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

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► To cite this version:

Didier Cailhol, Lisa Ciadamidaro, Delphine Dupuy, Séverine Allegra, Françoise Girardot, et al.. Fungal and bacterial outbreak in the wine vinification area in the Saint-Marcel show cave. *Science of the Total Environment*, 2020, 733, pp.138756. 10.1016/j.scitotenv.2020.138756 . hal-02903008

HAL Id: hal-02903008

<https://hal.inrae.fr/hal-02903008v1>

Submitted on 20 May 2022

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21 **Abstract**

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

37

38

39 **Keywords:** Fungal outbreak, Sequencing, Show cave, Bacterial communities,
40 Biodeterioration

41

42 **1. Introduction**

43 For centuries, people have been visiting caves for tourism or recreation. As evidence
44 of this activity, a signature dated from 1213 was found in the Postojna cave in Slovenia
45 (Cigna, 2012). Currently, 250 million tourists visit more than 800 show caves around the
46 world every year (Cigna, 2016). Tourist caves offer a wide range of activities to tourists (e.g.,
47 speleology, concerts, theatre, aquarium expositions, and wine tastings), generating 2.3 billion
48 dollars per year (Cigna and Burri, 2000). However, natural site anthropization leads to
49 microbial disturbances (Cennamo et al., 2012), even if ecological considerations become
50 increasingly important. In the case of natural caves, microorganisms such as some bacteria or
51 autotrophic protists can take advantage of the environmental changes (e.g., increased artificial
52 light, carbon dioxide and organic matter) to proliferate. For instance, lampenflora are well
53 known and widely described (Borderie et al., 2014; Cennamo et al., 2016). The first
54 observations were performed by Kyrle and Gams in 1923 and 1925, respectively (Cigna,
55 2011; Lamprinou, 2014). Since then, optical observations and the use of molecular methods
56 have permitted a better overview of these organisms, which live together in a matrix called a
57 biofilm. In addition to lampenflora, other microorganisms, such as fungi, have often been
58 involved in microbial infestation of caves, such as the infestation that happened in the world-
59 famous Lascaux Cave (Bastian et al., 2009a, 2009b, 2009c). The negative consequences of
60 lampenflora and fungal outbreaks are numerous. In addition to creating a visual disturbance
61 for tourists, microorganisms lead to limestone and wall painting degradation by physical or
62 chemical actions (Borderie et al., 2014). Moreover, some microorganisms, such as fungi, can
63 be dangerous for human health by inhalation of fungal spores (Jurado et al., 2010).

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

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

73 Furthermore, since April 2015, oenological activities have been undertaken in Saint-
74 Marcel cave. To vinify red wine, several producers from Saint-Marcel village have stored 300
75 litres of wine in wooden barrels and one thousand wine bottles. Wine tastings in the cave,
76 associated with guided speleological activities, are a part of an oenotourism project. However,
77 after one year, strong fungal growth started on the wooden barrels and on the bottles in the
78 wine storage section at the beginning of the tourist area, near the access gate and the “Gallery
79 of Painters”. Given the amount of mold, some other parts of the cave may be endangered by
80 displaced spores due to the strong air dynamics that affect the speleological system. Several
81 authors have described fungal communities in wine cellars (Simeray et al., 2000; Hass et al.,
82 2010) or in natural cavities (Stomeo et al., 2009; Taylor et al., 2014). However, as far as we
83 know, fungal communities related to wine vinification in show caves have never been
84 described.

