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

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15   **ABSTRACT**

16   Silking, a conservation technique which involved gluing silk gauze over the face of a

17   manuscript was popular in the mid-20th Century, especially for treating early Chinese

18   documents. The method is now little used, and the question as to whether silking

19   interventions should be reversed is controversial, given the high economic cost of active

20   intervention, and there are few scientific studies as to the long-term consequences of the

21   technique. Silk-facing materials from documents of the Stein collection were analysed using

22   scanning electron microscopy coupled with energy dispersive X-ray spectroscopy. The

23   mycobiota diversity was unravelled through the combination of culture dependent methods

24   and amplicon sequencing analyses. The SEM micrographs showed smooth regular nodules of

25   *ca.* 3-5 µm diameter on both silk threads and glue paste. This morphology differs from the

26   irregular and the crystalline morphologies of glue paste and inorganic crystallites,

27   respectively, but it is consistent with that of small-sized conidia (asexual spores of fungi) or

28   yeasts. Glue paste demonstrated three fungal strains: *Aspergillus tubingensis*, *Penicillium*

29   *crustosum* and *Chrysonilia sitophila* which display cellulolytic activity except the last.

30   Amplicon sequencing revealed that silk threads and glue paste host distinct mycobiota. Here,

31   we preliminary show that the silking method may be affecting the overall integrity of the silk-

32   faced manuscripts, principally due to contamination with cellulolytic fungal strains. Unless

33   the silk facing is removed, irreversible damage to the documents is highly probable.

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

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

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

80

81 **2. Materials and methods**
82 *2.1. Samples*
83 The manuscripts originate from Dunhuang, dated from the 5th to early 11th Centuries,
84 discovered by Yuanlu Wang in 1900 (Wang and Perkins, 2008). They were sealed in Cave
85 17, known as the Library Cave, in the Mogao Grottoes, where closely packed layers of
86 heaped bundles of scrolls were discovered, along with textiles, such as banners, as well as
87 figurines of Buddha (damaged) and other Buddhist artefacts. Mogao Grottoes enclose
88 important cultural heritage and have been listed officially as UNESCO World Heritage Site
89 in 1987 (Wu et al., 2017). The manuscripts, many of which reside in the British Library, are
90 referred to as the Stein Collection (Wang and Perkins, 2008).

91   Two representative manuscripts with silk-facing were selected for this study (Figure
92 1A and 1B), namely Or.8210/S.417 and Or.8210/S.316 (*n.b.* this is the British Library
93 registration system for manuscripts from Dunhuang in Stein Collection, and uniquely defines
94 a document), from which silk threads (BL1 and BL2, showing distinct yellowing of the
95 fibres) and glue (BL3-BL6) were removed and donated by the British Library.

96

97

98 *2.2. Surface analyses*
99 In order to investigate the deterioration of the silk facing materials, a scanning electron
100 microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDAX) was
101 employed: JEOL JSM-6500F Field Emission Scanning Electron Microscope and Oxford

102 instrument INCA X-sight 7558 (School of Mathematics and Physics at QUB). Silk samples
103 were sputter-coated with gold, and were affixed *via* copper tape to the SEM sample holders.
104

105 *2.3. Cultivable fungi isolation, identification and characterisation of cellulolytic activity*
106 Following the identification of structures in the SEM data, of similar size and shape to fungal
107 spores and/or yeasts, it was speculated that this may be due to fungal contamination. To
108 isolate any cultivable fungal strains, the samples were incubated in a sterile peptone solution
109 (2 %) for three days at room temperature followed by vortex cycles, and aliquots were then
110 directly inoculated onto Malt Extract Agar (MEA) and incubated at 27 ºC. The forming
111 colonies were monitored daily. Isolated colonies were selected for further purification by
112 consecutive sub-culturing onto fresh MEA. Morphological characterisation (microscopy)
113 allowed their preliminary identification. Negative controls, *i.e.* similar materials (unused
114 paper pieces collected inside the laboratory) manipulated alongside with the study samples,
115 were used to discard the possibility of cross-contamination during the analysis.

