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ORIGINAL ARTICLE

Characterization of the archaeal and fungal diversity associated with gypsum efflorescences on the walls of the decorated Sorcerer's prehistoric cave

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Abstract

Purpose This study focuses on analysis of the archaeal and fungal diversity associated with gypsum efflorescences damaging the walls of the Sorcerer's prehistoric cave registered as a world cultural heritage site.

Method Archaeal 16S rDNA and fungal internal transcribed spacer (ITS) clone libraries were constructed and analysed.

Results Two thaumarchaeotal OTUs belonging to the Nitrososphaeraceae family dominated the archaeal community (100% of clones). Nitrososphaeraceae are obligate aerobic, chemolithoautotrophic organisms that derive their energy from the oxidation of ammonia and may contribute to primary productivity in the cave. Seven fungal OTUs belonging to Ascomycota and one belonging to Basidiomycota were present. The Cordycipitaceae family, mainly represented by entomophilous fungi, dominated the analysis (66.7% of clones).

Conclusion We show that archaeal and fungal OTUs are associated with gypsum efflorescences damaging the walls of the Sorcerer's cave. The role of these microorganisms in the deterioration of the walls of the cave remains to be determined.

Keywords Cave · Gypsum · Biodeterioration · Archaea · Fungi · Diversity

Introduction

Characterization of the microorganisms associated with biodeterioration processes encountered in rock art caves and shelters is needed (Saiz-Jimenez et al. 2011, 2012; Lepinay et al. 2017, 2018), as also in mural painting from catacombs and tombs (Sanchez-Moral et al. 2005; Laiz et al. 2009; Vasanthakumara et al. 2013; Krakova et al. 2015). These works of art are constantly threatened by decay resulting from the interaction of biological and physicochemical environmental factors that can alter the rock substrate according to their mineralogical composition and structure (Chamley 2003; Saiz-Jimenez 2015).

Decorated caves and shelters are natural cavities subjected to natural deterioration processes. Natural cavities are nutrient-poor environments containing an abundant and diverse sessile biomass (Barton and Jurado 2007; Lavoie et al. 2010). Representatives of the 3 domains of life are recovered in these subterranean environments. As in most environments, the focus in the caves was on the diversity of bacteria. Among them, the most abundant and frequently retrieved phyla are as follows: Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Nitrospirae and Proteobacteria (Saiz-Jimenez 2015). Previous work in the Sorcerer's cave showed that Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes and Planctomycetes were the main bacterial inhabitants of areas of salt efflorescences damaging the walls (Lepinay et al. 2018). Actinobacteria was the most prevalent phylum, mainly represented by members of the Pseudonocardiaceae family, data consistent with other findings (Barton and Jurado 2007; Porca et al. 2012; Riquelme et al. 2015; Wu et al. 2015).

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Conversely, a limited number of investigations were carried out on Archaea, particularly for sites of cultural heritage preservation. However, many Archaea are adapted to nutrient-poor conditions and may contribute to nutrient cycling in oligotrophic environments through nitrogen fixation, methanogenesis, sulphur oxidation, nitrification and ammonia oxidation (Ettenauer et al. 2010; Jarrell et al. 2011; Meng et al. 2016, 2017). To date, the most frequently recovered archaeal phylum is Thaumarchaeota (Northup et al. 2003; Chelius and Moore 2004; Legatzki et al. 2011; Miller et al. 2012; Ortiz et al. 2013). Members of the Crenarchaeota have also been retrieved from steam vents and caves from volcanic national parks in the USA (Benson et al. 2011) and Euryarchaeota in caves (Macalady et al. 2006; Reitschuler et al. 2014, 2016). In addition to Bacteria and Archaea, fungi are very well represented in cave environments due to their high rate of spore production and air dispersion. They are important in the feeding strategies of cave fauna because they are important decomposers providing food for many organisms (Nováková 2009). As reviewed by Vanderwolf et al. (2013) and recently completed by Zhang et al. (2017), > 1150 fungal species in 550 genera have been discovered in caves and mines worldwide by 2017. The most frequently encountered genera (e.g. *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium*, *Trichoderma*, *Cladosporium*, *Alternaria*, *Paecilomyces*, *Acremonium*, *Engyodontium*) are air-borne and soil-borne fungi that come from outside the cave cavities due to air circulation (Saiz-Jimenez 2015). Several efforts have been made to characterize the diversity and distribution of fungi in caves, but there is little information on taxa associated with specific niches, such as oligotrophic conditions and mineral formations (Saiz-Jimenez 2015).

