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Biodeterioration in art: a case study of Munch's paintings

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Abstract Biocolonization and biodeterioration phenomena in Cultural Heritage is presently considered a relevant issue when planning conservation strategies and preservation measures in museum collections. Artworks such as easel paintings are source of various ecological niches for microbial communities' growth due to the presence of several organic resources. Therefore, the identification of proteinaceous materials may play an important role in the evaluation of their conservation status, in the characterisation of the artistic technique, and in the definition of compatible conservation/restoration processes. Another challenge is to understand the microbiota associated to the degradative processes when developing conservation strategies in CH artworks. For this study Edvard Munch paintings belonging to Munch Museum in Oslo presenting surface alterations were analysed to increase the knowledge about the materials used by the painter and try to understand the source and the dynamics of the associated colonising microbiota, helping in devising a conservation intervention plan. Immunoenzymatic assays was carried out in microsamples allowing the detection of casein as the binder used by the artist. The high throughput sequencing approaches allowed us to explore and characterise the microbial communities that colonise these artworks. Bacterial communities found in these artworks were mainly composed by species characterised by proteolytic capacity, an important biodeteriogenic characteristic for these paintings. Simulation assays performed in paint models prepared with casein as binder display signs of degradative action promoted by the proteolytic strains isolated from the damaged areas. This approach can be useful to promote effective intervention processes in E. Munch's paintings with the same pathologies.

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1 Introduction

Easel paintings, one of the most important art expressions with historic and cultural value, may present a diversity of organic materials, namely proteins, polysaccharides, siccative oils, plant resins, gums, mucilages, and waxes, as well as, support materials like wood, fabric, canvas, paper, metal or parchment [1]. This explains why these artworks are affected by microbial contamination that among other factors can be responsible for different aesthetic and structural alterations.

Among these, proteinaceous materials, abundant in Cultural Heritage (CH), have attracted the attention of the conservators given their obvious facility for undergoing morphological and chemical changes on ageing, playing an important role in their conservation status [2–5]. Used by artists as binders, adhesives in paint layers or as additives in coating layers, their identification allows to characterise the artist technique and is crucial in conservation/restoration processes decision making considering compatibility and authenticity. Immunological techniques have become a powerful diagnostic tool in CH, offering unique advantages over other analytical approaches used in conservation science: selectivity regarding the biological source, ability to resolve complex mixtures of proteins and high specificity of antibody-antigen binding, detecting protein at low quantities [3, 6].

On the other hand, biocolonization/biodeterioration phenomenon is a relevant issue for the preservation/conservation of CH artworks [7, 8]. The new molecular-based approach using metagenomic DNA by Next Generation Sequencing (NGS) allows the study of complex microbial communities without prior isolation of microorganisms and is a powerful tool for understanding the dynamics of the communities involved in degradation processes [9].

This study is in the scope of the research project "THE SCREAM—Touchstone for Heritage Endangered by Salt Crystallization: a Research Enterprise on the Art of Munch" (ALT20-03–0145-FEDER-031577) that intends to study several Edvard Munch' sketches on canvas from the Munch Museum collection (Oslo, Norway) where efflorescence's were developed and need to be removed in order to stabilise the colours in the art works.

Between 1909 and 1916 E. Munch produced around 140 sketches for the decoration of the festival hall, the Aula, of the University of Oslo [10]. Many of these sketches were preparations for this decorative project representing a testimony to Munch's efforts and the evolution of his creative process and are now in Munch Museum's inventory. Nevertheless, due to extended exposure to the outdoor elements in large outdoor studios constructed by the painter during the project, and inappropriate storage and handling, many of the sketches present evident chromatic and structural alterations. Beyond many residues founded such as, moss stain, areas with clay and soil, insect and bird residues, several of them also developed intense salt efflorescences, visible on their surfaces. Analyses to affected sketches by X-ray diffraction or scanning electron microscopy with energy-dispersive X-ray analysis suggested the presence of hydrated zinc sulphates, (namely $ZnSO_4{\cdot}4H_2O$, $ZnSO_4{\cdot}6H_2O$, and $ZnSO_4{\cdot}7H_2O$), magnesium sulphates ($MgSO_4$) and zinc-magnesium sulphates ($ZnMgSO_4\ 4H_2O$) [11]. These salts are the result of the degradation of materials probably due to exposure for four years to outdoor extreme conditions of rain, snow and low temperature during winter, sun radiation and pollution [12]. Although larger sketches, like *The Researchers/Alma Mater* (M 907), had seen mounted in his outdoor studio in photographs, the smaller sketches were probably not exposed, as some don't have tidelines or spots, but still large amounts of efflorescence. For these, unstable indoor storage could also have been ideal for crystal appearance. Since the efflorescence appeared on coloured layers as well as on the canvas where there was no paint or ground and was detected Zn and Mg in these areas indirectly suggest that Munch had possibly applied a casein solution to the canvases.

On the other hand, the microorganisms play an important role in deterioration phenomena, colonising several ecological niches present in this type of artworks. Since these sketches were exposed to the environment during several years, the biocolonisation is an important issue to take into consideration to develop a strategy focussed on the conservation intervention process.

All of Munch's decorations consist of large-format paintings with different types of canvases such as diverse qualities of linen and cotton canvases with the paint applied directly to the fabric without ground layers and some linen canvases with ground layers. Analyses and visual examinations indicate that E. Munch used different materials and application, including crayons and paints. The media detected were casein, linseed oil, and emulsion of oil in animal glue emulsion. An array of pigments has also been identified, such as ultramarine and Prussian blue, cobalt blue, zinc oxide, lead white, chrome yellow, yellow ochre, vermillion, red earth, Scheele or emerald green and green zinc chromate [11–13].

Microsamples of an *The Researchers/Alma Mater (M907)* painting sketch were collected in areas with evident structural damages and visible efflorescence to study the microbial communities involved in the deterioration process and microsamples from detachments were used for protein immunodetection. This approach has the vision to better understand the materials used by E. Munch that can be correlated with the high microbial contamination in the damaged areas and to contribute for novel conservation solutions based on the knowledge of the colonising microbial communities.