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

91

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 m³.s⁻¹ and relatively
114 high CO₂ 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 CO₂ and O₂. 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

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

144

145 2.4. Molecular methods and data analysis

146 The library creation, sequencing and data analysis described in this section were
147 performed by Microsynth AG (Balgach, Switzerland). The V3 and V4 regions of the bacterial
148 16S rRNA gene were sequenced in a two-step Nextera PCR library procedure using the
149 primer pair 341F (5'- CCT ACG GGN GGC WGC AG -3') and 802R (5'- GAC TAC HVG
150 GGT ATC TAA TCC -3'). To sequence the internal transcribed spacer (ITS2) regions of the
151 fungal 18S rRNA gene, two-step Nextera PCR libraries using the primer pair ITS3 (5'- GCA
152 TCG ATG AAG AAC GCA GC -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3')
153 were created. The Illumina MiSeq platform and a v2 500 cycle kit were used to sequence the
154 PCR libraries. Then, FastQC (v. 0.11.8) was used to assess read quality. The locus-specific
155 V34 and ITS2 primers were trimmed from the sequencing reads with the software Cutadapt
156 (v. 2.3). Paired-end reads were discarded if the primer could not be trimmed. Trimmed
157 forward and reverse reads of each paired-end read were merged to *in silico* reform the
158 sequenced molecule considering a minimum overlap of 15 bases using the software
159 USEARCH (v. 11.0.667). Merged sequences were then quality filtered, allowing a maximum
160 of one expected error per merged read and discarding those containing ambiguous bases.
161 From the remaining reads, the ITS2 subregions were extracted with the help of the ITSx
162 software suite (v. 1.0.11) and its included fungi database. Reads that contained ambiguous
163 bases or were outliers with respect to the amplicon size distribution were also discarded. The

164 remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to
165 form operational taxonomic units (OTUs), discarding singletons and chimaeras in the process.
166 The resulting OTU abundance table was then filtered for possible bleed-in contaminations
167 using the UNCROSS algorithm, and abundances were adjusted for 16S copy numbers using
168 the UNBIAS algorithm. OTUs were compared against the reference sequences of the RDP
169 16S and UNITE databases, and taxonomies were predicted considering a minimum
170 confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH.

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

177

178 **3. Results**

179 *3.1. Taxonomic composition of samples*

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

183 *3.1.1. Blue-grey mold on stairwell*

184 The sequencing results obtained from the fungal growth on the stairwell (Fig. 2 A)
185 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 brachytheticii*, 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 meyeriae* (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.

210

211 *3.1.3. PVC hose*

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

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

218

219 *3.1.4. Limestone wall*

220 *Guehomyces pullulans* was recorded as the major species on the limestone walls
221 (33.5%), while *Hypocreales* (23.4%, mainly the genus *Trichoderma*), *Mortierellales* (12.1%),
222 *Pleosporales* (9.6%), *Sordariales* (6.6%) and GS11 (6%, belonging to the phylum
223 Rozellomycota) were the other major fungal orders.

224 Similar to the barrel supports, the limestone walls were mainly colonized by Proteobacteria
225 (46.6%) and Actinobacteria (45.9%). The nine most abundant species that were recorded were
226 *Dongia mobilis*, *Segetibacter aerophilus*, *Pseudoxanthomonas yeongjuensis*, *Kofteria flava*,
227 *Bradyrhizobium icense*, *Gaiella occulta*, *Methyloceanibacter caenitepidi*, *Ferrithrix*
228 *thermotolerans* and *Hyphomicrobium hollandicum*.

229

230 *3.2. Indices of diversity*

231 The species diversity and evenness were calculated using the Shannon and Pielou
232 indices (Fig. 3). The results indicated higher fungal community diversity on limestone (4 and
233 0.7 bits.cell⁻¹, respectively) than metal (0.8 and 0.1 bits.cell⁻¹, respectively) and PVC
234 supports (1.1 and 0.2 bits.cell⁻¹, respectively). Thus, the fungal communities colonizing
235 wooden barrels and supports showed half the diversity of those on limestone. However, the
236 bacterial communities sampled on each type of material (metal, wood, limestone and PVC)
237 showed high diversity. In fact, the Shannon and Pielou indices ranged from 3 to 4.3 and 0.5 to
238 0.7 bits.cell⁻¹, respectively.