We have focused on the French Sorcerer's prehistoric cave ("La grotte du Sorcier"). The cave undergoes alternations of humidification and desiccation phases which induce cycles of dissolution and recrystallization of the salts leading to efflorescence development on the walls that threaten the engravings (Pigeaud et al. 2012). This work completes the previously published data corresponding to bacterial diversity following analysis of the archaeal and fungal diversity associated with saline efflorescences (Lepinay et al. 2018).

Materials and methods

Site description and sampling

The Sorcerer's cave, the Saint-Cirq cave, dug in a Turonian siliceous limestone, harbours several prehistoric engravings already described by different archaeologists (Fig. 1) (Delluc et al. 1987; Karnay et al. 1999; Pigeaud et al. 2012). Formation of gypsum efflorescences are present absolutely everywhere in the cave, from the entrance to the bottom. The gypsum deposits are located on the outer layer of the stone and make it a kind of extremely fragile "molasses" (Pigeaud et al. 2012). The same samples as those collected by Lepinay et al. (2018) in April 2015 (SCApr15) have been analysed. Sterile scalpels were used to collect 3 efflorescence areas (each ~ 2 cm^2) and the underlying rock from a depth of ~ 2 mm, (Fig. 1b, c). Samples were mixed together and homogenized in sterile mortars. Samples were stored at − 20 °C awaiting molecular analysis. Scanning electron microscopy observations of the samples and elemental and mineralogical analyses of the corresponding saline efflorescences have been previously published (Lepinay et al. 2018). This indicated that the formation of gypsum on the walls of the cave was mainly related to geochemical phenomena, efflorescences being mainly composed of calcium, sulphur and oxygen, with additional small amounts of nitrogen and carbon.

DNA extraction and purification

DNA extraction was done as previously described (Zhou et al. 1996; Lepinay et al. 2018). The protocol combines the use of enzymatic (proteinase K) and chemical (CTAB and SDS) components to ensure efficient cell lysis. Extracted, nucleic acids were purified with a Power Biofilm™ DNA Isolation Kit (MoBio) and were stored at − 20 °C until use.

PCR amplification of fungal ITS and archaeal 16S rDNA genes

Fungal ITSs were amplified using primers ITS1-F and ITS4. A 25-μl reaction comprising 20 ng of DNA, 100 μM of each dNTP, 0.4 μM of each primer, 1× PCR buffer and 1.5 U of Accusure DNA Polymerase (Bioline, France) was performed. Cycling conditions were as follows: 5 min at 95 °C, followed by 30 cycles of 20s at 95 °C, 15 s at 54 °C and 1 min and 30 s at 68 °C, and a final extension at 68 °C for 7 min. Amplicon was extracted from an agarose gel after electrophoresis using the Nucleospin Gel and PCR clean up kit (Macherey Nagel, France). Archaeal 16S rRNA gene sequences were amplified using a semi-nested PCR approach. Archaeal universal primers 340F-pSTC (5′-CCTTCgCCgACTgACCCTAY-GGGGYGCASCAG-3′) and 1000R (5′-GGCCATGCACYWCYTCTC-3′) (Gantner et al. 2011) were used as outer primers. Then, a semi-nested PCR was carried out using 340F-pSTC and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) (Stahl and Amann 1991). The PCR reaction was carried out as previously described, using an annealing temperature of 57 °C (Lepinay et al. 2017). One microlitre of the first PCR was used in the nested PCR, and an amplicon of ~ 580 bp was purified as described above.

