

Mini-Review

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The microbiology of Lascaux Cave

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Lascaux Cave (Montignac, France) contains paintings from the Upper Paleolithic period. Shortly after its discovery in 1940, the cave was seriously disturbed by major destructive interventions. In 1963, the cave was closed due to algal growth on the walls. In 2001, the ceiling, walls and sediments were colonized by the fungus *Fusarium solani*. Later, black stains, probably of fungal origin, appeared on the walls. Biocide treatments, including quaternary ammonium derivatives, were extensively applied for a few years, and have been in use again since January 2008. The microbial communities in Lascaux Cave were shown to be composed of human-pathogenic bacteria and entomopathogenic fungi, the former as a result of the biocide selection. The data show that fungi play an important role in the cave, and arthropods contribute to the dispersion of conidia. A careful study on the fungal ecology is needed in order to complete the cave food web and to control the black stains threatening the Paleolithic paintings.

Introduction

The conservation of Paleolithic paintings in caves is of great interest because they represent a priceless cultural heritage for all humankind. Among Paleolithic paintings, those at Chauvet and Lascaux, France, and in Altamira, Spain, show remarkable sophistication. However, some of the most important caves have suffered episodes of biological contamination that might damage the paintings (Schabereiter-Gurtner *et al.*, 2002; Dupont *et al.*, 2007).

The Cave of Lascaux was discovered in 1940. The importance of its paintings was recognized shortly after their discovery and they are now considered one of the finest examples of rock art paintings (Fig. 1). As soon as it was open to the public the cave attracted a large audience, which amounted to 1800 every day in the 1960s (Sire, 2006). This seriously disturbed the microclimate and had a strong impact on the cave ecosystem.

Rock art tourism started at the beginning of the last century. At that time, there was no scientific knowledge of conservation problems; therefore decisions adopted often resulted in fatal errors that marked the future of many caves. In the Cave of Lascaux the adaptation works established for visits in 1947–1948 and 1957–1958, as well as the impact of massive tourism thereafter, were two of the main problems for its conservation. The lighting was responsible for the growth of a green biofilm on the wall paintings in 1960, initially identified as *Chlorobotrys*, a xanthophyte alga. Years later, the observation of zoospore formation in one of the algal isolates not previously detected led to its proper

determination as *Bracteacoccus minor*, a member of the Chlorophyta (Lefèvre, 1974). This green biofilm, also called 'la maladie verte', was the first of the various outbreaks or 'microbial crises' suffered by the cave, which led to its closure in 1963 due to the damage produced by visitors' breath, lighting and algal growth on the paintings.

Sire (2006), in an historical report on Lascaux Cave management, stated that the treatments for defeating 'la maladie verte' in 1963 included a combined spray application of streptomycin and penicillin for bacteria and a subsequent treatment with formaldehyde for algae. These applications were effective until 1969 when it was necessary to start again and a programme of periodic maintenance and cleaning was adopted.

In July 2001 the first evidence appeared of an outbreak of the fungus *Fusarium solani*, and its associated bacterium *Pseudomonas fluorescens* (Allemand, 2003; Oriol & Mertz, 2006), which can be considered the second major microbial crisis. This growth appeared in the form of long white mycelia, with a fluffy appearance. The rapid extension of the outbreak promoted an intensive treatment in September 2001 based on benzalkonium chloride solutions (Vitalub QC 50) plus streptomycin and polymyxin. The sediments were treated with quicklime (Sire, 2006). In 2004 benzalkonium chloride treatments were replaced by mechanical cleaning, air extraction and recovery of cleaning debris. However, in 2006, the dispersion of black stains on the ceiling and passage banks became apparent. This constituted the third major microbial crisis (Fig. 2),



Fig. 1. Detail of the Black Cow Panel in the Main Gallery.

although some of these stains were already present in 2003 (Geneste, 2008). Dematiaceous hyphomycetes, producing olivaceous to black colonies, and species of the genera *Verticillium* and *Scolecobasidium* were isolated from the stains (Bastian & Alabouvette, 2009). Mechanical cleaning as well as new biocide treatments have been used since January 2008.

