituents [11] and provide different ecological niches that may be colonised by a large variety of microorganisms. A number of reviews provide a comprehensive picture of the role of microorganisms in the degradation of art objects, such as paintings, stone, wood, paper, masonry, leather, parchment, glass and metal [2, 5, 7, 15, 17]. Many of the components of paintings are biodegradable, and those binders and additives (glues, oils, plasticisers, emulsifiers, thickeners, etc.) that facilitate drawing or the application of paint layers are susceptible substrates for microbial colonisation. Furthermore, the support material (paper, wood, wool, silk, etc.) and many components such as polysaccharides, gums, proteins, oils, egg yolk, etc. may easily support microbial growth if favourable environmental conditions (humidity, temperature, pH, light) are met. By contrast, several components of paintings, such us the heavy metals in pigments, have antimicrobial properties and are important agents that inhibit or modulate the growth of microbial populations. In short, environmental conditions combined with rich nutrients in antique pictorial substrates create suitable conditions for the development of bacteria or the reproduction of quiescent fungal spores. As colonisation proceeds, the smooth surface of the painting is degraded and the pictorial layers became rough and susceptible to attack by new microorganisms [7]. Accordingly, the microbial flora is usually the result of successive colonisations by different microorganisms. The alterations suffered by the substrates are the result of modifications of their chemical composition, to which the microorganisms themselves may partially contribute. Microorganism growth on paintings may cause structural damage involving different processes, such us

cracking, exfoliation of paint layers, formation of paint blisters and detachment of the paint layer from the support. Strongly linked to the structural damage is the aesthetic damage, which involves the appearance of pigment discolouration and stains. According to the literature, aesthetic damage occurs earlier than structural damage and may precede serious corruption of the materials. The microbial deterioration of paintings is caused by degradation due, firstly, to the hydrolytic activities that the microorganisms undertake to sustain growth and, secondly, to the damage that excretion metabolites (solvents, acids, etc.) inflict. In addition to the physical stability of the paint, these compounds may also alter colours [7].

Many studies have reported on the microbial populations that are present in paintings, but they have often been limited to fungi [22–24, 31] and bacterial [37] or phototrophic microorganisms [6]. Only a few reports have described the majority of the microbial populations present in art objects [7, 25]. Such studies should start with a descriptive analysis, cataloguing which organisms are found on which substrate. This is a necessary starting point for any restoration process. Traditionally, microbiology research carried out in the field of biodeterioration has been based mainly on classical cultivation methods. Culture-based approaches, whilst extremely useful for understanding the physiological potential of isolated organisms, do not necessarily provide comprehensive information on the composition of microbial communities. Due to the well-documented disparity between cultivable and in situ diversity [3, 12, 13, 18, 38, 46], it is often difficult to assess the significance of cultured members in microbial communities. In addition, extensive cultivation strategies require more sample material than could be obtained from art objects. Microbiological investigations of the compositions of microbial communities such as soil or aquatic environments, mural paintings, wall frescoes, etc. have shown the versatility of denaturing gradient gel electrophoresis (DGGE) as a molecular analytical method [9, 14, 40, 41, 44]. Using both ribosomal DNA (rDNA) analyses by DGGE and culture techniques, it is possible to characterise the microbial diversity and culture characteristics of the isolated microorganisms in different environments, building up a more complete picture. These molecular techniques in combination with others, such as scanning electron microscopy (SEM)–energy-dispersive X-ray (EDX), are used in this work to gather knowledge on the relationship between the elemental composition of the paint layers and the microbial populations.

However, it is also important to understand the mechanisms responsible for the microbial attack. To that end, it will be necessary to follow up the variations in the microbial populations depending on the physicochemical conditions. In order to gain information on the microbial communities inhabiting biodeteriorated sixteenth century Spanish paintings, this research determines the microbial colonisation of an art object and explains the changes in the microbial populations as a function of the pictorial composition, giving a global view of the problem of microbial development in artworks.

Methods

The Oil Painting Studied

The artwork studied is an oil painting on wood called "Cristo con la Cruz a Cuestas". It belongs to the sixteenth century Spanish School (Fig. 1). It was stored for decades inside a dark damp cellar. Now, following its restoration, it is to be found at the Archbishopric of Toledo, Spain.

Sampling Sites, Microbial Isolation and Identification

Two different types of specimens from the sixteenth century oil painting were obtained in collaboration with the restorers. First, in order to culture the microorganisms present on the paint surface, specimens were obtained using sterile swabs; various 2-cm² areas of the painting were sampled. Second, for sample preparation for environmental scanning electron microscopy (ESEM), SEM–EDX, fluorescent in situ hybridisation (FISH) and DGGE studies, duplicate specimens were obtained by scraping off surface material to a depth of 1 to 3 mm with a sterile scalpel. Two representative specimens (1 and 2) were obtained from five different damaged areas (a, b, c, d and e; Fig. 1). These areas were selected in order to determine the microbial population present in different areas with particular problems (colour alteration, microbial growth, blistering, exfoliation, etc.). Sample "a" was taken from a well-conserved zone characterised by the presence of a clear pigment (probably white in origin) and deteriorated by the formation of small paint blisters. Sample "b" was obtained from a cyan-blue area reflecting the sky and characterised by the presence of cracking. Sample "c" was taken from a clear brownish area characterised by discolouration, probably due to the presence of humidity and fungal colonisation during storage. Sample "d" was located in an area characterised by the presence of cracking and a dense dust patina which was hiding the pictorial motifs and where it was possible to observe (after restoration) the presence of a reddish flag and a lance of the infantryman located at the background. Sample "e" was taken from a gold colour area which was relatively well conserved and located at the aureole surrounding Christ's head.

