Onsite advanced biocleaning system on ancient wall paintings using new agar-gauze bacteria gel

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ABSTRACT

Aims: In this study the results of the use of a new agar-gauze biogel system activated by viable bacterial cells applied on altered wall-paintings are reported.

Methods and Results: Biocleaning using agar biogel and agar-gauze biogel systems was performed onsite by direct application onto altered wall-painting artwork surfaces (25-1,000 cm^2). The treatments were performed for the restoration of two original Italian sites: i) at the Vatican Museums, Cristo che salva Pietro dalle acque - La Navicella wall-painting by Giovanni Lanfranco (1627-1628) and ii) at Pisa Cathedral Cupola, Incarnato wall-painting by Orazio Riminaldi (1593-1630) and his brother (Girolamo Riminaldi). The novelty of this study is the use of Pseudomonas stutzeri A29 viable cells using an advanced agar-gauze biogel system and that the bioapplications required short contact-times of between 3-12 hours. The ancient artworks were altered by lipid and protein residues from past restoration as confirmed by the Py-GC/MS and FTIR data. Assessment was made on the effectiveness of the biological treatment and general considerations were discussed.

Conclusions: The short contact-time of the bioapplications using advanced agar-gauze gel activated with P. stutzeri viable cells makes this biotechnology promising as an alternative method to the traditional onsite cleaning techniques currently in use for altered ancient wall-paintings.

Significance and Impact of the Study. In this study, we report the biocleaning of altered materials located in vertical and vaulted areas using agar-gauze biogel and short application times for the first time. These findings are of great significance for future restoration activities, being crucial for determining best preservation strategies in this field.

Keywords: *Onsite* biocleaning, biorestoration, wall-paintings, agar-gauze biogel, bacteria.

Introduction

Up to date biocleaning processes have been successfully applied on real-scale lithoid artwork, such as stoneworks and buildings (Antonioli *et al*. 2005; De Belie *et al.* 2005; De Graef *et al*. 2005; Ranalli *et al*. 2005; Cappitelli *et al*. 2006, Cappitelli *et al*, 2007; May *et al*. 2008; Polo *et al*. 2010; Alfano *et al*. 2011; Gioventù *et al*. 2011; Barbabietola *et al*. 2012; Lustrato *et al*. 2012; Bosch-Roig *et al*. 2013a, Bosch-Roig *et al.* 2013b; Troiano *et al*. 2013; Mazzoni *et al*. 2014; Barras 2018). In all these studies, the most common alterations were black crusts, nitration, sulphation, salt efflorescence and the deposition of dust and organic compounds as hydrocarbons as a consequence of atmospheric pollution (Gauri et al. 1989; Saiz-Jimenez 1995; Saiz-Jimenez 2004; Doehne and Price 2010). Recently, a characterization of biodegradation processes occurring in a 17th century easel painting and the potential decontaminating activity of a biocompound has been investigated to prevent biodeterioration of artworks (Caselli *et al*. 2018). Other successful biorestoration treatments have been recently carried out on frescoes at the Camposanto Monumentale in Pisa; in this case the specific aim was to remove the gauze, glued to the paint surface, which was used in the "strappo" technique during the World War II to detach frescoes from the original walls after a bomb (Ranalli *et al*. 2003a; Antonioli *et al*. 2005; Ranalli *et al*. 2005; Ranalli *et al*. 2018).

On the contrary, little has been done on wall-paintings; one of the main complications, frequently encountered, is the presence of organic compounds which have been applied and not completely removed during previous and/or inappropriate restoration interventions. These organic residues can be altered and weathered over time and can represent a danger to the conservation of artwork. Such residual compounds, under favourable environmental conditions, can, in fact, be an adequate growth substrate both for bacteria and fungi, which can alter and deteriorate surfaces, causing chemical, mechanical and aesthetic damage to the materials. Therefore, the removal of these organic residues is crucial and, together with the traditional cleaning methods, the use of enzymes has been also considered in some cases (Cremonesi, 1999; Wolbers, 2000).

In order to apply a biocleaning technology to a different type of artistic materials such as wall paintings, the procedure requires specific analytical investigations to identify and distinguish organic compounds originally present in the paintings from those deriving from restoration interventions.

The materials undergo transformation through natural ageing processes and these effects are no less important. Cultural Heritage artworks are obviously unique, so they are usually characterized by low homogeneity and the various compounds to be identified are generally present in low concentrations. Therefore, the use of sensitive, selective and, if possible, non-destructive techniques is crucial and, strongly recommended, such as the use of FTIR and gas chromatography-mass spectrometry (GC/MS) (Colombini *et al*. 1999, Colombini *et al*. 2003; Andreotti *et al*. 2009; Rampazzi *et al.* 2016). Thus, once the unwanted organic matter has been identified on wall paintings and the difficulty in removing them by traditional methods has been verified, the use of biological systems may represent a valid, suitable alternative method to use.