2 Methodology

2.1 Paintings and sampling

This work focuses on the study of Edvard Munch's easel paintings belonging to The Munch Museum in Oslo, Norway. A sketch of The Researchers/Alma Mater (M907 a,b,c—ID-numbers: M 961, 962, 963) with evident chromatic and structural alterations was selected for the study (Fig. 1). Several microsamples of this artwork were collected during the THE SCREAM—Touchstone for Heritage Endangered by Salt Crystallization—a Conservation Research (ALT20-03–0145-FEDER-031577) project campaign (September 2018), using non-invasive and micro-invasive methods, in semi-sterile conditions.

The sampling process was made according to the conservation ethics, collecting the minimum amount required for testing, near lacunae, edges or fissures, avoiding thus structural and aesthetic damage to the artwork. The use of a micro-destructive method is permissible only on fragments that cannot undergo conservation or cannot be reunited, such as fragments from the margins and reverse sides, biofilms and parts that will certainly be eliminated during restoration.

Three microsamples (M907 6P, 7P and 9P) collected in areas with evident structural damages and visible detachments, by scrapping off 0.1 mg of paint layer from the paintings surface with a micro-scalpel in sterile microtubes, were used for immunodetection assays. The microsamples were stored at − 80 ºC until analysis.

Several microsamples were collected in semi-aseptic conditions using sterile cotton swabs in the same three selected areas with visible superficial biofilms formation for evaluation of the microbial contamination by isolation and characterisation of the cultivable microbial population and characterisation of the total microbiota (including non-cultivable) by metagenomics analysis. The microsamples were conservated at 4 °C during the transport to the laboratory and until its utilisation [8].

Fig. 1 Schematic representation of the sampling process performed in Edvard Munch's sketch of The Researchers/*Alma Mater* (M907) (Munch Museum, Oslo)

2.2 Immunodetection of proteic binders

2.2.1 Reagents

Polyclonal Anti-Chicken Egg Albumin antibody produced in rabbit (whole antiserum) (C6534, Sigma-Aldrich) and Anti-Casein antibody produced in rabbit (SAB2100491, Sigma-Aldrich) and Monoclonal Anti-Collagen Type I antibody produced in mouse (C2456, Sigma-Aldrich) were used as primary antibodies for indirect ELISA assays.

Anti-Rabbit IgG (whole molecule) produced in goat (A3687, Sigma-Aldrich) and Anti-Mouse IgG (whole molecule) produced in rabbit (A2418, Sigma-Aldrich) were used as secondary antibodies.

Other solutions needed for performing the indirect ELISA assays were the same described in Salvador et al.(2017) [8].

2.2.2 *Paint microsamples protein extraction and detection*

Proteinaceous content extraction of three paint microsamples (M907 6P, 7P and 9P), was achieved using a previously optimised protocol [14] and according to Salvador et al.(2017) [8].

Protein extracts obtained from three paint microsamples with concentrations between 40 – 50 $\mu g\ mL^{-1}$ were used as antigens in an immunoenzimatic assay for protein binders (ovalbumin, collagen and casein) detection. Indirect ELISA immunoassays was carried out following a previously optimised procedure conditions, such as temperature and incubation time for the antigens binding step and selected concentrations of the primary antibodies [14]. Next sequential steps for indirect ELISA assays were performed according to Salvador et al.(2017) [8].

The specificity of the primary monoclonal and polyclonal antibodies was checked performing positive controls using commercial standard proteins of ovalbumin, collagen, and casein.

Detection results for each protein extract were validated performing three times the ELISA tests. Positive detection is assessed by a colorimetric enzymatic reaction of secondary antibody alkaline phosphatase conjugate with the substrate para-nitro-phenyl-phosphate (p-NPP, 71,768**,** Sigma-Aldrich), measured at 405 nm. Results were expressed in antibody units per millilitre being one antibody unit defined as the amount of antibody required to give a change in 1.0 unit of absorbance per 30 min of reaction, under the specified ELISA conditions [8, 15–17].

2.3 Characterisation and identification of microbial communities

The biofilm microsamples collected with cotton swabs were incubated overnight at 28ºC with agitation of 100 rpm with 1 mL of Maximum Recovery Diluent medium (MRD) for cells rehydration. These suspensions were used for the study of the microbial communities that are colonising this painting for isolation and identification of cultivable microorganisms. Analyses by high-throughput sequencing (HTS) were performed using the cotton swabs microsamples collected directly from de paintings for metagenomic DNA extraction.

2.3.1 *Characterisation of the microbiota*

For the study of complex microbial communities without prior isolation of microorganisms metagenomic DNA was extracted from microsamples (M907 6P, 7P, 9P) using Omega's E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Georgia, USA), with slight modifications from the manufacturer's instructions, such as improving the cells lysis increasing the incubation time step and using all supernatant obtained for purification step, trying to increase the DNA concentration.

Prokaryotic communities were characterised by HTS for the 16S rRNA V3-V4 region using Illumina Sequencing platform.

DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adaptors and dual indexes (Nextera XT Index Kit, Illumina, San Diego, CA). The first PCR reactions were performed with 0.2 μM of each PCR primer: forward primer Bakt_341F 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer Bakt_805R 5′-GACTACHVGGGTATCTAATCC-3′, using 2X KAPA HiFi HotStart Ready Mix in a total volume of 25 μl.

The amplicons were quantified by fluorimetry with QuantiFluor® ONE dsDNA kit (Promega, Madison, WI, USA), pooled at equimolar concentrations and paired-end sequenced with the MiSeq Reagent Kit V2 (2×250pb) in the MiSeq® sequencing system according to manufacturer's instructions (Illumina, San Diego, CA, USA) [18].