With a view to isolating viable microorganisms, both fungi and bacteria from swab samples were directly
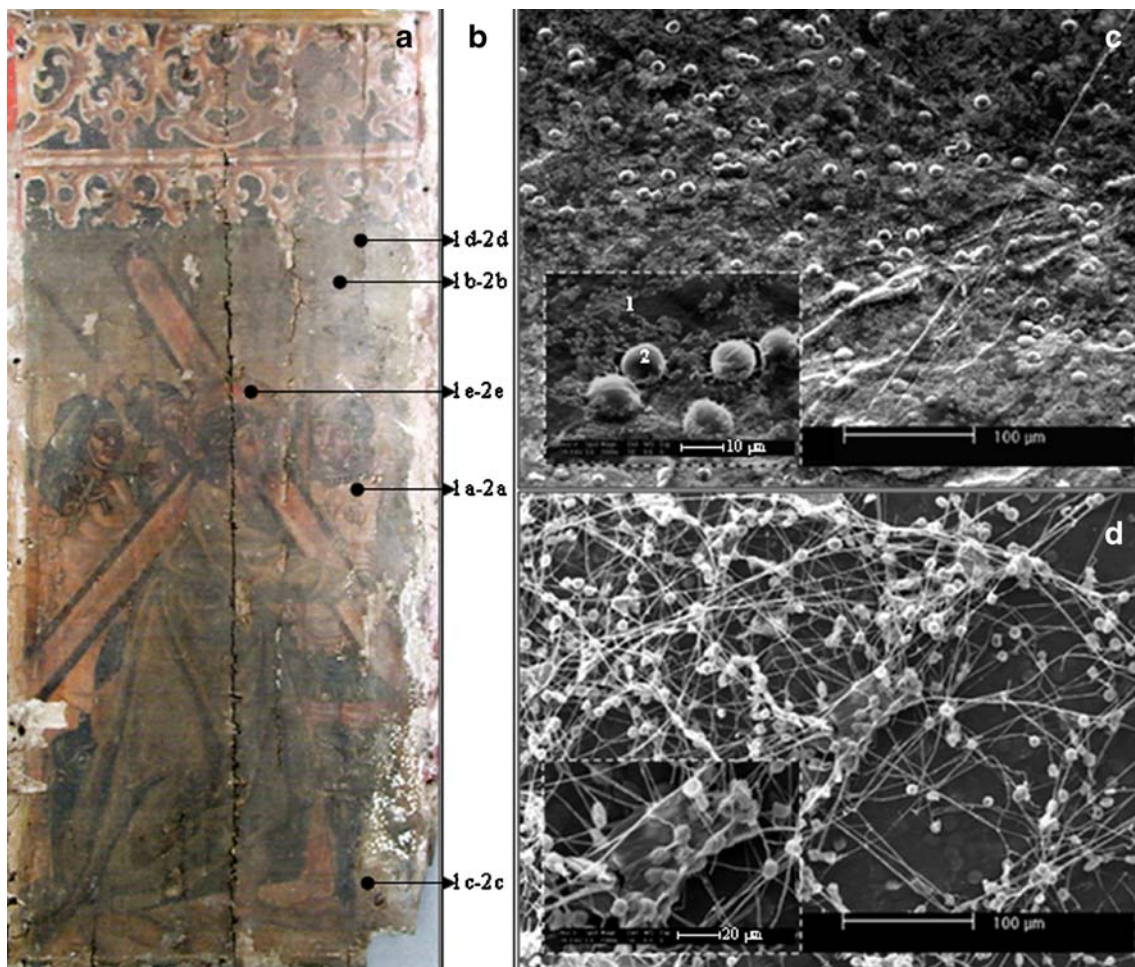
Figure 1 *a* The art work studied is a sixteenth century painting on wood called "Cristo con la Cruz a Cuestas". *b* Two samples (1 and 2) from every sampling area (areas *a* to *e*) were obtained. Electron micrographs of surface material from specimen *1b* (*c*) and specimen *1e* (*d*). The material shows a dense microbial colonisation. Bacteria (*1*) and fungal spores (*2*), which are attached to the surface material, are shown

inoculated on the surface of the appropriate media. Bacterial and fungal strains were identified in the Spanish Type Culture Collection by using BIOLOG technology (Biolog, Inc., CA, USA) and additional morphological and biochemical studies.

Culture Conditions

Bacterial strains were grown on 1/100 diluted tryptone soy agar and tryptone soy broth (TSB). Incubation was performed at 15°C and 30°C for a period of 15 days. For DNA extraction, 25-ml TSB cultures were harvested by centrifugation and then washed with sterile Milli-Q water. Pellets were transferred to microcentrifuge tubes, lyophilised and processed for DNA extraction.

All filamentous fungi were maintained and grown at 15°C and 30°C on Sabouraud glucose agar (Oxoid Ltd.) or Sabouraud glucose broth, respectively. For DNA extraction from fungal isolates, 25-ml Sabouraud broth cultures were harvested by centrifugation and then washed with sterile

Milli-Q water. The collected mycelia were transferred to microcentrifuge tubes, lyophilised and processed for DNA extraction as described in due course.

