Hétérogénéité spatio-temporelle du microbiote de la grotte de Lascaux

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Résumé

Les grottes sont des environnements oligotrophes présentant des conditions comparables de stabilité de température, d'humidité relative généralement élevée et d'absence de lumière, mais aussi des spécificités en termes de dimensions et d'architecture, de substrats minéraux et d'écoulement d'eau. L'anthropisation est la principale source de perturbations dans les grottes (aménagements, visites touristiques, traitements chimiques), et dans le cas de la grotte de Lascaux célèbre pour ses peintures et gravures rupestres cela a entrainé la prolifération de certains microorganismes et des altérations de paroi menaçant la conservation de ce site paléolithique.

L'objectif général de cette thèse était de mieux comprendre l'écologie des microorganismes colonisant la grotte de Lascaux, et plus particulièrement l'hétérogénéité spatio-temporelle du microbiome de cette grotte. Il s'agissait notamment d'identifier la communauté microbienne de la grotte à différentes échelles spatio-temporelles, de caractériser les facteurs qui structurent cette communauté et d'en étudier la dynamique fonctionnelle en utilisant le séquençage à haut débit d'acides nucléiques, une approche qui n'avait pas encore mise en œuvre à Lascaux. Ces recherches visaient à tester quatre hypothèses, à savoir (i) la diversité de la communauté microbienne sur les parois dépend du niveau d'anthropisation de la grotte, (ii) les altérations (taches noires) impactent plus que la nature du substrat minéral la diversité de la communauté microbienne, (iii) les taches noires et les zones sombres qui sont deux types d'altérations dans la salle de l'Abside présentent des communautés microbiennes différentes, mais dont les collemboles se nourrissent, participant dans les deux cas à la dissémination des microorganismes, et (iv) les activités transcriptionnelles des microorganismes diffèrent sur et autour des taches noires, les gènes potentiellement impliqués dans la production de pigments (mélanines) étant surexprimés dans les taches noires.

Nos travaux se sont tout d'abord portés sur une comparaison à l'échelle régionale de différentes grottes de Dordogne, plus ou moins anthropisées, puis à une échelle locale avec l'étude d'une salle positionnée de façon centrale dans la grotte (le Passage) pour évaluer le rôle des substrats minéraux, et celle d'une autre salle (l'Abside) qui présente à la fois des taches noires et des zones sombres. Nos résultats montrent que les grottes anthropisées (dont Lascaux) présentent des communautés microbiennes particulières. Dans le Passage, le substrat minéral structure davantage la communauté que la présence de taches. Concernant l'Abside, bien que les zones sombres soient visuellement différentes des taches noires, leurs communautés microbiennes présentent des similarités fortes ; les champignons noirs des taches noires sont disséminés par les collemboles, qui peuvent consommer des bactéries du genre *Pseudomonas* (qui peuvent d'ailleurs inhiber la croissance de champignons noirs) majoritaires en dehors des taches noires et des zones sombres. Enfin, le séquençage des ARN messagers a montré des profils métatranscriptomiques différents en fonction des salles et de la présence de taches, mais sans identifier de surexpression possible d'enzymes impliquées dans le métabolisme des mélanines.

Ce projet a permis de caractériser la diversité, la structure, la composition et les activités transcriptionnelles de la communauté microbienne de la grotte de Lascaux. Ces informations permettent de mieux comprendre le fonctionnement microbien de la grotte et d'alimenter la réflexion sur les stratégies à mettre en place pour optimiser la conservation de ce site paléolithique majeur.

Abréviations

ADN : Acide DésoxyriboNucléique
ANOSIM : Analysis of similarities
ARN : Acide RiboNucléique
BCA : Between-Class Analysis
COG : Clusters of Orthologous Groups
DGGE : Denaturing Gradient Gel Electrophoresis
EBI : European Bioinformatics Institute
EC : Enzyme Commission numbers
FROGS : Find, Rapidly, OTUs with Galaxy Solution
iTOL : iterative Tree Of Life
ITS : Internal Transcribed Spacer
NMDS : Non-Metric multi-Dimentional Scaling
NRI : Net Relatedness Index
NTI : Nearest Taxa Index
OTU : Operational Taxonomic Unit
PAST : PAleontological STatistics
qPCR : quantitative Polymerase Chain Reaction
RAPD : Random Amplified Polymorphic DNA
RDP : Ribosomal Database Project
RFLP : Restriction Fragment Length Polymorphism
RISA : Ribosomal Intergenic Spacer Analysis
UV : Ultra Violet

Introduction générale

Dans la nature, les êtres vivants ne sont distribués ni uniformément ni au hasard, ils sont plutôt répartis selon des gradients ou dans des zones particulières correspondant à des habitats homogènes (Legendre & Fortin, 1989). Ces êtres vivants interagissent entre eux et avec leur environnement, ce qui forme un écosystème. De façon générale, l'étude d'un écosystème a pour but de définir sa structure ainsi qu'expliquer son fonctionnement et sa dynamique, ce qui nécessite de prendre en compte ses fluctuations spatiales et temporelles. En écologie microbienne, la distance spatiale doit être prise en compte à différentes échelles (millimétrique, centimétrique, locale, régionale…) pour comprendre le fonctionnement du site étudié (sol de prairie, lac, etc.), car les microorganismes étant par définition microscopiques (0,02 à 200 µm) une petite variation de distance peut avoir une influence sur la communauté microbienne. La notion de temps est aussi essentielle car les activités microbiennes se produisent en quelques secondes (expression des gènes, catalyse enzymatique, etc.) ou plusieurs heures (croissance) (Prieur et al. 2015). Ces deux facteurs sont donc largement étudiés dans différents environnements comme les sols (Laverman et al. 2001), les lagons (Laque et al. 2010), les systèmes digestifs (Costello et al. 2009), pour comprendre leur impact sur la communauté microbienne.

Les grottes sont des environnements considérés comme extrêmes car pauvres en nutriments (Tomczyk-Żak & Zielenkiewicz, 2016). Elles présentent des conditions comparables comme un taux d'humidité élevé, une température stable et l'absence de lumière (Barton & Jurado, 2007). En revanche, les grottes sont différentes les unes des autres en ce qui concerne la composition des parois (e.g. calcaire, gypse) (Northup & Lavoie, 2001), leur taille, le nombre de salles, la faune présente et l'intérêt historique par exemple. Les grottes ont aussi une communauté microbienne qui a été de plus en plus étudiée depuis le début des années 90 grâce à l'avènement des nouvelles technologies de biologie moléculaire (Barton & Northup, 2007). En effet, les grottes étant oligotrophes, les chercheurs pensaient que la diversité microbienne était faible. Néanmoins, certains processus géochimiques étaient difficiles à expliquer seulement par des transformations abiotiques (Barton & Northup, 2007). Ainsi l'implication des microorganismes dans des processus biogéochimiques (précipitation du carbonate de calcium) a été établie et donc l'étude de ces microorganismes dans les grottes a progressé (Barton & Northup, 2007). Cependant, l'étude des microorganismes dans les grottes est plutôt descriptive et peu comparative. Aussi deux types de grottes sont étudiées, les grottes « naturelles » et les grottes ornées présentant de l'art rupestre préhistorique (peintures et gravures). Les grottes naturelles sont étudiées pour une meilleure compréhension de cet écosystème alors que l'étude des grottes ornées a également un but de conservation.

La principale cause de perturbation d'une grotte est d'origine anthropique (e.g. aménagements, visites touristiques, traitements chimiques) (Urzi et al. 2010). L'impact de l'anthropisation sur la communauté microbienne dans les grottes a été étudié au niveau

local, c'est-à-dire une comparaison des zones non anthropisées et anthropisées à l'intérieur d'une même grotte (Northup et al. 1997) ou de façon plus globale en comparant des grottes anthropisées et non anthropisées (Mulec et al. 2012) souvent par des méthodes de mise en culture. Les résultats montrent que le nombre de colonies microbiennes obtenues sur boite est plus important pour les zones perturbées d'une même grotte (Northup et al. 1997) alors que peu de bactéries sont trouvées dans la grotte anthropisée comparativement aux grottes non anthropisées (Mulec et al. 2012). Cependant la grotte anthropisée a une plus grande taille ce qui peut biaiser les résultats. Ainsi de nombreux facteurs peuvent impacter la structure ou la diversité de la communauté microbienne, c'est pourquoi il est important de prendre en compte ces facteurs conférant une hétérogénéité au système étudié, comme la spatio-temporalité, pour l'analyse de la communauté microbienne.

Lascaux

La grotte de Lascaux, classée au patrimoine mondial de l'UNESCO en 1979, est une grotte calcaire de Dordogne célèbre pour ces peintures et gravures rupestres (Bastian et al. 2009). Elle est composée de 11 salles de tailles et morphologies différentes. Les parois de ces salles sont composées de substrats minéraux différents allant du calcaire à l'argile. De plus, certaines salles n'ont jamais été accessibles au public et aujourd'hui elles ne sont pas accessibles aux scientifiques pour des raisons de conservation, donc le niveau d'anthropisation dans les salles est différent. La grotte de Lascaux a été largement visitée pendant 15 ans (1948-1963) avec un nombre de visiteurs allant de 1800 à 2000 par jour dans les années 60 (Bastian et al. 2010). Pour permettre ces visites, des aménagements ont été effectués (installation d'escaliers, système de renouvellement de l'air…), ces perturbations anthropiques (et ultérieurement les traitements chimiques des parois) ont engendré le développement de taches d'origines microbiennes sur les parois de la grotte sous différentes formes (Bastian et al. 2010). D'abord, des algues vertes puis un champignon blanc et enfin des taches noires. A chaque nouvelle apparition de taches des traitements chimiques ont été appliqués, les algues vertes et le champignon blanc ont disparu alors que les taches noires sont toujours présentes. Il existe aussi des taches jaunes situées dans la galerie du Mondmilch et des taches violettes dans l'Abside mais ces types de taches sont moins nombreuses que les taches noires. Les taches noires sont présentes essentiellement dans deux salles de la grotte de Lascaux, le Passage et l'Abside. Visuellement, les taches noires n'ont pas toutes la même morphologie et ne sont pas réparties de façon uniforme sur les parois. Cette hétérogénéité spatiale est un facteur intéressant pour la caractérisation de la communauté microbienne.

La grotte de Lascaux est donc un modèle d'étude intéressant pour comprendre l'écologie d'une communauté microbienne dans un environnement hétérogène ayant subi une forte pression anthropique. Elle a d'ailleurs fait l'objet d'un premier projet scientifique en 2008 pour analyser l'écologie microbienne de la grotte et caractériser ces taches noires. Ce projet coordonné par Claude Alabouvette (INRA Dijon) et Cesareo Saiz-Jiménez (INRAS-

SCIC, Espagne) a permis d'identifier et de caractériser les communautés microbiennes par des techniques de mise en culture, de clonage séquençage et de caractérisation moléculaire, ainsi que d'étudier la nature biochimique des taches noires, le rôle des collemboles dans la dispersion des taches noires, et de déterminer le niveau de contamination microbiologique dans l'air de la grotte (Alabouvette & Saiz-Jiménez, 2011). Ce projet a mis en évidence un écosystème complexe, la présence de nouvelles espèces fongiques (Martin-Sanchez et al. 2012) et apporté des premières réponses sur le comportement des macro et micro-organismes dans la grotte (Bastian et al. 2009b ; Bastian et al. 2009c). Afin d'enrichir cette première étude et de caractériser la communauté microbienne de façon plus complète, un deuxième projet a été mis en place en 2014 avec une approche d'identification des micro-organismes par séquençage à haut débit et un suivi spatio-temporel. Ce projet coordonné par Yvan Moënne-Loccoz (UMR 5557 Ecologie microbienne, Université Lyon 1 et CNRS Lyon) a été réalisé en collaboration avec l'équipe de Christophe Douady du laboratoire d'écologie des hydrosystèmes naturels et anthropisés (LEHNA UMR CNRS 5023).

Objectifs du projet 'Ecologie microbienne de la grotte de Lascaux'

Le projet 'Ecologie microbienne de la grotte de Lascaux' (2014-2017) a plusieurs objectifs, (i) caractériser la communauté microbienne de la grotte de Lascaux présente sur les taches noires et sur des zones non tachées en fonction du temps et dans différentes salles de la grotte, en utilisant le séquençage à haut débit des acides nucléiques et en ciblant plus particulièrement la salle du Passage de la grotte de Lascaux qui est une salle ayant subi une forte pression anthropique et possédant des taches noires, (ii) étudier la dissémination des microorganismes producteurs de taches par trois moyens possible, l'eau, l'air et les collemboles, (iii) caractériser les taches noires et les champignons noirs de la grotte de Lascaux afin d'identifier la composition chimique des pigments produits par les microorganismes. Ce dernier objectif a été réalisé par le centre d'étude des substances naturelles (CESN) du laboratoire d'écologie microbienne.

Afin d'étudier la communauté microbienne de façon globale, la technique de séquençage à haut débit des acides nucléiques a été utilisée par une approche de métabarcode en ciblant les marqueurs taxonomiques des trois domaines de la vie, l'ARNr 16S des bactéries, l'ARNr 16S des archées, l'ARNr 18S des micro-eucaryotes et l'Internal Transcribed Spacer 2 (ITS2) pour identifier plus finement les micro-eucaryotes correspondant aux champignons. C'est dans ce contexte qu'a été réalisée cette thèse, qui répond en partie aux deux premiers objectifs du projet.

Objectifs et démarche de la thèse

L'objectif principal de cette thèse était d'étudier l'hétérogénéité spatio-temporelle de la communauté microbienne de la grotte de Lascaux en utilisant les nouvelles technologies de séquençage à haut débit, avec pour **hypothèse générale** que l'hétérogénéité de l'habitat (différences en termes de salles, substrats géologiques et présence de taches) se concrétise par une hétérogénéité de la communauté microbienne sur les parois, avec un impact plus fort de l'hétérogénéité spatiale que de l'hétérogénéité temporelle vue la stabilité des conditions climatiques dans la grotte.

Pour atteindre cet objectif général, quatre objectifs particuliers ont été définis. **Le premier** est de comparer à l'échelle régionale la communauté microbienne de grottes calcaires anthropisées et non anthropisées dont la grotte de Lascaux, avec l'hypothèse que la diversité microbienne est liée au niveau d'anthropisation.

Le deuxième, déterminer le facteur qui structure la communauté microbienne présente et active du Passage en prenant en compte le temps, le substrat minéral et la présence de taches, sachant que les conditions environnementales sont plutôt stables dans le temps et que les taches sont le facteur d'hétérogénéité le plus récent. L'hypothèse est que les taches impactent plus que le substrat minéral et le temps la structure de la communauté.

Le troisième objectif particulier est d'étudier la communauté microbienne de l'Abside ainsi que les interactions potentielles des microorganismes avec les collemboles. L'Abside comporte des taches noires mais aussi de nouvelles altérations nommées les zones sombres. Les hypothèses sont que la communauté microbienne des taches noires est différente de la communauté microbienne des zones sombres et que les collemboles se nourrissent des microorganismes et ont un rôle dans la dissémination de ceux-ci.

Enfin, **le quatrième** objectif particulier est d'étudier la dynamique fonctionnelle de la communauté microbienne et de rechercher les séquences potentiellement impliquées dans la production de pigments noirs, avec pour hypothèse que les gènes impliqués dans le métabolisme de la mélanine ou autres pigments noirs soient surexprimés sur les taches noires en comparaison avec les zones non tachées.

Structure de la thèse

Le document de thèse est structuré en six parties. **La première partie** est une synthèse bibliographique la microbiologie des grottes puis la présentation du modèle d'étude ainsi que des techniques de biologie moléculaire utilisées.

La deuxième partie répond au premier objectif avec l'étude comparative à l'échelle régionale de la communauté microbienne de quatre grottes non anthropisées, quatre grottes anthropisées et la grotte de Lascaux. Dans ces grottes situées sur la même veine

calcaire ont été étudiées la diversité et la taxonomie de la communauté des bactéries, archées et micro-eucaryotes par séquençage à haut débit ciblant les marqueurs taxonomiques (les gènes codant les ARNr 16S bactériens et archéens, et les ARNr 18S) ainsi que l'abondance des bactéries et des micro-eucaryotes par PCR quantitative. Cette étude a pris en compte l'aspect temporel en réalisant l'échantillonnage sur deux dates et l'aspect spatial en prenant des échantillons à différents endroits des grottes (de l'entrée jusqu'au fond).

La troisième partie répondant au deuxième objectif a été réalisée en étudiant la diversité de la communauté présente et active des bactéries, des micro-eucaryotes et des champignons par séquençage à haut débit des marqueurs taxonomiques (ARNr 16S bactéries, ARNr 18S micro-eucaryotes, ITS2 pour les champignons) sur cinq dates différentes, sur trois substrats minéraux et sur les zones tachées et non tachées du Passage de la grotte de Lascaux. Afin de comprendre les interactions entre les bactéries et les champignons du Passage des réseaux de co-occurrence ont été également été réalisés.

La quatrième partie répondant au troisième objectif s'intéresse à la diversité des microorganismes présents sur les taches noires et en dehors des taches de manière globale en utilisant le séquençage à haut débit (gènes codant les ARNr 16S des bactéries, ARNr 18S des micro-eucaryotes, et ITS des champignons), mais aussi à l'interaction entre les microorganismes (bactéries et champignons) et les collemboles avec des tests *in vitro.* La consommation et la dissémination par les collemboles des champignons et des bactéries isolés de taches et de zones non tachées ont été testées, ainsi que l'inhibition de la croissance de champignons par des bactéries. Dans l'Abside, la diversité et la taxonomie des communautés microbiennes présentes sur les taches noires, sur les zones sombres et sur les zones non tachées ont été comparées en utilisant le séquençage à haut débit.

La cinquième partie est une étude métatranscriptomique de la dynamique fonctionnelle de la communauté microbienne. Deux substrats minéraux du Passage ont été prélevés à trois dates différentes, d'une part, et cinq situations (deux substrats minéraux du Passage et trois salles différentes) ont été prélevées à une même date, d'autre part, en prenant pour chaque situation une zone tachée (quand disponible) et une zone non tachée. Le transcriptome entier a été séquencé, puis le traitement des séquences a permis de ne conserver que les ARN messagers. Une analyse des familles fonctionnelles de gènes a été réalisée pour comparer les différentes situations, puis une analyse discriminante entre les zones tachées et non tachées pour identifier des enzymes spécifiques de ces zones.

La dernière partie est consacrée à la discussion générale de l'ensemble des travaux réalisés et aux perspectives de ces recherches.

Chapitre 1 : Synthèse bibliographique

A. Introduction : L'écosystème karstique

A1. Description et formation d'un karst

Un karst est un type de paysage trouvé sur des terrains carbonatés (calcaire, dolomie, marbre) ou des évaporites (gypse, sel de gemme, anhydrite), caractérisé par des reliefs particuliers résultant de la solubilisation des roches et d'une porosité secondaire très développée (fractures) (Bakalowicz, 1999). C'est aussi un système hydro-géologique, avec un réseau de drainage souterrain très développé et une pénurie de ruisseaux de surface. Le processus de mise en place du karst ou karstification s'étale typiquement sur quelques dizaines de milliers d'années (Ford & Williams, 1989). Les karsts se trouvent le plus souvent dans des régions relativement humides, mais de toutes sortes (méditerranéennes, tempérées, tropicales, alpines ou polaires), et le processus de dissolution souterrain profond peut également se produire dans des régions arides (Ford & Williams, 1989). Les karsts, en ce qui concerne leurs surfaces et affleurements, concernent 20% des terres émergées (Figure 1).

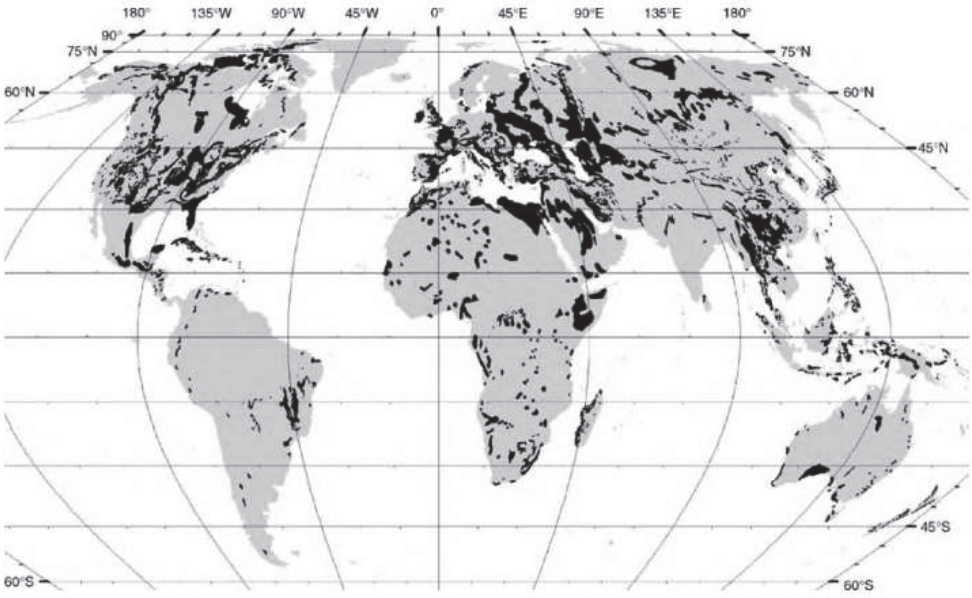

Figure 1. Distribution globale des affleurements de roches carbonatées (Ford & Williams, 2007)

L'infiltration d'eau à travers les roches carbonatées conduit à la dissolution chimique du calcaire et de la dolomie par le CO_2, ce qui peut entraîner la formation d'écosystèmes souterrains particuliers tels que les grottes karstiques, c'est la karstification. Trois types d'énergies entrent en jeu dans le processus de karstification : chimique, potentielle et mécanique. La transformation de l'énergie chimique est la dissolution de la roche mère souvent calcaire avec la production d'ions. L'énergie potentielle est l'évacuation des produits par les débits d'eau et les différences d'altitude entre la zone d'alimentation et la

zone de sortie. Enfin, l'énergie mécanique engendre la fracturation de la roche (Quinif et al. 2014).

Il existe plusieurs types de grottes, mais les plus communes sont celles formées dans la roche calcaire. D'autres types de grottes sont limités dans leur extension comme celles qui se forment dans le gypse, le granite, le grès ou la glace.

A2. Caractéristiques biotiques et abiotiques d'une grotte

Les grottes karstiques présentent des conditions environnementales particulières comme l'absence de lumière, une humidité très forte (proche de la saturation), des températures stables (Cuezva et al. 2009 ; Bastian et al. 2009b ; Pašic et al. 2009 ; De Leo et al. 2012) et un taux élevé de CO_2 (Banerjee et al. 2013). La concentration de minéraux est élevé (Barton & Jurado, 2007 ; De Leo et al. 2012) mais les grottes sont oligotrophes (Bastian et al. 2009b, Canaveras et al. 2001, Jurado et al. 2009, Cuezva et al. 2009). Un milieu oligotrophe comporte moins de 2 mg de carbone organique total par litre (Barton et al. 2007), et dans les grottes le taux de carbone organique total est souvent inférieur à 0,5 mg par litre (Barton & Jurado, 2007). La plupart du carbone organique provient de la surface et est transporté vers l'endokarst, c'est-à-dire la partie souterraine du karst (Figure 2). Il provient aussi des flux d'air transportant des particules organiques ou de déjections animales comme celles des chauves-souris (Saiz-Jimenez & Hermosin, 1999 ; Pašic et al. 2009 ; Barton & Jurado, 2007 ; Cuezva et al. 2009).

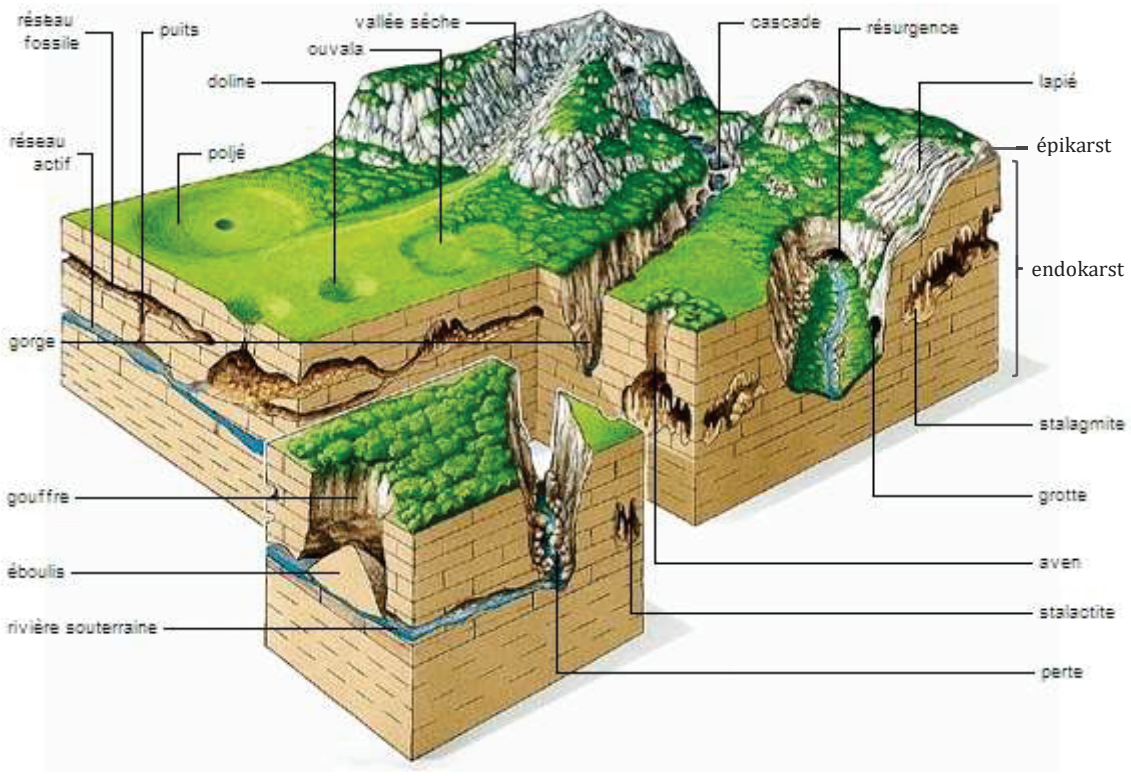

Figure 2. Schéma d'un relief karstique d'après le dessin de Dominique Sablons – Archive Larousse

Les grottes sont aussi des systèmes hétérogènes de par la nature et les propriétés de surface des différentes couches de roches, l'architecture interne des grottes, ainsi que les flux d'air et les échanges climatiques avec l'atmosphère extérieure. Les conditions climatiques extérieures impactent surtout l'entrée des grottes et donc les fluctuations climatiques dépendront de la distance par rapport à l'entrée de la grotte, et de l'épaisseur de l'épikarst, qui est la partie superficielle du karst, caractérisée par un réseau de fissures (Cuezva et al. 2009).

Les grottes contiennent de nombreux organismes animaux comme des chauves-souris, des araignées, des coléoptères, des collemboles (Gnaspini & Trajano, 2000) et aussi des microorganismes, à savoir bactéries, virus, champignons et protozoaires (Jurado et al 2010 ; Risse-Buhl et al. 2013 ; Tomczyk-Żak & Zielenkiewicz, 2016). Ces microorganismes ont d'ailleurs un rôle dans certains processus géochimiques (Lian et al. 2011). Les grottes sont hétérogènes et les facteurs engendrant cette hétérogénéité peuvent influencer la communauté microbienne et son fonctionnement (Tomczyk-Żak & Zielenkiewicz, 2016).

A3. Intérêt des communautés microbiennes dans les grottes

Les microorganismes sont retrouvés dans tous les environnements, mais certains facteurs physiques peuvent limiter leur croissance comme la température, le pH acide ou la sécheresse (Barton & Jurado, 2007). Le carbone organique, l'azote combiné et le phosphore sont également essentiels pour les microorganismes, et bien que les grottes soient des milieux oligotrophes, les microorganismes y sont nombreux (Barton & Jurado, 2007).

Les microorganismes interviennent dans le processus de karstification en altérant la roche carbonatée suivant trois processus : (1) altération chimique, dans lequel les acides organiques et les ligands sécrétés par le métabolisme microbien contrôlent la désintégration et la solubilisation des roches carbonatées (Lian et al. 2011), (2) altération physique, car lors de leur croissance les microorganismes induisent une pression physique capable de casser les particules minérales (Lian et al. 2011), (3) altération enzymatique, certaines enzymes et composés actifs sécrétés par les bactéries ou les champignons dégradent les roches carbonatées (Lian et al. 2011). Ainsi, les microorganismes pourraient employer un ou plusieurs de ces processus. Certaines études montrent que les champignons ont une plus grande capacité d'altération des minéraux que les bactéries. En effet, le mycélium fongique pénètre, enveloppe et fissure les particules minérales, en générant une pression mécanique (Kumar & Kumar, 1999 ; Sterflinger et al. 2000 ; Gorbushina et al. 2007). Les champignons produisent également de nombreuses enzymes et produits métaboliques qui peuvent contribuer à altérer les minéraux.

Avant les années 90, les études sur la microbiologie des grottes étaient descriptives et avaient pour but de démontrer la présence de microorganismes dans cet environnement (Barton & Northup, 2007). Certains chercheurs pensaient que peu d'espèces microbiennes étaient capables de vivre dans un milieu aussi pauvre en nutriments, et ils n'étaient pas

convaincus que les microorganismes pouvaient intervenir dans les processus de dissolution de la roche par exemple. Au début des années 90, de nouvelles techniques moléculaires permettent aux microbiologistes d'étudier un plus grand nombre d'environnements, de cibler les microorganismes indépendamment de leur cultivabilité, et de répondre à de nouvelles questions à l'interface entre la microbiologie et la géologie.

B. Colonisation des surfaces minérales par les microorganismes

B1. Dissémination de l'extérieur vers l'intérieur des grottes

Les grottes sont des environnements souterrains mais liés au monde extérieur (Anton et al. 2012), notamment via l'eau et l'air. Ainsi, les microorganismes peuvent pénétrer dans les grottes et les écosystèmes karstiques suite à une dissémination passive par infiltration d'eau, par des flux d'air correspondant à des convections (Engel & Northup, 2008 ; Cuezva et al. 2009), ou via l'Homme ou les animaux tels que les arthropodes (Dupont et al. 2007 ; Barton & Jurado, 2007). En effet, on considère que la plupart des microorganismes présents dans les grottes y ont été transportés, et ils ont souvent été considérés comme ne représentant pas un microbiote spécifique à une grotte (Palmer et al. 1991 ; Cunningham et al. 1993 ; Northup et al. 1994 ; Rusterholtz & Mallory, 1994).

Les premiers colonisateurs, et notamment dans les zones d'entrée des grottes ou bien dans les grottes touristiques éclairées artificiellement, sont des micro-organismes phototrophes comme les cyanobactéries ou les algues (Ortega-Calvo et al. 1993 ; Borderie et al. 2016). Ces communautés nommées « lampenflora » forment des biofilms sur les parois ou le sol des grottes (Lefèvre, 1974), qui sont bénéfiques aux micro-organismes hétérotrophes (bactéries et champignons) qui consomment la matière organique ainsi synthétisée (Akatova et al. 2007). Les champignons sont probablement disséminés sous forme de spores, tandis que les bactéries sont susceptibles d'entrer dans les grottes sous forme de cellules adhérant aux particules de poussière (Chen et al. 2009 ; Dupont et al. 2007 ; Garcia-Anton et al. 2014). Le ou les principaux processus conduisant à l'entrée des micro-organismes dans les grottes varient en fonction des spécificités de la grotte. Pour la grotte d'Altamira (Espagne), les échanges avec l'air extérieur sont considérés importants (Cuezva et al. 2009 ; Saiz-Jimenez et al. 2011) alors que dans la grotte de Lascaux, les arthropodes joueraient un rôle plus important (Bastian et al. 2010).

B2. Adhésion

Dans les grottes, l'établissement des microorganismes sur les parois peut se faire grâce à un phénomène d'adhésion (Cuezva et al. 2009). Le mécanisme d'adhésion des bactéries le plus étudié est la sécrétion d'exopolysaccharides (Gorbushina et al. 2007 ; Zucconi et al. 2012) qui conduit à la formation de biofilms et facilite la fixation d'autres microorganismes (Riding

et al. 2000). Les biofilms sont composés de différents microorganismes. Dans la grotte des Chauves-souris (Cave of Bats) en Espagne et dans la grotte des Moidons en France, les biofilms sont constitués majoritairement de microorganismes phototrophes (Urzì et al. 2010 ; Borderie et al. 2016) mais contiennent aussi des microorganismes hétérotrophes. Les conditions microclimatiques peuvent aussi faciliter l'adhésion de microorganismes comme la condensation sur les parois. La condensation forme des microparticules d'eau appelées hydroaérosols, qui contiennent des particules présentes dans l'air, des spores et des bactéries et qui peuvent adhérer à la paroi (Cuezva et al. 2009 ; Garcia-Anton et al. 2013). À son tour, l'adhésion microbienne aux surfaces rocheuses peut faciliter la biotransformation des minéraux qui fournissent des nutriments ou de l'énergie (Riding et al. 2000).

Concernant les champignons, leurs hyphes pénètrent directement dans les micropores des parois (Kumar & Kumar, 1999 ; Sterflinger et al. 2000 ; Gorbushina et al. 2007) leur permettant de coloniser différents types de substrats minéraux (Stupar et al. 2014).

B3. Croissance

Les conditions microclimatiques des grottes et la disponibilité des ressources sur la roche déterminent la susceptibilité des matériaux des grottes à l'adhésion microbienne, et surtout à la croissance et à la colonisation (Gorbushina, 2007 ; Cuezva et al. 2009). Les microenvironnements sur les parois des grottes susceptibles d'accueillir des microorganismes présenteront typiquement des biofilms plutôt que des microcolonies çà et là (Schabereiter-Gurtner et al. 2002 ; Portillo et al. 2009). Les champignons, les algues et les bactéries sont susceptibles de coexister au sein de ces communautés (Gorbushina 2007). Cependant, les surfaces rocheuses sont considérées comme des environnements oligotrophes, ce qui entraîne de faibles taux de croissance (Portillo et al., 2010). La croissance sur ces surfaces rocheuses est généralement limitée par la disponibilité en carbone organique (Stomeo 2009), mais dans certains cas la prolifération microbienne peut entraîner la formation de colonies visibles, et dans la grotte d'Altamira ces colonies peuvent être composées de dizaines de bactéries différentes (Schabereiter-Gurtner et al. 2002 ; Portillo et al. 2008).

C. Diversité microbienne des grottes et interactions écologiques entre organismes cavernicoles

Comme indiqué dans les paragraphes précédents, les microorganismes colonisent largement les parois des grottes. Ici, le but est de décrire la diversité des bactéries, des archées et des champignons dans les grottes et les interactions possibles entre ces microorganismes et avec les arthropodes. Cette description n'est pas exhaustive, mais s'intéresse aux taxons dominants car chaque grotte peut présenter une spécificité en termes de diversité (Barton & Jurado, 2007 ; Engel, 2010).

C1. Diversité bactérienne dans les grottes

Les taxons bactériens les plus abondants et les plus fréquemment retrouvés dans les grottes sont les Protéobactéries, les Nitrospirae, les Actinobactéries, les Acidobactéries, les Bacteroidetes et les Firmicutes (Tomczyk-Żak & Zielenkiewicz, 2016).

Les Protéobactéries forment un groupe taxonomique très large. Le succès de leur colonisation peut être attribué en partie à leur capacité à dégrader une large gamme de composés organiques (Tomczyk-Żak & Zielenkiewicz, 2016). Les Protéobactéries représentent la majorité des taxons trouvés dans la grotte d'Altamira (Portillo et al. 2007) et dans la grotte de Lascaux (Bastian et al. 2009b) qui sont des grottes anthropisées, et dans la grotte de Jinjia (Chine) non anthropisée (Wu et al. 2015).

Les Nitrospirae ont des capacités métaboliques diverses. Elles sont aérobies et chimiolithoautotrophes obligatoires, dérivent leur énergie (pour la croissance) de l'oxydation du nitrite ou du fer, avec une capacité de réduction anaérobie du sulfate (Pohlman et al. 1997). Les Nitrospirae sont souvent rencontrés dans les grottes, comme dans la grotte de Su Bentu en Sardaigne (Leuko et al. 2017) ou dans la grotte de Lechuguilla (Northup et al. 2003), mais sont encore peu caractérisés à ce jour (Tomczyk-Żak & Zielenkiewicz, 2016).

Les Actinobactéries sont impliquées dans des processus de biominéralisation (Canaveras et al. 2001 ; Laiz et al. 1999) car elles produisent de nombreux types de cristaux. Les Actinobactéries peuvent représenter une part majoritaire de la communauté bactérienne des grottes, par exemple 65% dans une grotte du nord de l'Inde (De Mandal et al. 2017) et 80 % dans la grotte de Carlsbad au Nouveau-Mexique (Barton et al. 2007), ou être le deuxième (22% dans la grotte de Llonin ; Schabereiter-Gutner et al. 2004) ou troisième phylum (9,8% à Tito Bustillo ; Schabereiter-Gutner et al. 2002).

Les Acidobactéries sont un groupe aussi divers que les Protéobactéries (Quaiser et al. 2003). Les Acidobactéries des grottes n'ont pas encore été isolées ou cultivées en laboratoire (Engel, 2015). Elles peuvent être associées à des microorganismes phototrophes autour des lampes installées dans les grottes (Engel, 2015). Les Acidobactéries sont retrouvées dans la grotte d'Altamira (Shabereiter-Gutner et al. 2004) et dans la grotte de Su Bentu (Leuko et al. 2017).

Les Bacteroidetes sont des microorganismes avec des phénotypes diversifiés. Elles sont aérobies ou anaérobies facultatives, et typiquement chimioorganotrophes. Ce groupe contient des bactéries mésophiles, psychrophiles et thermophiles (Tomczyk-Żak & Zielenkiewicz, 2016). Leur rôle fonctionnel dans les grottes semble être assez limité, mais les Bacteroidetes pourraient être impliquées dans les processus de fermentation et de circulation d'éléments métalliques (Chelius & Moore, 2004 ; Ikner et al. 2007). Elles représentent 8% de la composition des biofilms de grottes en Suisse (Pfendler et al. 2018) et 20% pour la grotte de Carter Saltpenter (Mississippi) (Carmichael et al. 2013).

Les Firmicutes sont hétérotrophes et chimiolithotrophes (Tomczyk-Żak & Zielenkiewicz, 2016). Quelques représentants sont photohétérotrophes anaérobies. Les Firmicutes sont capables de réduire et d'oxyder le soufre (Macalady et al. 2007). Ces bactéries prédominent dans les communautés bactériennes de la grotte des Chauves-souris (19.5% des séquences de gènes de l'ARNr16S) (Urzi et al. 2010) ou des grottes de Kartchner (52% des isolats) (Iker et al. 2007).

C2. Diversité des archées dans les grottes

La diversité des archées est variable en fonction des grottes étudiées. Dans la Wind Cave (Dakota du sud), la majorité des séquences retrouvées est affiliée à des Crenarchaeota alors que les Euryarchaeota sont minoritaires (Chelius & Moore, 2004), et dans la grotte Weebubbie, les Thaumarchaeota représentent 45% des séquences (Tetu et al. 2013). Dans la grotte de Lechuguilla, des Crenarchaeota et Euryarchaeotaont été identifiées (Northup et al. 2003). Bien que les archées soient de plus en plus étudiées, les informations disponibles sont encore faibles.

C3. Diversité fongique dans les grottes

Une synthèse sur les champignons dans les grottes et les mines recense 518 genres (Vanderwolf et al. 2013). Le plus souvent les champignons sont des parasites ou des décomposeurs (Vanderwolf et al. 2013), mais ils contribuent aussi à la formation de concrétions telles que les dépôts secondaires de carbonate de calcium (Bindschedler et al. 2012). Plusieurs espèces fongiques prolifèrent de façon opportuniste lorsqu'elles sont soudainement en présence de source de carbone (intrants végétaux, carcasses ; Cubbon, 1976). Min (1988) a noté que les débris organiques dans les grottes sont souvent rapidement couverts de conidies d'*Aspergillus*, de *Penicillium* et de *Mucor*.

Les flux d'airs permettent le transport et la dispersion des spores de champignons, qui proviendraient majoritairement de l'extérieur (Engel, 2015 ; Vanderwolf et al. 2013), en effet les taxons de champignons retrouvés dans les grottes sont typiques de milieux non souterrains (Vanderwolf et al. 2013).

Les genres fongiques fréquemment retrouvés dans les grottes sont *Aspergillus, Penicillium* (Engel, 2015 ; Mitova et al. 2017,). Certaines espèces de champignons ont été isolées exclusivement dans des grottes, par exemple *Ochroconis anomala* (Martín-Sánchez et al. 2012) et *Ochroconis lascauxensis* (Martín-Sánchez et al. 2012) qui ont été isolés de la grotte de Lascaux.

C4. Compétition et symbiose

En écologie, la compétition est le fait que des individus d'une même espèce (compétition intra spécifique) ou d'espèces différentes (compétition interspécifique) soient en

concurrence pour l'accès à des ressources nutritives et/ou territoriales, lorsque ces ressources sont exploitées au même endroit et de façon simultanée.

Dans les grottes, les nutriments sont limités et chimiquement complexes, or peu d'espèces microbiennes sont capables de réaliser toutes les réactions d'absorption et de catabolisme pour assurer leur croissance dans ce type de contexte nutritionnel. Certains chercheurs suggèrent que pour pallier cette limitation, la compétition pour les ressources est remplacée par de la coopération et des interactions mutualistes (Tomczyk-Żak & Zielenkiewicz, 2016) comme cela est observé dans les biofilms (Barton & Jurado, 2007). D'autres considèrent que lorsque les ressources sont limitées les micro-organismes produisent davantage de métabolites secondaires, y compris des toxines pour inhiber d'autres microorganismes, des biorégulateurs et des molécules signal qui apporteraient un avantage concurrentiel aux micro-organismes les produisant (Lavoie et al. 2017).

La symbiose est une association biologique, durable et réciproque profitable entre deux types d'organismes. Certains champignons épilithiques sont associés à des producteurs primaires comme des algues ou des cyanobactéries, par exemple sous la forme de lichens (Klappa, 1979 ; Jones & Wilson, 1986), et consomment la matière organique ainsi produite.

Le peu d'information disponibles sur les interactions entre les micro-organismes dans les grottes est lié au fait que la plupart des études sur la microbiologie des grottes reste descriptive.

C5. Microorganismes et collemboles

Dans de nombreuses grottes, les champignons et les bactéries constituent probablement les principales sources de nourriture pour les invertébrés cavernicoles tels que les isopodes et les collemboles (Walochnik & Mulec, 2009 ; Bastian et al. 2010). Certaines espèces de collemboles comme *Folsomia candida* ont une préférence pour les espèces de champignons mélanisés (Scheu & Simmerling, 2004), mais ils se nourrissent également d'autres microorganismes comme des bactéries du genre *Pseudomonas* (Thimm et al. 1998) ou des nématodes (Lee & Widden, 1996). Ainsi, les collemboles sont des prédateurs des microorganismes.

D. Problèmes liés aux microorganismes dans les grottes

D1. Altérations des parois des grottes

Certaines grottes sont attrayantes en raison des concrétions (e.g. stalagmites ou stalactites) ou de l'art préhistorique qu'elles contiennent (grottes ornées). Elles peuvent être souvent visitées, quelquefois dans le cadre d'opérations touristiques. Cet intérêt touristique n'est pas sans conséquences pour ces écosystèmes.

Dans différentes grottes ornées comme la grotte d'Altamira ou la grotte de Lascaux, des revêtements microbiologiques sont apparus sur les parois, menaçant la conservation des peintures préhistoriques qu'elles contiennent (Bastian et al. 2010 ; Portillo & Gonzales, 2010). Ces altérations sont apparues suite aux visites touristiques, qui parfois nécessitent des aménagements comme l'installation d'escaliers ou de systèmes de refroidissement de l'air, mais aussi entrainent une augmentation de la température, un apport de lumière et un apport de matière organique via les chaussures, les cheveux etc. (Adetutu et al. 2011 ; Barton, 2006 ; Ikner et al. 2007). L'ensemble de ces facteurs impacte la communauté microbienne, stimule le développement de certains microorganismes, et des altérations sur les parois apparaissent.

Les microorganismes peuvent se développer sous forme d'un revêtement multicolore jaune, gris, blanc, rose ou noirs pouvant atteindre 1 mm d'épaisseur sur les parois et les voûtes carbonatées et argileuses de certaines grottes (Pašić et al. 2010).

D2. Moyens de lutte

La croissance microbienne et le fonctionnement microbien des parois des grottes peuvent avoir un effet délétère sur les peintures et les gravures rupestres (Jurado et al. 2009 ; Wang et al. 2010), ce qui a entraîné la mise en œuvre de quatre types d'actions correctives (Tiano 2016). Premièrement, l'action corrective la plus courante est l'application de produits chimiques antimicrobiens tels que des biocides (formaldéhyde, peroxyde d'hydrogène, chaux vive, chlorure de benzalkonium, fongicides) ou antibiotiques (streptomycine, pénicilline, polymyxine) (Dupond et al. 2007 ; Bastian et al. 2009b ; Jurado et al. 2010). Les traitements sont appliqués à plusieurs reprises (Dupond et al., 2007) ou par rotations de produits (Langsrud et al. 2003 ; Bastian et al. 2009b). Cependant, l'utilisation d'antimicrobiens dans les grottes est devenue de plus en plus controversée (Diaz-Herraiz et al. 2014) car ils peuvent être utilisés comme substrats de croissance par certains microorganismes ou favoriser le développement de mécanismes de résistance favorisant ainsi la prolifération microbienne (Stomeo et al. 2009). De plus, certains de ces produits chimiques peuvent être dangereux pour les humains, ce qui représente un problème de santé pour le personnel des grottes touristiques et les visiteurs.

Deuxièmement, les conditions climatiques peuvent être régulées pour fournir un contrôle microbien indirect, en particulier avec la modification des voies de passage ou l'installation de systèmes de climatisation (Dupond et al. 2007). Troisièmement, le nettoyage mécanique avec des écouvillons ou des éponges est efficace pour éliminer la biomasse microbienne, en particulier lors de développement fongique abondant (Jurado et al. 2010). Quatrièmement, les populations microbiennes peuvent être traitées avec des méthodes physiques, telles que la lumière UV (Tiano, 2016).

D3. Survie et recolonisation après traitements

Les communautés microbiennes présentes sur les parois des grottes forment parfois des taches inesthétiques ou pouvant dégrader une œuvre du patrimoine culturel. Ainsi, les traitements fréquemment utilisés sont le formaldéhyde, les composés d'ammonium quaternaire (Sterflinger & Piñar, 2013) et le désinfectant le plus courant l'éthanol à 70%. Malheureusement, ces méthodes ne sont pas efficaces à moyen et long terme (Akatova et al. 2007) et certains microorganismes résistent à ces traitements en raison des mécanismes de résistance dont ils disposent. Dans la grotte de Lascaux, les traitements chimiques ont favorisé le développement du champignon *Fusarium solani*, et ultérieurement des champignons mélanisés tels que *Ochroconis lascauxensis* ou *Exophiala castellanii* (Martin-Sanchez et al. 2012).

La paroi épaisse et mélanisée des champignons ainsi que la production d'exopolysaccharides leur permettent une meilleure tolérance aux stress chimiques comme l'application de biocides ou de traitements antimicrobiens (Sterflinger, 2010), ainsi qu'aux stress physiques (Sterflinger, 1998 ; Onofri et al. 2008 ; Selbmann et al. 2011). Concernant les bactéries, la résistance aux traitements chimiques et aux antibiotiques peut être due aux changements de la membrane externe pour les Gram -, alors qu'elle pourrait être liée à la présence de plasmides portant des gènes de résistance à ces produits pour les Gram + (Russell, 1997 ; Blair et al. 2015). Il existe plusieurs mécanismes de résistance portés par les plasmides, comme l'inactivation de composés anti-microbiens, la diminution de l'absorption de ces composés, la modification de la membrane externe (ce qui diminue l'entrée des antimicrobiens) et les pompes à efflux (Russell, 1997 ; Blair et al. 2015).

Ces différents mécanismes de résistance sont bien connus grâce aux études cliniques et à l'importance des souches microbiennes multi-résistantes, mais les mécanismes de résistance privilégiés par les micro-organismes présents sur les parois des grottes ne sont pas documentés.

Certains micro-organismes résistent à l'application de biocides alors que d'autres meurent, à moyen terme la communauté microbienne est modifiée, et certains micro-organismes ayant motivé le déclenchement du traitement peuvent diminuer en importance mais proliférer de nouveau. A Séville, les tombes de Servilla qui se composent d'une galerie ouverte et d'une chambre funéraire dont les murs sont décorés par des peintures ont été restaurées et des biocides ont été appliqués. La communauté microbienne dont la structure a été modifiée a recolonisé les tombes au bout de 20 mois (Akatova, 2007).

E. Conclusion

La compréhension du fonctionnement d'une grotte nécessite de prendre en compte, à la fois des processus physicochimiques pour comprendre sa formation et des processus biologiques pour se rendre compte de la biodiversité qu'elle peut contenir. Même si de nombreuses études seront encore nécessaires pour mieux comprendre cet écosystème, le

lien entre la géologie et la microbiologie s'est renforcé depuis la prise de conscience de l'importance des microorganismes dans les grottes.

Chapitre 2 : Sites d'études et méthodologie générale

A. Description des sites d'étude : la grotte de Lascaux et autres grottes de Dordogne

A1. La grotte de Lascaux

Historique des crises microbiennes

La grotte de Lascaux a été découverte en 1940 et ouverte au public de 1948 à 1963, ce site paléolithique est célèbre pour ses gravures et peintures préhistorique datant d'environ 18 000 ans.

En 1960, un biofilm vert apparait sur les parois à cause de la lumière, c'est l'algue verte *Bracteacoccus minor* qui est à l'origine de cette première crise microbiologique appelée « maladie verte ». Pour éliminer cette algue, du formaldéhyde, de la streptomycine et de la pénicilline ont été appliqués.

En 2001, une seconde crise microbiologique apparait, un duvet blanc se forme dans la grotte et s'étend rapidement, il s'agit du champignon *Fusarium solani*. Ce dernier semblait associé à des bactéries de l'espèce *Pseudomonas fluorescens*, mais ce point n'est pas complètement clair. En effet, cette identification repose sur des données non publiées (Allemand & Bahn, 2005 ; Bastian et al. 2009a ; Orial & Mertz, 2006). Une autre étude a permis d'obtenir des clones (gènes de l'ARNr 16S) qui ont été affiliés à des espèces de *Pseudomonas,* à savoir *P. saccharophila* [EU770637], *P. lanceolata* [EU770635] et *P. fluorescens* [EU789833] (Bastian et al. 2009c). Néanmoins, la taxonomie des *Pseudomonas* était souvent défaillante à cette époque, et les bases de données étaient encore peu fournies et contenaient de nombreuses séquences mal affiliées. J'ai repris ces séquences, et leur analyse indique que *P. saccharophila* [EU770637] et *P. lanceolata* [EU770635] sont en fait affiliées à des bétaprotéobactéries, et *P. fluorescens* [EU789833] est effectivement un *Pseudomonas* mais qui n'appartient pas à l'espèce *P. fluorescens.* Sur cette base, il n'est pas clair si *Fusarium solani* était réellement associé à des *P. fluorescens.* Pour lutter contre les problèmes liés à *F. solani*, le traitement appliqué incluait du chlorure de benzalkonium, de la streptomycine et de la polymyxine, et le sol a été recouvert de chaux. En 2004, un nettoyage mécanique complète les traitements chimiques.

