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A MICROBIOLOGICAL SURVEY OF THE ETRUSCAN MERCARECCIA TOMB (ITALY): CONTRIBUTION OF MICROORGANISMS TO DETERIORATION AND RESTORATION

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ABSTRACT

The Necropolis of Tarquinia (Italy), included by Unesco in the world heritage since 2004, is notable for the large number of rock-cut tombs which retain on their walls paintings of daily life and of funeral rites and banquets. Solutions for the safeguard of these sites, that represent to some extent an unicum, are linked to the knowledge on colonising microorganisms supported by the peculiar ecological conditions of hypogeum environments. Aim of this work is a microbiological survey of the Etruscan Mercareccia Tomb and the selection of strains suitable for biotechnological application in the field of restoration. The Tarquinia tombs are mostly simple rectangular chambers, while the Mercareccia Tomb has an outer chamber (dromos) with a timber roof sloping to a central opening which reproduces the atrium of a house of the time as described by Vitruvius, so far in a rather poor state of preservation and now under restoration. 142 different heterotrophic colony morphotypes have been isolated, 16% of which result not yet described in relation to artistic heritage. The most represented bacteria belong to Actinomycetales and Bacillales; fungi belong to the taxa Sordariomycetes, Eurotiomycetes, Ascomycota and Dothideomycetes. The presence of unculturable microorganisms has been highlighted by means of t-RFLP analysis. The investigation has subsequently been focused on the selection of two opposite metabolic functions: the biomineralisation (71 strains) and the solubilisation of calcareous patina (only 5 strains). The most efficient strains in biomineralisation of calcium carbonate have been tested in vivo for consolidation experiments of "Pietra di Lecce", a calcareous stone. The calcite deposition has been monitored by capillary water absorption, colorimetry, biofilm formation (SEM microscopy). The best results have been obtained with the strain TSND13 Rhodoccoccus erythropolis, applied for the first time as agent for biorestoration. TSND13 reduced the water absorption by 20% compared to control, without altering perceptively the original colour of the stone. In order to overcome the problems arising from the use of living cells also the capacity to induce the formation of calcite by cellular fraction (BCF) has been tested in consolidation trials as an alternative technology.

INTRODUCTION

The notable historical-artistic Italian heritage encompasses several hypogee sites, like rock-cut churches, tombs and catacombs, that represent to some extent an *unicum*, housing on their walls thousands of precious paintings. Hypogees represent peculiar ecological niches where the environmental conditions become determinant factors supporting a microbial colonisation often responsible for a biodeterioration. Solutions for the safeguard of these sites are linked to many factors. The relevance of biotic factors is becoming better understood as the knowledge on ecology, structure, diversity and functioning of the colonising microorganisms increases and improves, since the biodeterioration is the result of the action of a whole microbial community. Microbial characterisation allows to determine which microorganisms are naturally able to colonise that monument in the course of time, providing a reasonable base for the development of monitoring protocols for the prevention of the biodeterioration and for the safeguard of the monument.

Additionally this knowledge enables to understand how to positively exploit these microorganisms, as a source for new biotechnological applications in the field of conservation and restoration.

Object of this study is an Etruscan tomb, the Mercareccia Tomb. The extensive Necropolis at Tarquinia (Italy), included in the World Heritage by Unesco since 2004, is notable for the large number of rock-cut tombs which retain on their walls paintings of daily life and of funeral rites and banquets. These tombs are mostly simple rectangular chambers, while the Mercareccia Tomb (one later tomb possibly third century BC), has an outer chamber (*dromos*) with a timber roof sloping to a central opening which reproduces the atrium of a house of the time, as described by Vitruvius, the roman writer and architect, active in the 1st century BC. Around the walls were friezes of carved reliefs and paintings, as documented by the drawings of Byres in 18th century The tomb, excavated in the "macco", a calcareous rock, was discovered in 1735 and during the wartime has been improperly used, contributing tothe current relatively poor state of preservation: the walls are covered by crusts and patinas. Presently a restoration project is under way, but unfortunately the paintings have completely disappeared.

In this work, besides an explorative characterisation of the etherotrophic microflora coloniser of the tomb's walls, is also described the selection of microbial strains potentially useful in the field of restoration, focusing the research on two opposite metabolic functions: the biomineralisation and the solubilisation of calcareous patina. The most efficient strains in biomineralisation of calcium carbonate have been then tested in laboratory trials for study of consolidation of "Pietra di Lecce", a calcareous stone.

