Unveiling microbial role in stone pink discoloration at Batalha Monastery

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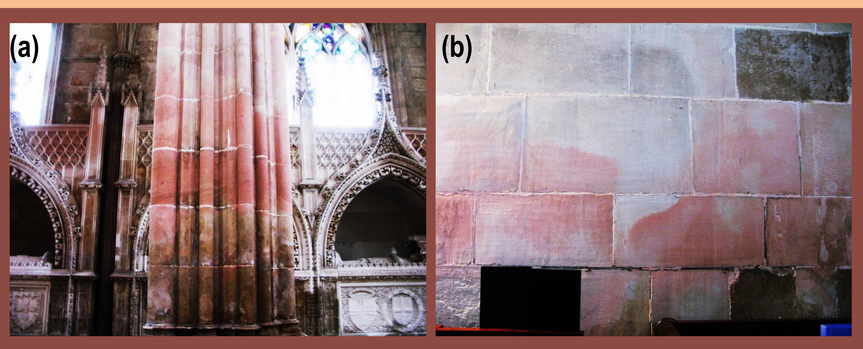
Introduction

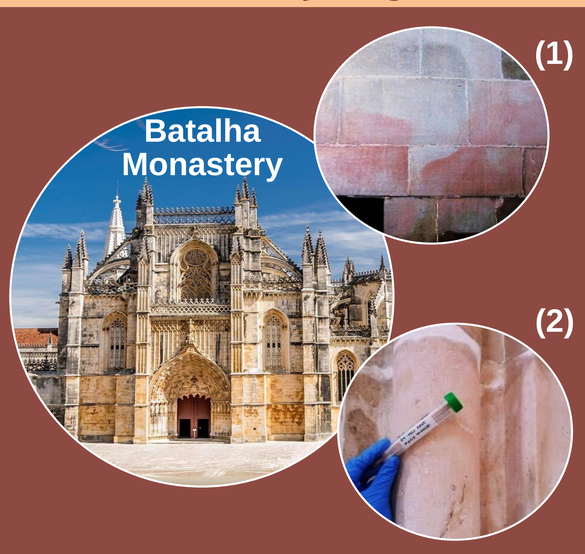
The microbial colonization of heritage buildings by different types of microorganisms is a well-known phenomenon, having a direct effect on the conservation of cultural assets [1]. **Pink discoloration** is an intriguing phenomenon in different historic buildings located in various parts of Europe. This colour change, which is often associated with conditions of high humidity and salinity, and moderate lighting, is thought to be due to the production of carotenes as a cellular protection mechanism against high UV radiation, chemical stress and/or saline stress. These compounds can modify colours leading to aesthetic problems, but also problems in terms of substrate surface stability [2]. The **Batalha Monastery** (Portugal), a UNESCO World Heritage Site, currently exhibits a high degree of surface alterations of the stone architectural elements both inside the Founder's Chapel (Fig.a) and the church (Fig.b), including an extensive pink coloration in the walls and columns.



Main goal

Characterize the biological colonization and subsequent biodeterioration of an architectural stone material (Ançã limestone) found in Batalha Monastery using a comprehensive multidisciplinary approach to help custodians, conservators, and restorers in determining the most effective cleaning technique to use for the monastery's preservation.

Sampling



Two sampling zones

1) Church columns and walls covered with pink biofilms (samples A1, B1 and B1-1)

2) Columns and walls of the Founder's Chapel with pink biofilms (samples A2, A3, B2, B3, B4, B4-1, B5 and B6)

Methodology

Reddish-pink biofilms

- Culture-dependent method (CDM)
  - Cultured in NA and MM
  - 30ºC (24–48h)
  - Bacterial growth
  - Prokaryotic DNA extraction
  - Sanger's PCR
  - Alignment and homology analysis
- High-throughput sequencing (HTS)
  - Metagenomic DNA extraction
  - Target region (V3 V4)
  - Amplicon preparation
  - Illumina sequencing platform

Concluding remarks

(1) Regarding the observed pink discoloration, and considering previous studies, we hypothesized that it is caused by biofilms formed mainly by bacteria of the genera *Bacillus* and *Halalkalicoccus* that produce pigments of the same color, particularly carotenoids.

(2) Microorganisms producing pink pigments were identified through both culture-dependent and non-culture-dependent methods tested.

(3) Through biocolonization tests on limestone, bacterial growth was observed in the form of pink biofilm, which allowed us to mimic the phenomenon that occurred at the monument.

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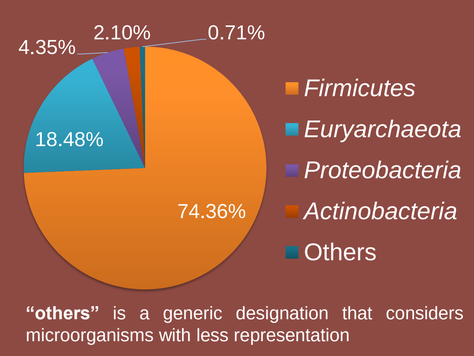
Results & Discussion