Cependant en 2006, des taches noires apparaissent sur la voûte et les banquettes du Passage, c'est la troisième crise microbiologique. Le nettoyage mécanique et l'application de traitements chimiques ont duré jusqu'en 2008.

Les traitements chimiques ont donc été stoppés en 2008 et une première étude scientifique a été initiée pour caractériser l'écologie microbienne de la grotte de Lascaux (projet Ecologie microbienne de la grotte de Lascaux coordonné par Claude Alabouvette et Cesáreo Sáiz-Jiménez).

Description de la grotte de Lascaux

La grotte de Lascaux est constituée de différentes salles décrites par la suite. L'entrée de la grotte est fermée par une lourde porte en bronze (Figure 3).

Figure 3. Entrée de la grotte de Lascaux

Ensuite se trouve le Sas-1 qui est divisé en 3 compartiments (C1, C2 et C3), le Sas-1 a été entièrement aménagé par l'Homme, puis se trouve le Sas-2 qui comprend un escalier menant à la Salle des Taureaux, dans le prolongement se trouve le Diverticule axial. La partie droite de la grotte mène au Passage puis à l'Abside, au fond de l'Abside se trouve l'accès à la Diaclase et au Puits du sorcier. Dans le prolongement du Passage se trouve la Nef, suivie de la galerie du Mondmilch et du Diverticule des félins (Figure 4).

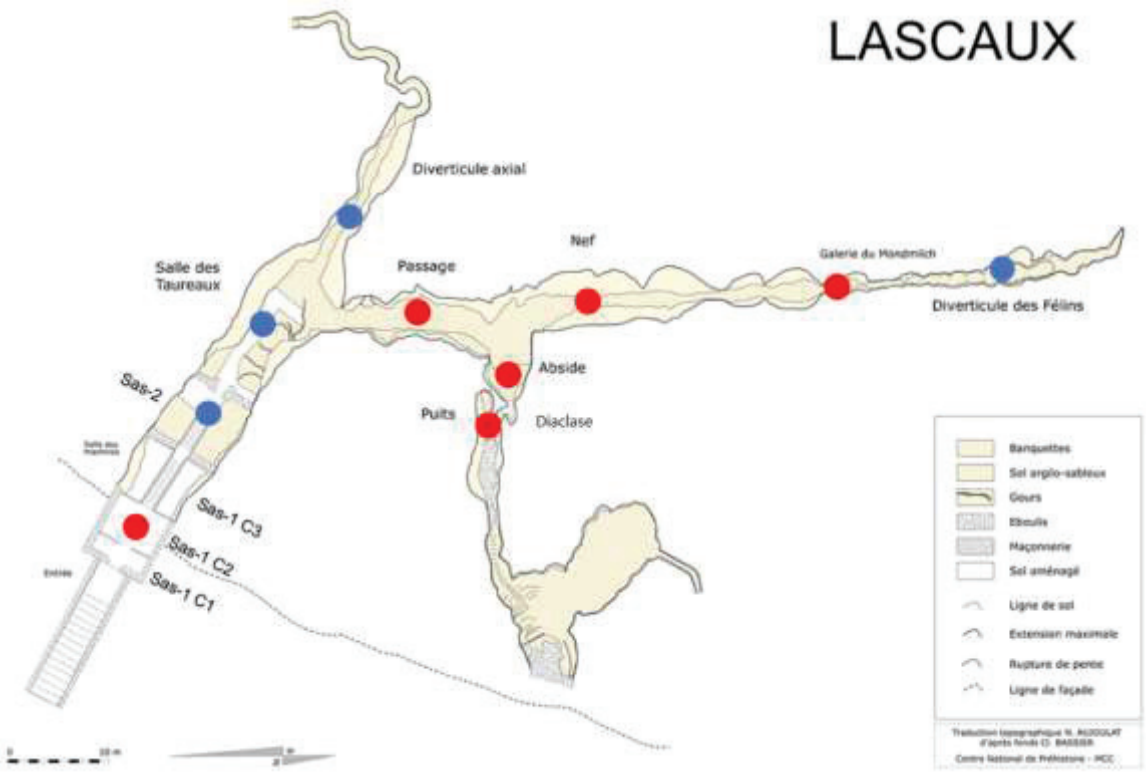

Figure 4. Carte de la grotte de Lascaux (D'après N. Aujoulat). Les points correspondent aux salles échantillonnées pour le projet Lascaux. Les points rouges correspondent aux salles étudiées dans cette thèse.

Le **Sas-1 C2** est une salle maçonnée avec des briques de calcaire similaire au calcaire de la grotte, une surface de 80 x 80 cm^2 a été mise à nue en avril 2014, c'est sur cette zone que les échantillonnages ont été effectués. Cette zone est considérée comme une zone test car des taches noires sont apparues courant 2014, ce qui nous a permis de faire un suivi temporel de l'évolution de cette zone.

Le **Passage**, salle centrale de la grotte permet la connexion entre la salle des Taureaux (vers l'entrée) et l'Abside et la Nef (vers le fond). Cette salle est composée de substrats minéraux différents (de bas en haut), le sol en terre battue constitué de sable calcaire, les banquettes sont en argile, les parois (plans inclinés) en calcaire et la voûte en calcaire et calcite. La voûte n'est pas étudiée car trop fragile. Des taches noires sont présentes de façon hétérogène sur les banquettes et les plans inclinés. Cette salle a été l'une des premières salles contenant des taches noires.

L'**Abside** est la salle dans laquelle sont apparues en 2008 de nouvelles altérations de parois nommées zones sombres et dans laquelle se trouvent des collemboles sur les zones sombres et sur les taches noires. Quelques zones sombres se sont développées vers l'entrée de la **Nef**, c'est pourquoi cette salle a été échantillonnée.

La **Diaclase** est une salle située en profondeur par rapport au reste de la grotte. C'est une zone dont l'accès est limité aux techniciens de la grotte et à quelques scientifiques, donc c'est une salle moins anthropisée qui ne contient pas de taches noires et a reçu peu de traitements chimiques.

La **galerie Mondmilch** est composée de Mondmilch qui est un substrat minéral particulier, c'est un dépôt poreux de calcite gorgé d'eau. En plus de contenir un substrat particulier cette salle contient des taches jaunes, ce qui caractérise cette salle en comparaison avec les autres salles.

Toutes ces salles comportent des caractéristiques particulières en termes de composition de substrat minéral, de taches ou d'historique, c'est pourquoi nous les avons choisis pour étudier l'hétérogénéité spatio-temporelle du microbiote de la grotte de Lascaux.

A2. Les autres grottes de Dordogne

Pour comparer la communauté microbienne de la grotte de Lascaux avec la communauté microbienne d'autres grottes de la région, nous avons choisi des grottes situées sur la même veine calcaire avec l'aide de Jean-Christophe Portais (Ingénieur du patrimoine, DRAC Nouvelle-Aquitaine) et si possible dans toutes les directions géographiques (Chapitre 3, Figure 1).

Les grottes non anthropisées sont Allas, la Reille, le Mouflon et le Pilier et les grottes anthropisées sont Combarelles, Rouffignac, Tourtoirac et Cap Blanc. Les caractéristiques de ces grottes sont listées dans le Chapitre 3, Tableau S4.

B. Conditions d'échantillonnages

La grotte de Lascaux étant un site paléolithique majeur des conditions particulières sont établies pour la préservation du site.

B1. Conditions d'entrée dans la grotte de Lascaux

Afin de limiter la présence humaine dans la grotte qui est une des conditions majeures pour la préservation, seulement deux ou trois personnes entraient dans la grotte en même temps pour l'échantillonnage, avec au moins un membre de l'UMR Ecologie microbienne accompagné de la restauratrice Diane Henry-Lormelle, avec un temps limité à environ 2h. Lors de la campagne d'échantillonnage de décembre 2014, la technicienne de recherche pour la conservation de la grotte de Lascaux Sandrine Géraud-Van Solinge faisait aussi partie du groupe d'échantillonnage, mais l'entrée de trois personnes engendrait trop de présence humaine donc pour les campagnes suivantes seulement deux personnes y participaient. Après une entrée dans la grotte, une période que je vais nommer « repos » était nécessaire pour que les conditions de température se stabilisent donc une campagne d'échantillonnage était dessinée comme cela : J0 échantillonnage dans Lascaux, J+1 repos, J+2 échantillonnage dans Lascaux, puis nous revenions après 12 jours de repos pour échantillonner à J+14 et J+16.

L'entrée dans la grotte s'effectuait avec un équipement particulier stérile, charlotte sur la tête, masque, combinaison, gants et surchaussures.

Tous les échantillons prélevés au cours du projet sont répertoriés dans l'annexe 1.

B2. Prélèvements des échantillons

La prise d'échantillon dans la grotte est répertoriée et documentée, pour chaque prélèvement une photographie est prise et une base de données recense l'ensemble des prélèvements.

L'ensemble des échantillons est prélevé à l'aide d'un scalpel ou d'un écouvillon stérile par la restauratrice. Puis les échantillons destinés à l'analyse par séquençage à haut débit sont placés dans un conteneur à azote liquide et les échantillons destinés à la mise en culture sont conservés à 4°C jusqu'au retour au laboratoire.

C. Méthodologie disponible pour l'étude de la diversité microbienne dans les grottes

C1. Approches culturales et observations microscopiques

Les premières études de la diversité des communautés microbiennes ont été réalisées en utilisant des techniques de culture traditionnelles associées à de la microscopie (Gounot, 1967 ; Northup, 1997 ; Cañaveras et al. 2001). Les grottes étant oligotrophes, les milieux de culture utilisés sont des milieux pauvres en nutriments, ou des milieux de culture classiques mais dilués. Cependant, cette approche prend en compte moins de 1 % des microorganismes présents (Amann et al. 1995) donc ne permet pas d'étudier la diversité totale.

Actuellement, certaines études utilisent la méthode culturale en complément d'autres méthodes moléculaires, pour isoler un microorganisme particulier par exemple (Engel et al. 2001).

C2. Approches de clonage séquençage et empreintes moléculaires

Les techniques de biologie moléculaire fondées sur l'utilisation de marqueurs moléculaires taxonomiques ciblant le gène de l'ARNr 16S ou des gènes fonctionnels comme *amoA* pour les microorganismes oxydant l'ammoniac ont permis d'élargir les connaissances sur la diversité microbienne dans les grottes (Tomczyk-Żak & Zielenkiewicz, 2016). Les gènes d'intérêt sont amplifiés à partir de l'ADN total isolé d'échantillons environnementaux, puis clonés et séquencés par la méthode Sanger, ou analysés par électrophorèse en utilisant des méthodes d'empreintes moléculaires telles que : DGGE (électrophorèse sur gel en gradient dénaturant), RFLP (polymorphisme de longueur de fragments de restriction), RAPD (*Random Amplified Polymorphic DNA*), RISA (*Ribosomal Intergenic Spacer Analysis*) et d'autres (Dorigo et al. 2005). Ce type d'approche s'affranchit du problème des microorganismes non cultivables, mais des biais techniques peuvent exister lors de l'extraction de l'ADN (qui peut

ne pas être complète) ou de l'amplification (qui n'est pas toujours efficace à cause d'inhibiteurs par exemple) (Wilson, 1997).

Une étude de la diversité fongique comparant des zones accessibles aux touristes à des zones non accessibles dans les grottes de Naracoorte en Australie, utilisant une approche de mise en culture et une approche DGGE montre que les résultats obtenus par ces deux méthodes ne sont pas les mêmes (Adetutu et al. 2011). L'approche cultivable indique une diminution microbienne de la diversité avec l'anthropisation alors que les données de DGGE indiquent que la diversité est plus forte dans les zones visitées que dans les zones non visitées. Cela montre que des biais existent, liés aux techniques utilisées.

C3. Séquençage à haut débit

A partir des années 2010, les études des communautés microbiennes dans les grottes utilisent un autre type de méthode qui permet d'identifier de manière détaillée la diversité de la communauté microbienne à partir d'un échantillon environnemental prélevé, c'est le séquençage à haut débit des acides nucléiques (Brannen-Donnely et al. 2015 ; Carmichael et al. 2015 ; De Mandal et al. 2017 ; Pfendler et al. 2018). Cette méthode permet d'appréhender la diversité totale d'une communauté.

Actuellement, la majorité des études utilisant le séquençage à haut débit cible un marqueur taxonomique particulier pour étudier une communauté spécifique, le gène de l'ARNr 16S pour les bactéries (Brannen-Donnely et al. 2015) ou l'ITS pour les champignons (Carmichael et al. 2015). D'autres utilisent plusieurs marqueurs, par exemple les gènes des ARNr 16S et ARNr 18S (pour Duan et al. 2017), les gènes de l'ARNr 16S et de l'ARNr 23S ainsi que l'ITS (pour Pfendler et al. 2018).

Le principal inconvénient de cette méthode est le manque d'informations dans les bases de données, ainsi la diversité des communautés est étudiée de façon globale mais certains taxons restent parfois non identifiés.

Ces différentes approches sont aussi complémentaires car elles n'ont pas les mêmes biais, il est donc intéressant de faire des études utilisant plusieurs approches. Dans le cadre du premier projet d'Ecologie microbienne de la grotte de Lascaux, des approches de mise en culture et de clonage séquençage ont été utilisées (Bastian et al. 2009b ; Bastian et al. 2009c ; Martin-Sanchez et al. 2012 ; Martin-Sanchez et al. 2012a).

D. Méthodologie suivie dans cette thèse

D1. Extraction des acides nucléiques

Mise au Point. Pour des raisons de conservation, les échantillons prélevés dans la grotte de Lascaux contiennent très peu de matière. Afin de ne pas perdre d'échantillons précieux de la grotte de Lascaux, nous avons mis au point le protocole d'extraction des acides nucléiques à

partir d'échantillons de paroi prélevés dans une grotte située dans le Bugey (proche de Lyon). Cinq protocoles d'extraction d'ADN ont été testés (NucleoSpin Soil, Macherey Nagel ; Xpedition™ Soil/Fecal DNA MiniPrep + Beadbeating avec le TerraLyzer™, Zymo Research ; NucleoSpin Triprep, Macherey Nagel ; FastDNA SPIN for Soil, MP Biomedicals) et deux protocoles d'extraction d'ARN (ZR Soil / Fecal RNA MicroPrep, Zymo Research ; extraction au phénol/chloroforme) avec des quantités de matières différentes pour établir la quantité de matière minimale requise. Les méthodes retenues sont le kit FastDNA SPIN for Soil, MP Biomedicals pour l'extraction d'ADN et le kit ZR Soil / Fecal RNA MicroPrep, Zymo Research pour l'extraction d'ARN avec une quantité minimale de 10 mg de matière.

Protocoles utilisés. Les échantillons destinés à une même analyse ont été traités avec les mêmes protocoles pour limiter les biais techniques.

L'extraction d'ADN a été réalisée en utilisant le kit FastDNA SPIN for Soil (MP Biomedicals, Illkirch, France), en suivant les instructions du fabricant. L'étape d'élution a été réalisée en utilisant deux volumes de 50 µl de tampon d'élution pour chaque échantillon. L'extraction d'ARN a été effectuée en utilisant le kit ZR Soil / Fecal RNA MicroPrep (Zymo Research, Irvine, CA), en suivant les instructions du fabricant. Le traitement à la DNase a été effectué pendant l'extraction avec la DNase I Set (Zymo Research). Les concentrations d'ADN et d'ARN ont été quantifiées en utilisant le kit de dosage Qubit ARN BR (Thermo Fisher Scientific, Eugene, OR) en suivant les instructions du fabricant. Les extraits d'ADN et d'ARN ont été conservés à -80 ° C jusqu'à analyse ultérieure.

D2. PCR quantitative

Pour évaluer le nombre de gènes d'ARNr 16S bactériens et de gènes d'ARNr 18S micro-eucaryotes, les PCR quantitative (qPCR) ont été réalisées sur un LightCycler 480 (Roche Diagnostics, Meylan, France) en utilisant les amorces 519F 5'-CAGCMGCCGCGGTAANWC -3' / 907R 5'- CCGTCAATTCMTTTRAGTTT -3' (Laiz et al. 2003) et EUK345F 5'- AGGAAGGCAGCAGGCG -3' / EUK499R 5'- CACCAGACTTGCCCTCYAAT -3 ' (Zhu et al. 2005), respectivement. Brièvement, les réactions des gènes ARNr 16S et 18S ont été réalisées dans des volumes de 20 µl contenant respectivement 0,6 µl (concentration finale 0,3 µM) ou 0,8 µl (0,4 µM) de chaque amorce, 4 µl d'eau de qualité PCR, 10 µl de LightCycler-ADM Master SYBR Green I mélange maître (Roche Applied Science) et 2 pi d'ADN de l'échantillon (5 ng). La PCR a été réalisée avec un protocole de 10 min à 95 ° C, suivi de 40 cycles de (i) 95 ° C pendant 15 s (ARNr 16S) ou 94 ° C pendant 15 s (ARNr 18S), (ii) 63 ° C pour 60 s (ARNr 16S) ou 60 ° C pendant 15 s (ARNr 18S), et (iii) 72 ° C pendant 30 s (ARNr 16S) ou 72 ° C pendant 15 s (ARNr 18S).

D3. Séquençage à haut débit

Pour étudier la diversité de la communauté microbienne de façon globale la méthode de séquençage à haut débit Illumina a été choisie. Quatre marqueurs génétiques de diversité microbienne ont été étudiés : les gènes ARNr 16S spécifiques aux bactéries, les gènes ARNr 16S spécifiques aux archées, les gènes ARNr 18S spécifiques aux micro-eucaryotes (y compris les champignons) et l'*Internal Transcribed Spacer* 2 (second espaceur interne transcrit ; ITS2) spécifiques aux champignons. Pour les amplifications du gène de l'ARNr 16S bactérien, nous avons utilisé les amorces 341F 5'-CCTACGGGNGGCWGCAG-3' et 805R 5'-GACTACHVGGGTATCTAATCC-3', qui ciblent les régions V3-V4 (Klindworth et al. 2013). Les amorces Arch519F 5'- CAGCCGCCGCGGTAA -3' et Arch915R 5'- GTGCTCCCCCGCCAATTCCT -3' (Hertfort et al. 2009) ont été utilisées pour l'amplification du gène de l'ARNr 16S archéen. Nous avons utilisé les amorces 18S_0067a_deg 5'-AAGCCATGCATGYCTAAGTATMA-3' et NSR399 5'-TCTCAGGCTCCYTCTCCGG-3' (Dollive et al. 2012) pour l'amplification du gène de l'ARNr 18S micro-eucaryote, et les amorces ITS3_KYO2 5'-GATGAAGAACGYAGYRAA-3' et ITS4 5'- TCCTCCGCTTATTGATATGC-3' (Toju et al. 2012) pour l'amplification fongique de l'ITS2. Le séquençage Illumina MiSeq des produits de PCR a été effectué sur 1 µg d'ADN avec des séquences de marqueurs spécifiques pour séquencer simultanément différents échantillons sur la même série. Les amplifications et le séquençage ont été réalisés par la société Fasteris (Genève, Suisse), en utilisant la chimie appariée Illumina MiSeq 2 × 300 pb pour obtenir 70 000 séquences en paire de chaque extrémité du fragment, par échantillon.

Chapitre 3 : Relation entre anthropisation et communautés microbiennes des grottes, en comparant Lascaux et d'autres grottes de Dordogne

Avant-propos

Le nombre de grottes (de tous types) dans le monde est estimé à des centaines de milliers (Romero, 2012). Certaines grottes sont seulement visitées par des passionnés de spéléologie et d'autres attirent un plus grand nombre de visiteurs par leurs contenus que ce soit des concrétions (e.g. stalagmites ou stalactites) ou de l'art préhistorique. Ces dernières sont définies comme des grottes ornées, c'est-à-dire des grottes portant le témoignage de l'art préhistorique (peintures, gravures, sculptures), mais elles représentent la minorité des grottes (environ 300). Le niveau d'intérêt pour ces grottes n'est pas le même, et le niveau d'anthropisation non plus, les grottes visitées par des petits groupes de spéléologues sont très peu anthropisées alors que les grottes ayant un intérêt touristique sont très anthropisées. Elles ont souvent été aménagées pour accueillir les visiteurs. Par exemple, le sol de la grotte du roi Salomons (en Tasmanie) et de la grotte de Lascaux ont été excavés pour faciliter l'accès (Russell & MacLean, 2008). Parfois, des escaliers, de la lumière artificielle et des systèmes de climatisation sont également installés (Bastian et al. 2009b), ce qui peut avoir un impact significatif sur les conditions environnementales dans les grottes en particulier les flux d'air et la température. Les visiteurs eux-mêmes peuvent provoquer de profonds changements dans le microclimat de la grotte, avec une augmentation de la température, du taux de CO_2 et de la condensation (Canaveras et al. 2001 ; Bastian et al. 2009 ; Diaz-Herraiz, 2014). En outre, ils peuvent également apporter de la matière organique de l'extérieur, avec leurs chaussures ou en y laissant des cheveux etc. (Canaveras et al. 2001), et permettre l'arrivée de microorganismes exogènes (Dupont el al. 2007 ; Bastian et al. 2009 ; Barton & Jurado, 2007). Dans certains cas, des produits chimiques sont appliqués sur les parois pour traiter les altérations microbiennes, les plus fréquentes étant dues à des algues ou des cyanobactéries autour des zones éclairées (Mulec et al. 2007 ; Smith & Olson, 2007).

L'anthropisation des grottes (aménagements, visites touristiques, traitements chimiques) impacte la diversité et l'abondance des communautés microbiennes (Northup et al. 1997 ; Ikner et al. 2007 ; Mulec et al. 2012 ; Griffin et al. 2013 ; De Mandal et al. 2017). Lorsque l'anthropisation augmente, l'abondance microbienne peut être plus élevée (Northup et al. 1997 ; Mulec et al. 2012), mais la diversité microbienne peut être plus grande (Adetutu et al. 2011) ou plus faible (Ikner et al. 2007). Néanmoins, les études réalisées jusqu'ici reposent sur un petit nombre de comparaisons.

La grotte de Lascaux est une grotte très anthropisée, sa communauté microbienne a donc subit de nombreuses perturbations. Cependant, aucun échantillon de la grotte avant l'anthropisation n'est disponible, donc formellement nous ne pouvons pas caractériser l'impact de l'anthropisation sur la communauté microbienne de Lascaux. En revanche, en comparant des grottes anthropisées (dont Lascaux) et non anthropisées de la même région

(Dordogne), nous pouvons déterminer si l'anthropisation correspond à des particularités au niveau des communautés microbiennes présentes dans ces grottes, avec l'hypothèse que la diversité microbienne sur les parois dépend du niveau d'anthropisation de la grotte.

Quatre grottes non anthropisées (Allas, Le Mouflon, La Reille, Pilier), quatre grottes anthropisées actuellement encore ouvertes au public (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) ont été choisies sur la même veine calcaire que la grotte de Lascaux, avec l'aide de Jean-Christophe Portais (Ingénieur du patrimoine, DRAC Nouvelle-Aquitaine). Afin d'étudier l'impact de l'anthropisation sur la biodiversité des grottes nous avons étudié la communauté des bactéries, archées, micro-eucaryotes et arthropodes, à deux dates différentes (Mai-Juin 2016 et Février 2017).

L'étude des arthropodes a été réalisée par le LEHNA. Les arthropodes ont été piégés avec 25 pièges contenant du propylène glycol à 30 % placés au niveau du sol à différents endroits de chaque grotte (de l'entrée jusqu'au fond), le propylène glycol étant un agent (faiblement) attractant pour les arthropodes. L'UMR Ecologie Microbienne a étudié les communautés microbiennes, et pour ce faire six à huit échantillons de paroi ont été prélevés à l'aide d'un scalpel stérile dans chaque grotte à chaque campagne. Les échantillons ont été traités comme indiqué dans le chapitre 2.

Les résultats de cette étude montrent que l'anthropisation a entraîné une diminution de la diversité et une modification de la structure de la communauté pour les bactéries et les archées sur les parois des grottes, mais avec un effet plus limité sur les micro-eucaryotes et les arthropodes. Les mêmes taxons majoritaires microbiens et arthropodes prédominent, mais la grotte la plus anthropisée (Lascaux) est unique car elle diffère des huit autres par une proportion plus élevée de bactéries Bacteroidetes et l'absence d'Euryarchaeota et de Woesearchaeota. Cette étude révèle que l'anthropisation a façonné la biodiversité des grottes indépendamment des caractéristiques naturelles des grottes.

Mon rôle dans ce travail a été le suivant : j'ai participé à la réflexion sur les objectifs scientifiques et la démarche expérimentale, à la préparation du matériel pour la pose des pièges d'arthropodes et le prélèvement d'échantillons de paroi, ainsi qu'à la mission d'échantillonnage en Mai-Juin 2016. J'ai traité l'ensemble des échantillons destinés à l'étude des microorganismes, de l'extraction d'ADN à l'analyse des séquences issues du séquençage à haut débit des acides nucléiques et aux analyses multivariées, et j'ai participé à la rédaction du manuscrit.

L'ensemble de ce travail a permis la rédaction de la publication « Regional biogeography of underground biota demonstrates anthropization of Lascaux cave microbiome ».

Article 1 : Regional biogeography of underground biota demonstrates anthropization of Lascaux cave microbiome

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Limestone areas across the world display karstic caves, some subjected to anthropization due to human frequentation and touristic management. Despite their cultural importance, the impact of cave anthropization on biodiversity is not known. Here, we tested the hypothesis that anthropization could be associated with specific cave biota alterations. We compared diversity in four pristine caves, four anthropized show caves and the iconic Lascaux Cave with even stronger anthropization. Anthropization resulted in lower diversity and altered community structure for bacteria and archaea on cave walls, but with a more limited effect on micro-eukaryotes and arthropods. The same microbial and arthropod higher taxa predominated, but the most anthropized cave (Lascaux) was unique as it differed from the eight others by a higher proportion of Bacteroidetes bacteria and the absence of Euryarchaeota and Woesearchaeota archaea. This study reveals that anthropization shaped cave biota irrespective of cave natural features.

Limestone areas are prone to the development of karstic underground systems following dissolution of rock by water. Caves represent particular environments for life[1] in terms of prevailing physicochemical conditions[2] including darkness, temperature stability, high relative humidity and CO_2 concentration[3–6]. Another prominent feature of caves is oligotrophy, with total organic carbon content below 2 mg per liter or even lower[7]. Despite nutrient limitations, however, caves are extensively colonized by all kind of organisms[8,9], which play a significant part in biogeochemical cycling and dissolution/precipitation processes[1].

Many limestone caves have been preserved from human impact, i.e. they can be considered in a pristine state[9]. They have not been subjected to tourism because they may correspond to caves undiscovered so far, documented but difficult to access, or under private land ownership and so seldom visited. In contrast to these non-anthropized caves, a large number of caves (several hundred in France and Spain alone) have been extensively used by mankind in the last 40,000 years for artistic purposes, with prehistoric parietal art forms ranging from engravings to drawings and paintings[10,11]. Other caves are renowned for their speleothems and other geologic features. Both types of caves attract considerable public attention and many of them have been opened and converted for touristic visits. In such cases, anthropization resulted in adaptation work to facilitate visits, which may have changed (micro)climatic conditions and brought lighting systems in the cave. Moreover, touristic frequentation itself implies the consumption of oxygen, the release of body heat, water vapor, carbon dioxide and potentially organic compounds, and possibly the introduction of allochthonous microorganisms[12,13]. Consequently, tourism-related anthropization can modify environmental conditions in caves and lead to the establishment and expansion of certain organisms e.g. in Lascaux Cave, which is characterized by a much more intense anthropization than most other show caves in terms of disease outbreaks, chemical treatments and microbial growth responses[11,14]. It is therefore likely that biological communities in anthropized caves present common features in comparison with pristine caves. However, the impact of anthropization on cave biota is little documented so far, despite the prime importance of Paleolithic caves as the cultural memory of humanity.

Here, we tested the hypothesis that cave anthropization strongly drives the diversity of communities established in these ecosystems. To this end, we compared four pristine caves with four reference anthropized caves (three of them on the UNESCO list) and two compartments of Lascaux Cave, i.e. the main compartment heavily anthropized and the other one much less anthropized (Table 1). All caves are located in Dordogne region in the South-West of France (Fig. 1) and correspond to the same Cognacien calcareous vein. To assess the global microbial community of cave walls, we used MiSeq Illumina sequencing of DNA fragments targeting the three main microbial groups, i.e. bacteria, archaea and micro-eukaryotes. The arthropod (Hexapod) community, which represents most of subterranean metazoan, was characterized using morphology analysis and *COI* barcoding after pitfall sampling.

Table 1. Anthropization features of the caves used in this study.

Caves	Man-made layout	Fittings	Tourism	Microbial outbreaks	Chemical treatments
Pristine					
Allas	-	-	-	-	-
Reille	-	-	-	-	-
Mouflon	-	-	-	-	-
Pilier	-	-	-	-	-
Anthropized					
Cap Blanc	Walls and shelter built, walkway	Lights	Open since 1910; up to 100 visitors per day ; UNESCO site	Rare and very localized	Rare and very localized
Combarelles	Excavation, walkway	Lights	Open since 1911; up to 70 visitors per day; UNESCO site	Rare and very localized	Rare and very localized
Rouffignac	Walls, railway, walkways	Lights	Open since 1959; up to 500 visitors per day; UNESCO site	Absent	Absent
Tourtoirac	Elevator, walkways	Lights	Open since 2010; up to 800 visitors per day	Absent	Absent
Lascaux[b]					
Diaclase	Excavation, metal ladder	Air extraction	-	Rare and very localized	Rare and very localized
Passage	Excavation, walkway	Lights, air extraction, system for condensation of water vapor	Open since 1948; up to 2000 per day, closed to the public in 1963; UNESCO site	Many (to treat microbial 'diseases')[a]	Repeatedly and extensively for several years[a]

[a] As described (4, 54) [b] Lascaux samples were taken from the Shaft of the Dead Man (in the Diaclase) and the inclined plane (in the Passage), whose walls correspond to the same Cognacien limestone sampled in the other caves.

Figure 1. Geographic location of the caves studied in Dordogne region (South-West of France). Blue squares represent pristine caves, pink squares reference anthropized caves and grey square Lascaux Cave.

Results

Prokaryotic but not eukaryotic diversity diminishes with cave anthropization

To assess microbial diversity, we obtained 4,010,813, 1,980,310 and 8,227,676 full-length non-chimeric Illumina sequences for respectively bacteria (16S rRNA genes), archaea (16S rRNA genes) and micro-eukaryotes (18S rRNA genes) colonizing cave walls. Most rarefaction curves computed using observed data reached an asymptote at genus level (Supplementary Fig. 1) indicating a good representation[15] of diversity in each cave. Microbial Illumina sequences along with arthropod taxonomic data were used to test the hypothesis that anthropization resulted in lower biodiversity, based on four complementary metrics.

First, the numbers of operational taxonomic units (OTUs) were significantly lower in reference anthropized caves than in pristine caves when considering bacteria (647-1107 versus 817-1211 OTUs, respectively; ANOVA's $F_{2,57} = 8.26$, $P = 0.0007$) and archaea (71-115 versus 124-173 ; $F_{2,56} = 50.96$, $P = 2 \times 10^{-16}$), whereas they were of the same order of magnitude for micro-eukaryotes (226-432 versus 203-426 OTUs) (Supplementary Table 1) and arthropods (7-12 versus 10-22 OTUs) (Supplementary Table 2). In the Passage of Lascaux

Cave, the number of OTUs reached only 537 for bacteria, 14 for archaea and 144 for micro-eukaryotes, versus respectively 735, 85 and 157 in the Diaclase.

Second, the Chao1 index (to estimate total genus/species richness) for bacteria in three of four reference anthropized caves (Cap Blanc, Rouffignac, Tourtoirac) was statistically lower than in three of four pristine caves (Allas, Mouflon, Reille) (Fig. 2). The pristine cave Pilier and the anthropized cave Combarelles showed a Chao1 index that did not differ significantly from the index found in certain anthropized or pristine caves, respectively. In both Lascaux compartments, the Chao1 index was statistically lower than in the pristine ($P = 8 \times 10^{-7}$) and reference anthropized caves for bacteria ($P = 3 \times 10^{-9}$). For archaea, the Chao1 index in the four reference anthropized caves except Combarelles was statistically lower than in the four pristine caves (Fig. 2), and indeed the differences was statistically significant when comparing both groups of caves (41.7 ± 17.4 versus 71.9 ± 25.4, respectively; $P = 4 \times 10^{-16}$). In both Lascaux compartments, the Chao1 index was statistically lower than in the pristine ($P = 8 \times 10^{-8}$) and reference anthropized caves for archaea ($P = 1 \times 10^{-7}$). For micro-eukaryotes and arthropods, however, differences were not significant when comparing pristine and reference anthropized caves, and in this context Lascaux did not stand out (Fig. 2).

Third, differences in Shannon diversity index (to measure diversity by taking into account the number of genera/species as well as their abundance) for bacteria were found between individual caves, but differences were not significant when comparing globally the four reference anthropized caves to the four pristine caves (Fig. 2). In both Lascaux compartments, the Shannon index was statistically lower than in two pristine caves (Mouflon, Reille) and three referenced anthropized caves (Cap Blanc, Combarelles, Tourtoirac). For archaea, the Shannon index in three of four reference anthropized caves (i.e., except Combarelles) was statistically lower than in the four pristine caves (Fig. 2). In both Lascaux compartments, the Shannon index was statistically lower than in the pristine ($P = 1 \times 10^{-7}$) and reference anthropized caves ($P = 1 \times 10^{-7}$). For micro-eukaryotes and arthropods, there was no statistical difference in Shannon index when comparing pristine and reference anthropized caves, and Lascaux did not stick out (Fig. 2).

Fourth, there was no difference in Simpson's index of diversity (this 1-D index represents the probability that two individuals randomly selected from a sample will belong to different genera/species) for bacteria when considering the four reference anthropized caves, three of four pristine caves (Allas, Mouflon, Reille) and the two Lascaux compartments, but the Simpson index for the group of reference anthropized caves was statistically higher than the one for the group of pristine caves ($P = 0.0006$) because of the low value of Pilier cave (Fig. 2). For archaea, the Simpson index was statistically lower than in the four pristine caves for two of four reference anthropized caves (Rouffignac, Tourtoirac), whereas for both Lascaux compartments it was even lower than for the reference anthropized caves ($P = 1 \times 10^{-7}$) (Fig. 2). For micro-eukaryotes and arthropods, the difference

between pristine and reference anthropized caves was not statistically significant, and Lascaux was comparable to some of these caves (Fig. 2).

In summary, the four metrics used to assess biodiversity demonstrated that anthropized caves displayed lower levels of taxonomic diversity for bacteria and archaea. In contrast with prokaryotes, however, anthropization did not result in lower eukaryotic diversity, both for micro-eukaryotes and arthropods.

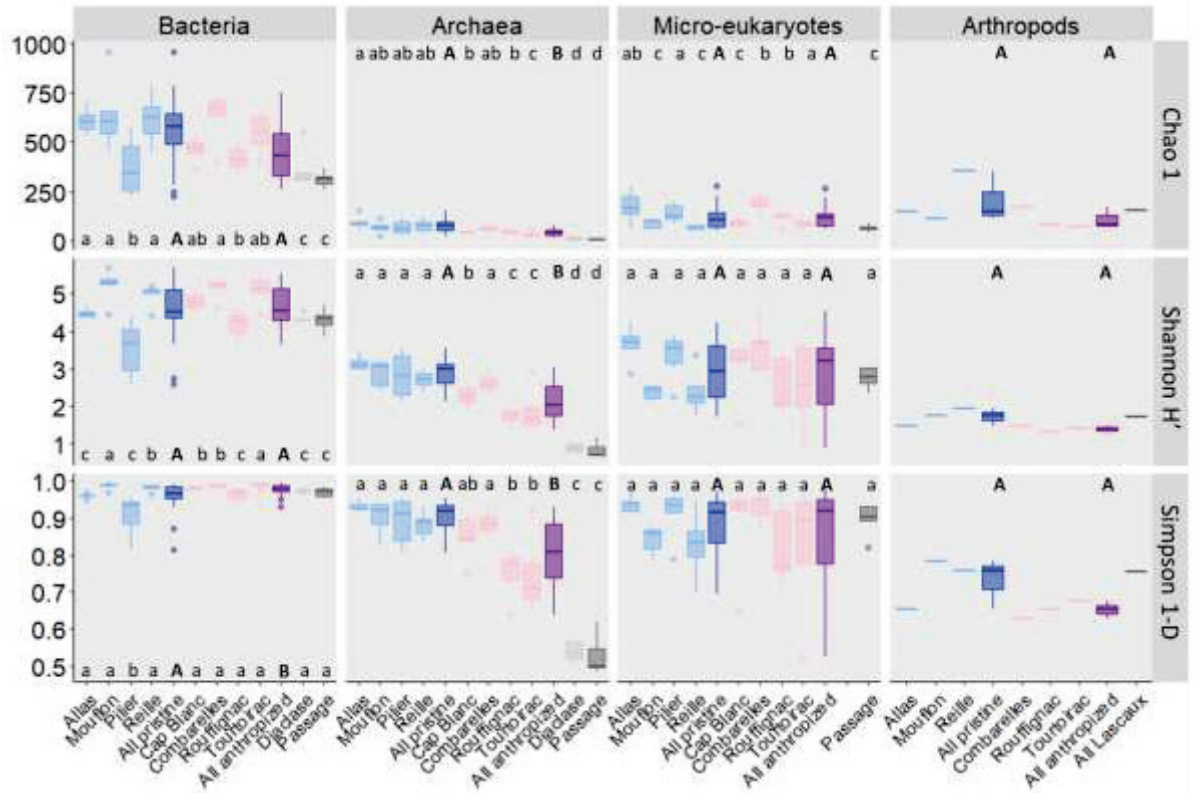

Figure 2. Chao1 index of genus/species richness (A-D), Shannon index of genus/species diversity (E-H) and Simpson's index of diversity (I-L) in cave wall samples from four pristine caves (Allas, Mouflon, Pilier, Reille ; in light blue), four reference anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac ; in pink) and Lascaux Cave (Diaclase and Passage ; in grey). Data are represented in dark blue for all pristine caves combined and in purple for all reference anthropized caves combined. The three indices were calculated for bacteria (A, E, I), archaea (B, F, J), micro-eukaryotes (except for Diaclase where sequencing failed for insufficient DNA levels ; C, G, K) and arthropods (D, H, L). Arthropods were not studied in Pilier, Cap Blanc and Diaclase, and in the case of Lascaux the pitfalls were placed in the various rooms of the main compartment including the Passage. Statistical differences between individual caves are shown with lowercase letters and between the two groups of caves with capital letters (based on ANOVA and Tukey's tests; $P < 0.05$).

Taxonomic community composition is cave specific

The bacterial community was composed of 15-23 phyla in pristine caves, 12-22 in reference anthropized caves and 12 in both Lascaux compartments (Supplementary Table 1). Proteobacteria (14-56%) and Actinobacteria (11-49%) were the most prevalent phyla in all caves (Fig. 3A). In comparison with the reference anthropized caves and pristine caves, Lascaux Passage displayed a higher proportion of Bacteroidetes (19% versus 1.4% and 0.2%,

respectively) and a lower proportion of Nitrospirae (0% versus 5.2% and 10.2%) (Chi-squared tests, $P < 2 \times 10^{-16}$). At genus level, the bacterial community was composed of 63-95 genera in pristine caves, 55-99 in reference anthropized caves, 77 in Diaclase and 94 in Passage (Supplementary Table 1). A large percentage of sequences could not be affiliated at genus level in pristine (57-83%) and reference anthropized caves (39-58%), whereas this percentage was only 13% in the Diaclase and 26% in the Passage. The number of bacterial genera representing more than 1% of sequences was 2-9 for pristine caves and 6-12 for reference anthropized caves, versus as many as 18 for each of the two Lascaux compartments. *Nitrospira* was among the three most prevalent genera in all caves but Rouffignac, Diaclase and Passage, whereas no other genus was prevalent across several caves (Fig. 4A). The phylum taxonomic profile of bacteria (i.e. the relative proportions of all bacterial phyla in the community) differed ($P < 0.05$) (i) from one cave to the next among pristine caves or reference anthropized caves, (ii) between the four pristine caves and the four reference anthropized caves, (iii) between the two compartments of Lascaux and (iv) between the two compartments of Lascaux and the eight other caves (Fig. 3A).

The archaeal community was composed of 5-6 phyla in pristine caves and in reference anthropized caves, versus 1 (i.e. Thaumarchaeota; Passage) and 3 phyla (Diaclase) in Lascaux, with the Thaumarchaeota predominating (74-99%) in all cases (Fig. 3B). The phylum profile of archaea did not differ between reference anthropized caves and pristine caves, e.g. with Euryarchaeota (7.2% and 7.1%, respectively) and Woesearchaeota (7.1% and 4.3%, respectively) at essentially comparable levels. The archaeal community was composed of 7-10 classes in pristine caves, 6-9 classes in reference anthropized caves, versus only 2 in the Diaclase and 4 in the Passage. The class profile of archaea differed when comparing (i) individual or groups of pristine caves versus reference anthropized caves ($P < 0.05$ for the group comparison), and (ii) the two compartments of Lascaux together as well as to the eight other caves ($P < 0.05$) (Fig. 4B). Archaeal genera were not considered because affiliation at genus level is not available.

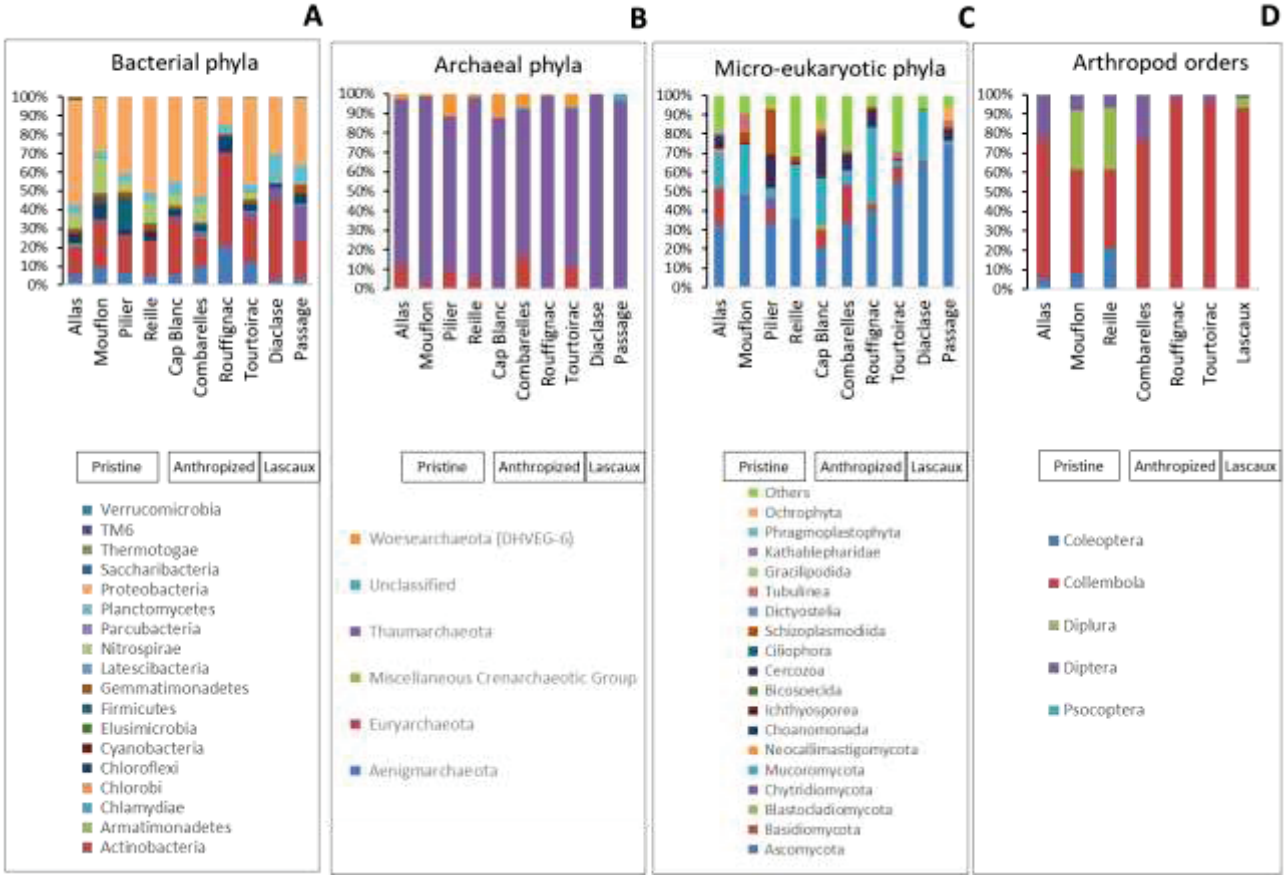

Figure 3. Community composition at phylum level for bacteria (A), archaea (B), micro-eukaryotes (C) and at order level for arthropods (D) in cave wall samples from four pristine caves (Allas, Reille, Mouflon, Pilier), four reference anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) and two compartments of Lascaux Cave (Diaclase, Passage). Arthropods were not studied in Pilier, Cap Blanc and Diaclase, and in the case of Lascaux the pitfalls were placed in the various rooms of the main compartment including the Passage.

The micro-eukaryotic community was composed of 14-23 phyla in pristine caves, 17-20 in reference anthropized caves, 10 in Diaclase and 17 in Passage. Ascomycota (20.6-60.8%) was the most prevalent phylum (Fig. 3C). The phylum profile of micro-eukaryotes differed from one cave to the next, regardless of anthropized or pristine status, including when considering Lascaux compartments. The minor phylum Ochrophyta was in higher proportion in the Passage (6.6%) than in reference anthropized caves (0.69%), pristine caves (0.17%) and Diaclase (0%). The micro-eukaryotic community was composed of 30-83 genera in pristine caves, 36-70 in reference anthropized caves, versus only 25 in the Diaclase and 27 in the Passage. The genus profile of micro-eukaryotes differed ($P < 0.05$) when comparing (i) individual pristine caves and reference anthropized caves, and (ii) the two Lascaux compartments together or to the eight other caves (Fig. 4C).

The arthropod (Hexapod) community was composed of 5 orders in pristine caves, 2 in reference anthropized caves and 3 in Lascaux Cave. Collembola was the most prevalent order in all caves and reached up to 98% of arthropod diversity in reference anthropized

caves and Lascaux. The Coleoptera order was found in all pristine caves, but in none of the other caves.

In summary, taxonomic community composition was cave specific. This finding was made both with prokaryotes and eukaryotes, regardless of the anthropization status of the caves.

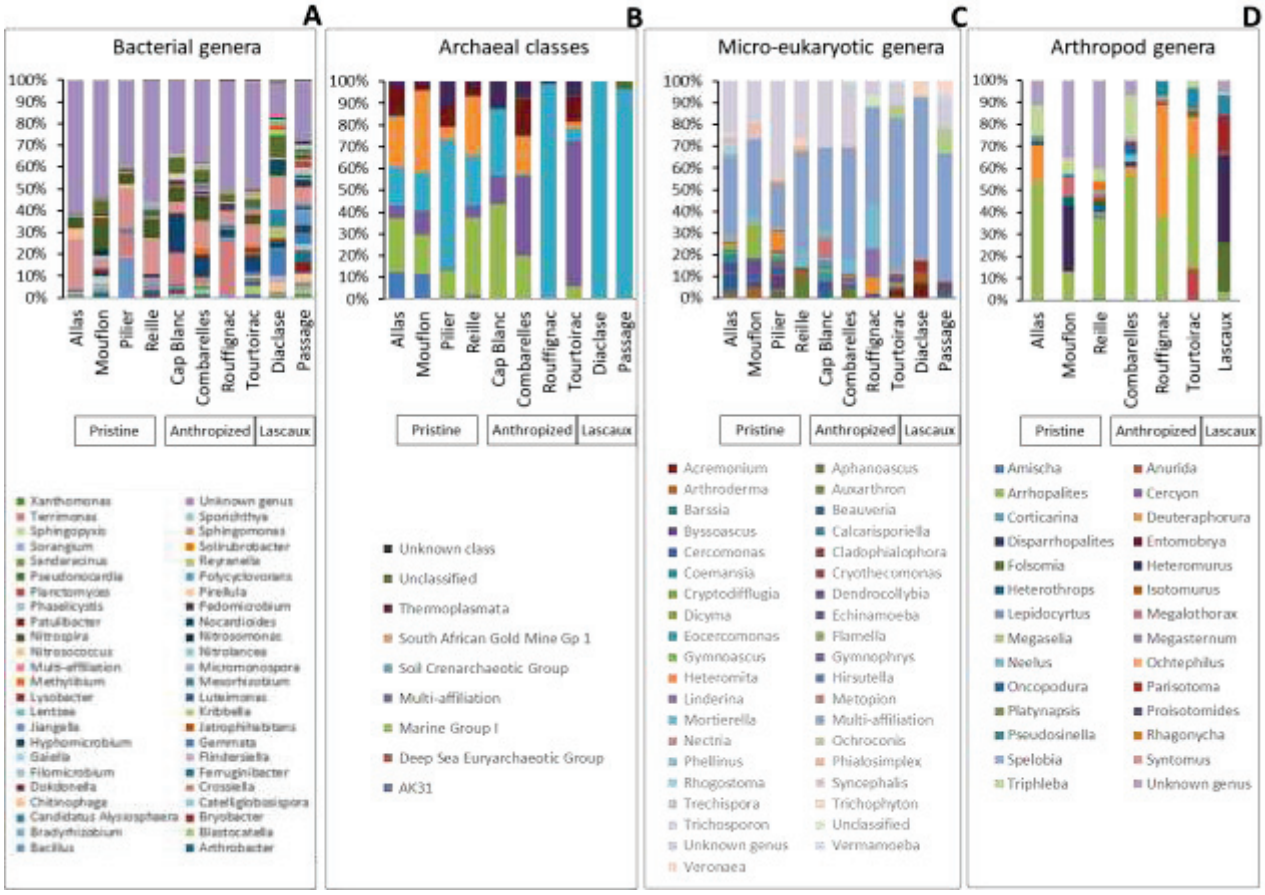

Figure 4. Community composition at genus level for bacteria (A), class level for archaea (B), genus level for micro-eukaryotes (C) and genus level for arthropods (D) in cave wall samples from four pristine caves (Allas, Reille, Mouflon, Pilier), four reference anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) and two compartments of Lascaux Cave (Diaclase, Passage). Arthropods were not studied in Pilier, Cap Blanc and Diaclase, and in the case of Lascaux the pitfalls were placed in the various rooms of the main compartment including the Passage. Genera or classes representing more than 1% of sequences are indicated.

