

# MAY METAGENOMICS DISCLOSE THE HIDDEN SECRETS OF THE ANCIENT DAMAGED PARCHMENTS?

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**Abstract** – The use of microbiological high-throughput molecular methods allowed an unprecedented level of knowledge about the microbial responsible for parchment deterioration and their dynamics. The aim of this study discussed how metagenomics helped to get insights into parchment biodeterioration so far, but also to get a perspective of their possible future use in the study and preservation of an important part of our cultural heritage.

## I. INTRODUCTION

Ancient parchments have been used as the main written support material for over two millennia, hence they preserve a huge part of our culture. Due to its animal origin and protein composition (collagen), parchment represent a perfect source of food for heterotrophic microorganisms, which are able to attack and biodeteriorate the matrix, sometimes with great damage to the cultural content. One of the most common signs of biodeterioration are the so-called purple spots [1, 2], which might compromise document readability not only for the discolouration but also for the detachment of the superficial (written) layer.

Many studies analysed purple-damaged parchments, however standard cultivation and basic molecular techniques were unsuccessful in finding out the causative agents of purple spots for many years [2, 3, 4].

Recently, a number of studies have explored the parchment biodeterioration dynamics by adopting microbiological high-throughput molecular methods [1, 5, 6, 7, 8, 9]. These techniques allowed an unprecedented level of knowledge on the complex microbial colonization responsible for parchment biodeterioration.

## II. MATERIALS AND METHODS

For the microbial community characterization, small pieces of two ancient parchments characterized by a different deterioration rate, a low deteriorated document dated 1244 d.C. and a set of three dramatically damaged documents dated XV-XVII Century from the Vatican Apostolic Archive (Vatican City). Pieces of about 2-4 mm<sup>2</sup> were collected from both the purple damaged and the white

undamaged areas of all documents, in sterile conditions. Each replica was composed of one piece of parchment: 3/4 replicates of purple areas and 3 of uncoloured ones (as a control) were utilized, respectively from low or high damaged documents. To extract metagenomic DNA, each parchment piece was directly processed using the Power Soil<sup>®</sup> DNA isolation kit (Mo Bio, Carlsbad, CA, USA), according to the manufacturer's instructions. Pure DNA extracts were analysed by both 454 pyrosequencing (the low damaged document) and Illumina-based techniques (the high damaged document). The metagenomic bioinformatic analysis of raw DNA sequencing data was performed by using MOTHUR (low damaged document) and Quantitative Insights Into Microbial Ecology (QIIME, high damaged document) [1, 5]. Bacterial community analyses were performed by unconstrained UPGMA clustering built using the UniFrac.weighted metric [1].

## III. RESULTS

The analysis of bacterial community structure, based on the OTUs (Operational Taxonomic Units) abundances (Fig. 1), showed a clear separation between the microbial community found on the purple damaged or the white undamaged samples in the low deteriorated parchment.

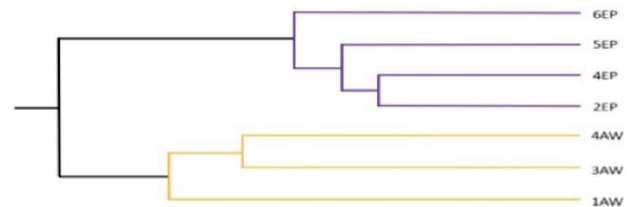


Fig. 1. Different microbial communities in the low deteriorated document. UPGMA tree of purple damaged (#EP) vs. uncolored undamaged (#AW) samples; UniFrac.weighted, WScore = 1; WSig = <0.001. From Migliore et al., 2017.

*Actinobacteria* and *Gammaproteobacteria* were dominant, the prevalent orders were *Pseudonocardiales* and *Vibrionales* in undamaged and purple damaged areas,

respectively (Fig. 3a). Hence, the main difference concerns the ratio between *Pseudonocardiales* and *Vibrionales*. No *Archaea* was found, although the purple stain was ascribable to bacteriorhodopsin, the haloarchaeal typical pigment.