239

240 *3.3. Influence of colonized substrate on communities*

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

256

257 **4. Discussion**258 *4.1. Microbiology in show caves*

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

274 In the Saint-Marcel cave, we assessed the bacterial and fungal communities, and
275 according to the results, we hypothesized that two types of communities may be
276 distinguished. The first community may be related to oenological activities and cave tourism.
277 In fact, the results of this study show that each type of colonized surface (wood, PVC, metal,
278 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 meyeræ*, 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

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

329

330 4.2.3. Microorganisms colonizing PVC

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

337

338 4.3. Non-related bacteria and fungi to wine ageing

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

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

355 Similar to the fungi, the most abundant bacterial species consisted of wine-related
356 bacteria. Among the other species, several known species were recorded, such as
357 *Pseudoxanthomonas spadix*, which was isolated from gasoline-contaminated sediment and
358 can metabolize all six BTEX (benzene, toluene, ethylbenzene, and o-, m-, and p-xylene)
359 compounds (Lee et al., 2012). One member of the genus *Streptomyces* (unknown species)
360 dominated the bacterial community on the PVC hose, followed by *Leifsonia antarctica*, a
361 bacterium isolated from a spade core sediment sample from the Antarctic Ocean (Pindi et al.,
362 2009), *Kofleria flava* and *Agromyces humatus*. *A. humatus* was previously found on a wall of
363 the Catacombs of Domitilla in Italy (Jurado, 2005).

364

365 4.4. Fungal outbreaks in show caves and treatment

366 Several outbreaks have been recorded in the literature, such as those in the Castañar
367 and Lascaux caves (Bastian et al., 2009a, 2009b, 2009c; Jurado et al., 2010). As already
368 demonstrated, fungal outbreaks could have serious consequences on wall and painting
369 preservation (Pfendler et al., 2017) and can lead to human pulmonary diseases (Pereira,
370 2011). To avoid potential uncontrolled and harmful proliferation, fungi should be eradicated
371 as soon as possible and, if possible, by means of an environmentally friendly method
372 (Grobbelaar, 2000; Faimon et al., 2003; Mulec, 2014). In the Saint-Marcel cave, fungi were
373 removed manually, and then a chemical product (highly concentrated sodium hypochlorite)
374 was applied. However, a few months later, the same outbreak was observed. The Saint-

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

382

383 **5. Conclusions**

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

398

399 **6. Acknowledgement**

400 First, we are grateful to the curators of the Saint-Marcel cave and the mayor of Saint-
401 Marcel village, who kindly gave us permission to access the cave and to carry out all our field
402 experiments. We also thank the laboratory EVS-ISTHME for the financial contribution. We
403 express our appreciation to the anonymous reviewers for helping to improve our paper.

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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
584 substrate (PVC in red, wooden barrels and their supports in blue and purple, respectively, stairwell in
585 green and limestone in orange).