MATERIALS AND METHODS

According to the standard method NORMAL 3/80 (1980), the sampling was made using sterile swabs on 10 x 15 cm areas. Ten areas of interest were selected: three in the Dromos (DFVB, DPBS, DAN) and seven in the inner chamber (TSC, TSND,TSG, TSNRS, TPSB, TPBF, TPID). The same abbreviation has been used to name the strains isolated from the correspondent area.

Total heterotrophic aerobic microorganisms were grown on agar plates (TSA for bacteria and PDA for fungi) and their identification was carried out by 16S and 18S rDNA full gene sequencing. Genomic DNAs were extracted from single colonies and amplified by PCR using P0 (GAG AGT TTG ATC CTG GCT CAG) and P6 (CTA CGG CTA CCT TGT TAC GA) primers for bacteria, and EF4f (GGA AGG GAT GTA TTT ATT AG) and EF3r (TCC TCT AAA TGA CCA GTT TG) primers for fungi. The sequences obtained were compared to data-base sequences using the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/) and deposited in the GenBank® genetic sequence database (Nucleic Acids Research 2007 Jan;35; Database issue D21-5). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar, Tamura, Nei 2004).

Calcium carbonate patina degradation test was performed according to the NORMAL 9/88 standard method, by spreading on agar plates a suspension of CaCO3 1%, pH 8.4.

The selection of bacteria capable to produce crystals of calcium carbonate (carbonatogenesis) was carried out on B4 medium (4 g/l calcium acetate, 4 g/l yeast extract, 0,5 % glucose, pH 8,00; Bouquet e al., 1979). Bacteria were grown at 28°C for 40 days (Urzì e al.; 1999). The crystals deposited on the colonies were analyzed for their carbonate content by HCl test (Bouquet e al., 1979) and by X-ray diffractometer HUBER – XCS after purification according to Ryvadeneira e al., 1994. The diffractometer data were analyzed in the databank PDF2 (ICDD: International Center for Diffraction Data), by using the software WinSearch 32 (Ital Structures).

Biomineralisation trials were performed on "Pietra di Lecce" stones (5 x 5 x 1 cm) according to Tiano et al. (1999). Each trial was performed in triplicate for each bacterial strain, and compared with a blank reference (B4 medium without cells). In order to follow the bacterial behaviour and to check that no contamination occurred, control stones were coated with a thin agar layer and inoculated with bacterial cells. Colonies growth was optically controlled by a stereomicroscope. The following parameters were evaluated: capillary water absorption before and after the trial (Tiano e al; 1999); colorimetric variations by colorimeter Minolta CM – 525i (Normal 43/93); biofilms formation by SEM analysis of stone surfaces and sections.

RESULTS AND DISCUSSION

Descriptive analysis of the colonising microorganisms. From 10 spots of the walls of the two chambers of the tomb, 142 heterotrophic strains have been isolated, whereof 127 have been identified by r-DNA 16S or 18S full-gene sequencing. The phylogenetic tree is shown in figure 1a,b. Among the bacteria Actinomycetales are predominant, specifically the genera *Streptomyces*, *Microbacterium* and *Rhodococcus*. Bacillales, among which *Bacillus* e *Paenibacillus,* are also abundant. A different distribution of the genera in the *dromos* and in the inner chamber is observed: Bacillales are predominant in the *dromos*, while Actinomycetales, well–known to be the first colonisers of hypogee environments, are predominant in the inner chamber. The fungi are more homogeneously distributed and belong to the taxa: Sordariomycetes, Eurotiomycetes, Ascomycota mitosporici and Dothideomycetes. On the whole one observes a higher biodiversity in the inner chamber, which is more protected from the exterior. Most of the isolated microorganisms have already been described in the literature as colonisers of similar environments of historical-artistic interest, while 16% of the isolated have not yet been described in association with such environments. Among the latter group, bacteria are represented by the genera *Isoptericola*, *Cellulosimicrobium*, *Stenotrophomonas*, *Ochrobactrum*, *Lysobacter* and the fungi by the genera *Lecanicillium*, *Tritirachium*, *Torrubiella*, *Microascus*, *Preussia*, *Paecilomyces.*

