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Hi-tech restoration by two steps biocleaning process of *Triumph of Death* fresco at the Camposanto Monumental Cemetery (Pisa, Italy)

Running Title

Hi-tech restoration by two steps biocleaning

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ABSTRACT

Aims: In this work, the "hi-tech" complex biocleaning and restoration of the 14th-century fresco *Triumph of Death* (5.6x15.0 m) at the Camposanto Monumental Cemetery (Pisa, Italy) is reported.

Since 2000, the restoration based on the biological cleaning of noble medieval frescoes, has been successfully utilized in this site.

Methods and Results: The novelty of this study is the 2-steps biocleaning process using *Pseudomonas stutzeri* A29 viable cells, previously applied for recovering other valuable frescoes. In this case, after the fresco detachment from the asbestos-cement support (eternity), both the animal glue and the residues of calcium caseinate were biologically removed respectively from the front and from the back of the fresco in 3 hours as indicated by GC-MS and PY/GC-MS analyses.

The data obtained during the monitoring of the bio-restoration process confirmed that the adopted procedure does not leave residual cells on the fresco surfaces as showed by plate count method, ATP determination and also SEM observation. In addition, to avoid the risk of condensation phenomena after the relocation of the restored fresco sections onto the original walls, the use of a new support has been set up together with the design of a control system that allows a continuous monitoring of environmental parameters for prevention and conservation purposes.

Conclusions: This large-scale biorestoration work clearly shows and confirms that this biotechnology is highly efficient, safe, non-invasive, risk-free and very competitive compared to the traditional cleaning methods, offering an unusual "resurrection" of the degraded artworks also in very complicated and delicate conditions such as the *Triumph of Death* fresco, defined for its dimension and artistic importance the 'Pisa's Sistina frescoes'. Significance and Impact of the Study. These findings can be of significant importance for other future new restoration activities and they are crucial for determining preservation strategies in this field.

Keywords Hi-tech restoration, frescoes biocleaning, *Pseudomonas stutzeri*, optimization of fresco support, anti-condensation system

INTRODUCTION

The Camposanto Monumental Cemetery in Pisa during the II World War in 1944 was badly damaged by a bomb; considering the extremely precarious conditions, a large number of frescoes was rapidly detached with the 'strappo' technique using animal glue and gauze to tear off the fresco from the original walls and stored, once rolled, without any rigid support. The frescoes (a total surface area of about 1,500 m^2) dated back to the 14^{th} century and were painted by Bonamico Buffalmacco, Antonio Veneziano, Benozzo Gozzoli, Taddeo Gaddi, Francesco Traini, and Spinello Aretino. Later, in the 1960's, some of them were placed onto an asbestos cement support (eternit) in order to carry on with the restoration intervention and to finally put them back onto the original walls, but in some cases alteration processes such as swelling and detachment of the fresco paint film were observed. Therefore, in the 2000's the frescoes were once again detached from the Camposanto Monumental Cemetery walls and submitted to a new delicate restoration work which foresaw the initial removal of the

adhering organic matter. Traditionally frescoes are restored both by traditional chemical and physical techniques, based on the removal of organic residual substances and salts with the use of ammonium carbonate solution and organic solvents, but these methods in some cases fail as in the case of Camposanto Monumental Cemetery frescoes (Ranalli *et al.* 2003a).