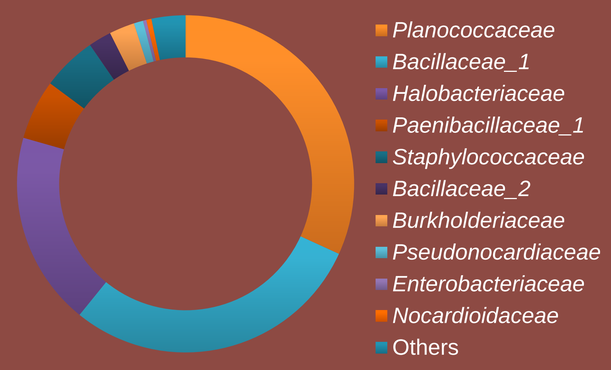
- **HTS analysis**

MOST IDENTIFIED PHYLA

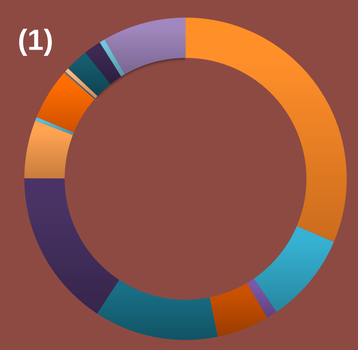


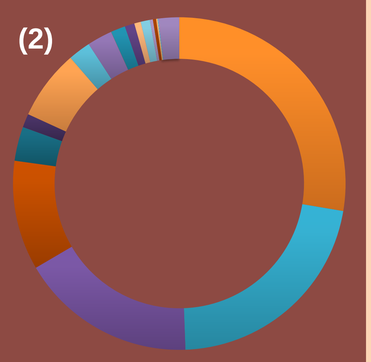
"others" is a generic designation that considers microorganisms with less representation

MOST IDENTIFIED FAMILIES



MOST IDENTIFIED GENUS



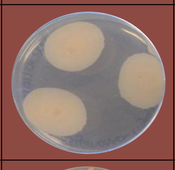
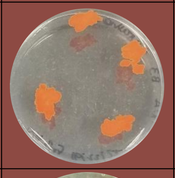
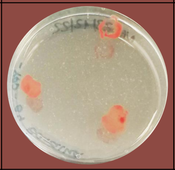
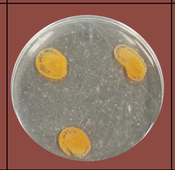
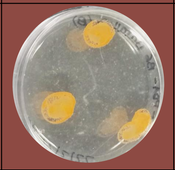


- *Bacillus*
- *Sporosarcina*
- *Paenibacillus*
- *Rummeliibacillus*
- *Ralstonia*
- *Lysinibacillus*
- *Domibacillus*
- *Halococcus*
- *Haloechinothrix*
- *Tissierella*
- Others
- *Halalkalicoccus*
- *Planococcaceae_incertae_sedis*
- *Staphylococcus*
- *Paenisporosarcina*
- *Paucisalibacillus*
- *Oceanobacillus*
- *Burkholderia*
- *Halorientalis*
- *Kribbella*
- *Methylobacterium*

Comparing the **main genera** identified in zone 1 and 2, the pink biofilm sampling from the church and Founder's Chapel, respectively: the church contains a greater relative abundance of *Bacillus* (31.41%) than the Founder's Chapel (27.64%). The same happens with the genera *Staphylococcus* and *Paenibacillus*. However, the Founder's Chapel contains a higher percentage of relative abundance of the genera *Halalkalicoccus* (21.77%) compared to the church (9.04%) – which includes **orange and pink-pigmented species**. The same happens with the genera *Sporosarcina*, *Planococcaceae_incertae_sedis* and *Rummeliibacillus.* We were also able to identified the genus *Methylobacterium* which most of the members are pink-pigmented, have strong biofilm-producing ability and frequently colonizers of stone substrates.

In both contaminated zones, prokaryotic genera producing pink pigments were identified.

- **CDM analysis**

Sample	Macroscopic features		Most probable identification
	Front	Back	
B2			*Bacillus megaterium* (MT322950.1)
B3			*Gordonia alkanivorans* (KU597074.1)
B4			*Methylorubrum extorquens* (KY622701.1)
B6			*Verrucosispora gifhornensis* (KJ571063.1)

CDM allowed to characterize the cultivable isolated population (table), composed of prokaryotic microorganisms → **producers of interesting pigments.**

Sample B2 presented high similarities with *B. megaterium,* which contain a **unique pink pigment** in their membranes that is not found in other species (role in the stability of the membranes of the spores) [6].

G. alkanivorans and *M. extorquens* are referred to as **high carotenoid-producer bacteria**, associated with reddish carotenoids (canthaxanthin and astaxanthin), which confer tolerance to UV radiation [7,8].

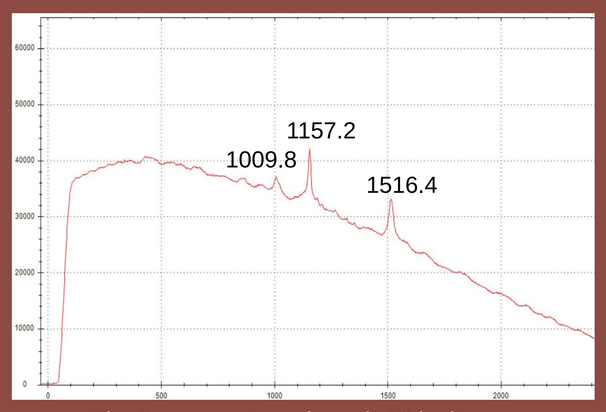
Lastly, *V. gifhornensis* is an **orange pigment**-producing actinobacteria [9].

- **Biocolonization tests**

Biocolonization tests are being performed in which stone mock-ups were prepared and inoculated with the bacteria isolated in the study, in order to simulate the natural conditions of the monastery and monitor the colonization process, to better understand the discoloration phenomenon.

Sample B3

We were able to demonstrate, by RAMAN, that the microorganism in sample B3 produces carotenes.



The Raman spectrum of sample B3 *in vitro*

Standard β-carotene peaks:
1005, 1156, 1523 cm^{-1}