Fig. 1 The Sorcerer's cave (Saint-Cirq-du-Bugue, Dordogne, France): **a** entrance (white arrow); **b** sampling areas 1, 2 and 3; **c** enlarged view of the sampling area no. 2 showing gypsum efflorescences. Credit photo: LRMH

Clone libraries

Purified PCR products were inserted into the pSTC1.3 vector of the StabyCloning kit (Eurogentec, Belgium), and recombinant plasmids were electrotransformed in *E. coli*. Transformed cells were plated onto LB agar containing 50 μg/ml ampicillin and incubated overnight at 37 °C. PCR amplifications of inserts using primers targeting the pSTC1.3 vectors: pSTC-F (5′-AATGCAGCGCGTTAGAA-3′) and pSTC-R (5′-CGCC CGGTTTATTGAAA-3′), followed by agarose electrophoresis in 1.5% agarose gel, allowed to select clones presenting inserts at the expected sizes for archaeal 16S rDNA and ITS libraries, respectively. Restriction fragment length polymorphism (RFLP) was used to screen these clones. Each PCR product was digested using the restriction enzymes, AluI and RsaI (Fermentas, France) for 16S rDNA amplicons or with the restriction enzyme BshfI (Fermentas, France) for ITS amplicons. At least one clone per RFLP profile was partially sequenced (Eurofins Genomics, Germany).

Sequence analysis

Sequences were first checked using the software FinchTV v1.4 (Geospiza, Inc.). Chimeric sequences identified with the Decipher program (Wright et al. 2012) were excluded. ITS and 16S rDNA sequences were clustered into OTUs (operational taxonomic unit) at an overlap percentage identity cut-off of 98% using ClustalW (Thompson et al. 1994). BLASTn was then used to compare DNA sequences with those in the GenBank database (Basic Local Alignment Search Tool, http://blast.ncbi.nih.gov/Blast.cgi). Sequences with no significant similarities were excluded from the analysis. The affiliation of each cloned sequence to a genus or species was based on a similarity ≥ 95 or ≥ 98%, respectively, with the closest identified phylogenetic sequence in GenBank (Yarza et al. 2008). Archaeal sequences were also classified using the Naïve Bayesian Classifier from the RDP project with a confidence threshold of 80% (https://rdp.cme.msu.edu/classifier/classifier.jsp). We assessed whether DNA clone libraries were large enough to be representative of OTU richness from samples. S-Chao1 and Good's C index of coverage were calculated as described by Kemp and Aller (2004).

Nucleotide sequence accession numbers

The nucleotide sequence data reported herein have been deposited in the NCBI nucleotide sequence database under accession numbers MK212376 to MK212382, MK590246 to MK590262 for fungi, and MK226534 to MK226535 and MK607017 to MK607025 for Archaea.

Results

OTU richness and its reliability

Analysis of RFLP patterns allowed grouping 178 archaeal 16S rDNA clones into 4 restriction patterns and 91 fungal ITS clones into 10 restriction patterns. When the number of clone inside a RFLP pattern was higher than 2, several randomly chosen clones were partially sequenced. After elimination of chimeric and low quality sequences, 172 archaeal 16S rDNA clones and 81 fungal ITS clones remained in the banks. Pairwise alignments of randomly chosen clone sequences using 98% similarity as the cut-off allowed discrimination of 2 and 8 OTU in the archaeal 16S rDNA and the fungal ITS libraries, respectively (Tables 1 and 2).

Estimated OTU richness (S-Chao1 estimator) was compared to the values of cloned libraries Kemp and Aller (2004). Predicted S-Chao1 values were of 2 and 8 phylotypes for the archaeal 16S rDNA and the fungal ITS libraries, respectively. Calculation of Good's C indices showed that most of the sample diversity was represented in the clone libraries. Indeed, coverage was 0.99 for both libraries, which indicates that the analysis of an increasing number of clones would have shown only a little further richness.