Analytical data on frescoes from the same site affirmed that some of the altered frescoes exhibited hydrophobic behavior related to weathering and protein polymerisation from past restoration works (Ranalli *et al*. 1996; Ranalli *et al*. 2000; Ranalli *et al.* 2003a; Ranalli *et al*. 2005; Lustrato *et al.* 2012) and therefore the removal of such compounds by using the traditional methods resulted ineffective. For these reasons, a crucial step in restoration is the chemical characterization in order to distinguish the original organic materials and those deriving from past restoration works and it is pivotal to define if the materials have undergone through transformation and weathering processes. The use of sensitive, selective and if possible, not destructive techniques is fundamental and this include techniques such as gas chromatography coupled with mass spectrometry (GC-MS) and pyrolysis/GC-MS (Lluveras *et al.* 2010; Rampazzi *et al.* 2012; Rampazzi *et al.* 2016; Orsini *et al.* 2017). Previous studies highlighted that, when traditional methods resulted ineffective, the use of enzymes, surfactants and solubilizing agents showed a great potentiality (Makes, 1988; Bellucci and Cremonesi,1994; Bonomi,1994; Wolbers, 2000; Polo *et al.* 2010; Troiano *et al.* 2013) together with the application of a selected viable bacterial cells as "bio-restoration and bio-cleaning" agents, which can be an alternative and innovative technique to clean both frescoes and altered stoneworks (Antonioli *et al.* 2005; Cappitelli *et al*. 2005; Cappitelli *et al*. 2007; May *et al*. 2008; Alfano *et al.* 2011; Gioventù *et al.* 2011; Bosch-Roig *et al.* 2012; Bosch-Roig *et al.* 2013a; Bosch-Roiget *et al.* 2013b; Bosch Roig *et al.* 2014; Bosch-Roig and Ranalli, 2014; Mazzoni *et al.* 2014; Bosch-Roig *et al.* 2016).

As previously mentioned, using the "*strappo*" or "tear-off" technique a notable quantity of organic compounds such as animal glue and casein was distributed on both surfaces; on the front, as a fixative or consolidation agent and on the back of the fresco as a support adhesive (Ranalli *et al.* 2003a; Ranalli *et al*. 2005; Bosch Roig *et al.* 2014). Therefore, at the Camposanto Monumental Cemetery, some fresco sections showed situations and conditions very complex and different.

Since 2000, the success of this technology have led the Technical Commission for Restoration (Pisa, Italy) to accept and approve the use of *Pseudomonas stutzeri* viable cells for biological applications. In fact, the first application of biocleaning technique on frescos at the Monumental Cemetery in Pisa was reported in pioneering studies, by Ranalli *et al.* 2005, where a protocol that put together the synergic combination of hydrolytic enzymatic action with microbial metabolism, was described for the fresco restoration. The case study featured the restoration of one of the frescoes in the Cemetery at Pisa, "*The Conversion of Saint Ephysius and Battle*" (Conversione di S. Efisio e Battaglia) by Spinello Aretino, which, as a result of the dramatic consequences of bombing during World War II had been removed from the walls using the "tear-off" technique, covering the surface with a strong cloth bound with generous layers of animal glue (Antonioli *et al.* 2005; Ranalli *et al*. 2005). When, 10 years later, restorers had attempted to remove the cloth, they had found that the glue had polymerised following historical addition of formaldeyde to the surface, thus prohibiting effective use of a number of surfactants and solubilizing agents. At that time, preliminary tests with single, or with a mixture of commercial and purified enzymes showed no positive effects. The use of bacterial cells was, thus, the only way to restore the altered noble fresco. The biocleaning technique was performed by direct application of whole viable bacterial cells of *Pseudomonas stutzeri*, A29 strain, onto the artwork surface, for 10-12 hours. The

biological pack removed a great amount of the glue, leaving only a little residue on a limited surface area which was then eliminated with the purified enzymes, collagenase and protease. Years later, another positive biocleaning was performed in only three 3 hours on a medieval fresco "*Stories of the Holy Fathers*" (Storie dei Santi Padri) altered by traces of animal glue (Lustrato *et al.* 2012). Another important matter is related to the safety of the application of microorganisms on artwork (Bosch and Ranalli 2014). Microbiological controls after the biocleaning processes are also very important to guarantee that no bacterial cells are left on the cleaned surface at the end of the treatment. Different techniques have been used such as ATP surface analysis and contact plate microbial counts. (Bosch-Roig *et al.* 2013a).

In this work, we report the *ex situ* large-scale biotreatment of *Triumph of Death* fresco (5.6 x 15.0 m) painted by Buonamico Buffalmacco in the period 1336-41. The conservation state of this fresco showed evident problems due to the color loss caused by pulverization processes, but also due to recent restoration practices that in the course of time resulted inappropriate. The study aimed to the setting up of a "hi-tech restoration process" based on a two-steps biocleaning of the proteinaceous residues (mainly animal glue and casein) present both on the front and back of the fresco paintwork, including the removal of the old asbestos cement support. In addition, the adoption of a new support with specific properties in terms of resistance, insulation and mechanical characteristics for the relocation on the original walls and lastly, the design of a continuous monitoring system of environmental conditions (temperature and humidity) on the fresco surface and in the surroundings are also illustrated.