There has been debate on the black stains in Lascaux Cave in several European and US media outlets in the last few years (e.g. Graff, 2006; Di Piazza, 2007; De Roux, 2007; Simons, 2007; Fox, 2008; Sire, 2008). In addition to an historical description of the works carried out in the cave since the discovery, comments on the problem were summarized in newspapers and magazines and the

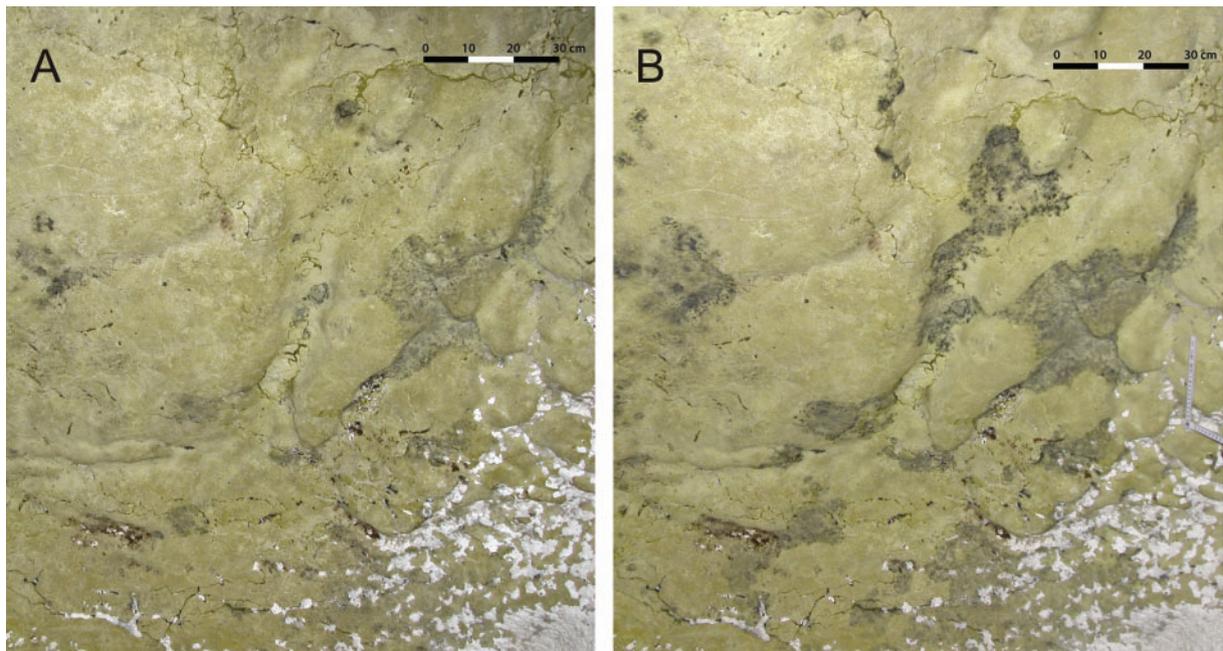


Fig. 2. Black stains in the vault of the Passage. Note the progression of stains. A, June 2008; B, December 2008. Pictures protected by copyright: Ministère de la Culture et de la Communication, DRAC d'Aquitaine and Centre National de la Préhistoire. Reproduced with permission.

appearance of black stains noted. Unfortunately, very few scientific data have been reported on the microbial problems of Lascaux Cave.

Cave ecology

Why has Lascaux Cave suffered successive biological invasions since its discovery? The problem derives from the public interest and pressure of rock art tourism and the erroneous concept that all rock art should be exposed to public view, a concept which generally opposes an effective conservation of rock art. If we accept that a statue or an oil painting can be exposed to mass tourism in a museum, the question is quite critical when it refers to rock art that has been confined in a closed subterranean environment for millennia. It is often forgotten that the opening of a cave immediately results in a sudden change of microclimate, deterioration of speleothems and rock art paintings, and implies a strong and irreversible aggression to cave biology and the whole ecosystem. Bacteria, fungi and arthropods have all constructed delicate and balanced trophic relationships between predator and prey, and the strength of interactions between species is frequently interrupted by adaptations for mass visits, which also include excavations and major destructive interventions. We must admit that science and technology have a limited field of action because no cave is ever completely restored to its former ecological state.