In this paper, we illustrate the application of a biocleaning technology on wall-paintings using agar-gauze, biogel-activated bacteria as an advanced delivery system. The agar-gauze gel, enriched with viable bacterial cells as "biorestoration" agents, was used in two different sites: the wall painting *Cristo che salva Pietro dalle acque - La Navicella* at the Vatican Museums and the wall painting *Incarnato* in the Cupola inside Pisa Cathedral.

Our main task was to perform advanced, fast, *onsite* biocleaning using agar-gauze biogel activated with *P. stutzeri* viable cells on the two artworks cited above in order to degrade the organic substances present as residues from past restoration and which were affecting the substratum layers on the ancient wall-paintings. The following steps were taken:

i) Chemical-characterisation analytical techniques were used to identify the original organic compounds, the substances produced by their degradation, organic matter used in previous restorations and compounds produced by inorganic decay, as well as their effectiveness and effects over time by Py-GC/MS and FTIR;
ii) *Onsite* fast bioapplication to remove the undesirable matter present on ancient wall-paintings by advanced agar-gauze gel activated with *P. stutzeri* viable cells.

Materials and methods

Description of the selected wall paintings

Two ancient wall paintings were selected for the *onsite* biocleaning studies: *Cristo che salva Pietro dalle acque* (also known as *La Navicella)* at the Vatican Museums in Rome, and the *Incarnato* in the Cupola at Pisa Cathedral (**Table 1**).

Cristo che salva Pietro dalle acque - La Navicella - The painting chosen from those present at the Vatican Museums in Rome was an oil-painting fresco called *La Tempesta Sedata* or *Cristo che salva Pietro dalle acque - La Navicella*. It was painted by the Italian artist Giovanni Lanfranco between 1627 and 1628 using a mixed technique (**Figure 1a**). Originally this wall-painting was in the Saint Peter Basilica in Rome, but it was detached from the wall in 1721 and transferred to the present location in the *Aula delle Benedizioni* at the Vatican Museums, where the *onsite* biocleaning application was carried out. Initially, this wall-painting was irregularly covered by residual substances which altered the paint surface and that were visible even to the naked eye in some areas.

Cupola wall paintings - *Incarnato.* Among the ancient paintings present in the Cupola inside Pisa Cathedral (**Figure 1b**), the *Incarnato* wall-painting (about 300 m^2) was painted by Orazio Riminaldi (1593-1630) and his brother (Girolamo Riminaldi). Since the end of the 17th Century, this wall-painting has been a complex example referred to as a "*style defined by whirling and radiant circular motion" (turbinoso e radioso movimento circolare*) due to the peculiarity of figures painted on the vertical, vaulted and oblique internal walls in the Cupola. This wall-painting showed alterations in terms of undesirable substances covering and altering the original noble paint layer and, therefore, the aim of the biocleaning application was to remove these undesirable residual substances.

Table 1 Works of art selected, samples, type of alterations and methodology.

Artworks	*Cristo che salva Pietro dalle acque* *La Navicella,* Vatican Museums, Rome	*Incarnato,* Cupola Cathedral, Pisa
Site of painting	On vertical wall, at 8 m from the floor	On vaulted wall, at 50 m from the ground
Surface (m^2)	11.0	350
Site of samples	2 (LN1-LN2)	4 (IN1-IN4)
Substance to remove	Protein residues	Protein residues and calcium oxalate
Biocleaning systems	Agar biogel, agar-gauze biogel	Agar-gauze biogel
Treatment times (min)	60, 150	720

Chemical characterization by Py-GC/MS and FTIR

Small-samples were taken from the surface of both artworks in representative, homogeneous areas with a micro-scalpel, before and after the bio-treatment (**Table 2**). A Pyroliser PY3030 by Frontier Laboratories Ltd (Fukushima, Japan) coupled on line with Gas Chromatograph 6890N with Mass Spectrometer detector 5973 (Py-GC/MS by

Agilent Technologies, Palo Alto, USA) was used for pyrolysis in order to analyse the lipid, proteinaceous fractions of the samples. This analysis was for fundamental identification of the altered substances present on the surfaces of the two artworks studied. A few µg of the sample mixed with 2 µl of hexamethyl-disilazane (HMDS) were placed in a quartz tube. Detailed working conditions are published elsewhere (Andreotti *et al*. 2009, Lluveras *et al.* 2010; Rampazzi *et al.*, 2012; Orsini *et al.* 2017).