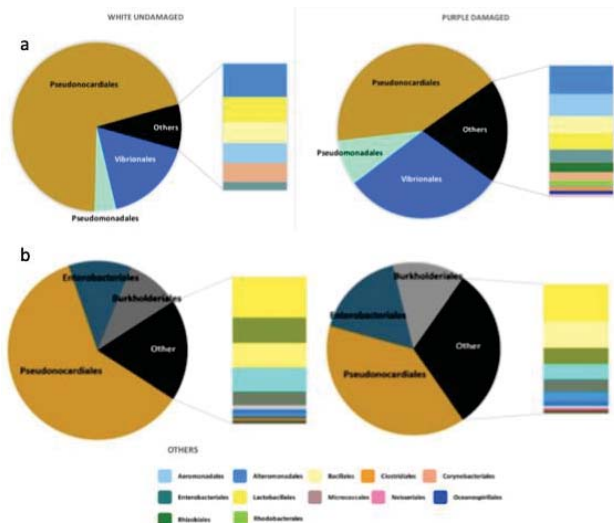


Fig. 3. Distribution of taxa at order level in: a. low damaged and b. high damaged parchments, analyzed separately for undamaged/less damaged (left) and purple damaged (right) areas of the documents. From Migliore et al., 2017 and 2019.

On the contrary, the analysis of bacterial community structure, based on the OTUs abundance in the high deteriorated parchments (Fig. 2), did not show a clear separation between the purple damaged and the less damaged areas of the documents. Haloarchaeal and bacterial sequences are similarly distributed in both areas of the parchments.

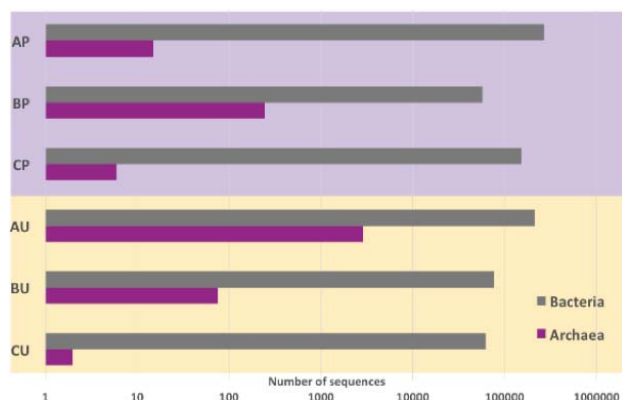


Fig. 2. Haloarchaeal and bacterial sequence distribution in purple damaged (P, purple box) or less damaged (U, warm yellow box) samples in the three parchments (A, B, C indicate each document). From Migliore et al., 2019.

In the three highly deteriorated parchments, the dominant

group in both sets of samples was *Pseudonocardiales*, environmental or human/animal associated bacteria, with *Actinobacteria* being the prevalent class (Fig. 3b). Furthermore, there were within both batches also halophilic and halotolerant marine microorganisms. Haloarchaeal OTU were found, belonging to the genus *Halobacterium*, with a 100% homology value with *H. salinarum* species sequences.

#### IV. DISCUSSION

The microbial colonizers of parchments suffering a different degree of deterioration showed a main difference between the damaged and undamaged/less damaged areas as regard the ratio between *Actinobacteria* (i.e. *Pseudonocardiales*) and marine halophilic (i.e. *Halobacteriales*) and halotolerant (i.e. *Vibrionales*) microorganisms [1, 5].

Since ancient times but also nowadays, in the Southern coastal regions (e.g. Italy), rawhides were treated with sea salt to prevent skins from decay [1]. Salting was carried out dry or in brine, and during brining NaCl gradually entered inside the skin [10], bringing both halophilic archaea and halotolerant bacteria (present in natural marine salt) [1] and making the parchment environment extremely salty [11]. In these conditions only halophilic and halotolerant microorganism may thrive. Nevertheless, in all samples *Pseudonocardiales* are the dominant group. This can be due to the metagenomic analysis which provides a flat picture (i.e. a two-dimensional representation) of the colonisation process that, instead, occurred on a three-dimensional perspective (in time), which imply the stratification of several generations of different microbes succeeded over centuries hence, an ecological succession.

With this general model of natural processes in mind, we proposed a heterotrophic ecological succession to explain the biodeterioration process; in the parchments this process consists of two clearly distinct phases.