Community structure in caves depends on anthropization

Non-metric multidimensional scaling (NMDS) analysis indicated that the genetic structure of the bacterial community (Fig. 5A) was similar amongst pristine caves, whereas Rouffignac was different in comparison with the three other anthropized caves (P = 0.0001). The structure of the bacterial community differed for the reference anthropized caves compared with the pristine caves (Supplementary Table 3). The structure of the bacterial community in Lascaux Cave (Diaclase and Passage) differed as well, in comparison both with the pristine caves and the other anthropized caves (Supplementary Table 3), and it differed between the two Lascaux compartments (P = 0.0001).

The genetic structure of the archaeal community was similar amongst pristine caves (Supplementary Table 3), except for Pilier where only the four samples from the end of the cave grouped with other pristine caves, the two samples taken in the room closer to the entrance bearing similarities with Lascaux's Diaclase (Fig. 5B). The genetic structure of reference anthropized caves was similar among each other, except for Rouffignac which was similar to pristine caves. The structure of the archaeal community differed for the reference anthropized caves compared with the pristine caves (Supplementary Table 3). The structure in the two Lascaux compartments differed as well, in comparison with the pristine caves (except the two samples from the entrance room of Pilier) as well as the reference anthropized caves (Supplementary Table 3).

The genetic structure of the micro-eukaryote community was similar amongst pristine caves and differed in comparison with reference anthropized cave (Supplementary Table 3) except for Rouffignac (Fig. 5C). The structure in the Passage differed as well, in comparison both with the pristine caves (Supplementary Table 3) and the other anthropized caves (Supplementary Table 3), but for NMDS analysis the stress value was high (0.26).

The difference in the genetic structure of the arthropod (Hexapod) community in pristine and reference anthropized caves (Fig. 5D) was not significant (Supplementary Table 3), but statistical power was restricted by sampling design constraints. Lascaux Cave was in an eccentric position in comparison with all other caves.

In summary, community genetic structure was significantly impacted by cave anthropization. The most anthropized cave, Lascaux, stood out for all communities investigated.

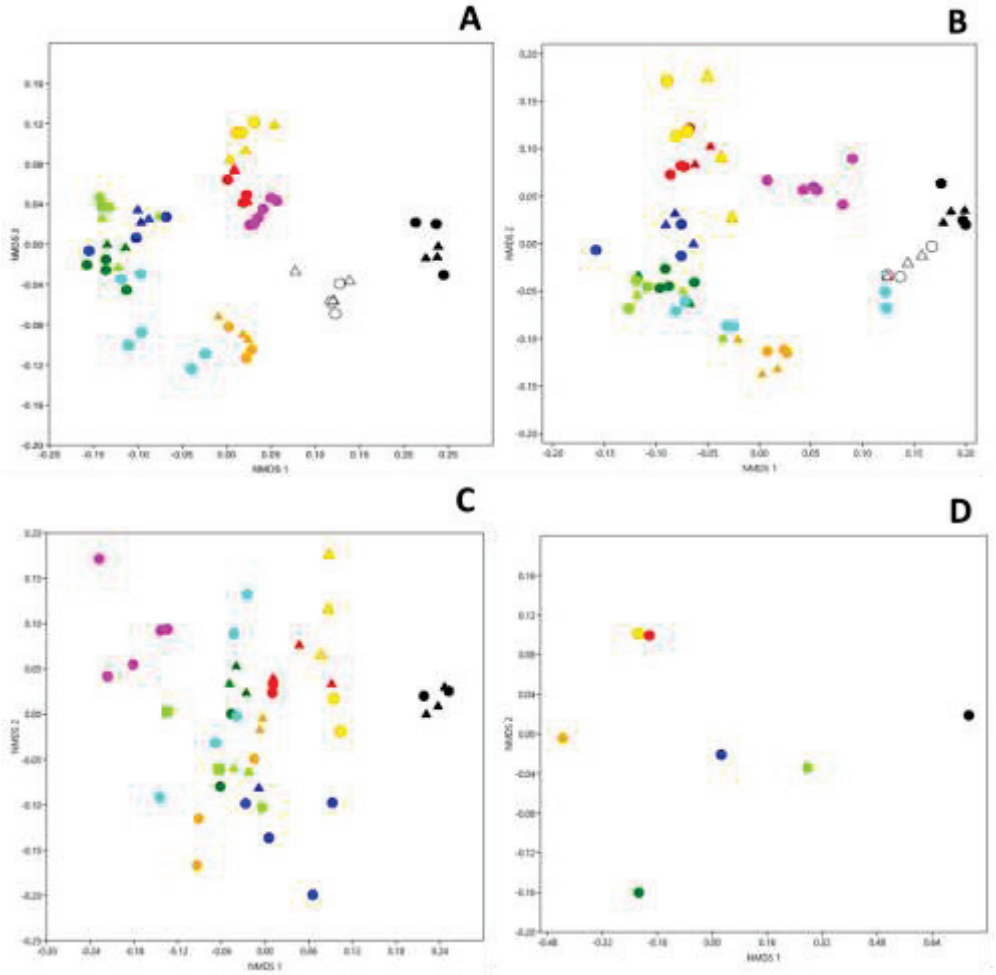

Figure 5. NMDS analysis of cave wall samples from four pristine caves (Allas, dark green symbols; Reille, dark blue; Mouflon, light green; Pilier, light blue), four reference anthropized caves (Cap Blanc, pink symbols; Combarelles, red; Rouffignac, orange; Tourtoirac, yellow) and two compartments of Lascaux Cave (Diaclase, white symbols; Passage, black), based on the relative proportion of both phyla and classes in the bacterial (A), archaeal (B), micro-eukaryotic (C) and arthropod communities (D). Squares represent the two Mouflon samples collected in June 2015, triangles the samples taken in May-June 2016 and circles those in February 2017. For archaea (B), the two Pilier samples (light blue) close to Diaclase samples were taken in the entrance room of the cave and the four others in the back room. Arthropods were not studied in Pilier, Cap Blanc and Diaclase, and in the case of Lascaux the pitfalls were placed in the various rooms of the main compartment including the Passage. Stress values were 0.06 for bacteria, 0.11 for archaea, 0.27 for micro-eukaryotes and 0.07 for arthropods.

Discussion

This work aimed at testing the hypothesis that tourism-related anthropization strongly drives cave community structure, comparing Lascaux Cave with a range of four pristine and four reference anthropized caves. Despite occurring in the same Dordogne area in south-western France and sharing the same Cognacien limestone, both sets of caves that were compared with Lascaux displayed inter-cave variability, regardless of the community studied (i.e., bacteria, archaea, micro-eukaryotes and arthropods) and the type of assessment

(community diversity, taxonomic composition, or genetic structure). This variability did not come as a surprise, since caves may differ from one another in size, morphology or water dynamics (e.g. Reille and Tourtoirac have active streams) (Table 1), and here it could be documented thanks to the unprecedented scale of this study (10 different caves/compartments studied, 165 Illumina sequencings carried out).

Clear differences in community features were found when comparing the group of four reference anthropized caves with the four pristine caves. While each cave is both heterogeneous and distinct from the others in biological terms, our results show that anthropization can be an overriding factor determining cave communities. This was the case even for Tourtoirac, although it was open to the public only a few years ago. Therefore, the consequences of anthropization materialize quickly, without requiring the implementation of cumulative effects.

In comparison with these reference anthropized caves, however, Lascaux Cave corresponds to an even higher scale of anthropization in terms of sediment/scree excavation, work on access infrastructures (air extraction, cooling system, lighting installation), past tourist numbers (up to 2000 visitors certain days in the 1960's), disease outbreaks, as well as chemical treatments[14]. Against this background, it can be anticipated that the particular anthropization of Lascaux Cave should lead to even more drastic modifications in the ecology of cave organisms. Preliminary assessments of Lascaux microorganisms have been carried out using culture-based and cloning/sequencing approaches[4,11,12,16,17], but without targeting the entire microbial community (the scale of cloning/sequencing being a limit), the arthropod phylum, or comparing Lascaux with other types of caves. Here, we show that the communities of Lascaux's Passage differed significantly from the ones found in the reference anthropized caves, and the differences were even larger when comparing with pristine caves. Therefore, communities were affected not only by the occurrence of anthropization (as indicated when comparing reference anthropized vs pristine caves) but also by the magnitude of anthropization (as shown when comparing Lascaux's Passage, reference anthropized caves, and pristine caves).

Within Lascaux Cave, the Passage was selected as a centrally-located area of the main, highly-anthropized part of the cave, and indeed the Passage was a suitable representative of that part of the cave as similar microbial and arthropod community findings were obtained when considering samples taken in neighboring rooms (data not shown). In this context, samples for this work were also taken in the other compartment of the cave (Diaclase), which displays a few paintings only, is difficult to access and separated from the rest by a trap door, and was never open for touristic visits (Supplementary Table 4). The Diaclase underwent much less anthropization than the Passage, yet an air extractor had been installed there and a few chemical treatments were carried out especially in the vicinity of the trap door. Therefore, it represented a useful external reference for the Passage. Our results show that microbial communities of the Diaclase did not differ much from those in the Passage but were in intermediate position between the Passage and

reference anthropized caves when considering bacterial NMDS data. This indicates that the Diaclase, although less subjected to human influence than the Passage, cannot be considered as an undisturbed area of the cave, as thought so far[4].

It is thought that pollution or stress may lead to a reduction in species richness and changes in community structure[18,19]. It can be explained by a diminution of rare species and a higher abundance of dominant species[18]. This is compatible with fragmented data available from cave ecosystems, e.g. for culturable bacteria[20] and fungi[21], but not with DGGE findings on fungi[21]. Furthermore, differences in microbial diversity after bleach treatment were not significant[22]. Here, the comparison of pristine and reference anthropized caves showed that anthropization was associated with reduced numbers of bacterial and archaeal OTUs, reduced estimated richness (Chao1) and diversity (Shannon) of archaea, and fewer arthropod predators especially Coleoptera (family Staphylinidae). When extending the comparison to the case of Lascaux's Passage, more intense anthropization coincided with a decrease in the numbers of bacterial, archaeal and micro-eukaryotic OTUs, and in the richness (Chao1) and diversity (Shannon) of both bacteria and archaea. In parallel, NMDS data for the bacteria and to a lesser extent the archaea also showed that the occurrence as well as the magnitude of anthropization could explain differences between caves. A clear difference between Lascaux Cave and the eight other caves was also revealed by NMDS data for micro-eukaryotes (in the Passage) and arthropods (sampled throughout the main, heavily-anthropized compartment of Lascaux Cave), even though such a difference did not show when comparing Chao1, Shannon and Simpson indices. Perhaps biocide tolerance was more prevalent amongst eukaryotes than prokaryotes[22]. Thus, the ability of communities to reveal anthropization effects depended on the community considered. Anthropization effects appeared unrelated to the levels of nutrient resources, as suggested by C content and C:N ratio values recorded in the floors of the caves investigated (Supplementary Table 4).

Most taxonomic groups identified here can be widespread in other caves (and other types of environments)[23-25]. For bacteria, the Passage and Diaclase stood out with a higher proportion of Bacteroidetes (respectively 19% and 6 % versus 2.8% for anthropized caves and 0.3% for pristine cave) and lower proportion of Nitrospirae (0% and 0.5% versus 5.2% and 10.3%). The Bacteroidetes represented 2.1% of the bacterial community in pristine Niu Cave, China[26] versus as much as 5.5% and 8.1% in anthropized Maijishan Grottoes, China[27] and anthropized French/Swiss caves[22]. For Nitrospirae phylum, the relative abundance was 8% in Niu Cave (and 14% in pristine Lechuguilla Cave[28]) vs less than 1% in Maijishan Grottoes and the French/Swiss caves. Thus, our results demonstrate that cave anthropization results in higher relative abundance of Bacteroidetes and lower abundance of Nitrospirae, a possibility raised in these previous studies. We can therefore extend to the case of karstic underground environments the status of Bacteroidetes as disturbance bioindicators[29-33] and Nitrospirae as a phylum well adapted to pristine ecological conditions[34-37]. Here, anthropization effects took place without an impact on total bacterial numbers, as indicated

by quantitative PCR of 16S rRNA genes (Supplementary Fig. 2). The archaeal community in caves is poorly documented[38–41], and the impact of anthropization on this community has not been studied before. Much more is known on cave micro-eukaryotes, with Ascomycota the most abundant fungal phylum in caves[21,22] and grottoes[27], but again with poorly-documented anthropization effects[21].

In conclusion, this large-scale investigation of regional cave biogeography demonstrated for the first time the effect of anthropization on the cave biota, based on direct comparison of a range of several pristine and anthropized caves. It also revealed the extent of cave microbiome variability within and across caves, filling a gap in our understanding of cave biodiversity. This work stresses the importance of better understanding cave ecology to optimize the management of Lascaux and other prominent cultural heritage landmarks such as Chauvet-Pont d'Arc, Altamira and others, and highlights the importance of limiting potential ecological disturbances in conservation management of caves.

Methods

Sampling

For microbial community assessments, Lascaux Cave (i.e., Passage and Diaclase compartments), four other anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) and four pristine caves (Allas, Reille, Mouflon, Pilier) from the Dordogne region (South-West of France) were sampled (Fig. 1, Table 1 and supplementary Table 4) in May-June 2016 and February 2017. The aim was to obtain a total of 6 samples per cave or compartment. In the case of micro-eukaryotes, PCR for sequencing did not succeed for certain samples, and thus the study was carried out with 5 samples successfully sequenced (except for Diaclase where this sequencing failed), and for Mouflon cave this was achieved by including two additional samples collected in a pre-study in June 2015. Cave wall samples (in the order of 100 mg each) were taken using sterile scalpels at the level of the Coniacien limestone vein, away from stained areas in Lascaux Cave to avoid comparison bias. Samples were collected at various distances (at least 30 m) from the cave entrance, and more specifically in the Passage and Diaclase compartments in Lascaux. The Passage is a central area in Lascaux Cave, which has been heavily visited and treated with biocides and is well connected to most other areas in the cave. On the contrary, the Diaclase is separated from the main part of Lascaux (including the Passage) by a trap door, it was not visited by tourists and was seldom treated with biocides. Thus, the Passage and Diaclase compartments can be considered as two different environments, each with a different level of anthropization.

Due to access limitations for Lascaux's Diaclase and Cap Blanc, arthropods were investigated in Lascaux's Passage, the three anthropized caves Combarelles, Rouffignac and Tourtoirac, and the three pristine caves Allas, Reille and Mouflon. The sampling was carried out twice (in February-March and May-June 2016), using non-baited pitfall traps as bait

could not be used in Lascaux for sanitary reason. In each campaign, 25 pitfall traps were set in each cave, arranged according to cave topography but always at the bottom of cave walls and with a minimum of 3 m between traps. Each trap consisted in a 40-ml polypropylene tube (opening diameter of 3 cm) with 15 ml 70% water- 30% propylene glycol solution, which was set in the soil so that its opening was flush with the ground surface. Such pitfall traps are meant to capture passing arthropods, which fall into the tubes and are trapped in the water-propylene glycol solution. At the end of each 7-day trapping campaign, 96% ethanol was added to the water-propylene glycol solution to fill the 40-ml tubes, in order to better preserve arthropods for subsequent identification.

DNA extraction, sequencing and bioinformatics for microbial community characterization

DNA extraction from cave wall samples was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), following the manufacturer's instructions. Briefly, the elution was made with two volumes of 50 µl for each sample. The resulting DNA concentration was quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) following the manufacturer's instructions. DNA extracts were stored at -20°C until further analysis. Three gene markers were analyzed, i.e. the 16S rRNA genes of bacteria, the 16S rRNA genes of archaea and the 18S rRNA genes of micro-eukaryotes (PCR primers in Supplementary Table 5). These genes were amplified by PCR and sequenced by Fasteris company (Geneva, Switzerland), using high-throughput Illumina MiSeq with 2 × 300 bp, paired-end chemistry to obtain 70,000 paired reads per sample.

Prokaryotic (bacterial and archaeal 16S rRNA genes) and eukaryotic (18S rRNA genes) paired-end reads were demultiplexed and cleaned by removing adaptators as well as sequences presenting two mismatches with primer sequences, using a Perl script from Fasteris company. They were then merged using FLASh (Fast Length Adjustment of Short reads) with a maximum of 10% mismatch in the overlapped region. Denoising procedures consisted in discarding reads without the expected length (i.e. 200-500 bp) or containing any ambiguous base (N). After dereplication of sequences, the clusterisation tool was run with SWARM[42], which uses a local clustering threshold rather than a global clustering threshold and an aggregation distance of 3 for OTU identification. The lower taxonomic level reached corresponded to (depending on taxa considered) the genus or the species, and was hereafter referred to as 'genus/species' level. Chimeras were then removed using VSEARCH[43] and low-abundance sequences were filtered at ≥ 0.005%, i.e. keeping OTUs representing at least 0.005% of all sequences[44], and discarding singletons from the datasets. Taxonomic affiliation was performed with both RDP Classifier[45] against the 119 SILVA database[46] for bacteria and archaea and the 123 SILVA database for micro-eukaryotes. This procedure was automated in the FROGS pipeline[47]. To compare samples, a normalization procedure was applied by randomly resampling down to 10028, 3287 and 7161 sequences in

the bacteria, archaea and micro-eukaryote datasets, respectively. The different sequencing datasets reported in this paper have been deposited in EBI under reference PRJEB24734. Raw sequence datasets contain merged paired-end reads.

Arthropod (Hexapod) community characterization

Arthropods were identified to species level whenever possible. Individuals were first sorted into morphospecies, followed by formal identification based on detailed morphology assessment and/or DNA barcoding (experimental protocols for extraction, PCR and sequencing given in Supplementary Methods). When no identification was possible because of insufficient DNA quality or lack of taxonomical expertise, morphospecies were retained. When it was impossible to even have clear morphospecies, identification was limited to order (especially for Acari, Opiliones, Araneae and Myriapoda). As a result, only hexapods were reliably sorted into morphospecies, with most individuals identified further to genus or species level. For Collembola, which made up the majority of captured individuals, identification was confirmed by DNA barcoding and formal morphological taxonomy for all individuals. As arthropod samples were relatively small due to sampling constraints in Lascaux, individuals from both campaigns were pooled for all analyses.

Statistical analyses

Rarefaction curves were calculated to assess sampling efficacy. OTU richness and diversity (at genus or species level) were estimated using Chao 1 index[48], Shannon's H'[49] and Simpson (1-D) index[50]. For community analysis, data were square-root transformed to minimize the impact of highly-dominant taxa, and community structures of different caves were assessed using the VEGAN package (http://cran.r-project.org/web/packages/vegan/index.html) in R 3.3.0. Communities were primarily compared with NMDS, using the Paleontological Statistics (PAST) software v3.14[51]. The procedure computes a stress value, which measures the difference between the ranks on the ordination configuration and the ranks in the original similarity matrix for each replicate. Stress values below 0.1 are considered without risk of drawing false inferences, values below 0.2 acceptable (especially those close to 0.1), while values above 0.2 indicate limited interpretation potential[52]. Analysis of similarity (ANOSIM) was conducted using the VEGAN package in R, to test differences ($P < 0.05$) in overall community composition between different caves and to further confirm the results observed in the NMDS plot. A Bonferroni correction was applied on *P* values to lower alpha risk. All analyses were based on similarity matrices calculated with the Bray-Curtis similarity index[53], using R. An analysis of variance (ANOVA) and post-hoc Tukey HSD tests were performed to compare the number of OTUs or diversity indices across the caves studied, based on comparison of all 10 caves as well as of the group of pristine caves versus the group of reference anthropized caves ($P < 0.05$). A Pearson's Chi-squared test in R was conducted to compare the proportions of taxa in different communities ($P < 0.05$).

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Contributions

T.P., C.J.D. and Y.M.L. conceived the study. Y.M.L. led and coordinated the study. L.A., A.D., D.C., J.D., T.P., C.J.D. and Y.M.L. performed the samplings, and L.A. and B.K. the measurements/identifications. L.A., A.D. and B.K. treated the data. L.A. and Y.M.L. wrote the manuscript with significant contributions from T.P., C.J.D. and B.K. All authors contributed intellectually throughout the study, commented on and edited the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary Methods. Molecular methods used for molecular identification of Arthropods.

In order to confirm morphological identifications, arthropod DNA extractions were performed as follows. After 12 h of digestion of whole individuals at 55°C in a solution consisting of 15 µl of proteinase K and 200 µl of digestion buffer (NaCl 0.1M, EDTA 0.01M, SDS 0.5%, Tris HCl 0.05M), the cuticles were removed and 250 µl of NaCl solution 2.6M were added to the solution. The solution was then vortexed and centrifuged (15 min, 13200 rpm), the supernatant was transferred in 400 µl chloroform (trichloromethane/chloroform Rotipuran; Roth, Karlsruhe, Germany). The solution was then vortexed again for 5 s, followed by centrifugation for 5 min at 13200 rpm. The supernatant was precipitated in a cold solution of -20°C, 600 µl ethanol, 2 µl glycogen (20 mg/ml; Euromedex, Souffelweyersheim, France), which was slowly agitated by hand, then incubated for 30 min at -80°C and centrifuged again for 20 min at 4°C and 13200 rpm. After elimination of the supernatant, the pellet was washed with 300 µl of 70% ethanol at -20°C, without resuspension, and then dried using a SpeedVac apparatus (Thermo Fisher Scientific, Waltham, MA) for 10 min. Finally, the dried pellet was re-suspended in15 µl of TE buffer (Tris-HCl 10mM, EDTA 1mM).

All Collembola, Diptera and Coleoptera were Sanger-sequenced for the COI region of the mtDNA. Following Hebert et al.[1], we used the primers LEP-F1 (5'-ATTCAACCAATCATAAAGATAT-3') and LEP-R1 (5'-TAAACTTCTGGATGTCCAAAAA-3') to amplify a cytochrome c oxidase subunit 1 (COI) region. For some individuals, additional primers (COILCO1490, GGTCAACAAATCATAAAGATATTGG and COIHCO2198, TAAACTTCAGGGTGACCAAAAAATCA[2]) were added to the LEP-F1 / LEP-R1 primers to increase PCR yield. In such cases, sequencing was carried out using the COILCO1490 primer.

PCR reactions were carried out in 30 µl solutions with 0.2 µM dNTPs, 0.1 µg/µl BSA (New England Biolabs, Ipswich, MA), 1.5 mM $MgCl_2$, 0.16 µM of each primer, 1.20 U Taq polymerase (GAETAQ00 ; Eurobio, Courtaboeuf, France), 1× PCR Buffer (Eurobio), and 0.8 µl of DNA. Cycling was conducted on a PTC-200 thermal cycler (MJ Research, Waltham, MA) with the following parameters: (i) initial denaturation for 5 min at 94°C, (ii) 40 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 48°C and extension for 30 s at 72°C; (iii) final extension for 5 min at 72°C. All PCR products were purified, sequenced and ran on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) by a service provider (BIOFIDAL, Vaulx-en-Velin, France).

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Table S1. Number of Illumina DNA sequences for bioinformatic analyses and number of microbial OTUs, phyla, genera (or classes for archaea).

	Pristine caves			Reference anthropized caves					Lascaux	
	Allas	**Mouflon**	**Reille**	**Pilier**	**Cap Blanc**	**Combarelles**	**Rouffignac**	**Tourtoirac**	**Diaclase**	**Passage**
Bacteria (16S rRNA)										
Raw sequences	167 043	355 786	335 876	204 829	286 842	286 475	233 404	444 392	386 979	463 275
Filtered sequences	85 863	213 726	157 556	101 453	128 601	135 352	144 162	238 770	295 109	359 747
OTUs	967	1211	1127	817	713	1107	647	1010	735	537
Phyla	21	23	22	15	14	22	12	22	12	12
Genera	71	95	81	63	60	99	55	94	77	94
Archaea (16S rRNA)										
Raw sequences	166 292	315994	248 952	189 082	221 588	211 596	234 461	260 578	188 514	327 136
Filtered sequences	79 071	176 565	75 133	130 850	51 375	67 396	168 333	110 750	132 792	42 236
OTUs	173	124	153	164	75	115	71	97	85	14
Phyla	4	5	5	3	4	4	1	4	1	3
Classes	8	10	10	7	8	9	6	9	2	4
Micro-eukaryotes (18S rRNA)										
Raw sequences	473 752	571 754	286 581	376 222	235 499	521 263	472 943	497 843	495 061	827 691
Filtered sequences	331 804	532 590	253 790	331 151	185 213	441 062	428 950	436 960	458 749	458 076
OTUs	426	223	203	382	226	432	276	283	144	157
Phyla	23	17	21	14	19	20	17	18	10	17
Genera	83	35	30	72	36	70	43	48	25	27

Table S2. Number of OTUs, orders and genera for arthropods.

	Pristine caves			Reference anthropized caves			Lascaux
	Allas	**Mouflon**	**Reille**	**Combarelles**	**Rouffignac**	**Tourtoirac**	**Main compartment**
OTUs	13	10	22	12	8	7	14
Orders	3	5	4	2	2	2	3
Genera	7	6	14	8	6	7	10

Table S3. Comparison of four pristine caves (Allas, Reille, Mouflon, Pilier), four reference anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) and two compartments of Lascaux Cave (Diaclase and Passage for bacteria and archaea, only Passage for micro-eukaryotes) by analysis of similarity of NMDS data.

	Bacteria		Archaea		Micro-eukaryotes		Arthropods[a]	
	R value	***P* value**	**R value**	***P* value**	**R value**	***P* value**	**R value**	***P* value**
Pristine vs anthropized caves	0.662	0.0001	0.380	0.0001	0.224	0.0001	0.018	0.4015
Pristine caves vs Lascaux	0.989	0.0001	0.902	0.0001	0.929	0.0001	-	-
Anthropized caves vs Lascaux	0.671	0.0001	0.639	0.0001	0.483	0.0002	-	-

[a]Arthropods were studied in three of four pristine caves (Allas, Reille, Mouflon; not Pilier), three of four reference anthropized caves (Combarelles, Rouffignac, Tourtoirac; not Cap Blanc) and in the main Lascaux compartment (including Passage) but not the Diaclase. Comparisons of Lascaux with other caves could not be performed since all Lascaux samples were pooled.

Table S4. List and general features of the caves used in this study.

Caves	**Geographic coordinates**	**Length (m)**	**Presence of stream**	**Discovery date**	**C content (%)[b]**	**C:N ratio[b]**
Pristine						
Allas	44°53’12” N and 1°10’03” E	130	No	Early 1900s	0.21 ± 0.14	7.2 ± 0.7
Reille	45°07’09.5” N and 1°07’11.2” E	2000	Yes	Early 1900s	0.21 ± 0.12	8.5 ± 1.2
Mouflon	44°55’ N and 1°01’ E	280	No	2008	0.12 ± 0.03	4.4 ± 0.3
Pilier[a]	44°55’ N and 1°5’ E	150	No	Early 1900s	0.41 ± 0.18	8.5 ± 1.4
Anthropized						
Cap Blanc	44° 56’ 44’’ N and 1° 05’ 49’’ E	13	No	1909	0.74 ± 0.42	9.3 ± 0.9
Combarelles	44°56’37” N and 1°02’32” E	300	No	1901	0.15 ± 0.12	7.3 ± 0.8
Rouffignac	45°00’31” N and 0°59’16” E	8000	No	1956	0.52 ± 0.33	12.5 ± 2.5
Tourtoirac	45°15’53.6” N and 1°04’28.4” E	3500	Yes	1995	0.15 ± 0.06	6.9 ± 1.1
Lascaux						
Diaclase	45°03’10.7” N and 1°10’12.6” E	30	No	1940	0.14 ± 0.012	12.3 ± 0.9
Passage	45°03’10.7” N and 1°10’12.6” E	17	No	1940	0.22 ± 0.024	9.4 ± 1.1

[a] Pilier cave consists of an entrance room connected to a back room by a man-size, 8-m-long crawling tunnel.

[a] Means ± standard deviation are shown for samples (n = 5 for pristine and anthropized reference caves, n = 3 for each Lascaux Cave room) taken in the floor of the caves. In other Lascaux rooms, C contents and C:N ratio were respectively 0.18 ± 0.13 % and 14.6 ± 3.0 in Airlock-2 entrance zone, 0.28 ± 0.20 % and 10.7 ± 0.9 in great Hall of Bulls, 0.16 ± 0.05 % and 11.3 ± 1.6 in painted Gallery, 0.16 ± 0.12 % and 9.7 ± 0.9 C in the Apse, 0.29 ± 0.06 % and 9.6 ± 1.1 C in Mondmilch Gallery, 0.16 ± 0.01 % and 12.0 ± 0.9 in the Nave.

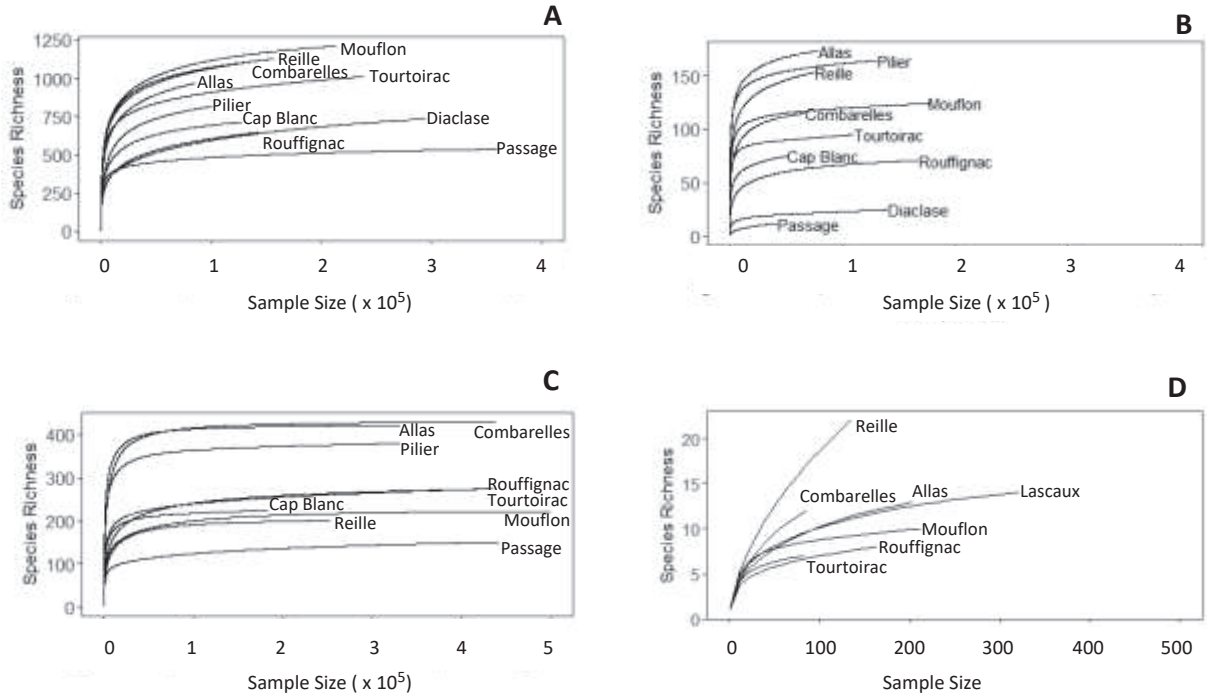

Figure S1. Rarefaction curves at genus level for cave samples in the case of bacteria (16S rRNA gene dataset) (A), archaea (16S rRNA gene dataset) (B), micro-eukaryotes (18S rRNA gene dataset) (C) and arthropods (*COI*) (D), based on observed data. Samples originated from pristine caves (Allas, Reille, Mouflon, Pilier), reference anthropized caves (Cap Blanc, Combarelles, Rouffignac, Tourtoirac) and two compartments of Lascaux Cave (Diaclase, Passage). Arthropods were not studied in Pilier, Cap Blanc and Diaclase, and in the case of Lascaux the pitfalls were placed in the various rooms of the main compartment including the Passage. Rarefaction curves reached an asymptote in 8 of 10 cases (bacteria), 10 of 10 cases (archaea), 9 of 9 cases (micro-eukaryotes) and 5 of 7 cases (arthropods).

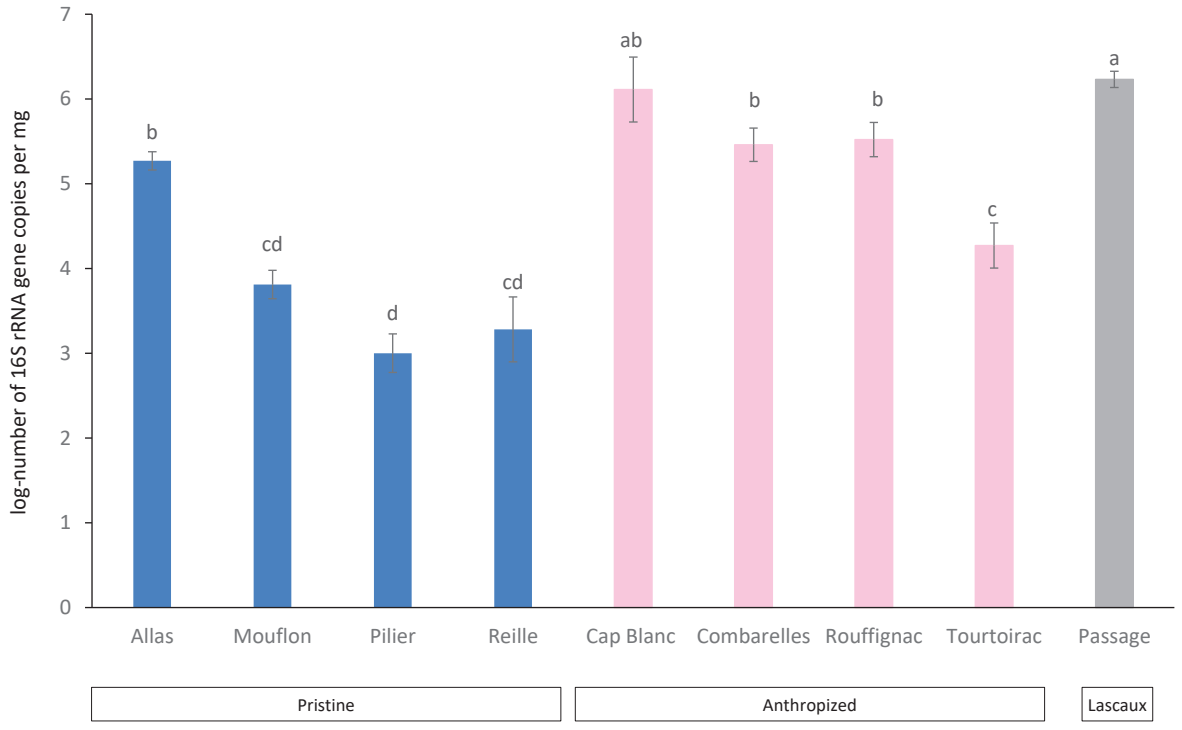

Figure S2. Abundance of the bacterial community colonizing cave walls. Pristine caves (Allas, Reille, Mouflon and Pilier) are represented in blue, anthropized caves (Combarelles, Rouffignac, Tourtoirac, Cap Blanc) in pink, and Lascaux Cave (Passage) in grey. The values shown are means with their standard errors. Statistical differences between caves are shown with letters a-d (Wilcoxon tests, $P < 0.05$). Quantitative PCR was done using primers 519F/907R[1] and a LightCycler 480 (Roche Diagnostics, Meylan, France). Briefly, the 16S rRNA gene reactions were carried out in 20-µl volumes containing 0.6 µl (final concentration 0.3 µM) of each primer, 4 µl of PCR-grade water, 10 µl of LightCycler-DNA Master SYBR Green I master mix (Roche Applied Science, Meylan, France) and 2 µl of sample DNA (5 ng). PCR was done with 10 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s, (ii) 63°C for 60 s, and (iii) 72°C for 30 s. Melting curve calculation and Tm determination were done using the Tm Calling Analysis module of Light-Cycler Software v.1.5 (Roche Applied Science). The number of copies of 16S rRNA genes varied between 10^2 and 10^6 per mg of cave wall sample. It was lower in Pilier (about 8×10^2 copies mg^{-1}) than in the three other pristine caves (about 1×10^4 to 5×10^5 copies mg^{-1}), whereas it was about 10^5 to 10^6 copies mg^{-1} for the four anthropized caves Cap Blanc, Combarelles, Rouffignac and Tourtoirac. In Lascaux Cave, the number of copies of 16S rRNA genes (about 10^6 copies mg^{-1}) was significantly higher than in the pristine caves.

Reference: [1]Zhu, F., Massana, R., Not, F., Marie, D. & Vaulot, D. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiol. Ecol.* **52,** 79–92 (2005).

Chapitre 4 : Le Passage, abondance et diversité de le communauté microbienne présente et active

Avant-propos

Comme nous l'avons vu dans le chapitre précédent, la grotte de Lascaux possède une communauté microbienne particulière. Cependant, la communauté microbienne qui a été caractérisée se situait sur les parois non altérées de la grotte, or des taches noires d'origine microbienne sont aussi présentes sur les parois (Bastian et al. 2009 ; Martin-Sanchez et al. 2012).

Le Passage est une salle centrale faisant le lien entre les autres salles de la grotte, plus particulièrement avec la salle des Taureaux (vers l'entrée), l'Abside et la Nef (vers le fond de la grotte). Le Passage est composé de plusieurs substrats minéraux différents, le sol en terre battue constituée de sable calcaire, les banquettes sont en argile, les parois (plans inclinés) en calcaire et la voûte en calcaire et calcite. Les taches noires présentes dans le Passage depuis 2006 sont réparties de façon hétérogène sur les parois et les banquettes.

Ce chapitre est consacré à l'analyse du Passage. Ainsi nous avons étudié l'abondance (PCR quantitative) et la diversité (en utilisant le séquençage à haut débit) de la communauté microbienne présente et active du Passage en prenant en compte différents facteurs d'hétérogénéité, à savoir le temps, la nature du substrat minéral et la présence de taches, pour déterminer lequel structure la communauté microbienne. L'hypothèse est que les taches impactent plus que le substrat minéral et le temps la structure de la communauté, car le développement des taches est un phénomène relativement récent et lié à la prolifération de certains microorganismes (Bastian et al. 2009b ; Martin-Sanchez et al. 2012), alors que les différents substrats minéraux présentent vraisemblablement des conditions oligotrophes, et que les caractéristiques climatiques (température et CO_2) varient peu dans le temps.

Le Passage a été échantillonné à 7 dates différentes (Décembre 2014, Juin-Juillet 2015, Janvier 2016, Mai-Juin 2016, Décembre 2016, Février 2017 et Mai 2017), ce qui nous a permis un suivi régulier de la communauté microbienne durant le projet, pour trois substrats minéraux (sol, banquettes, plans inclinés), qui ont été prélevés en prenant à chaque date 3 à 6 échantillons de taches noires (quand disponibles) et de zones non tachées adjacentes. Le sol ne possédant pas de taches noires seules des zones non tachées ont été prélevées. La voûte n'a pas été étudiée car c'est une zone trop fragile. De plus, des échantillons supplémentaires ont été prélevés en Janvier 2016 pour étudier la communauté microbienne active en extrayant les ARN. Enfin, les prélèvements en février 2017 et mai 2017 n'ont pas été pris en compte dans l'étude, car les données de séquençage ont été obtenues trop tardivement.

Les échantillons ont été prélevés par la restauratrice Diane Henry-Lormelle avec le support technique de l'UMR Ecologie Microbienne, en suivant le protocole (compatible avec les contraintes de préservation de la grotte) décrit dans le chapitre 2.

Les résultats de cette étude montrent que le temps ou le type de substrat minéral n'affecte pas l'abondance des bactéries et des micro-eucaryotes sur ou en dehors des

taches, mais que le substrat minéral plutôt que la présence de tache semble être le facteur qui influence le plus la structure et la diversité de la communauté microbienne, que l'on considère l'ensemble de la communauté présente (analyse ADN) ou seulement la communauté transcriptionnellement active (analyse ARN). Un signal phylogénétique a également été détecté en relation avec les types de substrats et la présence de taches mais pas la date de prélèvement, au niveau des OTU communes aux trois substrats. Aussi, les analyses de réseau de co-occurrence ont montré que la plupart des interactions bactériennes et fongiques étaient positives quel que soit le facteur testé (date, substrat minéral ou présence de taches), mais ces réseaux variaient en fonction des conditions écologiques et du temps. Les micro-organismes connus pour être capables de produire une pigmentation sont bien présents sur les taches noires mais aussi sur les zones non tachées, ce qui peut être une condition préalable à la formation ultérieure de taches.

Mon rôle dans ce travail a été le suivant : j'ai participé à la réflexion sur les objectifs scientifiques et la démarche expérimentale, à la préparation du matériel ainsi qu'aux différentes missions d'échantillonnage excepté en Février 2017. J'ai traité l'ensemble des échantillons en réalisant l'extraction d'ADN et D'ARN, puis l'analyse des séquences issues du séquençage Illumina. Ensuite, j'ai réalisé les analyses multivariées qui déterminent le facteur qui structure le plus la communauté microbienne, les arbres phylogénétiques des OTUs communs aux trois substrats minéraux ainsi que les calculs d'indices phylogénétiques pour quantifier la structure phylogénétique de la communauté. Puis, j'ai créé les réseaux de co-occurrence pour déterminer les interactions potentielles entre les bactéries et les champignons et j'ai participé à la rédaction du manuscrit.

L'ensemble de ce travail a permis la rédaction de la publication « Rock substrate rather than black stain alterations drives microbial community structure in the Passage of Lascaux Cave ».

Article 2 : Rock substrate rather than black stain alterations drives microbial community structure in the Passage of Lascaux Cave

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Abstract

Background: The World-famous UNESCO heritage from the Paleolithic human society, Lascaux Cave (France), has endeavoured intense microclimatic perturbations, in part due to high touristic pressure. These perturbations have resulted in numerous disturbances of the cave ecosystem, including on its microbial compartment, which resulted in the formation of black stains especially on the rock faces of the Passage. We investigated the cave microbiome in this part of Lascaux by sampling three mineral substrates (soil, banks and inclined planes) on and outside stains to assess current cave microbial assemblage and explore the possibility that pigmented microorganisms involved in stain development occur as microbial consortia.
Methods: Microbial abundance and diversity were assessed by means of quantitative PCR and high-throughput sequencing (Illumina MiSeq) of several DNA and cDNA taxonomic markers. Five sampling campaigns were carried out during winter and summer to embrace potential seasonal effect in this somewhat stable environment (based on measurements of temperature and CO_2 concentration).
Results: While the season or type of mineral substrate did not affect the abundances of bacteria and micro-eukaryotes on or outside stains, mineral substrate rather than stain presence appears to be the most significant factor determining microbial diversity and structuring microbial community, regardless of whether DNA or cDNA markers were considered. A phylogenetic signal was also detected in relation to substrate types, presence of stains but not with season among the OTUs common to the three substrates. Co-occurrence network analyses showed that most bacterial and fungal interactions were positive regardless of the factor tested (season, substrate or stain), but these networks varied according to ecological conditions and time. Microorganisms known to harbour pigmentation ability were well established inside but also outside black stains, which may be prerequisite for subsequent stain formation.
Conclusions: This first high throughput sequencing performed in Lascaux Cave showed that black stains were secondary to mineral substrate in determining microbiome community structure, regardless of whether total or transcriptionally-active bacterial and micro-eukaryotic communities were considered. These results revealed the potential for new stain formation and highlight the need for careful microbiome management to avoid further cave wall degradation.

Keywords: Lascaux Cave, Microbial degradation, Bacterial-fungal co-occurrence, Microbial community

Background

Lascaux cave is famous for its paintings dating from the Upper Paleolithic (ca. 18,000 BA) (Bastian & Alabouvette 2009). Since its discovery in 1940, this jewel of humanity (recognized as such by the UNESCO in 1979) suffered several ecosystem disturbances mostly associated with the development of tourism. The visits, which reached up to ~1,800 visitors per day in the 1960s, strongly modified the microclimatic conditions of the cave in terms of temperature, light conditions and CO_2 concentration (Bastian et al. 2009b). They resulted in several microbe-related "diseases", materialized by various developments of stains on the walls, i.e. green stains, later on white stains and more recently black stains (Bastian et al. 2010; Mauriac 2011).

The successive microbial diseases were treated with the application of biocides and antibiotics, which promoted the development of resistant microorganisms (Bastian et al. 2009a). In other environments, similar treatments have resulted in presence of adapted bacteria *e.g. Pseudomonas aeruginosa* and *Pseudomonas stutzeri* and drastic changes in microbial community structures (Hammesfahr et al. 2008; Tandukar et al. 2013). For instance in fish farm sediments, the number of bacteria declined with 50-67 % due to the application of antibiotics and in agricultural soils (Hansen et al. 1992), whereas application of sulfonamide antibiotics could affect bacterial community structure (Zielezny et al. 2006). Similarly, fungi are often sensitive to antifungal substances that dramatically constrain their community structure to the few resistant strains (Gleason & Marano 2011). However, fungi are generally more resistant to biocides than bacteria (Russell, 2003), which is mostly due to their composition of outer cell layers with chitin. Although little is known about the responses to fungicides and antibiotic treatments in cave environments, it is thought that such treatments had altered the microbial community of Lascaux Cave (Bastian et al. 2009a). However, since its pristine microbial community is not known, disentangling the relative impacts of the treatments from previous human activity is highly challenging.

Several studies have shown that microbial community is abundant and diverse in caves despite the fact that caves are oligotrophic environments (Barton & Northup 2007; Barton et al. 2004; Tebo et al. 2015). Indeed, in cave environments poor in nutrients, microorganisms are thought to cooperate to optimize resource utilization rather than competing (Tomczyk-Żak & Zielenkiewicz 2016). Some bacterial phyla are usually predominant in caves (*Proteobacteria*, *Actinobacteria*), but the overall microbial composition is specific to each cave and related to the type of cave (limestone, lava, ice) and their environmental conditions (pH, availability of nutrients, humidity) (e.g. Barton & Jurado 2007; Engel 2010). Lascaux Cave is a limestone cave with relative humidity near 100% and a mean annual temperature of 12.6°C. The Passage is a central node of communication between the twelve chambers of Lascaux Cave and harbors heterogeneous mineral substrates, i.e. a floor of beaten earth consisting of local calcareous sand, a bank of clay deposits, near-vertical walls (termed inclined planes) of limestone, and a vault where the limestone had been covered by a calcite layer (Fig. 1).

Since 2006, black stains appeared on the vault and the inclined planes, and then propagated on other mineral substrates (i.e. the banks) and other chambers, threatening paintings (Bastian et al. 2010). A previous culture-based microbiological study of this part of the cave after chemical treatments provided a large number of isolates, among which the most abundant bacteria belonged to *Ralstonia* and *Pseudomonas* genus and the most abundant fungi to *Exophiala* genus and *Fusarium solani* species (Bastian et al. 2009). Because these genera are rarely predominant in natural caves, it is thought that they have been selected by chemical treatments (Bastian et al. 2009). Some of the fungal taxa selected include strains with pigmentation potential, and black fungal isolates i.e. from *Ochroconis lascauxensis*, *Exophiala moniliae* and *Acremonium nepalense* have been obtained from the Passage (Martin-Sanchez et al. 2012). Some of them *e.g. Exophiala salmonis* have also been found on cave wall samples or in extreme environments e.g. desert areas (Sterflinger & Krumbein, 1995). Since fungal melanin (black coloration) can confer resistance to many types of environmental stress (Butler et al. 2001), these pigments may be an important asset to resist to chemical treatments in caves.

In the Passage, black stains cover a minority of cave wall surfaces, and the vast majority does not display surface alterations. The occurrence of black stains probably results from spatially-localized conditions promoting particular development or physiology of pigmented microorganisms, and the formation of black stains itself should lead, in turn, to specific surface conditions. On this basis, it may be anticipated that black stains could feature similar microbial communities even if they are located on different mineral substrates. If this hypothesis was true, it would mean that the presence of stain is as influential as the type of mineral substrate. This question was the focus of the present investigation.

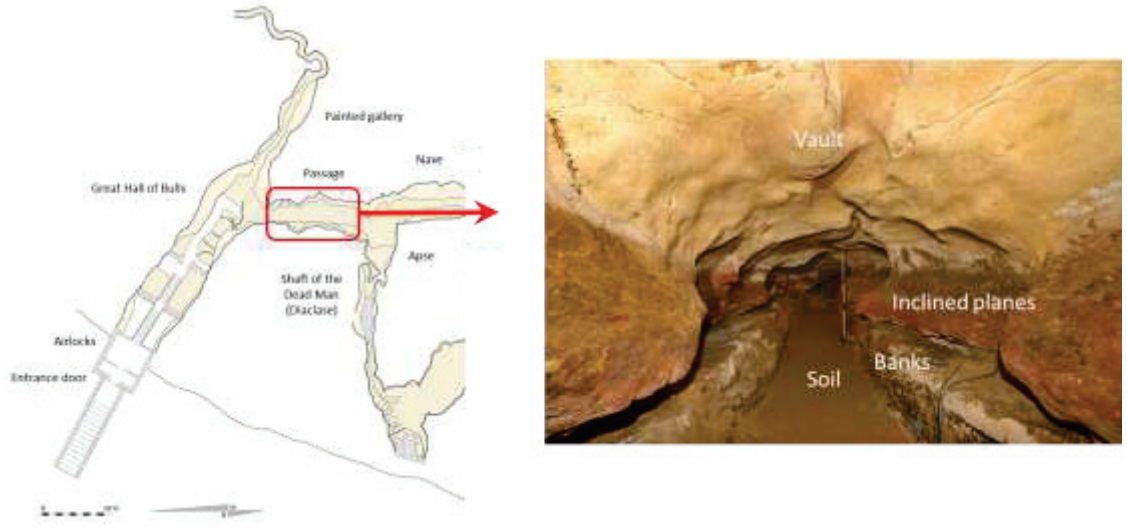


Figure 1. Map of Lascaux Cave with location of the Passage (source: MCC-CNP) and internal structure of the Passage with the three mineral substrates studied (soil, banks, inclined planes) (source: S. Konik, *Centre National de la Préhistoire*; photograph taken on 29 September 2017).

The objective of this work was to assess the relative importance of mineral substrate, presence of black stains and temporal variations in determining microbial community structure. To this end, we applied high throughput sequencing of bacterial,

eukaryotic and fungal taxonomic markers amplified from the DNA of 112 samples collected during five campaigns between 2014 and 2016 in the Passage. This aimed also at describing the microbial consortia associated with pigmented microorganisms in Lascaux Cave, and better understanding microbial successions in stable environments such as continental caves. We further compared these DNA-based microbial patterns with those obtained from RNA extracted from a selection of samples to analyze how the active community responded to the same environmental variables. Finally, we investigated whether co-occurrence networks were driven by environmental pressures and examined the associated phylogenetic patterns.