Sample Preparation for Environmental Scanning Electron Microscopy

As described above, specimens were taken with sterile scalpels and stored in sterile Eppendorf tubes. A Philips XL30 Environmental Scanning Electron Microscope with an EDAX X-ray analyser was used under a "wet mode" protocol at 2°C and a hydrostatic pressure of 5 Torr.

Scanning Electron Microscopy with Energy-Dispersive X-ray Microanalysis

An SEM was used in combination with EDX microanalysis to characterise the structure of the paint layers of specimens taken from the painting. The sequence of the various paint

layers, as well as the distribution of the elements present in the pigments, was obtained from the cross-sectioned specimens. SEM analysis of the cross sections was carried out with a JEOL JM-6400 SEM microscope with an acceleration voltage of 20 kV and a resolution of 35Å at a working distance of 8 mm at 35 kV. The microscope was fitted with a LINK exL energy dispersion X-ray spectrometer providing a resolution of 138 eV at 5.39 keV. For SEM–EDX analysis, the sample was previously coated with a conductor (graphite).

Purification of Fungal DNA and DGGE Analysis of Fungal Samples

Prior to DNA extraction, heavy metals were removed from the samples by using a Chelex-100 ion-exchange resin (Bio-Rad). Fifteen milligrammes of powdered samples (from fungal cultures or from pictorial specimens) was extracted by adding 300 μl 5% Chelex-100 resin and 6 μl (10 mg ml^{-1}) proteinase K (Sigma). The mixture was incubated at 55°C for 50 min and for 10 min at 96°C. DNA extracts were vortexed for 10 s and centrifuged for 2 min at 12,000 rpm. Aliquots of the samples were routinely stored at −20°C and then used for PCR amplification [26].

Fifty microlitres of supernatant from Chelex resin was incubated with 0.5 ml 0.5 M EDTA, 20 μl 10% sodium dodecyl sulphate and 40 μl proteinase K (10 mg ml^{-1}) at 37°C for 12 h with mixing. According to the manufacturer's protocols, albeit with slight modifications, 1 ml DeHybernation Solution A (Geneclean Kit, MP Biomedicals) was added to each sample and incubated for 2 h at 60°C with mixing. Samples were centrifuged at 12,000×g (5 min) to pellet particulate material. The supernatant was transferred to a microcentrifuge tube and then 300 μl of DNA Glassmilk suspension was added to each sample and mixed for 30 min at room temperature. The manufacturer's instructions were then followed to obtain the final DNA extracts.

In order to amplify 260-bp fragments from a variable region of the fungal 28S rRNA gene, two primers (U1 and U2) were used for PCR as reported [31]. Fungal 28S rDNA fragments from samples were amplified by nested polymerase chain reaction (PCR) using previously published primers [39]. Nested PCR assays were conducted according to Mölenhoff et al. [31]. All PCR products (5 μl) were analysed by electrophoresis in 1.5% agarose gels for 60 min at 100 V before DGGE was carried out.

PCR products of the fungal communities from paintings and corresponding fungal isolates were analysed by the DCode System (Bio-Rad) using 10% (w/v) acrylamide (37.5:1 acrylamide/bisacrylamide) gels. All reagents and techniques were as previously described [32, 34]. DNA from 11 identified isolates (Alternaria alternata, Aspergillus versicolor, Chaetomium globosum, Cladosporium cladosporoides, Aspergillus hollandicus, Eurotium chevalieri, Aspergillus terreus, Aspergillus oryzae, Aureobasidium spp., Penicillium spp. and Penicillium stoloniferum) were used as internal standards for DGGE.

Bacterial DNA Extraction Methods, DGGE and Sequence Analysis

DNA of bacterial microcosms from paintings or isolated from paintings were obtained according to previously described methods [10, 45]. Furthermore, bacterial DNA was purified using the Chelex-100 resin as described above. The amount and quality of DNA extracted were estimated by electrophoresis of 5-μl aliquots on an 0.8% agarose gel and compared to a molecular weight standard (stained using ethidium bromide). DNA extracts were stored at −80°C prior to analysis.

The PCR amplification of approximately 200 base pairs of the V2–V3 region, corresponding to positions 339 to 539 of Escherichia coli, of the 16S ribosomal DNA (rRNA gene) was performed by using the eubacterium-specific primers HDA1 and HDA2 used by Walter et al. [45], according to the methodology described. All PCR amplification products were analysed on 1% agarose gels.

PCR products, derived from bacterial microcosm community samples, were resolved as follows. A DCode Universal Mutation Detection System (Bio-Rad) with 16-by-16-cm and 1-mm-deep polyacrylamide gels (8%) run with Tris–acetate–EDTA buffer was used for these analyses. Gels were run for 6 h at 150 V at a temperature of 60°C. Initially, separation parameters were optimised by running PCR products from selected pure cultures of bacteria and PCR amplicons from extracted community DNA on gels with a 0% to 100% denaturing gradient perpendicular to the direction of electrophoresis (a 100% denaturing solution contained 40% [vol/vol] formamide and 7.0 M urea). Denaturing gradients were formed with two 8% acrylamide (acrylamide–bisacrylamide, 37.5:1) stock solutions (Sigma). On this basis, a denaturing gradient for parallel DGGE analysis ranging from 30% to 60% was selected. PCR amplicons from bacterial isolates (Pseudomonas alcaligenes, Nocardia asteroides, Arthrobacter spp., Bacillus subtilis, P. aeruginosa, Streptococcus spp., P. fluorescens, Micrococcus roseus, B. pumilus, Streptomyces spp. and M. luteus) were run on a parallel gel in order to validate the separation conditions and determine their DGGE signals. For community analyses, the gels also contained a 30% to 60% denaturing gradient. DGGE bands were visualised as described above.

