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Fungal and bacterial outbreak in the wine vinification area in the Saint-Marcel show cave

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Abstract

In the Saint-Marcel cave (France), wood barrels and thousands of bottles containing red wine were stored for vinification. After storage began, a fungal and bacterial outbreak occurred, and the area was invaded by numerous types of mold colonizing the cave ceilings and all objects related to human activities (the stairwell and oenological materials). In this study, using the metabarcoding approach, we have studied the microbial outbreak and have linked the identified microorganisms to oenological activity. Both 16S and ITS primers were used to sequence the samples collected from the cave. The results showed that the dominant microorganisms proliferating in the cave were related to wine vinification. For instance, *Zasmidium cellare,* a strain known for living in dark and ethanol-rich environments, was the dominant fungus on the cave stairwell. Furthermore, *Guehomyces pullulans*, a cold-adapted yeast used for juice clarification, was recorded as the major species on the blackened limestone ceilings. These findings reveal a complex community structure in the studied cave based on the assembly of bacteria and fungi. Finally, our results demonstrate that oenological activities could seriously affect cave preservation, changing the natural microbial communities populating cave environments.

Keywords: Fungal outbreak, Sequencing, Show cave, Bacterial communities, Biodeterioration

1. Introduction

For centuries, people have been visiting caves for tourism or recreation. As evidence of this activity, a signature dated from 1213 was found in the Postojna cave in Slovenia (Cigna, 2012). Currently, 250 million tourists visit more than 800 show caves around the world every year (Cigna, 2016). Tourist caves offer a wide range of activities to tourists (e.g., speleology, concerts, theatre, aquarium expositions, and wine tastings), generating 2.3 billion dollars per year (Cigna and Burri, 2000). However, natural site anthropization leads to microbial disturbances (Cennamo et al., 2012), even if ecological considerations become increasingly important. In the case of natural caves, microorganisms such as some bacteria or autotrophic protists can take advantage of the environmental changes (e.g., increased artificial light, carbon dioxide and organic matter) to proliferate. For instance, lampenflora are well known and widely described (Borderie et al., 2014; Cennamo et al., 2016). The first observations were performed by Kyrle and Gams in 1923 and 1925, respectively (Cigna, 2011; Lamprinou, 2014). Since then, optical observations and the use of molecular methods have permitted a better overview of these organisms, which live together in a matrix called a biofilm. In addition to lampenflora, other microorganisms, such as fungi, have often been involved in microbial infestation of caves, such as the infestation that happened in the world-famous Lascaux Cave (Bastian et al., 2009a, 2009b, 2009c). The negative consequences of lampenflora and fungal outbreaks are numerous. In addition to creating a visual disturbance for tourists, microorganisms lead to limestone and wall painting degradation by physical or chemical actions (Borderie et al., 2014). Moreover, some microorganisms, such as fungi, can be dangerous for human health by inhalation of fungal spores (Jurado et al., 2010).

The Saint-Marcel cave is an important cave system located at the end of the “Gorges de l'Ardèche” (France). In addition to numerous other show caves around the world, this cavity suffers from lampenflora growth inside the tourist area. In France, sodium hypochlorite

is commonly used as a treatment against phototrophic and heterotrophic organism proliferation (Pfendler et al., 2017b). However, despite several treatments each year and a new lighting strategy consisting of limiting illumination to tourist hours, lampenflora growth in the Saint-Marcel cave continues. In addition, ceilings, some walls and some remarkable speleothems remain inaccessible to direct treatment, leading to a densification of microorganisms and a strong visual impact.

Furthermore, since April 2015, oenological activities have been undertaken in Saint-Marcel cave. To vinify red wine, several producers from Saint-Marcel village have stored 300 litres of wine in wooden barrels and one thousand wine bottles. Wine tastings in the cave, associated with guided speleological activities, are a part of an oenotourism project. However, after one year, strong fungal growth started on the wooden barrels and on the bottles in the wine storage section at the beginning of the tourist area, near the access gate and the “Gallery of Painters”. Given the amount of mold, some other parts of the cave may be endangered by displaced spores due to the strong air dynamics that affect the speleological system. Several authors have described fungal communities in wine cellars (Simeray et al., 2000; Hass et al., 2010) or in natural cavities (Stomeo et al., 2009; Taylor et al., 2014). However, as far as we know, fungal communities related to wine vinification in show caves have never been described.

The aim of this study was to assess the bacterial and fungal communities present following the establishment of oenological activities in a natural cavity. Widespread fungal and bacterial growth was observed on several types of materials present (metal, wood, PVC, and limestone) inside the Saint-Marcel cave. Samples were taken, and high-throughput sequencing (Illumina MiSeq) was used to determine microbial community structures. The ecological role of the microorganisms and their relation with wine storage are discussed.

92

93 **2. Material and methods**

94 *2.1. Cave description*

95 The catchment of the karst system on the Saint-Remeze plateau covers 70 km². The
96 speleological network now extends more than 60 km, and five interconnected networks have
97 been explored by speleologists. The lowest is a phreatic system 17 km long and -114 m deep,
98 which has been explored by cave divers. Network I, studied in this present work, consists of
99 the historical galleries of the cavity, and the increase in elevation from the natural entrance to
100 the tourist part is 87 m. This area was the first part of the cave opened to tourism, which
101 started in 1839. Most of the cave explorations started in this way. The morphologies of the
102 galleries are typical of an old drainage system (Mocochain, 2007), 4 km long, in several
103 interconnected galleries. The current tourist part (104 m deep) dates from 1988 and is
104 accessible from a dug tunnel; this part ends at the top of the mason vault, where it joins the
105 cathedral, located 600 m from the natural entrance. The show cave in this area is four hundred
106 metres long.