Fungal outbreaks

The situation in Lascaux is particularly worrying because in the last few years the cave has suffered two different fungal outbreaks. In 2001 the presence of members of the *Fusarium solani* species complex was reported (Dupont *et al.*, 2007).

Fusarium solani is considered a natural cave mycobiont. We found this fungus in different Spanish caves (unpublished data) and in cave sediments from Slovakia (Nováková, 2009), and other authors have reported its presence in caves from the UK (Mason-Williams & Holland, 1967), the USA (Cunningham *et al.*, 1995) and India (Koilaraj *et al.*, 1999).

Dupont *et al.* (2007) found, in addition, representatives of six fungal genera in the cave: *Chrysosporium*, *Gliocladium*, *Gliomastix*, *Paecilomyces*, *Trichoderma* and *Verticillium*. However, no species identification was provided. The data reported by these authors suggest a strong correlation between cave fungi and arthropods because these fungal genera contain many entomopathogenic species (Samson *et al.*, 1988). Species of *Chrysosporium*, *Gliocladium*, *Paecilomyces* and *Verticillium* have been isolated from larval and adult cadavers of cave crickets (Gunde-Cimerman *et al.*, 1998), and an association between fungi and insects in caves was recently reported (Kubátová & Dvorák, 2005; Jurado *et al.*, 2008).

Molecular microbiology

We undertook a detailed molecular biology study aimed at deciphering the origin of the microbial communities thriving in the cave. Eleven samples were collected between April 2006 and January 2007 in different halls and galleries of Lascaux Cave. The Painted Gallery (samples 1–3, 14 and 15), Great Hall of Bulls (4 and 5), Chamber of Felines (26 and 27) and Shaft of the Dead Man (30 and 31) were selected, because they were representative of the different cave microenvironments (Fig. 3). In the samples white mycelia colonizations, black stains, areas not apparently colonized, and therefore considered references, and an area cleaned with biocides in 2004, without apparent colonization, were represented. Methodological details were published elsewhere (Bastian *et al.* 2009a, b).

From a total of 696 bacterial clones, the two most abundant taxa were *Ralstonia* and *Pseudomonas* with a total of 374 clones, which amounted to 53.7% of clones (Table 1). The most abundant bacterial phylotypes were