Figures 1a – 1b Images of artworks tested at onsite cleaning. A) *La Navicella* wall painting, Vatican Museums, Rome. B) Internal wall inside the Cupola at Pisa, Italy.

FTIR and Py-GC/MS analyses were carried out in order to identify the composition of the surface of the samples and to confirm either the increase or the decrease in the compounds present, depending on the intensities of their associated bands or peaks. FTIR spectra were recorded on KBr pellets (Sigma-Aldrich FTIR Grade) in transmission mode, by a BioRad Excalibur Series FTS 3000 spectrometer (detector DTGS) in the 4000–400 cm^{-1} range, with a resolution of 4 cm^{-1}. Pellets were prepared by mixing samples and KBr in an agate mortar, pouring the mixture into

a press and then applying a pressure of 6 t/cm^2 for 1 minute. The powders, from both the surface of micro-fragments and from the external layers of the polished cross-sections, were sampled under optical microscopy by means of micro-scalpel (Corti *et al*. 2016). Paper-based database was used to identify the species in each FTIR spectrum, by comparing experimental peaks with reference patterns (Derrick *et al*., 1999).

***Growth conditions of Pseudomans stutzeri* A29 strain**

P. stutzeri A29 strain (DIBT Microbial Collection, University of Molise, I) was used for all the bioapplications on the basis of the performances obtained in previous studies (Ranalli *et al*. 2005; Bosch-Roig *et al*. 2012; Lustrato *et al*. 2012). Furthermore, growth conditions of the selected strain and its biodegradation activities were tested before the biocleaning was undertaken (Bosch-Roig and Ranalli, 2018; Ranalli *et al.* 2018).

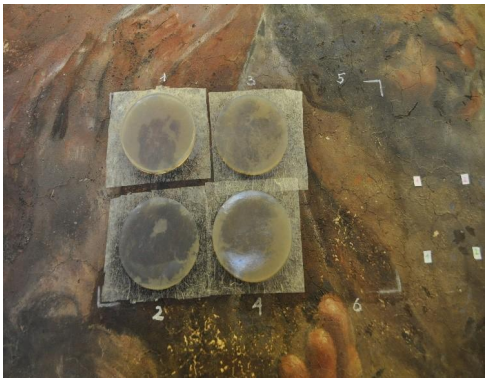
The strain was incubated at 28°C for 24-36 hours, on Tryptone Soya broth medium containing pancreatic digest of casein, 17.0 g l^{-1} (CM0129, Oxoid Ltd., Basingstoke, England); bacterial growth was monitored by OD_{560}, and cultures were stored on TS Agar medium (Oxoid Ltd., Basingstoke, England).

Bacterial cell and new biogel system for onsite biocleaning

Suspensions containing exponentially-growing bacteria, of approximately 10^8 CFU ml^{-1}, were obtained by inoculating 10 ml of an overnight broth-culture into 1,000 ml of fresh broth medium (in a flask with 3,000 ml total volume) and incubating it in a shaker (200 rpm) for 24 hours at 28°C. The cells were centrifuged at 7,000 *g* for 10 minutes at 4°C, washed twice with phosphate-buffered saline solution, and re-suspended in sterile distilled water. Final cell concentration was around 10^8 cells ml^{-1}, corresponding to an $O.D._{560}$ nm of about 1.6; pH was around 7.0 and the solution conductivity was 947.1 μs cm^{-1}. Cells were stored at 4 °C during transportation from the laboratory to the site for *onsite* application.

Two types of delivery systems were used to bioclean the selected areas: Agar biogel (Bosch-Roig *et al*. 2014) for vertical surfaces, as shown in **Figure 2a**, and advanced agar-gauze biogel for vaulted surfaces, **Figure 2b.**

Both the biogels were carefully brushed with the suspension cells (*P. stutzeri* A29 bacteria cells at a concentration of 2-5 x 10^6 viable cells/cm^2) to favour cell contact and adhesion. For the preparation of the agar-gauze biogel, under sterile cabinet conditions, a thin, sterile, cotton gauze was added at the bottom of sterile plastic vessels covered by hot agar broth (at more than 80°C), obtaining sheets of standard dimensions (25 x 40 cm^{-1} and 1,000 cm^2) of advanced agar-gauze biogel. After agar solidification (below 45°C), the larger agar-gauze gels were protected by a double layer of plastic PET film (Domopak, Volpiano, Italy), and stored at 4 °C. When required, the plastic cover was removed and the agar-gauze biogels were applied onto the surface of the artworks. If required, the large agar-gauze biogels were cut using a sterile lancet or scissors in order to obtain sheets of specific dimensions according to the areas to be treated. **(Figures S1a-b).**



Figures 2a – 2b Images of artwork tested at onsite cleaning, La Navicella wall painting, Vatican Museums, Rome. Detail of biocleaning by traditional agar biogel disks (2a) and advanced agar-gauze biogel activated with *P. stutzeri* (2b).