107 Two main modes of air circulation and intermediate modes operate according to
108 seasonality. During the winter, the traffic mainly proceeds from the natural entrance and other
109 low entrances towards the higher parts of the system connected with the surface (Aven du
110 Deyspesse, etc.). During the summer, the direction of circulation is from high parts to low
111 parts. During spring and fall, transitional systems are established over short periods of time
112 depending on temperature differences between the surface and the galleries of the cave. The
113 flow of air passing through the natural entrance has values of 7 to 11 l $m^3.s^{-1}$ and relatively
114 high CO_2 values from 1.5 to 2.5%. The airflow velocity was monitoring with a hot wire
115 anemometer Testo 405 and an ibrid MX 6 Gas sensor with infrared CO_2 and O_2. The weekly

116 measurements led for 3 months. In April 2015, red wine vinification started in the Saint-
117 Marcel cave. Thirteen wine barrels were stored 200 m from the cave entrance at 11 to 13.5°C
118 (Bondil, 2019). More than 2,500 bottles per year were maintained in zinc lockers, and 3,600
119 litres of wine were aged in oak barrels.

120

121 *2.2. Fungal outbreak observations*

122 In the Saint-Marcel show cave (Fig. 1), a fungal outbreak was detected in February
123 2019. In the entrance, substantial blue-grey molds were observed on the metal stairwell (Fig.
124 2 A). Below the stairwell, 13 stored wine barrels (Fig. 2 B) were contaminated with molds of
125 different colours (*e.g.*, green, yellow, white, and grey). These barrels were placed on wooden
126 and limestone supports, which were also colonized with fungal mats. The fungal colonies
127 showed several colours, sizes and structures (*e.g.*, filamentous or cottony). For instance, some
128 proliferations were 20-30 cm high and 15-20 cm wide (Fig. 2 C). Moreover, a fine and white
129 mold was proliferating (Fig. 2 C) on a PVC garden hose, which was stored on the floor
130 behind the barrels. Fig. 2 D shows black fungal proliferation on the limestone ceiling next to
131 and above the wine barrels. Finally, green or white molds (Fig. 2 E) colonized wine bottles,
132 more precisely on bottle corks.

133

134 *2.3. Sampling in the wine storage area*

135 Fifteen samples were collected in March 2019 in different contaminated parts of the
136 wine storage area. Samples were taken in triplicate from stairwells, barrels, the PVC hose, and
137 limestone and barrel supports, corresponding to the tourist pathway, wine equipment, cave
138 soil and ceilings, respectively. In accordance with the sequencing platform (Microsynth AG,
139 Balgach, Switzerland) protocol, at least 1 g of fresh matter was taken from each microbial

growth instance. To avoid unwanted contamination, samples were directly scraped and collected in 50-ml sterile tubes. To keep the samples dry, 2 silica-gel bags were subsequently added to each tube. Samples were then maintained at room temperature during two days until total DNA extraction, amplification steps and sequencing.

2.4. Molecular methods and data analysis

The library creation, sequencing and data analysis described in this section were performed by Microsynth AG (Balgach, Switzerland). The V3 and V4 regions of the bacterial 16S rRNA gene were sequenced in a two-step Nextera PCR library procedure using the primer pair 341F (5′- CCT ACG GGN GGC WGC AG -3′) and 802R (5′- GAC TAC HVG GGT ATC TAA TCC -3′). To sequence the internal transcribed spacer (ITS2) regions of the fungal 18S rRNA gene, two-step Nextera PCR libraries using the primer pair ITS3 (5′- GCA TCG ATG AAG AAC GCA GC -3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC -3′) were created. The Illumina MiSeq platform and a v2 500 cycle kit were used to sequence the PCR libraries. Then, FastQC (v. 0.11.8) was used to assess read quality. The locus-specific V34 and ITS2 primers were trimmed from the sequencing reads with the software Cutadapt (v. 2.3). Paired-end reads were discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to *in silico* reform the sequenced molecule considering a minimum overlap of 15 bases using the software USEARCH (v. 11.0.667). Merged sequences were then quality filtered, allowing a maximum of one expected error per merged read and discarding those containing ambiguous bases. From the remaining reads, the ITS2 subregions were extracted with the help of the ITSx software suite (v. 1.0.11) and its included fungi database. Reads that contained ambiguous bases or were outliers with respect to the amplicon size distribution were also discarded. The

remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form operational taxonomic units (OTUs), discarding singletons and chimaeras in the process. The resulting OTU abundance table was then filtered for possible bleed-in contaminations using the UNCROSS algorithm, and abundances were adjusted for 16S copy numbers using the UNBIAS algorithm. OTUs were compared against the reference sequences of the RDP 16S and UNITE databases, and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH.

All statistical tests were performed at a significance level of 0.05. Indices of diversity were calculated using the Vegan package (Legendre et al., 2011) in R software v. 1.0.136 (R Development Core Team, 2016) and statistically tested using Kruskal-Wallis test. Phylogenetic trees were obtained using SHAMAN software. Principal component analyses (PCAs) were performed using R software and statistically tested using the Kruskal-Wallis test.

3. Results

3.1. Taxonomic composition of samples

DNA of the bacteria and fungi was sequenced using an Illumina MiSeq device. A total of 539 fungal OTUs and 1319 bacterial OTUs were recorded. The 50 most abundant bacterial and fungal OTUs are listed in Tables S1 and S2.