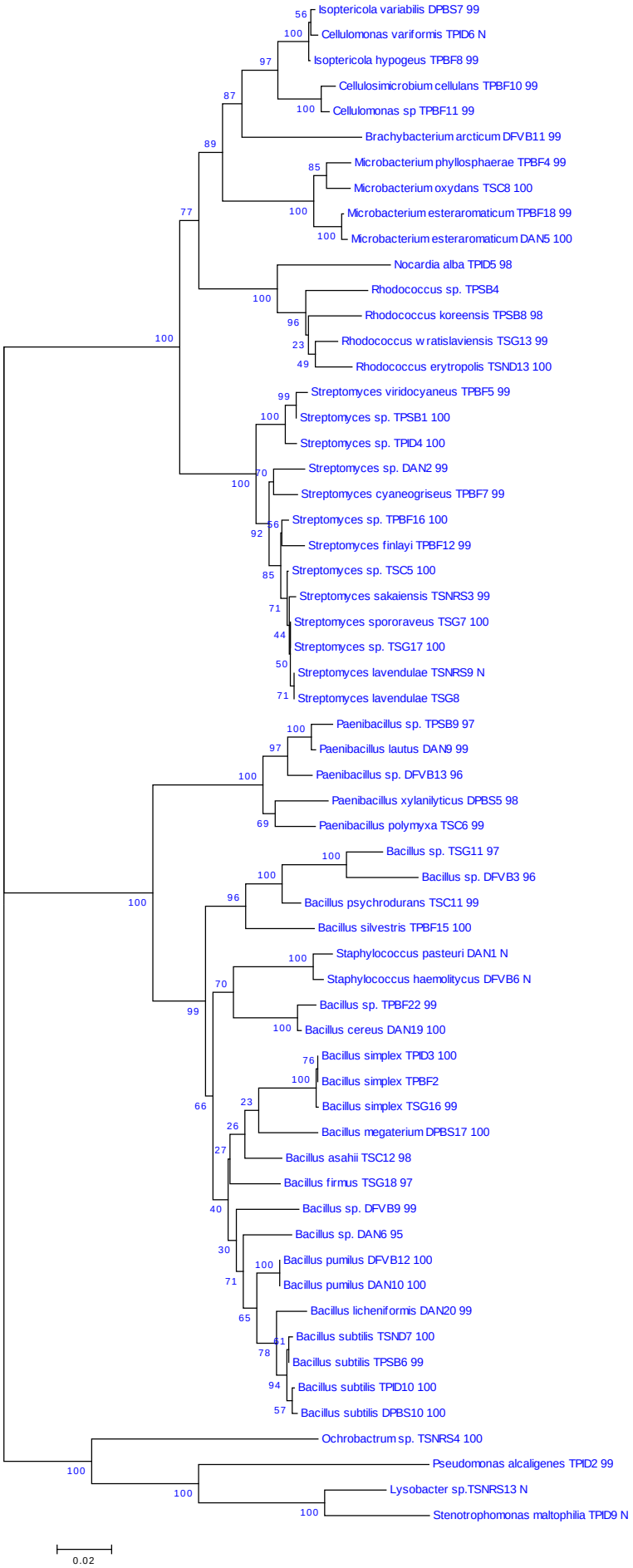


Figure 1a. Phylogenetic tree of bacteria isolated in the Mercareccia Tomb

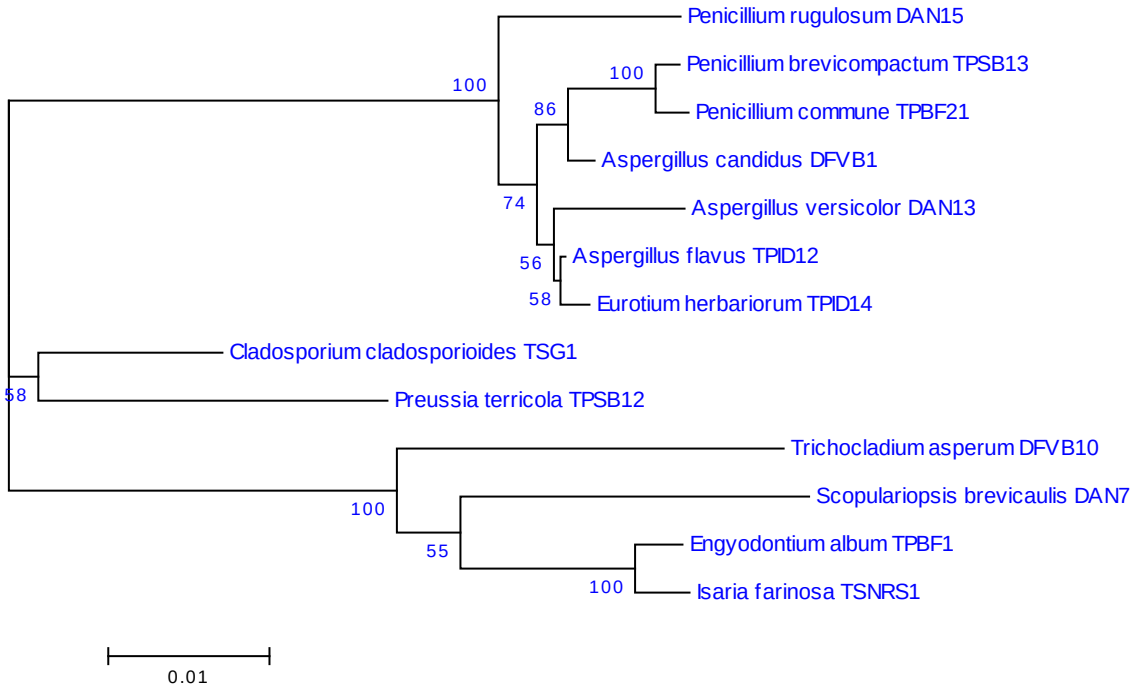
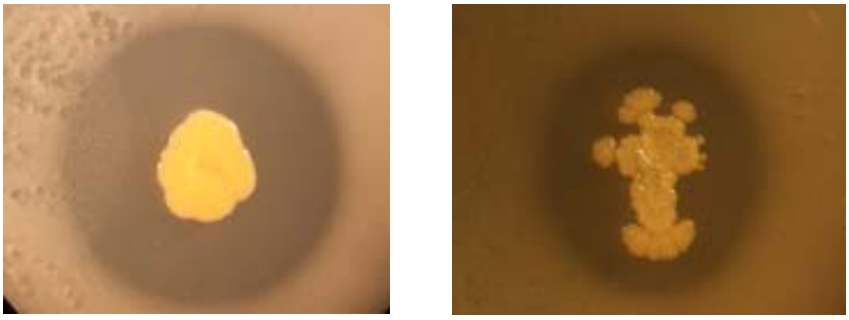


Figure 1b. Phylogenetic tree of fungi isolated in the Mercareccia Tomb

Selection of bacterial strains as potential agents for biorestoration. The ability to solubilise carbonates is a metabolic function poorly represented among the isolated strains, only five showed this ability: DFVB6 (*Staphylococcus haemoliticus*), TPBF 10 e TPID 1 (*Cellulosimicrobium cellulans)* TPBF 11 *(Cellulomonas cellulans)* and DPBS 7 (Actinomycetaceae). The strain DFVB 6, *Staphylococcus haemolyticus*, is the most active in solubilising, but the strain TPBF 11 *Cellulomonas cellulans*, though slower, shows a more persistent activity (fig. 2 a,b). This ability to solubilise $CaCO_3$ is interesting in relation to this environment, since the tomb is excavated straight in the "macco", a calcareous rock. In time, this microbial community may have contributed to the degradation of this site. These strains can be a part of strains as potential degrader of unwanted patinas and crusts.