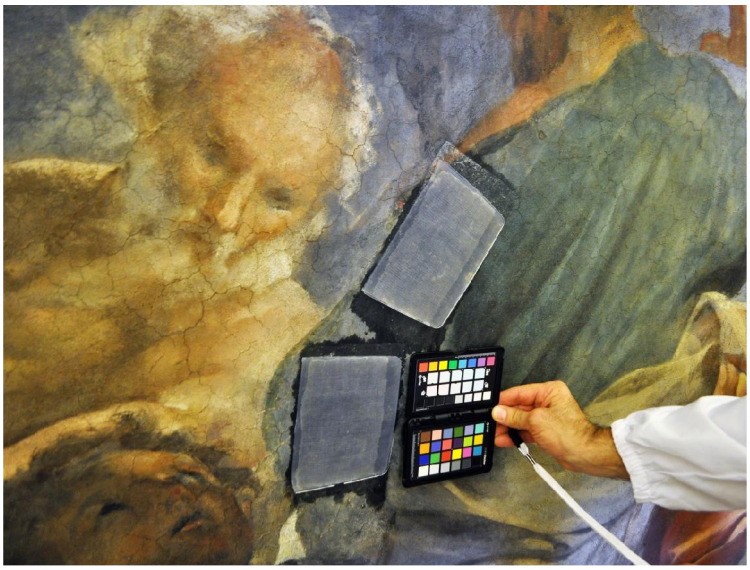
The bioapplications on wall-paintings were performed on small, representative areas of the entire altered wall-painting surfaces. Representative areas of from around 60 cm^2 to around 4,000 cm^2 were selected and the biocleaning-treatment contact times varied between 10 and 150 minutes for the *Navicella* (**Figures 3a-3b**) and were up to 12 hours for the Cupola wall-paintings (**Figures S2a-d**). The tests were performed as follows: i) Natural, white, unbuffered, 9.0 g/m^2 Japanese paper (Klug Conservation, Walter GmbH & Co. KG., Immenstatd, Germany) was applied onto the vaulted and vertical wall-painting surfaces; ii) *P. stutzeri* A29 viable cell-suspension was used as a biocleaning agent at the bacterial concentration of 2-5 x 10^6 viable cells/cm^2 as reported above; iii) New agar-gauze biogel, activated by viable bacterial cells, as an advanced delivery system to favour biocleaning; iv) Agar-gel or agar-gauze gel with sterile water instead of viable bacterial cells were used as negative control.

Monitoring of the effectiveness of the biorestoration process

One very important aspect of biorestoration is the monitoring of the effectiveness of the process. On the basis of previous studies performed by the same research group, at the end of the biocleaning, treated and control areas were washed with sterile distilled water (3 soft manual applications by sponge) and dried at room temperature (Ranalli *et al.* 2003b; Ranalli *et al.* 2005, Ranalli *et al.* 2009).

After the final washing and drying steps, to check the possible presence of residual bacterial cells, sterile swabs (Fissan, Milano, Italy) were used to ensure that no treatment residues were left on the artworks and microbiological

analyses by plate counting and ATP content determination were performed. Three replicates were always considered for both the biocleaning and the control tests. Temperature was also monitored at both sites by portable data loggers (176 T3 model, Testo SpA, Settimo Milanese, I).



Figures 3a-3b Images of artwork tested at onsite cleaning, La Navicella wall painting, Vatican Museums, Rome. Detail of biocleaning by agar-gauze biogel activated with *P. stutzeri*.

Results

Chemical and physical analyses before the biocleaning

Py/GC-MS results confirmed the presence of fatty acids and protein residues on the *La Navicella* and *Incarnato* wall paintings. In particular, pyrolisis of the superficial layer of the sample from the *Navicella*, confirmed the presence of numerous materials: proteinaceous material with the presence of traces of hexadecanenitrile and octadecanenitrile, confirming the presence of egg; cholesterol and fatty acids, such as azelaic acid, myristic acid, palmitic acid and stearic acid, revealed the presence of a lipid component. In fact, in addition to the lipid component of the egg, other sources of lipid material have also been identified like long fatty acid chains and their isomers C15, C17 and C19, which are characteristic of animal-fat material, as shown by the extract ions m/z 299, 327, 355 in **Figure 4**.