Archaeal diversity

Only the Thaumarchaeota phylum was recovered in the archaeal 16S rDNA clone library (Table 1); this phylum was

Table 1 Assignment of archaeal 16S rDNA sequences to their closest cultured representative

OTU	Representative clones*	% of clones**	RDP family***	Closest cultured representative (percentage similarity)	Closest Candidatus organism (percentage similarity)
Thaumarchaeota					
OTU-ARC-01	SC-Arc-09 (MK607017); SC-Arc-30 (MK226534); SC-Arc-47 (MK607019); SC-Arc-64 (MK607018); SC-Arc-69 (MK607020); SC-Arc-95 (MK607021); SC-Arc-B2-12 (MK607022); SC-Arc-B2-35 (MK607023); SC-Arc-B2-56 (MK607024); SC-Arc-B2-74 (MK607025)	99.4% (171/172)	Nitrososphaeraceae (100%)	*Nitrososphaera viennensis* strain EN76 (95.8 to 96.3%)	*Candidatus* Nitrosocosmicus oleophilus strain MY3 (99.4 to 100%)
OTU-ARC-02	SC-Arc-54 (MK226535)	0.6% (1/172)	Nitrososphaeraceae (100%)	*Nitrosopumilus maritimus* SCM1 strain SCM1 (96.2%)	-

*Number in brackets indicates the GenBank accession number

**In parentheses: ratio between the number of clones of the corresponding OTU and the total number of clones

***Bacterial family determined using the Naïve Bayesian Classifier from the RDP project website. The confidence threshold is 80%

represented by 2 phylotypes belonging to the archaeal family, Nitrososphaeraceae. One of them dominated the library and presented as closest cultured representative: *Nitrososphaera viennensis* strain EN76 (99.4% of clones, 95.8 to 96.3% sequence similarity, SS); and as closest *Candidatus* organism: *Candidatus* Nitrosocosmicus oleophilus strain MY3 (99.4 to 100% SS). The second one presented as closest cultured representative: *Nitrosopumilus maritimus* SCM1 strain SCM1 (0.6% of clones, 96.2% SS).

Fungal diversity

ITS sequences mainly belonged to the phylum Ascomycota (Table 2), with most represented family being the Cordycipitaceae (66.7% of clones). Cordycipitaceae were represented by 2 phylotypes, one being affiliated with *Isaria fumosorosea* strain BCMU PF01 (51.9% of clones, 99.8 to 100% SS), and the other related to *Engyodontium album* isolate MC_A31 (14.8% of clones, 95.5 to 95.6% SS). The second most abundant OTU belonged to the Mycosphaerellaceae family, being distantly related to *Pseudocercosporella* sp. 09CT02 (18.5% of clones and 92.9 to 93.1% SS). The fourth most abundant OTU belonged to the Cladosporiaceae family and corresponded to *Cladosporium pulvericola* CPC: 22403 (6.2% of clones, 100% SS). Three other ascomycotal families were recovered in the library, each representing 2.5% of clones that had sequence identities between 92 and 95% with their closest relative in Genbank, namely the Ophiocordycipitaceae, Diatrypaceae and Teratosphaeriaceae. Finally, Basidiomycota were represented by one clone belonging to the Polyporaceae family closely related to *Trametes hirsuta* isolate BHI-F579a (1.2% of clones, 99.8% SS).

Discussion

This study aims to improve the understanding of biological phenomena associated with the formation of gypsum efflorescences on the walls of the French Sorcerer's prehistoric cave. We have previously shown that these areas of gypsum efflorescences harbour a variety of bacterial communities and a dense subjacent biofilm (Lepinay et al. 2018). We continued the investigations of the microbial communities living in these zones of efflorescence by analysing Archaea and fungi.

Concerning Archaea, all the clones belonged to the phylum Thaumarchaeota, a phylum, previously referred to "mesophilic Crenarchaeota", being proposed by Brochier-Armanet et al. (2008). Thaumarchaeota have been detected by molecular studies in many subterranean habitats (Takei et al. 2001; Northup et al. 2003; Chelius and Moore 2004; Legatzki et al. 2011; Miller et al. 2012; Ortiz et al. 2013; Barton et al. 2014; Anda et al. 2017) and in many different environments, such as seawater (e.g. Church et al. 2003), freshwater sediments (e.g. Schleper et al. 1997) and soils (e.g. Ochsenreiter et al. 2003). All known ammonia-oxidizing archaea (AOA) belong to Thaumarchaeota and Thaumarchaeota are receiving much attention regarding their ability to proliferate in environments with low nutrient availability (Martens-Habbena et al. 2009; Pester et al. 2011; Ortiz et al. 2014). This phylum can represent the main source of

Table 2 Assignment of fungal ITS sequences to their closest cultured representatives and closest sequence matches