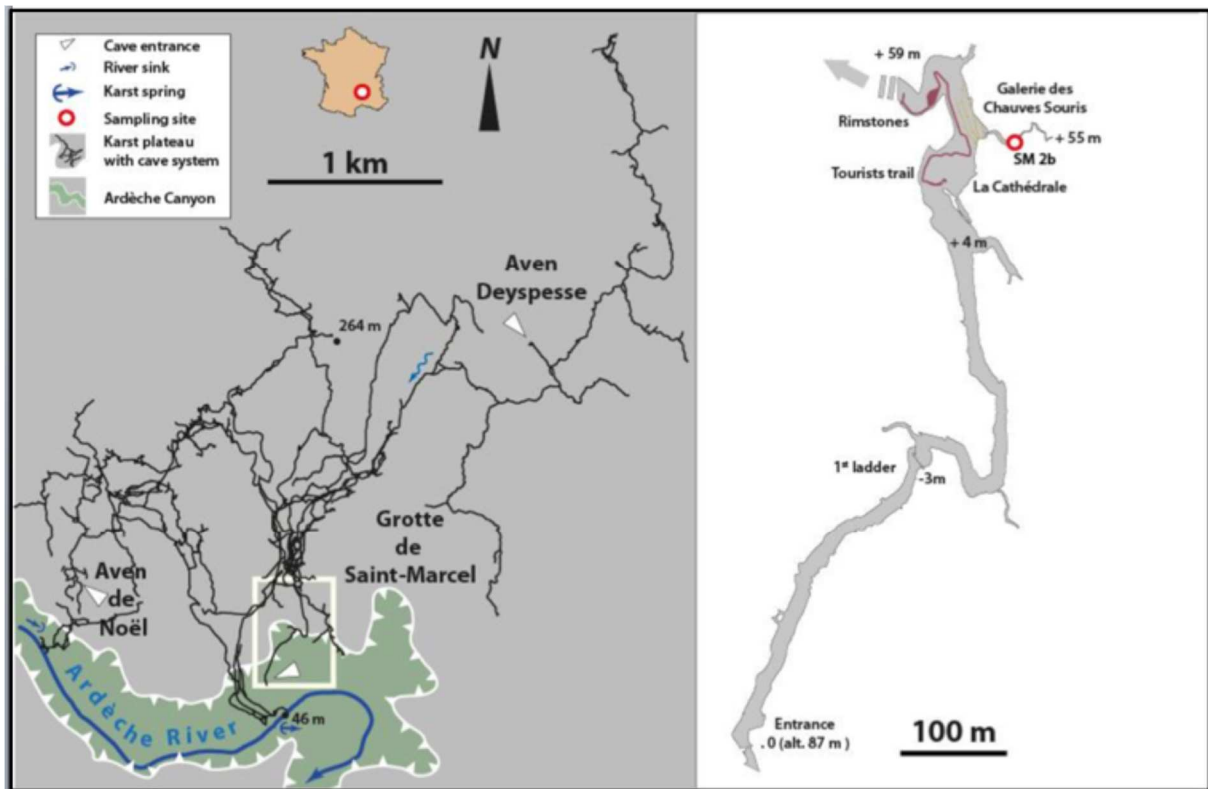


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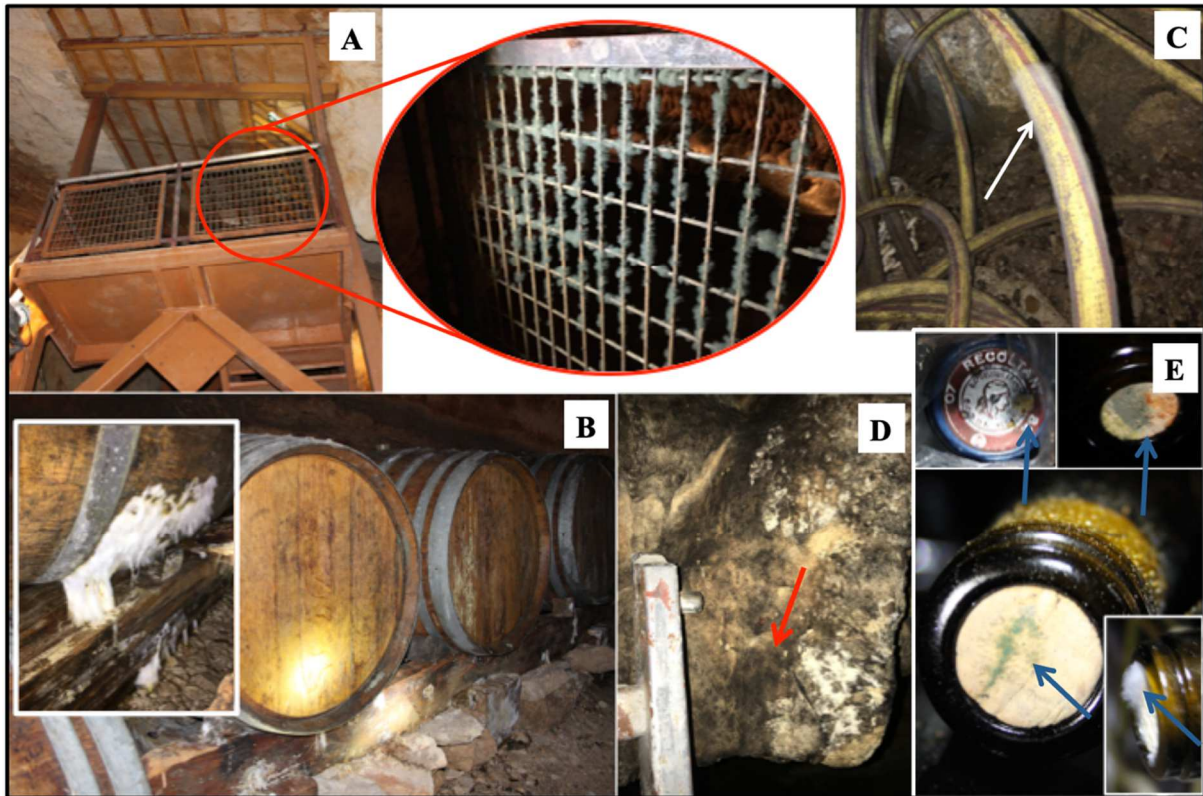