In FTIR spectra of the *La Navicella* samples, the absorbance bands around 1435, 874 and 713 cm^{-1} (C=O asymmetric stretching, out-of-plane bending, and in-plane bending vibrations, respectively) and around 1623 and 1315 cm^{-1} (CO stretching vibrations) showed the presence of calcite and calcium oxalate as whewellite $CaC_2O_4*H_2O$, respectively (Petrov and Šoptrajanov 1975; Derrick *et al.* 1999; Bugini *et al.* 2017; Rampazzi *et al.* 2018). The S-O asymmetric stretching signals at 1145, 1116 and bending mode at 669 cm^{-1} also revealed small amounts of gypsum, and the absorbance around 1385 cm^{-1} suggested an alkaline nitrate (Petrov and Šoptrajanov 1975; Derrick *et al.* 1999). Calcite derives from the carbonatic substrate unintentionally sampled, while calcium oxalate, gypsum and alkaline nitrate are neo-formation compounds from well-known decay phenomena of stone materials (Amoroso and Fassina 1983; Rampazzi 2019). Weak bands around 2915 and 2850 cm^{-1} were observed, probably due to CH stretching signals of traces of organic compounds, but definitive identification was not possible (Derrick *et al*. 1999). Silicate signals were present in the spectrum of sample LN1, which may be ascribed to unintentional substrate sampling. In sample LN2 peaks representing basic copper carbonate indicated the presence of the pigment azurite.

For the *Incarnato* samples, FTIR analyses of the external surface of the 4 samples (IN1a-IN4a) showed the specific calcite signals at 1433 (asymmetric C-O stretching band), 874 (out-of-plane bending vibration) and 713 cm^{-1} (in-plane bending vibration) and those of silicates at around 1000-1100 cm^{-1} (Derrick *et al.* 1999; Rampazzi *et al*. 2011), both deriving from the unintentionally sampled substrate. The spectra also showed characteristic aliphatic carbon hydrogen stretches (methyl and methylene groups) at 2923 and 2852 cm^{-1} and an absorbance-signal band around 1700 and 1246 cm^{-1}, which may be associated with carbonyl stretching and N-H deformation/C-N stretching, respectively. The pattern of the bands suggests that the organic substance is a proteinaceous compound (Derrick *et al.* 1999).

Biocleaning treatments of the selected areas

The complete removal of organic compounds depended on the thickness of the layer and on the duration and type of application. Some areas were completely cleaned, while others still showed the presence of residues at the end of the biotreatment. The results of the *onsite* trials using agar-gauze biogel enriched with *Pseudomonas stutzeri* cells at different application times (12 hours for the Cupola at Pisa Cathedral; 60 and 150 minutes at the Vatican Museum) are shows in **Table 3**. An evaluation of the advantages of the biocleaning treatments is also reported: the performance of the agar-gauze biogel adopted in this study shows good adhesion on different surfaces, dehydration and detachment and it is easy to apply and to remove. Rare instances of leakages (*colature*) were observed during the adhesion on the vaulted/oblique internal wall-surfaces at the Cupola (**Figures S2a-d**).

As regards *La Navicella* wall-painting, two areas of the painting were tested; a small area of the artwork featuring the red cape of the lower left figure, was initially treated with *P. stutzeri* for approximately 150 minutes. Approximately 25 cm^2 of agar gel was applied in triplicate over a layer of protective Japanese paper, both with bacteria and without, using water instead of the bacterial solution, as a control (see Materials and methods).

Once the results of the biocleaning had been verified, a larger area of about 1,000 cm^2 (on the green cape of the upper right figure) was treated. In this case, we used agar-gauze biogel, which allowed better adhesion during the bioapplication on vertical walls. In fact, the conservators were able to adapt the agar-gauze biogel and to cut it according to the size and shape of the areas selected, for the presence of painted objects or figures. Additional treatments lasting 60 minutes were tested with control tests in parallel.

Table 2 *Onsite* results of biocleaning treatments on vertical wall painting artwork *Cristo che salva Pietro dalle acque - La Navicella* and vaulted wall painting on the Cupola *Incarnato* at Pisa, affected by proteinaceous residues.