Methods

Sampling site and environmental data measurements

Lascaux Cave is located in Dordogne in South-West France (N 45° 03′ 13″ and E 1° 10′ 12″) and has been closed for touristic visits since 1963 due to cave wall degradations. Since then, human presence is highly restricted and restrained to scientific campaigns and official visits. The Passage in Lascaux Cave was selected for sampling due to its central location and because black stains occurred early in this part of the cave before similar stains also formed in neighbouring chambers of the cave. The Passage spans 16.6 m in length and 1.6 m in width. Temperature and CO_2 concentration are monitored continuously with sensors, and records are processed every minute using an AMR WinControl (Akrobit®) software. For this study we used the monthly average temperature and CO_2 concentration during the month preceding sampling. The Passage presents four main zones (from bottom to top: soil, banks, inclined planes, vault) that are defined according to their mineral substrate and topography: beaten-earth soil (calcareous sand), banks (clay sediments), inclined planes (limestone), and vault (limestone covered by calcite).

To avoid degradation of Palaeolithic art, samples were only taken on the soil, the banks and art-free parts of the inclined planes, but not on the vault (too fragile). Five sampling campaigns were performed in December 2014, June-July 2015, January 2016, May-June 2016 and December 2016 to study the present microbial community (DNA analyses). In January 2016, supplementary samples were also taken to assess the active microbial community (RNA analyses). In each campaign, samples were taken in four different days spread over a 16-day period to limit the impact of human presence, in accordance with cave rules and regulations. For each mineral substrate and each sampling campaign, 3-6 samples of ~ 50 mg material were taken on (when present) and outside black stains using sterile scalpels. In total 136 samples were collected for this study. To avoid community changes during storage, the samples were immediately placed in liquid nitrogen and transferred latter to -80°C until DNA and RNA extraction.

Nucleic acids extraction, quantitative PCR and high-throughput sequencing

DNA extraction was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), following the manufacturer's instructions and adapted to low amounts of sample. The elution step was achieved using two volumes of 50 µl elution buffer for each sample. RNA extraction from cave samples was performed using ZR Soil/Fecal RNA MicroPrep kit (Zymo Research, Irvine, CA), following the manufacturer's instructions. The DNase treatment was performed during extraction with DNase I Set (Zymo Research). The resulting DNA and RNA concentrations were quantified using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) following the manufacturer's instructions. DNA and RNA extracts were stored at -80°C until further analysis. The reverse transcription of RNA extracts into complementary DNA (cDNA) was carried out by Fasteris compagny (Geneva, Switzerland) before high-throughput sequencing (Illumina MiSeq).

To assess the numbers of bacterial 16S rRNA genes and micro-eukaryotic 18S rRNA genes, quantitative PCR (qPCR) was performed on a LightCycler 480 (Roche Diagnostics, Meylan, France) using primers 519F 5'- CAGCMGCCGCGGTAANWC -3' / 907R 5'- CCGTCAATTCMTTTRAGTTT-3' (Laiz et al. 2003) and EUK345F 5'- AAGGAAGGCAGCAGGCG -3' / EUK499R 5'- CACCAGACTTGCCCTCYAAT-3' (Zhu et al. 2005), respectively. Briefly, the 16S and 18S rRNA gene reactions were carried out in 20-µl volumes containing respectively 0.6 µl (final concentration 0.3 µM) or 0.8 µl (0.4 µM) of each primer, 4 µl of PCR-grade water, 10 µl of LightCycler-DNA Master SYBR Green I master mix (Roche Applied Science) and 2 µl of sample DNA (5 ng). PCR was done with 10 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s (16S rRNA genes) or 94°C for 15 s (18S rRNA genes), (ii) 63°C for 60 s (16S rRNA genes) or 60°C for 15 s (18S rRNA genes), and (iii) 72°C for 30 s (16S rRNA genes) or 72°C for 15 s (18S rRNA genes). Melting curve calculation and Tm determination were done using the Tm Calling Analysis module of Light-Cycler Software v.1.5 (Roche Applied Science). Statistical analysis of qPCR data was performed with Kruskal-Wallis tests and post-hoc Wilcoxon pairwise tests, or with ANOVA and post-hoc Tukey-HSD using VEGAN package (http://cran.r-project.org/web/packages/vegan/index.html) (Oksanen et al. 2017) in R 3.3.0.

Three gene markers of microbial diversity were analyzed using high throughput sequencing: the 16S rRNA genes specific for bacteria, the 18S rRNA genes specific for micro-eukaryotes (including fungi), and the second internal transcribed spacer (ITS2) specific for fungi only. For bacterial 16S rRNA gene amplifications, we used primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3', which target the V3-V4 regions (Klindworth et al. 2013). We used the primers 18S_0067a_deg 5'-AAGCCATGCATGYCTAAGTATMA-3' and NSR399 5'- TCTCAGGCTCCYTCTCCGG -3' (Dollive et al. 2012) for eukaryotic 18S rRNA gene amplification, and primers ITS3_KYO2 5'- GATGAAGAACGYAGYRAA-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (Toju et al. 2012) for fungal ITS2 amplification. Illumina MiSeq sequencing of the PCR products were conducted on 1 µg DNA with specific tag sequences to concurrently

sequence different samples on the same run. The amplifications and sequencing were realized by Fasteris compagny (Geneva, Switzerland), using Illumina MiSeq 2 × 300 bp, paired-end chemistry to obtain 70,000 paired reads per sample.

Processing and analyses of sequencing data

Prokaryotic (bacterial 16S rRNA genes) and eukaryotic (18S rRNA genes and fungal ITS2) paired-end reads were demultiplexed in the different samples according to exact match to adaptors (subsequently removed). Reads presenting one or more nucleotide mismatch to adaptator or at least two mismatches with primer sequences were discarded (Martin 2011). The resulting reads were then merged using FLASh (Fast Length Adjustment of Short reads) (Magoc & Salzberg 2011) with a maximum of 10% mismatch in the overlapping region. Denoising procedures consisted in discarding reads exhibiting length outside the expected 200-500 bp range, and those containing any ambiguous bases (N). After sequence dereplication, clusterisation was performed using SWARM (Mahé et al. 2014), which uses a local clustering threshold rather than a global clustering threshold and an aggregation distance of 3 for identifying operational taxonomic units (OTUs). Chimeras were removed using VSEARCH and low-abundance sequences were filtered out at 0.005% (i.e. keeping OTUs representing at least 0.005% of all sequences) (Bokulich et al. 2013), discarding singletons from the datasets. Taxonomic affiliation of OTUs at phylum, class, genus and/or species level was performed with RDP Classifier (Lan et al. 2012) against the SILVA database v. 119 (Pruesse et al. 2007) for bacterial 16S rRNA genes, the SILVA database v. 123 for micro-eukaryotes 18S rRNA genes and the UNITE database for fungal ITS2. This procedure was automated in the FROGS pipeline (Auer et al. 2015). Alpha diversity indices including Chao 1 (Chao 1987), Shannon's H' (Shannon 1948) and Simpson 1-D (Simpson 1949) were measured using the Paleontological Statistics (PAST) software v3.14 (Hammer et al. 2001). The diversity indices were assessed with Kruskal-Wallis tests and post-hoc Wilcoxon pairwise tests, or with ANOVA and post-hoc Tukey-HSD using VEGAN package in R 3.3.0. Community structure was analyzed using a matrix that was square root-transformed to minimize the impact of highly-dominant phyla or classes, and then subjected to statistical analyses to compare samples and ecological conditions. Microbial communities were primarily compared by non-metric multidimensional scaling (NMDS), and NMDS analysis of RNA data was carried out considering the most abundant OTUs (> 0.1%) only. The stress value calculated to measure the difference between the ranks on the ordination configuration and the ranks in the original similarity matrix for each replicate was considered acceptable when below 0.1 (Ramette, 2007). Non-parametric statistical test of analysis of similarities (ANOSIM) was conducted to test differences in overall microbial community composition in phyla or classes between different mineral substrates, seasons or occurrences on or outside stains, and to further confirm the results observed in the NMDS plot. All analyses were based on similarity matrices calculated with the Bray-Curtis similarity index (Bray & Curtis, 1957) and statistical

analyses were performed using PAST. The different sequencing datasets reported in this paper have been deposited in EBI under reference PRJEB27064.

Phylogeny and networks analysis

Common OTUs of the three mineral substrates were selected to build a phylogenetic tree using MOTHUR v.1.36.1 (Schloss et al. 2009) and the Interactive Tree Of Life (iTOL) (Letunic & Bork 2016) was used to the display, annotate and manage the phylogenetic tree. To quantifying the community phylogenetic structure, the net relatedness index (NRI) and the nearest taxa index (NTI) were calculated as described in the Phylocom manual (Webb et al. 2002) using Qiime relatedness command (Caporaso et al. 2010). To calculate the co-occurrence patterns between microbial communities in the Passage, we used Spearman correlation coefficient based on work of Williams et al. (Williams et al. 2014). Correlation values > 0.60 with $P < 0.001$ were selected. For network construction, correlation values > 0.85 were used. The network visualization was carried out by Gephi 0.9.1. The network was constructed with the spatialization of Fruchterman Reingold, which highlights the complementarities (Fruchterman & Reingold 1991). Two statistics tests were calculated. The average weighted degree, which calculated the mean number of links for each node and the Eigenvector centrality which distinguished important nodes. Data were filtered according to the degree range, which represents the minimum number of links for each node inside the network. The filter was fixed to 5, which was the number of links displayed by 75% of the dataset

Results

Microbial abundances according to time, mineral substrate and in relation to black stain presence

The copy number of bacterial 16S rRNA genes varied between $4.00 \bullet 10^7$ and $8.56 \bullet 10^7$ copies per mg of sample. There was no significant variation of bacterial abundance between samples collected on or outside stains in banks (Wilcoxon test, $P = 0.065$) (Fig. S2), or in time (Wilcoxon test, $P = 0.069$) (Fig. 2A). However, bacterial abundance was significantly lower (Wilcoxon test, $P < 2 \bullet 10^{-16}$) in the banks ($4.48 \bullet 10^6$ copies per mg) than in soil and inclined planes ($1.02 \bullet 10^9$ and $9.75 \bullet 10^7$copies per mg, respectively) when considering all samples together (Fig. 2B). The number of copies of 18S rRNA genes varied between $2.53 \bullet 10^4$ and $1.65 \bullet 10^6$ copies per mg of sample, but it did not vary significantly according to stain presence/absence (Wilcoxon test, $P = 0.98$) (Fig. S2). Against this background, eukaryotic abundance was significantly lower in May-June 2016 and higher in December 2016 than at other samplings (Wilcoxon test, $P = 6.3 \bullet 10^{-16}$) (Fig. 2A), and in inclined planes ($1.96 \bullet 10^6$ copies per mg) than in soil or banks samples ($9.25 \bullet 10^5$and $4.31 \bullet 10^5$ (Wilcoxon test, $P = 0.013$) (Fig. 2B). There was no correlation between microbial abundance and microclimatic parameters (i.e. monthly average temperature and CO_2 concentration prior to sampling campaign; Table 1) in the Passage (Table 2).

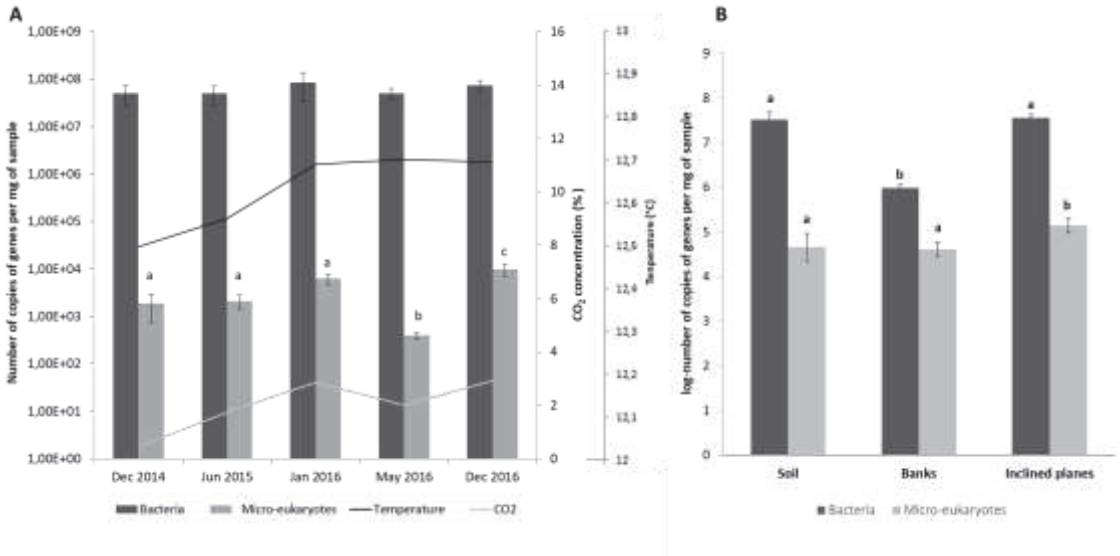


Figure 2. Bacterial and micro-eukaryotic abundances according to time (along with mean temperature and CO_2 concentration) (A) or mineral substrate (B). Abundance data are shown as mean log number of 16S rRNA and 18S rRNA genes copies ± standard errors.

Variations in microbial richness and diversity indices

For each bacterial, micro-eukaryotic and fungal community sampled between December 2014 and December 2016, Chao1 richness index (which estimates total genus/species richness) were estimated, as well as two diversity indices i.e. Shannon H' index (which takes into account both the numbers of individuals and of genera/species; Fig. 3) and Simpson 1-D evenness index (which represents the probability that two individuals randomly selected from a sample belongs to different taxa ; Fig. 3). None of the three indices showed any temporal variation for the three communities studied (Fig. S1), but bacterial richness/diversity indices were always higher than for micro-eukaryotes or fungi (Fig 3).

Richness/diversity indices were also computed according to the mineral substrate and stain presence. Compared with unstained parts, Chao1 index and Shannon H' index of bacteria on black stains did not differ on inclined planes but was statistically higher on the banks (Wilcoxon test, $P = 3.3 \bullet 10^{-7}$ and $P = 0.0002$, respectively). Both Chao1 and Shannon H' indices were statistically higher on the soil than the inclined planes ($P = 6.4 \bullet 10^{-6}$ and $P = 1.6 \bullet 10^{-8}$, respectively) or the banks ($P = 3.6 \bullet 10^{-7}$ and $P = 1.6 \bullet 10^{-6}$, respectively). The Simpson 1-D index did not differ when considering black stains and unstained parts, both on inclined planes and banks. Simpson index on soil and inclined planes was higher than on banks ($P = 2.9 \bullet 10^{-5}$ and $P = 0.0063$, respectively).

For micro-eukaryotes, the Chao1, Shannon H' and Simpson indices did not differ when considering black stains and unstained parts of the inclined planes, but they were statistically higher in black stains than in unstained parts of the banks (Wilcoxon test, $P = 0.01$, $P = 0.01$ and $P = 3.1 \bullet 10^{-10}$, respectively). Chao1, Shannon H' and Simpson indices were statistically higher on soil than on banks ($P = 1.9 \bullet 10^{-9}$, $P = 1.4 \bullet 10^{-12}$ and $P = 4.5 \bullet$

10^{-12}, respectively) and inclined planes (P = 2.8 • 10^{-9}, P = 3.2 • 10^{-8} and P = 5 • 10^{-4}, respectively).

For fungi, Chao1, Shannon H' and Simpson 1-D indices did not differ when considering black stains and unstained parts of inclined planes, whereas for the banks Shannon H' and Simpson 1-D indices were statistically higher in black stains than in unstained parts (Wilcoxon test, P = 0.0003 and P = 1.8 • 10^{-5}, respectively). Chao1, Shannon H' and Simpson 1-D indices were statistically higher on soil than on banks (Wilcoxon test, P < 2 • 10^{-16}, P = 4.7 • 10^{-9} and P = 3.9 • 10^{-9}, respectively) and inclined planes (P < 2 • 10^{-16}, P = 6.3 • 10^{-7} and P = 2. • 10^{-6}, respectively).

In summary, several microbial diversity indices were higher for black stains than unstained parts on the banks (but not on inclined planes). Soil showed higher Chao1, Shannon H' and Simpson 1-D indices than banks and inclined planes for the three communities.

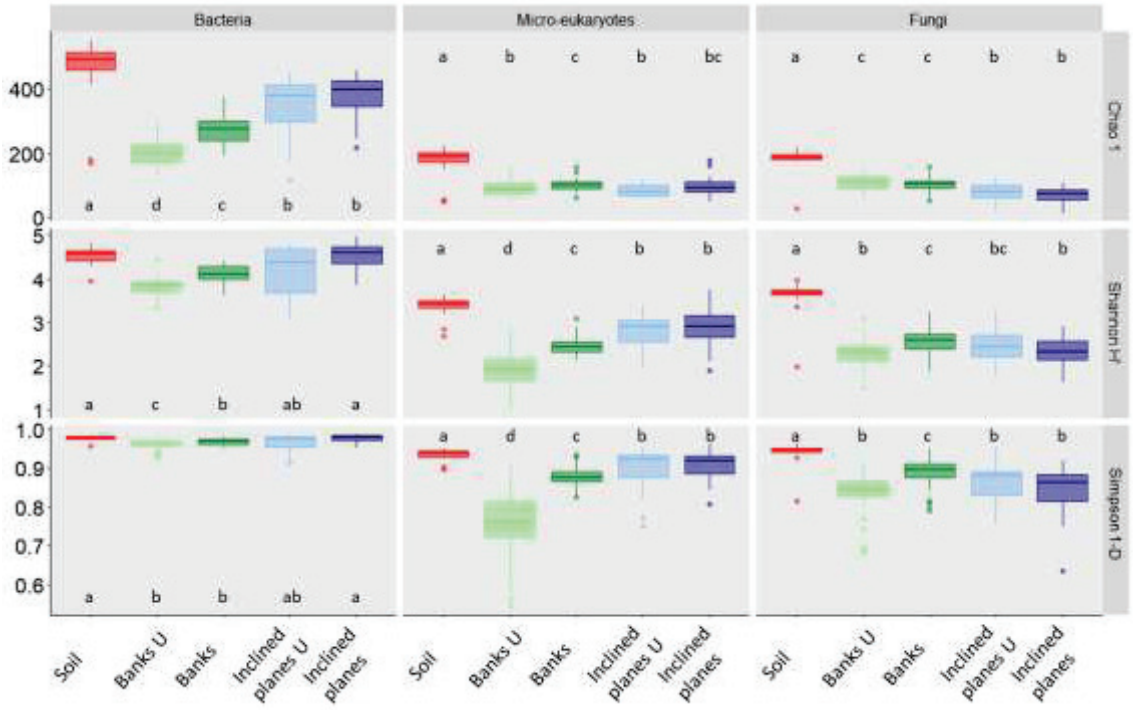


Figure 3. Biodiversity of microbial communities according to mineral substrate and stain presence, based on estimated richness (Chao 1 index), diversity (Shannon H' index) and evenness (Simpson index). For each index × community combination, differences according to mineral substrate and stain presence are shown with letters (Wilcoxon tests, P < 0.05). The same findings were obtained when using the observed number of taxa instead of Chao 1 index (not shown).

Differences in microbial community structures

To compare the structure of microbial communities, non-metric multidimensional (NMDS) analyses were conducted for bacterial (Fig. 4A), micro-eukaryotic (Fig. 4B) and fungal (Fig. 4C) OTUs retrieved from DNA. Time, mineral substrate and the presence or absence of stain were assessed as grouping parameters. For the bacterial community,

NMDS distinguished three groups of samples corresponding to the three mineral substrates (stress value = 0.09, ANOSIM test, R = 0.91, *P* = 0.0001), whereas the effects of time and stain presence were not significant (ANOSIM test, R = 0.19 and 0.03, *P* = 1 and 0.03, respectively). Similarly, for micro-eukaryotes and more specifically fungi, communities were significantly different between the three mineral substrates (stress value = 0.12, ANOSIM test, R = 0.70, *P* = 0.0001 and stress value = 0.16, ANOSIM test, R = 0.80, *P* = 0.0003, respectively) but some samples of the inclined planes and the banks appeared close to each other. Time and stain presence did not structure the micro-eukaryotic (ANOSIM test, R = 0.10 and 0.02, *P* = 1 and 0.1, respectively) and fungal communities (ANOSIM test, R = 0.1 and 0.07, *P* = 0.1 and 0.001 respectively).

Comparing community structures for bacterial, micro-eukaryotic and fungal OTUs, obtained from RNA analysis (performed at one date for banks and inclined planes samples) allowed to assess the effects of mineral substrate and stain presence for the transcriptionally-active microbial community (Fig. 5A,B,C). NMDS showed results similar to those observed for the entire communities, in that the effect of mineral substrate was significant but the effect of stain presence was not, regardless of whether bacteria (stress value = 0.18, ANOSIM test, R = 0.50, *P* = 0.0001), all micro-eukaryotes (stress value = 0.19, ANOSIM test, R = 0.12, *P* = 0.01) or only fungi (stress value = 0.19, ANOSIM test, R = 0.28, *P* = 0.0002) were considered. Nonetheless, the structure of active and entire communities showed different patterns for micro-eukaryotes on the inclined planes (ANOSIM test, R = 0.54, *P* = 0.001) and banks (R = 0.28, *P* = 0.006), and for fungi on the inclined planes (ANOSIM test, R = 0.23, *P* = 0.0078) but not on banks (ANOSIM test, R = 0.15, *P* = 0.08).

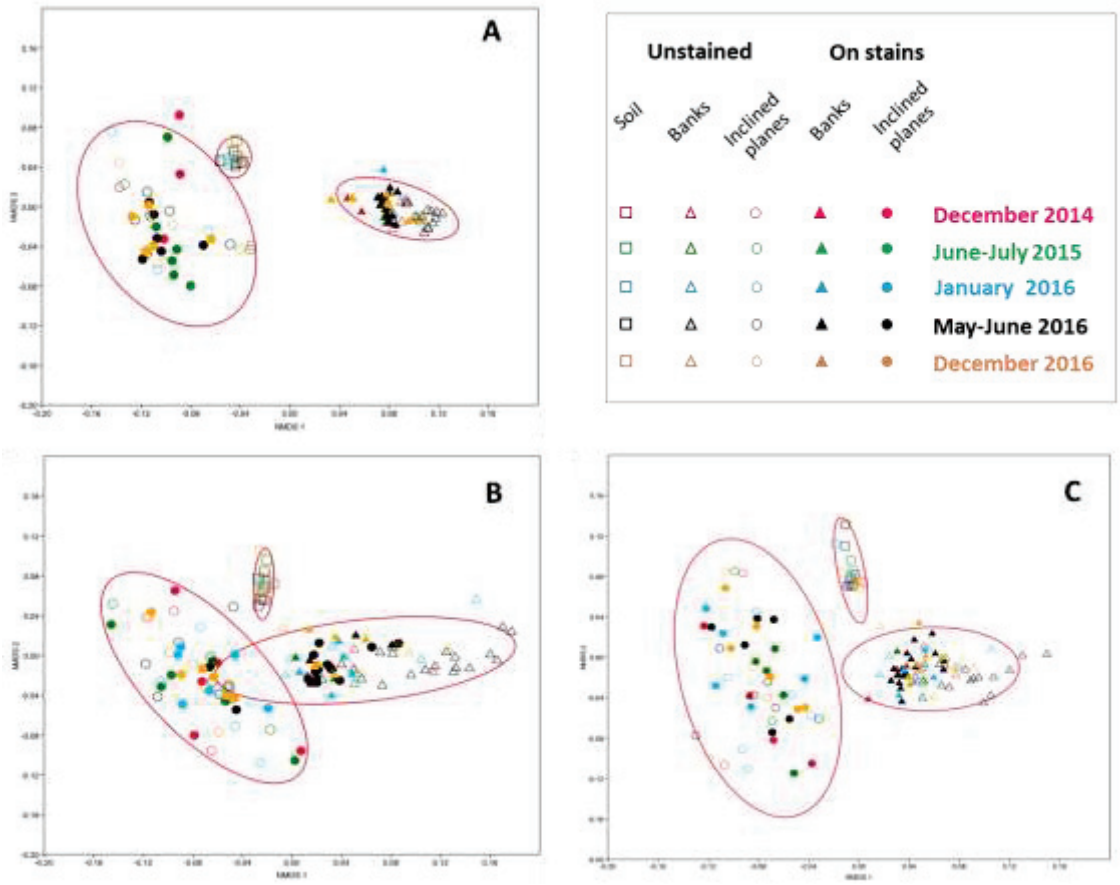


Figure 4. Non-metric multidimensional scaling (NMDS) analysis of microbial community structure in the Passage according to time, mineral substrate and stain presence. Results are shown for bacteria (A), micro-eukaryotes (B) and fungi (C), and ellipses (95% confidence intervals) indicate the different mineral substrates.

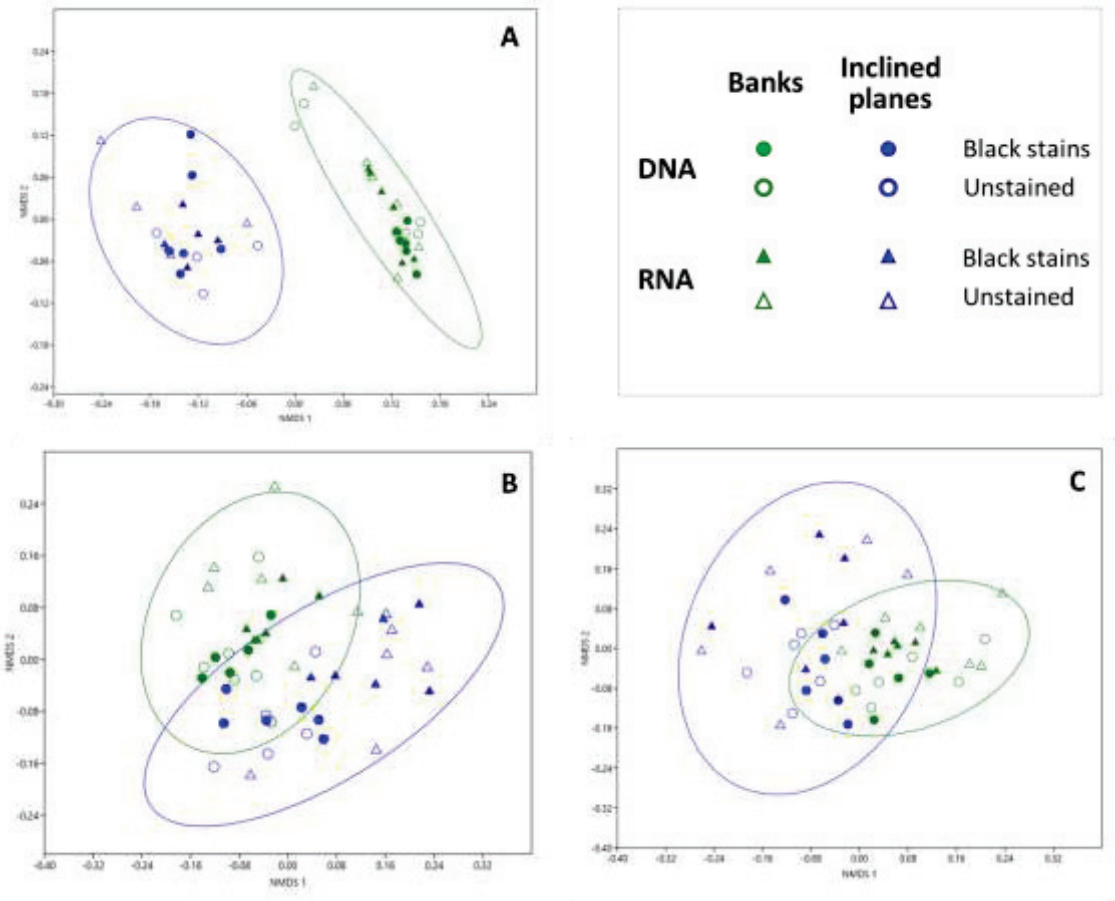


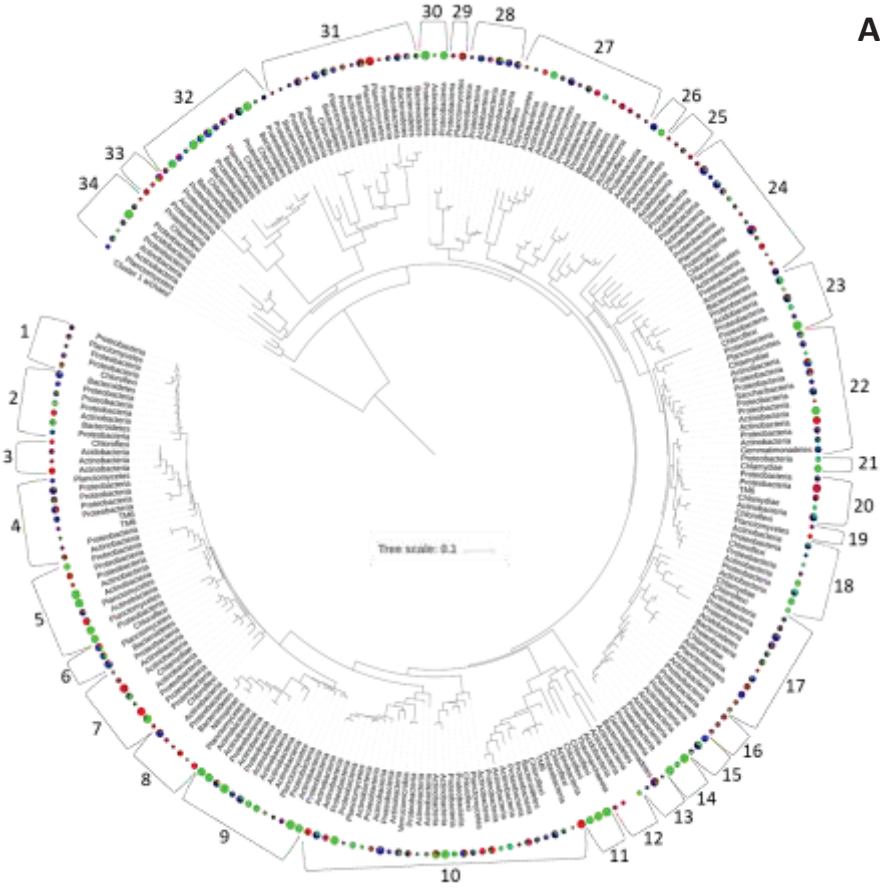
Figure 5. Non-metric multidimensional scaling (NMDS) analysis of the entire (DNA analysis) and transcriptionally-active (RNA analysis) microbial communities in the Passage according to mineral substrate (banks and inclined planes) and stain presence. Ellipses correspond to 95% confidence intervals for each mineral substrate.

Common OTUs and structuring characteristics of microbial community

Because mineral substrate was a parameter structuring microbial communities, we built the phylogeny of the OTUs common to the three mineral substrates to assess if these OTUs were selected by phylogeny or by environmental factors in each mineral substrate (Fig. 6). The number of common OTUs was 266 for bacteria, 113 for micro-eukaryotes (among them 88.4% were fungi) and 59 for fungi only (including black fungi *Acremonium*, *Ochroconis*, *Exophiala* and Herpotrichiellaceae). For each of the three microbial communities, the weighted UniFrac was significantly different in all mineral substrate comparisons (Table 3), indicating that the phylogenetic structures of the common bacteria, micro-eukaryotes and fungi was also related to mineral substrate. The occurrence of black stain was also a significant factor when considering these common OTUs (Table 3).

For bacteria, the phyla *Proteobacteria* (37.4%) and *Actinobacteria* (30.9%) were the most represented, followed with the three phyla *Bacteroidetes* (12.5%), *Chloroflexi* (7.0%) and *Planctomycetes* (4.9%). Micro-eukaryotes included mostly *Opisthokonta* (88.6%), a very large group that includes fungi. For fungi, the fungal classes *Sordariomycetes* and *Eurotiomycetes* were the most represented (55.5% and 28.8%,

respectively) in ITS data. One OTU corresponding to *Sordariomycetes* was abundant in the three mineral substrates. The net relatedness index (NRI) and the nearest taxa index (NTI) were calculated to quantify community phylogenetic structure and highlight the drivers of community assembly. Each index was calculated for groups of OTUs that were arbitrarily defined based on close phylogenetic relatedness (Fig. 6) and/or significant abundance in one particular mineral substrate. For bacteria, 33 of the 34 groups defined had positive indices, meaning that they tended to cluster on a phylogenetic rather than an ecological basis. For micro-eukaryotes, all 14 groups of OTUs had positive indices except the one with a high relative abundance in inclined planes samples, thus the inclined planes community tended to be overdispersed. In contrast, OTU groups of fungi with high relative abundance in inclined planes samples had high positive index values (Table S1). Thus in general, the bacterial, micro-eukaryotic and fungal OTUs common to the three mineral substrates were not phylogenetically driven by substrate types.



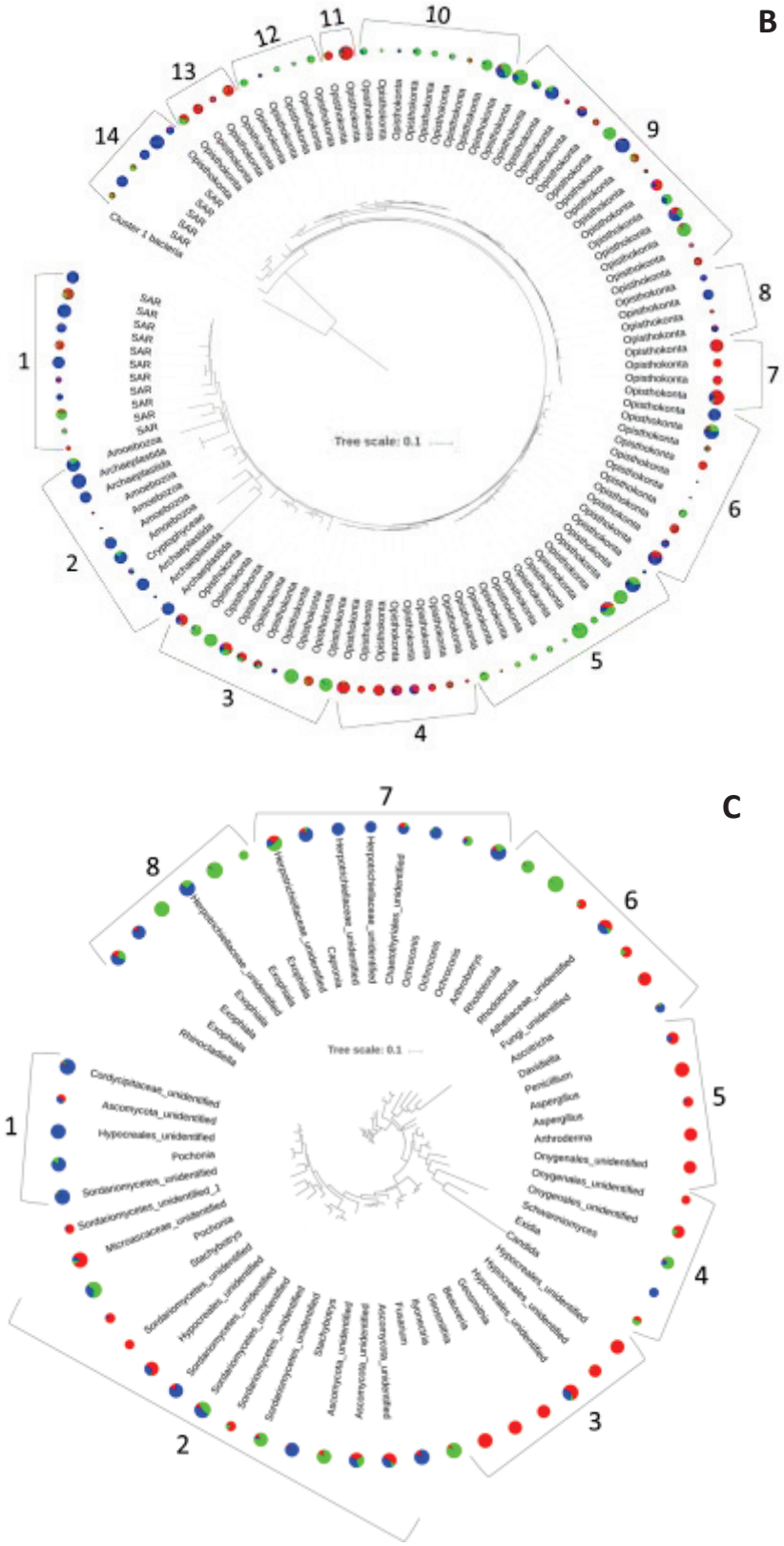


Figure 6. Phylogenetic tree of OTUs common to the three mineral substrates based on analysis of 16S rRNA genes (A), 18S rRNA genes (B) and fungal ITS (C). The size of pie charts represents the relative abundance of each OTU, and colors the distribution across mineral substrates (soil in red, banks in green and inclined planes in blue). Groups of OTU (i.e. groups 1-34 in A, 1-14 in B and 1-8 in C) used to compute NRI and NTI values listed in Table S1 are indicated on the outside.

Co-occurrence networks

The co-occurrence of bacterial and fungal OTUs found on all three mineral substrates was represented using network analyses. These networks identified microorganisms for which sequence abundances were significantly correlated with sampling time, mineral substrate or stain presence, which may reveal potential microbial interactions and specific consortia. Each node represented a microbial genus (bacterial in blue or fungal in green) connected by edges that were weighted by the significance of their association (positive in grey or negative in red). Regarding temporal changes, co-occurrence relations differed between the three sampling dates i.e. December 2014, June-July 2015 and December 2016 (Fig. 7). All co-occurrence relations were positive, regardless of the date. The fungal genera with highest numbers of connections in the network were *Lecanicillium* (Fig. 7A), *Exophiala* (Fig. 7B) and *Rhodotorula* (Fig.7C) for December 2014, June-July 2015 and December 2016, respectively. The date networks were different by their size, their composition in microorganisms and the main nodes that built them. The networks illustrated possible interactions in different mineral substrates, and they showed different patterns (Fig. 7). In soil, all co-occurrence relations were positive. A single fungus was present in that network, and the highest number of edges was displayed by unclassified *Acidimicrobiaceae* bacteria (Fig. 7D). In the banks, 62 taxa including 5 fungi made up the network, with 157 positive and 17 negative edges. The five main nodes corresponded to bacteria (Fig. 7E). The inclined planes network had 163 positive and 12 negative edges. Four bacterial genera (*Asanoa*, *Pseudonocardia*, unclassified *Micromonosporacea*, and unclassified *Phyllobacteriaceae)* presented the highest numbers of edges, while the network included only three fungi (*Acremonium*, *Pochonia* and unclassified *Ateliaceae*) that were noticeably negatively correlated with the network (Fig. 7F). Finally, we built networks on and outside stains. The network outside stains was composed of one group of bacteria and another, disconnected group, which contained only fungi, had the highest numbers of connections and displayed only positive co-occurrence (Fig. 7G). The network of co-occurrence on stains had only positive co-occurrence of bacterial genera (Fig. 7H). All described networks did not include the same actors, which pointed to the complexity of interactions between microorganisms according to seasons, mineral substrate or stain presence.

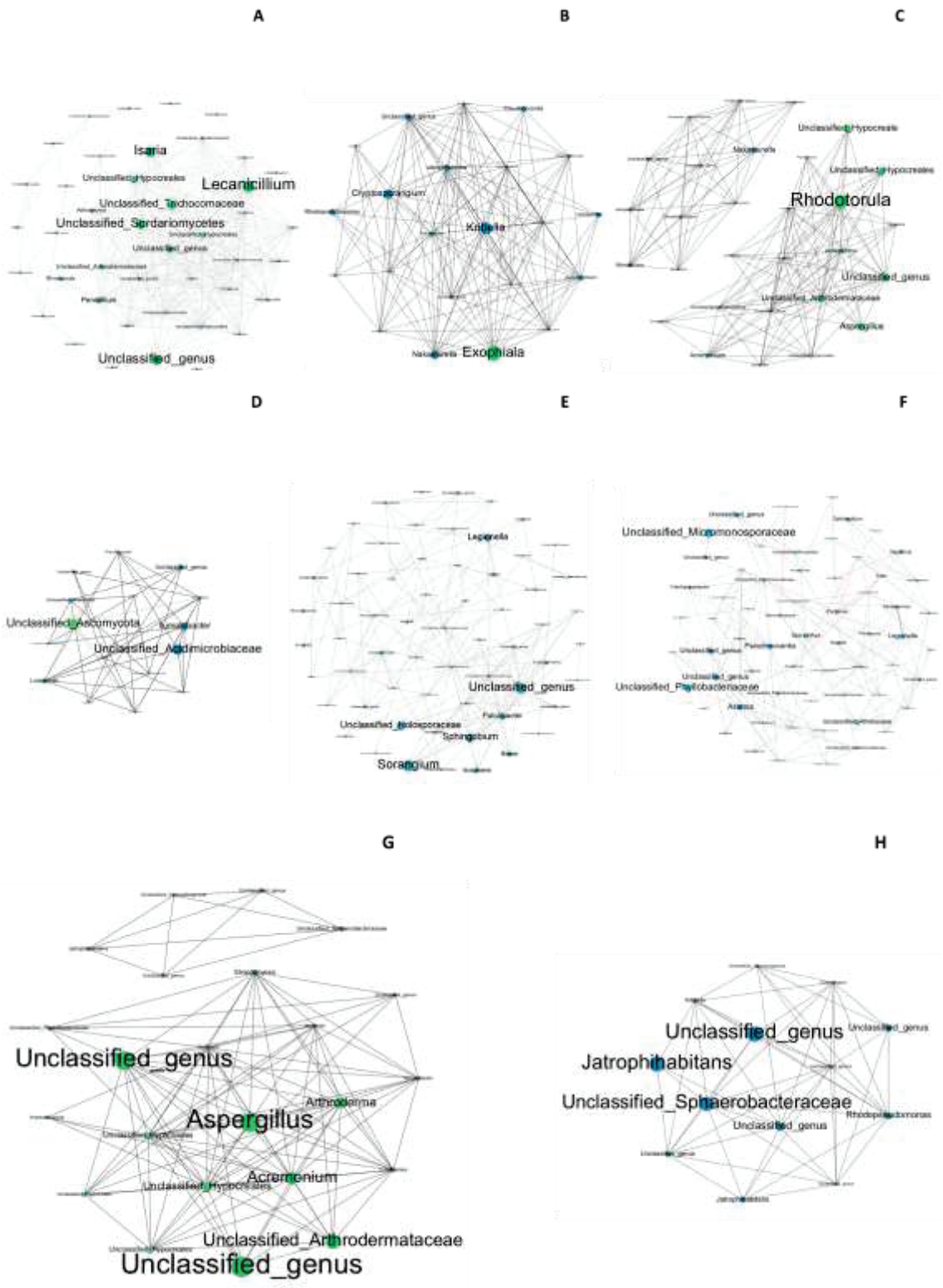


Figure 7. The co-occurrence networks of bacteria and fungi based on 16S rRNA and ITS MiSeq Illumina sequences. Connections materialize strong (Spearman's $|\rho| > 0.85$) and significant ($P < 0.001$) correlations. Co-occurrence networks are shown after combining all data for December 2014 (A), June-July 2015 (B), December 2016 (C), soil (D), banks (E), inclined planes (F), unstained parts (G) and black stains (H). Blue nodes depict bacterial taxa and green nodes fungal taxa. Links in gray indicate positive co-occurrence and links in red negative co-occurrence. The size of nodes is scaled to their Eigenvector centrality.

Discussion

This work focused on the Passage, the central area of Lascaux Cave connecting the entrance and Hall of the Bulls to the Apse, Nave and Chamber of the Felines located deeper in the cave, and where black stains started to form in the early 2000s (Bastian et al. 2009b). Black stains represent advanced cave wall alteration processes, linked to the development of black fungi that may resist to certain toxic organic chemicals through synthesis of melanin pigments (Martin-Sanchez et al. 2012) while being able to catabolize the others (Bastian et al. 2009). Black stains should therefore represent very specific ecological conditions resulting from both fungal development as well as biotransformation of toxic chemicals and melanin synthesis. Indeed, Chao1 (for micro-eukaryotes), Shannon H' (for bacteria, micro-eukaryotes and among them fungi) and Simpson indices (for micro-eukaryotes including for fungi) were statistically higher on black stains than unstained parts, for the banks (but not the inclined planes). Contrarily to expectations, however, the presence of black stains did not have a significant impact overall on microbial communities on the banks or inclined planes of the Passage. This was indicated by comparisons targeting the bacterial community, the fungal community and/or the micro-eukaryotic community at large, based on qPCR data of 16S and 18S rRNA genes, microbial richness and diversity indices, NMDS comparisons of microbial community structure (DNA analysis) and NMDS comparisons focusing of transcriptionally-active microbial communities (RNA analysis). In particular, emblematic black fungi isolated from Lascaux (Martin-Sanchez et al. 2012) and associated to melanin synthesis (De la Rosa et al. 2017) were also evidenced outside of black stains and at levels similar (or sometimes higher) to those within stains. This was for instance the case for *Ochroconis lascauxensis* on the banks (1.8% in stains and 3.7% outside stains) and inclined planes (11.2% in stains and 6.5% outside stains), *Herpotrichiellaceae* on the banks (8.1% in stains and 2.4% outside stains) and inclined planes (11.6% in stains and 10.5% outside stains) and *Exophiala moniliae* on the banks (17.9% in stains and 43.8% outside stains) and inclined planes (0.6% in stains vs 0.1% outside stains). This suggests that precedent chemical treatments in Lascaux Cave could have selected similar types of microorganisms on different mineral substrates of the Passage, independently of black stain formation. The current situation regarding black stain alterations is rather stable in the Passage, but one implication of these findings is that the establishment of pigmented fungi outside stains might enable future formation of new black stains provided fungal physiology is directed towards melanin synthesis pathways. Hence the need to avoid any further chemical treatments in the Passage.

This investigation relied on Passage monitoring over two years, and it showed that seasonal successions had little impact if any on cave microorganisms in the Passage. This is in accordance with the modest climatic variations (temperature and CO_2) that were recorded during all sampling campaigns (from December 2014 to December 2016) and the overall stability of cave wall surface quality in the Passage in recent years. Indeed, climatic conditions were relatively stable through time (12.66 ± standard

deviation 0.09 °C and 1.99 ± 1.00 % CO_2), even though temperatures in December 2014 and June-July 2015 were significantly lower (Wilcoxon test, $P < 2 \bullet 10^{-16}$) than at other dates (by 0.2°C) and CO_2 concentrations significantly higher (Wilcoxon test, $P < 2 \bullet 10^{-16}$) in January and December 2016 than in May-June 2016 (by 1%). Cave climatic conditions are often considered to be stable in time (Schabereiter-Gurtner et al. 2002; Northup & Lavoie 2001), and when fluctuations are monitored typically they are not connected with microbiology assessments (Forbes et al. 1998; Buecher et al. 1999; Houillon et al. 2017). Martin-Sanchez et al. (2012) had also taken cave samples at different dates, over 5 years, but without resampling at similar locations, which did not allow to characterize the effect of time.

The main effect evidenced in this work was the strong impact of mineral substrates on the microbial community structure in the Passage. These three mineral substrates differ in their physicochemical composition and history. In terms of chemical treatments, all three received biocides during the microbial crises (Bastian et al. 2009a). While the soil was covered with quicklime, banks and inclined planes were treated with benzalkonium chloride solutions and antibiotics (Bastian et al. 2010). Noticeably, black stains occurred only once (early 2000's) on the surface of the soil, but never came back after removal, most likely due to its composition, physical protection from benzalkonium chloride and/or microbial particularity. The soil was also the only mineral substrate in direct contact with visitors during touristic operations, thus it potentially received organic matter such as hair, external ground traces, etc. These various factors contributed to the differences between mineral substrates in the Passage and therefore to different microbial communities. Accordingly, microbial abundance and diversity on soil was higher than the two other mineral substrates. Similarly, Brewer et al. (2017) found that microbial community composition on tales from tombs were driven by rock type and particularly by differences in physical (porosity) or chemical (pH) characteristics. Despite the application of chemical treatments on all three mineral substrates, black pigmentation was spatially heterogeneous, resulting in patches (black stains).

As indicated by Unifrac comparison, mineral substrates strongly structured microbial community, as well as its phylogenetic structure. Nevertheless we observed no particular phylogenetic patterns associated to the mineral substrates, as determined by measuring NRI and NTI indexes that were in most cases highly clustered. This suggests a predominance of clustering process throughout all observed taxa, which might reveal general pressure of previous antimicrobial treatments. None of the measured environmental factors seem to drive microbial community assembly, but taking samples for physico-chemical analyses was not possible and only temperature and CO_2 concentration were available. However, most pairwise interactions (as assessed by co-occurrence networks) between bacteria and fungi in these communities were positive, independently of date, mineral substrate and stain presence. Although not causal, such positive interactions might be indicative of cooperative interactions, as oligotrophic environments imply that high-energy reactions need to be performed by

cooperative microorganisms to enable growth (Tomczyk-Żak & Zielenkiewicz 2016). The co-occurrence networks differed in time (Fig. 7A-C), which suggests that the participation of the microbial taxa to these networks might may be optional and form temporarily according to the needs of the time, e.g. depending on resource availability or effects of chemical residues. Fungi with black pigmentation potential displayed contrasted status in relation to these networks, and in any case they did not belong to the same co-occurrence networks. *Acremonium* was found in co-occurrence networks outside stains (Fig. 7G), where it was linked only with other fungi, but *Exophiala* found in June-July 2015' network was linked only with bacteria (Fig. 7B). In contrast, *Ochroconis* did not show in appear in any co-occurrence network, except when focusing outside stains on banks (Fig. S3A). This raises the possibility of contrasted ecological strategies for these fungi. Cooperation can be associated with metabolite conversion in certain cases (Frey-Klett et al. 2011) and this might be important for melanin synthesis in certain fungal taxa, but perhaps not in other taxa. Therefore, it could be that black stain formation might not require a particular consortium of microorganisms, or at least a single consortium. Rather, the advent of fungi with pigmentation potential only in co-occurrence networks outside stains when considering specific mineral substrates, i.e. *Acremonium* and *Ochroconis* on banks (Fig. S3A) and *Exophiala* on inclined planes (Fig. S3C), raises the possibility that microbial interactions with these fungi might be important to avoid melanin synthesis, and this deserves further research attention.

Conclusion

We found that mineral substrate was an important driver structuring the bacterial, micro-eukaryotic and fungal communities in the Passage of Lascaux Cave, more so than the presence of black stains did. This study highlights the potential for multiple interactions between bacteria and fungi, and shows that a global approach on microbial communities can help better understand the conditions of stain formation. A promising perspective will be to assess the transcriptomic activities of microbial community and determined the functional profiles and metabolic pathways implicated in black stain formation.

Acknowledgement

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Supplementary data

Table S1. NRI and NTI values for groups of OTUs of bacteria (groups 1-34), micro-eukaryotes (groups 1-14) and fungi (groups 1-8).