(a) ***(b)***

Fig.2 $CaCO_3$ solubilisation rings by strains DFVB 6 Staphylococcus haemolyticus (a) and TPBF11 Cellulomonas cellulans (b), after 7 days of growth on agar plates spread with a suspension of $CaCO_3$ 1%

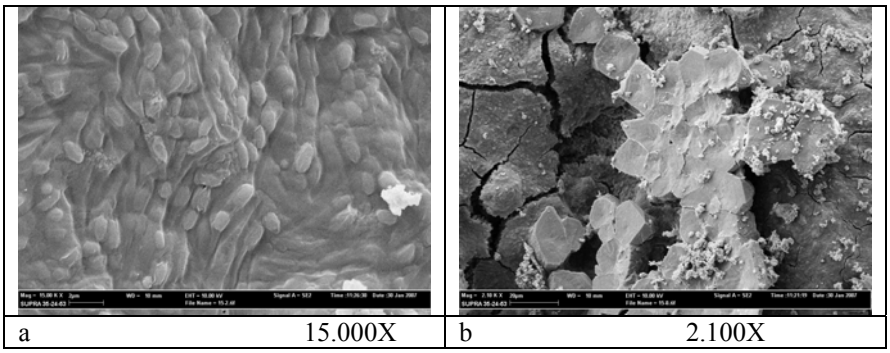
The biomineralisation is rather diffused among this community, as it is in nature, indeed 71 bacterial strains able to precipitate carbonates have been detected (corresponding to 65%). The most represented genera are *Bacillus* (24 strains) and *Streptomyces* (18 strains), moreover *Rhodoccoccus* (7 strains), *Paenibacillus* (2 strains) and *Pseudomonas* (1 strain), well-known in literature to be able of calcinogenesis. Other bacterial strains, belonging to the genera *Microbacterium* and *Stenotrophomonas,* (3 strains), *Ochrobactrum* (2 strains) and *Lysobacter* (1 strains), genera never included as biomineralisers, have been highlighted for their ability to

