Mycobiota of silk-faced ancient Mogao Grottoes manuscripts belonging to the Stein collection in the British library

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ABSTRACT

Silking, a conservation technique which involved gluing silk gauze over the face of a manuscript was popular in the mid-20th Century, especially for treating early Chinese documents. The method is now little used, and the question as to whether silking interventions should be reversed is controversial, given the high economic cost of active intervention, and there are few scientific studies as to the long-term consequences of the technique. Silk-facing materials from documents of the Stein collection were analysed using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy. The mycobiota diversity was unravelled through the combination of culture dependent methods and amplicon sequencing analyses. The SEM micrographs showed smooth regular nodules of *ca.* 3-5 µm diameter on both silk threads and glue paste. This morphology differs from the irregular and the crystalline morphologies of glue paste and inorganic crystallites, respectively, but it is consistent with that of small-sized conidia (asexual spores of fungi) or yeasts. Glue paste demonstrated three fungal strains: *Aspergillus tubingensis, Penicillium crustosum* and *Chrysonilia sitophila* which display cellulolytic activity except the last. Amplicon sequencing revealed that silk threads and glue paste host distinct mycobiota. Here, we preliminary show that the silking method may be affecting the overall integrity of the silk-faced manuscripts, principally due to contamination with cellulolytic fungal strains. Unless the silk facing is removed, irreversible damage to the documents is highly probable.

Key words: the Stein collection; manuscripts; conservation science; silking; scanning electron microscopy (SEM); energy dispersive X-ray spectroscopy (EDXS); mycobiota; culture dependent methods; amplicon sequencing analyses; conidia

1. Introduction

The conservation and preservation of ancient manuscripts is an area of huge social, historical, religious, and cultural significance, and yet one which has attracted little scientific study. In the field of conservation, there has been a *volte-face* in acceptable techniques with the new guiding principle being minimalist intervention and reversibility (The Institute of Conservation, 2015). In the field of analysis, there have been tremendous advances in the past decades. It is critical that these "two cultures", of classical historical contexts and analytical science, are brought together to the advantage of preserving our culture (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012).

The preservation of ancient manuscripts - invaluable information carriers - in modern libraries and archives currently benefit from advanced environmental control systems that efficiently block the impact of numerous exogenous factors like acidity, heat, UV light, humidity, oxygen and pollutants (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012). However, other influential issues during historical conservation treatments perhaps are easily neglected, which could cause unforeseen detrimental effect.

Restoration of historical manuscripts and paper documents can be traced back to China, almost 2000 years ago - the birth of well-known techniques including mounting, remounting, backing, lining, *etc*. The first evidence of such techniques appearing in the Western world dates back to 1837 in the United States of America, and to 1858 in Europe (Marwick, 1964). The silking technique was first applied in the 1940s, and consists of the use of fresh silk gauze as an ideal solution to strengthen the manuscript pages (Marwick, 1964). This technique has been formerly used, extensively, to preserve numerous manuscripts in a wide range of institutions. In particular, it constituted the major conservation effort for thousands of manuscripts belonging to the Stein collection in the British Library in the 1960s-1970s. However, scientific studies on silk-faced manuscripts are lacking, especially on the detrimental impacts. This constitutes a serious omission, because water, starch paste and animal glue paste, which were often used, might increase the manuscripts ability to be colonised by living organisms upon silking. Such potential vulnerabilities through the

decades might have opened the door for microbial colonisation, mainly fungi (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012). Moreover, microbial colonisation can provoke serious damage/degradation of the affected manuscripts (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012).

The silk facing procedure is, of course, no longer used in an era defined by minimal intervention. However, the question remains "how diverse is the community now colonising the silk-facing materials?" This present study aims at a evaluating the presence of fungal contamination on ancient Chinese manuscripts from the Mogao Grottoes that have been submitted to the silking conservation technique and are currently requiring further conservation (Figure 1A), and weight arguments on whether silk should or not be removed. The data obtained provide sufficient evidence of both the physical damage and the fungal contamination of the ancient Chinese silk-faced manuscript selected for study.