MATERIALS AND METHODS

Triumph of Death fresco description

The *Triumph of Death*, Buonamico Buffalmacco's fresco (1336-41), is the first of a series of three scenes for the Camposanto Monumental Cemetery and was painted on Dominican monk commission. The fresco, based on the theme of the end of the world, is divided in different scenes dominated by the horrid, grotesque, comic and serenity images. The best preserved areas are those located in the higher part of the fresco (in particular the battle between angels and demons) and the two side scenes. As previously described, the fresco was already extremely damaged by pulverization processes and after the bomb in 1944 (1947-1960) it was detached from the walls and glued in a framework screwed onto asbestos cement eternity supports. The whole *Triumph of Death* fresco (about 85 m^2) was divided totally in 14 sections.

Microorganism, media and bacterial cells production

Pseudomonas stutzeri A29 strain (DISTAM-DISTAAM Strains Collections, University of Milan and University of Molise, Italy) was used for the biocleaning process. Two different growing conditions where used:

- *bacterial cells suspension F* for front treatment: the strain was incubated at 28°C for 24-36 hours on Standard Plate Count broth medium (CM463, Oxoid Ltd., Basingstone, England) containing pancreatic digest of casein, 5.0 g l^{-1} supplemented by animal glue in pearl (CTS srl, Altavilla Vicentina, Italy) at 0.5% w/v;
- *bacterial cells suspension B* for back treatment: 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., Basingstone, England).

Bacterial suspensions containing exponentially growing bacteria, approximately 10^8 CFU ml^{-1}, were obtained by inoculating 10 ml of an overnight broth-culture into 1,000 ml fresh broth medium (in flask of 3,000 ml total volume) and incubating it in a shaker (200 rpm) for 24 h at 28°C. The cells were centrifuged at 7,000 *g* for 10 min at 4°C, washed twice with phosphate-buffered saline, and re-suspended in sterile bi-distilled sterile water, pH 6.9. Final cell concentration was above 10^8 cells ml^{-1}, corresponding to an O.D.560 nm of about 1.6 and the cells were used immediately or stored at 4 °C during conservation and/or transport from the laboratory to the field application. (Ranalli *et al.* 2005). A laboratory batch fermenter (20 l volume), fully computerized (mod. Biostat C, B. Braun, Biotech International, Germany), was used to produce a suitable amount of viable bacterial cell biomass to apply in the biocleaning treatment of the whole *Triumph of Death* fresco.

Present day 14th-century fresco restoration

The present restoration procedure involves several steps performed on the front (F) and on the back (B) of the fresco: the biological and chemical cleaning treatments and the fixing phase are reported in detail in Table S1.

Artificially altered specimen biotreatments

In order to evaluate the effectiveness of the biocleaning process, artificial altered specimens, simulating the presence of animal glue and casein residues were prepared, separately. Adequate water solutions prepared using animal glue in pearl (CTS srl, Altavilla Vicentina, Italy) at 5-0 g l^{-1} and pancreatic digest of casein 17.0 g l^{-1} dissolved at 80°C for 15 min under agitation (100 rpm), was used. These solutions were applied by soft manual brush as a thin layer directly on the upper surface of gauze specimens (20 x 20 cm). Once the animal glue or casein formed a thin film, about 30 min, the artificially altered specimens were weighted

using analytical balance (mod. Crystal, Gibertini, Elettronica, Novate Milanese, Italy) and submitted to the biocleaning tests using the same conditions for frescoes. The change in weight of organic materials (animal glue and casein residues, added separately) before vs after biocleaning process was calculated and related to a removal efficacy of the ancient frescoes.