3.1.1. Blue-grey mold on stairwell

The sequencing results obtained from the fungal growth on the stairwell (Fig. 2 A) showed that 83.6% of the total OTUs matched with *Zasmidium cellare*, 8.2% with

186 *Neodevriesia lagerstroemiae* and 4.1% with *Lecanicillium* sp. Twenty-two other species were
187 recorded but represented less than 1%.

188 Actinobacteria (57.4%), Bacteroidetes (23.5%) and Proteobacteria (19.2) were the
189 three dominant bacterial phyla recorded on the stairwell. *Methylobacterium brachythecii*, a
190 Proteobacteria, was the most abundant species (11.9%), followed by *Promicromonospora*
191 *xylanilytica* (9.7%), *Olivibacter soli* (9.7%), *Pseudosphingobacterium domesticum* (8.5%)
192 and *Promicromonospora iranensis* (8.1%). In a less extensive proportion, *Amycolatopsis*
193 *lexingtonensis* (2.8%) was also recorded.

194

195 *3.1.2. Wine barrels and their supports*

196 The fungal communities on wine barrels were predominantly composed of
197 *Talaromyces minioluteus* (36.4%), *Moristroma quercinum* (30.4%), *Penicillium corylophilum*
198 (14.3%) and *Zygoascus meyerae* (4.7%). Regarding bacteria, Proteobacteria (82%, mainly
199 *Pseudoxanthomonas spadix*, *Acidisoma sibiricum, Dyella jiangningensis and Tanticharoenia*
200 *sakaeratensis,* and *Coxiella burnetii*), Actinobacteria (9.4%), Acidobacteria (5.9%) and
201 Bacteroidetes (2.7%) were the four phyla recorded on wine barrels.

202 The barrel supports were made of wood and placed on stones (Fig. 2 D). Samples were
203 collected on both wood and limestone supports. The results indicated that 25.6% of the OTUs
204 matched with *Crustoderma dryinum*, 22.5% with Sordariomycetes class (undefined species),
205 18.2% with *Kendrickiella phycomyces*, 9.1% with *Talaromyces rademirici* and 3.7% with
206 *Penicillium spinulosum*. Other OTUs were represented in a less extensive proportion.

207 Actinobacteria (51.1%) and Proteobacteria (48.9%) were the only two phyla representing
208 more than 1% of the OTUs. *Promicromonospora* sp. (Actinobacteria) and *Pseudomonas* sp.
209 (Proteobacteria) represented 19.8% and 26.8%, respectively.

3.1.3. PVC hose

The major species recorded on the PVC hose was *Candida railenensis* (65.1%), followed by *Sistotrema* sp. (22.4%), *Apiotrichum laibachii* (5%) and 5 species (6.9%) of Penicillium (*P. corylophilum, P. thomii, P. kongii, P. spinulosum,* and *P. marinum*).

Actinobacteria (79.5%), Proteobacteria (17.1%) and Bacteroidetes (3.4%) represented the most abundant phyla on the stored hose. These phyla mainly consisted of the genera *Streptomyces*, *Leifsonia*, *Kofleria*, *Agromyces* and *Pseudonocardia*.

3.1.4. Limestone wall

Guehomyces pullulans was recorded as the major species on the limestone walls (33.5%), while *Hypocreales* (23.4%, mainly the genus *Trichoderma*), *Mortierellales* (12.1%), *Pleosporales* (9.6%), *Sordariales* (6.6%) and GS11 (6%, belonging to the phylum Rozellomycota) were the other major fungal orders.

Similar to the barrel supports, the limestone walls were mainly colonized by Proteobacteria (46.6%) and Actinobacteria (45.9%). The nine most abundant species that were recorded were *Dongia mobilis, Segetibacter aerophilus, Pseudoxanthomonas yeongjuensis, Kofleria flava, Bradyrhizobium icense, Gaiella occulta, Methyloceanibacter caenitepidi, Ferrithrix thermotolerans* and *Hyphomicrobium hollandicum.*

3.2. Indices of diversity

The species diversity and evenness were calculated using the Shannon and Pielou indices (Fig. 3). The results indicated higher fungal community diversity on limestone (4 and 0.7 bits.cell^{-1}, respectively) than metal (0.8 and 0.1 bits.cell^{-1}, respectively) and PVC supports (1.1 and 0.2 bits.cell^{-1}, respectively). Thus, the fungal communities colonizing wooden barrels and supports showed half the diversity of those on limestone. However, the bacterial communities sampled on each type of material (metal, wood, limestone and PVC) showed high diversity. In fact, the Shannon and Pielou indices ranged from 3 to 4.3 and 0.5 to 0.7 bits.cell^{-1}, respectively.

3.3. Influence of colonized substrate on communities

The results of phylogenetic trees (Fig. 4) and principal component analysis (Fig. 5) showed that the fungal communities were specific to the colonized support. The metallic stairwell and wood barrels were mainly colonized by Ascomycota, while a higher proportion of Basidiomycota were recorded for both the PVC hose and limestone supports. In fact, 93% of the metal-colonized fungi belonged to the order Capnodiales, while the PVC hose was colonized by 68% Saccharomycetales. Furthermore, 72% of the Eurotiales and Phaeomoniellales inhabited the wood, and 90% of the Hypocreales, Cystofilobasidiales and Pleosporales were recorded on limestone. The principal component analysis results were in accordance with phylogenetic trees. Indeed, the FL1 axis (40.93%) and FL2 axis (17.69%) showed clear fungal community differences between the limestone, metallic stairwell, wood and PVC. Moreover, the PCA results distinguished two types of samples: the first samples were collected near the wine on the floor (PVC hose and barrel supports), and the second samples were collected from above the wine storage area (stairwell and limestone ceiling), in contact with alcohol released from the wine barrels. Similar results were obtained for the bacterial communities (data not shown).