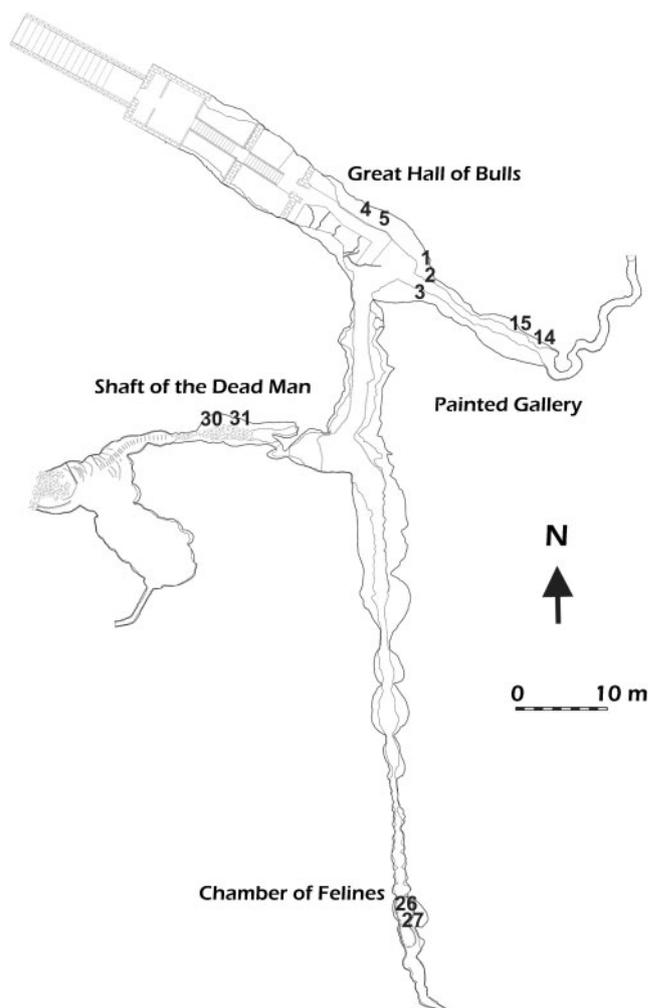


Fig. 3. Map of Lascaux Cave and location of sampling points.

Table 1. Most abundant bacterial taxa in Lascaux Cave

Genus	No. of clones from a total of 696 (%)	Most representative species, clones number and (%)
<i>Ralstonia</i>	207 (29.7)	<i>R. mannitolilytica</i> 77 (11.1), <i>R. pickettii</i> 47 (6.8)
<i>Pseudomonas</i>	167 (24.0)	<i>P. saccharophila</i> 35 (5.0), <i>P. lanceolata</i> 26 (3.7)
<i>Escherichia</i>	28 (4.0)	<i>E. coli</i> 26 (3.7), <i>E. albertii</i> 2 (0.3)
<i>Achromobacter</i>	25 (3.6)	<i>A. xylosoxidans</i> 25 (3.6)
<i>Afipia</i>	22 (3.2)	<i>Afipia</i> genospecies-14 18 (2.6)
<i>Ochrobactrum</i>	20 (2.9)	<i>Ochrobactrum</i> sp. R26465 12 (1.7)
<i>Legionella</i>	19 (2.7)	<i>Legionella</i> sp. OA32 3 (0.4), <i>L. yabuuchiae</i> 2 (0.3)
<i>Alcaligenes</i>	15 (2.2)	<i>Alcaligenes</i> sp. R21939 4 (0.6)
<i>Stenotrophomonas</i>	15 (2.2)	<i>S. maltophilia</i> 13 (1.9)
<i>Symbiobacterium</i>	15 (2.2)	<i>Symbiobacterium</i> sp. K438 8 (1.1)

Ralstonia mannitolilytica and *Ralstonia pickettii*, which all together represented 17.9% of clones (Bastian *et al.*, 2009a). These *Ralstonia* species are pathogens (Daxboeck *et al.*, 2005; Stelzmueller *et al.*, 2006). It is noteworthy that only five clones of *Pseudomonas fluorescens*, a species which was previously reported to be abundant in the cave (Oriol & Mertz, 2006), were recorded. In contrast, 77 and 47 clones corresponding to *R. mannitolilytica* and *R. pickettii*, respectively, were detected by Bastian *et al.* (2009a).

Two possibilities can be considered to explain the scarce representation of *P. fluorescens* in the study of Bastian *et al.* (2009a). First, the biocide treatments in the past were effective in eliminating this bacterium. This explanation can be disregarded in light of the results of Nagai *et al.* (1996), which showed that *Pseudomonas* spp., and particularly *P. fluorescens*, were resistant to biocides. Second, the original *P. fluorescens* isolates could have been misidentified. In fact, the biochemical identification of *Ralstonia* species with commercially available tests is problematic. *R. pickettii* is easily confused with *Burkholderia cepacia* and *P. fluorescens* (Daxboeck *et al.*, 2005), and *R. mannitolilytica* is often misidentified as *P. fluorescens* when using API 20NE (Vanechoutte *et al.*, 2001).

Although unfortunately no report on the bacteria present in Lascaux Cave before benzalkonium chloride treatments is available, and therefore the composition of pristine microbial communities is unknown, the data suggest that years of benzalkonium chloride treatments in Lascaux Cave might have selected a population of *Ralstonia* and *Pseudomonas* highly resistant to the biocide (Bastian *et al.*, 2009c).