Chemical and physical analyses

GC-MS and PY/GC-MS analyses

Reagents, apparatus, analytical procedure based on selective extraction and hydrolysis assisted by microwave and GC-MS analysis are detailed in the literature (Lluveras *et al.* 2010). Twelve samples were collected by scalpel from the different sections of *Triumph of Death* fresco and a characterisation of the organic adhesive used both to detach the fresco from the wall and the adhesion onto the asbestos support, was performed by GC-MS and PY/GC-MS (Orsini *et al.* 2017) analytical procedures. Moreover, to identify the natural organic compounds, their decay products and the effectiveness over the time, the analytical procedures previously adopted by the same authors were used (Ranalli *et al.* 2005; Lustrato *et al.* 2012). The thickness of the glue layers was measured in different areas of the fresco using a laser sensor micrometer, Thrubeam LX2-V mod. (Keyence Corp., Osaka, Japan).

Scanning Electron Microscopy analyses

The morphology of some micro-fragments taken from the fresco after the biocleaning and the treatment with the anionic resin was observed with a FEI/Philips XL30 ESEM (Corti *et al.* 2013; Corti *et al*. 2016). Every sample was analyzed "as is" in low vacuum mode (1 torr) at 20kV, by using GSE and BSE detectors.

Monitoring of viable cell counts and ATP determination during and after the biocleaning

At time 0 and 3 hours of the biocleaning step, the evaluation of the total viable cells has been performed. Small samples taken from the wet cotton layer (1.0 g), were analyzed using the plate counting technique in Standard Plate Count Agar medium (SPCA, Difco), with incubation at 28°C for 48 hours and ATP content (see below). In addition, residual bacterial cell presence both on the front and back of the biocleaned surfaces was monitored immediately after the washing steps with distilled sterile water, and at time 0, 30 and 180 days. For the fresco microbiological monitoring, sterile velvet cloths were pressed onto the cleaned fresco surface and then onto SPCA medium (Difco) plates. After the incubation at 28 °C for 48 hours the colony forming units (CFU) per 25 cm^2 were expressed.

At the same time, bacterial activity was measured by total ATP assay using sterile cotton swabs allowing a not destructive sampling of the surface of ancient frescoes. A Biocounter P1500 luminometer (Lumac B.V.) equipped with a photomultiplier tube set at 7,200 Relative Luminescence Unit (RLU) with 200 pg ATP in 100 μl^{-1} of Lumit buffer and Lumit-QM reagent, was used (Ranalli *et al*. 2003b; Ranalli *et al.* 2005; Bosch-Roig *et al.* 2013a).

Choice and utilization and of a new support for the relocation of the recovered fresco sections

The previous support (glass-resin) used for other frescoes from the same site showed some limits and evidenced the need to optimize a new system with better physico-chemical characteristics and properties. The new fresco's support is composed by the restored fresco mounted on canvas, aluminum-centered honeycomb heating fabric of polyester and carbon fibers (Termotex srl, Vicenza; Studio Progetti, Galliera Veneta, PD, Italy), **(Figure S1).** Moreover, an additional metallic structure in aluminum grid (De Simone, Firenze) on the

back of canvas can guarantee the fixing of large final panels on the original mural walls. More detailed information of the new support is reported in result section.

Statistical analysis

Data of ATP content and viable bacterial cell counts, in triplicate media were expressed as mean and standard deviation compared statistically by the Tukey's *b* test at the 5% level with SPSS 11.5 (SPSS for Windows, Version 11.5, USA). Differences between values at $P \leq 0.05$ are considered not statistically significant.

RESULTS

The "hi-tech" complex restoration and biocleaning of the 14th century fresco *Triumph of Death* is summarized in **Table S1** and here reported in detail.

Two-steps biocleaning treatment at the front and back of the fresco

The *ex situ* biorestoration treatments were performed by a two steps application (front and back) of the *P. stutzeri* strain A29 bacterial suspension on the altered fresco as indicated in Materials and Methods section **(Figure 1).**

After 3 hours of biotreatment using bacterial suspension F (at the front of the fresco) and B (at the back of the fresco), the bacterial activity was so intense that the treated areas were largely biocleaned and no residues of proteinaceous materials were presence. The treatment was soft and delicate and did not show any structural damage **(Figure 2).**

Residues of glue from the front and casein from the back of the fresco, present as thin layer at the beginning of the treatments, were completely cleaned. At the back only where small spots of calcium caseinate were thicker (as 1-2 mm), some residues were still noticed, but no

greater than around 0.4 mm. Anyway for the Restorers the residual traces of glue and casein were at very low level not visible to the naked eye and by use of a magnifying glass.