Excised DGGE bands were sequenced directly from PCR products which were re-amplified with the primer set used above. Prior to sequencing, the PCR products were analysed by DGGE to confirm the band positions relative to the original sample. Sequencing was performed with an

automatic sequencer 3730×l (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of DGGE were deposited in the GenBank database under accession numbers from FJ968640 to FJ968650. All sequencing results obtained were analysed using the BLAST programme and the results were analysed in order to corroborate the bacterial identification.

Fluorescent In Situ Hybridisation for Bacteria and Calcofluor White Staining of Fungi

In brief, fixed paraffin-embedded samples from paintings were cut (thickness 50 μm) using a sterile approach, and the sections were mounted on poly-L-lysine-coated slides. The slides had previously been cleaned with 95% ethanol and allowed to air dry. Slides were run through a graded series of 50%, 70% and 95% ethanol and then fixed in a solution of 10% formaldehyde–90% ethanol. For hybridisation, 50 μl of hybridisation solution (0.9 M NaCl, 20 mM Tris–HCl, pH 8, 0.1% sodium dodecyl sulphate 8 ng of fluorescein isothiocyanate (FITC)-labelled oligonucleotide per microlitre and 25 μM calcofluor white) was dropped onto the slide. Incubation was performed with a domain Eubacteria-specific probe to 16S ribosomal RNA (5′-F-ACTGCTGCCTCCCGTAGGAGTTTATTCCTT-3′). Primers were synthesised commercially and 5′-labelled with FITC (Operon Technologies). A coverslip was sealed on each slide with rubber cement. Slides were placed in an MJ Research PTC-16S thermal cycler with a petri dish of distilled deionised water to maintain humidity for 16 h at 45°C. Coverslips were removed and slides were washed in 50 ml of hybridisation solution for 15 min at 45°C.

As stated above, in order to simultaneously detect filamentous fungi, a stain was made with calcofluor white (Sigma), which is a fluorescent probe that has a high affinity for chitin-forming hydrogen bonds with free hydroxyl groups. The excitation wavelength for the calcofluor white stain was 346 nm and the signal acquired is blue.

After FISH and calcofluor white staining, slides were examined on a Leica DMRB epifluorescence microscope equipped with excitation filter cubes for fluorescein and calcofluor white. PTI ImageMaster 5.0 Software was used. FISH showed the spatial localisation of prokaryotic microorganisms whilst the calcofluor white method revealed filamentous fungi localisation.

Results

Structural Analysis of the Painting by SEM–EDX

An example of the application of SEM–EDX to the study of paint and ground layers from a sixteenth century painting is shown in Table 1 and Fig. 2, displaying the results of the element distribution of the different specimens studied in this work. As expected, the analysis of the different samples revealed high heterogeneity in the structure and composition of the pictorial layers of the different specimens, most of them related to the different pigments used in drawing. However, the preparative ground layers were more homogeneous in composition, especially the *gesso sottile* (preparative layer of highly refined gypsum), in the specimens studied from different areas. Pictorial layers were mainly composed of Pb, Cu, Hg, Au and several others, all of which are representative elements of known pigments such as vermilion, verdigris, lead white, etc. Ground layers were composed of a wide variety of elements present in sulphates, carbonates and aluminium silicates, originating in natural sources used in the past in these preparative ground layers (Table 1).
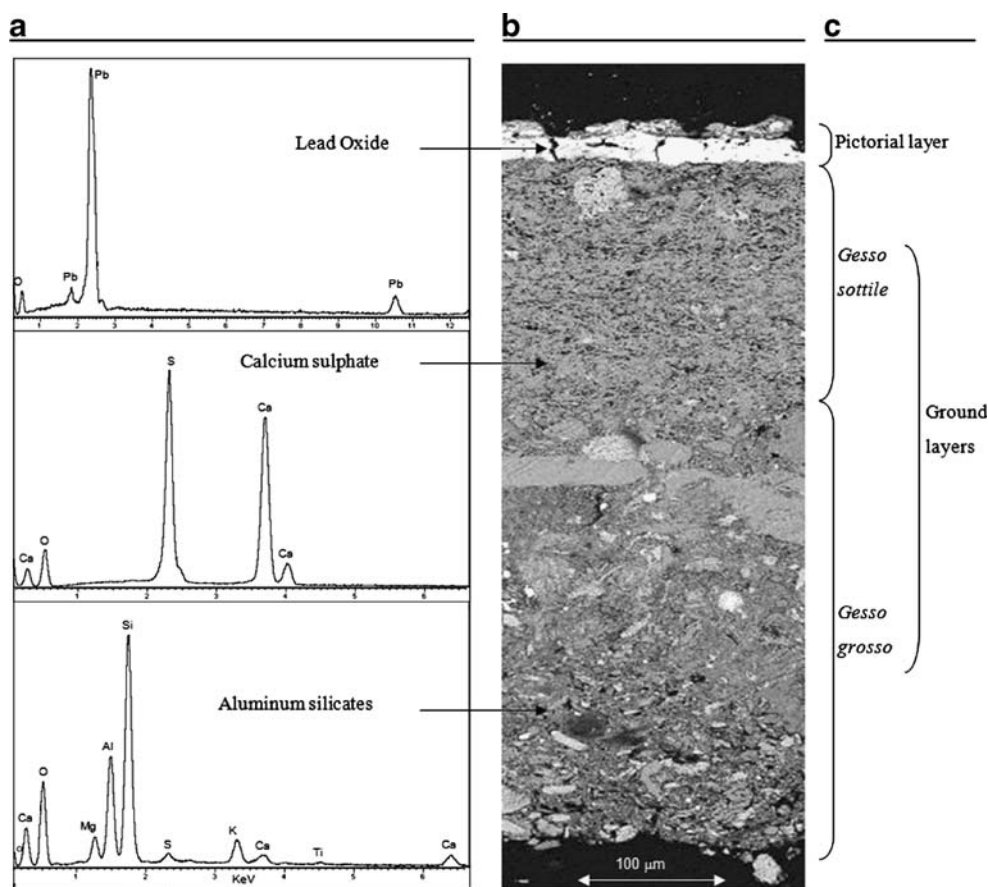
The backscattered electron image in Fig. 2 clearly reveals the structure of one of the studied paint layers due to the different electronic densities and particle morphologies. The study was conducted on the cross section of a microsample so as to retain the original paint layer structure. The sample had two ground layers and one white paint layer. The predominant component of the ground layers (*gesso grosso* and *gesso sottile*) was shown to be gypsum ($CaSO_4$), mainly $CaSO_4 \cdot 2H_2O$ in *gesso sottile* together with carbonates and aluminium silicates. The paint layer was composed entirely of lead white [$Pb_3(CO_3)_2(OH)_2$].