The bacterial communities were characterised for the 16S rRNA V3-V4 region using the Illumina Sequencing platform, according Dias et al. (2020) and Ding et al. (2021) [19, 20]. The run was performed using the MiSeq Reagent nano Kit V2 in the Illumina MiSeq System, where the sequencing was done using a 2×250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) directly on the MiSeq instrument (Illumina, San Diego, CA, USA) and multiplexed automatically by the Miseq sequencer using the CASAVA package (Illumina, San Diego, CA, USA). The forward and reverse reads were merged by overlapping paired-end reads using the Adapter Removal v2.1.5 [21] software with default parameters. The QIIME package v1.8.0 [22] was used for the generation of Operational Taxonomic Units (OTU), in taxonomic identification, against the Greengenes v13.8 database [23] OTUs were selected at a similarity of 97%. Data were visualised using Calypso (version 8.84) (http://cgenome.net/calypso/). The sequences were deposited at NCBI Sequence Read Archive (SRA) database, uploaded on bioproject PRJNA610727 with accession numbers SRR16234985-90 (https://submit.ncbi.nlm.nih.gov/about/sra/).

2.3.2 Cultivable microbial population isolation and characterisation

The microbial suspensions were aseptically cultured in Nutrient Agar (NA: 1% peptone, 1% meat extract, 0.5% sodium chloride and 1.8% agar, HIMEDIA) and Tryptic Soy Agar (TSA: 1.5% peptone 0.5% peptone, 0.5% sodium chloride and 1.5% agar, HIMEDIA). Plates were sealed with cling film and incubated at 30ºC in the dark during 24–48 h for bacteria growth [7, 24]. To detect slow microbial growth, the inoculated Petri dishes stayed in incubation more time, up to 30 days and monitored periodically.

Pure bacterial strains were obtained after successively subculture of the colonies that grew during this period with different macro-morphological characteristics.

Standard classical methods were used to characterise the bacterial isolates based on macro- and micro-morphological features such as diameter, texture, colour of the colonies, dimensions, and morphology of cells [25, 26]. Microscopic characteristics were observed using light microscopy (Motic BA410E) coupled with a resolution camera (MoticamPro 282B) [8].

Bacterial strains isolated from paint microsamples were catalogued and stored on the culture collection HERCULES-Biotech laboratory, University of Évora, and were maintained on fresh TSA slants at 4ºC for using as test microorganisms during the assays next described.

2.3.3 Bacterial DNA extraction and 16S ribosomal DNA sequence analysis

Identification of bacterial isolates was performed by sequencing 16SrDNA region. The genomic DNA extraction was carried out by the method for natural samples described by Rinta – Kanto, J. [27] and with some modifications. The cells of the bacterial strains (10 mg of sample) that have shown proteolytic activity were collected from fresh slants and suspended in lysis buffer (40 mM EDTA, 400 mM NaCl, 50 mM Tris-hydrochloride, pH 9.0). The cells were disrupted by chemical and mechanical processes adding glass beads (200 µL v/v) and lysozyme to a final concentration of 2 mg/mL followed by strong vortex (5 min) and

next incubation at 37 °C for 40 min. After was added α-Chymotrypsin in 10% (w/v) Sodium Dodecyl Sulphate (SDS) aqueous solution was added to a final concentration of 4 mg/mL and SDS to a final concentration of 0.5% (w/v) and the cell suspension was then incubated at 50 °C for 1 h.

DNA was extracted by first adding a phenol/chloroform/isoamyl alcohol (25:24:1) volume equal to the aqueous phase, with a subsequent extraction of the aqueous phase using an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated overnight (-20 °C) after the addition of absolute ethanol (2 × aqueous volume) and 3 M sodium acetate (0.3 × the volume of the aqueous phase). After this period DNA was collected by centrifugation the next day by centrifugation at 11 900 g for 25 min and the pellet was resuspended in sterile 1 × TE buffer, pH 8.

The extracted genomic DNA was amplified in 16S rDNA region using the oligonucleotides universal primers 27F 5'—(AGA GTT TGA TCM TGG CTC AG)—3'and 785R 5'- CTACCAGGGTATCTAATCC—3' and the PCR reactions were carried out in a 25 μL reaction volume containing 1 μL of genomic DNA, 2.5 μL 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTP mix, 0.04 μM of each primer and 1U Taq DNA polymerase. The amplification was conducted by polymerase chain reaction in a PCR thermal cycler (MJ Mini Bio-Rad; Laboratories Inc., USA) and were carried out as follow: an initial denaturing step at 95ºC for 3 min followed by 36 cycles at 94ºC each 50 s, 55ºC for 50 s, and 72ºC for 1 min. The reaction was completed by a 10 min extension at 72ºC. PCR products were analysed by agarose gel (1.5%) electrophoresis stained by GreenSafe Premium (Nzytech, Portugal), quantified by fluorimetry with QuantiFluor® ONE dsDNA kit (Promega, Madison, WI, USA), purified with the NucleoSpin Extract II Kit (MachereyeNagel) and sequenced by capillary electrophoresis using the ABIPRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDTv1.1 (Applied Biosystems).

The nucleotide sequences were aligned with those retrieved from the GenBank (NCBI-National Centre for Biotechnology Information) databases for the homology analysis using the Basic Local Alignment Search Tool Nucleotide- BLASTN 2.10.0 programme (http://blast.ncbi.nlm.nih.gov).

The sequences were deposited at NCBI database through GenBank repository (https://submit.ncbi.nlm.nih.gov/about/genbank/).

2.4 Selection and characterisation of bacterial isolates with proteolytic capacity

2.4.1 Screening of proteolytic capacity

To evaluate the proteolytic capacity of the isolated bacterial strains, a qualitative test was performed on TSA supplemented with 1% (v/v) of skim milk (Molico, Nestlé, Portugal) medium. A loop of cells from a TSA fresh slant incubated at 30 ºC for 48 h was applied onto the Petri dishes in 3 equidistant points and next incubated at 30 ºC for 48 h [28].

The formation of clear zones around the colonies indicates proteolytic capacity of the bacterial strains with the production of the extracellular proteases that degrade the substrate casein in the culture medium. The clear zones diameter was considered as a measure of the activity and the proteolytic index (PI) of the isolates was calculated as the next equation [29]:

$$PI = \frac{clear\ zone\ diameter - colony\ diameter}{colony\ diameter}$$

Pure bacterial colonies with the highest PI were chosen for the next step with their characterisation in liquid cultures. All the assays were performed in triplicate.