Even if a prolonged bacterial treatment (6 hours) was possible, we excluded this duration in order to avoid any risks to the fresco; a longer contact of the moist cotton with the fresco surfaces could lead indeed to the possibility of swelling and detachment of paint fragments.

The optimized biocleaning process is here reported in detail.

1st -step biocleaning on the front of fresco paint layer for the removal of glue residues

The selected procedure involves the following cleaning steps:

1. soft cleaning step to remove dirt and dust from the surface using Japanese paper and water (40°C). The Japanese - silk paper, 9 g per m^2, natural white un-buffered (Klug Conservation, Walter GmbH & Co. KG., Immenstatd D), was applied to protect the front of painting layer;
2. biocleaning step using *P. stutzeri* A29 viable cells previously adapted in animal glue. Bacterial cell suspension F (about 2.0 liters per m^2 of fresco) was distributed to the artwork surface covered by paper and the hydrophilic cotton layer (0.5 cm high, 40 cm wide, 6-10 m long) utilized as delivery system, was abundantly embedded with the viable cells and then applied to the selected area for 3 hours. Sterile water and cotton was the negative control (Ranalli *et al.* 2005). Great care was taken to avoid fractures, creases on the thin silk paper;
3. final soft cleaning step on the front of the fresco surface by distilled water (3 soft manual sponge applications);
4. final chemical cleaning step using an anion exchange resin (Ionex OH, CTS srl, Altavilla Vicentina) to remove the organic residues.

2nd -step biocleaning on the back of the fresco for the removal of casein residues

1. mechanical removal from the asbestos support. Japanese - silk paper, 9 g per m^2, natural white un-buffered (Klug Conservation, Walter GmbH & Co. KG., Immenstatd D) was applied in this case to protect the back of painting;
2. biocleaning of the casein residues using bacterial cell suspension B (see above) previously adapted for casein degradation. The cotton layer as delivery system was abundantly brushed with the living cells and then applied to the selected area for 3 hours;
3. final soft cleaning step on the back of the fresco surface by distilled water (3 soft manual sponge applications).

Chemical analyses and SEM observation

The analyses by GC-MS and PY/GC-MS techniques evidenced the presence of proteinaceous material, mainly animal glue, used in the past to glue the gauze onto the front of the fresco ("*strappo*" technique) and casein from the calcium caseinate used on the back of the painting. In a few samples in which the paint layer was most abundant respect to the overlaying patina, egg was also present, suggesting his use as organic binder of a few details of the fresco or in the retouching. **Figure 3a** shows the pyrogram of one of the samples, with the specific markers identified for each protein. Benzeneacetonitrile and indole for casein, pyrrole for animal glue, and hexadecanenitrile, octadecanenitrile and cholesterol for egg (other peaks in the pyrogram are not labeled for simplicity, but they are typical rearrangement products of the pyrolysis of these proteinaceous materials). Finally, the GC-MS amino acidic profiles confirmed that all the proteins had undergone degradation. After 3 hours of biotreatment with bacterial cells suspension F and three gently manual washing steps, the proteinaceous materials were bio-dissolved and not more detectable since below the instrumental limit and

sensitivity of the method adopted (see the absence of the main markers of proteinaceous material (**Figure 3b**).

In a preliminary study, the biocleaning removal efficiency was calculated in weight respect to the control. On the basis of the specific enzymatic activities, the removal efficiency of *P stutzeri* A29 cells (2 liters per m^2 fresco, time 3 hours, on casein and collagen substrates) was 2.31 and 3.83 mg μl^{-1} h^{-1} for caseinolytic and collagenolytic, respectively (Antonioli *et al.* 2005). In this work and in our real operative conditions, it means that the maximum amount removal of proteinaceous materials was 13.8 g and 21.6 g per m^2, respectively for caseinolytic and collagenolytic activity.

The effectiveness was confirmed by the data obtained with the artificially specimen.

As already indicated in Materials and Methods section, at the end of the restoration, the front fresco surface was treated with anionic resin to remove traces of organic residues. Some representative micro-samples from the fresco were observed by SEM, at higher magnifications, and confirmed the absence of microorganisms after the treatment with the anionic resin and the large presence of spherical particles. In fact, as we observed from the low-magnification picture **(Figure 4)**, spherical particles were detected with the diameter-around 10 μm; the same morphology has been previously reported in literature in SEM analyses of anionic resins similar to those used at the end of the restoration work (Gupta *et al.* 2006; Albuszis *et al.* 2016).

