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Microbial induced stone discoloration in alcobaça monastery: A comprehensive study

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ABSTRACT

The Alcobaça Monastery (Portugal), a UNESCO World Heritage Site, currently exhibits a high degree of surface alterations of the stone architectural elements inside the church, including an extensive pink coloration in the walls and columns, bacteria biofilms, and salt efflorescences. The main goal was to identify the microbiota that colonizes the walls and columns of this monument, to help custodians and conservators-restorers in the selection of the correct procedure to be adopted for the conservation of the monument. Regarding the observed pink discoloration, and considering previous studies, we hypothesize that is caused by biofilms formed by bacteria or other microorganisms that produce pigments of the same color, particularly carotenoids. Curiously, a distinct phenomenon was noticed: the pink discoloration always seems to appear at a very similar height in most of the columns and walls, starting at 40 cm to the floor and associated with the presence of salts on the walls. Using high-throughput sequencing approaches, we were able to characterize the microbial community present. We identified several bacteria that are producers of pink pigments and halotolerant such as *Rubrobacter radiotolerans, Domibacillus robiginosus, Bacillus licheniformis* and *Halalkalicoccus* sp. that develop in areas of high salinity.

Introduction

Biodeterioration of Cultural Heritage is the result of interactions between living organisms, material support and environmental conditions [1]. The process is related to surface bioreceptivity, that is, "the totality of properties or characteristics of a material that controls its probability of being colonized by one or more groups of living microorganisms" [2–5]. Microorganisms often find a suitable habitat for their growth, whether in cultural artifacts made of different types of material such as paper, fabrics, glass, wood, metal, textiles, ceramics and plastic, in indoor environments (public museums, private art collections, churches, caves), as well as in external environments (stone monuments, architectural surfaces) [2,6,7] which has a direct effect on the conservation of cultural assets. This not only results in the irreversible and irreparable loss of the materials physical strength, aesthetic appearance, value, and information but also, occasionally, the destruction of the structural integrity of the heritage asset. The phenomenon of biodeterioration is often overlooked and goes unnoticed unless overgrowth of the biofilm and discoloration or weakening of the physical integrity of the material occurs [8].

Classified by UNESCO as a World Heritage Site in 1989, the imposing Alcobaça Monastery, located in the city of Alcobaça (Portugal), is one of the best and most impressive examples of Cistercian architecture in Europe [9]. Currently, this monument presents a high degree of surface alteration of the stone architectural elements inside the church, mainly observing the appearance of pink biofilms on the walls and columns (Fig. 1a, 1b), associated with the presence of saline efflorescence (Fig. 1c). Biofilms, described as collections of microbial cells and extracellular polymeric substances, are considered a generic survival mechanism used by microorganisms, as it gives them advantages such as resistance to

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Fig. 1. (a) distinct phenomenon observed at the altar of the monastery church; (b) pink coloured biofilms on the walls; (c) evidence of the presence of salts on the walls.

environmental stress, mediates intracellular communications, increased recovery of nutrients and water in their matrix, thus offering protection against toxic substances (biocides and antibiotics) and against desiccation [10]. Its development weakens the structure of stone materials, leading to proliferation in depth and acting as a physical barrier against biocides, making mitigation treatments difficult [11].

Pink discolorations and biofilms of this color have rarely been associated with a biological origin, as in the past their genesis was predominantly attributed to chemical processes. The biological origin of these biofilms on old building walls was first proposed exactly a century ago, although without a precise ethology. More recently, several studies have already shown that stone monuments or works of art and wall paintings represent a common habitat for halotolerant bacteria, and, consequently, pink discoloration is an intriguing phenomenon, having already been observed in several monuments around the world [12,13]. This color change, which is often associated with conditions of high humidity, high salinity, and moderate lighting, is thought to be due to the production of carotenoids as a cellular protection mechanism. These compounds can modify colors leading to aesthetic problems, but also problems in terms of substrate surface stability [14]. The function of carotenoids is primarily photoprotection but also: (i) species-specific coloring; (ii) stabilization of specific protein pigment complexes, (iii) elimination of reactive oxygen species (antioxidant function), (iv) stabilization of cell membranes, (v) precursors of hormones and vitamins in more complex organisms, and (vi) defense against unfavorable environmental conditions like UV radiation, hypersalinity and sublethal levels of ionizing low wavelength radiation [7,15–17].

Furthermore, a distinct phenomenon was noticed in Alcobaça Monastery (Fig. 1a): the pink discoloration always seems to appear at a very similar height in most of the columns and walls, starting at 40 cm to the floor and associated with the presence of salts on the walls. Generally, the appearance of saline efflorescence furthers the proliferation of halotolerant/halophilic microorganisms, which can lead to discoloration caused by pigments released or contained in the microorganisms, of which the pink color is an example. These biogenic pigments are usually very stable on the materials, even if the causative microorganisms are already dead [18].

To help custodians and conservators-restorers in the selection of the correct cleaning procedure to be adopted for the conservation of the monument, it is crucial to identify the stone colonizing microorganisms. This work aims to encompass a comprehensive multidisciplinary approach to characterize the biological colonization and consequently biodeterioration of an architectural stone material (Ançã limestone) present in Alcobaça Monastery.

This stone type has been extensively employed in significant Portuguese monuments and are known as soft, pure, and homogeneous calcium carbonate limestones. Biochemically, the stone does not have significant nutritional qualities because it exhibits a basic mineralogy made of 99 % calcite and 1 % quartz. Because of these features, it is extremely vulnerable to natural weathering and conservation/restoration procedures. The large porosity and capillary absorption properties make the Ançã limestone present the highest primary bioreceptivity to microbial colonization among the many Portuguese lithotypes analysed. Consequently, it is described as one of the types of stone that suffers the most deterioration caused by microorganisms [11,19–22].

Due to the known limitations of culture-dependent methods for the purpose of this study, a phylogenetic metagenomic approach was used in this work, through Next Generation Sequencing (NGS). This technique utilizes small subunit ribosomal DNA genes such as 16S, universally present in all prokaryotes, which provide an efficient means of identifying microorganisms. These ribosomal sequences have variable and highly conserved regions, which are used as phylogenetic markers to identify and distinguish different microorganisms at all phylogenetic levels. The NGS has evolved exponentially in recent years, allowing the characterization of microbial diversity, but also a better understanding of the functions, activities, and dynamics of microbial communities. It is a culture-independent technique, requires a smaller amount of DNA, makes it possible to extract information at the level of genes and biochemical functions, and allows to sequence several samples simultaneously, reducing analysis time [2,6,16–18].