The study of the fungal population (607 clones) revealed that the 10 most abundant phylotypes represented 59.2% of the total clones (Bastian *et al.*, 2009b). Only two of these 10 phylotypes can be labelled as soil fungi, while the rest can be classified as entomophilous fungi, including the well-known entomopathogen *Isaria farinosa* (Table 2) (Bastian *et al.*, 2009b). Only seven clones of *Fusarium solani* were found in the samples, which suggests that after prolonged biocide treatment the *F. solani* population decreased drastically, but in turn, this was replaced by an abundant population of entomophilous and other fungi.

It is interesting to note the presence of *Geosmithia putterillii* in the cave (Table 2). *Geosmithia* species are encountered rather rarely. Kubátová *et al.* (2004) found *Geosmithia* species in most stages of the life cycle of the beetle *Scolytus intricatus* under oak bark and they asked

Table 2. Most representative fungal phylotypes found in Lascaux Cave

Representative clone	Closest identified phylogenetic relatives	Accession no.	Similarity (%)	No. of clones from a total of 607 (%)
S26-24	<i>Penicillium namyslowskii</i>	EU811181	100	79 (13.0)
S4-222	<i>Isaria farinosa</i>	EU811178	99	53 (8.7)
S5-226	<i>Aspergillus versicolor</i>	EU811179	99	37 (6.1)
S4-12	<i>Tolyposcladium cylindrosporium</i>	EU811177	99	32 (5.3)
S26-8	<i>Tricholoma saponaceum</i>	EU811180	99	30 (4.9)
S1-12	<i>Geomyces pannorum</i>	EU811175	100	28 (4.6)
S30-88	<i>Geosmithia putterillii</i>	EU811182	100	28 (4.6)
S2-118	<i>Engyodontium album</i>	EU811176	100	28 (4.6)
S5-43	<i>Kraurogymnocarpa trochleospora</i>	EU811403	98	28 (4.6)
S2-9	<i>Clavicipitaceae</i> sp.	EU811240	99	17 (2.8)

why *Geosmithia* species, which produce a high number of conidia, are rarely found outside the subcorticolous niche. The topsoil above Lascaux Cave is covered by a forest where oaks predominate over pines, a system favourable to beetle colonization. Our data support a niche for *Geosmithia* species in the cave as a consequence of either water infiltration and conidia transport, or alternatively arthropods having carried this entomophilous species into the cave. The overarching question is: are the *Geosmithia* from Lascaux Cave associated with beetles, as previously reported by Kolarík *et al.* (2008), or are different classes of arthropods involved? The question could be answered if a study on the mycoflora associated with the arthropods present in the cave were carried out.

The black stains

After the *F. solani* outbreak, several black stains were found in the cave vault. The stains have extended in the last few years to the walls and have showed an alarmingly accelerated rate of colonization (Fig. 2). While between the years 2004 and 2007 a large number of fungi were isolated from the cave, the occurrence of melanized fungi was discrete. However, in the last two years *Scolecobasidium tshawytschae* has been frequently isolated from black stains (Fig. 2). This fungus synthesizes a characteristic melanin and could be responsible for the black stain formation and dissemination, as was reported for other fungi (Saiz-Jimenez *et al.*, 1995).

Previous reports on *Scolecobasidium* species indicated that they constitute a very small proportion of the fungal biota in natural habitats, including soil and decaying leaves, and are particularly active in oil-contaminated soils (Pinholt *et al.*, 1979). In addition, *S. tshawytschae* is a known fish pathogen (Doty & Slater, 1946).

We can hypothesize that the reason why *S. tshawytschae* appeared in the cave is linked to the availability of carbon sources, and benzalkonium chloride or their degradation products might be a possible carbon source. Benzalkonium chloride, a cationic surfactant, is rapidly and strongly sorbed onto sediments, clays and minerals. Benzalkonium chloride is transformed into alkyl dimethylamines through a nucleophilic substitution at low temperatures (0–40 °C) in an abiotic reaction in the presence of nitrites (Tezel & Pavlostathis, 2009). Nitrites and ammonium are abundant in Lascaux Cave (Lastennet *et al.*, 2009), and it is expected that nitrites are as well; however, the last was not analysed. When benzalkonium chloride is degraded by bacteria, formation of benzyldimethylamine, benzylmethylamine, benzylamine, benzaldehyde and benzoic acid occurs (Patrauchan & Oriol, 2003). Nobuo (2005) found that a *Scolecobasidium* sp. was dominant in washing machines using synthetic detergent. Laundry detergents typically contain cationic surfactants. It is possible that *S. tshawytschae* utilizes some of the benzalkonium chloride intermediates as nitrogen and carbon sources. This is a hypothesis to be tested.