Isolation of Bacteria and Fungi from Painting Samples

We were able to isolate 22 different strains of heterotrophic bacteria and fungi. With some exceptions, bacterial isolates were obtained in quantities ranging from 10^1 to $10^4 CFU/cm^2$ and fungal isolates were detected in ranges from 10^1 to $10^5 CFU/cm^2$. No significant differences were observed between cultures developed at 15°C or 30°C. An important characteristic of microorganisms biodeteriorating art work is the colony pigmentation amongst the purified bacterial and fungal strains which varied between isolates, showing the typical and characteristic colours described previously for these microorganisms. The positively identified strains belonged to the following bacterial and fungal species: *P. alcaligenes*, *N. asteroides*, *Arthrobacter* spp., *B. subtilis*, *P. aeruginosa*, *Streptococcus* spp., *P. fluorescens*, *M. roseus*, *B. pumilus*, *Streptomyces* spp., *M. luteus.*, *A. alternata*, *A. versicolor*, *C. globosum*, *C. cladosporoides*, *A. hollandicus*, *E. chevalieri*, *A. terreus*, *A. oryzae*, *Aureobasidium* spp., *Penicillium* spp. and *P. stoloniferum*. These species have been previously reported on paintings by standard cultivation techniques [7]. Identification of bacteria was also corroborated by BLAST analysis of the 16 rDNA sequence obtained from excised DGGE bands as described.

Table 1 Element composition of pictorial and ground layers determined by SEM–EDX

Samples	Pictorial layer		Ground layers			
			Gesso sottile		Gesso grosso[a]	
	Elements	Relative abundance (% weight)	Elements	Relative abundance (% weight)	Elements	Relative abundance (% weight)
1a	Pb	92.83	S	23.65	Al	11.10
	O	7.17	Ca	29.27	Si	27.15
			O	47.08	O	47.33
					Others[b]	14.1
1b	Cu	69.95	S	22.29	Al	10.40
	O	22.68	Ca	28.39	Si	24.55
	Others[b]	7.36	O	46.29	Fe	5.68
			Others[b]	3.82	O	46.31
					Others[b]	12.64
1c	Pb	92.83	S	22.95	Al	8.49
	O	7.17	Ca	30.52	Si	19.59
			O	46.53	S	5.87
					Ca	6.93
					O	46.42
					Others[b]	12.7
1d	S	11.76	S	21.91	Al	10.95
	Hg	63.61	Ca	30.26	Si	25.37
	Others[b]	1.61	O	46.23	Fe	8.94
			Others[b]	1.6	O	45.31
					Others[b]	9.43
1e	Au (Bol)	100	S	22.44	Al	9.19
			Ca	30.35	Si	21.66
	Al	12.54	O	46.43	Ca	13.96
	Si	23.99	Others[b]	0.77	O	43.28
	Fe	9.29			Others[b]	11.9
	O	44.61				
	Others[b]	9.57				

[a] Gesso grosso is composed by a mixture of gypsum and aluminium silicates. The table shows the elemental composition of aluminium silicates
[b] Elements in a percentage less than 5%