In order to understand the deterioration processes that are affecting the stone, the combination of scanning electron microscopy with energy-dispersive X-ray spectroscopy analysis (SEM-EDS) and NGS was envisaged to study samples from specific zones of the Alcobaça Monastery church, showing different alteration processes to (a) confirm bacterial contamination of the samples; (b) characterize the microorganisms that are colonizing the stone surfaces and (c) evaluate the distribution patterns of the microbiota in the different sampling zones.

Research aim

Biodeterioration can be caused by biochemical processes, for example, through biocorrosion, due to microbial excretion of organic acids, however, the aesthetic effects are those that appear more frequently in countless stone monuments. At this time, the Monastery of Alcobaça currently presents a high degree of surface alteration of the stone architectural elements inside the church, mainly observing the appearance of pink stains on the walls and columns, associated with the presence of saline efflorescences.

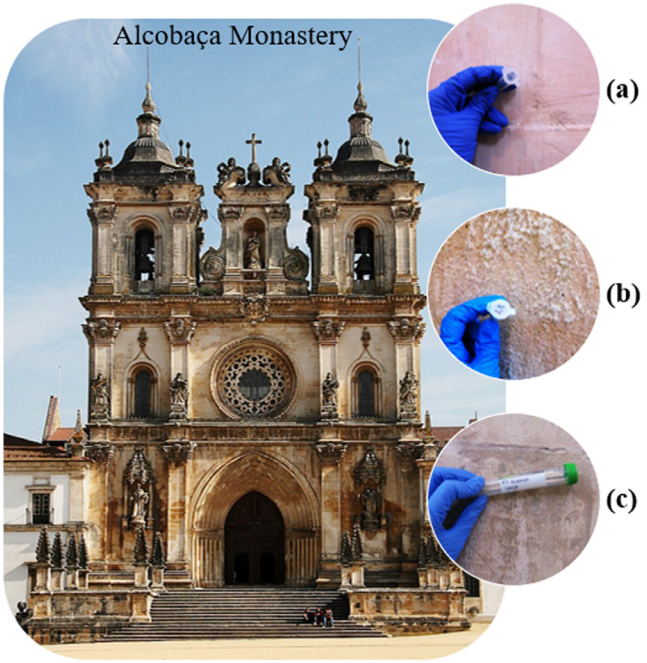

Fig. 2. Sampling process details in Alcobaça Monastery.

The main goal of this work was to identify the microbiota that colonizes the walls and columns of this monument, using culture-independent techniques, to help custodians and conservators-restorers in the selection of the correct cleaning procedure to be adopted for the conservation of the monument.

Materials and methods

Sampling process

The Alcobaça Monastery is a Catholic monastic complex located in the town of Alcobaça in central Portugal, some 120 km north of Lisbon (39°32′54″N 8°58′48″W). Due to its artistic, cultural, and historical relevance, it was included in UNESCO's World Heritage Site, and it is the one of the most impressive and beautiful testimonies of the Cistercian architecture throughout Europe.

The sampling process was carried out in the monastery's walls and columns, built with Ançã limestone. This is a pure white limestone with a high $CaCO_3$ content (>96.5 %) having poor compressive strength and hardness, significant porosity (18.7 - 22.4 %), real density between 2.72 and 2.73 g/cm3 and thermal conductivity between 1.64 and 1.78 W/m·K. [22,23].

Sample collection was performed in three representative areas of the monastery church with significant signs of contamination and alteration, shown in Fig. 2: (a) corresponds to columns and walls covered by pink biofilms (samples A4-A7); zone (b) concerns the walls with the presence of saline efflorescence and simultaneously pink biofilms (samples A8-A9) and in zone (c) the samples were collected from columns with pink coloration near the altar of the church (samples B7, B8). Sampling was done under the coordination of conservators-restorers, complying with the requirements of conservation and minimization of the structural and aesthetic impact of the work of art, collecting the minimum amount necessary for the assays. Non-invasive methods and semi-invasive methods were used for sample collection, under semi-aseptic conditions (collection performed with sterile material but in outdoor environment). The collection of stone microfragments was done close to losses or cracks in order to avoid further damage [1,24].

Characterization of salt efflorescences

X-ray diffraction (XRD) was used to characterize salt efflorescences. Collected samples were placed in a silicon low background sample holder and data were collected in Bragg–Brentano ($\theta-2\theta$) geometry by means of a BRUKER AXS D8 Discover micro-diffractometer in micro-mode. Operating conditions were: CuKα radiation, 40 kV accelerating voltage and 40 mA current; 3–75° 2θ angular range, step scan of 0.05° 2θ/2 s. Mineralogical phases were identified using the EVA Bruker-AXS software and the PDF-2 ICDD database.

Biocolonization assessment

The stone microfragments collected were analyzed by SEM and the samples were air-dried, coated with Au/Pd during 30 s. The micrographics were obtained using a Field Emission Scanning Electron Microscopy (TESCAN Clara, Czech), operating under high vacuum at 10 kV accelerating voltage, 30 pA current, and 10–11 mm working distance. The detector used was the Everhart–Thornley secondary electron detector.

The EDS experiments were performed with an X-ray spectrometer Bruker XFlash 6130 SDD detector with 126 eV spectral resolution at the FWHM/Mn Kα coupled to the previous instrument. The map and point analyses were acquired at 15 kV accelerating voltage, 300 pA current, and 10 mm working distance. The data were collected and processed using the software Esprit 2.5.

Characterization of microbiota through culture independent methods

To use the NGS approach, metagenomic DNA was extracted from original microsamples using the Soil DNA Purification Kit (OMNI, Inc.), according to the manufacturer's instructions. The DNA was quantified by a fluorometric technique with the Quantus™ Fluorometer equipment (E6150, Promega) using the QuantiFluor® dsDNA System Kit (E487, Promega).

Bacterial communities were characterized by Illumina Sequencing for the 16S rRNA V3-V4 region. DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR (Polymerase Chain Reaction) to add sequencing adaptor and dual indexes. First, PCR reactions were performed for each sample using 2X KAPA HiFi HotStart Ready Mix. In a total volume of 25 µl, 12.5 ng of template DNA and 0.2 µM of each PCR primer were used. For bacteria, the following primers were used: forward primer Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer Bakt_805R (5′- GACTACHVGGGTATCTAATCC-3′) [11,25].