Parameters	*The Navicella* wall painting (Vatican Museum)		*Incarnato* - Internal wall inside the Cupola, Pisa	
Treatments	Control - agar gauze (no bacteria)	Agar gauze-biogel activated bacteria	Control - agar gauze (no bacteria)	Agar gauze-biogel activated bacteria
FTIR				
Before	Organic compounds (≈ 5% w/w)	Organic compounds (≈5% w/w)	Proteinaceous compounds (≈11% w/w)	Proteinaceous compounds (≈11% w/w)
After	Organic compounds (≈5% w/w)	Organic compounds ≈ LOD	Proteinaceous compounds (≈11% w/w)	Proteinaceous compounds ≈ LOD
Py/GC-MS	Organic substances (Abundance ≈140.000)		Organic substances (Abundance ≈350.000)	Organic substances (Abundance ≈350.000)
Before	Organic substances (Abundance ≈140.000)	Organic substances (Abundance ≈140.000)	Organic substances (Abundance ≈350.000)	Organic substances (Abundance ≈ 20.000)
After		Organic substances (Abundance ≈ 20.000)		

Legend – LOD, Limit of detection: FTIR (≈ 0.5% w/w); Py/GC-MS Limit of detection of the Ion Current Abundance of the extract ion m/z 194/208 and 222/236, characteristic for the markers of egg (Abundance ≈ 20.000) (Colombini et al., 2003; Ranalli et al., 2005).

Microbiological monitoring of the biorestoration process

The microbiological monitoring performed immediately after the removal of the agar biogels at the Vatican Museum, and two months after the biotreatment on the Cupola at Pisa Cathedral, showed the absence, or very low presence, of viable cells (ranging from 0 to 8 CFU/ 100 cm^2); the bacterial colonies detectable were not attributable to the bacterial strain used for the biocleaning. In fact, on the Cupola the bacterial colonies grown on the agarized plates were characterized by pigmentation (yellow and orange color) ascribable to outdoor environment. Similar results were found over time in the biotreated and untreated control areas by determining ATP contents, suggesting that the treated artworks do not show any significant changes in viable microbial counts. Temperature monitoring during the biocleaning indicated that the recorded temperature was stable at around 21$\pm$2.0ºC at the Vatican Museum room. On the contrary, recorded temperatures during the biotreatment carried out inside the Cupola at Pisa Cathedral ranged from 17 to 24ºC (with day / night variation); these environmental conditions, however, ensured favourable bacterial metabolic activity.

Chemical and physical analyses after the biocleaning

After the treatment, some micro-fragments of the painting films were taken, in the same homogeneous areas as the previously analyzed samples (**Table 2, Figure 4)**, in order to determine the composition. The results confirmed the absence or very low amounts of organic materials, which, at least, could be below the limits of instrument sensitivity. The Py/GC-MS limit of detection of the Ion Current Abundance of the extract ion m/z 194/208 and 222/236, characteristic for the markers of egg was Abundance < 20.000. The Py-GC/MS analysis in fact attested to the effectiveness of the cleaning in removing the proteinaceous material, as visible from the comparison of the extract ion pyrogram of the material collected before and after cleaning, of m/z 194/208 and 222/236, which are characteristic of the markers for egg hexadecanenitrile and octadecanenitrile, respectively. (**Table 3, Figure 5).**

In *La Navicella* FTIR spectra too the weak signals of organic compounds previously observed were no longer detected. With regards to the *Incarnato*, FTIR signals were quite similar to those observed before the biocleaning. Specific calcite and silicate signals were still present. The spectra also showed very weak signals at 2923, 2852 cm-1 and at around 1700 cm-1, which are characteristic of aliphatic carbon hydrogen stretches (methyl and methylene

groups) and carbonyl stretching, respectively. The absorbance pattern indicated the same proteinaceous compound determined in the painting film before the biocleaning, but at very low concentration. The limit of detection for FTIR was 0.5% w/w. **(Table 3).**