Arthropod ecology

In the last few years, the springtail *Folsomia candida* (Collembola, Isotomidae) has been found on and around black stains (Fig. 4). This is a cosmopolitan opportunistic troglophile (a facultative cavernicole, frequently completing its whole life cycle in caves, but not confined to this habitat), recorded in caves all over the world (Palacios-Vargas, 2002). It is generally accepted that this springtail is found in cave sites which are not occupied by cave-adapted species and in disturbed and artificial areas, and we must admit that Lascaux Cave is an example of an ecologically disturbed site where *Fol. candida* was probably attracted from the topsoil litter to the cave by the food source that the different fungal outbreaks represented.

DNA of the collembolae *Fol. candida* and *Hypogastrura* sp. was also retrieved in the cave, but specimens of the latter were not found (Bastian *et al.*, 2009b). Several authors have reported the presence of trogliphilic *Hypogastrura* spp. in North American and Romanian Caves (Gruia, 2003; Elliott, 2007; Skarzynski, 2007).

Collembolae are largely mycophagous, and numerous *Fol. candida* specimens were observed feeding on black stains (Fig. 4). *Fol. candida* prefers melanized fungal species (Scheu & Simmerling, 2004) over hyaline fungi as food, but *Fusarium* hyphae (Sabatini & Innocenti, 2000), several *Pseudomonas* spp., including *P. fluorescens* (Thimm *et al.*, 1998) and nematodes (Lee & Widden, 1996), have also been shown to be eaten.

We used the collembolan *Fol. candida* and the two fungi from Lascaux outbreaks (*Fusarium solani* and *S. tshawytschae*) to answer the following questions: Does *Fol. candida* show feeding preferences for these fungi? Can the feeding preferences be responsible for the fungal dispersion? Live specimens of *Fol. candida* from Gombasecka Cave in Slovakia were collected. Thirty specimens were placed in



Fig. 4. A black stain on sediments with *Folsomia candida* specimens (the collembolae are about 1 mm long).

Petri dishes containing either a fresh culture of *F. solani* (4-day-old colonies) isolated from Domica Cave, Slovakia, or a culture of *S. tshawytschae*, isolated from black stains in Lascaux Cave. Both were deposited onto a cave sediment or plaster of Paris (gypsum). After 24 h, all specimens in the gypsum dishes were feeding on *Fusarium* or in the immediate vicinity and they were eating mycelia, while in cave sediment dishes most specimens were in the environs of *Fusarium* although visible signs of feeding were observed.

After 10 days, strong grazing of *Fol. candida* on the fungi was observed, especially on *Fusarium*, where the mycelium was eaten off and *Folsomia* eggs were present (Fig. 5A). Even further more evident changes were found after 20 days of the experiment and more than one-third of *Fusarium* mycelia on both substrata (cave sediment and gypsum) were eaten off (Fig. 5B). *Scolecobasidium* was also attacked and the mycelia on gypsum were completely eaten while on the cave sediment distinct parts were eaten off

(Fig. 5C). These results show that the two fungal species which caused the most serious outbreaks in Lascaux Cave were a very suitable food for *Fol. candida*.

We have observed *Fol. candida* feeding on black stains in Lascaux Cave, as well as in the laboratory on peat debris or fungi. In both cases *Fol. candida* produced black faecal pellets whose dissemination was extensive, as can be seen in Fig. 5D. Sawahata (2006) found considerable production of black faecal pellets when collembolae consumed the hymenial area of agaric fruit bodies. Faecal pellets contain fungal conidia that germinate once deposited on a wet surface. Whilst the germinability of conidia may be reduced by gut passage, it is not uncommon for almost all faecal pellets produced by arthropods to contain some germinable conidia (Thimm *et al.*, 1998; Williams *et al.*, 1998). Sabatini *et al.* (2004) showed that some collembolae preferred *Fusarium* as food and that a few colonies of *Fusarium* developed from their faecal pellets.