OTU	Representative clones*	% of clones**	Fungal family	Closest cultured representative (percentage similarity)	Closest sequence match (percentage similarity)***
Ascomycota					
OTU-FUNG-01	SC-ITS-04 (MK590248); SC-ITS-14 (MK590250); SC-ITS-28 (MK590251); SC-ITS-38 (MK212376); SC-ITS-42 (MK590252); SC-ITS-48 (MK590253); SC-ITS-49 (MK590254); SC-ITS-58 (MK590258); SC-ITS-74 (MK590260)	51. 9% (42/81)	Cordycipitaceae	*Isaria fumosorosea* strain BCMU PF01 (99.8 to 100%)	-
OTU-FUNG-02	SC-ITS-02 (MK590246); SC-ITS-50 (MK212378); SC-ITS-76 (MK590261)	18.5% (15/81)	Mycosphaerellaceae	*Acrodontium crateriforme* strain CPC 11509 (91.3 to 91.5%)	*Pseudocercosporella* sp. 09CT02 (92.9 to 93.1%)
OTU-FUNG-03	SC-ITS-39 (MK212377); SC-ITS-57 (MK590257); SC-ITS-79 (MK590262)	14.8% (12/81)	Cordycipitaceae	*Engyodontium album* isolate MC_A31 (95.5 to 95.6%)	Uncultured fungus clone RS-Apr-ITS37 (99.9 to 100%)
OTU-FUNG-04	SC-ITS-13 (MK590249); SC-ITS-55 (MK590256)	6.2% (5/81)	Cladosporiaceae	*Cladosporium pulvericola* CPC: 22403 (100%)	
OTU-FUNG-05	SC-ITS-30 (MK212379)	2.5% (2/81)	Ophiocordycipitaceae	*Thyronectria asturiensis* strain MA3 (92.5%)	-
OTU-FUNG-06	SC-ITS-44 (MK212380)	2.5% (2/81)	Diatrypaceae	*Eutypa consobrina* culture-collection CBS: 122678 (95.1%)	-
OTU-FUNG-07	SC-ITS-33 (MK212381)	2.5% (2/81)	Teratosphaeriaceae	*Teratosphaeria knoxdaviesii* strain CBS 122898 (94.7%)	Uncultured fungus clone RS-Apr-ITS18 (99.3%)-
Basidiomycota					
OTU-FUNG-08	SC-ITS-52 (MK590255)	1.2% (1/81)	Polyporaceae	*Trametes hirsuta* isolate BHI-F579a (99.8%)	Uncultured fungus clone S98 (100%)

*Number in parenthesis indicates the GenBank accession number

**In parentheses: ratio between the number of clones of the corresponding OTU and the total number of clones

***The "-" indicates that the closest sequence corresponds to the one of the closest cultured representatives

nutrient in closed or semi-closed environments, e.g. caves (Hathaway et al. 2014).

The library we reached was dominated by Thaumarchaeota of the Nitrososphaeraceae family and was closely related to genera *Nitrososphaera* and *Nitrosopumilus*. Members of the Nitrososphaeraceae family are AOA that derive energy from the oxidation of ammonia to nitrite that can be a source of nitrogen for other organisms in close and semi-close environments, such as the Sorcerer's cave. Unlike rosy saline efflorescences found in many subterranean environments (reviewed by Piñar et al. 2014), the archaeal diversity associated with gypsum efflorescences did not correspond to the halophilic archaeal genera *Halococcus*, *Halobacterium* and *Halalkalicoccus*. Our most abundant Thaumarchaeota sequence showed similar percentage > 99% with *Candidatus* Nitrosocosmicus oleophilus strain MY3, a *Candidatus* organism retrieved from contaminated soils. No archaea with metabolic functions associated with sulphur cycling were detected, showing that Thaumarchaeota were not directly involved in the formation of gypsum efflorescences. Meng et al. (2017) showed a high occurrence of AOA closely related to genera *Nitrososphaera*, *Nitrosopumilus* and *Nitrosotalea* on Angkor monuments. AOA may play an important role in the biodeterioration of Angkor monuments by nitrogen cycling and nitric acid production.