4. Discussion

4.1. Microbiology in show caves

Since bacteria and fungi play a crucial role in natural caves, several studies have assessed the microbial communities of such caves (Jurado et al., 2009; Pfendler et al., 2018; Long et al., 2019). For instance, microorganisms are involved in cave and speleothem formation (Kondratyeva et al., 2016), in limestone deterioration (Coutinho et al., 2019) and in bioactive molecule production (Groth et al., 1999). In our previous studies, we showed the high diversity of microorganisms present in natural caves and their high adaptation and plasticity depending on the sampled cave section, especially for bacteria and autotrophic protists (Pfendler et al., 2018a). However, few studies have been carried out on fungal outbreaks in natural caves. Fungi naturally live in caves but are less abundant than bacteria. Thus, they are mostly found as degraders and have mainly been recorded on organic matter, such as dead insects, bat guano, decaying wood or plant roots penetrating into shallow caves (Gunde-Cimerman et al., 1998; Vanderwolf et al., 2013). Understanding the microorganism communities living in show caves is essential to understand cavity dynamics and operation. Using modern technology, such as high-throughput sequencing, microorganism community assessment can give valuable information regarding cave ecology.

In the Saint-Marcel cave, we assessed the bacterial and fungal communities, and according to the results, we hypothesized that two types of communities may be distinguished. The first community may be related to oenological activities and cave tourism. In fact, the results of this study show that each type of colonized surface (wood, PVC, metal, limestone) was mainly inhabited by wine storage-related communities. The second

279 community has no direct link with wine ageing or tourism and may be considered a non-
280 anthropized cave community.

281

282 *4.2. Wine storage-related communities*

283 *4.2.1. Microorganisms inhabiting the metallic stairwell and limestone ceilings*

284 A thick blue-grey mycelial mat colonized the stairwell at the entrance of the cave. This
285 mycelium was identified as *Zasmidium cellare*, which is well known as cellar mold and lives
286 in dark and ethanol-rich environments, such as wine and brandy cellars in central and
287 southern Europe (Hass et al., 2010). As demonstrated in a previous study (Tribe et al., 2006),
288 alcohol that has leaked out of wine barrels may be used by *Z. cellare* as volatile carbon
289 source. The use of ethanol by some mold species has been known for several decades and has
290 been further demonstrated under laboratory conditions (Tribe and Mabadeje, 1972). As a
291 result of this process, all available surfaces may be covered by a thick mycelial mat (Tribe et
292 al., 2006). Accordingly, in our study, we observed a high proliferation of *Z. cellare* compared
293 to other strains. It is well known that such proliferation can lead to an increase in spore
294 concentrations in the air, as reported by Hass et al. (2010), which are dangerous for cave
295 preservation, limestone degradation and human health by inhalation (Jurado et al., 2010)

296 In addition to *Z. cellare*, *Guehomyces pullulans* was detected in both metallic- and
297 limestone-colonized samples. This strain was found by Golden et al. (1987) on individually
298 shrink-wrapped peaches. *G. pullulans* is described as a cold-adapted yeast capable of
299 producing pectinases at low temperatures, leading to the clarification of fruit juice (Cavello et
300 al., 2017), such as grapes, by the breakdown of the substrate pectin. When pectin is
301 decomposed by enzymes of microorganisms, the methyl esters combine with water to produce
302 methanol. Then, the methanol can be oxidized by some prokaryotes, such as

303 *Methyloceanibacter caenitepidi*, a methanol-oxidizing bacterium (Takeuchi et al., 2014)
304 mainly recorded on the limestone walls and ceilings of the Saint-Marcel cave.

305

306 *4.2.2. Microbial communities on oak barrels*

307 One-third of the fungal communities present on the oak wine barrels consisted of
308 *Talaromyces minioluteus*. Since *T. minioluteus* has been described as a dextranase producer
309 (Sufiate et al., 2018), we can hypothesize that this strain is a wine-related fungus. In fact, the
310 by-product of dextranase is dextran, which was discovered by Louis Pasteur as a microbial
311 product in wine (Pasteur, 1861). In our study, the second most dominant strain recorded on
312 the oak barrel samples was *Moristroma quercinum*, consisting of >30% of the total species.
313 *M. quercinum* was found by Nordén et al. (2005) on hard heartwood from attached or shed
314 branches from *Quercus robur* and *Q. petraea*. This species can also be found on old oak
315 stumps. This result is not surprising, knowing that in general, wine barrels, and specifically
316 the wine barrels present in the Saint-Marcel cave, are made using oak wood. Finally, the
317 fourth most abundant fungus, *Zygoascus meyerae,* is a yeast belonging to the class
318 Saccharomycetes and has been isolated from environmental sources such as damaged grapes
319 (Ioakimidou et al., 2011).

320 The oak barrels were also colonized by wine-related bacteria, such as *Acidisoma sibiricum*,
321 the second most abundant bacteria on the wood barrels. This species is an acidophilic (pH
322 3.0–7.6) and psychrotolerant (2–30 °C) bacterium (Belova et al., 2009) that can grow under
323 the optimal conditions on wine barrels in the Saint-Marcel cave (wine pH=3-4 and cave
324 temperature of 14°C). A second potential wine-related bacterium was *Dyella jiangningensis*,
325 which represented 9% of the total bacteria and was isolated by Zhao et al. (2013) from the
326 surfaces of weathered potassic trachytes in China. We can thus hypothesize that this

bacterium is linked to wine, given that potassium is found in an amount of 0.7 to 2% per litre in wine. Moreover, potassium bicarbonate is used for the deacidification of wine.