Monitoring of the bio-cleaning process

The monitoring is one of the most crucial aspect, both during and at the end of the treatment. To determine both the presence and the viability of bacterial cells plate count technique and ATP content determination by bioluminescence have been used. At the end of the biocleaning of the fresco surface, and after the final washing with sterile distilled water, these analyses

were performed on the fresco surface to check the presence of residual bacterial cells. Once both front and back of the fresco surfaces were in fact completely dry, the presence of viable bacteria was checked immediately at time 0, 30 and 180 days following the biocleaning treatment in order to verify that no live bacteria remained on the surface of the artwork **(Table 1).**

The results of the plate count monitoring confirmed the absence of microbial cells detectable. These data were also confirmed by the total ATP content values, which were 0.015 - 0.012 pg per 25 cm^2 immediately after biocleaning, both adopting bacterial suspension F or B (respectively for removal of animal glue or casein), 0.015-0.016, and 0.017-0.018 pg per 25 cm^2 at time 30 and 180 days. While *P. stutzeri* A29 bacterial cells used for the biocleaning treatment of altered animal glue (front) or casein residues (back fresco) showed an ATP average value over 21,000 pg ml^{-1} related to a viable cell value of $2*10^8$ CFU ml^{-1}.
The average of colony forming units (CFU) and total ATP content as expression of microbial activity immediately after biocleaning (T0), at T30 and T180 days did not show significant differences ($P \leq 0.05$) both for the front and the back of the biocleaned artwork.

Microclimatic monitoring and new type of support

The microclimatic and environmental characterization study of Pisa's Camposanto highlighted in 2011 the need to provide a protection system against the formation of condensation of atmospheric humidity and the deposition of fine particles on the surface of the frescoes already restored and waiting for installation on the original walls. The solution adopted is based on the back heating of the frescoes in order to maintain the pictorial surface at temperature values slightly above the dew point temperature or the temperature at which of the water vapor must condense into liquid water.

The new support, known as Compolam or Hexelite, is a panel of aluminum-centered honeycomb used extensively in aircraft industry because of their extreme rigidity, strength and lightweight. The sandwich panel has outer surfaces in fiberglass reinforced epoxy resin (**Figure S1**). Compolam is a complex material, which requires particular processes and therefore its production is limited in quantities and customized for unique use. The cost indicatively ranges between 80 and 120 €/m^2. In addition, to complete the final relocation on the original wall, all parts of the fresco were fixed on a special aluminum structure with an additional cost ranging between 100-150 €/m^2 (De Simone, Firenze, IT). The protection of the frescoes occurs through heating of the painted surfaces that is achieved using heating fabric of polyester and carbon fibers adopted for military and civilian heated clothing. The low power supply voltage, the absence of metallic conductors and the reduced energy consumption, guarantee the long time reliability of this application. The heating sheets are based on a traditional manufacturing way, that is a longitudinal warp and a transversal weft, mainly in polyester yarns. At the ends of the fabric, or in the warp (lengthwise), tinned copper filaments are inserted to form two bands, about one cm wide each, which serve to feed the fabric. Transversely, or in the weave (in the sense of width), carbon filaments are inserted into continuous fibers that intersect the two copper bands at the ends of the warp. There are therefore many parallel strands, spaced apart by a few centimeters. The carbon fiber behaves like a current conductor characterized by a specific resistance such as to generate heat (due to Joule effect) when it is crossed by an electric current. Therefore, the carbon fibers inserted into the fabric behave as parallel resistors that take power from the two copper bands. The result was a fabric that, once powered by an appropriate electrical source, generated homogeneous heat over the entire surface, according to the desired temperature, the size of the fabric and the supply voltage. The fabric thus produced was then coupled on both sides with a thin polyurethane film which guaranteed an electrical insulation, an adequate