For the DNA amplification reaction of prokaryotic microorganisms, 5 µL of forward primer, 341F (1 mM) and 5 µL of reverse primer, 805R (1 mM), 10 µL of Bioline MyTaq HS Mix 2X and, finally, 5 µL of each sample was added to the PCR tubes, making a total volume of 25 µL in each tube. The PCR conditions involved 3 min of denaturation at 95 °C, followed by 25 cycles of 98 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Negative controls were included for all amplification reactions. Electrophoresis of the PCR products was undertaken on a 2 % (w/v) agarose gel, and the ~490 bp V3–V4 amplified fragments were purified using AMPure XP beads (Agencourt, Beckman Coulter) according to the manufacturer's instructions. Second, PCR reactions added indexes and sequencing adaptors to both ends of the amplified target region by the use of 2X KAPA HotStart Ready Mix, 5 µl of each index (i7 and i5) (Nextera XT Index Kit, Illumina) and 5 µl of the first PCR product in a total volume of 50 µl. The PCR conditions involved a 3-min denaturation at 95 °C, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The Amplicon PCR products were analyzed by electrophoresis agarose gel (2 %,w/v), and the amplified fragments were purified using the HighPrep™ PCR Clean-up System (AMPure XP beads, MagBio Genomics) according to the manufacturer's instructions. The amplicons were quantified by fluorimetry with a Quantus Fluorometer ONE dsDNA quantitation kit (Invitrogen, Life Technologies), pooled at equimolar concentrations and paired end sequenced with the V3 chemistry in the MiSeq® ac-cording to the manufacturer's instructions (Illumina) at Genoinseq. They were multiplexed automatically by the Miseq® sequencer using the CASAVA package (Illumina) and quality-filtered with PRINSEQ software using the following parameters: (a) bases with average quality lower than Q25 in a window of five bases were trimmed, and (b) reads with less than 220 bases were discarded for V3–V4 samples. The forward and re-verse reads were then merged by overlapping paired-end reads using the Adapter Removal v2.1.5 software with default parameters. The QIIME package v1.8.0 was used for operational taxonomic unit (OTU) generation, tax nomic identification, sample diversity, and richness indexes' calculation. Sample IDs were assigned to the merged reads and converted to FASTA format (split_libraries_fastq.py, QIIME). Chimeric merged reads were detected and removed using UCHIME against the Greengenes v13.8 database for V3–V4 samples. OTUs were selected at 97 % similarity threshold using the open reference strategy. First, merged reads were prefiltered by removing sequences with a similarity lower than 60 % against the Greengenes v13.8 database for V3–V4 samples. The remaining merged reads were then clustered at 97 % similarity against the same databases listed above. Merged reads that did not cluster in the previous step were again clustered into OTU at 97 % similarity. OTUs with less than two reads were removed from the OTU table. A representative sequence of each OTU was then selected for taxonomy assignment (pick_rep_set.py, assign_taxonomy.py; QIIME) [26].

Results and discussion

Characterization of salt efflorescences

The appearance of saline efflorescence is a frequently encountered problem in cultural heritage and may result in a loss of historic information. The migration of water, mainly through the walls, produces further crystallization of salts on the surfaces. Due to changes in physical parameters, like temperature or humidity, salts can precipitate on the exposed surfaces leading to the formation of salt efflorescences [27]. Salts are known to damage porous materials through a range of mechanisms, such as the production of physical stress resulting from the crystallization of salts in pores, differential thermal expansion, hydration pressure, and enhanced wet/dry cycling caused by deliquescent salts [28]. They thus produce an additional pressure that may lead to material loss and destruction due to cracking and detachment of the mineral support [18].

Therefore, a chemical analysis is crucial to determine the composition of efflorescences, in order to prevent their development and avoid the degradation of cultural assets [29]. XRD was used in this study to characterize the salt efflorescences. XRD is a practical and helpful instrument for the identification of crystalline compounds, to examine the mineralogical composition of stone substrate, salts, and alteration products, which has been applied in the preservation of cultural heritage [30].

The XRD diffractogram collected from efflorescences (Fig. 3) demonstrated the typical diffraction pattern of potassium nitrate (KNO_3). From these results, we can establish that the efflorescences that are damaging the stone materials in Alcobaça Monastery are mainly made of saltpetre. The crystallization of saltpetre is a typical process in many historic buildings, previously well-documented [29,31–33].

Detection of microbial colonization

To assess the deterioration degree of the support and the presence of biocolonization, microfragments of stone were analyzed by SEM-EDS.

The SEM technique in the analysis of heritage materials has grown exponentially and is one of the few techniques suitable for showing bacteria in their natural habitat [26,34]. It has been used to characterize the surface structure of biomaterials and to measure cell adhesion and changes in bacterial morphology [35], mainly because it has several advantages associated with its use, namely a large depth of field; high resolution at the nanoscale; ease of sample preparation; the variation of the examined region and a large sample chamber space [36,37]. When coupled to the EDS technique it is possible to determine the types of elements present in the target analyte, allowing the investigation of the surface microstructure and offering the chemical composition of the deteriorated area [37,38].

SEM-EDS analysis confirmed microbial contamination of the samples and enabled the detection of organic material on the surface of the stone (Fig. 4a). It was possible to identify carbon (C), sulfur (S), nitrogen (N) and oxygen (O) as the main constituents of the stone materials but also the concomitant presence of potassium (K) and sodium (Na) (Fig. 4b). The presence of C, N, O and S on the stone surface can be indicative of the presence of biological material, being further confirmed by SEM analysis (Fig. 5). On the other hand, the presence of Na and K are related to the presence of saline efflorescences [39–42]. Furthermore, salt efflorescences simulate saline environments that provide optimal growth conditions for halotolerant microorganisms containing carotenoid pigments that cause the phenomena of rose discoloration [27].

SEM micrographs showed mainly the presence of bacteria biofilms (Fig. 5a,b) in areas on the surface of the stone altered by the presence of stains and pink pigmentation.

After the identification of microbiological proliferation, analyses for microbiota characterization were performed. The results are shown below.

Characterization of microbiota through culture independent methods

Currently, metagenomic studies are frequently applied to estimate the microbial contamination of cultural heritage, in an attempt to understand degradation phenomena and prevent them from happening [4,43]. In this study, a phylogenetic metagenomic approach is used, in which NGS is used as a culture-independent technique to identify and characterize the prokaryotic communities causing the biodeterioration associated with the pink discoloration observed. A detailed analysis was performed at the phylum, family, genus, and species level that are presented below, in Fig. 6-10.