The Cordycipitaceae family was the most represented fungal family in the cave. Fungi from this family are particularly known to have a significant negative impact on global human and animal health (Menzies and Turkington 2015). The most abundant OTU recovered in the library was related

to the entomophilous fungi, *I. fumosorosea*, a pathogen of 7 insect orders (Humber and Hansen 2005). Entomophilous fungi are the most abundant fungal phylotypes in the Lascaux cave, where they may have an important ecological role (Bastian et al. 2009; Martin-Sanchez et al. 2015). *Isaria fumosorosea* was identified as a facultative oligotrophic species able to adapt to particular conditions of caves by Jiang et al. (2017). Martin-Sanchez et al. (2015) described the presence of entomophilous fungal phylotypes in caves, which may be due to cave-dwelling arthropods feeding on fungal mycelia and disseminating their spores in their excrement during their movements, and by their bodies acting as a support for the attachment of spores. Moreover, as *I. fumosorosea* is used as a microbial insecticide to control several pests around the world (Zimmermann 2007a, b, 2008; Gurulingappa et al. 2011), its abundance in the cave could also be the consequence of the air circulation into the cave. The second most abundant Cordycipitaceae phylotype is distantly related to *Engyodontium album* a widespread species that can also be harmful to the health of humans and mammals (Siegel and Shadduck 1990; Goettel et al. 2001; Nucle Tucker et al. 2004; Balasingham et al. 2011). Interestingly, this phylotype has already been retrieved from areas of gypsum crystallization on the walls of a decorated shelter located only some kilometres from the Sorcerer's cave (Lepinay et al. 2017).

The second most represented OTU corresponded to an uncultivated *Pseudocercosporella* sp. from the Mycosphaerellaceae family. Mycosphaerellaceae include thousands of species of phytopathogenic fungi (Aguilera-Cogley et al. 2017). An OTU related to *Pseudocercosporella* sp. previously identified in the air of the carbonate cave located in China's Shuanghe National Geographic Park is well adapted to these oligotrophic conditions (Jiang et al. 2017).

The next most represented OTU was the cultivated *Cladosporium pulvericola* species, already recognized as inhabitants of caves. Indeed, Pusz et al. (2015) showed that *Cladosporium* spp. were the dominant fungi of the internal atmosphere of the Jarkowicka cave in Poland.

All other ascomycotal OTUs had similar percentages < 96% with any other existing Genbank sequence and thus corresponded to fungal species never previously recovered in any other environment. These phylotypes belonged to 4 different families: the Ophiocordycipitaceae, a family of parasitic fungi (Sung et al. 2007); the Diatrypaceae, a family related to wood decay (de Almeida et al. 2016); Teratosphaeriaceae, rock-inhabiting fungi (Ruibal et al. 2008); and the previously described *Cordycipitaceae*.

Finally, one clone corresponded to the Basidiomycota specie, *Trametes hirsuta*. Basidiomycota are often found in caves; however, they were in lower abundance and diversity than Ascomycota (Vanderwolf et al. 2013; Zhang et al. 2017). This may be explained by the scarcity of nutrient-rich substrates in caves, such as plant debris and animal excrements (Vanderwolf et al. 2013; Zhang et al. 2017).

Conclusion

This study highlighted that gypsum efflorescences damaging the walls and the engravings of the French Sorcerer's cave harbour several archaeal and fungal phylotypes. Analyses indicated the dominance of ammonia-oxidizing archaea belonging to the Nitrososphaeraceae archaeal family and the entomophilous member of the Cordycipitaceae fungal family. Equivalent results were obtained from other cave environments not associated with gypsum efflorescences (Northup et al. 2003; Chelius and Moore 2004; Bastian et al. 2009; Legatzki et al. 2011; Miller et al. 2012; Ortiz et al. 2013; Martin-Sanchez et al. 2015). With the exception of the 2 most abundant Cordycipitaceae phylotypes, most of the other archaeal and fungal phylotypes had similarity percentages below 97% with their closest cultured representatives. This showed that they correspond to yet uncultured microorganisms. Effort to characterize the physiology of these microorganisms is needed to gain more insight into their roles and behaviour in this particular ecosystem.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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