Bacteria	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
NRI	4.44	5.42	3.99	4.98	5.57	2.76	5.29	3.18	5.74	3.09	1.62	1.27	-0.43	3.36	3.20	3.41	5.39	5.08	2.47	4.45	2.54	6.88	3.55	4.17	3.80	1.88	2.30	4.98	2.52	2.20	2.53	5.69	3.30	1.48
NTI	3.11	3.49	2.99	3.33	3.42	2.35	3.15	2.99	5.00	4.17	1.25	1.00	-0.59	2.80	2.48	2.83	3.92	3.46	2.39	3.05	2.50	4.17	2.86	1.98	2.87	1.84	2.00	3.33	2.48	2.05	2.94	4.27	2.80	1.31

Micro-eukaryotes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NRI	0.82	-3.14	1.39	2.22	2.72	2.39	1.73	1.59	3.5	1.86	1.19	1.97	0.35	0.90
NTI	0.36	-2.96	1.61	2.09	2.19	1.87	1.68	1.63	2.47	2.06	1.22	1.92	0.55	0.69

Fungi	1	2	3	4	5	6	7	8
NRI	3.15	4.19	2.97	-1.76	2.99	0.85	2.45	3.43
NTI	2.81	2.67	3.33	-2.03	3.03	0.03	2.90	3.05

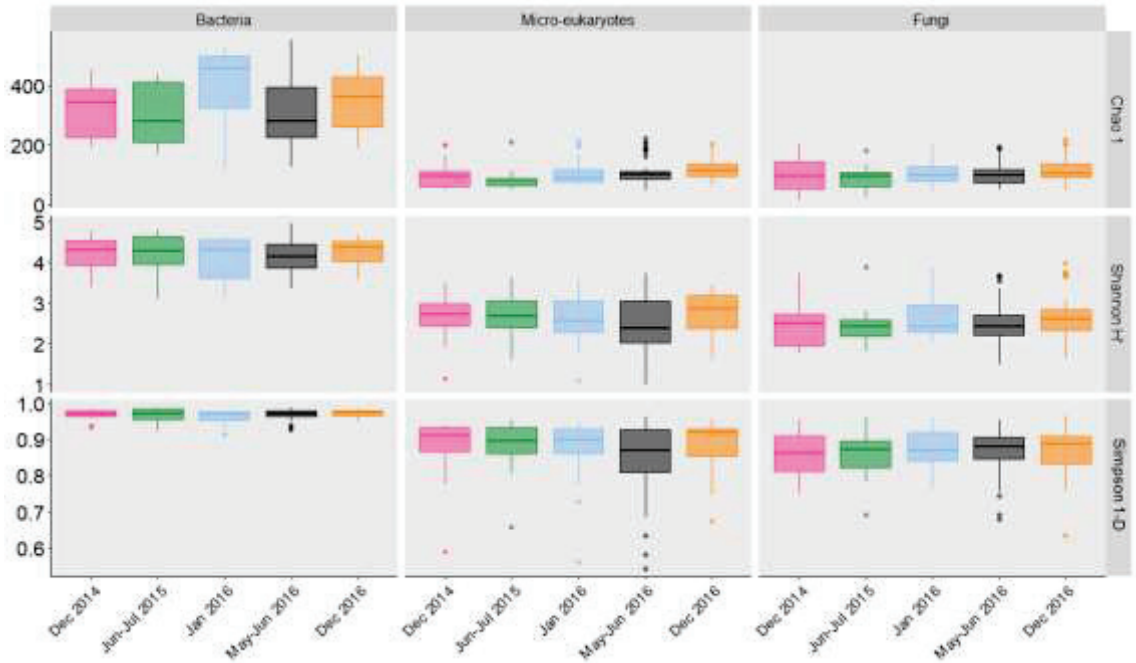

Figure S1. Biodiversity of microbial communities in the Passage according to sampling time. Biodiversity was considered using Chao1 richness index, Shannon H' index and Simpson evenness index. Variations were not significant for Chao1 (P = 0.15) and Shannon H' (P = 0.29) (ANOVA), and for Simpson 1-D (P > 0.05) (Wilcoxon test).

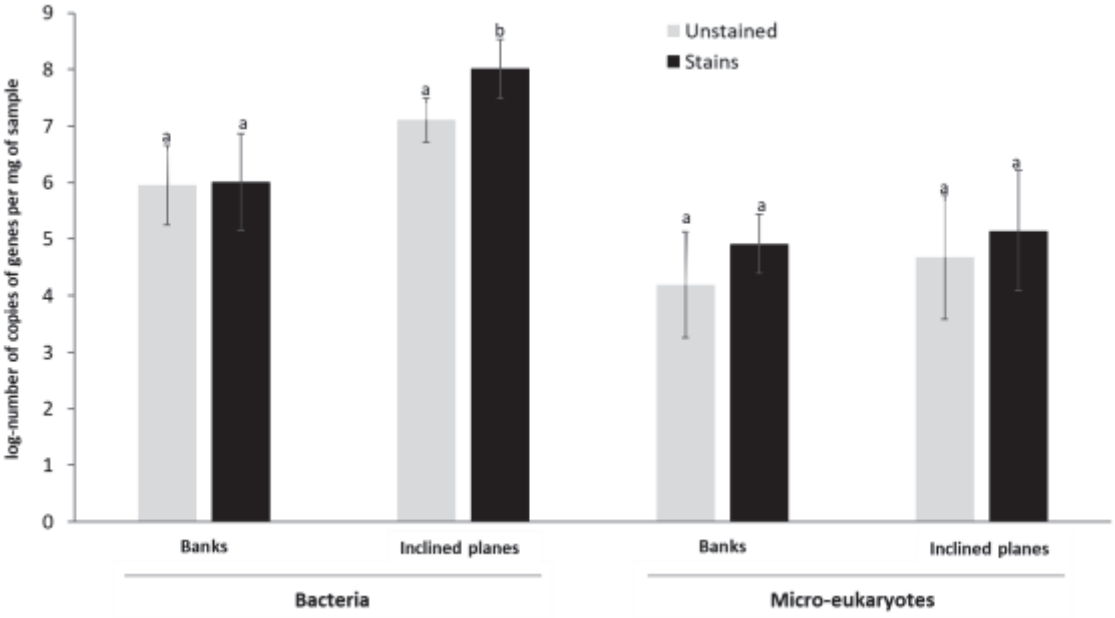

Figure S2. Bacterial and micro-eukaryotic abundances according to presence of black stains. Abundance data are shown as mean log number of 16S rRNA and 18S rRNA genes copies ± standard errors.

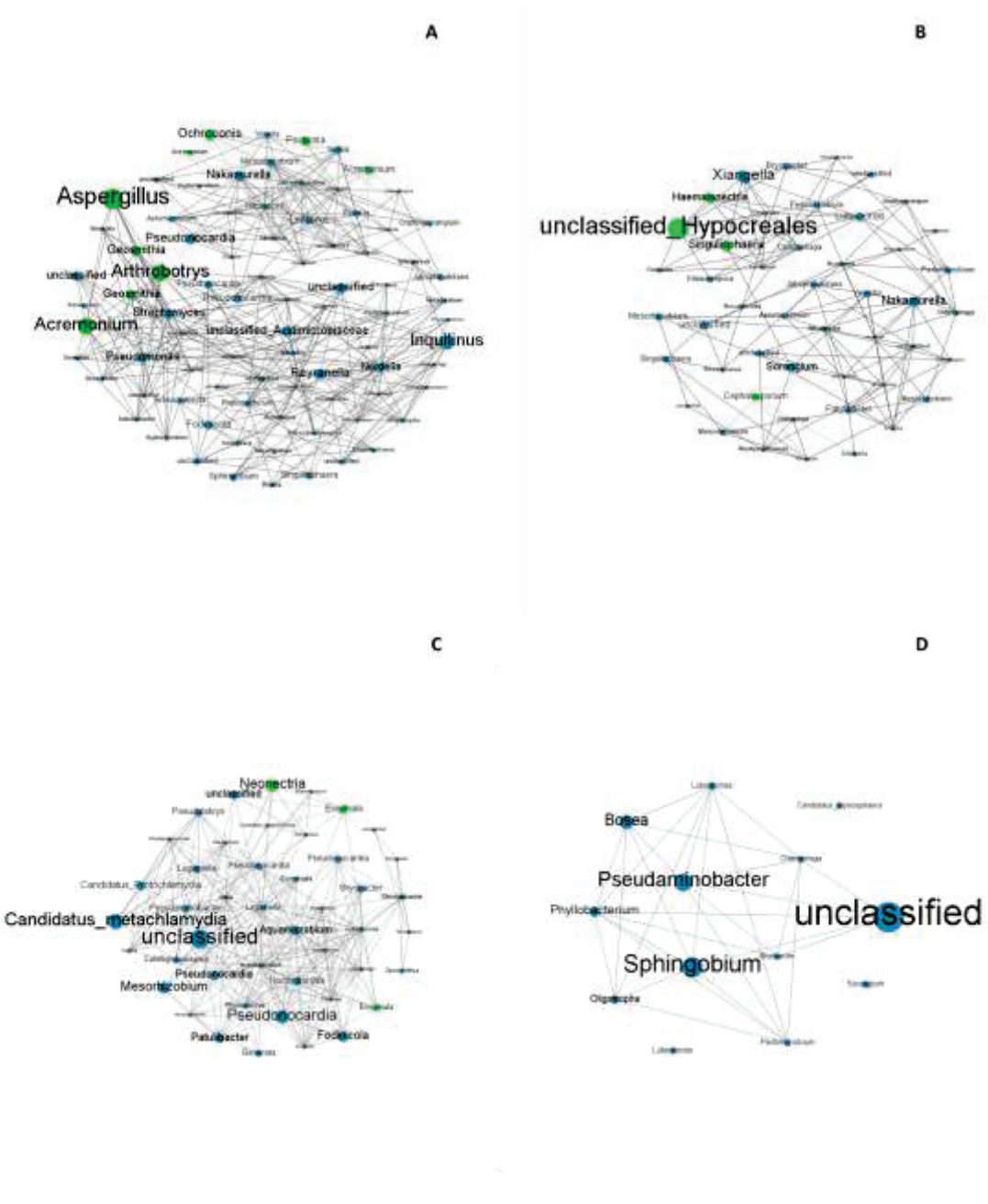

Figure S3. The co-occurrence networks of bacteria and fungi based on 16S rRNA and ITS MiSeq Illumina sequences. Connections materialize strong (Spearman's |ρ| > 0.6 for banks and Spearman's |ρ| > 0.75 for inclined planes) and significant ($P < 0.001$) correlations. Co-occurrence networks are shown after combining all data (December 2014, June-July 2015 and December 2016) for unstained parts (A) and black stains (B) of the banks, and for unstained parts (C) and black stains (D) of the inclined planes. Blue nodes depict bacterial taxa and green nodes fungal taxa. Links in gray indicate positive co-occurrence and links in red negative co-occurrence. The size of nodes is scaled to their Eigenvector centrality.

Chapitre 5 : Dynamique microbienne des altérations récentes de l'Abside

Avant-propos

Dans le chapitre précédent, nous nous sommes intéressés au Passage mais une autre salle de la grotte de Lascaux comporte de nombreuses altérations sur les parois, c'est l'Abside. L'Abside est située après le Passage, elle contient la majorité des gravures rupestres de la grotte. L'Abside comporte aussi des taches noires et depuis 2008 de nouvelles altérations nommées les zones sombres. Ces zones sombres sont visuellement très différentes des taches noires. L'Abside est aussi la seule salle dans laquelle, actuellement, on peut trouver visuellement des collemboles, qui sont localisés sur les taches noires mais aussi sur les zones sombres.

Les collemboles se nourrissent de matière organique morte et de microorganismes, ont une influence sur l'écologie microbienne des sols (Culik & Zeppelini, 2003) et ont un rôle dans la dispersion de champignons entomopathogènes (Dromph, 2001). Les résultats *in vitro* de la première étude sur l'Ecologie microbienne de la grotte de Lascaux (Alabouvette & Saiz-Jiménez, 2011) montrent que des collemboles (*Folsomia candida*) collectés dans la grotte Gombasecka en Slovaquie consomment des champignons isolés de la grotte de Lascaux (*Ochroconis lascauxensis* et *Fusarium solani*), comme l'indiquent (i) l'observation visuelle des boites de Petri contenant une culture des champignons *O. lascauxensis* ou *F. solani* déposée sur du sédiment de la grotte de Lascaux et confrontée à des collemboles, et (ii) l'analyse par spectroscopie de type *Surface-enhanced Raman scattering* (SERS) des taches et des fèces de collemboles issus des boites citées précédemment (Martin-Sanchez et al. 2012b). Ainsi, les collemboles pourraient avoir un rôle majeur dans l'écologie de ces microorganismes. Ici, nous avons mis en place une approche quantitative pour estimer le potentiel de consommation des champignons par les collemboles, et analyser la diversité des microorganismes associés à ces collemboles.

Ce chapitre est consacré à l'analyse de l'Abside. Les objectifs sont, d'une part, d'identifier la communauté microbienne présente sur les taches noires de l'Abside et caractériser l'interaction tripartite entre les champignons, les bactéries et les collemboles, et d'autre part, étudier la diversité et la taxonomie des communautés microbiennes présentes sur les taches noires, les zones sombres et les zones non tachées en utilisant le séquençage à haut débit. Les hypothèses sont que les collemboles se nourrissent des microorganismes présents sur les parois, qu'ils jouent un rôle dans la dissémination de ces microorganismes, et que la communauté microbienne des taches noires est différente de celle des zones sombres.

L'Abside a été échantillonnée en Juin 2015, Janvier 2016, Mai 2016 et Décembre 2016.

La procédure d'échantillonnage était la suivante pour chaque zone étudiée (taches noires, zones sombres) : les collemboles étaient échantillonnés à l'aide d'aspirateurs à bouche par les membres de l'UMR Ecologie microbienne ou du LEHNA, puis à l'aide d'un scalpel ou d'un écouvillon stérile la tache noire ou zone sombre associée était prélevée par la restauratrice Diane Henry-Lormelle, ainsi qu'une zone témoin adjacente.

La mise au point et l'extraction d'ADN des collemboles ont été réalisées par l'équipe Dynamique microbienne et transmission virale de l'UMR Ecologie microbienne. L'analyse des collemboles a été réalisée sur l'organisme entier (et typiquement plusieurs individus à la fois) et pas seulement sur le microbiote intestinal, ce qui ne permet pas de distinguer les microorganismes présents à la surface de l'animal de ceux qui sont consommés ou naturellement présents dans le système digestif, car les collemboles n'ont pas pu être stérilisés en surface à cause de leur petite taille. J'ai réalisé l'extraction d'ADN des échantillons de parois destinés à l'étude de la communauté microbienne. L'ensemble de ces échantillons a ensuite été séquencé à haut débit. Le séquençage des échantillons de collemboles a été effectué en ciblant les marqueurs taxonomiques bactériens (ARNr 16S) et fongiques (ITS2), et les échantillons de parois ont été séquencés en ciblant en plus les marqueurs taxonomiques des archées (ARNr 16S) et des micro-eucaryotes (ARNr 18S).

Afin de réaliser les tests *in vitro*, de consommation et de dissémination des microorganismes, des champignons noirs provenant de taches noires de la grotte de Lascaux ont été isolés par l'équipe Adaptation des microorganismes eucaryotes à leur environnement de l'UMR Ecologie microbienne et j'ai isolé avec l'aide de Flavien Maucourt (stagiaire M1 que j'ai encadré) les bactéries du genre *Pseudomonas* provenant des taches noires et de zones non tachées de l'Abside. Les *Pseudomonas* ont été ciblées car elles sont prédominantes sur les zones non tachées.

Les collemboles utilisés pour les expériences proviennent d'une lignée d'élevage, de la même espèce que ceux de la grotte de Lascaux, et ont été fournis par Thomas Tully (Sorbonne Université) car nous n'avons pas réussi à maintenir en élevage les collemboles prélevés dans la grotte de Lascaux. L'étude de la consommation des microorganismes par les collemboles a été réalisée par une analyse isotopique du carbone effectuée par Laurent Simon (LEHNA). Le test de dissémination des champignons noirs par les collemboles a été suivi par Flavien Maucourt et Jeanne Doré, les tests d'inhibition des champignons noirs par les *Pseudomonas* ont été effectués par Jeanne Doré, et les tests d'inhibition entre les différents isolats de *Pseudomonas* ont été effectués par David Chapulliot (UMR Ecologie microbienne).

Les résultats de la première étude **(Article 3)** concernant la caractérisation de la communauté microbienne des taches noires et l'interaction entre les collemboles et les microorganismes montrent que la communauté microbienne des taches noires et des zones non tachées est différente, le champignon *Ochroconis* et d'autres champignons noirs sont prévalents dans les taches noires alors que le genre *Pseudomonas* a une abondance relative beaucoup plus faible dans les taches noires que dans les zones non tachées. Les *Pseudomonas* présents sur les zones non tachées correspondent à différentes lignées de *Pseudomonas*, et certains peuvent inhiber *in vitro* la croissance des champignons noirs. Les collemboles présents sur les taches de la grotte de Lascaux ont été identifiés comme *Folsomia candida*, et l'analyse des isotopes du carbone a indiqué qu'ils pouvaient se nourrir de taches noires de la grotte ainsi qu'assimiler et disséminer les champignons noirs. Le séquençage Illumina a montré que le champignon noir *Ochroconis* était présent sur tous les

collemboles prélevés. Ce travail montre que le développement de champignons noirs est associé à la formation de taches noires dans l'Abside, et indique un rôle clé de *F. candida* dans la dissémination microbienne, la consommation probable des *Pseudomonas* antagonistes des zones non tachées, et le recyclage de la biomasse microbienne dans les taches noires.

Les résultats de la deuxième étude **(Article 4)** concernant la comparaison de la communauté microbienne des taches noires et des zones sombres montrent que les taches noires, les zones sombres et les zones non tachées adjacentes possèdent des communautés microbiennes distinctes. En ce qui concerne les taches noires, cependant, les champignons noirs tels que *Ochroconis* étaient plus fréquents et les bactéries *Pseudomonas* moins fréquentes dans les zones sombres que dans les zones non tachées. Le collembole *Folsomia candida* présent sur les taches noires a également été trouvé sur les zones sombres. En ce qui concerne les taches noires, le séquençage Illumina a mis en évidence la présence d'*Ochroconis* dans tous les échantillons de collemboles provenant des zones sombres. Cette étude démontre que les propriétés microbiennes des zones sombres ressemblent à celles des taches noires malgré différentes propriétés visuelles, et suggère un rôle similaire des collemboles dans l'altération microbienne des surfaces des parois.

Mon rôle dans ce travail a été le suivant : j'ai participé à la réflexion sur les objectifs scientifiques et la démarche expérimentale, à la préparation du matériel ainsi qu'aux différentes missions d'échantillonnage. J'ai participé à l'élevage des collemboles avec l'aide de Tran-Van Van et Flavien Maucourt (UMR Ecologie microbienne) ainsi qu'aux différentes expériences de consommation et inhibition. J'ai traité et analysé les séquences issues du séquençage Illumina des échantillons de parois. Ensuite, j'ai réalisé les analyses multivariées, l'arbre phylogénétique des *Pseudomonas* avec l'aide de Christophe Douady et j'ai participé à la rédaction des manuscrits.

L'ensemble de ce travail a permis la rédaction de deux publications « Microbial populations of Lascaux's Apse in relation to collembola and black stains on cave walls » et « Microbial populations of Lascaux's Apse in relation to collembola and black stains on cave walls ».

Article 3 : Microbial populations of Lascaux's Apse in relation to collembola and black stains on cave walls

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Abstract

Lascaux Cave is famous for its Paleolithic art, but has undergone wall surface alterations linked to the development of collembola and black pigmented fungi, in particular in the Apse room. Here, we assessed the microbial features of black stains formed in Lascaux's Apse room and collembola present on stains. Illumina sequencing showed that the microbial community of black stains and neighboring unstained parts differed. We found a higher prevalence of *Ochroconis* and other pigmented fungal taxa and a much lower level of the proteobacterial genus *Pseudomonas* in black stains than in unstained parts. The *Pseudomonas* colonizing unstained wall samples fell into different *Pseudomonas* lineages, and some could inhibit in vitro the growth of black fungi. The collembola thriving on black stains were identified as *Folsomia candida*, and carbon isotope analysis indicated that they could feed on black stain material in situ and assimilate the black fungi they were fed with in vitro. *F. candida* could disseminate black fungi in vitro when tested with black fungal isolates or black stain samples from Lascaux. Illumina sequencing showed that *Ochroconis* was retrieved from all collembola sampled on black stains. This work shows that the development of black pigmented fungi is associated to black stain formation in the Apse of Lascaux, and points to a key role of *F. candida* collembola in microbial dissemination, probable elimination of antagonistic pseudomonads from unstained parts, and recycling of microbial biomass in black stains.

Introduction

In regions with calcareous bedrocks, limestone dissolution by percolating water has led to the formation of karstic caves (Cuezva et al. 2012). In the past 180,000 years (Jaubert et al. 2016), hundreds of caves have been used by humans for shelter or to develop parietal art, including carving, drawing and painting of various motifs (Schabereiter-Gurtner et al. 2002; Dupont et al. 2007). During the last century, many caves with prehistoric art forms have been opened for touristic visits. However, touristic operations can change environmental conditions in caves, as a consequence of transformations (including light systems) meant to assist visits as well as of human physiology and behavior. The latter result in oxygen depletion, the discharge of carbon dioxide, body heat, water vapor and organic constituents, and the introduction of allochthonous microorganisms and arthropods (Bastian et al. 2009a; Jurado et al. 2010). These anthropic effects can stimulate the proliferation of certain microorganisms and lead to alteration of prehistoric art, and several of these caves had to be closed to the public, in particular the iconic UNESCO caves Altamira in Cantabria (Spain) and Lascaux in Dordogne (France).

Lascaux Cave, discovered in 1940, was operated as a tourism facility from 1948 to 1963. It received as many as 2000 visitors in peak days, giving more than one million visitors in about 15 years. As early as 1960, green stains (termed 'green disease') appeared on walls,

presumably as a consequence of light and CO_2 conditions on algal development (Bastian et al. 2010). These stains were treated with formaldehyde (to control algae) as well as streptomycin and penicillin antibiotics (to control bacteria). Posterior to cave closure, white stains involving the fungus *Fusarium solani* developed extensively in the cave in 2001, and they were treated with benzalkonium chloride and antibiotics (Dupont et al. 2007). From 2004 on, sporadic microbial outbreaks were cleaned mechanically, including black stains (termed 'black disease') that formed on walls in 2006 and against which chemical treatments were used again (Bastian et al. 2010). In parallel, the air extraction system was replaced in 2011 and phased out in 2015, while the system for condensation of water vapor was shut down in 2015. The management strategy implemented in Lascaux Cave in recent years consists in minimizing human intervention, in terms of chemical treatment, climatic control and human presence. Most black stains that had not been removed have been stable in the last years and some of them attenuated naturally, e.g. in the Chamber of Felines.

The Apse is a room of particular significance within Lascaux Cave, because (i) it presents a high density and quality of Paleolithic engravings, and (ii) it is the only room still displaying microbial development in recent years, and where collembola can be readily observed on cave walls (Fig. S1). Indeed, black stains reminiscent of those that had formed in other rooms of Lascaux and attributed to the growth of melanin-producing fungi (Martin-Sanchez et al. 2011) are present in lower parts the Apse walls (below the ornate part with the engravings).

While collembola with *Folsomia candida* morphology used to be seen in various stained parts of Lascaux Cave, currently they are mainly observed in the Apse especially on black stains. In previous work, collembola observed on black stains in Lascaux Cave or raised in vitro on plates where black fungi were grown displayed gut darkening, suggesting fungal consumption by the arthropods (Bastian et al. 2010). In addition, fungal conidia can be present in their feces (Bastian et al. 2010), raising the possibility that collembola could disseminate bacteria and fungi (Bastian et al. 2009b). However, their ability to feed on black fungi needs to be confirmed in quantitative terms, and whether a relation exists between collembola-associated microorganisms and black stain microbiota remains to be shown.

Therefore, the objective of this work was to characterize the microbial features of black stains and collembola in the Apse of Lascaux, and to identify potential roles of collembola as predators and disseminators of microorganisms in relation to the formation of these surface alterations.

Results

Fungal diversity in black stains

In Lascaux Cave, black stain formation is attributed to pigmented fungi (Bastian et al. 2010) capable of melanin synthesis (De la Rosa et al. 2017), and on this basis the fungal community colonizing cave walls of the Apse was investigated. NMDS showed that the structure of the fungal community differed in black stains and neighboring unstained parts (PERMANOVA

$F_{1,27}$ = 28.4, *P* = 0.001, R^2 = 0.31), as well as when comparing black stains on the left wall in June 2015 to (i) the same stains at other sampling dates (PERMANOVA $F_{2,27}$ = 14.5, *P* = 0.001, R^2 = 0.31), or (ii) black stains on the right wall at any of the samplings carried out (January 2016, June 2016 or December 2016), whereas unstained parts next to black stains did not differ on left vs right walls (Fig. 1A). Largely similar NMDS findings were obtained when considering the micro-eukaryotic community at large, i.e. (i) black stains differed from neighboring unstained parts (PERMANOVA $F_{1,30}$ = 36.8, *P* = 0.001, R^2 = 0.40), (ii) black stains (but not neighboring unstained parts) differed on left vs right walls (PERMANOVA $F_{1,30}$ = 3.8, *P* = 0.01, R^2 = 0.04), and (iii) black stains (but not neighboring unstained parts) from different sampling events could differ (PERMANOVA $F_{2,30}$ = 7.6, *P* = 0.001, R^2 = 0.16) (Fig. 1B).

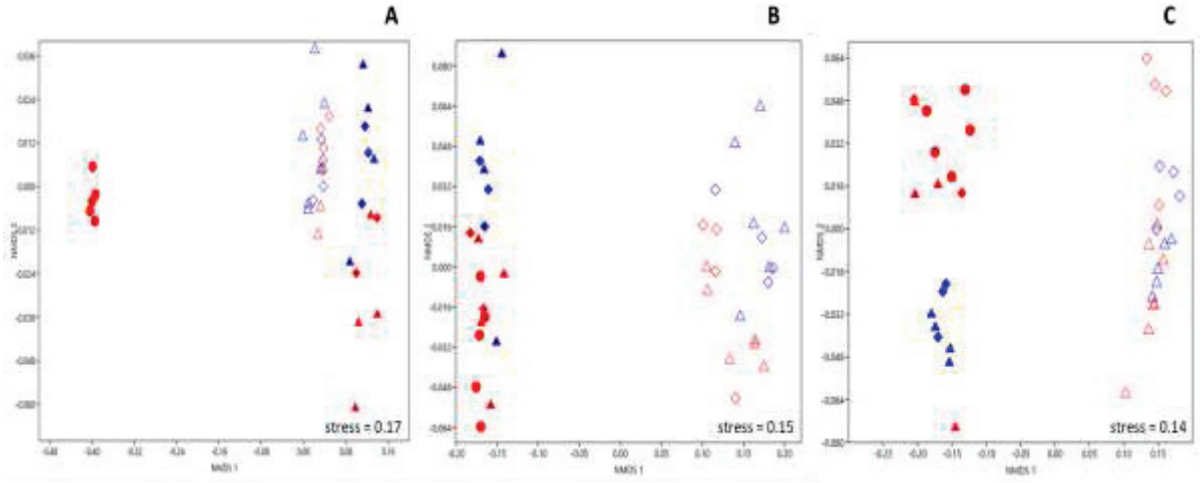

Figure 1. NMDS analysis of microbial communities present in black stains (full symbols) and nearby unstained parts (empty symbols) from the left (red) and right (blue) walls of Lascaux's Apse taken in June 2015 (circles), June 2016 (triangles) and December 2016 (diamonds), based on the relative proportion of both phyla and classes in the fungal (A), micro-eukaryotic (B) and bacterial communities (C).

Not all fungi produce black pigments, which prompted us to monitor more specifically the taxonomic composition of the fungal community. In unstained parts of the Apse sampled in the vicinity of black stains, results indicated that the genus taxonomic profile of fungi (but not the NMDS position which is based the totality of the sequences) differed when comparing left vs right wall samples (Chi-squared test, *P* < 0.0001), but comparatively it was less variable in time across the 1.5 years of the study (Fig. 2A). Against this background, the genus taxonomic profile of fungi differed strongly in black stains vs neighboring unstained parts (*P* = 0.0001), regardless of the Apse wall investigated (e.g. with fewer *Pseudogymnoascus* in black stains), but some differences were found as well when comparing black stain samples (i) from different sampling events and especially (ii) from left vs right wall samples. These findings were also made when considering the subsets of (i) pigmented fungal taxa (e.g. *Ochroconis* represented 7.1-29.6% of fungal sequences in left-wall black stains, 1.7-4.1% in right-wall black stains, vs only 0.7-1.4% in left- and right-wall unstained parts) and (ii) fungal taxa containing both pigmented and non-pigmented strains (as *Exophiala* and Herpotrichiellaceae represented a total of 31.6-67.5% of fungal sequences

in black stains vs only 0.07-0.87% in unstained parts) (Fig. S3). The genus taxonomic profile of all micro-eukaryotes pointed to the same results (Fig. S4).

The differences in black fungus prevalence might be accounted for if black stains had contained the same amounts of pigmented fungi but lower amounts of total fungi than unstained parts, but quantitative PCR data of 18S rRNA genes showed that black stains were equally populated than unstained parts (Fig. S5), meaning that the higher predominance of pigmented fungal taxa in black stains was not a bias linked to lower amounts of total microorganisms but entailed fungal proliferation instead. This higher predominance translated into statistically lower diversity levels of the fungal community in black stains than in unstained parts, as indicated by smaller number of taxa, Chao1 richness index, Simpson index and Shannon index (Fig. 3). In contrast, fungal diversity indices did not differ significantly when comparing sampling times or right and left walls (Fig. S6).

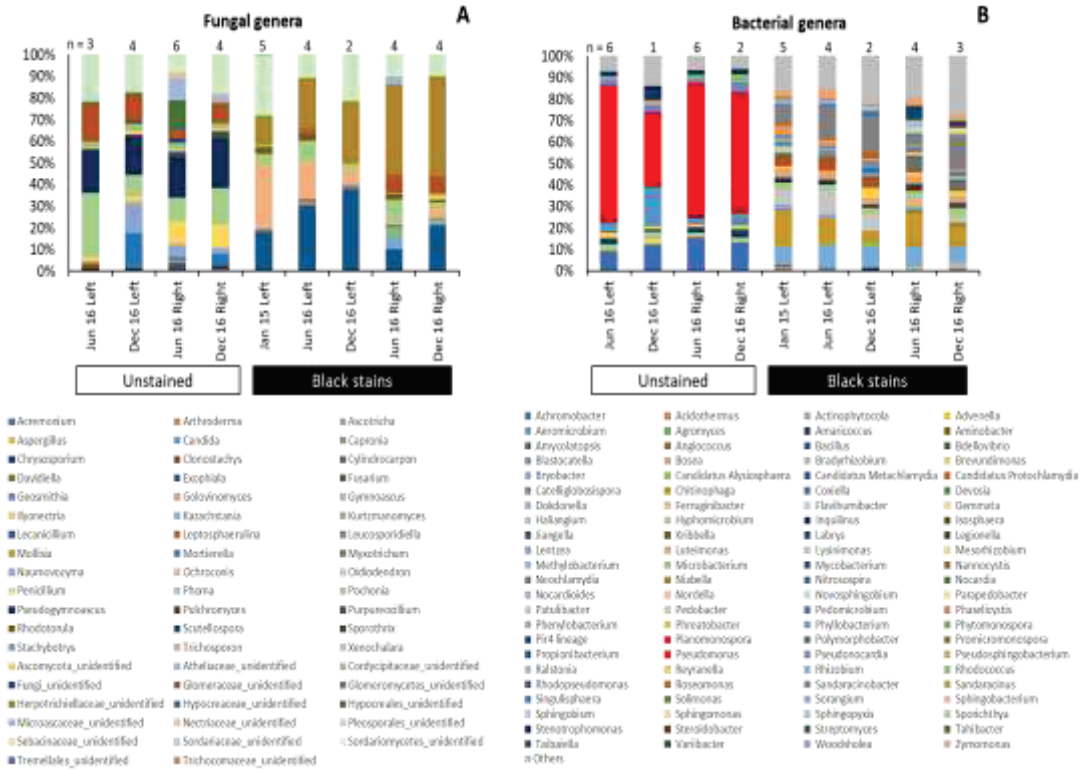

Figure 2. Community composition at genus level for fungi (A) and bacteria (B) in black stains and nearby unstained parts sampled from the left (L) and right (R) walls of Lascaux's Apse in June 2015 (15J), June 2016 (16M) and December 2016 (16D). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 1-6 samples (indicated in each case).

Bacterial diversity in black stains

Since fungal dynamics may be influenced by interactions with bacteria (Frey-Klett et al. 2011), the bacterial community was investigated as well. The bacterial community of Apse cave walls differed when comparing black stains to neighboring unstained parts (PERMANOVA $F_{1,29}$ = 53.9, P = 0.001, R^2 = 0.53) or left walls to right walls (PERMANOVA $F_{1,29}$ = 3.73, P = 0.018, R^2 = 0.04) for black stains (but not for unstained parts), whereas the effect of the sampling date was not significant (Fig. 1C).

In unstained parts of the Apse, the bacterial community was dominated by the proteobacterial genus *Pseudomonas*, which represented a large part of the sequences (i.e. 34-63%), regardless of the wall or sampling date (Fig. 2B). *Pseudomonas* aside, the rest of the genus taxonomic profile of bacteria was rather comparable in all unstained part samples. In contrast, the importance of *Pseudomonas* was much lower in black stains, as the genus accounted for only 0.01-0.11% of all bacterial sequences. In addition, the genus taxonomic profile of bacteria in black stains differed somewhat between cave walls, with e.g. a higher level of *Neochlamydia* in right walls than left walls (3.2-6.2% vs 0-1.2%), but otherwise differences between different black stains from a same wall or different sampling dates for a same black stain were minor i.e. not consistent or typically < 1.5%.

As for fungi, the findings made with bacteria did not coincide with biologically-lower bacterial numbers in black stains, as indicated by quantitative PCR data of 16S rRNA genes (Fig. S5). The much lower prevalence of *Pseudomonas* in black stains enabled proliferation of many other bacterial taxa, as indicated by higher Simpson and Shannon indices in comparison with unstained parts (Fig. 3).

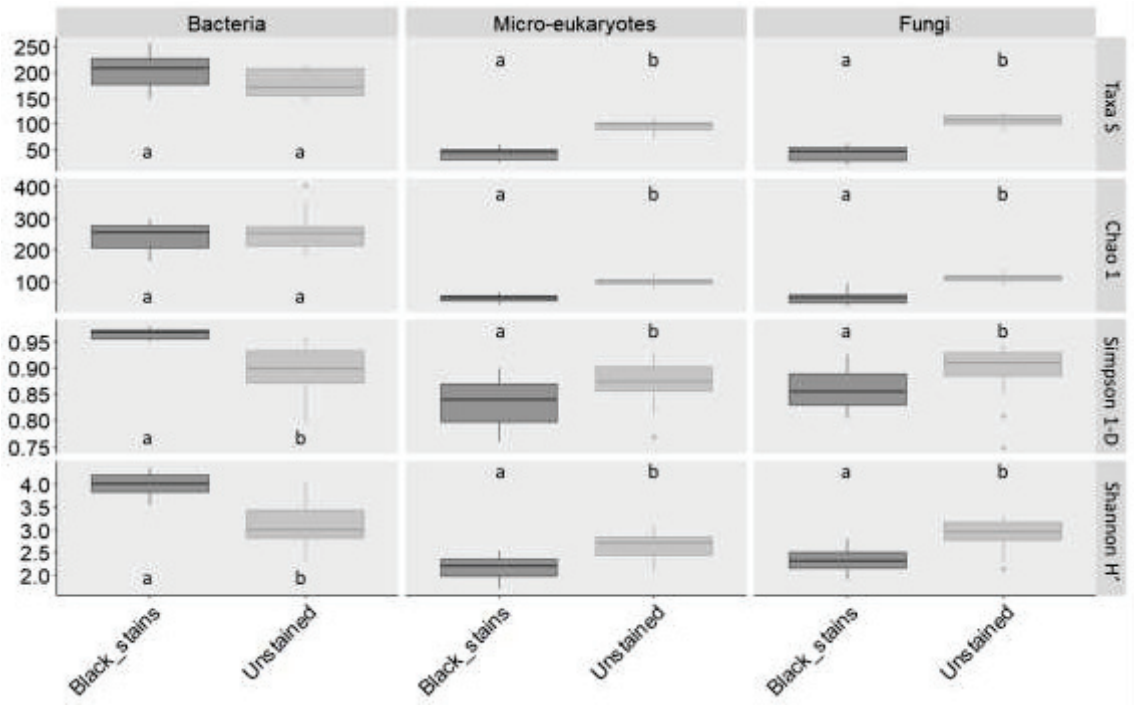

Figure 3. Number of taxa, Chao1 index of OTU richness, Simpson's index of diversity and Shannon index of OTU diversity for bacteria, micro-eukaryotes and fungi in black stains and nearby unstained parts sampled in Lascaux's Apse walls. Data were combined for the left and right walls sampled in June 2015, June 2016 and December 2016. Significant differences between conditions are shown with lowercase letters (based on ANOVA and Tukey's tests; $P < 0.05$).

Taxonomy and inhibitory potential of Pseudomonas strains

Considering the large differences in the prevalence of the *Pseudomonas* genus, isolation of these bacteria was attempted to investigate their potential interactions with black fungi. *Pseudomonas* isolates were readily obtained from unstained wall samples, but only one could be found from black stains. Taxonomic assessment of *Pseudomonas* 16S rRNA

sequences obtained from individual isolates (1440-1470 bp) or MiSeq data (465 bp) for the Apse showed that these pseudomonads belonged to 10 different *Pseudomonas* clades (Fig. 4). Some were related to *P. glareae*, *P. aeruginosa* and *P. anguilliseptica* (clade 1), *P. straminae* and *P. seleniipraecipitans* (clade 2), *P. alkylphenolica*, *P. vranovensis*, *P. donghuensis* and *P. wadenswilerensis* (clade 3), *P. extremaustralis*, *P. lurida* and *P. trivialis* (clade 4; including the only black stain isolate), *P. fluorescens* and *P. canadensis* (clade 5), *P. libanensis*, *P. synxantha*, *P. gessardii*, *P. lactis* and *P. cedrina* (clade 6), *P. yamanorum* (clade 8), or *P. sesami* and *P. saponiphila*, *P. protegens* (clade 9), while the others were not closely related to any described species (clades 7 and 10). Clade 7 to belongs to the '*P. gessardii*' subgroup outlined in Gomila et al. (2015) and clade 10 is close or belongs to the '*P. corrugata*' or '*P. chlororaphis*' subgroups, both clades being members of the larger '*P. fluorescens'* group as defined by Peix et al. (2017). Analysis of *Pseudomonas* MiSeq sequences indicated that (i) clades 6 and 8 were found only in Apse stains, clade 2 only in unstained parts of the Apse and clades 4, 5, 7 and 9 in both, and (ii) clades 4, 5, 6, 8 and 9 were also evidenced in collembola of Lascaux, clades 5, 6 and 10 elsewhere in Lascaux (Passage walls, Airlock-2 and Airlock-1, respectively), clades 1, 3, 8 and 10 in other Périgord caves (i.e. Mouflon, Tourtoirac for clade 1, Mouflon, Reille, Combarelles for clade 3, Allas, Mouflon, Reille, Combarelles, Tourtoirac for clade 8, and Allas, Mouflon, Reille, Combarelles, Tourtoirac, Pilier for clade 10).

Since the high prevalence of pseudomonads outside black stains coincided with a low prevalence of black fungi (Pearson correlation coefficient of -0.98 between sequence percentages of *Ochroconis lascauxensis* and *Pseudomonas* spp., $P = 0.025$), it raised the possibility that the former could limit the establishment of the latter in unstained parts of the Apse walls. Indeed, in dual confrontation experiments carried out on agar plates with 8 black fungi from Lascaux and 7 *Pseudomonas* isolates (all from clade 6) from unstained parts of the Apse, 1 pseudomonad inhibited a single black fungus only (i.e. *Ochroconis lascauxensis*) but the 6 others inhibited 3 to 5 black fungi each (among *Acremonium nepalense*, *Alternaria alternata, Doratomyces* sp., *Exophiala angulospora*, *Ochroconis lascauxensis* and/or *Minimelanolocus* sp.) (Fig. S7, Table 1). In comparison, the black stain *Pseudomonas* isolate (from clade 10) inhibited only *Doratomyces* sp., thus its inhibition potential was low. Moreover, all seven *Pseudomonas* isolates from unstained parts that were tested could inhibit the black stain *Pseudomonas* isolate in dual confrontation experiments on agar plates (data not shown).

Table 1. Effect of *Pseudomonas* isolates from the Apse on growth of Lascaux black fungi (All Ascomycota). Isolate La912c was obtained from a black stain (indicated by grey background) and the seven others from untained parts of Apse walls. The 'plus' indicate fungus inhibition by *Pseudomonas* and 'minus' a lack of inhibition. NC, test not conclusive due to bacterial swarming in presence of certain fungi.

Black fungi tested	Higher fungal taxonomy	La912c	La914b	La914c	La914e	La914g	La914h	La914i	La914j
Exophiala angulospora	Class Chaetothyriomycetes, Order Chaetothyriales, Family Herpotrichiellaceae	-	-	-	-	-	+	-	-
Exophiala castellanii	Class Chaetothyriomycetes, Order: Chaetothyriales, Family Herpotrichiellaceae	-	NC	-	-	-	-	-	-
Exophiala sp.	Class Chaetothyriomycetes, Order: Chaetothyriales, Family Herpotrichiellaceae	-	-	-	-	-	-	-	-
Ochroconis lascauxensis	Class Dothideomycetes, Order Venturiales, Family Sympoventuriaceae	-	-	-	+	+	+	+	+
Doratomyces sp.	Class Sordariomycetes, Order Microascales, Family Microascaceae	+	+	+	+	+	-	NC	+
Acremonium nepalense	Class Sordariomycetes, Order Hypocreales, Family Hypocreaceae	-	+	+	+	+	+	-	-
Minimelanolocus sp.	Class Eurotiomycetes, Order Chaetothyriales, Family Herpotrichiellaceae	-	+	+	+	NC	+	-	+
Alternaria alternata	Class Dothideomycetes, Order Pleosporales, Family Pleosporaceae	-	+	-	+	-	-	NC	+

E. angulospora was isolated from a black stain in the second compartment of Airlock-1 entrance zone, *E. castellanii* from a purple stain in the Apse, *Exophiala* sp. from a black stain in the second compartment of Airlock-1 entrance zone, *O. lascauxensis* from a black stain in the second compartment of Airlock-1 entrance zone, *Doratomyces* sp. from unstained part on wall in Passage, *A. nepalense* from a black stain in the second compartment of Airlock-1 entrance zone, *Minimelanolocus* sp. from a black stain in the Nave (Alonso et al. submitted d), and *A. alternata* from the air (LRMH isolate).

Figure 4. Phylogenetic tree of 16S rRNA genes from 82 *Pseudomonas* type strains, 8 Lascaux isolates and 39 *Pseudomonas* short Illumina sequences. *Cellvibrio japonicum* Ueda107 was used as outgroup. AGi stands for others caves including Allas, Mouflon, Reille, Combarelles, Tourtoirac and Pilier, NGSi for Illumina sequences, and Co_i for collembolan (I indicates sequence number). La912c is the *Pseudomonas* isolate from a black stain of the Apse and FB3_clone a sequence from Bastian et al. (2009c).

Organic carbon utilization by collembola in situ

A common feature of black stains is the occurrence of collembola resembling *Folsomia candida*, and indeed their predicted taxonomic status was confirmed by *cox1* sequencing of 11 collembola samples from black stains. Experiments carried out in vitro had suggested that *F. candida* was able to consume black fungi, based on gut darkening and regression of fungal colony (Bastian et al. 2010), but these findings had been obtained under laboratory conditions and evidence of assimilation of fungal constituents was lacking. Here, the $\delta^{13}C$ values measured in black stains of the Apse ($\delta^{13}C$ ranging from -28.0 to -23.8‰) and in *F. candida* collected from the same black stains ($\delta^{13}C$ ranging from -28.1 to -23.5‰) showed

that the carbon isotope signatures of stains and collembola were similar, strengthening the hypothesis that *F. candida* is actually feeding on these fungi (Fig. 5).

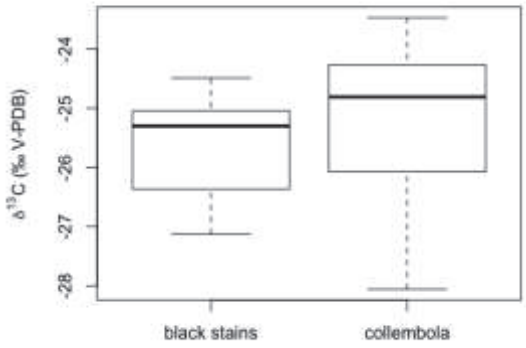

Figure 5. $\delta^{13}C$ values of 13 black stains from the Apse and 11 *Folsomia candida* samples (of at least 5 collembola individuals each) collected on black stains.

Experimental feeding of collembola on Lascaux black fungi, black stains and pseudomonads

Direct evidence for feeding of *F. candida* on black fungi was sought by monitoring of C assimilation in collembola, following artificial ^{13}C enrichment of the animals (using ^{13}C-labelled glucose) during rearing. Subsequent in vitro feeding experiment of collembola on non-labeled, laboratory-grown black fungi *Exophiala castelanii* ($\delta^{13}C$ = -24.6‰ ±1.2‰) isolated from a Lascaux purple stain, *Exophiala angulospora* ($\delta^{13}C$ = -23.1‰ ± 0.5‰) and *Ochroconis lascauxensis* ($\delta^{13}C$ = -21.9‰ ± 0.1‰) isolated from Lascaux black stains showed a significant decrease (Welch *t*-tests with Bonferroni correction for multiple comparisons, all $P < 0.001$) in the $\delta^{13}C$ values of *F. candida* from 54.2‰ ± 15.8‰ to 9.4‰ ± 15.7‰, 25.9‰ ± 19.5‰ and 19.8‰ ± 9.9‰ respectively (Fig. 6A), demonstrating that collembola had assimilated non-labelled carbon from the fungi. Gut darkening was also observed visually during the experiment (Fig. S1), as expected.

When the experiment was repeated with black stain samples taken from different rooms of Lascaux (including the Apse) ($\delta^{13}C$ = -25.5‰ ± 0.5‰), a similar and significant decrease in the $\delta^{13}C$ values of ^{13}C-enriched collembola was found (along with gut darkening) for stains from the Passage and the Nave ($P < 0.0001$ each) although it was not statistically significant for the stain from the Apse (Fig. 6B). Overall, these results provide direct indications on the ability of *F. candida* to feed on Lascaux black stains.

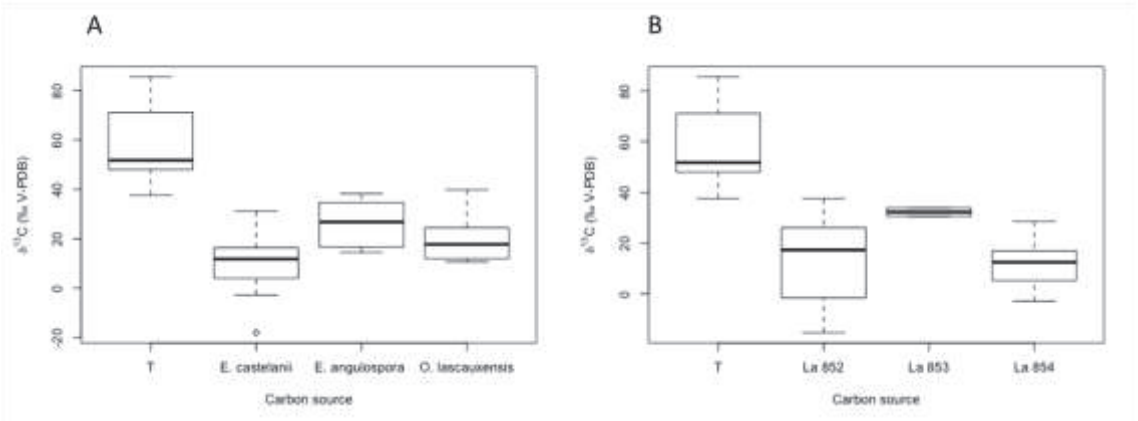

Figure 6. $\delta^{13}C$ values of collembola *Folsomia candida* line HA following ^{13}C enrichment (i.e., Control) and after subsequent feeding experiment with Lascaux black fungi *Exophiala castellanii*, *E. angulospora* and *O. lascauxensis* (A) or black stain samples La852 (from the banks of the Passage), La853 (from the far end of the Apse) and La854 (from the bottom of the wall of the Nave) (B).

Since fewer and distinct pseudomonads were found on black stains compared with adjacent unstained parts, an exploratory assessment of these pseudomonads as potential carbon source of *F. candida* was also carried out. The $\delta^{13}C$ value of ^{13}C-enriched collembola (37.8‰ ± 23.3‰) was only 16.5‰ ± 14.8‰ after exposure 3 d to the black stain pseudomonad La912c (itself at $\delta^{13}C$ = -23.7‰ ± 0.1‰) or the clade-9 pseudomonad La914h (12.2‰ ± 15.5‰), but values of similar magnitude were found with any of the five other *Pseudomonas* isolates originating from unstained walls (Fig. 7).

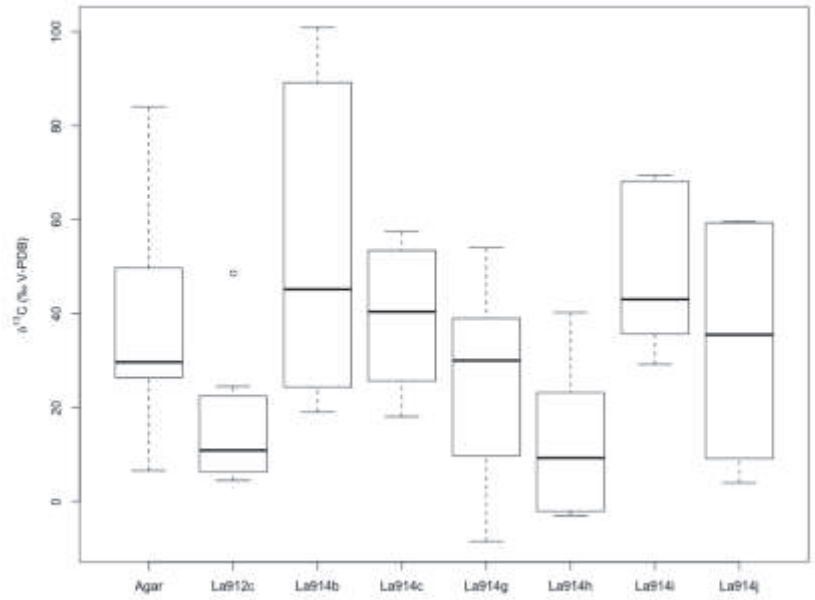

Figure 7. $\delta^{13}C$ values of for collembola *Folsomia candida* line HA following ^{13}C enrichment (i.e., Control) and after subsequent feeding experiment with Lascaux pseudomonads La912c (clade 10) isolated from black stain and La914b, La914c, La914g, La914h, La914i and La914j (clade 9) isolated from unstained parts of the Apse.