4.2.3. Microorganisms colonizing PVC

Both *Candida railenensis* and *Sistotrema* sp. were the most abundant fungi found on the PVC hose. Isaeva et al. (2009) reported that *C. railenensis* is a yeast colonizing oak fruits, but this fungus has also been described in decaying organic matter, such as trunks (Ramírez et al., 1984). Finally, the heavy metal-, tannin- and acid-tolerant microorganism *P. spinulosum* (Hujslová et al., 2017) may also be linked with oenological activity. However, no direct link has been established between the recorded bacteria and cave oenological activities.

4.3. Non-related bacteria and fungi to wine ageing

The second type of microbial community we propose consists of microorganisms that have no direct link with oenological activities. The obtained results show that these microorganisms were not the dominant microorganisms that were sequenced. Several entomopathogenic fungi were recorded on the stairwell, oak barrels and PVC. The presence of entomopathogenic fungi is not surprising since they are commonly described in show caves (Vanderwolf et al., 2013). For instance, the genus *Lecanicillium* is known and used worldwide for the biological control of insects (de Faria and Wraight, 2007). *P. corylophilum* is a pathogen of mosquitoes that produces alkaloids such as epoxyagroclavine and citrinin (Moraes et al., 2004). Other abundant fungi were recorded, such as those inhabiting wine barrels. One of the most abundant was *Penicillium corylophilum*, which is a species occurring in damp buildings in the United States, Canada and Western Europe (Bok et al., 2009). It was reported that *P. corylophilum* is the completely dominant mould species in crawl spaces in

Sweden (Bok et al., 2009). On PVC, several members of the genus *Penicillium* were recorded, such as the food spoiler *P. thomii* (Jones et al., 1996), *P. kongii*, which lives on plant leaves (Wang and Wang, 2013), and the roquefortine C producer *P. marinum* (Wigley et al., 2008).

Similar to the fungi, the most abundant bacterial species consisted of wine-related bacteria. Among the other species, several known species were recorded, such as *Pseudoxanthomonas spadix,* which was isolated from gasoline-contaminated sediment and can metabolize all six BTEX (benzene, toluene, ethylbenzene, and o-, m-, and p-xylene) compounds (Lee et al., 2012). One member of the genus *Streptomyces* (unknown species) dominated the bacterial community on the PVC hose, followed by *Leifsonia antarctica,* a bacterium isolated from a spade core sediment sample from the Antarctic Ocean (Pindi et al., 2009), *Kofleria flava* and *Agromyces humatus*. *A. humatus* was previously found on a wall of the Catacombs of Domitilla in Italy (Jurado, 2005).

4.4. Fungal outbreaks in show caves and treatment

Several outbreaks have been recorded in the literature, such as those in the Castañar and Lascaux caves (Bastian et al., 2009a, 2009b, 2009c; Jurado et al., 2010). As already demonstrated, fungal outbreaks could have serious consequences on wall and painting preservation (Pfendler et al., 2017) and can lead to human pulmonary diseases (Pereira, 2011). To avoid potential uncontrolled and harmful proliferation, fungi should be eradicated as soon as possible and, if possible, by means of an environmentally friendly method (Grobbelaar, 2000; Faimon et al., 2003; Mulec, 2014). In the Saint-Marcel cave, fungi were removed manually, and then a chemical product (highly concentrated sodium hypochlorite) was applied. However, a few months later, the same outbreak was observed. The Saint-

Marcel cave is a new example showing that the use of chemical compounds is unsuitable in show caves. Oenological activities contribute to increasing the carbon amount in the cave, necessarily leading to microbial proliferation. To protect the cave ecological balance, we suggest that oenological activities should be limited to wine testing and that wine vinification should be avoided as soon as possible. Further studies should be implemented to detect the presence of these wine-linked fungi and bacteria in the other parts of the 54-km-long Saint-Marcel cave.

5. Conclusions

This work has shown how human activities (oenology) have directly affected the Saint-Marcel cave microbiology. In fact, two types of microorganism communities were recorded. The first community consisted of unrelated wine cave bacteria and fungi, while the second community, which was dominant, was involved in wine vinification. Wine-related microorganisms were dominant and able to colonize all types of supports present in the cave (PVC, wood, limestone, and metal). This study has demonstrated that most of the recorded species are well known for their ability to use alcohol as a carbon source, for their involvement in wine fermentation or for their ability to degrade wood barrels. Therefore, a clear involvement of human activities in microorganism communities has been established. Caves are mainly composed of carbonate rocks such as limestone, which over time undergoes significant deterioration phenomena via microorganism colonization. This deterioration may be amplified by numerous organisms from anthropogenic activities such as wine vinification. Indeed, the darkening of the Saint-Marcel cave ceilings is an illustration of the consequences of human activities.

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573 **List of figures:**

574 **Fig. 1.** Map of Saint-Marcel show cave (Saint-Marcel, France).

575 **Fig. 2.** Illustration of the Saint-Marcel outbreak. (A) The tourist tour starts on the top of the stairwell.
576 Below, wine barrels (B) were stored on wood supports. The PVC hose (C) was stored behind wine
577 barrels. The limestone wall and roof next to the storage area were colonized by black mold (D). On
578 wine bottles (E), green, orange and white fungi proliferated.

579 **Fig. 3.** Shannon and Pielou indices for all types of colonized materials. Statistical analyses were
580 carried out to compare limestone, metal, PVC and wood samples for both 16S and ITS primers.
581 Significant differences are indicated using the letters a/*a*, b/*b* and c/*c*.

582 **Fig. 4.** Phylogenetic trees obtained with fungal metagenomic data.