protection of the fabric and a good mechanical stability. One of the two sides was then coupled with an expanded material and finally with a reflecting film to ensure good thermal insulation on the back side (passive) thus maximizing the heat transfer on the active face of the sheet which is placed in contact with the back fresco surface. In addition, to the conductive component, a high radiating component is obtained which also heats by infrared irradiation. So, the final support produced looks like a sandwich of several layers, of a uniform overall thickness of about 5 mm, robust but light, cut to size with a common scissor, able to develop specific powers up to a few hundred Watts / m^2 and temperatures up to one hundred Celsius degrees. In our specific case at Pisa, after relocation of the frescoes mounted on the new heated panels (**Figure 5**), the protocol adopted guaranteed to obtain a surface temperature of the frescoes at least 2°C higher than the air temperature, so as not to give the water vapor of the air a cold surface on which to condense. Through a constantly microclimatic monitoring, a new-casting model automatically provides the signals required to activate the heating of the frescoes and prevents the beginning of vapor condensation and the dust deposition on the painted surfaces.

The charts **(Figure 6** and **Figure 7)** show the frescoes surface temperature Ts and the dew point temperature Td. The vapor condensation conditions occur when the dots/yellow curve (Td) exceeds the solid/green curve (Ts). The unheated panel shows the condensation conditions during the same period. The variation in surface temperature between heating power-on and off is about 2.5°C.

Anti-condensation system

The water vapor condensation events are frequent in winter period (about ten) from November to February, when the moist air masses (above 90% RH) comes from the

Tyrrhenian Sea, SW direction, and can last for a long time up to one week. During the microclimatic monitoring period (2007-2017) these phenomena mainly affected the frescoes of the south gallery, particularly the southeast corner, and northwest corner of the north gallery (Mandrioli et al., 2013) as described previously (P. Mandrioli and P. De Nuntiis, presented at 23th Fair of the Conservation, Technology of Cultural and Environmental Heritage, Ferrara, IT, 22-24 March 2017).

DISCUSSION

The "hi-tech" complex biocleaning of the 14th-century fresco *Triumph of Death* reported in this paper is a new case of biorestoration in which the use of bacteria has been successfully applied to Cultural Heritage. The novelty in this study is the 2-steps biocleaning process using two different bacterial suspensions (F and B) of *Pseudomonas stutzeri* A29 viable cells; this biocleaning allowed the removal in 3 hours of both the animal glue and the residues of calcium caseinate, respectively from the front and the back of the fresco.

These results confirm the efficiency and degradation ability of the selected bacterial strain previously applied for recovering other valuable frescoes from the same site, as the Last Judgment and Hell (Colombini and Bonaduce 2003; Ranalli *et al.* 2005) and the Stories of the Holy Fathers (Lustrato *et al.* 2012) by Bonamico Buffalmacco. The monitoring of the bacterial cell counts and cell viability during the bio-cleaning process and after the washing steps at time 0, 30 and 180 days shows that residual bacteria on both the surfaces (front and back) are not detectable after the biotreatment. Therefore, all the biocleaned frescoes monitored (five sections of *Triumph of Death* fresco) did not show any risk of biological re-contamination in the conservation period of about 8 months under controlled indoor conditions before their relocation onto the original wall at the Camposanto Monumental Cemetery (OpaPisa).

These data confirm that the adopted procedure does not leave residual cells on the fresco surfaces as we already showed in previous works and therefore it is a fast, safe, selective and free-risk methodology that can be utilised in Cultural Heritage restoration field for the removal of different stone alterations (Ranalli *et al.* 2003a; Lustrato *et al.* 2012). In fact, bio-restoration through the use of not pathogenic selected bacteria shows no risk for human health and the environment, and represents a great potentiality for removing undesirable compounds from the surface of works of art also in complicated cases as the *Triumph of Death* fresco where two steps treatment (front and back) are required. Therefore, in all these years, the "bio-restoration" technology has been continuously employed on about 1,500 m^2 of the medieval frescoes, where the critical conditions of several of them imposed further studies and changes/optimization in the working protocols.

Therefore, the *Triumph of Death* fresco biorestoration represents a novel and important case of a large scale application of viable bacterial cells and shows that this biotechnology can be extremely useful even when situations are more complex and related to the both side of the fresco (front and back).