Fig. 2. Illustration of Saint-Marcel outbreak. (A) Tourist tour starts on the top of the stairwell. Below, wine barrels (B) were stored on woody supports. PVC hose (C) was stored behind wine barrels. Limestone wall and roof next to the storage area were colonized by black mold (D). On wine bottles (E), green, orange and white fungi proliferations.

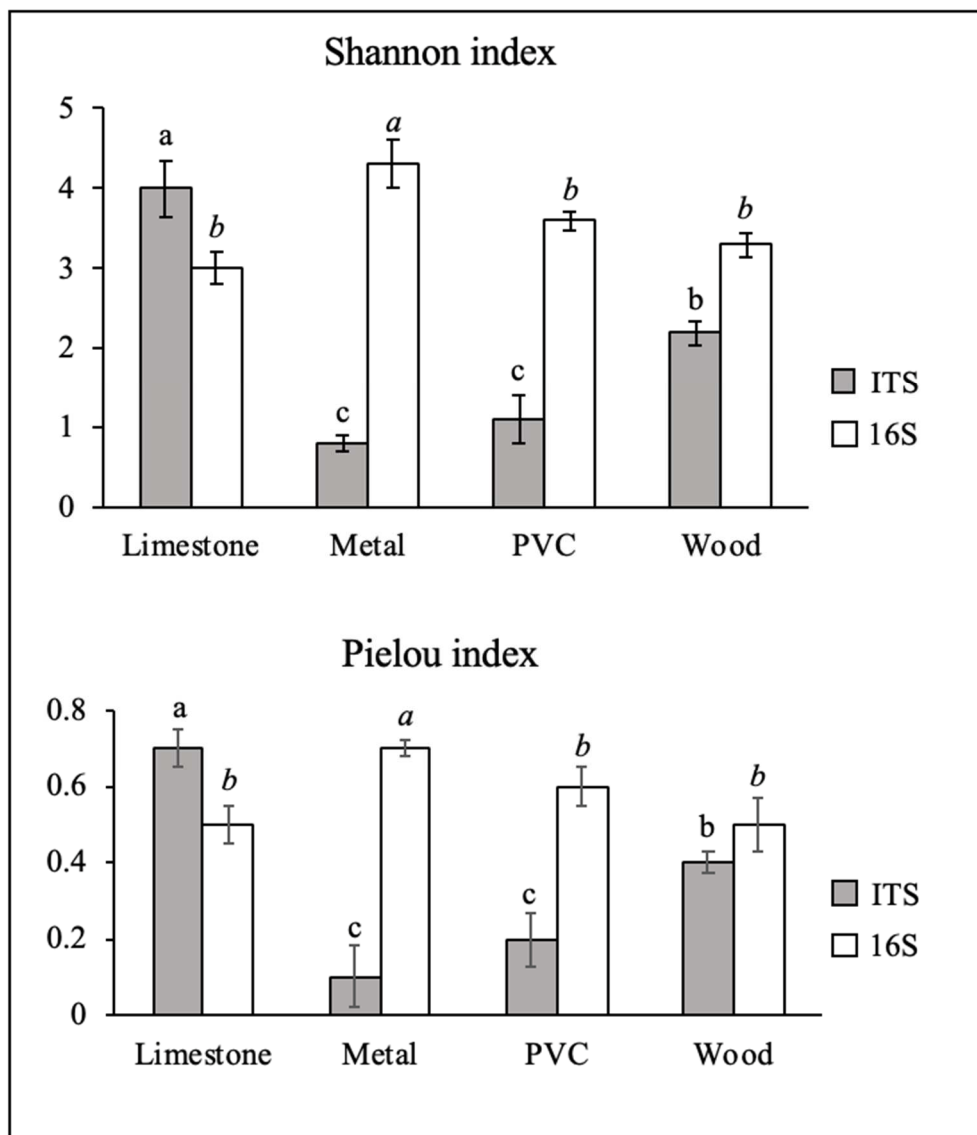


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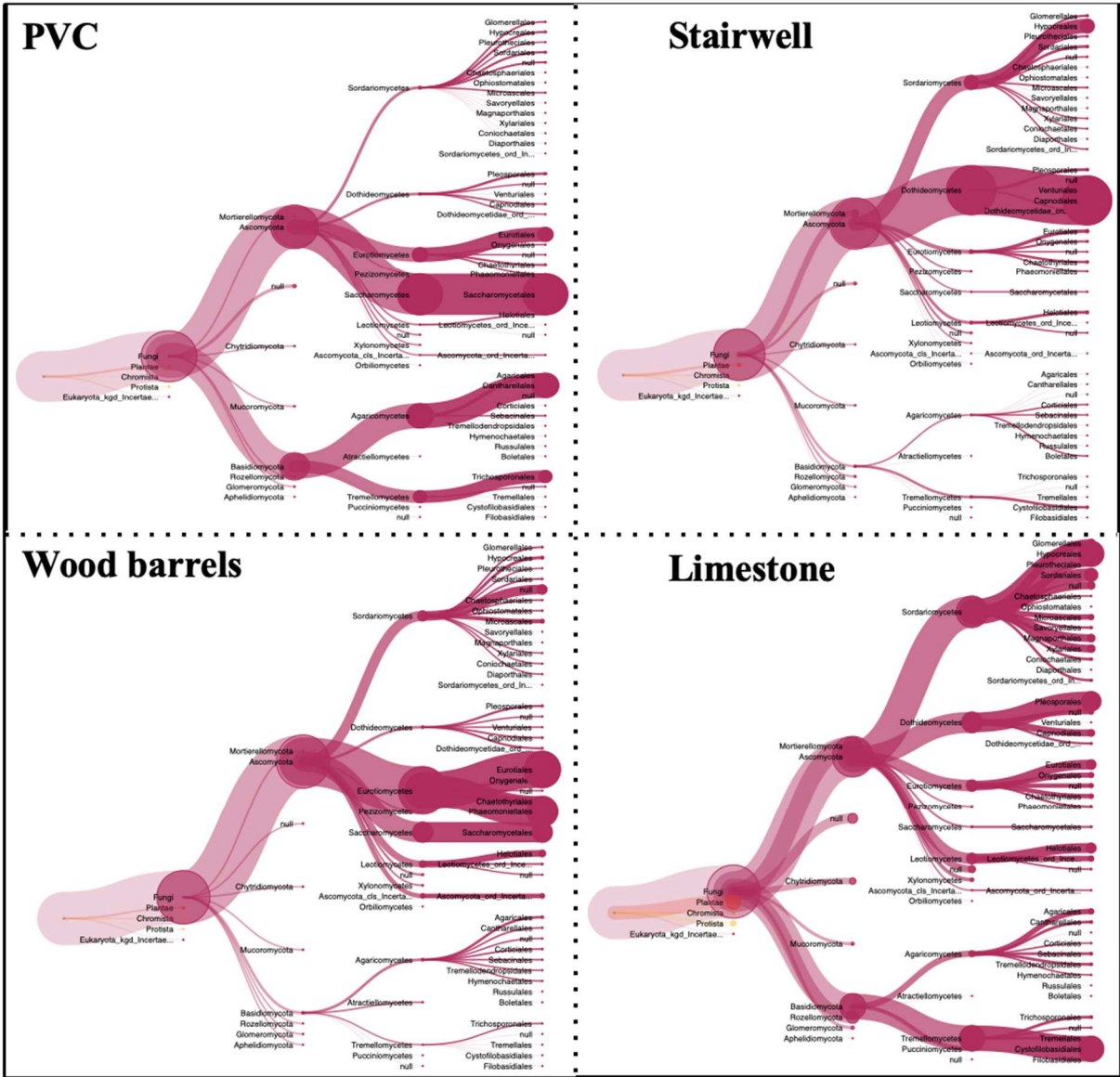