Experimental dissemination of Lascaux microorganisms by collembola

Previous work had suggested that collembola had the potential to disseminate fungi, based on presence of fungal conidia in collembola feces (Sabatini et al. 2004) and dissemination of faecal pellets of *F. candida* (Bastian et al. 2010). Here, this approach was taken one step further by feeding *F. candida* on laboratory-grown black fungi or black stain samples placed on plates (stage 1) and subsequently transferring them on fresh plates (stage 2). First, analysis of plates from both stages of the experiment showed that three of four test fungi could be disseminated on stage-1 plates (for *E. castellanii*) or both stage-1 and stage-2 plates (for *Ochroconis lascauxensis* and *Exophiala angulospora*). Additional culturable microorganisms presumably associated with collembola surfaces or guts were also disseminated, i.e. bacteria (not characterized taxonomically) and fungi (belonging to genera *Alternaria*, *Cladosporium*, *Engyodontium* or *Sistotrema*). Second, the experiment carried out using black stain samples also showed dissemination of bacteria (not characterized taxonomically) and fungi on plates, including the fungi *Alternaria chlamydosporigena*, *Exophiala* sp. and *E. castellanii*, *Ilyonectria* sp., *Mortierella* sp., *Ochroconis* sp. and *Pyrenochaeta acicola* on stage-1 plates, *Pochonia* sp. on stage-2 plates, and *Minimelanolocus* sp. on both. Six of these fungal taxa (*Ochroconis, Exophiala*, *E. castellanii, Mortierella*, *Minimelanolocus* sp*., Pochonia*) had been identified in MiSeq data from black stains of the Apse.

Microorganisms associated with Lascaux collembola

Since collembola were pinpointed by Bastian et al. (2009c) and/or in this work as potential disseminators of bacteria and fungi present in black stains, MiSeq sequencing was carried out to document the range of microorganisms associated to *F. candida* residing on black stains of the Apse. A total of 470 bacterial OTUs associated to collembola were thus identified, including the endosymbiont *Wolbachia* and *Pseudomonas*. Based on 16S rRNA gene MiSeq data, the *Pseudomonas* clades 4 (close to *P. lurida, P. trivialis* and *P. extremaustralis*), 5 (close to *P. fluorescens* and *P. canadensis*), 6 (close to *P. cedrina, P. lactis, P. gessardii, P. synthaxa* and *P. libanensis*), 8 (close to *P. yamanorum*), 9 (close to *P. sesami, P. saponiphila and P. protegens*) and 7 (close to the '*P. gessardii*' subgroup) were found in collembola from the Apse. In comparison with wall samples taken underneath, 369 of 382 bacterial OTUs found in black stains (i.e. 97%) were also evidenced in collembola sampled on these black stains (Fig. 8A).

In addition, 148 fungal OTUs were evidenced, including *Acremonium nepalense*, *Exophiala castellanii*, *Ochroconis lascauxensis* (all with black pigmentation potential), *Alternaria eichhorniae* and *metachromatica* (of uncertain pigmentation potential). Several types of fungi transported by collembola in the dissemination experiment were also evidenced in 16S rRNA gene MiSeq data for Apse collembola, i.e. the two test fungi *Exophiala castellanii* and *Ochroconis lascauxensis*, as well as the genera *Alternaria*, *Engyodontium*, *Exophiala*, *Ilyonectria*, *Ochroconis* and *Pochonia* directly associated with collembola and/or observed on plates when black stain samples were studied for

dissemination. More generally, in the comparison with wall samples taken underneath, 21 of 24 fungal OTUs found in black stains (i.e. 88%) were also evidenced in collembola sampled on these black stains (Fig. 8B).

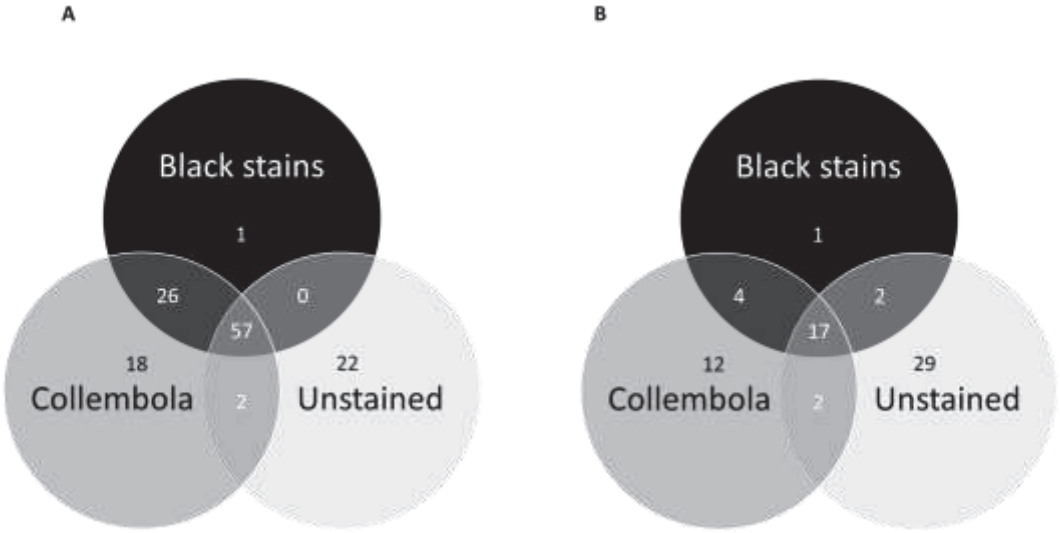

Figure 8. Venn Diagram showing unique and shared bacterial genera (A) and fungal genera (B) in collembola from black stains, black stains and unstained parts.

Discussion

This work established that the microbial community differed strongly between Lascaux's black stains and neighboring unstained parts. Microbial diversity of black stains has been investigated before, but this had been done (i) prior to the development of new-generation sequencing technology (which now enables large-scale analysis) and (ii) using a very limited number of samples (without direct comparison of stains and neighboring unstained parts) (Bastian et al. 2009a, 2009b; Martin-Sanchez et al. 2012). The occurrence of black fungi in black stains formed on Lascaux limestone rocks is well documented (Saiz-Jimenez et al. 2012), including in the Apse (Martin-Sanchez et al. 2012), and was quantified by real-time PCR for *O. Lascauxensis* (Martin-Sanchez et al. 2012). Here, we found a higher prevalence of *Ochroconis* (and other pigmented fungal taxa) in black stains than unstained parts, which provides evidence that the development of black pigmented fungi is associated to black stain formation in the Apse of Lascaux. Bacterial diversity was also important to document, because certain bacteria were found to provide the fungus *Cryptococcus neoformans* with the melanin precursor dopamine, thereby promoting melanization (Frases et al. 2006), and indirect bacterial effects on stain formation have been neglected so far.

White collembola (presumably *F. candida*) occurring on Lascaux's black stains have been reported for years (Bastian 2010) and 18S rRNA sequences pointing to *Folsomia* had been obtained by cloning-sequencing from a black stain in the Painted Gallery (Bastian et al. 2009a). They are thought to feed on black stains, based on high numbers present and gut darkening (Bastian 2010). Accordingly, these collembola were confirmed as *F. candida* by *cox1* sequencing, and isotopic analysis demonstrated that, under controlled conditions, they can assimilate black fungus biomass as well as organic C from Lascaux's black stains, pointing

to an important role played by these collembola in the recycling of microbial biomass in black stains. Feeding habits of *F. candida* are complex, but indeed they enjoy black fungi (Scheu & Simmerling 2004; Böllmann et al. 2010) over the others, whereas they can be repelled by certain fungi (Böllmann et al. 2010). Accordingly, exposure to *Aspergillus nidulans* fungi with the ability to produce secondary metabolites resulted in stress-related transcriptional responses in these collembola (Janssens et al. 2010). *F. candida* can also feed on bacteria, and bacteria could be seen in the gut and fecal pellets by scanning electron microscopy (Thimm et al. 1998).

F. candida also has the potential to disseminate the black fungi ingested, as indicated by the production of black fecal pellets (Sawahata 2006; Bastian 2010) and the possibility of fungal growth out of these pellets (Sabatini et al. 2004; Bastian 2010). In addition, cave *F. candida* displays fungal conidia in the gut based on histological imaging (Smrž et al. 2015) and many dominant fungal phylotypes found in stains corresponded to taxa typically associated with arthropods (Bastian et al. 2009a), whereas Fluorescence In Situ Hybridization (FISH) enabled detection of distinct bacteria in *F. candida* gut (Thimm & Tebbe 2003) and high bacterial diversity in these animals was documented by sequencing of 16S rRNA SSCP bands (Czarnetzki & Tebbe 2004). However, experimental demonstration of microbial dissemination potential from black stains was lacking, and in this work a range of taxa (including black fungi) present in Lascaux's black stains were indeed spread away when *F. candida* individuals were present. Furthermore, we established that many microbial taxa thriving in black stains were also found associated to collembola collected from the same black stains. These microorganisms might have been present within the animals, and indeed *Wolbachia* endosymbionts were evidenced. More likely, however, a number of these microbial taxa were present on animal surfaces such as legs and buccal parts (as pointed by Dromph, 2001), based on (i) the recovery of taxa usually not associated with arthropod guts e.g. *Sandaracinobacter, Bryobacter, Dokdonella* and *Davidiella*, and (ii) the high number of microbial OTUs common to collembola samples and black stains underneath. Collembola might thus represent a microbial reservoir, with the potential of starting new microbial pioneer colonies as they wander on cave walls, contaminating unstained surfaces with each footstep and depositing fecal pellets containing microorganisms, since geographic dissemination of fecal pellets can be extensive (Bastian 2010).

The *Pseudomonas* genus has been evidenced in Lascaux before (Bastian et al. 2009a, 2009b), but bacteria of the Apse had not been investigated. Here, we found a high diversity of *Pseudomonas* taxa on unstained walls of the Apse, and the isolates obtained displayed inhibition potential against black fungi. This might be a key phenomenon explaining why black stains do not develop on a large scale within Lascaux's Apse. However, new black stains have formed in the last ten years, indicating that foundation effects resulting from collembola-mediated microbial dissemination can be successful at times. The elimination of antagonistic pseudomonads taking place once black stains are established probably results from maladaptation to the new abiotic conditions prevailing in the stain, as (i) collembola did not feed significantly on most (5 of 6) antagonistic pseudomonads from unstained parts and

(ii) black fungi did not interfere with growth of these pseudomonads when tested *in vitro* (Fig. S7). In black stains, other types of pseudomonads were found, with (for the sole isolate available) (i) the ability to grow in presence of black fungi but also (ii) the potential to be grazed by *F. candida* and so to undergo significant mortality during gut passage (Thimm et al. 1998), resulting in very low prevalence in stains.

In conclusion, this study revealed the drastic ecological changes resulting from the collembola-fungus partnership on the walls of Lascaux's Apse. The establishment of *F. candida* collembola is favored by grazing on pigmented fungi and black stain constituents, while the collembola promote (i) dissemination and development of black pigmented fungi and more generally of a stain-specific fungal community, and (ii) counter-selection of the diversified fungal-antagonistic *Pseudomonas* guild otherwise prevalent on unstained walls. The success of this collembola-fungus cooperation is thus essential for the extension of existing black stains and the formation of new ones in the Apse of Lascaux. It will need to be targeted if ecological strategies are to be designed for conservation of cave wall quality in the Apse, which displays thousands of entangled engravings and represents one of the key paleolithic heritages in Europe.

Materials and methods

Sampling

Lascaux Cave is located near Montignac in Périgord in South-West France. The cave has been closed for touristic visits since 1963. Human presence is now highly restricted and restrained to scientific campaigns and official visits. The Apse in Lascaux Cave was selected for sampling due to the presence of black stains and collembola on different walls of the room. Five sampling campaigns were performed in June 2015, June 2016, December 2016 and (for additional collembola) May 2017, using several areas selected on the left and right walls of the room. Due to Lascaux management rules related to cave ecosystem vulnerability, sampling was distributed over several days to limit the duration of human presence on a given day.

Collembola and black stains underneath were sampled for metabarcoding characterization of their associated microorganisms, isolation of selected bacteria (from wall samples), and isotopic analysis. To this end, collembola present on black stains were sucked (17 black stain samples, making respectively 710 individuals in total) using sterile insect mouth aspirators (Rose Entomology, Benson, AZ). Wall samples were collected i.e. black stains (using scalpels when possible, otherwise sterile swabs ; ~ 50 mg material) and equivalent unstained control areas at about 10 cm of each black stain (using sterile swabs). At a given each sampling date, metabarcoding was carried out using 3-6 samples per wall surface condition. *Pseudomonas* isolation was performed in February 2017, using 5 rock wall samples (2 black stains and 3 unstained areas).

Samples of collembola and rock walls (black stains and controls) for sequencing were placed immediately into liquid nitrogen and later transferred at -80°C until DNA extraction, whereas samples for microbial isolation or isotopic analysis were kept à 4°C.

Extraction of DNA, collembola identification and Illumina sequencing

All DNA extractions from collembola were performed in a sterile environment under a laminar hood to avoid contamination. Pools of collembola (from 1 to 42 individuals per pool = per black stain) were crushed using 1-mm diameter beads in ATL lysis buffer (Qiagen, Hilden, Germany) containing 20 $mg.ml^{-1}$ lysozyme (Euromedex, Strasbourg, France) and homogenized for 10 s in a Mini-beadbeaterTM (BioSpec Products, Bartlesville, OK). After 2 h at 37°C, 20 µl of proteinase K (Qiagen, 20 $mg.ml^{-1}$) was added and samples incubated 4 h under constant agitation (300 rpm) at 56°C to lyse arthropod tissues completely. DNA was then extracted with Qiagen DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's recommendations for both Gram-negative and Gram-positive bacteria. Elution was performed using 12 µl, final concentration was measured using Nanodrop Safas UV-mc^2 (SAFAS, Monaco), and DNA stored at -20°C.

Extraction of DNA from cave wall samples was carried out using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), following the manufacturer's instructions. Elution was performed using two 50-µl volumes for each sample, and final DNA concentration was measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) according to manufacturer's instructions. DNA extracts were stored at -20°C.

For taxonomic identification of collembola (carried out on 11 samples), a 708-bp fragment flanking the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene was amplified by PCR using 50 ng of DNA matrix and primers LCO1490 33 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). Amplification was performed in 25-µl volumes containing 1× Q5 buffer (New England Biolabs, Ipswich, MA), 0.2 µM of each primer (Invitrogen), 40 µM of dNTP (Applied Biosystems, Waltham, MA), 0.2 $mg.ml^{-1}$ of Bovine Serum Albumin (New England Biolabs) and 0.35 U of Q5 DNA polymerase (New England Biolabs). PCR was performed at 94°C for 5 min, followed by 40 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 50 s and 72°C for 10 min. PCR products were sequenced using Sanger methodology (Biofidal company, Lyon, France) and sequences blasted against NCBI nr-database (Johnson et al. 2008). These sequences were deposited in Genbank with accession numbers XXX.

For Illumina MiSeq sequencing of each cave wall and collembola DNA extract, PCR was carried out using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') for the V3-V4 region of bacterial 16S rRNA genes (Klindworth et al. 2013), and ITS3_KYO2 (5'-GATGAAGAACGYAGYRAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the second fungal internal transcribed spacers (ITS2; Toju et al. 2012). In addition, cave wall DNA extracts were also assessed using primers 18S_0067a_deg (5'-AAGCCATGCATGYCTAAGTATMA-3') and NSR399 (5'-

TCTCAGGCTCCYTCTCCGG-3') for eukaryotic 18S rRNA genes (Dollive et al. 2012). High-throughput sequencing was performed by Fasteris company (Geneva, Switzerland), using 1 µg DNA and Illumina MiSeq (2 × 300 bp, paired-end chemistry), so as to reach 70,000 paired reads per sample.

Bioinformatic analysis of Illumina data

For each gene marker studied, the paired-end reads were demultiplexed, by removing adaptators and all primer-complementing sequences with two mismatches (or more) with original primer sequences, with the help of a proprietary Perl script from Fasteris company. The sequences selected were merged using Fast Length Adjustment of Short reads (FLASh; Magoc and Salzberg 2011) with a maximum of 10% mismatch in the overlapped region. Denoising was done by removing reads without the expected 200-500 bp length or exhibiting one ambiguous base (N) or more. Sequences were dereplicated and clustered using SWARM (Mahé et al. 2014), based on a local clustering threshold level (instead of a global clustering threshold) and an aggregation distance of 3 to identify operational taxonomic units (OTUs). The finer taxonomic level that was reached corresponded to the genus or the species depending on the taxa, and it was hereafter referred to as the 'genus/species' level. The chimeras were discarded using VSEARCH (Rognes et al. 2016). Singletons and low-abundance sequences were removed (keeping only the OTUs representing 0.005% or more of all sequences; Bokulich et al. 2013). Taxonomic affiliation of OTUs at phylum, class, genus and/or species level was carried out automatically in the FROGS pipeline (Escudié et al. 2017), using RDP Classifier (Lan et al. 2012) against (i) the 119 SILVA database (Pruesse et al. 2007) for bacteria, (ii) the 123 SILVA database for micro-eukaryotes, and (iii) the UNITE database for fungal ITS2 (Kõljalg et al. 2013). Sequence datasets of wall and collembola samples (containing merged paired-end reads) have been deposited in EBI under references PRJEB27258 and PRJEB27257, respectively.

Isolation and characterization of Pseudomonas

For isolation of *Pseudomonas*, swab were vortexed in 0.9% NaCl solution, followed by spread plating onto S1 selective medium (Gould et al. 1984) and incubation of plates at 12°C. Bacterial colonies were then grown in King's B medium (King et al. 1954) and DNA extraction was carried out using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions with support protocol for bacteria. Bacterial 16S ribosomal fragments were amplified using primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al. 1989) in 50-µl reaction volumes containing 10× PCR buffer, 50 mM $MgCl_2$, 2 mM dNTP, 10 µM of each primer, 1 unit of Taq polymerase (Invitrogen, Cergy-Pontoise, France) and 1 µl of DNA solution. PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 62.4°C for 30 s, 72°C for 40 s and 72°C for 3 min. PCR products were sequenced using Sanger methodology (Biofidal company, Lyon, France) and sequences were blasted against NCBI nr-database (Johnson et al. 2008).

Sequence datasets of *Pseudomonas* isolates have been deposited in EBI under reference PRJEB27281.

All *Pseudomonas* sequences obtained in this study with Illumina MiSeq or Sanger sequencing were aligned against *Pseudomonas* sequences from other areas in Lascaux Cave (the second compartment of Airlock-1 entrance zone, the Airlock-2 and the Passage) or other Périgord caves (Alonso et al. submitted a), 82 type strains of *Pseudomonas* from GenBank and 3 previous Lascaux clones (Bastian et al. 2009c). However, 2 of these 3 clones (EU770637 and EU770635) were removed as current taxonomic knowledge affiliates them to Betaproteobacteria. Sequences were aligned using MAFFT version 7.205 (Katoh & Standley 2013) with the mafft–qinsi method. Multiple alignment was refined visually and ambiguous sites trimmed using Gblocks (Castresana 2000) using options for a less stringent selection, as implemented in Seaview software version 4 (Gouy et al. 2010). The most likely tree was reconstructed using PhyML version 3.1 (Guindon & Gascuel 2003) assuming a GTR+G+I model of evolution with optimized nucleotide equilibrium frequency, proportion of invariant sites and alpha parameter of the Gamma distribution and an SPR heuristic for tree searching.

Inhibitory effects of Pseudomonas on black fungi and other Pseudomonas

The inhibitory effect of 8 *Pseudomonas* isolates on 10 Lascaux black fungi (listed in Table 1) was assessed with a dual culture protocol on solid medium. Each bacterium was grown 24 h in King's B broth and 5 µl of culture streaked 2 cm away from the center of CYM plates (Raper & Hoffman 1974), making a 2-cm-long line. A 5-mm CYM fungal plug was then placed at the center of each plate. As positive control, CYM plates were inoculated only with fungi. The plates (two per treatment) were placed at 12°C in the dark during 15-30 days and growth inhibition was measured.

The inhibitory effect of 8 *Pseudomonas* isolates on one another was assessed with a drop test. Each bacterium was grown 24 h in King's B broth, cultures were adjusted a DO_{600} of 1, and 200 µl spread on King's B plates. For each of the 7 other pseudomonads, five 5-µl drops of King's B culture was deposited 10 min later on each of two plates, and the 8 × 14 = 112 plates were incubated 7 days at 12°C before measuring inhibition halos.

Isotopic analyses and assimilation/dissemination experiment

A stable isotope analysis was performed on black stains from the Apse in June 2015. Five black stains colonized by collembola, four black stains colonized years ago but not since and four black stains without documented colonization history were sampled (using sterile scalpels), along with collembola (using sterile insect mouth aspirators) present on the five colonized stains. Black stain samples were acidified with 2N HCl to remove inorganic carbon, rinsed with ultrapure water and oven dried (70°C during 48 h), and weighed in tin capsules until analysis. Collembola were oven dried at 50°C for at least 48 h and each was weighed in a tin capsule (dry mass ranging from 10 to 200 µg.individual^{-1}).

The assimilation of organic carbon by collembola *Folsomia candida* line HA (kindly provided by Thomas Tully, iEES UPMC, Paris, France) was quantified using $^{13}C/^{12}C$ ratio. As

^{13}C labelling of black stains was not possible, collembola were ^{13}C enriched by feeding on ^{13}C-labelled glucose during 10 d (at 12°C) and placed 3 d at 12°C on water agar (15 $g.l^{-1}$) on which a black stain sample, a plug of black fungus, or 5 µl of *Pseudomonas* culture (isolated from the Apse) in King's B medium had been placed. The black stain samples (approximately 2 cm^2) were taken with a scalpel from the banquette of the Passage (sample La852), the bottom of the wall of the Nave (La854) or of the far end of the Apse (La853). The black fungi were Lascaux isolates identified (ITS2 sequencing) as *Exophiala angulospora* (from a black stain in the second compartment of Airlock-1 entrance zone), *Exophiala castellanii* (from a purple stain in the Apse), *Ochroconis lascauxensis* (from a black stain in the second compartment of Airlock-1 entrance zone), and *Minimelanolocus* sp. (from a black stain in the Nave). Two Petri dishes were used per treatment. The collembola from a given Petri dish were transferred onto a new Petri dish containing water agar (15 $g.l^{-1}$) and the plates incubated 3 d in the dark at 12°C. Collembola were harvested and used for stable isotope analysis, and both series of plates were assessed for microbial growth after 2 days of incubation at 12°C.

Stable isotope ratios of carbon ($^{13}C/^{12}C$) were measured by continuous flow stable isotope ratio mass spectrometer (CF-IRMS) using a Isoprime 100 (Elementar UK, Manchester, UK) mass spectrometer interfaced with a Vario PyroCube elemental analyzer (Elementar Analysensysteme, Hanau, Germany). $^{13}C/^{12}C$ ratios were expressed as δ in parts per thousand (‰) and referenced to Vienna Pee Dee Belemnite (VPDB) standard. Data were calibrated against IAEA-CH3 and IAEA-CH6 international reference materials. The analytical precision achieved for aspartic acid and casein in-house standards analyzed along with the samples was better than 0.1 ‰ (± standard deviation).

For analysis of dissemination potential, four fungal colonies were randomly chosen per plate (i.e. per treatment, 8 colonies from the first series of plates and 8 others from the second). Each was transferred (as agar plug) on Dichloran Rose Bengal Chloramphenicol selective medium for 1 month at 12°C. For strains that did not grow on this medium, the agar plug was transferred onto CYM agar for 1 month at 12°C. All fungi were then grown on CYM medium at 22°C for 15 d to produce sufficient biomass. DNA extraction, PCR amplification of fungal ITS2 and Sanger sequencing were performed, as described above.

Statistical analyses

To compare cave wall samples, a normalization procedure was applied for randomly resampling down to 6000, 26674 and 21074 Illumina sequences in the bacteria, micro-eukaryote and fungi datasets, respectively. Sampling efficacy was assessed by computing rarefaction curves (Fig. S2). Alpha diversity at OTU level was assessed using Chao 1 index (Chao 1987), Shannon's H' index (Shannon 1948) and Simpson 1-D index (Simpson 1949), which were computed with Paleontological Statistics (PAST) software v3.14 (Hammer et al. 2001). Microbial community structure was investigated based on the Bray-Curtis similarity index (Bray & Curtis 1957) after square-root transformation of data (to avoid over-dominance effects), using VEGAN package (http://cran.r-

project.org/web/packages/vegan/index.html) in R. First, microbial communities were compared by non-metric multidimensional scaling (NMDS), using PAST v3.14. NMDS stress values below 0.1 are considered without risk of drawing false inferences, and values below 0.2 acceptable (Clarke 1993). Second, permutation multivariate analysis of variance using distance matrices (PERMANOVA) was carried out using adonis in VEGAN package to identify differences ($P < 0.05$) in overall microbial community composition in phyla or classes and to confirm NMDS findings.

Analysis of variance (ANOVA) and post-hoc Tukey HSD tests were performed to compare the number of OTUs or microbial diversity indices inside versus outside black strains ($P < 0.05$). Pearson's Chi-squared tests in R were used to investigate the proportions of phyla and genera in different microbial communities ($P < 0.05$). *t*-tests (with Bonferroni correction for multiple comparisons) were used to compare stable isotope ratios ($P < 0.05$).

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Supplementary data

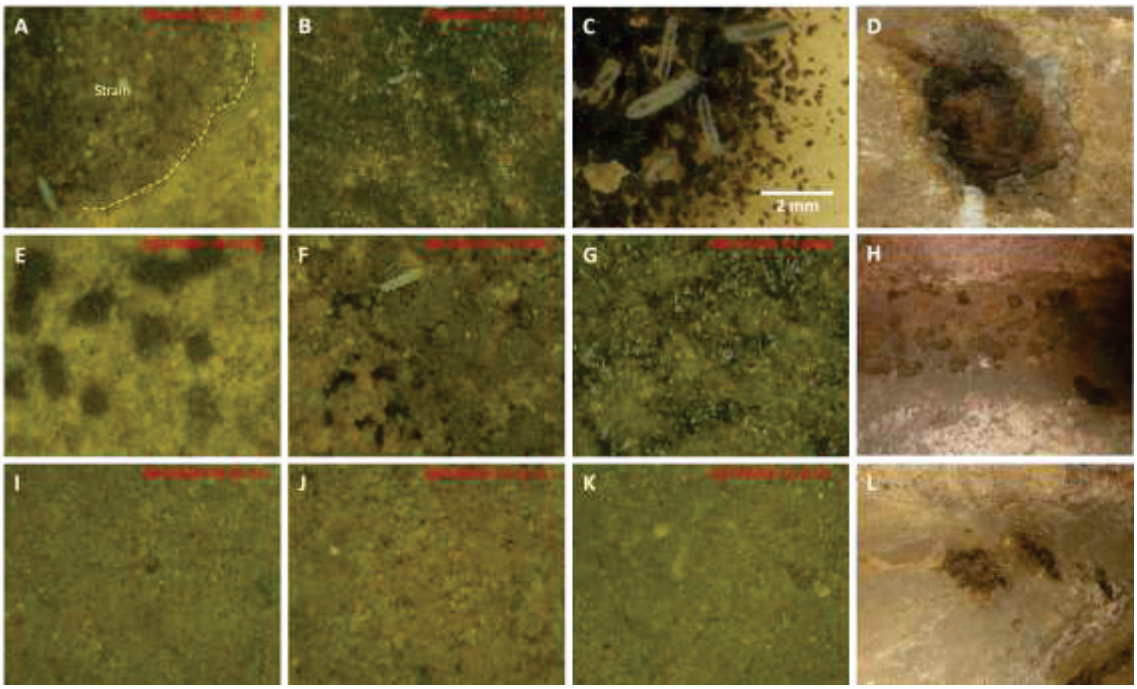

Figure S1. Photographs of black stains in the Apse (D, H, L), Dino-Lite microscope photographs of black stains (A, B, E, F, G) and neighboring unstained parts (I, J, K) on right walls (A, E, I), left walls (B, F, J) and the Absidiole area (G, K) of the Apse, and binocular microscope photograph of *Folsomia candida* collembola on a black fungal colony on laboratory medium (C). Collembola can be seen on black stains (A, B, F). Macroscopically, many black stains are entirely black whereas others also contain areas of lighter color (visible also in microscope photograph F), yet most display some level of patchiness when examined by microscope. During the feeding experiment *in vitro*, darkening of collembola gut can be seen thanks to the transparency of the animal tissues, as the black fungus was grazed (C). In D, collembola can be seen as small white spots on the black stain.

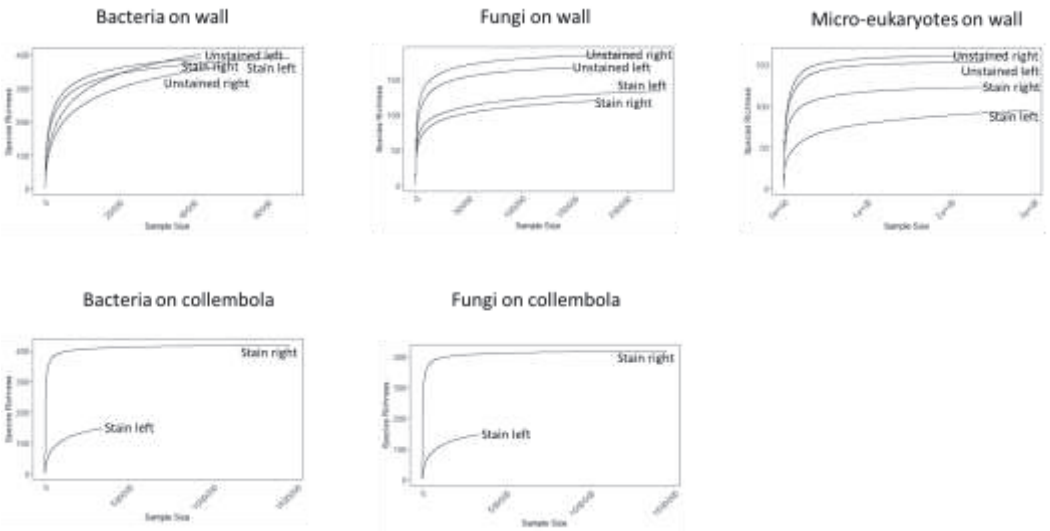

Figure S2. Rarefaction curves at OTU level for cave wall samples in the case of bacteria (16S rRNA gene dataset), micro-eukaryotes (18S rRNA gene dataset), fungi (ITS2 dataset) and for collembola samples in the case of bacteria (16S rRNA gene dataset) and fungi (ITS2 dataset), based on observed data. Wall samples originated from black stains and neighbouring unstained parts (from the left and right walls), whereas collembola samples originated from black stains (both left and right walls combined). Rarefaction curves reached an asymptote in 2 out of 4 cases (wall bacteria), all 4 cases (wall micro-eukaryotes), all 4 cases (wall fungi), 1 out 2 cases (collembola bacteria) and 1 out 2 cases (collembola fungi).

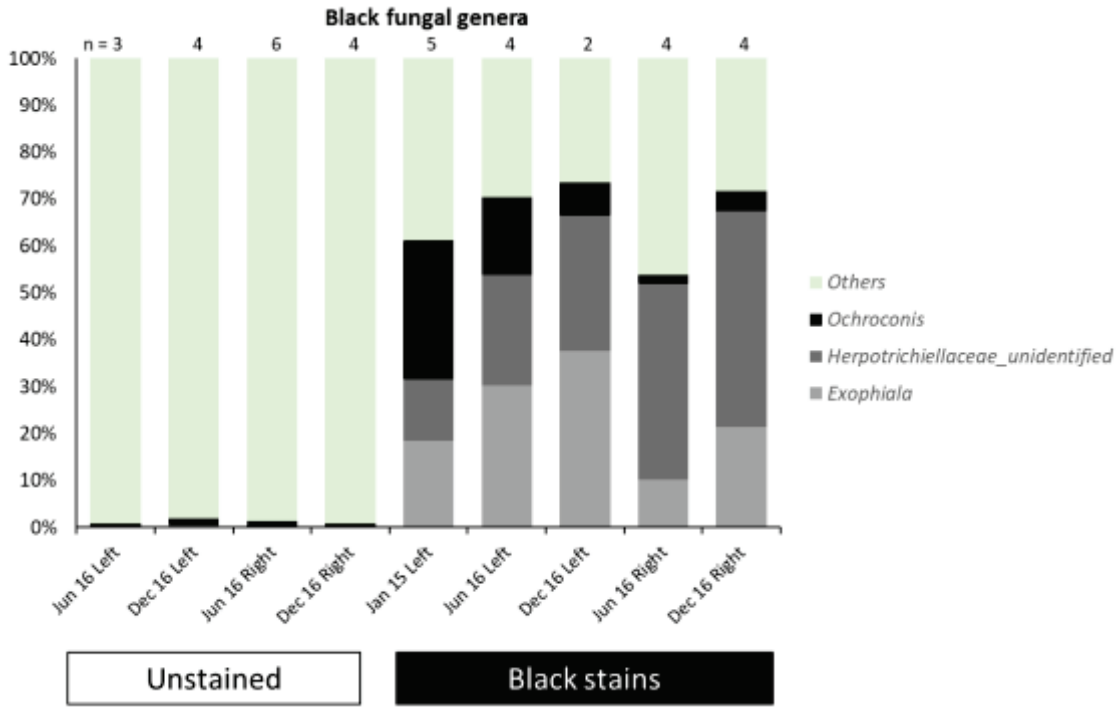

Figure S3. Community composition at genus level for fungi in black stains and nearby unstained parts sampled from the left (L) and right (R) walls of Lascaux's Apse in June 2015 (15J), January 2016 (16J), June 2016 (16M) and December 2016 (16D). Black fungi (i.e. *Ochroconis*) are shown in black and taxa containing both pigmented and non-pigmented strains in grey (Herpotrichiellaceae) or orange (*Exophiala*). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 1-6 samples (indicated in each case).

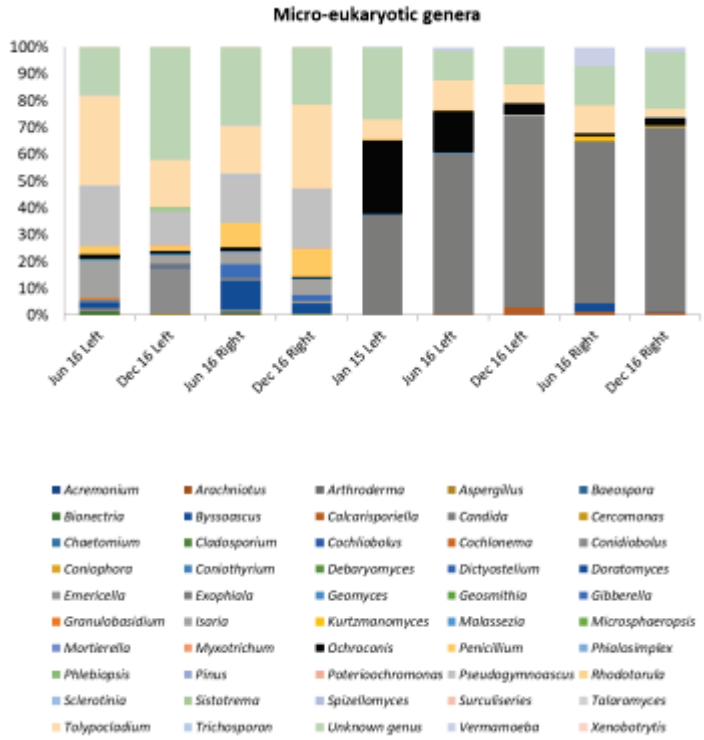

Figure S4. Community composition at genus level for micro-eukaryotes in black stains and nearby unstained parts sampled from the left (L) and right (R) walls of Lascaux's Apse in June 2015 (15J), January 2016 (16J), June 2016 (16M) and December 2016 (16D). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 2-6 samples (indicated in each case).

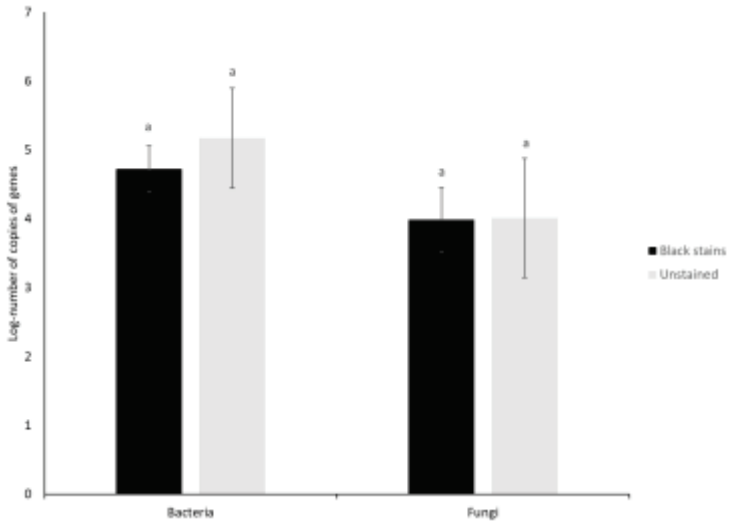

Figure S5. Abundance of the bacterial and micro-eukaryotic communities colonizing cave walls in the Apse. The values shown are means with their standard errors. There was no statistical difference between black stains and unstained parts (Wilcoxon tests, $P > 0.05$). Quantitative PCR was done using 16S rRNA primers 519F/907R (Laiz et al. 2003), 18S rRNA primers EUK345F/EUK499R (Zhu et al. 2005) and a LightCycler 480 (Roche Diagnostics, Meylan, France). Briefly, the 16S rRNA and 18S rRNA genes reactions were carried out in 20-µl volumes containing 0.6 µl (final concentration 0.3 µM) of each primer, 4 µl of PCR-grade water, 10 µl of LightCycler-DNA Master SYBR Green I master mix (Roche Applied Science, Meylan, France) and 2 µl of sample DNA (5 ng). PCR was done with 10 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s, (ii) 63°C for 60 s, and (iii) 72°C for 30 s for 16S rRNA genes, and 2 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s, (ii) 60°C for 15 s, and (iii) 72°C for 15 s for 18S rRNA genes. Melting curve calculation and Tm determination were done using the Tm Calling Analysis module of Light-Cycler Software v.1.5 (Roche Applied Science).

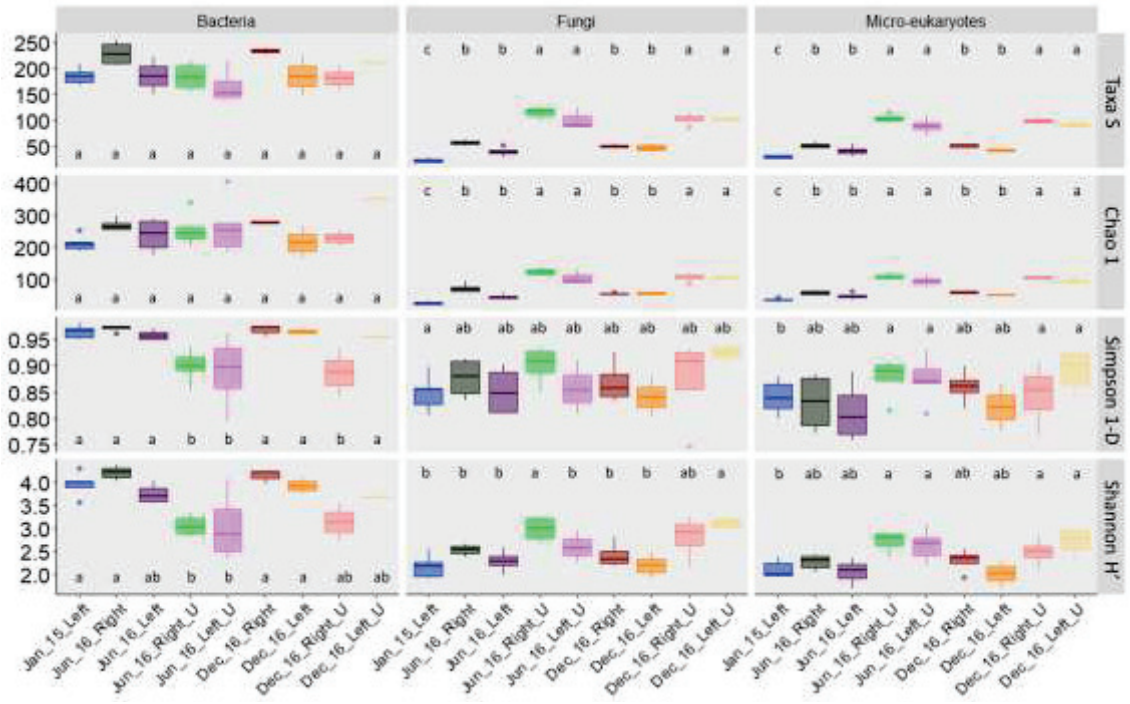

Figure S6. Number of OTU, Chao1 index of OTU richness, Simpson's index of diversity and Shannon index of OTU diversity for bacteria, micro-eukaryotes and fungi in Lascaux's Apse walls according to sampling time i.e. June 2015 (Jun_15), January 2016 (Jan_16), June 2016 (Jun_16) and December 2016 (Dec_16) of the left (Left) and right (Right) walls for black stains and nearby unstained parts (U). Differences between conditions are shown with lowercase letters (based on ANOVA and Tukey's tests; $P < 0.05$).

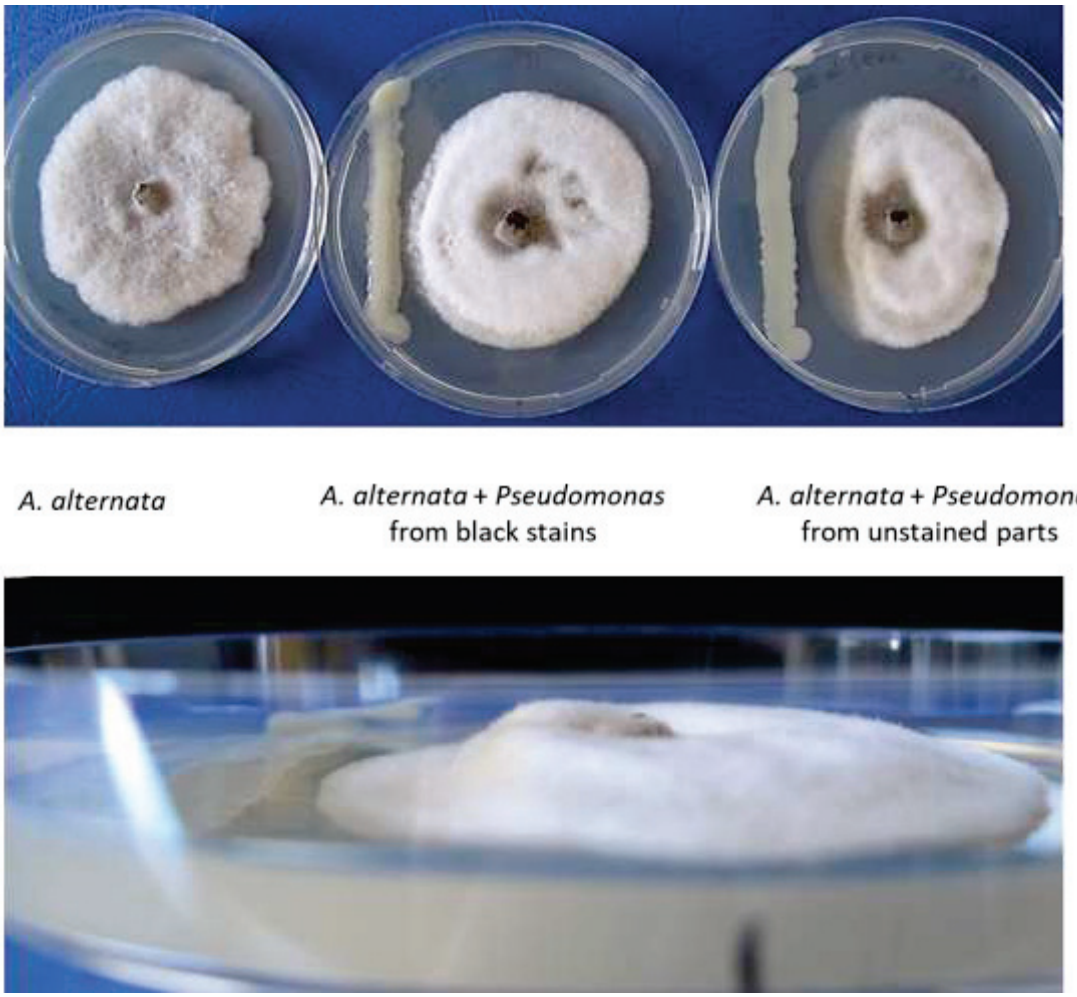

Figure S7. Set-up of dual confrontation experiments between *Pseudomonas* isolates from the Apse and black fungi on plates. Many of the black fungi tested become black at later stages of fungal growth, as illustrated here with *Alternaria alternata* that starts forming a white colony (shown with inhibition effects of *Pseudomonas* at the top right and at the bottom). Top left: the fungus is introduced in the plate as a square plug from which the mycelium develops and forms a large colony).

Article 4 : Microbial analysis of recent cave wall alterations in the Apse of Lascaux Cave

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Introduction

The calcareous region of Périgord in south-western France displays a wide range of karstic caves, and many of them are renowned for their exceptional parietal carving, drawing and painting (Schabereiter-Gurtner et al. 2002; Dupont et al. 2007). Some of these caves can be visited by the general public, but tourism-related anthropization can lead to significant changes in microbial and arthropod diversity in comparison with pristine caves (Alonso et al. submitted a). In most of these caves, tourism did not threaten cave wall quality and Paleolithic features. However, in the case of Lascaux Cave, anthropization resulted in various cave wall alterations and the cave was closed to the public in 1963.

Cave wall alterations in Lascaux Cave included green (in the 1960s) and white stains (in 2001), which were treated mechanically and with chemicals such as formaldehyde (to control algae), antibiotics (to control bacteria and fungi) and benzalkonium chloride (to control fungi) (Dupont et al. 2007; Bastian et al. 2010). From 2006 on, stains that developed on cave walls were black, and black fungi such as *Ochroconis lascauxensis* have been evidenced (Martin-Sanchez et al. 2012). The current management scheme in Lascaux Cave aims at minimizing human intervention. Most wall surface alterations have been stable in recent years and natural attenuation was even observed for some of them, for instance black stains in the Chamber of Felines. Within Lascaux, however, the Apse remains a monitoring priority as this room still undergoes microbial development.

In Lascaux's Apse, black stains resembling those present at other locations within the cave and thought to result from fungal synthesis of melanin (Bastian et al. 2010; Martin-Sanchez et al. 2012; De la Rosa et al. 2017) have been characterized microbiologically. These black stains occurring in lower parts of the Apse walls (below the ornate parts) are colonized by *Folsomia candida* collembola and display different microbial communities in comparison with unstained wall samples taken nearby (Alonso et al. submitted c). It is thought that *F. candida* plays a key role in stain microbial dynamics by disseminating black fungi and feeding them with their feces (Bastian et al. 2010; Alonso et al. submitted c).

The Apse walls also display small (centimetric) areas that are not black but are visually different from the rest of the wall, and that have been termed dark zones by Lascaux monitoring staff. Based on distinct visual properties of black stains and dark zones (Fig. 1D,H), the current paradigm is to consider both phenomena as separate alteration processes. Whether these intriguing dark zones could represent an initial stage in the formation of black stains is not clear, and longer-term monitoring will be useful to answer this question. They are also colonized by collembola, and the occurrence of these dark zones in the same part of the cave raises questions on their origin, formation and microbiological properties.

The objective of this investigation was to characterize the microbial community of dark zones developing in the Apse of Lascaux. In particular, we aimed at determining microbiota specificities of dark zones vs black stains, and whether microorganisms

associated to dark zones were also present in collembola to gain insight into their dissemination potential via these arthropods.

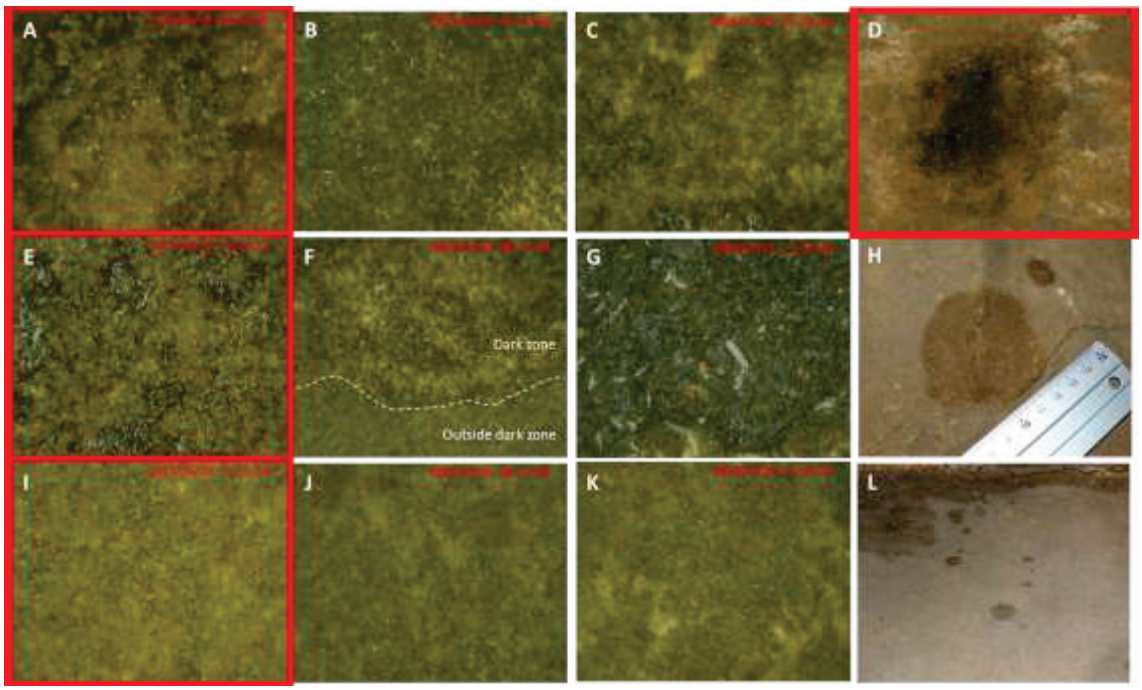

Figure 1. Photographs of dark zones (H, L) from the Apse compared with a black stain (D), and Dino-Lite microscope photographs of dark zones (B, C, F, G) compared with outside parts near dark zones (J, K), as well as black stains (A, E) and outside parts near a black stain (I). The reference situation of Apse black stains is shown in red frames. B, F (showing the limit of a dark zone) and J correspond to left wall photographs, and C, G (showing *F. folsomia* collembola) and K to right wall photographs.