583 **Fig. 5.** Principal component analysis (PCA) of fungal communities depending on the colonized
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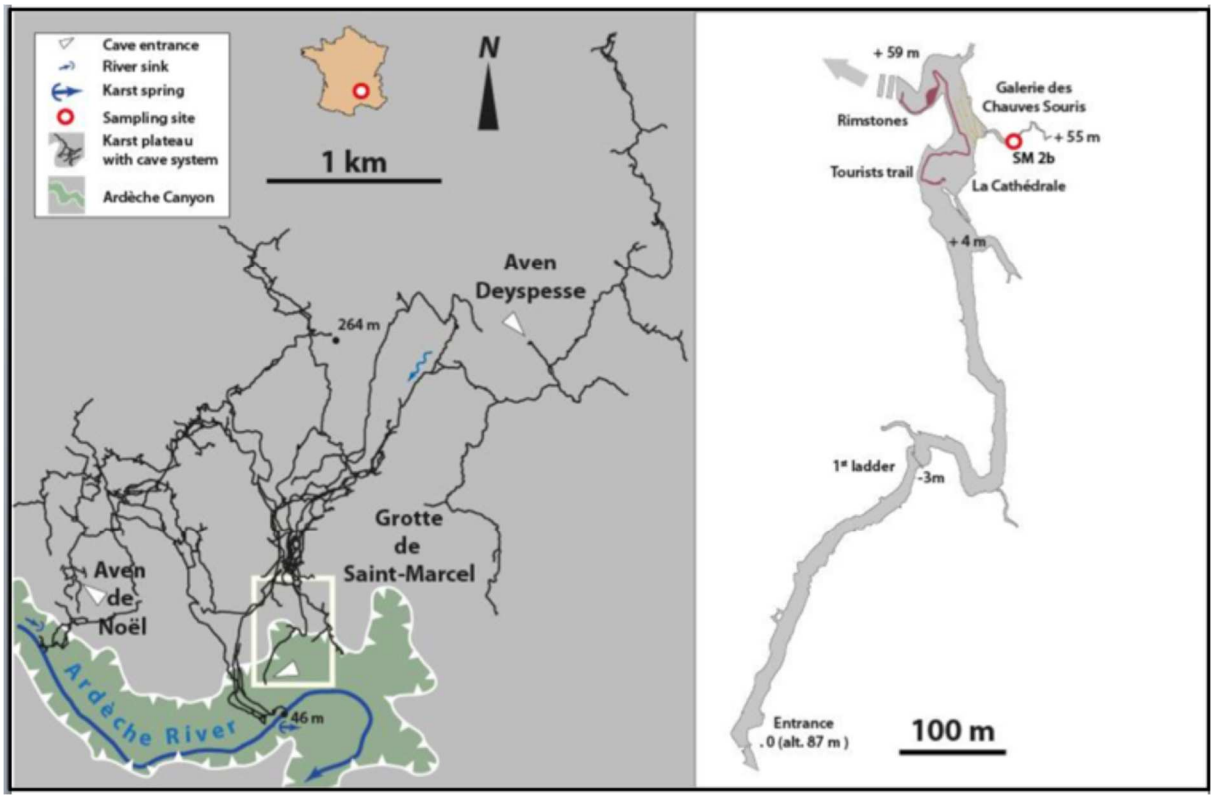

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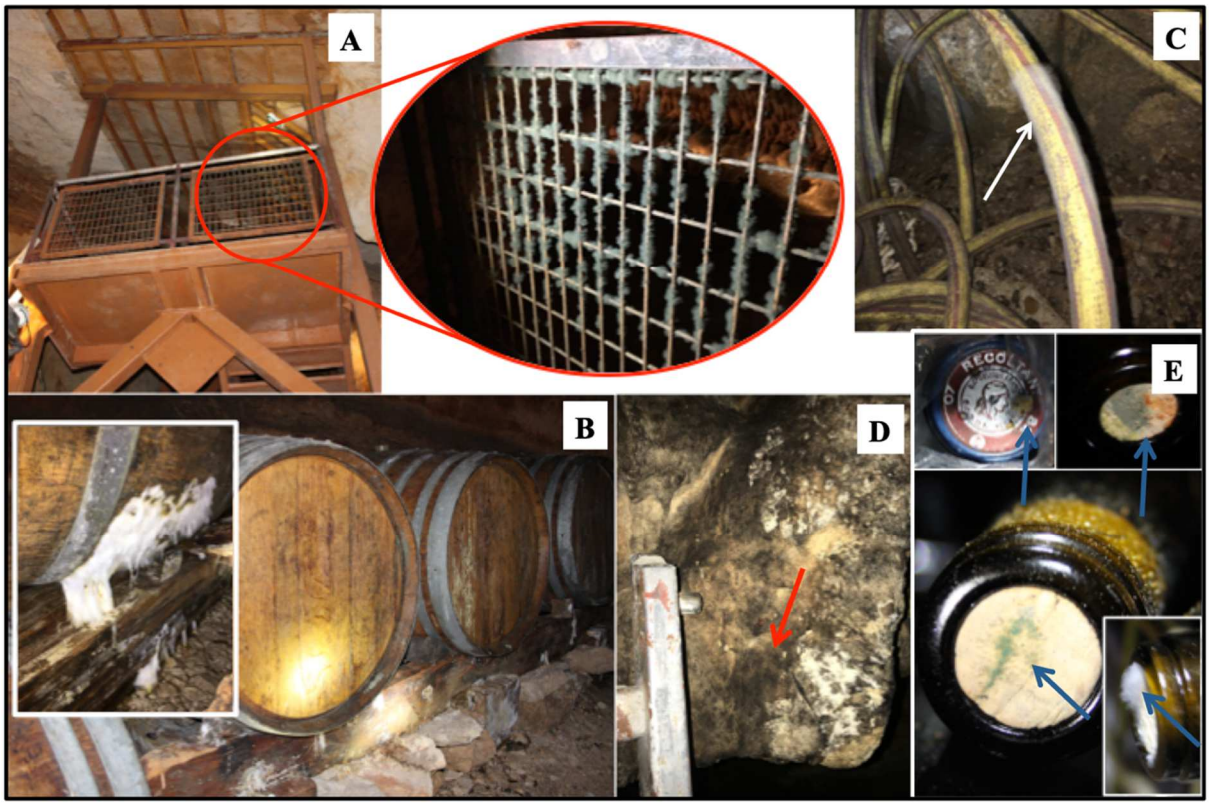