2. Materials and methods

2.1. Samples

The manuscripts originate from Dunhuang, dated from the 5th to early 11th Centuries, discovered by Yuanlu Wang in 1900 (Wang and Perkins, 2008). They were sealed in Cave 17, known as the Library Cave, in the Mogao Grottoes, where closely packed layers of heaped bundles of scrolls were discovered, along with textiles, such as banners, as well as figurines of Buddha (damaged) and other Buddhist artefacts. Mogao Grottoes enclose important cultural heritage and have been listed officially as UNESCO World Heritage Site in 1987 (Wu et al., 2017). The manuscripts, many of which reside in the British Library, are referred to as the Stein Collection (Wang and Perkins, 2008).

Two representative manuscripts with silk-facing were selected for this study (Figure 1A and 1B), namely Or.8210/S.417 and Or.8210/S.316 (*n.b.* this is the British Library registration system for manuscripts from Dunhuang in Stein Collection, and uniquely defines a document), from which silk threads (BL1 and BL2, showing distinct yellowing of the fibres) and glue (BL3-BL6) were removed and donated by the British Library.

2.2. Surface analyses

In order to investigate the deterioration of the silk facing materials, a scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDAX) was employed: JEOL JSM-6500F Field Emission Scanning Electron Microscope and Oxford

instrument INCA X-sight 7558 (School of Mathematics and Physics at QUB). Silk samples were sputter-coated with gold, and were affixed *via* copper tape to the SEM sample holders.

2.3. Cultivable fungi isolation, identification and characterisation of cellulolytic activity

Following the identification of structures in the SEM data, of similar size and shape to fungal spores and/or yeasts, it was speculated that this may be due to fungal contamination. To isolate any cultivable fungal strains, the samples were incubated in a sterile peptone solution (2 %) for three days at room temperature followed by vortex cycles, and aliquots were then directly inoculated onto Malt Extract Agar (MEA) and incubated at 27 ºC. The forming colonies were monitored daily. Isolated colonies were selected for further purification by consecutive sub-culturing onto fresh MEA. Morphological characterisation (microscopy) allowed their preliminary identification. Negative controls, *i.e.* similar materials (unused paper pieces collected inside the laboratory) manipulated alongside with the study samples, were used to discard the possibility of cross-contamination during the analysis.

DNA extractions were undertaken from the fungal isolates mycelia using a DNeasy extraction kit (Qiagen). The DNA samples were stored at -20 ºC until further analysis. Amplifications of a part of the β-tubulin and calmodulin genes, and the ITS regions (including 5.8S rDNA) were done in a GeneAmp PCR system 2720 (Applied Biosystems) thermocycler using the primers Bt2a and Bt2b, CMD5 and CMD6, and V9G and LS266, respectively (Deive et al., 2011). Primer sequences are as follows: Bt2a, 5'-GGT AAC CAA ATC GGT GCT GCT TTC-3'; Bt2b, 5'-ACC CTC AGT GTA GTG ACC CTT GGC-3'; CMD5 3' - CCG AGT ACA AGG ARG CCT TC; CMD6 - CCG ATR GAG GTC ATR ACG TGG; V9G, 5'-TTA CGT CCC TGC CCT TTG TA-3'; LS266, 5'-GCA TTC CCA AAC AAC TCG ACT-3' (Deive et al., 2011).

The PCR products were purified using the NZY Gelpure kit (NZYTech) and then sequenced at StarSEQ (Mainz, Germany). Sequence similarity searches were performed in public databases of GenBank (http://www.ncbi.nlm.nih.gov/) with BLAST (version 2.2.30).

To assess cellulolytic activity, each strain was platted onto carboxymethylcellulose (CMC) agar (0.2% NaNO3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone, and 1.7% agar) and incubated at room temperature. At the third and tenth day of incubation, the plates were flooded with Gram's iodine (binds to CMC) for 5 min and the excess of reagent removed. The formation of a decolouration halo indicates the production of cellulases, as previously described (Kasana et al., 2008).

2.4. Next generation sequencing (NGS)

DNA was extracted from the peptone extracts of each sample (see above: Cultivable fungi isolation and identification) using a DNeasy extraction kit (Qiagen). The DNA samples were stored at -20 ºC until further analysis. Amplifications of the ITS2 region were done in a GeneAmp PCR system 2720 (Applied Biosystems) thermocycler using barcoded gITS7 and ITS4 (Ihrmark et al., 2012) in three PCR reactions per sample. The PCR reactions were set as previously described (Žifčáková et al., 2016). Primer sequences are as follows: gITS7, 5'-GTG ART CAT CGA RTC TTT G-3'; ITS4, 5'- TCC TCC GCT TAT TGA TAT GC-3'. The PCR products were then tested using gel electrophoresis and finally pooled for each sample and sequenced on Illumina MiSeq. NGS analysis was performed by Gene Expression Unit at Instituto Gulbenkian de Ciência (Oeiras, Portugal)