2.4.2 Proteolytic bacteria growth profile characterisation in liquid cultures

Liquid cultures of the bacterial strains that have shown proteolytic potential were performed in 100 mL of Nutrient Broth (NB: 0.3% meat extract and 0.5% meat peptone, HIMEDIA) medium supplemented with 1% of skim milk.

Cell's suspensions were prepared in NB medium from a TSA fresh cultures incubated at 30ºC for 48 h and adjusted to the concentration of 10^6 CFU/mL.

To monitor the microorganism's growth profile, the cultures were incubated for 72 h at 30ºC in an orbital shaker at 150 rpm (IKA KS 4000 I control), samples were collected periodically, and the absorbance was monitored at 600 nm (HITACHI U-3010 *spectrophotometer*).

The kinetics of the bacterial populations growth was fit by the modified Gompertz model that can be written as follows [30, 31]:

$$Ln\frac{N}{No} = Ae^{\left(-e^{(b-cx)}\right)},$$

where N is the decimal logarithm of microbial cells (Abs 600 nm) at time t; N0 is the asymptotic log count as time decreases indefinitely; A is the number of log cycles of growth; b is the relative growth rate at time, t (h^{-1}), c is the time required to reach the maximum growth rate (h) and e is the Euler's number (2718).

Assays were carried out in triplicate and nonlinear regression modules of the software SigmaPlot (Version 12.0.0, Germany) were used to fit the data.

2.4.3 Proteolytic activity assessment

Liquid cultures samples collected in different culture times were centrifuged at 6000 rpm at 4 °C for 30 min and a supernatant with a crude protease enzyme, was recovered.

Activity test of protease enzyme measurement of proteolytic activity was carried out according to Kunitz method [32]. 0.250 mL of supernatant and 0.250 mL of substrate (1% casein in phosphate buffer pH 7) were added into a test microtube, then incubated at 50 °C for 60 min. After that, 0.750 mL of 10% trichloroacetic acid (TCA) solution was added to stop the reaction and the supernatant recovered. The proteolytic activity was determined measuring tyrosine residues, using the Folin & Ciocalteu's method [33] and the amount of tyrosine in the extract was determined using a linear equation of a tyrosine standard curve [34] with tyrosine standards in a range concentration of 5–80 μg/mL.

All samples and standards were applied in 96 well microplates and the absorbance was measured using a microplate reader (Multiskan GO, FI-01620 Thermo Scientific, Vantaa, Finland) at 760 nm. The proteolytic activity was expressed as units of tyrosine equivalents (U) per mL, being one unit defined as the amount of enzyme that release tyrosine to produce colour equivalent in 1.0 micromole per minute in the assay conditions of pH 7.5 at 37 °C (colour by Folin & Ciocalteu's reagent) [28].

Specific proteolytic activity was obtained by quotient of units of tyrosine by total protein content in the initial samples quantified by Bradford method and using BSA as standards [35]. All measurements were performed with six replicates.

At the same time proteolytic capacity of the liquid cultures samples collected in different culture times containing the crude protease enzyme was also tested in solid medium. For that 20 μL of supernatants were applied in filter paper discs (Macherey–Nagel 827 ATD) in Petri dishes containing TSA supplemented with 1% of skim milk and were incubated at 30 ºC for 48 h. The formation of clear zones around the discs indicates proteolytic capacity and

the PI of the isolates was calculated as described in 3.4.1 section [29]. All the assays were performed in triplicate.

2.5 Evaluation of proteolytic potential of bacterial strains in simulation assays

2.5.1 Paint models' construction

Paint models of Easel paintings were prepared with similar materials that were used by the artist in order to guarantee the representativeness of the simulation assays [13]. Glass slides covered by linen were used as support of the painting layer mimicking the real canvas support [10]. Single paint layers with thickness around 50–150 μm were prepared with proteins used as binders as commercial casein and casein extract from bovine milk or by mixing proteins with two different pigments commonly used in Easel paintings such as chrome yellow and ultramarine blue [36](kremer, Aichstetten, DE) in the ratio of pigment:protein binder; 3:1 (Table 1) [37, 38]. All paint models with different constitution shown in Table 1 are prepared in triplicate.

2.5.2 Inoculation of bacterial strains on paint models

Paint models were inoculated with two different isolated bacterial strains that had shown higher proteolytic activity, such as CCLBH-6PB31 and CCLBH-9PA31.

Bacterial cells suspensions were prepared from the fresh cultures and 1 mL of each suspension was applied separately in a lot of 6 paint models with the different constitutions (Table 1). One similar lot were left without any inoculation to act as a control. All paint models were subjected to an incubation period of 15 days at 30 °C in order to allow the microorganisms growth and making possible the analysis of the degradation signals and proteolytic activity assessment.

Subsequently, an artificial ageing process of the paint models was carried out for 6 months using an ageing chamber with controlled conditions of high humidity of 85% and low temperature ranging between − 5 °C and 10 °C.

2.5.3 Proteolytic activity assessment in paint models

For evaluation of proteolytic activity promoted by bacteria in paint models, a piece with about 9 mg was removed in each one, resuspended in 1 mL of sterile distilled water and was performed the same protocol as described in Sect. 2.4.3.

The proteolytic activity was expressed in Units of tyrosine (micromolar of tyrosine per min) and in Units of tyrosine per mg of microsample in the same conditions assay.

3 Results and discussion

3.1 Proteinaceous binders' detection by ELISA

The correct paint materials identification in microsamples collected from artworks is imperative in order to better understand the artist's techniques providing relevant information for a focussed strategy in an eventual conservation and restoration intervention [39].

In this study three microsamples of one part of *The Researchers/Alma Mater* sketch were analysed by immunological assay in order to access an identification of protein material

Table 1 Constitution of different paint models made in triplicates.