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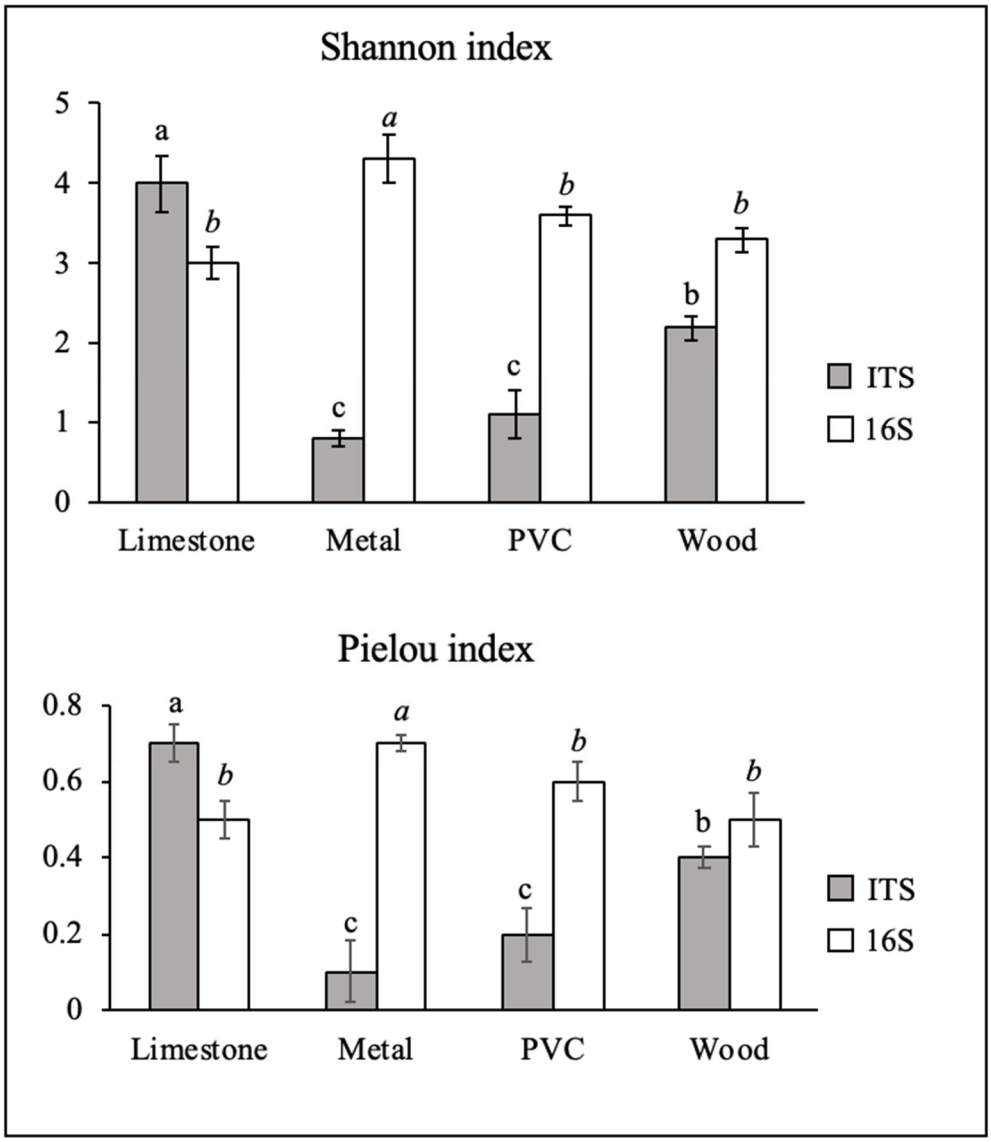