2.5 Data processing

The amplicon sequencing data were processed using the pipeline SEED 2.0.4 (Větrovský and Baldrian, 2013). Briefly, pair-end reads were joined using FASTQ-join (Aronesty, 2013). The ITS2 region was extracted using ITS EXTRACTOR 1.0.11 (Nilsson et al., 2010) before processing. Chimera search was done using USEARCH 8.1.1861 and deleted. Sequences were clustered using UPARSE implemented within USEARCH (Edgar, 2013) at a 97% similarity level. Consensus sequences were constructed for each cluster, and the closest hits were identified using BLASTn against GenBank. Sequences with less than 10 reads were discarded. The phylogenetic relations between the OTUs identified were estimated using Bayesian approximate branch support at PhyML 20120412, and further visualised and exported using the FigTree 1.4.2. Descriptive statistics were performed using XLSTAT 2009.1.02, and histogram analysis took into account the number of reads of each OTU at each sample, as weights. The data herein presented have been deposited in the Sequence Read Archive (NCBI) with the submission code SUB2308714.

3. Results and Discussion

3.1 Physical damage and elemental composition of the silk thread

Morphological degradation of the silk threads, as well as the presence of glue attached to the fibres, were evident in the SEM images (Figure 1C and 1D, sample BL2 as an example). Both the progressive weakening of the silk threads and the stiffness of the aged glue may aggravate the friction with the manuscripts. Elemental analyses (EDAX) of the silk threads showed the presence of both calcium and aluminium in parts with attached glue (Figure 1E

and 1G), but only organic content (apart from traces of copper from the support) in those devoid of glue residues (Figure 1F and 1G). The presence of calcium likely originates from the glue itself, maybe reflecting its animal origins, whereas that of aluminium is consistent with the use of a weighting process of the silk block (Des Barker et al., 2006).

3.2 Microbiological contamination

The silking technique might have also impacted the manuscripts ability to be colonised by living organisms. The SEM micrographs showed smooth regular nodules of *ca.* 3-5 µm diameter on both silk threads and glue paste (Figure 2B). This morphology differs from the irregular and the crystalline morphologies of glue paste and inorganic crystallites, respectively, but it is consistent with that of small-sized conidia (asexual spores of fungi) or yeasts. With the exception of BL6 glue that provided three fungal strains, the remaining silks and glues here analysed have not provided any cultivable isolate. These were *Aspergillus tubingensis*, *Chrysonilia sitophila* and *Penicillium crustosum* (Figure S2, Supplementary information). *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp. and *Mucor* spp. have been reported before as the prevalent cultivable taxa in antiques stored in old libraries (Cappitelli et al., 2010; Nevalainen et al., 2015; Sterflinger and Pinzari, 2012). Moreover, *Penicillium sp.* and *Aspergillus sp.* have both been identified in the air (Wang et al., 2011) and the walls (Ma et al., 2015) of the Mogao Grottoes, and were also isolated from old textile artefacts in Eastern Europe (Ljaljević-Grbić et al., 2013). *Penicillium crustosum* and *A. tubingensis* are considered common indoor fungi (Nevalainen et al., 2015). They produce small-size conidia of *ca.* 3 µm, hence similar to those observed in the SEM micrographs (Figure 2B). Both species are able to tolerate very low water activities (a_w) with optimal growth at *ca.* 0.83-0.85. Their occurrence in the British Library raises serious clinical concerns, since they can produce neurotoxins, particularly penitrem A, and act as an opportunistic pathogen of immunocompromised/competent patients (*e.g.* bone osteomyelitis or keratitis), respectively (Bathoorn et al., 2013; Moldes-Anaya et al., 2012). On the other hand, even if less frequently, *C. sitophila* (optimal a_w *ca.* 0.9) has been also identified in the indoor environment of historical buildings, libraries and museums (Ljaljević-Grbić et al., 2013). Despite its abundant, readily airborne conidia, systematic evidence that this fungus is the causal agent of any disease, infection or significant allergies is still lacking, with the exception of its association with suberosis (*viz*. hypersensitivity pneumonitis) (Cordeiro et al., 2011).