Proteic binders	Pigments		
	--	Yellow chromium: $PbCrO_4$	Ultramarine blue: $Na_{8\text{-}10}Al_6Si_6O_{24}S_{2\text{-}4}$
Commercial casein			
Bovine milk			

present. These microsamples collected in areas with visible damages were intensively studied trying to correlate this with the presence of proteins and colonising microbiota.

The protein content was extracted from microsamples of the paintings using a previously optimised protocol [14]. Table 2 shows the protein content in the analysed microfragments. Although the sampling and protein extraction processes have been performed similarly for all samples collected, different protein contents were yielded ranging between 39.9 ± 0.8 and 63.2 ± 4.0 μg protein/mg microsample, as stated in Table 2.

For analysing the extracted proteins, we used an optimised immunoassay based on indirect ELISA, with commercial antibodies Anti-ovalbumin, Anti-Collagen and Anti-Casein. This procedure was previously optimised and the conditions of the ELISA assay were adjusted allowing increased detection signals with low levels of proteins and aged protein [14, 15]. The dose–response curves for each antibody were made to select the optimal concentrations of primary antibodies for their application in this type of samples [14]. Detection results for protein extracts obtained from the paintings microfragments are summarised in Table 2. The results show that it was possible to detect and quantify the protein binders of casein in the three samples analysed with values between 11.07 ± 2.26 and 36.45 ± 0.03 U per mg of microsample. These microsamples did not present protein binders of ovalbumin from hen's egg or collagens from rabbit, pig, rat, bovine, deer and human according to the specifications of the Anti-collagen antibody.

The specificity of the immunological assay data allowed the identification and discrimination of the protein binder's origin which is one of the major difficulties in paintings analysis [8, 14]. This is a major breakthrough and can be used as complementary approach in protein binder analysis.

3.2 Microbial communities' assessment

Until recently, microbial communities' identification living in cultural assets were achieved only by classical microbiology methods requiring the isolation of microorganisms in pure cultures followed by multiple physiological and biochemical tests. However, those culture-dependent methods underestimate the microbial diversity thus becoming inadequate for studying the whole microbial communities, being the major gap in biodeterioration studies associated to Cultural Heritage. More recently the DNA-based typing methods have been developed to study the microbial diversity of colonising communities from various ecological

Table 2 Protein content extracted from the paintings microfragments, and protein binder's detection of ovalbumin, collagen and casein. Values are mean ± SD of the 6 replicates

Microsample	μg protein /mg microsample	Anti-Ovalbumin U/mg microsample	Anti-Casein	Anti-Collagen
M907 A6P	63.2 ± 4.0	–	22.69 ± 0.13	–
M907 A7P	49.1 ± 3.8	–	36.45 ± 0.03	–
M907 A9P	39.9 ± 0.8	–	11.07 ± 2.26	–

–: negative detection results

niches [9]. Advances in high-throughput technology (HTS) has allowed a deep and extensive knowledge of the microbial diversity present on colonising communities complex systems, a better understanding on the role of biodeteriogenic microorganisms and the community's dynamics.

These breakthroughs regarding microbial community's knowledge can provide the scientific community with new tools for focussed interventions in Cultural Heritage assets.

In this study, we applied Illumina-based deep sequencing on 16S rDNA amplicons to characterise the Prokaryotic community that colonise the Edvard Munch selected sketch which present high levels of deterioration associated to biocolonization. In the future differences in the distribution pattern of the microbiota of different sketches, that present similar signs of deterioration, will be evaluated.

The bacterial microbiota thriving on these altered areas of the sketch where the three microsamples (M907 6P, M907 7P, M907 9P) were collected is quite similar. The most abundant taxonomic classifications at phylum level belong to the phyla *Proteobacteria* (63.34–66.97%) and *Firmicutes* (30.16–30.45%), as presented in Fig. 2. It was also possible to classify other phyla with less than 1% of abundance, namely *Bacteroidetes* (0.28–0.33%) and *Actinobacteria* (0.15%), and four phyla with less 0.1% of abundance.

This barchart represent the top 20 of 773 to 893 genera classifications of the bacterial community composition for the three samples collected. The predominant genus achieved were Paenibacillus (29.69–29.93%), *Reyranella* (17.96–19.12%), *Phenylobacterium* (10.74–19.05%), *Caulobacter* (7.11–7.25%), *Variovorax* (6.68–6.78%), *Sphingomonas* (3.92–4.20%), *Mesorhizobium* (2.86–3.07%) and *Roseomonas* (2.79–2.97%). In terms of species level, it was possible to identify between 966 and 1231 classifications. The analyses show all classifications above 1% of relative abundance.

Using culture-dependent methods, it was possible to identify the cultivable population present on the degraded areas of the analysed painting.

The HTS approach revealed to be complementary to conventional microbiological techniques providing detailed and quality information. Although, the isolated strains obtained through cultivation do not match with the top 20 of the genera obtained in the NGS data, it is still imperative to continue to characterise the cultivable population to perform laboratory simulation assays.

This approach allowed the identification of 8 different bacterial isolates as Gram-positive coccus, *Micrococcus* sp., belonging to Actinobacteria phylum, Proteobacteria like *Paracoccus* sp. and Firmicutes like *Staphylococcus* sp.. The isolated strains were identified as *Micrococcus sp.*1 (CCLBH-6PA2) *Micrococcus sp.2* (CCLBH-6PB31), *Micrococcus sp.3* (CCLBH-9PA31), *Paracoccus* sp.1 (CCLBH-7P12), *Paracoccus* sp.2 (CCLBH-7P21),