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

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

129 To assess cellulolytic activity, each strain was platted onto carboxymethylcellulose
130 (CMC) agar (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium
131 salt, 0.02% peptone, and 1.7% agar) and incubated at room temperature. At the third and
132 tenth day of incubation, the plates were flooded with Gram's iodine (binds to CMC) for
133 5 min and the excess of reagent removed. The formation of a decolouration halo indicates the
134 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 ℃ 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

169 and 1G), but only organic content (apart from traces of copper from the support) in those

170 devoid of glue residues (Figure 1F and 1G). The presence of calcium likely originates from

171 the glue itself, maybe reflecting its animal origins, whereas that of aluminium is consistent

172 with the use of a weighting process of the silk block (Des Barker et al., 2006).

173

174 *3.2 Microbiological contamination*

175 The silking technique might have also impacted the manuscripts ability to be colonised by

176 living organisms. The SEM micrographs showed smooth regular nodules of *ca.* 3-5 µm

177 diameter on both silk threads and glue paste (Figure 2B). This morphology differs from the

178 irregular and the crystalline morphologies of glue paste and inorganic crystallites,

179 respectively, but it is consistent with that of small-sized conidia (asexual spores of fungi) or

180 yeasts. With the exception of BL6 glue that provided three fungal strains, the remaining silks

181 and glues here analysed have not provided any cultivable isolate. These were *Aspergillus*

182 *tubingensis*, *Chrysonilia sitophila* and *Penicillium crustosum* (Figure S2, Supplementary

183 information). *Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp. and *Mucor* spp. have been

184 reported before as the prevalent cultivable taxa in antiques stored in old libraries (Cappitelli

185 et al., 2010; Nevalainen et al., 2015; Sterflinger and Pinzari, 2012). Moreover, *Penicillium sp.*

186 and *Aspergillus sp.* have both been identified in the air (Wang et al., 2011) and the walls (Ma

187 et al., 2015) of the Mogao Grottoes, and were also isolated from old textile artefacts in

188 Eastern Europe (Ljaljević-Grbić et al., 2013). *Penicillium crustosum* and *A. tubingensis* are

189 considered common indoor fungi (Nevalainen et al., 2015). They produce small-size conidia

190 of *ca.* 3 µm, hence similar to those observed in the SEM micrographs (Figure 2B). Both

191 species are able to tolerate very low water activities (a_w) with optimal growth at *ca.* 0.83-

192 0.85. Their occurrence in the British Library raises serious clinical concerns, since they can

193 produce neurotoxins, particularly penitrem A, and act as an opportunistic pathogen of

194 immunocompromised/competent patients (*e.g.* bone osteomyelitis or keratitis), respectively

195 (Bathoorn et al., 2013; Moldes-Anaya et al., 2012). On the other hand, even if less frequently,

196 *C. sitophila* (optimal a_w *ca.* 0.9) has been also identified in the indoor environment of

197 historical buildings, libraries and museums (Ljaljević-Grbić et al., 2013). Despite its

198 abundant, readily airborne conidia, systematic evidence that this fungus is the causal agent of

199 any disease, infection or significant allergies is still lacking, with the exception of its

200 association with suberosis (*viz.* hypersensitivity pneumonitis) (Cordeiro et al., 2011).

201 Importantly, the three vital strains isolated from sample 6 were tested for their

202 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

237   (human skin origin). On the contrary, the presence of OTUs associated with ubiquitous fungi

238   may have originated from past contamination during any stage of their cross-continental

239   transport and storage (Wood and Barnard, 2010).

240

241   4. Conclusions

242   Our data report the historic existence of multiple sources of fungal contamination of the

243   ancient Chinese manuscript. The analysed silks/glues contained cellulolytic fungal strains

244   which reinforce a potential risk of deterioration if conditions of humidity and temperature

245   favour fungal growth. Although this is a preliminary study on a restricted sample set, it

246   constitutes one of the few reports on the total mycobiota associated with ancient manuscripts,

247   and the first report focussing on NGS amplicon sequencing for genomic fingerprinting.

248   Further studies are necessary to unveil the impacts of fungal contamination in the long-term

249   conservation of silk-faced ancient Mogao Grottoes manuscripts, which might further favour

250   the need of removing its silk facing.

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