DGGE Analysis

The five duplicated samples taken from different areas of the painting were analysed by using the methodological approach described above. DGGE analysis was performed with the DNA obtained from the pictorial samples and, separately, from all isolates. The migration of the DGGE bands was compared. DGGE analysis showed that the bands from either type of sample matched the controls (Fig. 3). Unless there is band comigration, these results confirm that the isolated strains are an important part of the microbial population inhabiting the pictorial layers. Band comigration was discarded by sequencing partial 16S rRNA genes of 11 bands excised from DGGE gels. BLAST analysis revealed that the sequence similarity between cultured isolates and uncultured samples was very high, ranging from 99% (*Streptomyces* spp. and *Arthrobacter* spp.) to 100%. These results, obtained from sequences, confirmed the identity of the bacterial isolates that were present in the pictorial surface and indicated that many of the cultured representatives were found to be dominant in the molecular profiles of DGGE. Furthermore, the PCR amplification of DNA obtained from pictorial layers was not observed to be affected by the complex matrix of inorganic salts and organic substances present in the sampling material. A surprising number of DNA bands from bacteria and fungi were obtained as members of microbial communities colonising the painting, although

Figure 2 EDX analysis of specimen 1a. *a* Energy-dispersive X-ray microanalysis showing the relative abundance of elements in the difference of pictorial and preparative ground layers. *b* Backscattered electron image of the cross section of the specimen. *c* Description of layers


only a small number of them were isolated. Assuming that one band of DGGE represents one unique ribotype, DGGE band patterns from pictorial samples contained a high number of different ribotypes and major differences between samples were found. Different band patterns were detected in which different bands were dominant and some bands were variable. The percentage (obtained by comparing the number of DGGE bands between samples) of the bacterial and fungal ribotype numbers of pictorial samples with metals or heavy metals (Pb, Cu or Hg) was lower (approximately between 10% and 25%) than that found in the samples with inert metals such as gold (samples 1e and 2e; Figs. 3 and 4). These results are consistent with the idea that some heavy metals inhibit the microbial populations. Although, the comparison between bacterial and fungal DGGE analysis must be carefully done, it was interesting to observe that the fungal ribotypes in surfaces with copper stains (samples 1b and 2b) were reduced, but the bacterial population was less affected, showing that fungal cells are more sensitive to copper than their bacterial counterparts [8]. This assumption must be taken with care because the presence of a band in DGGE could represent a microorganism that is not necessarily viable, although SEM and

FISH photographs of these samples (Figs. 1 and 5) revealed the presence of a high level of bacterial colonisation and the presence of a low number of fungal cells (conidia), corroborating the findings of DGGE analysis.

FISH Analysis

DGGE and FISH analyses are known to be useful tools for monitoring the microbial composition in enrichment cultures as well as for isolating pure cultures from cocultures [30]. The study of the biofilm using FISH with rRNA-targeted oligonucleotide probes provided novel insights regarding the structure of the microbial communities present. Samples were stained with calcofluor white and FITC for the determination of fungal and bacterial populations, respectively. The analysed painting samples showed the prevalence of fungal mycelium located on the aerial part of the biofilm, and bacterial cells were preferentially situated in close contact with the pictorial layers. As stated in Fig. 5, a dense FITC green colour was observed in specimen 1a, revealing the presence of a complex biofilm composed mainly of bacteria of different morphologies. Fungal structures, mycelium or fungal

Figure 3 Negative image of DGGE gels showing fungal community fingerprints from biofilm samples (1a, 2a, 1b, 2b, 1c, 2c, 1d, 2d, 1e and 2e) and a mixture of amplicons from pure cultures of isolated fungal strains (M). (*1*) *A. alternata*, (*2*) *A. versicolor*, (*3*) *C. globosum*, (*4*) *C. cladosporoides*, (*5*) *A. hollandicus*, (*6*) *M. rouxii*, (*7*) *A. niger*, (*8*) *A. oryzae*, (*9*) *Aureobasidium* spp., (*10*) *P. chrysogenum* and (*11*) *P. stoloniferum*
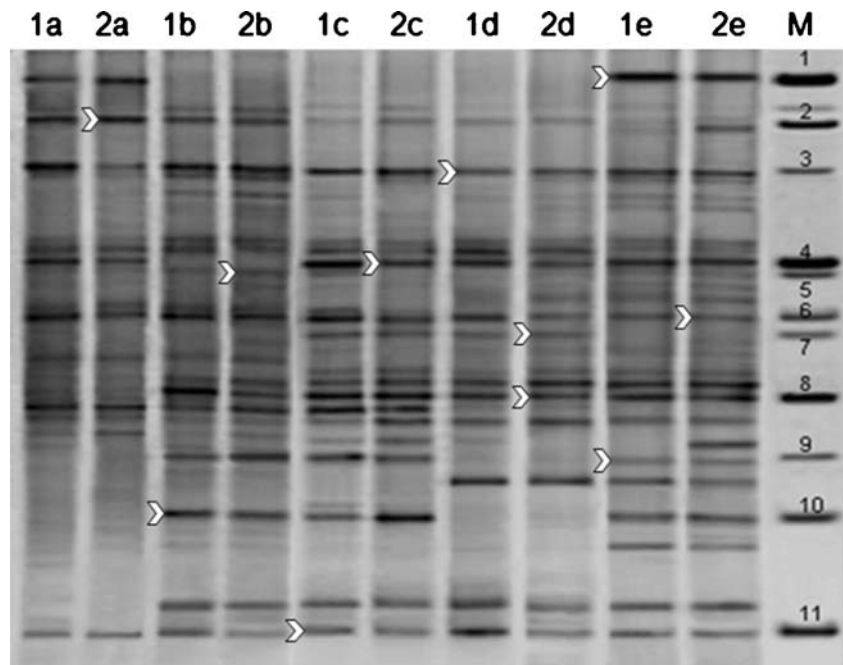

spores, were not clearly visualised by using calcofluor white, although it was possible to observe a blue colour covering the bacterial biofilm, confirming the presence of fungi (Fig. 5).