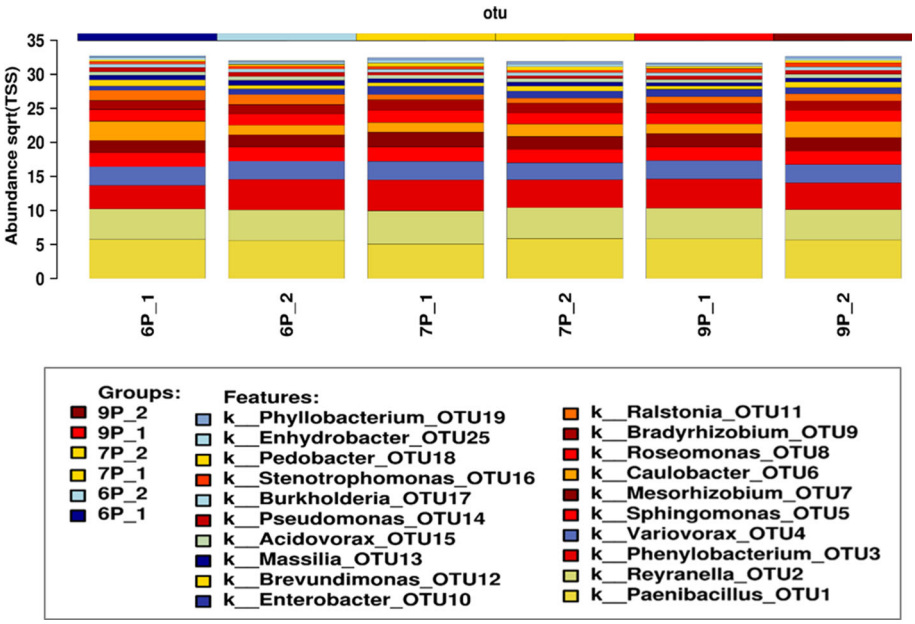

Fig. 2 Barchart including the top 20 most abundant prokaryotic population at genus level present on the damaged areas of the painting. The analyses were performed using Calypso Software (version 8.84) excluding taxa that have less than 1% of relative abundance across all microsamples

*Staphylococcus sp.*1 (CCLBH-6PB12), *Staphylococcus sp.*2 (CCLBH-6PC21) and *Staphylococcus sp.*3 (CCLBH-9PB1) (Table 3).

The strains of *Micrococcus* sp., among these *Micrococcus luteus* (CCLBH-BP501, CCLBH-9PA31), received our attention according to their proteolytic capacity described for several species of this genus [28, 34, 40].

The identification of different *Micrococcus* sp. strains in the casein paintings, as identified by the immunoassays, indicates the possible relation of these species proteolytic activity

Table 3 Identification of the bacterial isolated, based on BLAST comparison in GenBank, and corresponding accession numbers of the deposited sequences

Microsample	Bacterial strain	Genus Identification	GenBank Accession number
M907 A6P	CCLBH-6PA2	*Micrococcus sp.1*	OK416030
	CCLBH-6PB12	*Staphylococcus sp.1*	OK416071
	CCLBH-6PB31	*Micrococcus sp.2*	OK415803
	CCLBH-6PC21	*Staphylococcus sp.2*	OK422198
M907 A7P	CCLBH-7P12	*Paracoccus sp.1*	OK421318
	CCLBH-7P21	*Paracoccus sp.2*	OK416100
M907 A9P	CCLBH-9PA31	*Micrococcus sp.3*	OK416065
	CCLBH-9PB1	*Staphylococcus sp.3*	OK440874

with the damage observed in the E. Munch sketch. So, the characterisation of these bacterial isolates seamed to us imperative, in order to understand if these species can be the main cause of deterioration and biofilms formation.

3.3 Screening of proteolytic capacity of the bacterial strains

The screening test of proteolytic capacity performed for all isolated bacterial strains allow to understand their capacity to degrade casein substrates once this protein had been detected in the three microsamples analysed by the immunoassay.

Since the microsamples analysed are representative of areas of the painting with high visible aesthetic and structural damage, from which these bacterial strains were isolated, the positive results for proteolytic capacity can reveal the presence of some bacterial communities with biodeteriogenic potentialities associated to the degradation of proteins present in different paint layers.

This assay of proteolytic bacteria was performed on skim milk medium to evaluate the capacity of the bacteria to produce proteases capable to digest protein compounds, in this case casein contained in skim milk. The positive results were easily detected with the clear zones formed around the colonies resulting from casein hydrolysis.

The isolated bacteria were subjected to screening for proteolytic activity, with the results summarised in Table 4. Five strains showed clear zones in the culture medium, with different activity levels, two bacteria isolated from M907 A6P microsample, 2 bacteria from M907 A7P and one from M907 A9P.

Proteolytic activity of bacteria CCLBH-7P12 and CCLBH-7P21 that belongs to *Paracoccus* genus had a small clear zone or weak activity, corresponding to low proteolytic index. Bacteria CCLBH-6PA2 *Micrococcus* sp,1 present evident clear zone corresponding to a more expressive PI of 1.40 ± 0.17. The two other bacteria, CCLBH-6PB31 and CCLBH-9PA31 that belongs to *Micrococcus* genus showed a large clear zone corresponding to a strong activity and similar PI of 2.03 ± 0.06 and 2.15 ± 0.27. The five strains with positive results of proteolytic activity were selected for further tests.

The described qualitative assay capable to detect extracellular proteases directly in the culture medium have been used in several methods for biochemical studies and in clinical

Table 4 Results of qualitative test of protease-producing bacteria

Bacterial strain	Clear zone	Proteolytic index
CCLBH-6PA2	+ +	1.40 ± 0.17
CCLBH-6PB12	–	–
CCLBH-6PB31	+ + +	2.03 ± 0.06
CCLBH-6PC21	–	–
CCLBH-7P12	+	0.47 ± 0.02
CCLBH-7P21	+	0.48 ± 0.06
CCLBH-9PA31	+ + +	2.15 ± 0.27
CCLBH-9PB1	–	–

– Absence of clear zone around the colony: non – caseinolytic strain. + Presence of clear zone around the colony: caseinolytic strain. Width (cm) of proteolysis: +++(>1); ++(0.5–1); +(0.1–0.5); – (0)

laboratory for identification of bacteria providing a simple, inexpensive, straight forward method to assess the presence of proteolytic activity of a given bacterial colony [29, 41, 42].

3.4 Characterisation of proteolytic bacteria in liquid cultures

The growth of bacterial cultures involves a succession of phases that allow to characterise the growth profile of the strains and analyse their affinity for different substrates [43].