Considering the set of all samples, from the three sampling zones, the dominant phylum (Fig. 6a) is *Firmicutes* (42.22 %), followed by *Proteobacteria* (22.35 %), *Actinobacteria* (15.70 %), *Euryarchaeota* (10.52 %) and 9.21 % for "others" (generic designation corresponding to species with lower representation).

The results show that the prokaryotic population present on Alcobaça Monastery belongs to the following families (Fig. 6b): *Bacillaceae-1* (30.51 %), *Halobacteriaceae* (10.56 %), *Pseudonocardiaceae* (7.17 %), *Planococcaceae* (5.22 %), *Enterobacteriaceae* (5.09 %), *Moraxellaceae* (3.07 %), *Methylobacteriaceae* (2.51 %), *Chloroplast* (2.26 %), *Lachnospiraceae* (2.21 %), *Sphingomonadaceae* (2.20 %), *Porphyromonadaceae* (1.66 %), *Rubrobacteraceae* (1.23 %), *Paenibacillaceae-1* (1.21 %), *Nocardioidaceae* (0.97 %) and

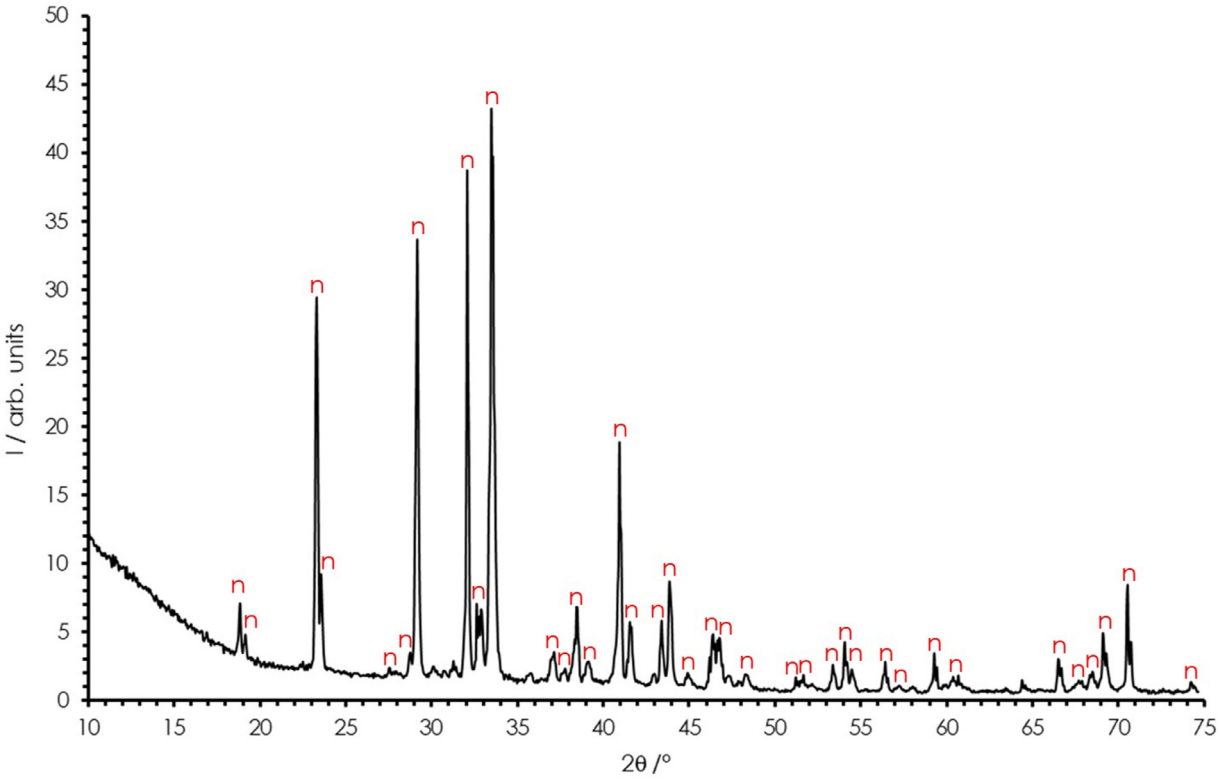

Fig. 3. XRD diffractogram of saline efflorescence from the stone micro fragments with identification of the main potassium nitrate signals (n).

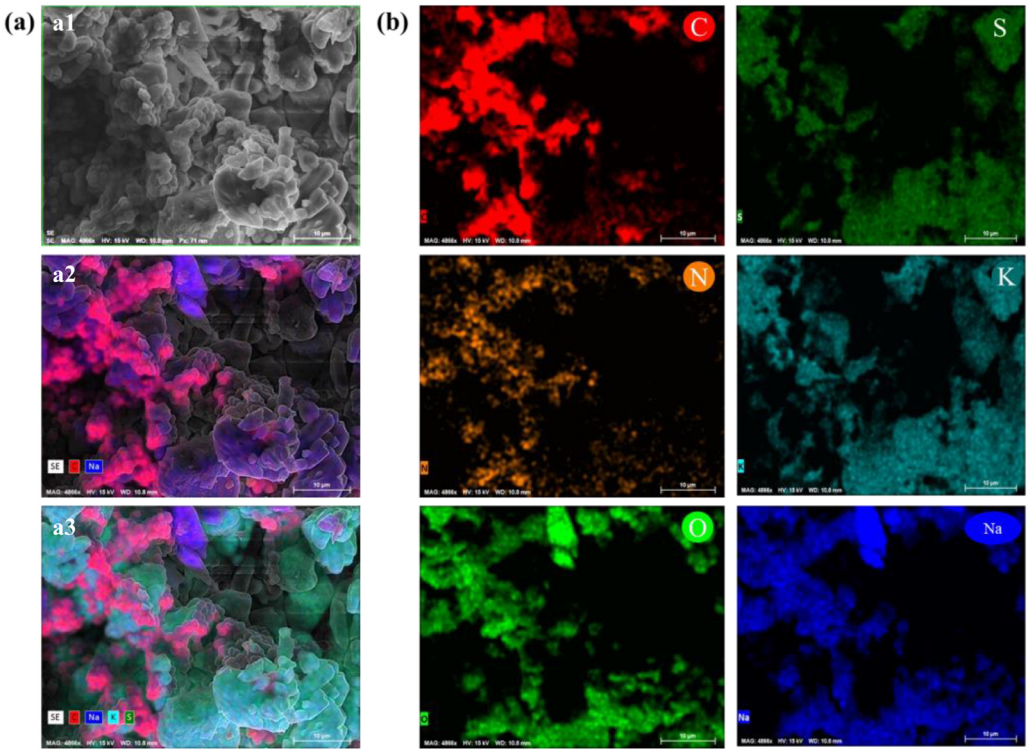

Fig. 4. Analysis of stone microfragments from the Alcobaça Monastery by SEM observation in secondary electron mode (a1) and EDS 2D elemental maps (a2-a3) with individual element distribution (b) of carbon (C), nitrogen (N), oxygen (O), sulfur (S), potassium (K) and sodium (Na).