Discussion

Microorganisms can be responsible for the destruction of cultural heritage, together with several environmental con-ditions, ageing and the chemical structure of the substrate. Restoration efforts do not always obtain the expected result, and sometimes they even accelerate the deterioration process. Restoration work should take in account biodecay as an integral part of the overall deterioration process. Therefore, an inventory of existing microorganisms associated with the damage to the selected objects of art should be included in those restoration projects where there were evidences that the microbial activity could be responsible of the deterioration. On the other hand, sometimes damages

Figure 4 Negative image of DGGE gels showing bacterial community fingerprints from biofilm samples (1a, 2a, 1b, 2b, 1c, 2c, 1d, 2d, 1e and 2e) and a mixture of amplicons from pure cultures of isolated bacterial strains (M). (*1*) *P. alcaligenes*, (*2*) *N. asteroides*, (*3*) *Arthrobacter* spp., (*4*) *B. subtilis*, (*5*) *P. aeruginosa*, (*6*) *Streptococcus* spp., (*7*) *P. fluorescens*, (*8*) *M. roseus*, (*9*) *B. pumilus*, (*10*) *Streptomyces* spp. and (*11*) *M. luteus*. *White arrows* indicate those bands selected for sequencing in order to corroborate the bacterial identification
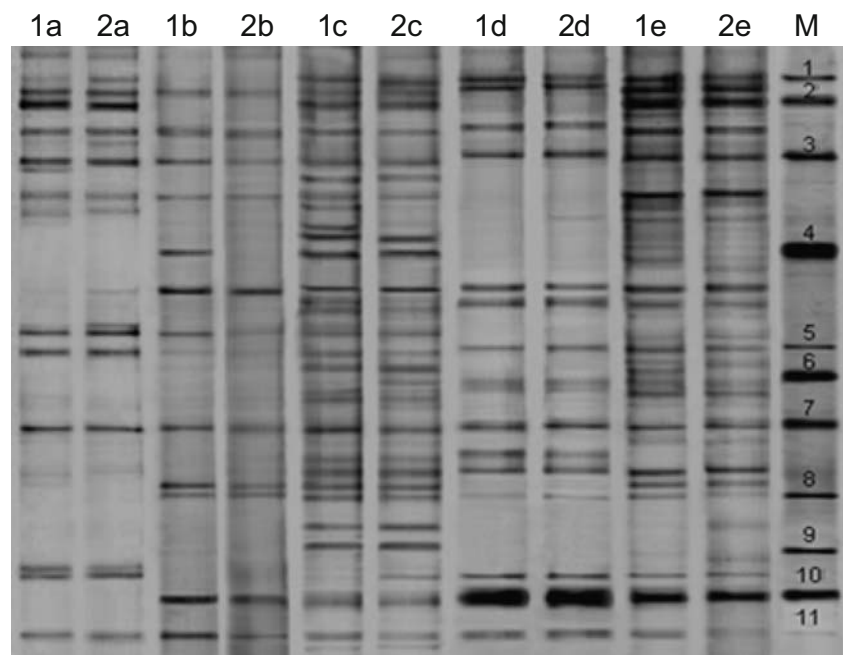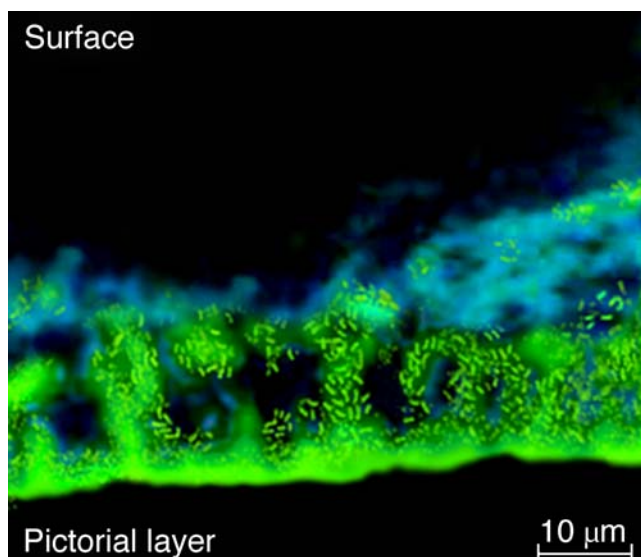
Figure 5 Fluorescent in situ hybridisation of a cross-sectioned specimen (1a) of the studied painting showing the existence of dense bacterial colonisation (FITC stain, *green*) in close contact with the pictorial surface and fungal biomass (calcofluor white stain, *blue*) covering and surrounding the bacterial biofilm

occurring in artworks are clearly not a consequence of a microbiological attack and in consequence these studies are not necessary.