precipitate calcite. These 71 strains allow to have a bank available of varied crystals (different coloring and sizes) useful for applications on lapideous polychrome materials.

On the base of the number of crystals precipitated on the colonies, the time of formation, the colour and the response to the HCl test, five strains have been selected to carry out bioreinforcement trials on the calcareous stone "Pietra di Lecce". The strains selected were TPBF 2 (*Bacillus simplex*), TSG 16 (*Bacillus simplex*), TSND 13 (*Rhodoccoccus erytropolis*), TPBS 4 (*Rhodoccoccus sp*.) and TSC 8 (*Microbacterium sp*.).

The characterisation of crystals, performed by RX Difractometry, revealed the nature of calcite for the crystals produced by the strains TSG 16, TPBF 2, TSND 13 and TSC 8 and of vaterite mixed to calcite, by the strain TPBS 4. Vaterite is a metastable form of $CaCO_3$, rather rare in nature, since it tends towards a more stable form of carbonates, calcite or aragonite, but it is very diffused as constituent of biogenic crystals produced by bacteria (Sanchez-Moral et al., 2003).

SEM analysis showed that all the 5 strains produce calcified mucous biofilms (figures 3a,3b,4a,4b) where polysaccharides (EPS) encompass the bacterial cells and promote the bacterial adhesion to the surface. The production of EPS is supposed to favour the precipitation of $CaCO_3$, through the formation, in response to the metabolic activity, of alkaline gradients attracting calcium ions (Riding, 2000). EDS analysis of biofilms show for all the samples the presence of Calcium, Carbon and Oxygen, attesting the carbonatic nature of the biological patina formed. Differences on crystal forms and deposition is evidenced in figure 3 and 4.



a 15.000X b 2.100X

Figure 3. Biofilm formed by the strain TPBF 2 Bacillus simplex.
Bacterial cells, partially included in EPS matrix (a) and tubular calcite crystals (b).(SEM)

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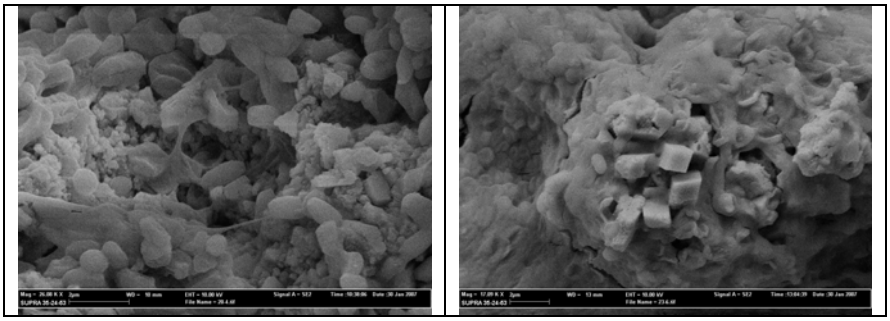

Figure 4. Biofilm formed by the strain TSND 13 Rhodoccoccus erytropolis.
Bacterial cells (a) and like-rhombus calcite crystal absorbed in the biofilm (b). (SEM)

As a consequence of the treatment with calcinogenic bacteria in B4 medium, the capillary water absorption of the stone decreases by values ranging from 45 to 73% (table 1), included the control treated only with the medium B4. The treatments causing a significant difference,

compared to the control, are those with the strains TSND 13 (*Rhodoccoccus erytropolis*) that reduces the water absorption by 67,43% *(18% vs control)* and TPBF 2 (*Bacillus simplex*), reducing the absorption by 73,12 % *(23% vs control)*.