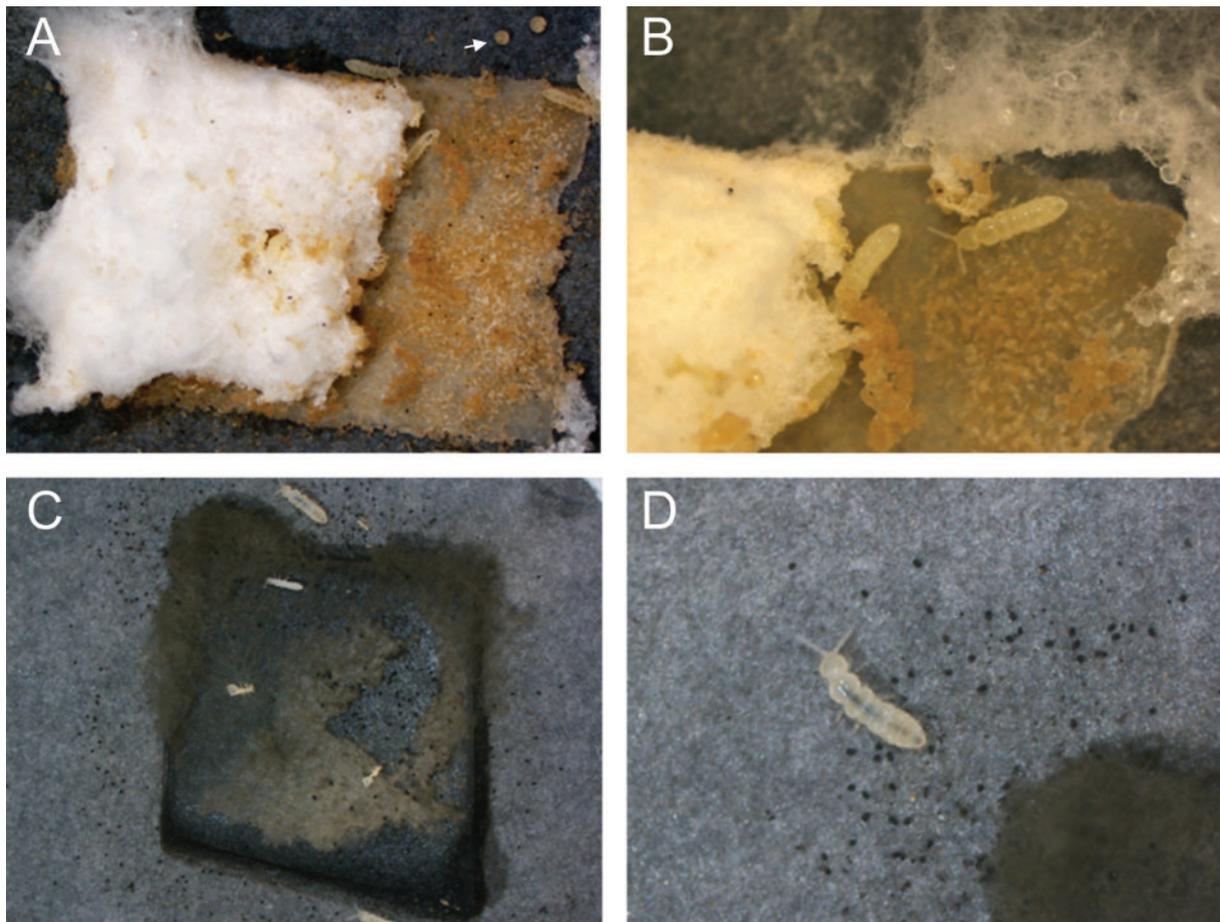


Fig. 5. Grazing of *Folsomia candida* on the two main fungi reported in Lascaux Cave. (A) Grazing on *Fusarium solani* grown in a microcosm with plaster of Paris. Some eggs are evident. (B) *Fusarium solani* mycelia have been eaten off. (C). Grazing on *Scolecobasidium tshawytschae* grown in a microcosm with cave sediment. Note two exuviae on the fungus and the faecal pellets. (D) *Folsomia candida* grazing on *Scolecobasidium tshawytschae* and faecal pellets. Note the black colour of the gut due to fungal melanin consumption.