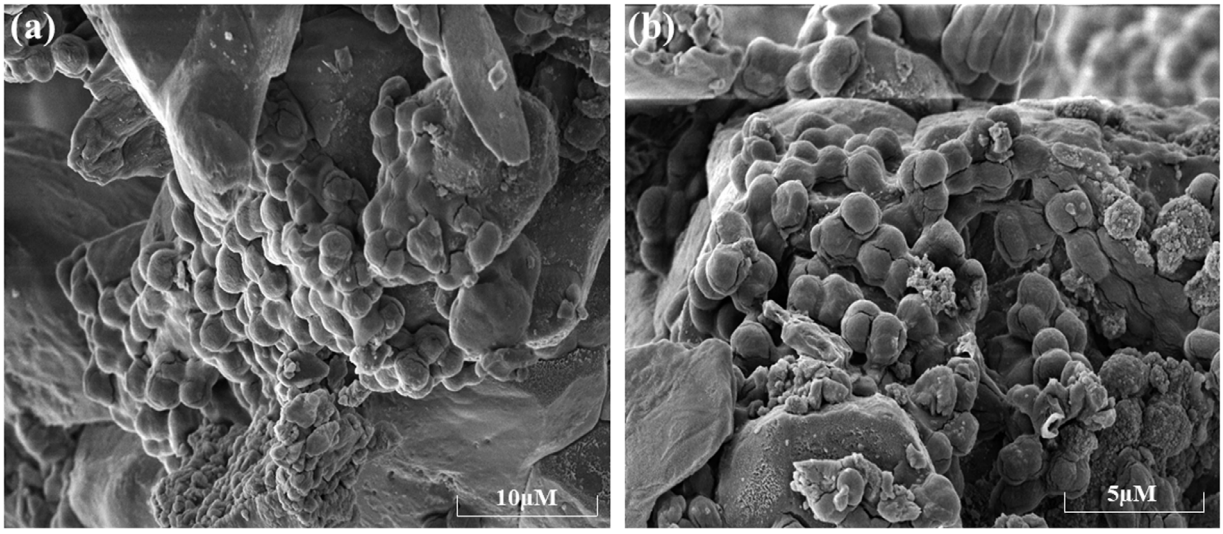

Fig. 5. SEM micrographs of microbial population that thrive on stone altered areas of the monastery.

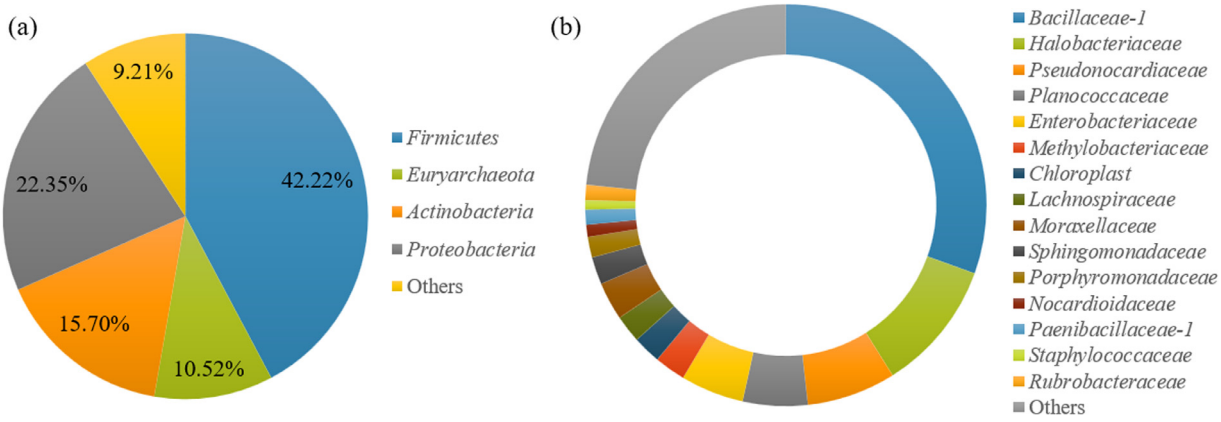

Fig. 6. Predominant phyla (a) and families (b) of prokaryote population present on pink biofilms.

Staphylococcaceae (0.75 %). Families classified as "others" represented 23.36 % of the population.

A detailed analysis was carried out at the level of genus (Fig. 7) and species (Fig. 8-10), considering the observable differences in each sampling zone.

Regarding to genus level, the sampling zone (a) (Fig. 7a) is dominated mainly by *Halalkalicoccus* (20.48 %), *Haloechinothrix* (9.56 %) and *Bacillus* (6.71 %). The genus *Planococcaceae_incertae_sedis* (4.74 %), *Methylobacterium* (3.33 %), *Sporosarcina* (3.32 %), *Barnesiella* (2.15 %), *Rubrobacter* (1.82 %), *Saccharopolyspora* (1.80 %), *Paenibacillus* (1.75 %), *Acinetobacter* (1.62 %), *Staphylococcus* (1.14 %), *Kribbella* (1.10 %), *Euzebya* (0.41 %) and *Domibacillus* (0.16 %), and were also identified. "Others" comprised 39.89 % of the genus. Thus, this sampling zone contains the highest number of pink biofilms collected, so it is not surprising that the majority genera are *Bacillus* and *Halalkalicoccus*, as they include several species that produce pink carotenoids. The last genus is red-pink pigmented and requires a high concentration of salt to develop [44].

In zone (b) (Fig. 7b), the mostly identified genera were *Bacillus* (12.88 %), *Acinetobacter* (7.65 %) and *Methylobacterium* (4.32 %). Since this is a sampling area where saline efflorescences predominate, and the genera mentioned above include several halotolerant species, the results are in line with expectations. The genera with minority relative abundance were *Rubrobacter* (1.72 %), *Barnesiella* (1.43 %), *Paenibacillus* (1.33 %), *Halalkalicoccus* (1.15 %), *Haloechinothrix* (1.04 %), *Kribbella* (0.93 %), *Saccharopolyspora* (0.83 %), *Sporosarcina* (0.30 %), *Planococcaceae_incertae_sedis* (0.28 %), *Staphylococcus* (0.13 %), *Domibacillus* (0.05 %) and 62.72 % were classified as “others”.