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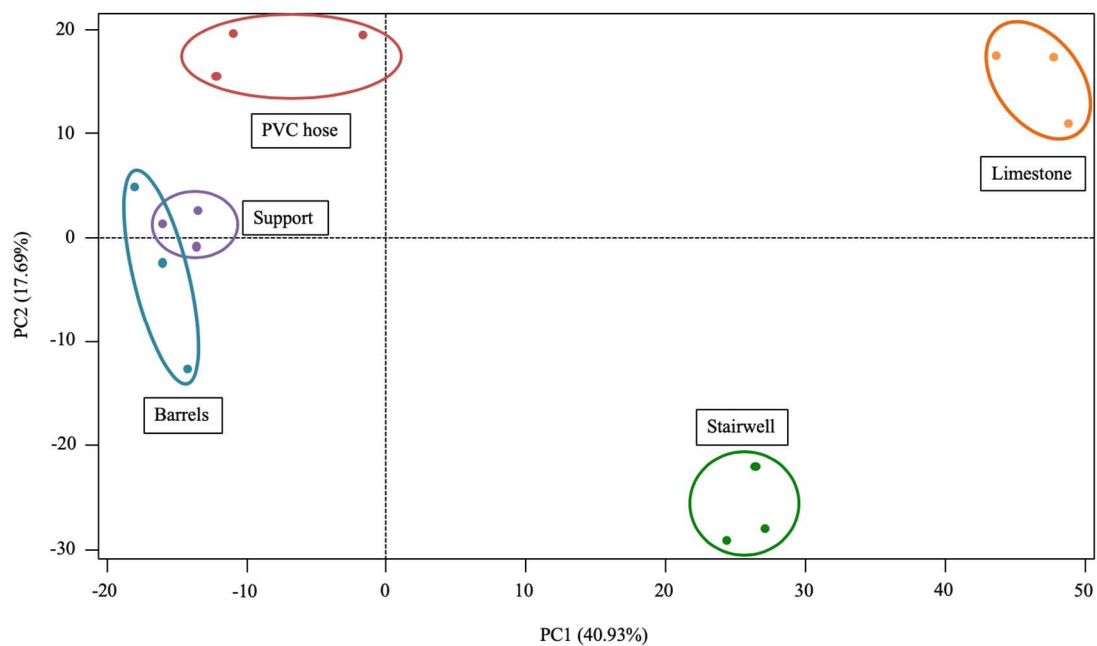
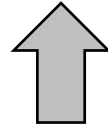


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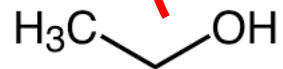
Fungal proliferation on limestone



Dominant microorganisms are related to vinification



Metabarcoding



Ethanol release

Fungal and bacterial outbreak