On the other hand, *Fol. candida* is a vector for micro-organisms. Dromph (2003) reported that collembolae are vectors of entomopathogenic fungi, and Greif & Currah (2007) isolated species of the fungal genera *Acremonium*, *Beauveria*, *Cladosporium*, *Cryptendoxyla*, *Geomyces*, *Gliocladium*, *Hormiactis*, *Leptographium*, *Oidiodendron*, *Penicillium* and *Verticillium* from collembolae.

The high density of collembolae in contact with bacterial cells, mycelial fragments and conidia suggests that these arthropods significantly increase the dispersal rate of bacteria (Scheu & Simmerling, 2004) and fungi (Thimm *et al.*, 1998) by carrying conidia on their bodies. In addition, the gut of *Fol. candida* is a selective habitat and a vector for micro-organisms (Thimm *et al.*, 1998).

The *Fol. candida* population in Lascaux Cave is related to its feeding preferences, which explains the presence of this collembola species after the first fungal outbreak. Fungi produce volatile compounds that are potentially attractive to collembolae (Bengtsson *et al.*, 1988). At present, grazing on the black stains might be a consequence of the presence of melanized fungi and, particularly, of *S. tshawytschae* in the last 2 years. This fungus is probably disseminated through the cave by the collembolae and their faecal pellets, thus contributing to the appearance of black stains. Interestingly, unidentified members of the family Campodeidae (Diplura) were also observed feeding on the black stains. Most diplurids are predators and their diet includes collembolae and mites (Lock *et al.*, 2009). They may also survive on vegetable debris and fungal mycelia. Indeed melanized fungi are also the most palatable fungi for mites (Schneider & Maraun, 2005). A detailed survey on different classes of arthropods should be carried out to confirm the presence of other cavernicole populations in the cave.

Conclusions and perspectives

This study raises intriguing questions about the past, present and future management of caves, and shows how human activities have interrupted the ecological balance and directly affected cave biodiversity. At present, micro-organisms in Lascaux Cave grow as biofilms which consist of assemblages containing many species of bacteria, fungi, protozoa, etc. Biocide treatments, including quaternary ammonium derivatives, were extensive for a few years, and were used again periodically from January 2008. As a result of years of treatments, the indigenous microbial communities of Lascaux Cave were replaced by microbial populations selected by biocide application. Lascaux Cave now contains potentially pathogenic bacteria and protozoa (Bastian *et al.*, 2009a), which might be the result of many years of human impact (visits, air-conditioning system, biocide treatments).

The composition of the fungal community is largely influenced by the colonization and activity of arthropods, with collembolae considered to be of primary significance.

Other arthropods, such as coleoptera, could play a role in fungal community structure; however, further studies are needed to verify this and to determine the degree of association of some of the fungal species identified in the cave with coleoptera.

The appearance of black stains in Lascaux Cave might be related to the presence of *S. tshawytschae*, probably promoted by biocide applications, and the grazing effects of the cavernicole population. How can we explain the fast development of black stains in the last 2 years, if not by the presence of abundant organic matter in the cave and the imbalance produced by antifungal treatments, which were revealed not to be fully effective? Are the black stains due to the production of melanin by *S. tshawytschae*? What are the carbon sources for this fungus? The utilization of benzalkonium chloride as a probable carbon and nitrogen source for cave micro-organisms has to be assessed.

There are still many unanswered questions on the microbial ecology of this cave that research should clarify in the coming years. A careful study on the arthropods, the ecology of foreign fungi and their dispersion patterns is needed in order to complete the food web and to control the black stains.

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