Looking at zone (c) (Fig. 7c), from columns with pink coloration near the altar of the church, the main genus identified was *Bacillus* (84.91 %), which stands out significantly from the other zones, followed by *Domibacillus* (11.54 %), which also includes rose-pigmented species [45]. *Halalkalicoccus* (0.85 %), *Staphylococcus* (0.61 %), *Paenibacillus* (0.21 %), *Sporosarcina* (0.20 %), *Planococcaceae_incertae_sedis* (0.12 %), *Methylobacterium* (0.05 %), *Rubrobacter* (0.04 %), *Acinetobacter* (0.03 %), *Barnesiella* (0.02 %), *Haloechinothrix* (0.01 %), *Saccharopolyspora* (0.01 %), e *Euzebya* (0.01 %) were also identified. "Others" comprised 1.40 % of the genus.

Concerning species level, from samples A4 to A7 (Fig. 8), it can be observed that the most discrepant sample is A7, standing out for having a much higher predominance of *Halalkalicoccus tibetensis* (71.61 %), which differs from all the others. *H. tibetensis* is a haloalkaliphilic bacterium (grows preferentially in conditions of high pH and high salinity) and produces orange pigments [46].

In A5 and A6 the microbiota is quite similar, which would be expected given that the sampling was carried out in very close areas. The detected species of prokaryotic community consists mainly of *Psychrobacillus soli* (8.97 and 12.45 % for sample A5 and A6, respectively) that is a halotolerant bacterium, *Muribaculum intestinale* (5.22 and 3.02 %) and *Rubrobacter radiotolerans* (2.61 and

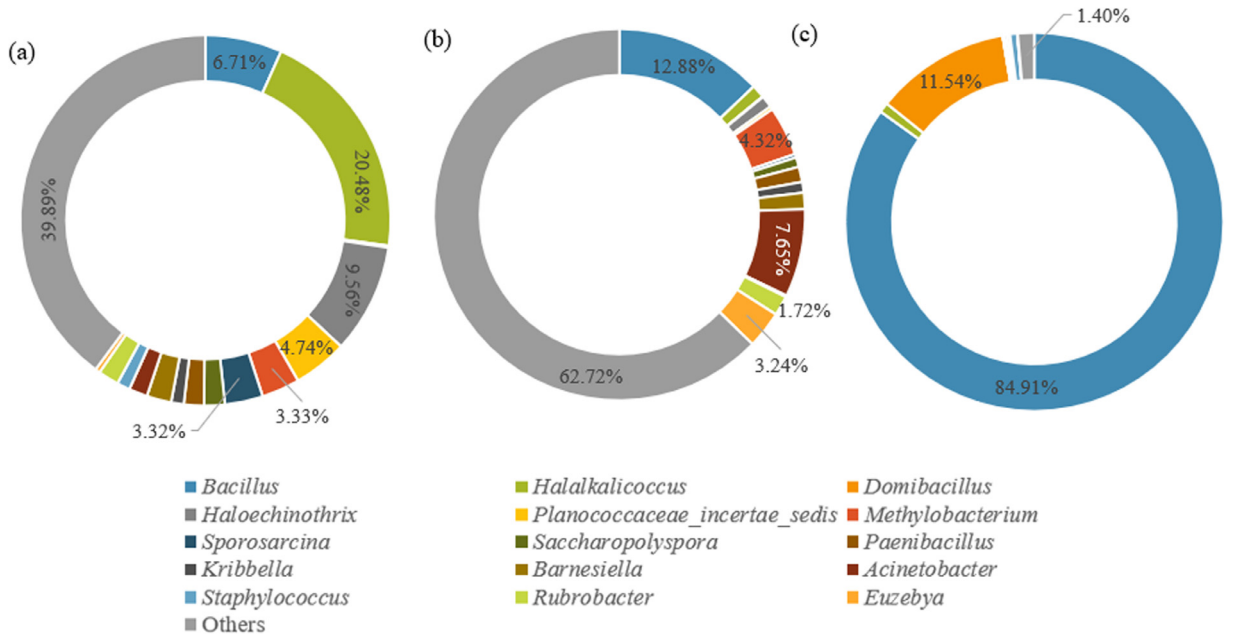

Fig. 7. Predominant genus of prokaryote population present in zone (a), (b) and (c) respectively.

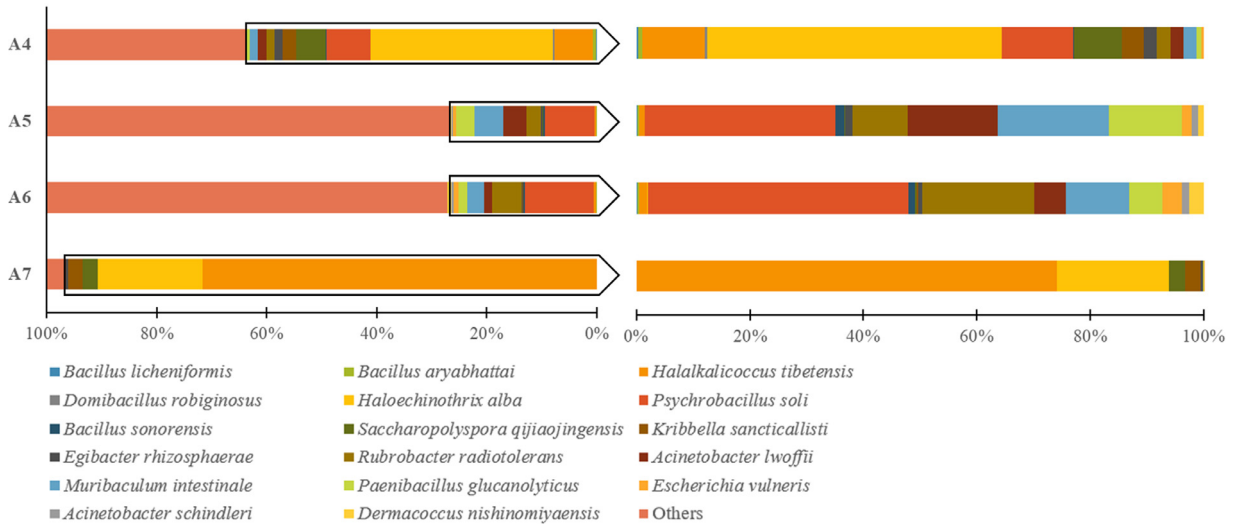

Fig. 8. Main species identified in zone (a) - samples A4-A7.