Concerning the chromatic effect induced by the treatments , the colorimetric measures show that stones undergo a yellowish alteration and are subject to a decrease of reflectivity values, most likely due to the combination of medium composition, as attested by the ΔE* value of the control, and microbial metabolism products. As one observes in table 1 the colorimetric alterations vary with the strains. The strain TSC 8 *Microbacterium sp*. alters the colour more, because it produces pigmented colonies, while the strain TPBS 4 (*Rhodoccoccus sp.)* attenuates the alteration also with respect to the control. The colour variation is just visible in the case of treatments with the strains TSND 13 (*Rhodoccoccus erytropolis)* and the control. Considering the global effects of the treatments the more suitable strain seems to be TSND13 *Rhodoccoccus erythropolis*, reducing the capillary water absorption by 18% with respect to the control, without altering the pristine colour of the "Pietra di Lecce". The quantitative results are comparable with those obtained by other authors with strains of *Bacillus simplex* (Tiano et al.,1999, Tiano Project Bioreinforce). The peculiarity of this work consists in the strains used, that all are native to a calcareous rock-cut tomb and that they have been tested for the first time in such an experimental work, with the exception of *Bacillus simplex*.

MICROBIAL STRAIN	Δ H_2O ABSORPTION (%)	ΔE *	BCF
TPBF 2 *Bacillus simplex*	73,12 ± 4,62	6,43 ± 0,23	++++
TSG 16 *Bacillus simplex*	45,12 ± 4,79	7,39 ± 0,71	+++
TPBS 4 *Rhodococcus sp.*	53,60 ± 5,29	3,79 ± 0,59	++++
TSND 13 *Rhodoccoccus erytropolis*	67,43 ± 3,25	5,89 ± 0,62	++++
TSC 8 *Microbacterium sp.*	52,57 ± 1,95	18,19 ± 7,83	+++
Control	50,17 ± 6,63	5,55 ± 1,28	+

*Table1. Capillary water absorption reduction, colorimetric coordinates variation (ΔE *) and production of crystals by the bacterial cells fraction (BCF*)

In table 1 is also tentatively reported the crystals production by the cellular fractions (BCF), obtained by applying a method proposed by some authors (Barabesi e al; 2006), in order to overcome the disadvantages of the direct application of living cells (new products from the bacterial metabolism reacting chemically with the stone minerals or secondary colonisation by other microorganisms due to the presence of organic nutrients). Preliminary results (non specifically reported) show that BCF act as a crystallisation nucleus, favouring the calcite precipitation for all the strains used. Crystals precipitation occurs in a different measure for different strains, although the inoculum is identical for all the strains, attesting that carbonates precipitation depends not only by the quantity of cellular fraction, but it is also affected by chemical-physical composition of the cell wall and membrane, containing chemical compounds that can vary from strain to strain influencing the carbonate precipitation.

CONCLUSIONS

The microbiological investigation carried out on the Mercareccia Tomb (Etruscan Necropolis of Tarquinia, Italy), although only partially, documents how the heterotrophic microbial community has developed in time within this environment, in absence of any conservation intervention and can form the basis for a future monitoring protocol, in order to establish a conservation plan, after the restoration, now under way.

The investigation lead to the isolation of microbial strains not yet reported in association with artistic heritage: *Isoptericola, Cellulosimicrobium, Stenotrophomonas, Ochrobactrum, Lysobacter* among the bacteria and *Lecanicillium, Tritirachium, Torrubiella, Microascus, Preussia, Paecilomyces* among the fungi.

Some species have the capacity to solubilise carbonates and others to produce calcite, both could have had some role in the biodeterioration processes, although it could be arbitrary to establish a correlation between their presence and the biodeterioration. Thus, we considered it more profitable to look for some positive exploitation of these strains in the field of biotechnology for the conservation and restoration.

Some of the species to which the isolated strains in this work belong have already been used for biotechnological applications, others represent a novelty in this field. For instance among the calcinogenic strains this study highlighted four genera for which the carbonatogenesis capacity was unknown: *Microbacterium Stenotrophomonas, Ochrobactrum e Lysobacter.* This work permitted, on the whole, the creation of a strains bank producing carbonate crystals of different colours and sizes, potentially useful for restoration of polychrome lapideous materials. The strain TSND 13 *Rhodoccoccus erythropolis* revealed to be a potential candidate for stone bioreinforcement applications, although this kind of application still needs to be carefully evaluated.

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