In the first-phase, ‘pioneer’ colonizers of this succession are halophilic microorganisms, as *H. salinarum*. If present inside the parchment they can thrive in a range of humidity able to ensure a high salinity, supported by the bacteriorhodopsin which convert light in chemical energy. As humidity increases and salinity decreases they collapse giving rise to the purple spots and allowing the growth of halotolerant bacteria (*Vibrionales*), which grow by consuming the haloarchaeal debris. This first phase is predictable (as mainly driven by rawhide salt-curing), common to all the damaged parchments, and triggered by *Halobacterium* conveyed by rawhide salt-curing. On the contrary, the second-phase depends on the individual history of each parchment, which determines the recruitment of colonisers from those available in the specific environment where the parchment was kept (libraries, bookcases, scholars, etc.; Fig. 4).

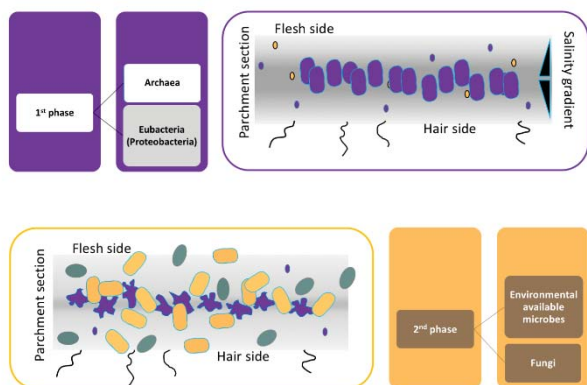


Fig. 4. The two-phase model of parchment colonization. The microbes involved in the parchment damage are: halophilic Archaea (purple, the cells are intact in the 1st phase draw or collapsed in the 2nd one); Gamma-Proteobacteria (gold) and Fungi (teal). From Migliore et al., 2017.

At the beginnings of the microbial attack, the *H. salinarum* forms the core of the purple spot damage, growing inside the hides and producing proteolytic and lipolytic enzymes able to attack and degrade the parchment collagen matrix. When the salt concentration within the parchment is lowered below the limiting value, *halobacteria* lysed, in the hot-spot where they had grown up, releasing the bacteriorhodopsin. Bacteriorhodopsin is responsible for the purple stains, and the haloarcheal cellular content provides nutrient and energy supplement to the subsequent colonizers. Such a boost allowed the halotolerant colonizers, such as *Gammaproteobacteria*, to rapidly utilize the nutrients made available by haloarcheal colonization and to continue the breaking down of the collagen matrix.

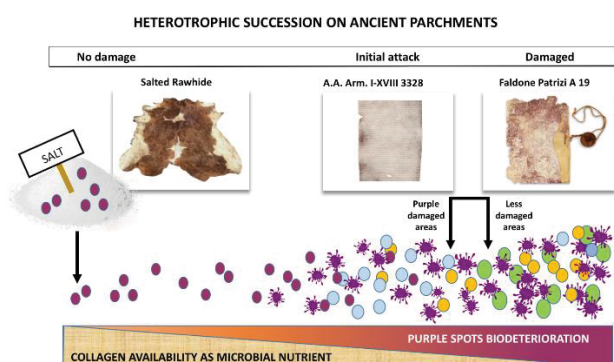


Fig. 5. Dynamics of the microbial succession in the parchment. In the deterioration process the main actors are in the sequence: haloarchaea (purple), halotolerant bacteria (light blue), actinobacteria (gold) and fungi (green). From Migliore et al., 2019.

The halotolerant colonizers grow and are able to wipe away all the halobacterial traces (i.e. DNA), except for the

persistent bacteriorhodopsin. Thus, the intrinsic mechanism of the ecological succession explains the absence or the low percentage of the *Haloarcheal* OTUs in the less deteriorated parchment and its presence or high abundance in the most dramatically deteriorated parchments. A summary of the process and its dynamics in ancient documents is reported in Fig. 5.

## V. PERSPECTIVES

The application of high-throughput techniques to the study of historical parchments allowed to uncover this intriguing process and to put the basis for a different perspective for the study of parchment biodeterioration. Nevertheless, these updated techniques have also weaknesses, for example the flattening of the historical perspective which can give rise to misunderstanding. The aim of this study is to open the discussion about the possible perspectives that metagenomics may open in the studies of parchment biodeterioration, in the light of what has been already done, and to get a perspective of their use in the study (environmental positioning, use, manipulation, etc.) or preservation of a huge number of documents.

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