5.35 %). The latest specie, extremely resistant to gamma and UV radiation [47] has been documented in the literature as an important cause of biodeterioration essentially (i) by the production of pink carotenoids (bacterioruberin and monoanhydrobacterioruberin) [48] and the formation of colored spots of the same color, in various substrates [49]; (ii) for the active role it plays in efflorescence phenomena and mineral precipitation [12] and (iii) for having the benefit of growing on stone surfaces even in direct sunlight due to its halotolerance and UV radiation resistance [13].

The microbiota of A4 is slightly different from the samples mentioned above, as it contains a high relative abundance of the halotolerant species *Haloechinothrix alba* (33.14 %). Previously, this species was isolated from a soil sample from the Qijiaojing Lake in China, classified as an interstitial brine, showing that *H. alba* has evolved specifically to survive in hypersaline conditions [50].

Concerning of saline efflorescence zone (Fig. 9), in sample A8, the main species that were identified was *Acinetobacter schindleri* (14.63 %), *Dermacoccus nishinomiyaensis* (12.20 %), *Escherichia vulneris* (9.76 %), *Acinetobacter lwoffii* (4.88 %) and *Bacillus sonorensis* (2.44 %).

A. lwoffii has the ability to produce reddish-pink pigments, using methanol as an energy source. According to Ghosh et al. (2007), based on information obtained through spectral mass analyses, the pigment was characterized as a carotenoid molecule, probably with a structure similar to bacterioruberin. It's interesting to note that the pink pigment produced by *A. lwoffii* appears to serve two different functions for the microorganism. First, it defends against oxidative stress by combating the produced free radicals (an antioxidant role); second, it keeps the membrane's structural integrity by being able to connect with the fatty acid bilayer [51].

In sample A9 the majority species was identified as *Egibacter rhizosphaerae* (9.95 %). This is an aerobic, obligate halophilic species that forms pale yellow-pink colonies [52]. In A9 also stand out the species *Acinetobacter lwoffii* (4.04 %), *Haloechinothrix alba* (3.19 %), *Paenibacillus glucanolyticus* (3.11 %), *Rubrobacter*

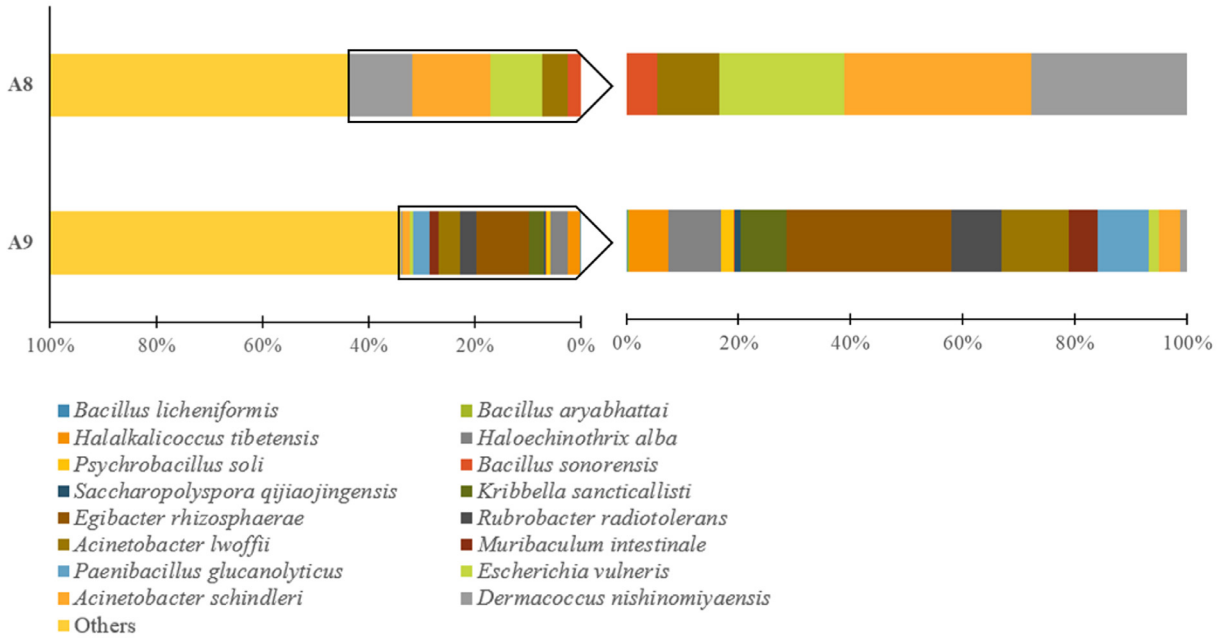

Fig. 9. Main species identified in zone (b) - samples A8-A9.