Figure 3 shows the bacterial growth profiles of the three independent cultures of each of the five isolated bacterial strains, in NB medium supplemented with skim milk and monitored during 72 h. The culture medium was supplemented with skim milk because the Munch paintings from which these bacteria were isolated revealed the presence of casein in the paint composition. All strains grew in the presence of casein in the culture medium.

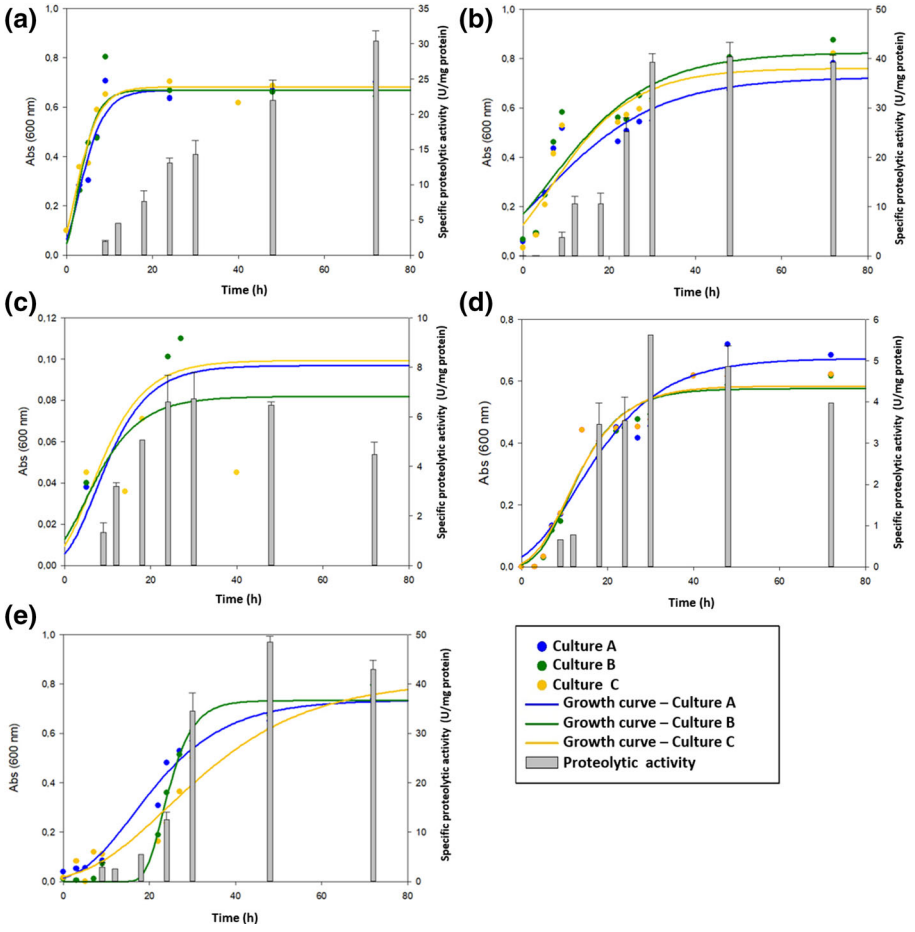

Fig. 3 Growth profile of the proteolytic bacteria in liquid culture with NB medium supplemented with skim milk and monitoring of the proteolytic activity at several culture times. **a** – CCLBH-6PA2; **b** – CCLBH-6PB31; **c** – CCLBH-7P12; **d** – CCLBH-7P21; **e** – CCLBH-9PA31. Specific proteolytic activity values presented are the mean ± SD of 3 replicates

At the same time, while bacterial cells growth was monitored, the proteolytic activity was quantified in the free cell's supernatants for the different strains. The results of proteolytic activity obtained are presented in the graph representations of the growth profiles (Fig. 3) in 10 different culture times. Proteolytic activity profiles seem to follow the bacterial growth profiles, with zero values or not detected for the samples collected at the times: 0, 3 and 6 h corresponding to the *lag* phase (when visible) and the initial period of the exponential phase of the cultures. During the period between 9 and 30 h the proteolytic activity increases for all bacterial strains, corresponding to exponential phase. With exception for *Micrococcus* sp.1 (CCLBH-6PA2) where the proteolytic activity values increase until 72 h, all other bacteria present constant values or small decrease after 30 h.

With these results it is possible to confirm the results of the proteolytic activity qualitative screening, since the two bacterial strains with greater activity are the strains *Micrococcus* sp.2 and sp.3 (CCLBH-6PB31 and CCLBH-9PA31) with higher value obtained at 48 h of culture with 40.33 ± 3.01 and 48.50 ± 6.20 U/mg of protein, respectively. The *Micrococcus* sp.1 strain (CCLBH-6PA2) presents the highest value at 72 h of 30.39 ± 1.44 U/mg of protein. The *Paracoccus* sp.1 and sp.2 (CCLBH-7P12 and CCLBH-7P21) present the lowest values of proteolytic activity with a maximum at 30 h with values of 6.73 ± 1.67 and 5.62 ± 1.57 U/mg of protein, respectively.

This assay of protease production characterisation was complemented with the qualitative proteolytic assay in TSA supplemented with skim milk. All 10 samples collected in different times of culture were tested and the clear zones formed around the discs were measured, corroborating the results of proteolytic activity profiles.

Proteases produced by bacteria have two types, alkaline and neutral, being active in narrow pH range (pH 5 to 9) and have relatively low thermo tolerance, presenting optimal temperatures for proteases production between 27 and 47 °C [34, 42]. *Micrococcus* species are described as alkaline proteases producers (pH 5 to 11) in temperatures until 45 °C [28]. *Micrococcus luteus* (*Micrococcus* sp.3, CCLBH-9PA31) are reported to have a maximum of proteases production close to 48 h of culture in a close range of temperatures of 30 to 37 °C with an optimal pH 7 [34].