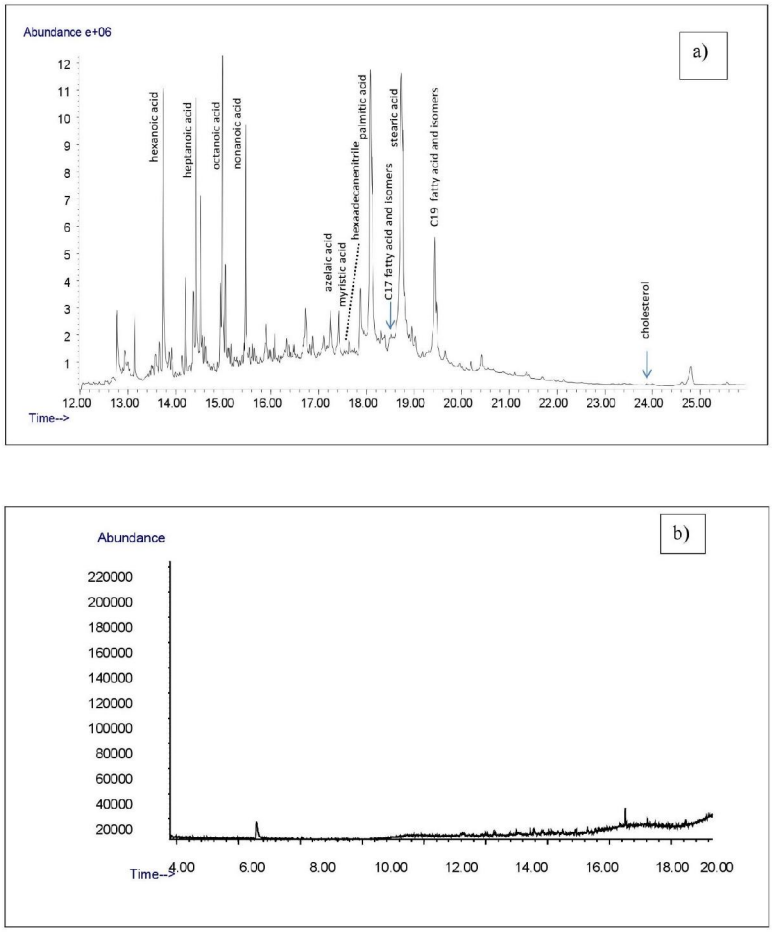


Figure 4 Pyrogram in extract ions m/z 299, 327, 355 of *La Navicella* sample, before biocleaning, characteristic of C15, C17 and C19 and their isomers.

Discussion

The success of the biotreatments performed using new biogel systems activated with viable bacterial cells shows that biocleaning is also highly-promising for wall-paintings and for the removal of various alterations. This is attributable to the great versatility of the bacteria and their wide range of enzymatic activities. Bacteria are known to produce not only constitutive, but also inducible enzymes that can attack and degrade different types of molecules. The synthesis of inducible enzymes only takes place in the presence of a substrate, creating a regulatory effect. Thus, the use of microorganisms is more effective than the use alone of a single enzyme that only attacks specific bonds. Moreover, constitutive and induced enzymes of viable cells of versatile bacteria like *Pseudomonas* strains are able to degrade a wide spectrum of organic compounds (Bosch-Roig *et al.* 2016). Several factors, including temperature, relative humidity, pH, carbon and energy sources and inhibitors (metal ions, etc.) can influence metabolic activity. Thus, in order to confirm predicted metabolic efficiency, laboratory-scale experiments on artificial specimens are useful to optimize conditions before onsite application.

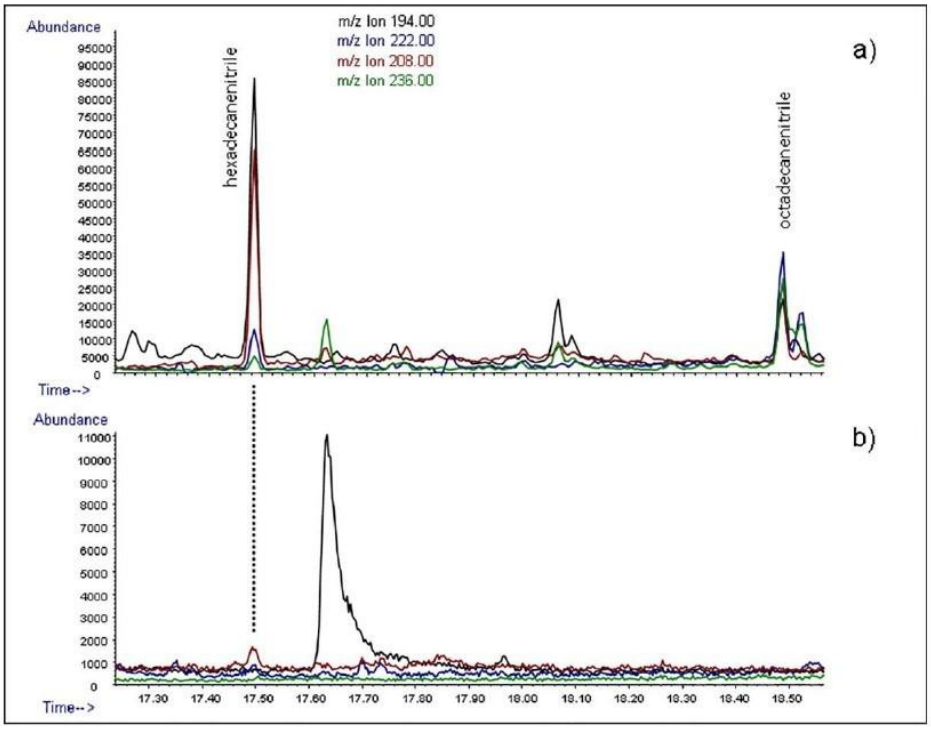


Figure 5 Pyrogram of the material collected before (a) and after cleaning (b) of the m/z 194/208 and 222/236, characteristic of the markers for egg hexadecanenitrile and octadecanenitrile, respectively.