Importantly, the three vital strains isolated from sample 6 were tested for their cellulolytic activity at room temperature to assess the risk of biodegradation of the ancient

manuscripts studied herein. Cellulolytic activity was only observed in the plates inoculated with *A. tubingensis* and *P. crustosum* (Figure 2, i and iii). Even after 10 days of incubation, *C. sitophila* failed to degrade CMC (Figure 2, D_{iii}). These results show that two out of the three vital strains isolated from the silk facing materials possess cellulolytic activity, therefore posing a real threat to the manuscripts.

To fully disclose the mycobiota diversity of the silks or the glues, the DNA content of the corresponding peptone extracts was recovered, then the highly conserved ITS2 regions were amplified and, finally the ensuing amplicons were sequenced using NGS (Figure 3). Four out of the six samples contained DNA sequences matching that of fungal genomes, namely BL1, BL2, BL5 and BL6 for 4, 18, 25 and 15 Operational Taxonomic Units (OTUs), respectively (reads >10) (Figure 3A, Table S1, Supplementary Information). Most of the identified OTUs are associated with fungi capable of growing in standard solid media regardless that only from BL6 glue three strains could be isolated. Accordingly, most DNA harboured in the silk-facing materials likely originated from fungal debris and/or spores which are no longer viable or are viable but in a nonculturable state. The DNA extracted from the silk BL1 retrieved only 4 distinct OTUs with a clear domination by *Solicoccozyma* spp. (common soil yeast), followed by *Umbelopsis* spp. and *Mortierella* spp. (widespread Zygomycota). In the BL2 silk, a more diverse and abundant community was detected, still with *Solicoccozyma* spp. as the dominant taxa yet comprising additional Basidiomycota (*e.g.* Agaricomycetes and *Malassezia* spp.) and Ascomycota (*e.g. Saccharomyces* spp., and Sordariomycetes such as *Chrysonilia* spp., *Fusarium* spp. and *Trichoderma* spp.). The very low amount and/or integrity of DNA extracted from BL3 and BL4 glues, which were removed from the same manuscript as the silks (Or.8210/S.417), retrieved no robust sequencing data. On the other hand, BL5 and BL6 glues (removed from the manuscript Or.8210/S.316) showed similar diversity of Basidiomycota OTUS though higher diversity and abundance of Ascomycota OTUs (namely of Sordariomycetes and Eurotiomycetes) were detected in the last. Within the Ascomycota OTUs associated to BL6 glue, some sequences matched those of the isolates found in this glue, namely *A. tubingensis* (1 OTU), *C. sitophila* (1 OTU) and *P. crustosum* (3 OTUs) (Figure 3B and Table S1, Supplementary information). The most abundant OTUs found in the DNA extracted from the glues (BL5 and BL6) matched *Malassezia* spp., whereas *Solicoccozyma* spp. dominated the silks (BL1 and BL2). The presence of *Malassezia* spp. may be due to the animal origin of the glues (consistent also with the detection of calcium in this samples, Figure 2C and 2E), although one cannot disregard the mishandling of Or.8210/S.316 manuscript during the silk-facing procedure

(human skin origin). On the contrary, the presence of OTUs associated with ubiquitous fungi may have originated from past contamination during any stage of their cross-continental transport and storage (Wood and Barnard, 2010).

4. Conclusions

Our data report the historic existence of multiple sources of fungal contamination of the ancient Chinese manuscript. The analysed silks/glues contained cellulolytic fungal strains which reinforce a potential risk of deterioration if conditions of humidity and temperature favour fungal growth. Although this is a preliminary study on a restricted sample set, it constitutes one of the few reports on the total mycobiota associated with ancient manuscripts, and the first report focussing on NGS amplicon sequencing for genomic fingerprinting. Further studies are necessary to unveil the impacts of fungal contamination in the long-term conservation of silk-faced ancient Mogao Grottoes manuscripts, which might further favour the need of removing its silk facing.

Acknowledgements

We wish to thank Mr. Stephen McFarland (QUB) for use of the SEM-EDAX instrument, and Dr. G. Srinivasan for useful discussions. C.M. and C.S.P. are grateful to the Fundação para a Ciência e a Tecnologia (FCT), Portugal, for funding, namely through the grant SFRH/BD/118377/2016 (C.M.) and grant UID/Multi/04551/2013 (Research unit GREEN-it "Bioresources for Sustainability").

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