Materials and methods

Sampling

Sampling in Lascaux Cave (near Montignac, South-West France) was carried out mainly in the Apse, and was completed with a few samples from adjacent Nave room in February 2017. Sampling followed rules and regulation implemented to protect the cave, including the distribution of sampling activities over several days to limit human presence duration on a given day and wall samples taken by qualified restoration personnel. Five sampling campaigns were performed in late June-early July 2015, January 2016, June 2016, December 2016 and (for additional collembola) May 2017, using several areas selected on the left and right walls of the Apse.

Collembola and dark zones underneath were sampled for metabarcoding assessment of their associated microorganisms. A total of 17 dark zone samples of collembola (201 individuals in total) were obtained by sucking using sterile insect mouth aspirators (Rose Entomology, Benson, AZ), whereas dark zones underneath and equivalent unstained, control areas located about 10 cm away were sampled using sterile swabs (3-6 samples per wall

surface condition at each sampling date). All samples were placed into liquid nitrogen and transferred at -80°C once in the lab prior to DNA extraction.

DNA extraction, identification of collembola and Illumina sequencing

DNA extractions for collembola were carried out under a laminar hood to avoid contamination. The 17 collembola samples (1 to 42 individuals per dark zone) were crushed using 1-mm beads in ATL lysis buffer (Qiagen, Hilden, Germany) that contained 20 $mg.ml^{-1}$ lysozyme (Euromedex, Strasbourg, France), following homogenization 10 s in a Mini-beadbeaterTM (BioSpec Products, Bartlesville, OK). After 2 h at 37°C, 20 µl of proteinase K (Qiagen, 20 $mg.ml^{-1}$) was added and samples were maintained 4 h with agitation (300 rpm) at 56°C to achieve collembola lysis. DNA was extracted using Qiagen DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's recommendations for both Gram-negative and Gram-positive bacteria, and elution was done using 12 µl. Final DNA concentration was measured using Nanodrop Safas UV-mc^2 (SAFAS, Monaco), and DNA kept at -20°C.

DNA extraction from cave wall samples was done using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), following the manufacturer's instructions. Elution was performed using two 50-µl volumes that were later combined, and final DNA concentration was measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) according to manufacturer's instructions. DNA extracts were kept at -20°C.

Taxonomic identification of collembola was done on four collembola samples, based on Sanger sequencing of a 708-bp fragment flanking gene *cox1*, after PCR amplification using 50 ng of DNA matrix and primers LCO1490 33 and HCO2198 (Folmer et al. 1994), as described in Alonso et al. (submitted c). Sequencing was done by Biofidal company (Lyon, France) and sequences were blasted against NCBI nr-database (Johnson et al. 2008).

DNA extracts from cave walls and collembola were used to amplify the V3-V4 region of bacterial 16S rRNA genes with primers 341F and 805R (Klindworth et al. 2013), the second fungal internal transcribed spacers (ITS2) with primers ITS3_KYO2 and ITS4 ((Toju et al. 2012) and (for cave wall DNA extracts only) the eukaryotic 18S rRNA genes with primers 18S_0067a_deg and NSR399 (Dollive et al. 2012). Illumina sequencing was implemented by Fasteris company (Geneva, Switzerland) from 1 µg of DNA extract, based on MiSeq paired-end chemistry (2 × 300 bp), with the goal of 70,000 paired reads per sample.

Bioinformatic treatment of Illumina sequence data

Paired-end reads were demultiplexed. The adaptators were removed, as well as all sequences whose primer-complementing regions displayed at least two mismatches with the primer sequences, using a proprietary Perl script from Fasteris company. The sequences were then merged using Fast Length Adjustment of Short reads (FLASh; (Magoc and Salzberg, 2011) with a maximum of 10% mismatch in the overlapped region. Denoising was performed by removing all reads that did not display the expected 200-500 bp length or exhibited ambiguous base(s) (N). The sequences were dereplicated and clustered using SWARM (Mahé et al. 2014), on the basis of a local clustering threshold level and an

aggregation distance of 3 to identify operational taxonomic units (OTUs). The finer taxonomic level thus obtained (hereafter referred to as 'genus/species' level) was the genus or the species depending on the taxa. Chimeras were removed using VSEARCH (Rognes et al. 2016), as well as singletons and low-abundance sequences so as to keep only OTUs representing at least 0.005% of all sequences (Bokulich et al. 2013). Taxonomic OTU affiliation at phylum, class, genus and/or species level was performed in the FROGS pipeline (Escudié et al. 2017), using RDP Classifier (Lan et al. 2012) against (i) the 119 SILVA database (Pruesse et al. 2007) for bacteria, (ii) the 123 SILVA database for micro-eukaryotes, and (iii) the UNITE database for fungal ITS2 (Kõljalg et al. 2013).

Statistical analyses

For normalization, random resampling was carried out down to 6000 (bacteria), 26674 (micro-eukaryotes) and 21074 Illumina sequences (fungi), to enable comparisons between cave wall samples. The efficacy of sampling was evaluated using rarefaction curves (Fig. S1). Alpha diversity at OTU level was measured with Chao 1 index (Chao, 1987), Shannon's H' index (Shannon, 1948) and Simpson 1-D index (Simpson, 1949), using Paleontological Statistics (PAST) software v3.14 (Hammer et al. 2001). Microbial community structure was studied using the Bray-Curtis similarity index (Bray and Curtis, 1957) after square-root transformation of data (to avoid over-dominance effects), using VEGAN package (http://cran.r-project.org/web/packages/vegan/index.html) in R. Comparison of microbial communities was done by non-metric multidimensional scaling (NMDS), using PAST v3.14. NMDS stress values below 0.1 are considered very good and those between 0.1 and 0.2 acceptable (Clarke, 1993). Then, analysis of variance using distance matrices (adonis) was performed, in VEGAN, to assess significant differences ($P < 0.05$) in overall microbial community composition in phyla or classes and to strengthen NMDS results.

Analysis of variance (ANOVA) followed with Tukey's HSD tests were carried out to compare the number of OTUs or microbial diversity indices in or outside dark zones ($P < 0.05$). Pearson's Chi-squared tests in R were used to investigate the proportions of phyla and genera in different microbial communities ($P < 0.05$).

Results

Fungal community diversity in dark zones

The hypothesis that dark zones could correspond to a different wall alteration process in comparison with black stains was assessed by Illumina sequencing, to determine whether the same fungal community was present in both. NMDS comparison of Apse cave walls indicated that the structure of the fungal community differed clearly when comparing (i) dark zones to neighboring unstained parts (adonis $F_{1,68} = 44.7$, $P = 0.001$, $R^2 = 0.25$), (ii) dark zones in June-July 2015 vs January or December 2016 (adonis $F_{3,68} = 7.99$, $P = 0.001$, $R^2 = 0.06$), and (iii) dark zones to black stains (adonis $F_{1,69} = 15.13$, $P = 0.001$, $R^2 = 0.11$) (Fig. 2A).

Some differences were also found between (i) unstained parts next to dark zones vs next to black stains (adonis $F_{3,38}$ = 8.82 P = 0.001, R^2 = 0.33), and (ii) dark zones from left vs right walls (but not for unstained parts nearly) (adonis $F_{1,68}$ = 11.6, P = 0.001, R^2 = 0.06). NMDS analysis carried out at the scale of the whole micro-eukaryotic community confirmed the findings made with the fungal sub-compartment, but with (i) a clearer distinction between dark zones from left or right walls and (ii) less marked differences between sampling dates (Fig. 2B). When considering OTUs, 60 of 109 fungal OTUs found in dark zones (i.e. 55%) were also present in black stains, which displayed only 20 (i.e. 25%) specific fungal OTUs (Fig. 5B).

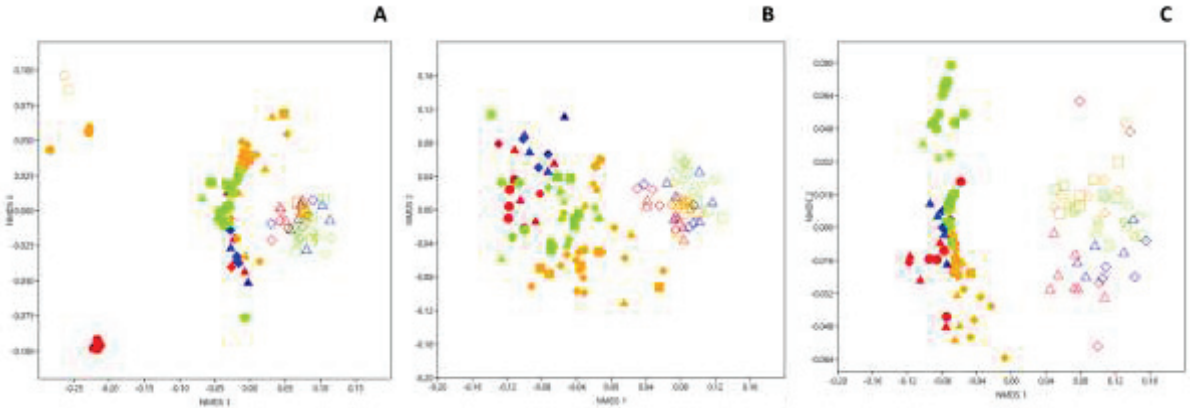

Figure 2. Microbial structure comparison of black stains (left wall samples in red and right wall samples in blue), dark zones (left wall samples in green and right wall samples in orange) and nearby unstained parts (empty symbols) from the left and right walls of Lascaux's Apse taken in June-July 2015 (circles), January 2016 (squares), May-June 2016 (triangles) and December 2016 (diamonds), with NMDS analysis, based on the relative proportion of both phyla and classes in the fungal (A), micro-eukaryotic (B) and bacterial communities (C). Data used for black stains and their neighboring unstained parts are those from Alonso et al. (submitted c). Stress values were 0.22, 0.19 and 0.17 for fungi, micro-eukaryotes and bacteria, respectively.

Fungal community composition in dark zones

In Lascaux, the development of black stains is attributed to melanin-producing fungi (Bastian et al. 2010; De la Rosa et al. 2017), but the fungal communities of dark zones and black stains are not identical. Therefore, the occurrence of black fungi in dark zones was investigated, based on the genus taxonomic profile of fungi. First, the genus taxonomic profile of fungi in dark zones strongly differed from the one found in neighboring unstained parts (Chi-squared test, P < 0.0001), with noticeably *Pseudogymnoascus* less present in dark zones (Fig. 3A). For these dark zones, the genus taxonomic profile also differed (i) between left vs right wall samples (P < 0.0001) (e.g. *Kazachstania* more prevalent on the right wall), and to a somewhat lesser extent (ii) between sampling times. Second, the genus taxonomic profile of dark zones was clearly distinct from the one of black stains, and for instance an unidentified *Cordycipitaceae* genus and *Kazachstania* were more prevalent in dark zones (Fig. 3A). Third, as for black stains, the prevalence of pigmented fungal taxa was much higher in dark zones than in neighboring unstained parts, i.e. *Ochroconis* represented 37.6-47.8% of fungal sequences in dark zones in winter, 2.6-20.4% in dark zones in summer, and only 0.7-2.1% in winter/summer neighboring unstained parts (Fig. S2). In addition, fungal taxa containing both pigmented strains and non-pigmented strains (i.e. in *Exophiala* and

Herpotrichiellaceae) amounted to 0.6-13.6% of fungal sequences in dark zones vs 0.05-0.92% in unstained parts (Fig. S2). These trends were also observed when considering the genus taxonomic profile at the scale of the entire micro-eukaryotic community (Fig. S3).

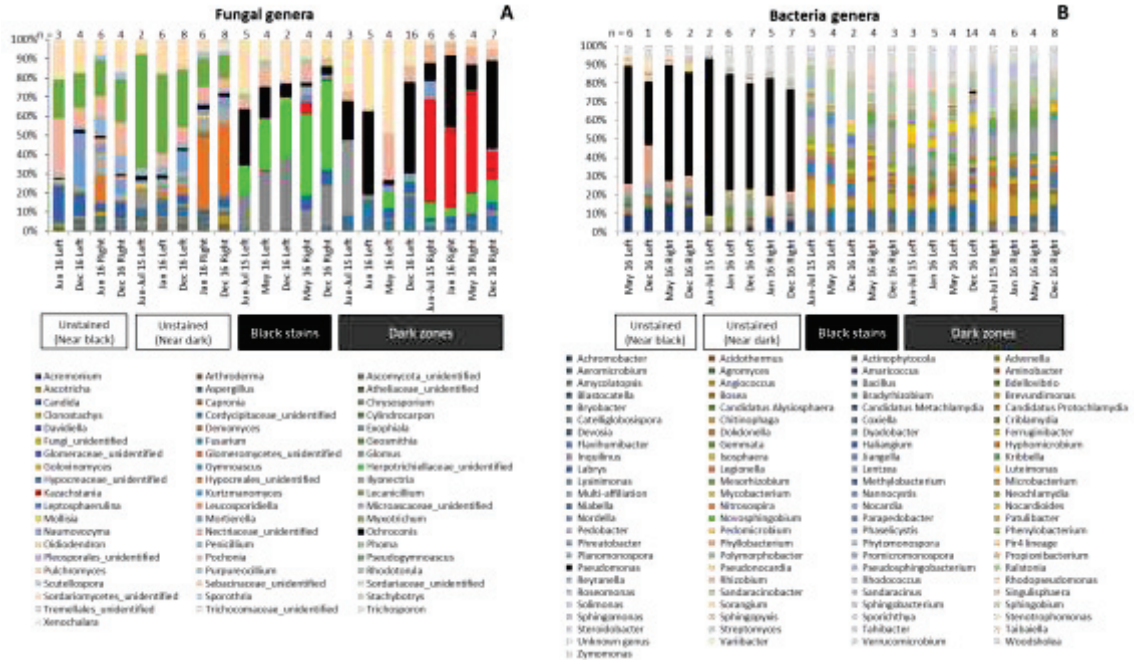

Figure 3. Community composition at genus level for fungi (A) and bacteria (B) in dark zones (Dark), black stains (Black) and nearby unstained parts (respectively Near Dark and Near Black) sampled from the left (L) and right (R) walls of Lascaux's Apse in June-July 2015 (15J), January 2016 (16J), May-June 2016 (16M) and December 2016 (16D). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 1-16 samples (indicated in each case). Data used for black stains and their neighboring unstained parts are those from Alonso et al. (submitted c).

Fungal community size in dark zones

Whether taxonomic findings made with fungi in dark zones could result from a bias linked to lower amounts of microorganisms there was explored by quantitative PCR of 18S rRNA genes, which pointed to microbial population levels in dark zones that were similar to those in unstained parts (Fig. S4B) and in black stains (Alonso et al. submitted c). Since some of the fungi increased in prevalence in dark zones, it resulted in lower fungal diversity in dark zones than in unstained parts, as shown by smaller number of taxa, Chao1 richness index, Simpson index and Shannon index (Fig. 4), which were at comparable levels as those in black stains (Alonso et al. submitted c). These fungal diversity indices for dark zones did not differ significantly when comparing sampling times (Fig. S5) or right and left walls (Fig. S6).

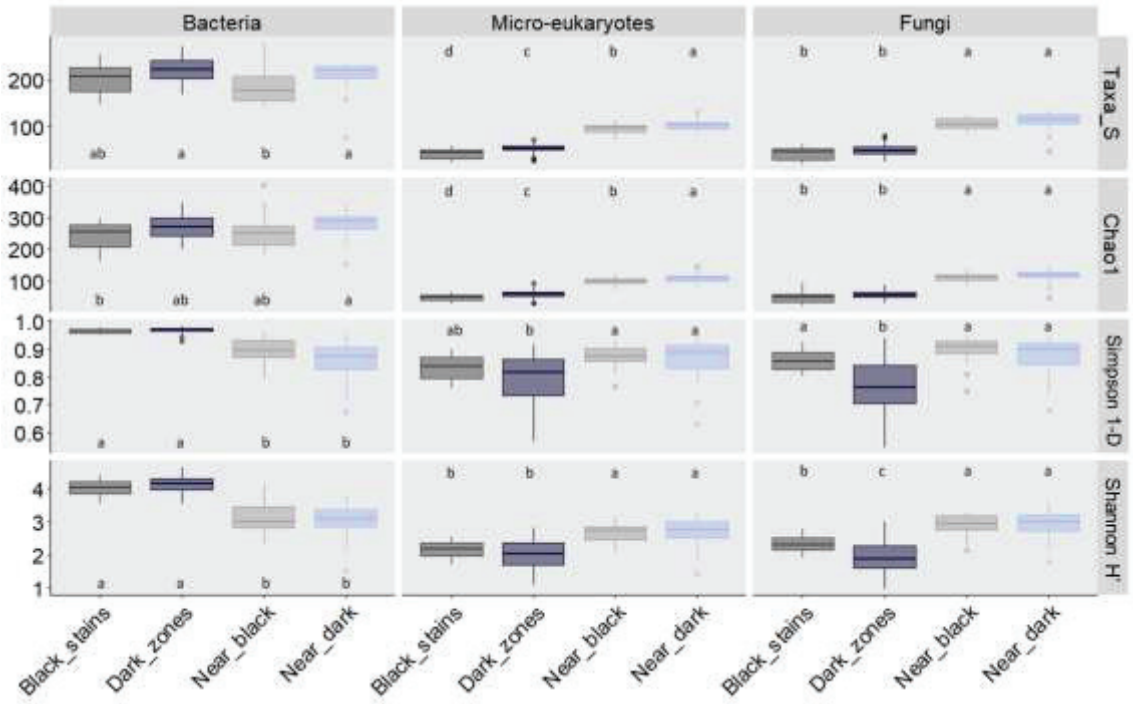

Figure 4. Number of taxa, Chao1 index of OTU richness, Simpson's index of diversity and Shannon index of OTU diversity for bacteria, micro-eukaryotes and fungi in dark zones (Dark), black stains (Black) and nearby unstained parts (respectively Near Dark and Near Black) sampled in Lascaux's Apse walls. Data were combined for the left and right walls sampled in June-July 2015, January 2016, May-June 2016 and December 2016. Differences between conditions are shown with lowercase letters (based on ANOVA and Tukey's tests; $P < 0.05$). Data used for black stains and their neighboring unstained parts are those from Alonso et al. (submitted c).

Bacterial community diversity, composition and size in dark zones

A key feature of the bacterial community in black stains was the very low amount of pseudomonads, which predominated in unstained parts (Alonso et al. submitted c). Whether this observation was also valid for dark zones was investigated by NMDS. This comparison of Apse cave walls indicated that the structure of the bacterial community differed when comparing (i) dark zones to neighboring unstained parts (adonis $F_{1,69} = 115.5$, $P = 0.001$, $R^2 = 0.49$) and (ii) dark zones to black stains (adonis $F_{1,69} = 8.69$, $P = 0.001$, $R^2 = 0.08$), as well as when comparing (i) unstained parts next to dark zones vs next to black stains (adonis $F_{1,41} = 9.78$, $P = 0.001$, $R^2 = 0.13$), (ii) left vs right walls (adonis $F_{1,69} = 13.58$, $P = 0.001$, $R^2 = 0.06$) for dark zones (but not for unstained parts), and (iii) different samplings (adonis $F_{3,69} = 5.62$, $P = 0.001$, $R^2 = 0.07$) (Fig. 2C). When considering OTUs, 334 of 368 bacterial OTUs found in dark zones (i.e. 91%) were also present in black stains, which displayed only 48 (i.e. 13%) specific bacterial OTUs (Fig. 5A).

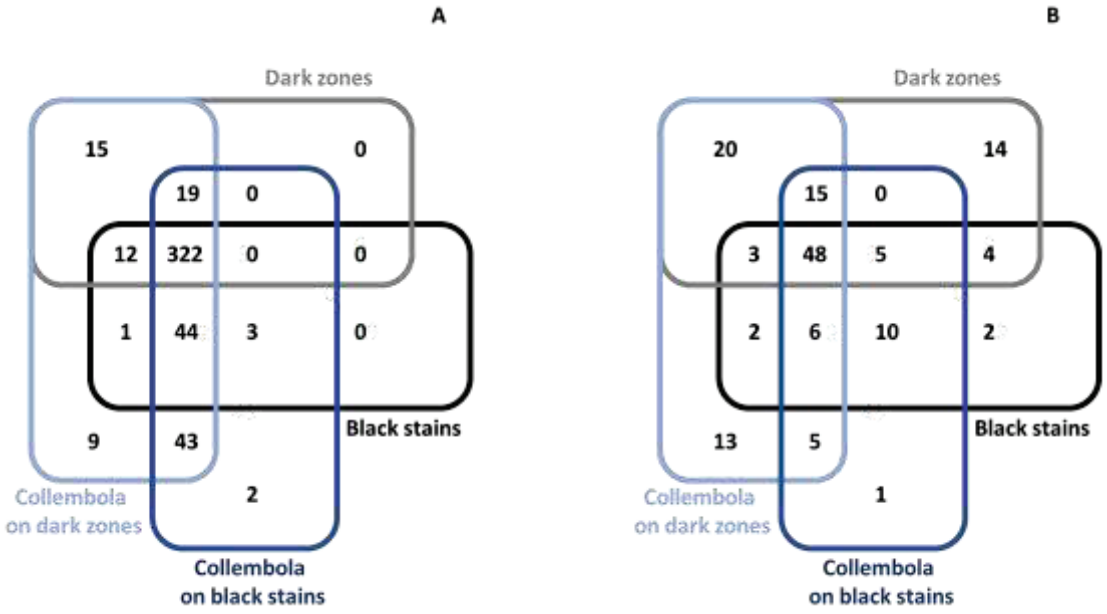

Figure 5. Venn Diagram showing unique and shared bacterial OTUs (A) and fungal OTUs (B) in collembola from dark zones, collembola from black stains, dark zones and black stains. Data used for black stains and black stain collembola are those from Alonso et al. (submitted c).

In unstained parts of the Apse, the genus taxonomic profile of bacteria was rather comparable in all samples, with minor differences for samples taken close to dark zones vs close to black stains. As for black stains, levels of *Pseudomonas* were low in dark zones (0.01-1.6%), levels of *Neochlamydia* within dark zones were higher in right walls than left walls (2.5-11% vs 0.01-2.5%), and minor differences were found between different dark zones from a same wall or different sampling dates for a same dark zone (Fig. 3B).

As for fungi, the findings made with bacteria did not coincide with biologically-lower bacterial numbers in dark zones, as indicated by quantitative PCR data of 16S rRNA genes (Fig. S4A). As in black stains, the much lower prevalence of *Pseudomonas* in dark zones than in unstained parts enabled proliferation of many other bacterial taxa, as indicated by higher Simpson and Shannon indices in comparison with these unstained parts (Fig. 4).

Comparison with dark zones in the Nave

A few dark zones have also been evidenced on walls of the Nave, a room contiguous but perpendicular to the Apse, and they were sampled in February 2017 and processed as for Apse samples. NMDS showed that the fungal community in dark zones did not differ when comparing Apse and Nave samples, whereas the comparison was not possible for neighboring unstained parts as ITS2 sequencing for these samples was not successful (Fig. 6A).

When considering more globally the micro-eukaryotic community, NMDS indicated that dark zones did not differ in Apse and Nave, whereas unstained parts in Apse and Nave did differ (Fig. 6B). Importantly, the micro-eukaryotic community was not the same in dark zones vs neighboring unstained parts, both in the Apse and the Nave.

When considering the bacterial community, NMDS indicated that dark zones differed from unstained parts in the Nave, and differences (of lower magnitude) were also found between Nave and Apse samples, regardless of whether dark zones or unstained parts were studied (Fig. 6C).

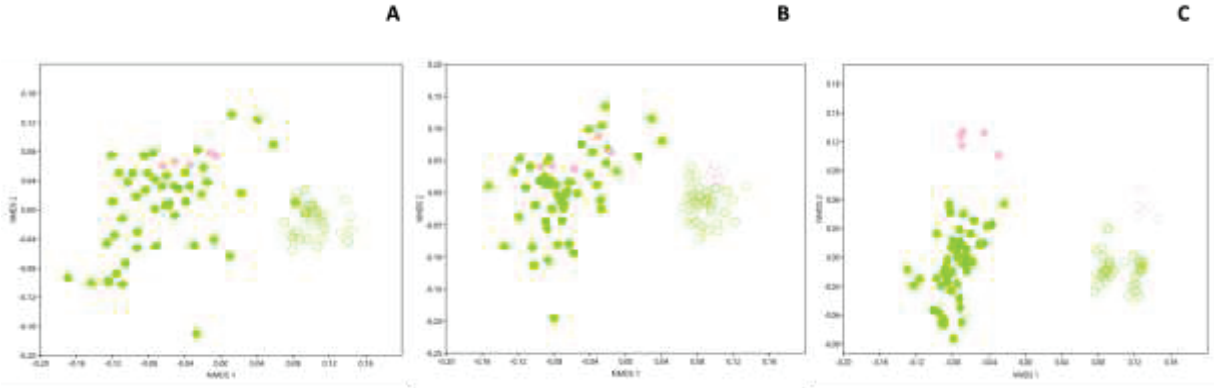

Figure 6. NMDS comparison of dark zones and nearby unstained parts from the walls of Lascaux's Apse taken in June-July 2015, January 2016, May-June 2016, December 2016 and from the Nave in February 2017, based on the relative proportion of both phyla and classes in the fungal (A), micro-eukaryotic (B) and bacterial communities (C). Apse data for dark zones and their neighboring unstained parts are those already shown in Figure 2 for the same sampling dates. Stress values were 0.19, 0.19 and 0.15 for fungi, micro-eukaryotes and bacteria, respectively. Green circles represent Apse samples, pink circles Nave samples, and empty symbols unstained parts of the Apse and Nave.

Dark zone microorganisms associated with collembola

Collembola have been identified as potential disseminators of bacteria and fungi located in black stains (Bastian et al. 2009a; Alonso et al. submitted c), and indeed may black stain taxa were also found in collembola samples (Alonso et al. submitted c). Whether the same applies also to dark zones was investigated by MiSeq sequencing. A total of 368 bacterial OTUs were documented based on 16S rRNA gene data, including the endosymbiont *Wolbachia*. The *Pseudomonas* clades 4 (close to *P. lurida, P. trivialis* and *P. extremaustralis*), 5 (close to *P. fluorescens* and *P. canadensis*), 7 (close to the '*P. gessardii*' subgroup) and 9 (close to *P. sesami, P. saponiphila* and *P. protegens*) previously evidenced for unstained parts and black stains of the Apse as well as (except clade 7) collembola on black stains (Alonso et al. submitted c) were also found here in collembola taken from dark zones (Table 1). All 368 bacterial OTUs found in dark zones were also present in collembola sampled from these dark zones (Fig. 5A). In addition, collembola from dark zones versus black stains shared 428 bacterial OTUs, which represented 92% of the 465 OTUs found with dark zone collembolan (and 99% of the 433 OTUs found with black stain collembolan).

A total of 109 fungal OTUs were found, including *Acremonium nepalense*, *Exophiala castellanii*, *Ochroconis lascauxensis* (all with black pigmentation potential), *Alternaria eichhorniae* and *metachromatica* (of uncertain pigmentation potential). More generally, 86 of the 109 fungal OTUs found in dark zones (i.e. 79%) were also evidenced in collembola sampled on these dark zones (Fig. 5B). In addition, collembola from dark zones versus black stains shared 74 fungal OTUs, which represented 66% of the 112 OTUs found with dark zone collembola (and 82% of the 90 OTUs found with black stain collembola).

Table 1. Taxonomic features of *Pseudomonas* sequences retrieved in dark zones, unstained parts near dark zones and *F. folsomia* collembola taken from dark zones in the Apse, and comparison with results from the Nave and from Apse black stains.

Clade[a]	Related *Pseudomonas* species	Apse black stains			Apse dark zones			Nave dark zones	
		Black stains	Collembola	Near stains	Dark zones	Collembola	Near zones	Dark zones	Near zones
Clade 4	*P. lurida, P. trivialis* and *P. extremaustralis*	NGS19(3)[b]		NGS17(1611) NGS19(2031)	NGS17(1672)[c] NGS19(1677)	Co10(3620)[d]	NGS17(986) NGS19(1042)	NGS17(354) NGS19(372)	NGS17(3012) NGS19(3091)
Clade 5	*P. fluorescens* and *P. canadensis*	NGS5(1)	Co12(71)	NGS6(3) NGS8(5) NGS5(314) NGS1(333) NGS7(2) NGS23(1)	NGS6(1)	Co12(169)	NGS6(5) NGS8(9) NGS5(532) NGS1(432) NGS7(1) NGS23(1)		NGS6(6) NGS8(9) NGS5(532) NGS1(432) NGS7(1) NGS23(1)
Clade 7	Other species in the '*P. gessardii*' subgroup	NGS25(23) NGS16(8)	Co2(11)	NGS25(28152) NGS3(12) NGS16(25105)	NGS25(12) NGS16(179)	Co2(35130)	NGS25(4585) NGS3(24) NGS16(110794)	NGS25(662) NFS16(513)	NGS25(110253) NGS3(24) NGS16(111486)
Clade 9	*P. sesami, P. saponiphila* and *P. protegens*	NGS4(7) NGS9(6) NGS2(1)	Co8(1)	NGS4(2865) NGS9(2251) NGS2(3646) NGS24(3003)	NGS4(77) NGS9(122) NGS2(16) NGS24(12)	Co8(7522)	NGS4(2912) NGS9(2272) NGS2(5412) NGS24(4585)	NGS4(3135) NGS9(4241) NGS2(151) NGS24(93)	NGS4(6124) NGS9(6635) NGS2(5579) NGS24(4690)

[a] In other Lascaux rooms, clade 5 was also evidenced on Passage walls.
[b] MiSeq sequences from cave walls are indicated as NGS, followed by the sequence number identified in Alonso et al. (submitted c), the number in parenthesis indicating the number of sequences obtained.
[c] MiSeq sequences only found in samples related to dark zone analysis (dark zones themselves or collembola from dark zones) are indicated in blue, whereas sequences evidenced both in black stain and dark zone investigations are in black.
[d] MiSeq sequences from collembola are indicated as Co, followed by the sequence number identified in Alonso et al. (submitted c), the number in parenthesis indicating the number of sequences obtained.

Discussion

Dark zones and black stains represent major wall surface alterations in lower areas of the Apse. They are visually very different, but both are largely colonized by collembola belonging to the same species *Folsomia candida*. Whether dark zones present the same microbial features as *bona fide* black stains was investigated by Illumina sequencing, which highlighted remarkable similarities between both, i.e. (i) microbial communities that differed clearly from neighboring unstained parts in both cases, (ii) the prevalence of *Ochroconis* and other pigmented fungi in contrast to the situation in unstained parts, (iii) the occurrence of these fungal taxa (and of numerous other microbial taxa colonizing altered surfaces) in *F. candida* sampled from the same dark zones or black stains, (iv) very sparse populations of *Pseudomonas* spp. even though these bacteria were predominant in unstained parts, (v) similar numbers of taxa, Chao1 richness indices, Simpson indices and Shannon indices, both for fungi and for bacteria, and (vi) similar quantitative PCR levels, both for 18S and for 16S rRNA genes. However, some microbial differences were also shown between dark zones or black stains, especially (i) in the structure of the fungal community and of the bacterial community (NMDS analysis), (ii) in OTU composition of surface alterations since 45% of fungal OTUs and 9% of bacterial OTUs found in dark zones were not present in black stains, and (iii) in OTU composition of collembola taken from surface alterations since 34% of fungal OTUs and 8% of bacterial OTUs found in dark-zone collembola were not present in black-stain collembola samples.

This study also showed that microbial communities differed when comparing unstained parts sampled next to dark zones and unstained parts close to black stains. On one hand, these differences could merely result from the spatial heterogeneity of microbial

geographic distribution within Lascaux, as suggested when comparing different areas on a same geological substrate in the Passage e.g. within the limestone inclined planes (Alonso et al. submitted b). This hypothesis is strengthened by NMDS findings for unstained parts of the Nave, sampled on the same substrate and in contiguous locations in comparison to the Apse, which showed also differences with Apse unstained parts. On the other hand, the possibility exists that these differences could reflect variability of (micro)environmental conditions on cave wall surfaces, which might translate into different conditions of microbial colonization and functioning and lead to different types of alteration processes. This possibility is substantiated by the fact that dark zones and black stains do not occur exactly at the same level of the walls in the Apse (Fig. S8). Both scenario are not mutually exclusive, which highlight the need to better understand the microbial mechanisms involved in wall surface alteration(s).

Whereas dark zones and black stains developed as separate visual alterations for years (since 2006 and 2008, respectively), different dynamics were noticed in recent years as black stains remained with the same visual appearance once they were formed, but certain dark zones evolved and a black stain (Fig. S9) progressively formed in the middle (without leading there to distinct microbial communities, however; Fig. S7). This may seem surprising, since (i) visual properties of dark zones and black stains (Fig. 1D,H) point to distinct formation processes, and (ii) all black stains monitored so far, in the Apse and elsewhere, formed without going through a preliminary dark-zone stage. However, this observation raises the possibility that a novel, multi-stage process of black stain formation had been started in the Apse, resulting perhaps from different priority effects in community assembly (Hiscox et al. 2015) which might, in turn, account for differences in early microbial functioning (Fukami et al. 2010). Further monitoring will be needed anyway to determine whether all dark zones are bound to form a black stain at some point of time, or if this process is restricted to a few dark zones present in specific Apse locations.

An intriguing feature of both dark zones and black stains is the abundance of *F. candida*, whereas these collembola are very seldom found outside of wall surface alterations in the Apse. The ecological significance of collembola in Lascaux has already been investigated (Bastian et al. 2009a; Bastian et al. 2010; Martin-Sanchez et al. 2012), including in the specific case of black stains formed in the Apse (Alonso et al. submitted c). Results point to two roles played by collembola in relation to black stain formation. First, *F. candida* collembola could be involved in dissemination of particular microorganisms including *Ochroconis* spp. and other black fungi, based on their splattering on black stains (Bastian et al. 2009a; Alonso et al. submitted c), ingestion of black fungus biomass and presence of viable conidia in fecal pellets (Bastian et al. 2009a), deposition of fecal pellets away from fungal colonies (Bastian et al. 2009a) and dissemination of black stain microorganisms themselves (Alonso et al. submitted c). This possibility is likely for dark zones as well, since the same collembola species was found there, microbial taxa present on black stains and potentially disseminated by *F. candida* (especially black fungi) were also established in dark zones, and many of the latter were evidenced in collembola sampled on dark zones. Second,

F. candida collembola could also be involved in recycling of microbial biomass and differential selection of microbial taxa, based on assimilation of microbial C and black stain constituents (Alonso et al. submitted c), decay of fungal colonies (Bastian et al. 2009a) and taxa-specific predatory trimming of bacteria when in high numbers (Thimm et al. 1998) *in vitro*. These *F. candida* effects are thought to promote establishment of black fungi (Bastian et al. 2009a) and subsequent melanin synthesis (Bastian et al. 2009a), but obviously this does not take place as such in dark zones, where the black color associated to melanin is not observed (at least as long as dark zones remain without any black stain center). Instead, visual observations suggest consumption of unstained microbial biofilms rather than microbial build-up. Therefore, further microscopy and transcriptomics-based assessments would be needed to decipher microbial processes in action.

In conclusion, this investigation showed that largely similar microbial dynamics were at play in dark zones compared with black stains of Lascaux's Apse, with probably the same role of collembola in promoting microbial dissemination and driving microbial selection. It also showed that very different visual properties could result from the functioning of microbial communities with seemingly-minor differences in structure and composition and colonizing similar types of limestone surfaces in Lascaux's Apse. These differences in microbial functioning need to be targeted to better understand cave wall alterations in Lascaux Cave.

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Supplementary data

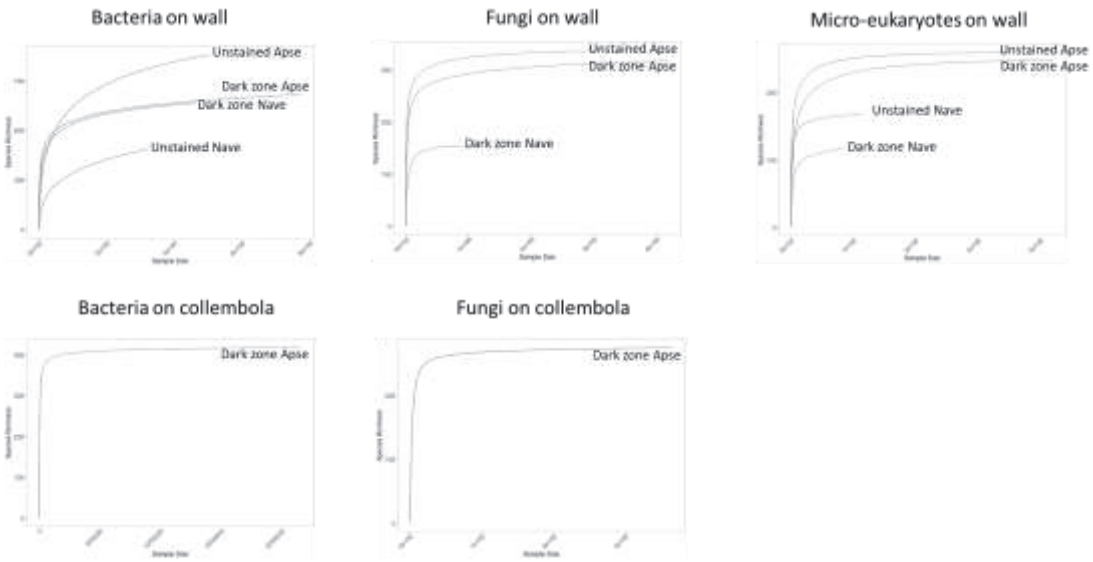

Figure S1. Rarefaction curves at OTU level for cave wall samples in the case of bacteria (16S rRNA gene dataset), micro-eukaryotes (18S rRNA gene dataset), fungi (ITS2 dataset) and for collembola samples in the case of bacteria (16S rRNA gene dataset) and fungi (ITS2 dataset), based on observed data. Wall samples originated from dark zones and neighbouring unstained parts (from the left and right walls), whereas collembola samples originated from dark zones (both left and right walls combined). Rarefaction curves reached an asymptote in 2 out of 4 cases with wall bacteria, 3 of 4 cases with wall micro-eukaryotes, all 3 cases with wall fungi, with collembola bacteria and with collembola fungi. Data for black stains and neighboring unstained parts are those from Alonso et al. (submitted c).

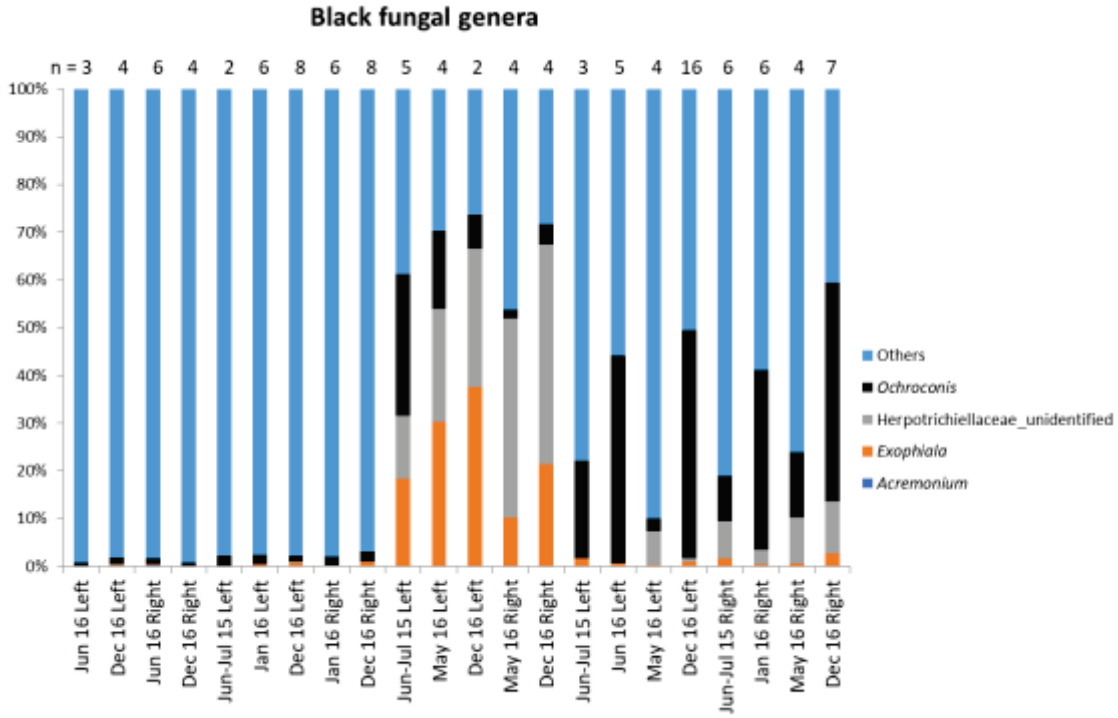

Figure S2. Community composition at genus level for fungi in dark zones (Dark), black stains (Black) and nearby unstained parts (respectively Near Dark and Near Black) sampled from the left (L) and right (R) walls of Lascaux's Apse in June-July 2015 (15J), January 2016 (16J), May-June 2016 (16M) and December 2016 (16D).

Black fungi (i.e. *Ochroconis*) are shown in black and taxa containing both pigmented and non-pigmented strains in grey (Herpotrichiellaceae) or orange (*Exophiala*). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 2-16 samples (indicated in each case). Data for black stains and neighboring unstained parts are those from Alonso et al. (submitted c).

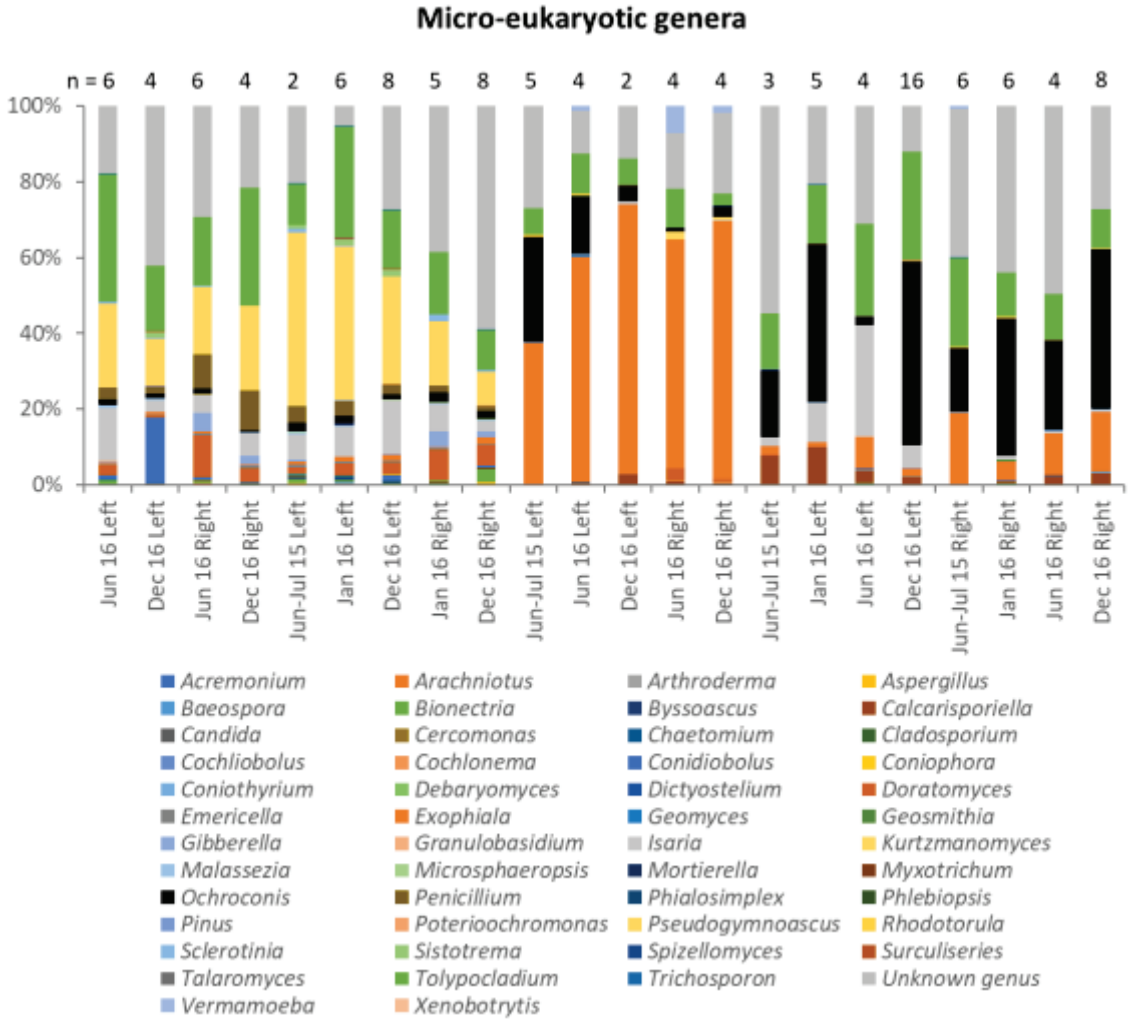

Figure S3. Community composition at genus level for micro-eukaryotes in dark zones (Dark), black stains (Black) and nearby unstained parts (respectively Near Dark and Near Black) sampled from the left (L) and right (R) walls of Lascaux's Apse in June-July 2015 (15J), January 2016 (16J), May-June 2016 (16M) and December 2016 (16D). Genera representing more than 1% of sequences are indicated. Each histogram is the average from 2-16 samples (indicated in each case). Data for black stains and neighboring unstained parts are those from Alonso et al. (submitted c).

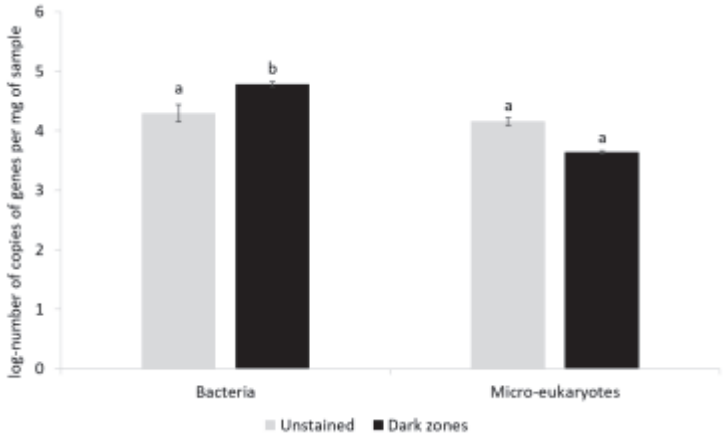

Figure S4. Abundance of the bacterial and micro-eukaryotic communities colonizing cave walls in the Apse. The values shown are means with their standard errors. Statistical differences between dark zones and unstained parts are shown with letters a-b (Wilcoxon tests, $P < 0.05$). Quantitative PCR was done using 16S rRNA primers 519F/907R (Laiz et al. 2003), 18S rRNA primers EUK345F/EUK499R (Zhu *et al.*, 2005) and a LightCycler 480 (Roche Diagnostics, Meylan, France). Briefly, the 16S rRNA and 18S rRNA genes reactions were carried out in 20-µl volumes containing 0.6 µl (final concentration 0.3 µM) of each primer, 4 µl of PCR-grade water, 10 µl of LightCycler-DNA Master SYBR Green I master mix (Roche Applied Science, Meylan, France) and 2 µl of sample DNA (5 ng). PCR was done with 10 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s, (ii) 63°C for 60 s, and (iii) 72°C for 30 s for 16S rRNA genes, and 2 min at 95°C, followed by 40 cycles of (i) 95°C for 15 s, (ii) 60°C for 15 s, and (iii) 72°C for 15 s for 18S rRNA genes. Melting curve calculation and Tm determination were done using the Tm Calling Analysis module of Light-Cycler Software v.1.5 (Roche Applied Science).

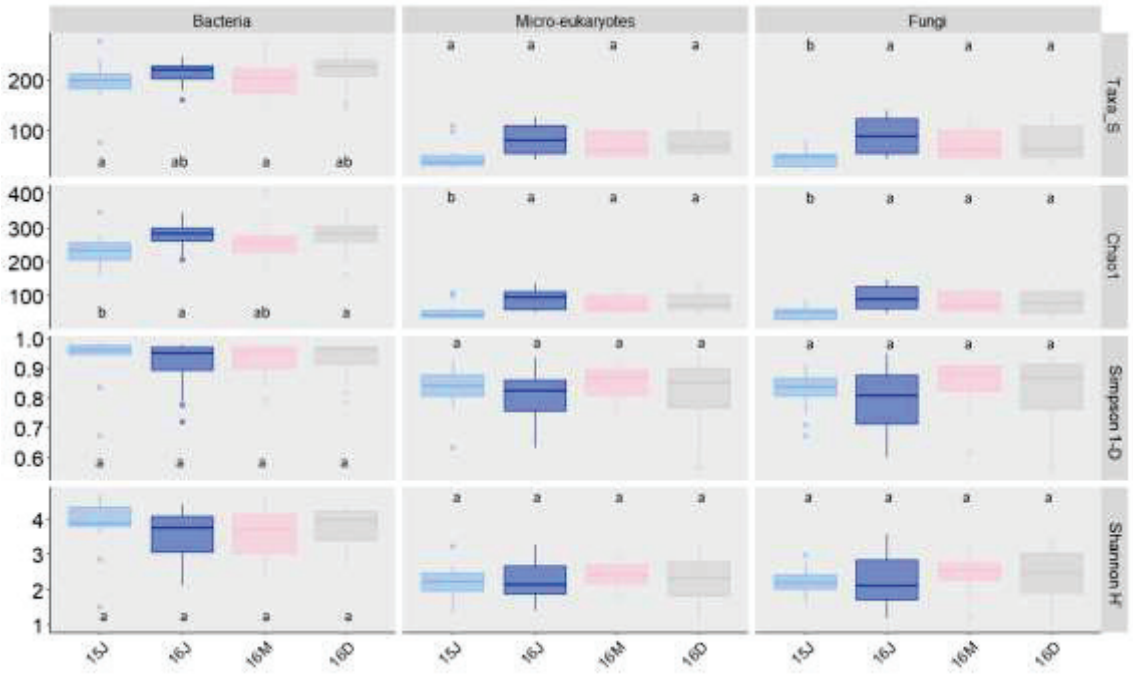

Figure S5. Number of OTU, Chao1 index of OTU richness, Simpson's index of diversity and Shannon index of OTU diversity for bacteria, micro-eukaryotes and fungi in Lascaux's Apse walls according to sampling time i.e. June-July 2015, January 2016, May-June 2016 and December 2016. Data were combined for wall conditions (dark zones and nearby unstained parts) of the left and right walls. Differences between conditions are shown with lowercase letters (based on ANOVA and Tukey's tests; $P < 0.05$).