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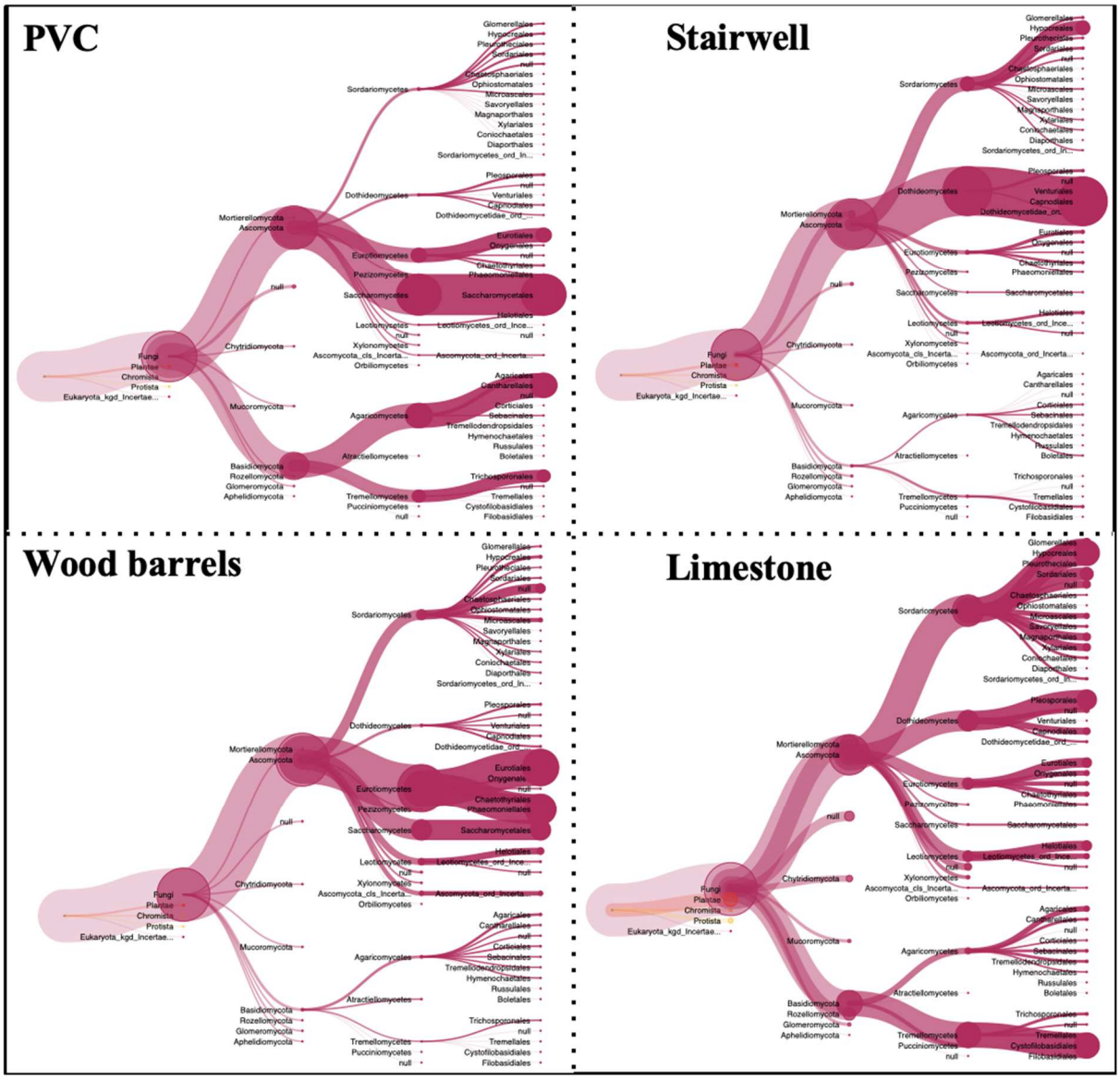

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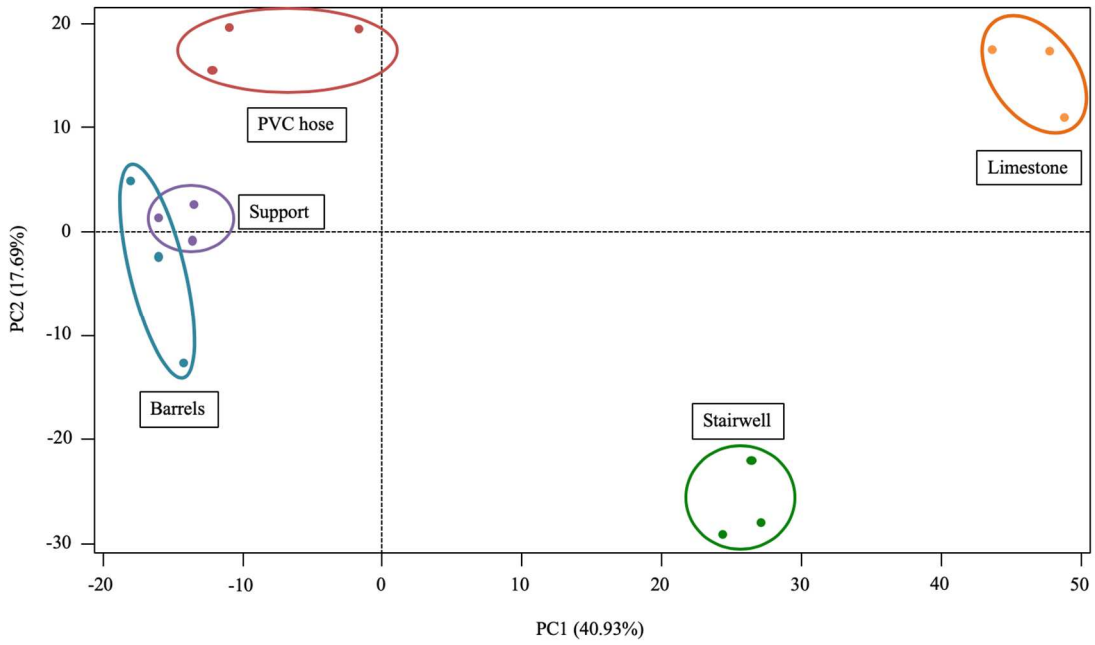

Fig. 5. Principal Component Analysis (PCA) of fungal communities depending to the colonized substratum (PVC in red, wooden barrels and their supports in blue and purple, stairwell in green and limestone in orange).

Fungal proliferation on limestone

H_3C OH

Ethanol release

Dominant microorganisms are related to vinification

Metabarcoding

Fungal and bacterial outbreak