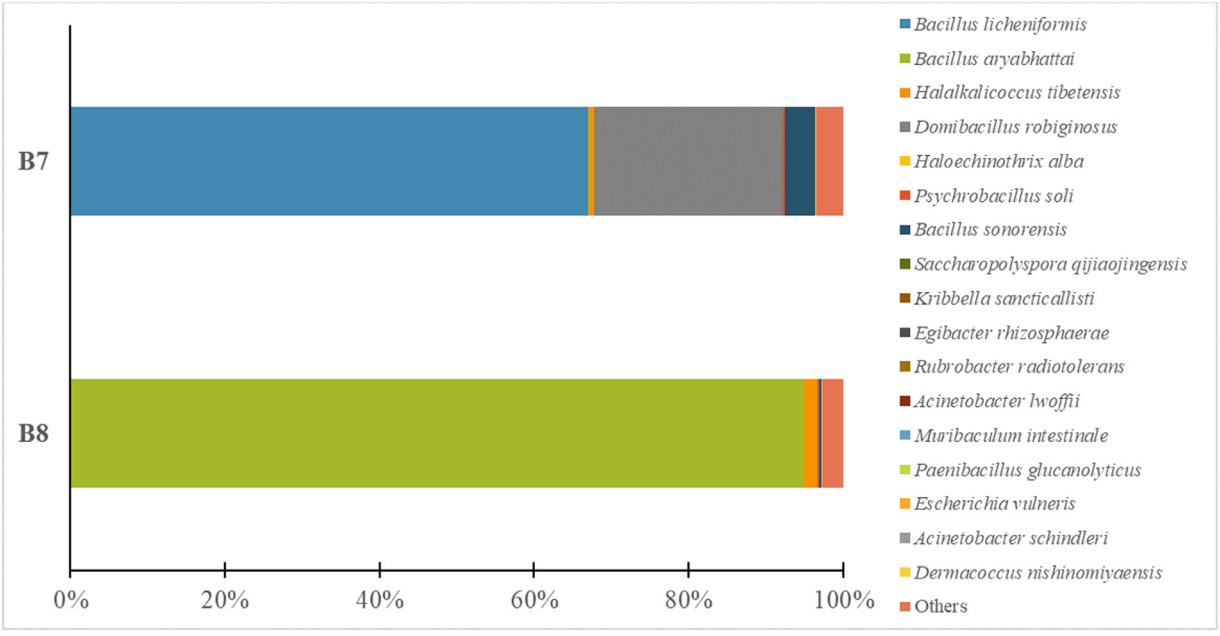

Fig. 10. Main species identified in zone (c) - samples B7-B8.

radiotolerans (3.07 %), *Kribbella sancticallisti* (2.80 %) and *Halalkalicoccus tibetensis* (2.37 %).

Since the samples were taken straight from a salty environment, it is expected that the microbial diversity is lower there than it is in the other sampling areas since fewer microorganisms are susceptible to growth there. Therefore, it is not unexpected that the organisms found in these samples are halotolerant.

In case of sampling zone c (Fig. 10), from columns with pink coloration near the altar of the church, sample B8 differs significantly from B7, since the first is constituted by 94.79 % of *Bacillus aryabhattai* while in B7 the species *Bacillus licheniformis* (66.97 %) and *Domibacillus robiginosus* (24.45 %) predominate.

B. aryabhattai, the major species identified in sample B8, is a species resistant to UV radiation and halotolerant, capable of forming biofilms under conditions of high salinity [53]. There is still no scientific evidence that this bacterium has an influence on heritage deterioration; the small number of published studies on this bacterium describe its activities in the area of bioremediation of heavy metals such as zinc and chromium and in the health area as a producer of asparaginase [54]. *B. aryabhattai* is a pink carotenoid-producing species [55].

The other species detected in sample B8 were Halalkalicoccus tibetensis (1.72 %), Bacillus sonorensis (0.26 %), Psychrobacillus soli (0.13 %), Bacillus licheniformis (0.12 %), Domibacillus robiginosus (0.09 %), Haloechinothrix alba (0.05 %), Escherichia vulneris (0.04 %), Saccharopolyspora qijiaojingensis (0.02 %), Rubrobacter radiotolerans (0.02 %), Egibacter rhizosphaerae (0.01 %), and 2.75 % of the species were considered as “others”.

Regarding to the main species detected in sample B7, *B. licheniformis* is a saprophytic bacterium widely distributed in nature

and halotolerant which produces a reddish-pink carotenoid pigment called pulcherrimina [56,57], has an enormous ability to form biofilms, and is also involved in the formation of saline efflorescence [58]. What happens is that nitrifying bacteria, such as *B. licheniformis*, oxidize ammonium into nitrite and nitrate ions, which lead to the formation of nitric acid. This process causes the stone to dissolve and soluble nitrate salts to form, appearing as efflorescence [59]. *B. licheniformis* has been isolated from the surfaces of several heritage objects [60]. According to Skipper et al. (2022), this bacterium is significantly associated with deteriorated stone surfaces. Based on the results of the study, *B. licheniformis* was considered to be a major contributor to biocorrosion as it was present in 100 % of the sampling sites with evidence of biodeterioration. The ability of this species to form biofilms and patinas has also been demonstrated [61].

The second species with the highest relative abundance in sample B7 corresponds to *D. robiginosus* which is a new bacterial strain with reddish pigmentation [45].

The other species identified in sample B7 were Bacillus sonorensis (3.92 %), Halalkalicoccus tibetensis (0.61 %), Bacillus aryabhattai (0.21 %), Psychrobacillus soli (0.15 %), Acinetobacter lwoffii (0.06 %), Paenibacillus glucanolyticus (0.03 %), Rubrobacter radiotolerans (0,03 %), Muribaculum intestinale (0.02 %), Egibacter rhizosphaerae (0.01 %) and 3.55 % of species were considered as "others".

It was also observed that areas with pink pigmentation and discolored areas had a similar microbiota, with the relative abundance of halotolerant and pigment-producing microorganisms varying, being significantly lower in regions without coloration, namely *Haloechinothrix alba, Halalkalicoccus tibetensis, Domibacillus robiginosus* and *Bacillus licheniformis.*

Thus, in order to define strategies to protect assets from biocolonization and develop approaches to safeguard the monuments, advances in high-throughput analysis have generated new tools for the identification of microorganisms present in complex systems. It is essential that NGS technology be implemented in cultural heritage workflows to fully characterize the microbiota. The understanding of biocolonizers as a whole and their impacts allows for the establishment of better intervention strategies to carry out the preservation of stone structures, avoid the loss of their aesthetic and historical value, and maintain their structural integrity [11].

Conclusions

One of the biodeterioration phenomena most frequently found in stone monuments is pink discoloration, which Alcobaça Monastery is a great example of an important stone building that has suffered significant alterations apparently caused by biocolonization.

Stains and biofilms, two aesthetic defects that have spread across the monastery's stone, appear to be caused by pink-pigmented bacteria, mainly by the species *Rubrobacter radiotolerans, Domibacillus robiginosus, Bacillus licheniformis, Bacillus aryabhattai, Halalkalicoccus* sp. and *Acinetobacter lwoffii*. This microbial colonization was previously confirmed using the SEM-EDS technique, where it was possible to observe the bacterial morphology and the presence of the main marker elements of organic material. Additionally, XRD allowed the determination of the chemical composition of the efflorescences: they are primarily composed of nitrate in the form of saltpetre. The appearance of colored biofilms is associated with the presence of saline efflorescence and, the halophilic and halotolerant bacteria that live in these saline conditions, typically develop pink biofilms because the creation of carotenoids leads to the synthesis of orange to red pigments.

To prevent the devastation of this cultural asset and to encourage its preservation and protection, we believe that this study is a step in the right direction for the implementation of an accurate and effective plan of conservation and intervention (including monitorization and mitigation treatment) of a UNESCO World Heritage monument, the Alcobaça Monastery.

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