DGGE technology is primarily used to fingerprint microbial communities in diverse environments and can be of great value for microbial identification in mixed populations, as it can detect both cultivable and as-yet-uncultivated microorganisms, and, in many cases, it can be a good alternative to more expensive, laborious and time-consuming cloning procedures. With this aim, DGGE analyses of PCR-amplified DNA with bacterial- and fungal-specific primers without prior cultivation of the microorganisms were carried out in combination with the identification of viable isolates of the microbial community. Unfortunately, many approaches address only culturable microorganisms, which are thought to represent only a small proportion (0.1% to 10%) of the total microbial population present in different habitats [16, 20, 27, 32, 33, 38, 43, 45, 46]. In combination with other techniques (FISH, SEM–EDX and ESEM) included in this research, this molecular technique provided an insight into the structure and complexity of the bacterial and fungal populations present in the biodeteriorated surface of the studied artwork. The diversity of the microbial population was observed by DGGE to be high, but when compared with other communities such as waters [42], soils [35, 43], rhizosphere [41], oral cavity [28] or rumen samples [21, 45] it was found to be the simplest and formed by a lower number of microorganisms. The same comparison is more

obvious when viable counts were taken into account. The number of cultivable microorganisms was found to be small and with high variability between different sampling zones. These findings indicate that the pictorial surface, even though susceptible to microbial colonisation, is a very restrictive substrate and only microorganisms with special metabolic (presence of hydrolysing enzymes such as collagenases, proteases, lacases or cellulases) and physiological capacities (presence of spores, resistance to heavy metals, resistance to xeric environments, etc.) are able to grow or survive [10, 19, 31]. Comparing our DGGE results with those obtained by other groups [15, 31, 37] involving mural paintings, frescoes and cave paintings revealed that the microbial populations inhabiting those systems are generally different, although some microbial populations coincide. Certain microorganisms known to belong to different bacterial genus (*Arthrobacter*, *Micrococcus*, *Pseudomonas*, *Streptomyces*) or fungal genus (*Alternaria*, *Aspergillus*, *Chaetomium*, *Penicillium*) seem to be the most common inhabitants of these niches; however, wide, quantitative and qualitative variations are evident. For instance, more than 33 different species of fungi were isolated from a fresco in St. Damian's Monastery in Assisi, whereas only one fungal species was isolated from a mural in Canterbury Cathedral [7]. The wide range of organic and inorganic molecules present in different types of paintings, as well as the variable environmental conditions (humidity, temperature, light, etc.) in which they are kept, means that the development of a microbial flora differs widely. Likewise, if temperature, moisture and light are not controlled, the microbial communities on two paintings produced with exactly the same materials will differ considerably if they are maintained in different environments, latitudes or orientations [7].

Advanced analytical methods also allow us to conduct research studies or contribute to the development of the simple diagnostic techniques necessary for practical applied conservation [1, 4, 36]. The extremely high magnification images of SEM–EDX together with localised chemical information mean that the instrument is capable of resolving a great deal of common research issues such as particle analysis and identification of materials [29]. In SEM–EDX, layers containing pigments of heavy elements, such as the lead present in lead white, appear bright; those containing primarily elements with medium or low atomic numbers are dark. Using these element distribution images, the main elements in the different layers were correlated with the pigments used for the pictorial layers. There was a high variability of pigments in the studied specimens: red-Hg-based pigments (vermilion), copper-based green pigments such us azurite and verdigris and white lead, amongst others. White lead was present in different proportions in some specimens because it was used in the past (before the

discovery of the low toxic pigment titanium white) to clarify pure pigments. When we correlated these results with the DGGE analysis and isolates, the microbial colonisation was found to be modulated by the element composition of the pictorial layer. The comparison between bacterial and fungal communities must be carefully done, but it was interesting to observe that specimens with copper-based pigments were observed to be mainly contaminated by bacteria, whereas there was a small representation of fungal communities. Vermilion induced a reduction in the number of ribotypes detected by DGGE, and the number of fungal and bacterial isolates was also lower.

In addition, FISH analyses were carried out to obtain more information on the distribution and abundance of bacteria and fungi on painting samples. Cross-sectioned specimens were observed to present biofilms with a high variability in height (0 to 50 μm) and complexity and composed of combinations of bacterial and fungal communities (Fig. 5). These results were also observed in ESEM studies (Fig. 1). This result, reported for the first time, suggests that the bacterial colonisation of the surface might have occurred prior to fungal colonisation, making the pictorial surface more attractive for a secondary fungal colonisation. Another hypothesis might be that bacteria were more resistant and were able to develop in close contact with the pictorial surface. Our results show that the direct fungal colonisation of surfaces occurred especially in those areas where wood, the support material used in this artwork, was exposed to air due to the deterioration and detachment of pictorial and ground layers (not shown).

Cutting-edge techniques developed in physics, chemistry and biology have a commonality of application to both antique and modern materials since problems encountered in the fields of both advanced technology and cultural heritage are similar. In conclusion, here, we report the identification and characterisation of the microbial populations of a particular artwork and their relationship with the composition of pictorial layers. Further characterisation of the biochemical capacities of these isolates is likely to provide important new insight into the mechanisms involved in microbial colonisation of paintings. New molecular analyses are currently being investigated to determine the function and spatial distribution of the main bacteria and fungi responsible for the microbial deterioration of the studied artwork.

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