3.5 Evaluation of proteolytic potential of bacterial strains inoculated in paint models

To evaluate the action of the bacterial isolates on materials used in easel paintings simulations assays in paint models constructed in our laboratory with some materials similar at used by E. Munch' paintings were performed. For that, 18 paint models consisting in a linen as support material, commercial casein or milk casein as binder and ultramarine blue or chrome yellow as pigments were made. The two bacterial strains *Micrococcus* sp.2 and sp.3 (CCLBH-6PB31 and CCLBH-9PA31), which exhibited higher proteolytic activity were selected to perform the simulation assay.

After inoculation with different pure bacterial cells suspensions, the paint models were incubated for 15 days to promote the microorganism's growth and subsequently subjected to ageing in a climatic chamber for 6 months, as described in the methods section. The development of the bacterial strains was evaluated under these simulated environmental conditions (with a controlled temperature and humidity levels) and analysed their damage effects on the surfaces.

Structural and aesthetic alterations were easily detected in paint models by direct visualisation such as alteration of the pigment coloration, stains formation and detachments of paint layer. Figure 4 shows a set of paint models, all with commercial casein and with the 2 pigments, tested after the incubation time in which is possible to distinguish these alterations

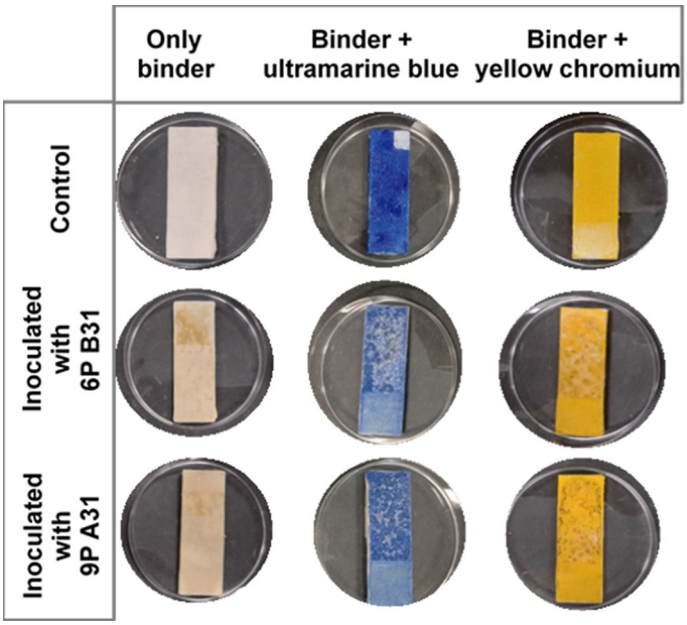

Fig. 4 Record of the alteration signs in a set of paint models of commercial casein without pigment, with ultramarine blue and with chrome yellow pigment after inoculation and incubation with bacterial strains CCLBH-6PB31 (*Micrococcus* sp.2) and CCLBH-9PA31 (*Micrococcus* sp.3) comparing with a control group

Table 5 Proteolytic activity of bacterial strains on paint models. The values are mean ± standard derivation of 6 replicates of 2 paint models

Bacterial strains	Proteolityc activity (U/mL)		Proteolityc activity (U/mg of microsample)	
Paint models	CCLBH-6PB31	CCLBH-9PA31	CCLBH-6PB31	CCLBH-9PA31
Commercial casein	0.39 ± 0.02	1.26 ± 0.20	0.09 ± 0.00	0.28 ± 0.04
Casein from bovine milk	0.51 ± 0.02	0.45 ± 0.03	0.11 ± 0.00	0.10 ± 0.01
Commercial casein + ultramarine blue pigment	0.43 ± 0.02	1.21 ± 0.10	0.10 ± 0.00	0.27 ± 0.02
Casein from bovine milk + ultramarine blue pigment	0.69 ± 0.04	0.64 ± 0.11	0.15 ± 0.01	0.14 ± 0.02
Commercial casein + chrome yellow pigment	0.17 ± 0.02	0.21 ± 0.02	0.04 ± 0.00	0.05 ± 0.00
Casein from bovine milk + chrome yellow pigment	0.26 ± 0.02	0.23 ± 0.01	0.06 ± 0.00	0.05 ± 0.00

in paint models inoculated with the two bacteria. These paint models were monitored during 6 months, being stored in controlled conditions.

The degradative action of the bacterial strains in paint models was confirmed by proteolytic activity assessment by testing inoculated and non-inoculated paint models. These results are presented in Table 5 with mean values of proteolytic activity of the two paint models subjected at the same conditions.

With these results it is possible to confirm that the strains present a greater proteolytic activity in the paint models with commercial casein or casein from bovine milk with ultramarine blue. In fact, the paint models with blue pigment present greater degradation signs comparing with yellow pigment, showing high cracking and some detachments of the paint layer. The *Micrococcus* sp.3 (CCLBH-9PA31, *M. luteus*) strain seems most active in paint models with commercial casein and *Micrococcus* sp.2 (CCLBH-6PB31) for paint models in the presence of milk casein.

4 Conclusions

Edvard Munch sketch of *The Researchers/Alma Mater* from the Munch museum with high levels of chromatic and structural decay and visible microbial colonisation was analysed in order to identify the protein materials used by the artist and access to the biocontamination source.

Protein compounds were extracted and quantified by immunological assays that allowed to identify the presence of casein protein as the paint layers binder.

Several bacteria found in these artworks were characterised revealing proteolytic capacity, an important biodeteriogenic characteristic for these casein paintings. Two bacterial strains with greater proteolytic potential were inoculated in paint models prepared with casein as binder, the same used by E. Munch, exhibiting degradation signs caused by these microorganisms' activity, during the period of 6 months of monitoring.

The knowledge of the materials used by E. Munch and the microbial populations thriving in the surface are fundamental to understand the degradative processes associated and to develop appropriate mitigation strategies against the biodeteriogenic agents.

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Author contributions CS involved in manuscript writing, data interpretation, concept and experimental analysis and supplying the paint models; IS and ES involved in sampling; AC involved in supervision; ATC involved in sampling, concept, and supervision.

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