With regards to the fresco support, the conservation scientists noticed undesired aspects following the utilization of the old glass-resin support. After the re-location to the original wall, a visible deformation of the glass resin has been observed with a high risk for the fresco surface caused by mechanical forces and absence of uniformity. In addition, phenomena of condensation on frescoes surfaces were frequently reported due to the fact that the walls of the galleries are facing the open-air interior courtyard. A further novelty in this work is represented by the special effort focused on the optimization of a new support with high technological properties and the planning of a continuous monitoring of the environmental temperature and humidity for the prevention and conservation of the recovered frescoes. The new support, known as Compolam or Hexelite, used for the first time, together with the

design of an environmental monitoring system represent an innovative anti-condensation system for the solution for these problems and guarantee indeed a better preservation and conservation of these valuable frescoes.

In conclusion, the findings obtained in the Pisa Camposanto Monumental Cemetery have a significance and relevant impact in fresco restoration field and represent a real example of "active" preventive conservation where the biorestored and relocated frescoes, after 74 years since their detachment from the walls, are fixed on a new and innovative support and protected by condensation phenomena and deterioration processes.

This study is of a great importance since the Camposanto Monumental Cemetery will be fully re-opened in June the 17th 2018 for its 750th construction anniversary.

The remarkable results achieved in the large scale investigations on medieval frescos, using the proposed advanced biocleaning techniques, clearly show the success of this methodology for an extraordinary “resurrection” of degraded works of art, also in very complicated and delicate conditions such as the *Triumph of Death* fresco, defined for its dimension and artistic importance the ‘Pisa’s Sistina frescoes’. The biotechnology proposed is highly efficient, safe, non-invasive, risk-free and very competitive compared to the traditional cleaning methods. These findings can be of significant importance for further future new restoration activities and they are crucial for determining preservation strategies in this field.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

GR, EZ 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, AA, MPC, PDN, PM, LR contributed to design the experiment, analysis of the data, and contributed in manuscript preparation and its critical revision, PB, GL contributed in the execution of part of the experimental work and in the acquisition of the data, CC contributed to the chemical analysis of samples, CG, DZ coordinated and supervised all the conservation activities. All authors reviewed the results and approved the final version of the manuscript.

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TABLES

Table 1 - Total viable bacterial counts (CFU) per 25 cm^2 and activity measured by ATP content (pg) per 25 cm^2 present on front and back of the of the *Triumph of Death* fresco surface, at time 0, 30 and 180 days after the 2-steps biocleaning. Standard Deviation (SD), n=15.

SUPPORTING INFORMATION

Figure S1 - The new fresco's support composed by restored fresco mounted on canvas, aluminum-centered honeycomb, heating fabric of polyester and carbon fibers.

Table S1 - Phases of the restoring intervention of the *Triumph of Death* fresco which included the two-steps biocleaning of altered animal glue and casein removal on front (F) and back (B), respectively, carried out by the application of *P. stutzeri* A29 cells.

Table 1 - Total viable bacterial counts (CFU) per 25 cm^2 and activity measured by ATP content (pg) per 25 cm^2 present on front and back of the of the *Triumph of Death* fresco surface, at time 0, 30 and 180 days after the 2-steps biocleaning. Standard Deviation (SD), n=15.

	Time (days)	Organic residues removal by 2-steps frescoes biocleaning	
		Front: animal glue (bacterial cell F)	Back: caseine (bacterial cell B)
Bacterial cell suspension adopted in biocleaning frescoes (CFU/ml^{-1})	0	$2*10^8$	$2*10^8$
ATP content (pg/ml^{-1})	0	21.000 (400)	21.000 (300)
Total viable microbial counts (CFU/25 cm^2) after biocleaning	0	3 (2.2)[a]	3 (4.0)[a]
	30	2.5 (3.0)[a]	2 (2.7)[a]
	180	3 (3.1)[a]	3.5 (2.6)[a]
ATP content (pg/25 cm^2)	0	0.015 (0.002)[b]	0.012 (0.002)[b]
	30	0.015 (0.004)[b]	0.016 (0.003)[b]
	180	0.017 (0.003)[b]	0.018 (0.004)[b]

Comparison of data were carried out by the Turkey test: mean values and standard deviation (SD) with the same superscript letter were not significantly different ($P \leq 0.05$).