In the biorestoration of artworks, biocleaning requires the use of selected microorganisms on the altered surface. The success of the application depends on the type of alteration, the artwork material and its condition, the location of the areas undergoing treatment and the metabolic activity of the selected microflora (aerobic and/or anaerobic processes). In addition, the complete removal and the overall effectiveness of the biological process can be influenced by the duration of the treatment (Ranalli *et al.* 2005). The state of conservation of the artwork, which also depends on the original materials used, can be critical for many reasons; in this phase it is crucial to reduce to the minimum both the volume of water added to the material and the contact time on the artwork surface. While mechanical and chemical methods commonly used to clean and restore artwork surfaces are not always satisfactory (not efficient enough and/or too invasive), short and fast biocleaning processes based on the use of a selected strain of viable cells represent a promising method and a more versatile alternative.

When artwork undergoes a restoration process, generally an accurate cleaning phase is required at the end of the biocleaning process to carefully remove the bacterial cells used as biocleaning agents and to avoid undesired metabolic processes. Therefore, an adequate strategy for the future protection of artwork needs to include an accurate cleaning phase, because, if the microorganisms remain viable and active, they may cause material loss or damage, either directly, or as a consequence of their metabolic by-products (Bosch-Roig and Ranalli, 2014).

Possible damage due to prolonged bacterial treatment on artworks has been also demonstrated in previous studies by Ranalli *et al.* (2003a) and Ranalli *et al.* (2003b) and, therefore, it is very important to appropriately define the duration of the application. Rapid analyses are also fundamental for the monitoring of the biorestoration processes and therefore, fast analyses that measure the microbial activity like the monitoring of ATP content as viable biomass indicators, are of great benefit.

In this study, *P. stutzeri* viable cells have been applied to clean two different wall-paintings using an advanced delivery system as agar-gauze biogel. The results reported in this paper show that this biotechnology, until now used for the recovery of frescoes, can be safely applied *onsite* to ancient paintings to bioclean vertical and vaulted wall-surfaces. Before proceeding the appropriate conditions, the ideal cell-carrier system (in this case the agar-gauze gel activated bacteria) and the appropriate duration of the application should be evaluated and optimized, in order to avoid potential inactivation of the biogel by de-hydration and possible detachment from the artwork surface.

This study also shows how this biotechnology can be useful and appropriate for the removal a mixture of mainly proteinaceous residues from wall-painting surfaces. The degradation of the residual compounds by bacterial activity (without the use of chemicals) leads to the natural release of carbon dioxide and water and the method can be therefore considered to be environmentally friendly (Bosch-Roig *et al.* 2016; Junier and Joseph, 2017). The performance of the agar-gauze biogel adopted in this study shows good adhesion on different surfaces, low risks of leakage, dehydration and detachment and it is easy to apply and to remove.

Recently the potential use of selected microorganisms to prevent biodeterioration of artworks has been also reported; the results suggested and indicated the ability of biocompound to counteract the growth of contaminating microorganisms potentially dangerous to paintings (Caselli *et al*. 2018).

Our results confirm the vast potential of biorestoration treatments as soft, but targeted, biotechnology aiming to recover altered artwork, being non-destructive and using non-pathogenic microorganisms and their natural metabolic activity. Therefore, biorestoration represents the development, under optimal conditions, of processes that spontaneously occur in natural environments and its application can be added to the ever-growing list of biotechnologies applied to the environment as promising and useful alternative methods in the conservation and preservation of our historic and artistic patrimony.

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Author contributions

G.R., E.Z. coordinated the project and participated in the conception of the work, in the analysis of results, and in the writing the draft of the manuscript, A.A., M.P.C., L.R. contributed to design the experiment, analysis of the data, and contributed in manuscript preparation and its critical revision, P.B., G.L. contributed in the execution of part of the experimental work and in the acquisition of the data, C.C. contributed to the chemical analysis of samples, P.V., C.G., D.Z. coordinated and supervised all the conservation activities. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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