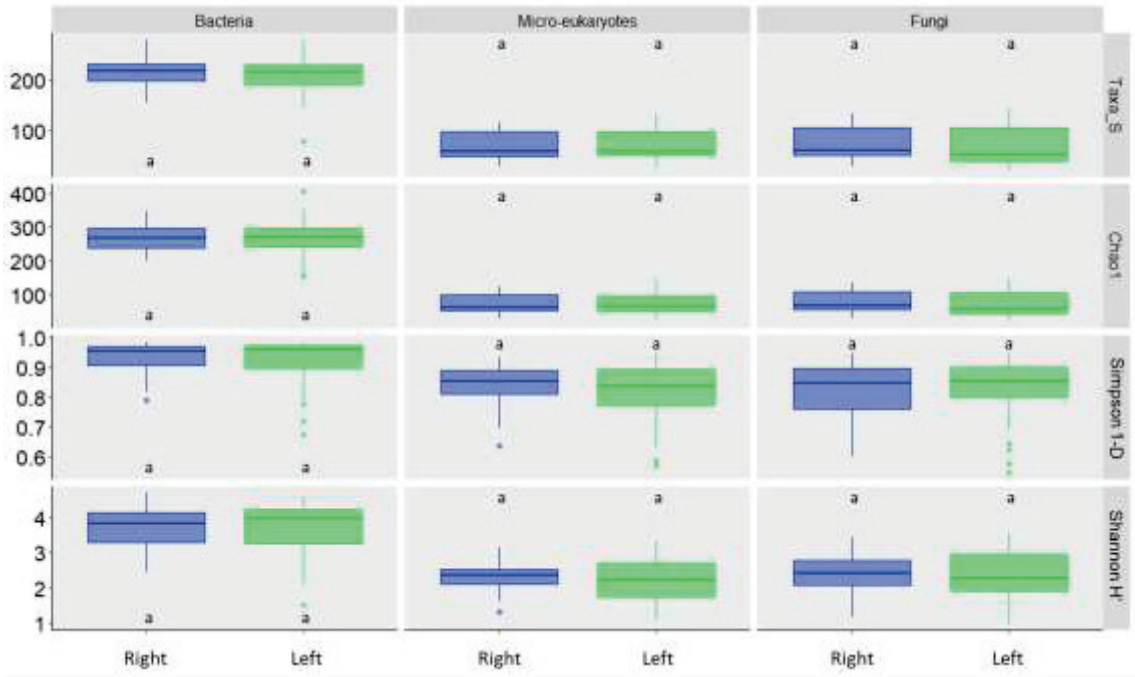

Figure S6. Number of OTU, Chao1 index of OTU richness, Simpson's index of diversity and Shannon index of OTU diversity for bacteria, micro-eukaryotes and fungi in Lascaux's Apse walls for the left vs right walls. Data were combined for the different sampling times (June-July 2015, January 2016, May-June 2016 and December 2016) and wall conditions (dark zones and nearby unstained parts). Differences between conditions are shown with lowercase letters (based on ANOVA and Tukey's tests; $P < 0.05$).

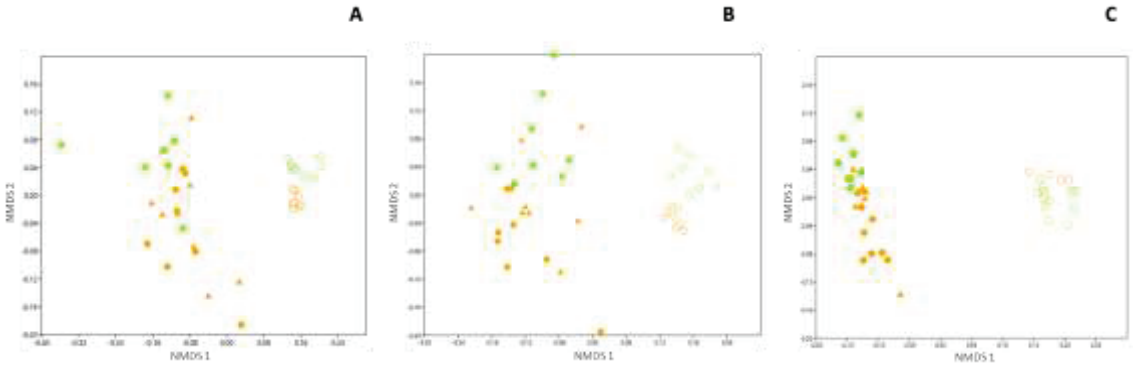

Figure S7. Microbial community structure comparison of black centers of dark zones (all of them sampled on the left wall), their remaining dark zones around black centers, full dark zones (from the right wall), and nearby unstained parts from the walls of Lascaux's Apse taken in June-July 2015, January 2016, May-June 2016 and December 2016, based on the relative proportion of both phyla and classes in the fungal (A), micro-eukaryotic (B) and bacterial communities (C). Apse data for dark zones and their neighboring unstained parts are those already shown in Figure 2 for the same sampling dates. Stress values were 0.15, 0.16 and 0.13 for fungi, micro-eukaryotes and bacteria, respectively. Left wall samples are represented with full orange triangles (black center of dark zones), full orange circles (remaining dark zones around black centers) or empty orange circles (unstained parts). Right wall samples are represented with full green circles (dark zones) or empty green circles (unstained parts).

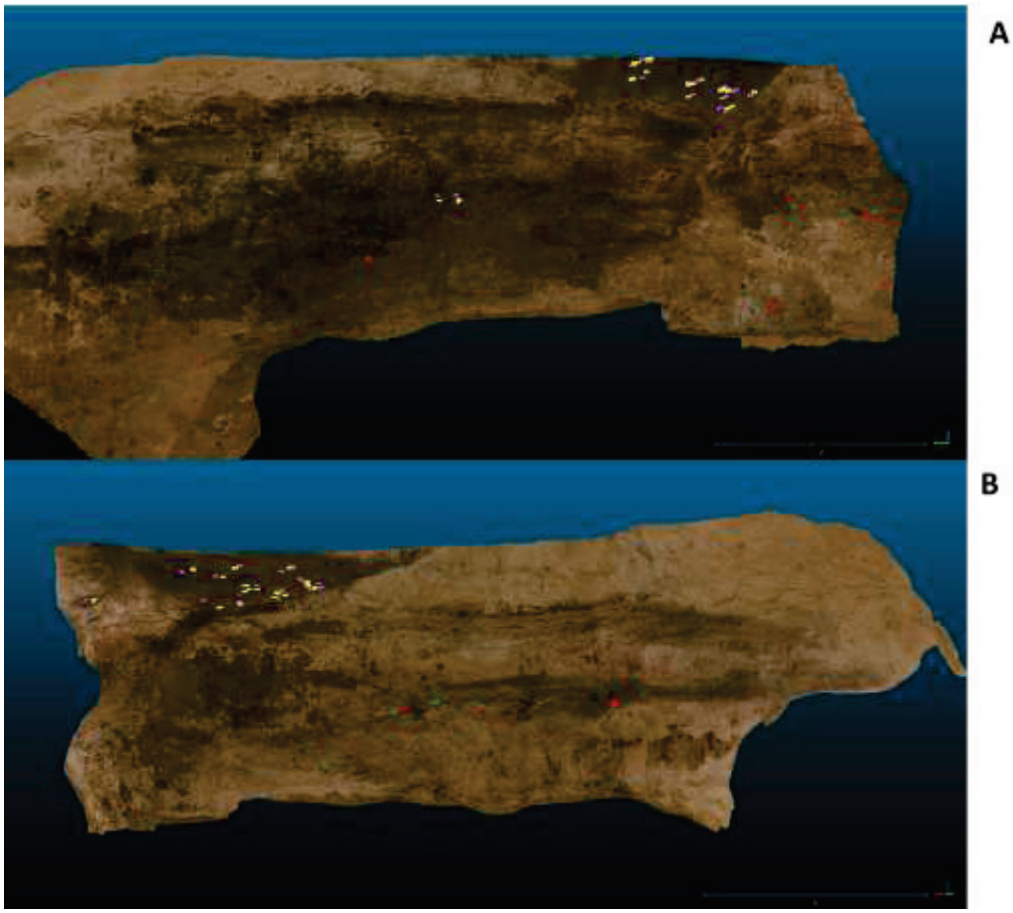

Figure S8. Location of samples taken to assess dark zones, black stains and unstained parts on a 3-dimension map, illustrated for the left (A) and for the right (B) wall of the Apse. Black stains are represented in red, unstained parts near black stains in green, dark zones in yellow and unstained parts near dark zones in purple.

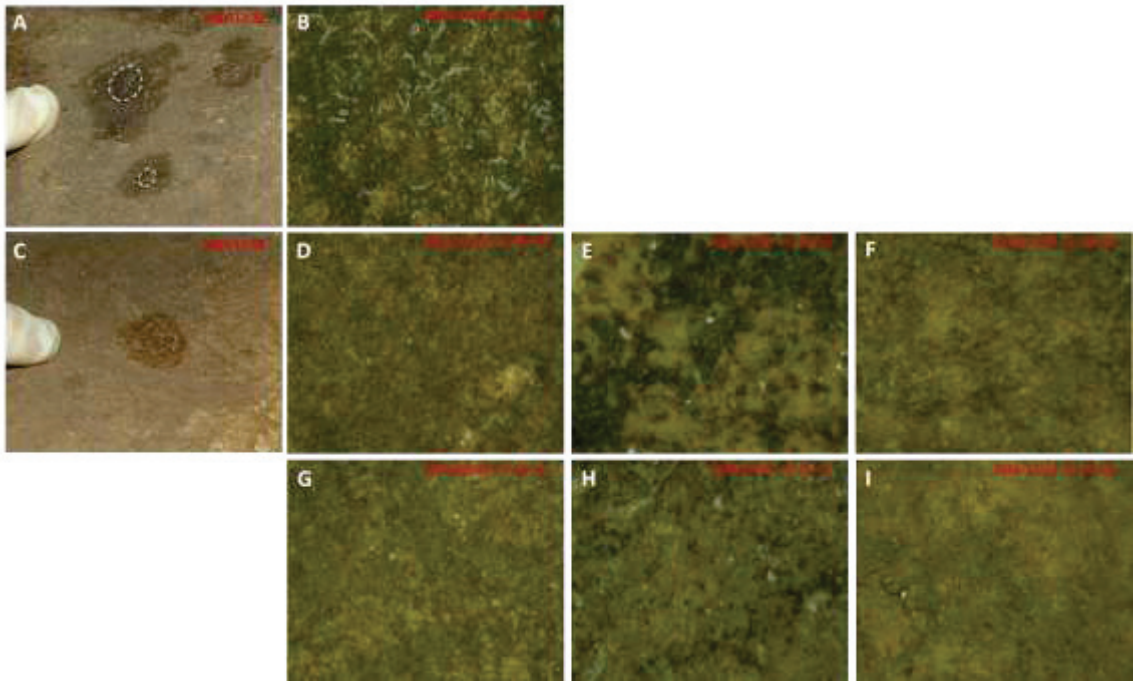

Figure S9. Photographs of a homogeneous dark zone (C) and a dark zone with a black stain developed in the middle (A) in the Apse (20th December 2016), and Dino-Lite microscope photographs of this central black stain (B, showing collembola remains) and of the rest of the dark zone (D) compared with outside parts nearby (G), as well as dark zones (E, F) and outside parts nearby (respectively H, I) in the Nave.

Chapitre 6 : Métatranscriptomique de la communauté microbienne

Avant-propos

Les chapitres précédents étaient orientés sur l'analyse de la diversité de la communauté microbienne de façon globale, sans prendre en compte son rôle fonctionnel. Ils montrent que les champignons noirs sont présents sur les taches noires et parfois en dehors de ces taches. Les altérations de parois ne sont pas seulement présentes dans le Passage et dans l'Abside mais aussi dans d'autres salles de la grotte, et par exemple dans le Sas-1 se trouvent des taches noires et dans la galerie du Mondmilch des taches jaunes.

Les taches noires du Sas-1 sont particulières car elles se trouvent sur une surface artificielle (paroi maçonnée constituée de blocs calcaire) de 80 × 80 cm^2 qui a été remise à nue en avril 2014. Cette zone est donc considérée comme une zone test car elle permet de faire un suivi de la formation des taches noires qui sont apparues sur cette surface dans les mois qui ont suivi.

Le mondmilch est un dépôt blanchâtre riche en eau et en calcite assez plastique (Borsato et al. 2000 ; Maciejewska et al. 2017), ce substrat minéral est situé dans la galerie du Mondmilch dans laquelle les taches jaunes se trouvent. La formation du mondmilch est attribuée à des processus biologiques (Maciejewska et al. 2017). Les bactéries peuvent être impliquées dans le processus de formation du mondmilch car elles génèrent du carbonate de calcium nécessaire à la précipitation de calcite lorsqu'elles dissolvent le substrat rocheux (Sweeting, 1973 ; Canaveras et al. 2006).

Ces différents types de taches se forment potentiellement de différentes façons, en outre à ce jour aucune information n'est disponible sur le fonctionnement des communautés de la grotte de Lascaux. Ainsi, en étudiant la dynamique fonctionnelle de différentes salles de la grotte à différentes dates et pour différentes situations (taches et zones non tachées) nous prenons en compte l'hétérogénéité présente dans la grotte pour comprendre le fonctionnement de la communauté microbienne et potentiellement la formation des taches. L'hypothèse est que les gènes impliqués dans le métabolisme de la mélanine ou autres pigments noirs soient surexprimés sur les taches noires en comparaison avec les zones non tachées.

Dans un premier temps, la structure et la composition de la communauté microbienne ont été étudiées par séquençage à haut débit en ciblant les marqueurs taxonomiques pour les bactéries, les archées, les micro-eucaryotes et les champignons afin de déterminer le facteur (temps, salle, présence de tache) qui façonne cette communauté. Les particularités ainsi observées pourront ensuite servir de base pour interpréter les différences fonctionnelles obtenues ultérieurement. Dans un second temps, une analyse métatranscriptomique a donc été réalisée sur deux substrats minéraux (banquettes et plans inclinés) du Passage prélevés à trois dates différentes (Décembre 2014, Juin-Juillet 2015 et Mai 2016), d'une part, et cinq situations (les banquettes et plans inclinés du Passage et trois salles différentes, le Sas-1, la Diaclase et le Mondmilch) ont été prélevées à une même date (Mai 2016), d'autre part, en prenant pour chaque situation une zone tachée (quand disponible) et une zone non tachée. Trois à six échantillons pour chaque situation ont été prélevés à l'aide d'un scalpel stérile. L'ARN des échantillons a ensuite été extrait et le séquençage à haut débit a été réalisé sur les ARN eucaryotes et procaryotes. L'analyse des séquences a été réalisée avec l'aide de la plateforme iBio de l'UMR Ecologie microbienne. L'assemblage des séquences et l'analyse discriminante du jeu de donné a été réalisé par Danis Abrouk, l'annotation des séquences par l'outil PRIAM a été effectué par Daniel Kahn et

l'expression différentielle des gènes a été réalisée avec l'aide d'Audrey Dubost (tous UMR Ecologie microbienne).

Les résultats de cette étude montrent que la diversité bactérienne des taches noires du Sas-1 différait de celle dans les zones non tachées et variait dans le temps. Plus généralement, lorsque l'on considère les différentes salles et taches, les niveaux de diversité microbienne varient principalement en fonction de la salle ou du substrat minéral, et dans une moindre mesure avec la présence de taches. La structure de la communauté différait en fonction de la salle de Lascaux ou du substrat minéral, ainsi que de la présence de taches, en particulier pour les banquettes du Passage. Ces différences sont encore plus grandes pour la composition taxonomique des communautés, et l'effet des taches était significatif pour le Mondmilch (taches jaunes) et les plans inclinés et les banquettes du Passage (taches noires), pour les bactéries et champignons (mais pas pour les archées). Les profils métatranscriptomiques basés sur les catégories fonctionnelles de gènes COG ne variaient pas en fonction des salles, du substrat minéral ou de la présence de taches sur la base des NMDS, mais des différences ont été trouvées lors de la comparaison des compositions de profils métatranscriptomiques. L'analyse inter-classe (BCA) a mis en évidence des profils spécifiques lors de la comparaison des taches et des zones non tachées, et divers types d'enzymes discriminantes pour les deux conditions. Cette analyse transcriptomique préliminaire de la grotte de Lascaux suggère que les différences dans la structure et la composition de la communauté peuvent se traduire par des différences dans le fonctionnement microbien.

Mon rôle dans ce travail a été le suivant : j'ai participé à la réflexion sur les objectifs scientifiques et la démarche expérimentale, à la préparation du matériel et aux prélèvements d'échantillons. J'ai réalisé l'extraction des ADN et des ARN de l'ensemble des échantillons. J'ai analysé les séquences destinées à l'analyse de l'ADN et participé à l'analyse des séquences destinées à l'analyse du métatranscriptome. J'ai participé aux analyses d'expression différentielle des gènes, et j'ai participé à la rédaction du manuscrit.

L'ensemble de ce travail a permis la rédaction de la publication « Metatranscriptomic analysis reveals functional dynamics of microbial communities in Lascaux Cave ».

Article 5 : Metatranscriptomic analysis reveals functional dynamics of microbial communities in Lascaux Cave

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Abstract

Excessive anthropization of Lascaux Cave during the last century has resulted in the development of stains in several rooms, in relation to proliferation of black fungi, but microbial composition and functioning in these stains is poorly documented. Here, microbial community structure and composition inside versus outside stains was investigated by Illumina sequencing of taxonomic DNA markers for bacteria, archaea, fungi, and micro-eukaryotes at large, including during pioneer colonization of a refreshed limestone surface in the airlock entrance zone. Illumina sequencing of mRNA extracts was performed and differentially-expressed genes were investigated.

NMDS indicated that bacterial diversity in new black stains on the refreshed limestone surface of Airlock-1 differed from that in unstained samples and varied in time, along with stain evolution. More generally, when considering different rooms and stains, microbial diversity levels varied mainly with room or geologic substrate, and to a lesser extent with stain presence. NMDS showed that community structure differed according to Lascaux room or geologic substrate, as well as stain presence especially for Passage's banks. These differences were stronger when assessing taxonomic community composition, and there the effect of stains was significant for Mondmilch (yellow stains) and Passage's inclined planes and banks (black stains), for bacteria and fungi (but not for archaea).

Simka-based multivariate analysis of raw mRNA sequences showed that metatranscriptomic data were structured according to Lascaux room or geologic substrate, but the effect of stain presence was significant only in Airlock-1. When focusing on the main COG gene categories, metatranscriptomic profiles did not vary with Lascaux room, geologic substrate or stain presence based on NMDS comparisons, but differences were found when comparing metatranscriptomic profile compositions. For instance, the presence of stains was associated with higher levels of gene categories J (ribosomal structure and biogenesis), L (replication, recombination and repair) and U (intracellular trafficking, secretion and vesicular transport) on Passage's inclined planes as well as banks. Between-class analysis evidenced specific profiles when comparing stains and unstained parts, and various types of discriminant enzymes for the two conditions.

This preliminary transcriptomic analysis of Lascaux Cave suggests that differences in community structure and composition may translate into differences in microbial functioning, which deserves further research attention.

Introduction

Underground ecosystems are well developed in calcareous areas, and many caves have been used by humans for shelter and/or prehistoric art (Jaubert et al. 2016; Hoffmann et al. 2018). Hundreds of caves have been operated for touristic activities owing to Paleolithic drawings, paintings and engravings (Schabereiter-Gurtner et al. 2002; Dupont et al. 2007) or outstanding speleothems. These show caves have undergone substantial environmental change as a result of adaptation work, light system functioning, and the presence of visitors consuming oxygen, releasing carbon dioxide, body heat and various organics, and facilitating the arrival of external microorganisms (Bastian et al. 2009; Jurado et al. 2010). Such anthropization may lead, in turn, to changes in cave microbial ecology (Pfendler et al. 2018), and alterations in cave wall communities especially prokaryotes were evidenced when comparing a range of pristine and touristic caves in the same Périgord area of southwestern France (Alonso et al. submitted a).

In some cases, anthropization may occasionally lead to erratic microbial growth and stain formation on cave walls. This is typically managed using mechanical removal and/or targeted chemical treatments, which can control microbial outbreaks but might also disturb other microbial community members (Mitova et al. 2017). In Lascaux Cave, a Paleolithic art landmark open to the public until 1963, anthropization and in particular chemical control methods resulted in a series of microbial crises in the last five decades. In 1960, a green algal biofilm (termed 'green disease') formed on wall paintings, most likely due to changes in light and CO_2 conditions (Bastian et al. 2010). A combination of streptomycin, penicillin (against bacteria) and formaldehyde (against algae) were sprayed to curb microbial development, but treatments were not satisfactory as resistant microorganisms appeared. In 2001, white mycelium of the fungus *Fusarium solani* invaded a large part of the cave, which was handled using benzalkonium chloride with antibiotics (Dupont et al. 2007). Since 2004, occasional microbial outgrowths were removed by mechanical cleaning, and the air extraction system was replaced. Black stains (termed 'black disease') were noticed on cave walls in 2006, and they were treated using mechanical cleaning and chemical treatments (Bastian et al. 2010). The air extraction system was shut down in January 2015.

The unusual anthropization level of Lascaux Cave resulted in more drastic effects on cave microbial communities in comparison with other touristic caves of the same area, even when analyses were restrained to unstained areas (Alonso et al. submitted a). When focusing on black stains that formed in Lascaux's Passage, it appeared that microbial community diversity depended on the presence of black stain as well as the geologic substrate underneath (Alonso et al. submitted b), suggesting that spatial heterogeneity is a key issue to consider to understand Lascaux's microbial ecology. Black fungi, such as *Ochroconis lascauxensis*, were evidenced in black stains, but they were also present in unstained cave wall samples. On this basis, it is clear that the occurrence of black fungi on Lascaux walls is not a sufficient criterion for black stain development, and it indicates that the functioning of the microbial community must be taken into account to understand the

formation of these black stains. However, no information is available to date on microbial functioning within Lascaux Cave.

The objective of this work was to assess the transcriptional activities of microorganisms colonizing the walls of Lascaux. Wall samples were taken in different rooms and/or on different geologic substrates of Lascaux Cave, and they included both black stains and neighboring unstained parts when available. In particular, we took advantage of restoration work in the airlock entrance zone, which generated a refreshed limestone surface where the establishment of pioneer microorganisms could be monitored. First, microbial community structure and composition was investigated using Illumina sequencing of taxonomic DNA markers for bacteria, archaea, fungi, and micro-eukaryotes at large. Second, Illumina sequencing of mRNA extracts was carried out to compare the transcriptomic profiles of cave wall microbial communities according to the Lascaux room/geologic substrate or the presence of stain. Third, differentially-expressed genes were investigated to identify potential microbial pathways associated with stain formation. This is the first microbial transcriptomic analysis of cave ecosystems.

Materials and methods

Sampling

Rock wall surface of Lascaux Cave was sampled in four different areas (the second section of Airlock-1 entrance zone, the Passage and the Mondmilch Gallery in the main Lascaux compartment, as well as the Shaft of the Dead Man in the Diaclase compartment located underneath), two kinds of mineral substrates (limestone and plastic material corresponding to clay deposits or mondmilch speleothems) and/or two surface qualities (stains and unstained parts) (Table 1). In Airlock-1, which is formed by cemented vertical walls comprised of cut limestone stones chemically analogous to Lascaux limestone, a 80 × 80 cm^2 area of stone was scrapped off (~2 cm depth) on April 2014 to monitor microbial recolonization by pioneer taxa, and this scrapped area was used here to sample black stains developing since mid-2014 as well as neighboring unstained surfaces (Fig. S1).

Samples to assess microbial community transcriptomics were taken in May 2016. Since the main difference in environmental conditions is between winter and spring (drier seasonal conditions), the effect of seasonal variations was investigated in the Passage, by taking another set of Spring samples (June 2015) and sampling also in winter (December 2014). Three to six replicate samples were taken for each condition, which gave a total of 60 samples for transcriptomics. The same types of samples were taken, at the same time, to assess microbial community structure and composition, except that Mondmilch samples were not available in May 2016 and previous samples from December 2014 were used instead. For the Airlock-1 entrance zone, additional samples were also collected in June 2015 and January 2016 to monitor stain development (DNA analysis). A total of 3 to 18 replicate samples were taken for each condition, which gave a total of 108, 44, 94 and 92 samples for

structure and composition analysis of bacterial, archaeal, micro-eukaryotic and fungal communities, respectively.

Samples were taken using sterile scalpels (~ 50 mg). They were placed in liquid nitrogen shortly afterwards, until DNA or RNA extraction.

Table 1. Number of samples for which sequencing was successful and resulted in sequences used in the study (based on initial numbers of samples of n = 3, 6 or 18).

Room	Substrate	Surface quality[b]	December 2014	June 2015	January 2016	May 2016	December 2016
DNA analyses							
Airlock-1	Limestone (built wall)	Unstained parts	-	3 / 3[a]	5 / 6	0 / 6	0 / 6
		Black stains	2 / 4	5 / 6	5 / 6	6 / 6	3 / 6
Passage	Limestone (inclined planes)	Unstained parts	3 / 3	2 / 3	-	6 / 6	-
		Black stains	5 / 6	6 / 6	-	6 / 6	-
	Clay (banks)	Unstained parts	3 / 3	3 / 3	-	18 / 18	-
		Black stains	2 / 3	2 / 3	-	18 / 18	-
Mondmilch	Mondmilch	Unstained parts	3 / 3	-	-	-	-
		Yellow stains	3 / 3	-	-	-	-
Diaclase	Limestone	Unstained parts[b]	-	-	-	4 / 6	-
RNA analyses							
Airlock-1	Limestone (built wall)	Unstained parts	-	-	-	3 / 3	-
		Black stains	-	-	-	3 / 3	-
Passage	Limestone (inclined planes)	Unstained parts	3 / 3	3 / 3	-	6 / 6	-
		Black stains	6 / 6	6 / 6	-	6 / 6	-
	Clay (banks)	Unstained parts	3 / 3	3 / 3	-	3 / 3	-
		Black stains	3 / 3	3 / 3	-	3 / 3	-
Mondmilch	Mondmilch	Unstained parts	-	-	-	3 / 3	-
		Yellow stains	-	-	-	3 / 3	-
Diaclase	Limestone	Unstained parts	-	-	-	3 / 3	-

[a] 3/3 means sequencing was successful for 3 samples out of 3.

[b] Monitoring of stain development in Airlock-1 was done using blue DNA sequences. No stain was found in the Diaclase.

DNA extraction and sequencing

DNA extraction was carried out with the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), following the manufacturer's instructions. Briefly, elution was performed using two 50-µl volumes for each sample, and the final DNA concentration was measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) following the manufacturer's instructions. The DNA extracts were kept at -20°C. Four gene markers were used to assess bacteria (16S rRNA genes), archaea (16S rRNA genes), fungi (ITS) and micro-eukaryotes at large (18S rRNA genes), after PCR amplification (Table S1) and sequencing done by Fasteris company (Geneva, Switzerland). Illumina MiSeq sequencing was performed using 2 × 300 bp, paired-end chemistry to reach 70,000 paired reads for each sample.

RNA extraction and sequencing

RNA extraction was performed using ZR Soil/Fecal RNA MicroPrep (Zymo Research, Irvine, CA), following the manufacturer's instructions. Briefly, samples were weighed and placed with beads in lysis buffer. Cells were lysed, DNA was retained in the first column (discarded), RNA was bound in the second column, washed and eluted, and inhibitors were removed. RNA concentration was determined using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Eugene, OR) following the manufacturer's instructions. RNA extracts were stored at -80°C until further analysis. A DNAse treatment was applied on RNA extracts, followed by

rRNA depletion using Ribo-Zero (Bacteria) protocol (Illumina, San Diego, CA) carried out by Fasteris sequencing Company (Geneva, Switzerland). RNA was reverse transcribed with custom primers for bacteria (805R; Klindworth et al. 2013), micro-eukaryotes (NSR399; Dollive et al. 2012) and fungi (ITS4; Toju et al. 2012), and a library was prepared by Fasteris using Illumina TruSeq stranded mRNA kit. The library was sequenced on Illumina HiSeq with 2 × 125 paired-end chemistry, to obtain 22-28 million reads per sample.

Bioinformatic analysis

For metabarcoding, paired-end reads of all four genes were demultiplexed. To this end, adaptators were removed and sequences with two mismatches with primer sequences were also discarded, using a Fasteris Perl script. Sequences were merged using Fast Length Adjustment of Short reads (FLASh) with a 10% mismatch maximum in the overlapping region. Denoising involved discarding reads without the expected 200-500 bp length or containing an ambiguous base (N). After sequence dereplication, clusterisation was performed using SWARM (Mahé et al. 2014), based on a local clustering limit (rather than a global clustering limit) and an aggregation distance = 3 to define operational taxonomic units (OTUs). The lower taxonomic identification obtained was at genus or species level, which was hereafter referred to as the 'genus/species' level. The chimeras were removed using VSEARCH tool (Rognes et al. 2016) and low-abundance sequences were filtered at ≥ 0.005%, which means OTUs representing ≥ 0.005% of all sequences were retained (Bokulich et al. 2013), and also the singletons were removed from the datasets. Taxonomic affiliation was carried out using RDP Classifier (Lan et al. 2012) against the 119 SILVA database (Pruesse et al. 2007) for the bacteria and archaea, as well as the 123 SILVA database for micro-eukaryotes at large and the UNITE database for fungi (Kõljalg et al. 2013). The set of procedures was implemented in FROGS (Escudié et al. 2017). For sample comparisons, normalization was performed by resampling randomly to 3000 (for bacteria), 2108 (for archaea), 14252 (for fungi) and 14966 sequences (for micro-eukaryotes at large).

Microbial communities were primarily compared with non-metric multidimensional scaling (NMDS), using the Paleontological Statistics (PAST) software v3.14 (Hammer et al. 2001). The procedure computes a stress value, which measures the difference between the ranks on the ordination configuration and the ranks in the original similarity matrix for each replicate. Stress values below 0.1 are considered without risk of drawing false inferences, values below 0.2 acceptable (especially those close to 0.1), while values above 0.2 indicate limited interpretation potential. Analysis of similarity (ANOSIM) was conducted using the VEGAN package in R 3.3.0, to test differences ($P < 0.05$) in overall community composition between different caves and to further confirm the results observed in the NMDS plot. A Bonferroni correction was applied on *P* values to lower alpha risk.

For transcriptomics, the first steps of bioinformatic analysis were performed on a Galaxy web platform 0.9.6.0 (Afgan et al. 2016). Paired reads were merged using PEAR (Paired-End read merger) (Zhang et al. 2014). The quality control procedure was carried out using FastQC, and sequences were filtered and trimmed with PRINSEQ (Schmieder and

Edwards 2011). Sequences smaller than 60 bp were removed. Then, strictly identical sequences were merged using VSEARCH tool (Rognes et al. 2016) and the remaining rRNA fragments were filtered using SortMeRNA (Kopylova et al. 2012) and discarded. A k-mer based classification with Simka (Soft Independent Modeling of Class Analogy) was performed to identify local models for possible groups and choose the assembly strategy. Finally, transcript sequences were assembled sample by sample with Trinity (Grabherr et al. 2011), and transcript expression was quantified using Salmon tool (Patro et al. 2017). Proteins families were assigned based on homology with the database of Clusters of Orthologous Groups (COG) (Tatusov et al. 2000) and predicted enzymes of our dataset were detected using PRIAM (Claudel-Renard et al. 2003). NMDS analysis (see above) was carried out using RNA sequences to compare treatments based on functional families of proteins. Enzymes corresponding to mRNA sequences found on stains and outside stains were compared with Between-Class Analysis (BCA) and Monte-Carlo tests with 10,000 permutations using ADE4 R package. The 20 most discriminating enzymes for stains and 20 most discriminating enzymes for neighboring, unstained parts were identified, and they are listed using only the first three numbers (i.e. without serial numbers) to simplify comparisons.

Results and discussion

Microbial diversity varies with black stain development in Lascaux's Airlock-1 entrance zone

Restoration work was carried out in the airlock-1 entrance zone, with the objective of assessing whether a refreshed limestone surface (which displayed a few small black stains) could also undergo the development of new black stains. This indeed was the case after surface work was completed, which enabled to monitor the establishment of pioneer microorganisms involved in stain formation. It is striking that new black stains formed within months of restoration work, since little took place in recent years in Lascaux in terms of new stain formation or enlargement of existing black stains, and certain black stains even underwent attenuation.

Analysis of unstained parts of the refreshed limestone surface proved difficult, as DNA yield was often insufficient for Illumina sequencing, yet analysis of DNA microbial markers was successful at two of the five sampling dates and NMDS showed that the corresponding bacterial communities were similar (Fig. 1). In comparison, DNA yields were better for black stain samples (pointing to enhanced microbial biomass), and NMDS analysis showed that the bacterial community in black stains differed (i) from unstained samples available and (ii) in time, at least when considering later sampling dates. In Airlock-1, the phylum taxonomic profile of bacteria (i.e. the relative proportions of all bacterial phyla in the community) in black stains compared with unstained parts displayed higher levels of Bacteroidetes (5.9-15.2% vs 2.9-4.1%), Chlamydiae (1.4-3.5% vs 0.4-0.6%), Planctomycetes (7.2-8.8% vs 1.3-4.1%), Verrucomicrobia (2.2-3.8% vs 0.3-0.5%) and lower levels of Acidobacteria (1.4-3.0% vs 3.4-3.6%), Proteobacteria (35.3-36.8% vs 42.5-45.5%), SBR1093

(0.01-0.7% vs 1.1-1.8%) (Fig. 2B). These profiles evolved in time for black stains but remained largely the same for the two samplings available outside stains. The changes in microbial features are consistent with changes in the appearance of the stains that were observed in the course of the study (Fig. S1).

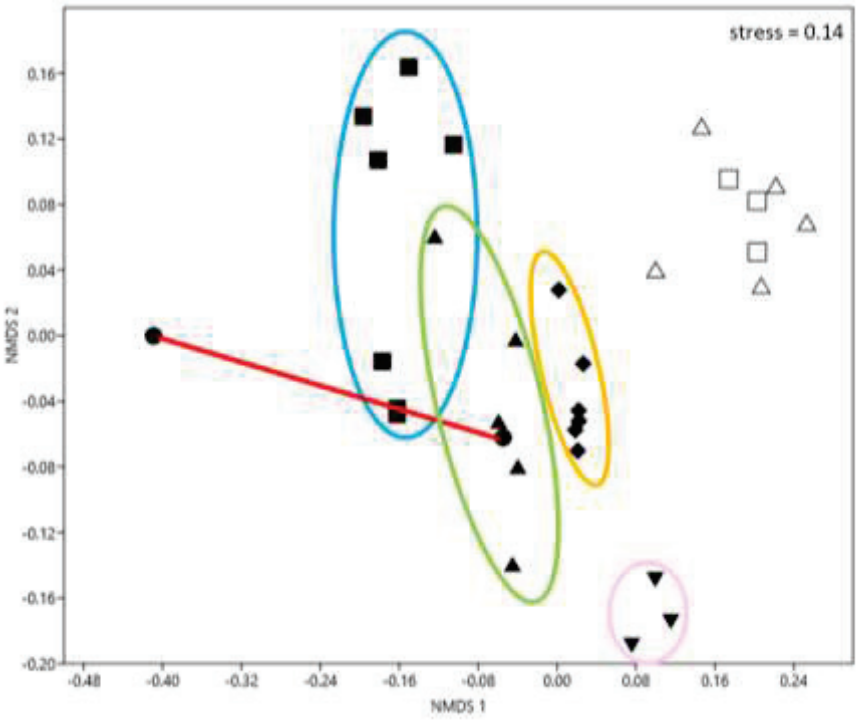

Figure 1. NMDS comparison of Airlock-1 samples in time. The full symbols represent samples from black stains and empty symbols the samples from unstained parts. Circles represent the 2 samples successfully analyzed in December 2014, squares the 10 samples in June 2015, triangles the 10 samples in January 2016, diamonds the 6 samples in June 2016 and inverse triangles the 3 samples in December 2016.

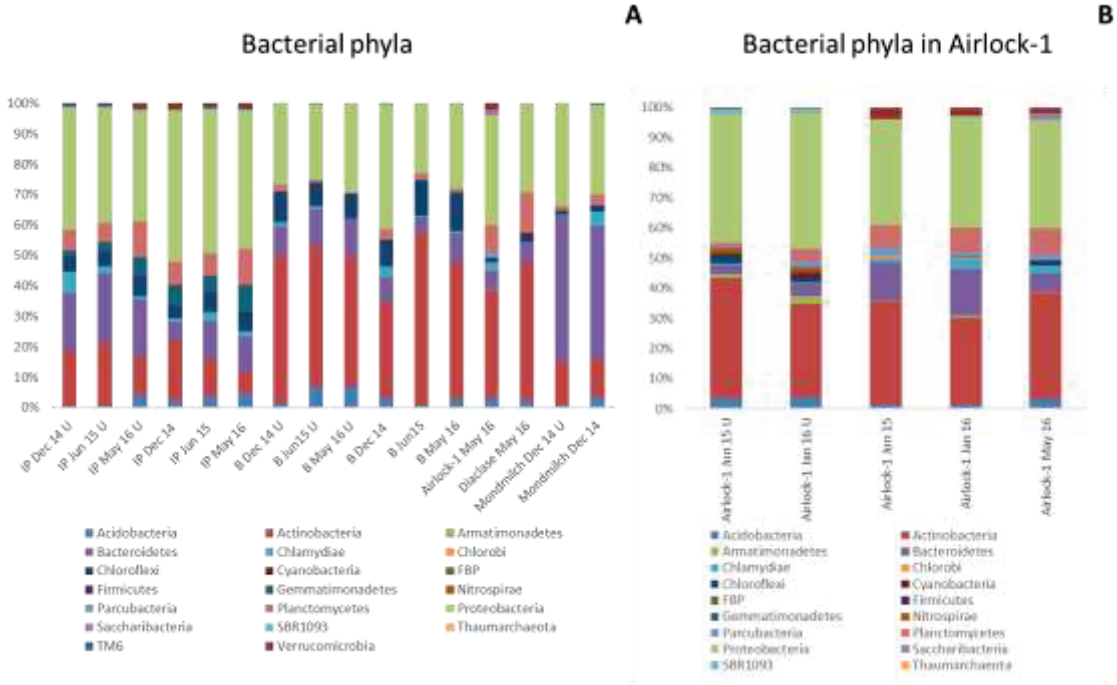

Figure 2. Bacterial community composition at phylum level for Passage's inclined planes and Passage's banks on December 2014, June 2015, May 2016, Airlock-1 and Diaclase on May 2016 and Mondmilch on December

2014 (A) and Airlock-1 samples at different dates (B). Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks), date (abbreviated month and year) and sample type (U for unstained parts).

Microbial diversity level varies with Lascaux room or geologic substrate, as well as stain presence

Since gene expression patterns are likely to depend strongly on the proportion of the different community members present (Moller et al. 1998), microbial community structure and composition of all the ecological conditions considered in the RNA study was investigated as a first step. A total of 1 795 302, 491 382, 5 824 435 and 5 163 029 full-length non-chimeric Illumina DNA sequences were obtained for cave wall bacteria (16S rRNA genes), archaea (16S rRNA genes), fungi (ITS) and micro-eukaryotes at large (18S rRNA genes), respectively. All rarefaction curves derived from OTU data were asymptotic except for bacteria (Fig. S2). Biodiversity was assessed based on Chao1 index (for genus/species richness), Shannon H' diversity index (which considers the numbers of both individuals and genera/species), and Simpson's 1-D index of diversity (which represents the likelihood that two randomly-selected individuals belongs to different genera/species). These diversity criteria indicated that for bacteria, Chao1 index was higher (i) on stains than outside stains for banks and (ii) on inclined planes than on banks of the Passage for unstained parts, while Shannon H' and Simpson's 1-D indices were similar in all rooms. For archaea, Chao1 index did not vary according to room, geologic substrate or stain presence, and both Shannon H' and Simpson's 1-D indices were higher in Airlock-1 (for unstained parts in June 2015 and black stains in May 2016) than in other Lascaux conditions studied (Fig. 3). For micro-eukaryotes, Chao1 index did not vary between the conditions tested. Shannon H' and Simpson's 1-D indices were higher on inclined planes and lower on Airlock-1 walls than in other rooms, and presence of stain resulted in higher Shannon H' and Simpson's 1-D indices when considering banks. For fungi, Chao1 index was higher on banks than in other conditions, and Shannon H' and Simpson's 1-D indices were lower in Airlock-1 than in other rooms. The three indices did not vary according to presence of stains.

Thus, biodiversity indices depended largely on room and/or geologic substrate, and to a lower extent on stain presence, but they did not differ in time. These results are consistent with previous findings derived from detailed analysis of the Passage, as stain presence proved less influential that geologic substrate in determining microbial community features (Alonso et al. submitted b).

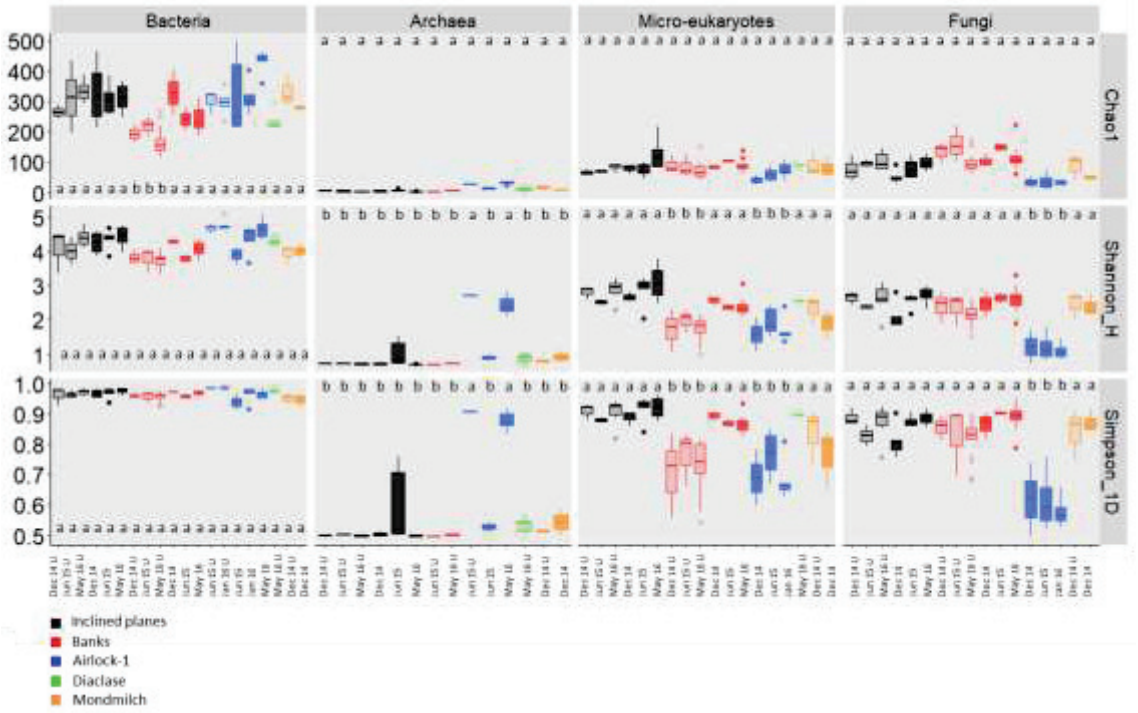

Figure 3. Diversity indices of cave wall samples according to time and presence of stains. Passage's inclined planes are represented in black, Passage's banks in red, Airlock-1 in blue, Diaclase in green and Mondmilch in orange. Dark colors correspond to stains and light colors to unstained parts. Treatment information includes the date (abbreviated month and year) and sample type (U for unstained parts).

Community structure varies with Lascaux room or geologic substrate, as well as stain presence

Based on NMDS, the structure of the bacterial community of (i) Airlock-1, (ii) Passage's banks and (iii) Passage's inclined plane, Diaclase and Mondmilch formed three distinct groups (Fig. 4A). The effect of stain presence was significant on Airlock-1 wall (ANOSIM R = 11.6, *P* = 0.004) and Passage's banks (ANOSIM R = 12.1, *P* = 0.004), but not for inclined plane or Mondmilch. The structure of the archaeal community differed between Airlock-1, Passage's banks, and all three other locations together (Fig. 4B). The effect of stain presence was not significant, but sequencing was successful only for one unstained sample in Airlock-1. The structure of the fungal community differed between Airlock-1, Passage's banks, Passage's inclined plane and Mondmilch, whereas Diaclase samples were in intermediate position (Fig. 4D). The effect of stain presence was significant on Passage's banks (ANOSIM R = 0.39, *P* = 0.002) but not on Passage's inclined plane or Mondmilch, whereas sequencing was unsuccessful for unstained samples in Airlock-1. The structure of the micro-eukaryotic community at large differed between Airlock-1, Passage's banks and Passage's inclined plane, whereas Mondmilch and Diaclase samples were in intermediate position (Fig. 4C). The effect of stain presence was not significant for Passage's inclined plane or Mondmilch, but was significant for Passage's banks (ANOSIM R = 0.45, *P* = 0.003).

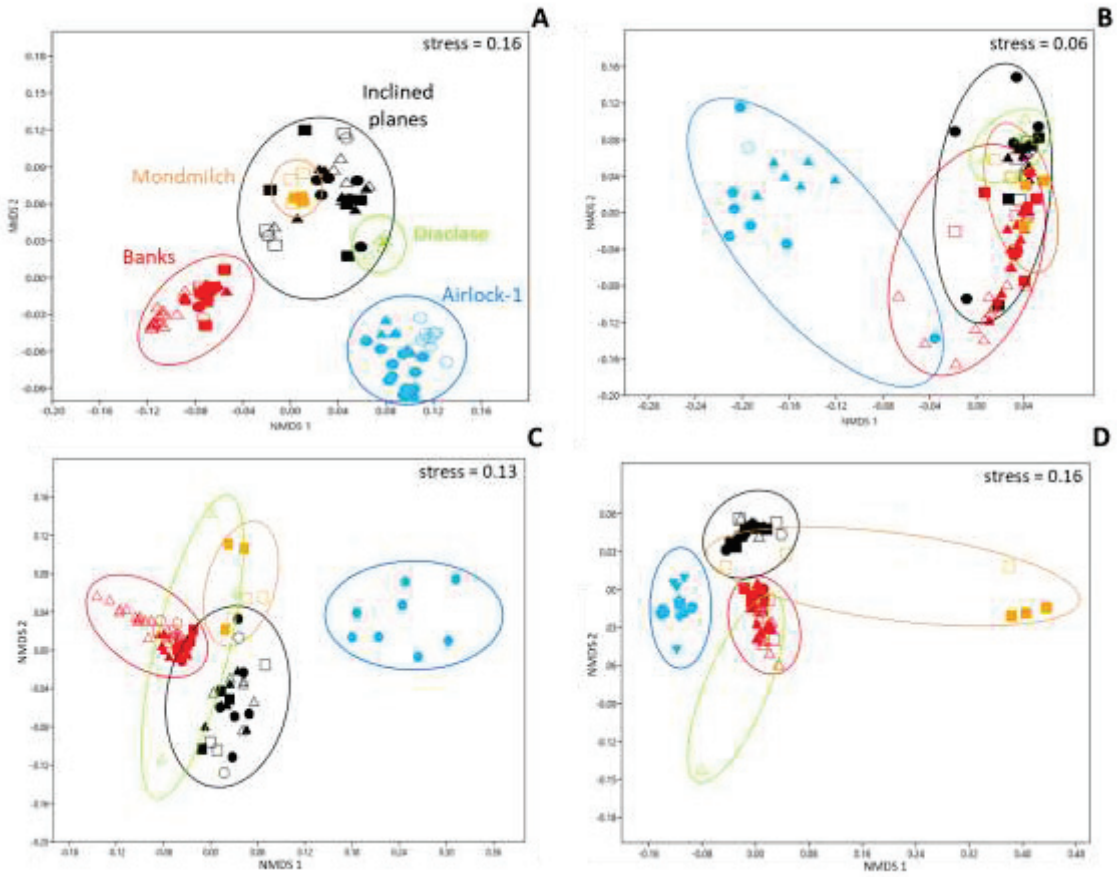

Figure 4. NMDS comparison of cave wall samples from Airlock-1, Passage's banks, Passage's inclined planes, Mondmilch and Diaclase based on the relative proportion of both phyla and classes in the bacterial (A), archaeal (B), micro-eukaryotic (C) and fungal communities (D). Samples are represented by squares (December 2014), circles (June 2015), diamonds (January 2016), triangles (May 2016), inversed triangles (December 2016) and crosses (May 2017). Full samples correspond to stains and empty samples to unstained samples. Stress values were 0.16 for bacteria, 0.06 for archaea, 0.13 for fungi and 0.16 for micro-eukaryotes at large. Arbitrary ellipses were drawn by hand to facilitate treatment identification.

In the Passage, microbial community structure differed according to geologic substrate (Alonso et al. submitted b) and here these findings could be considered at a larger scale by comparing different rooms of Lascaux. The results obtained point to room-specific microbial communities, but with a certain level of overlap. The proximity of Passage's inclined planes and Diaclase in the case of prokaryotes was not unexpected, based on similarities in geologic substrate properties and anthropization levels (Alonso et al. submitted a), even though it did not show up with fungi. Most Airlock samples tended to group away from samples from other milestone surfaces (i.e. Passage's inclined planes and Diaclase), pointing to the particular conditions of pioneer colonization and perhaps also climatic specificities of the entrance zone. Similarities might have been expected also between Passage's banks and Mondmilch based on physical surface properties, but surprisingly Mondmilch samples were closer to samples from inclined planes than from banks. The results also suggest somewhat distinctive environmental effects on the four microbial communities studied, as indicated by the different relations between

room/substrate conditions and the different variability levels of microbial communities within room/substrate conditions.

The different room/geologic substrate conditions investigated also enabled the analysis of stain effects, in the Passage and Airlock-1 for black stains and Mondmilch for yellow stains. Stain development might be the result of very particular micro-spatialized conditions and probably leads, in turn, to the establishment of novel micro-environmental conditions following the development of pigmented microorganisms and the presence of pigments themselves. Against this background, however, the relative effects of stain presence on microbial community structure were rather modest, as differences were significant only for bacteria in Airlock-1, and for bacteria, microeukaryotes and fungi on Passage's banks. This suggests that the occurrence of pigmentation does not necessarily require a drastic change in microbial community structure, pointing to the importance of microbial physiology changes in relation to stain formation. In addition, there was no evidence for convergent microbial community structures in stains, even when considering black stains only, which means that stains can be formed in microbial communities of contrasted genetic structure.

Taxonomic community composition varies with Lascaux room or geologic substrate, as well as stain presence

Overall, 20 phyla and 198 genera were evidenced for bacteria, 3 phyla and 5 classes for archaea, 23 phyla and 80 genera for micro-eukaryotes, and 5 phyla and 65 genera for fungi (58 of these 65 already found in the micro-eukaryote dataset). The phylum taxonomic profile of bacteria differed according to the room (Airlock-1 vs Passage vs Mondmilch vs Diaclase), the geologic substrate (banks vs inclined plane in the Passage) and the presence of stain (for the Mondmilch, for inclined planes and banks in the Passage), whereas the effect of time was comparatively neglectable (Fig. 2A). For room × geologic substrate combinations (unstained samples), these differences were linked to the relative prevalence of particular phyla (> 48% Bacteroidetes in the Mondmilch, > 46% Actinobacteria on Passage's banks and Diaclase, > 45% Proteobacteria on Passage's inclined planes) or to particular phyla combinations. In comparison with unstained samples, the presence of stains was associated with higher levels of Proteobacteria and lower levels of Bacteroidetes (on Passage's inclined planes), as well as higher levels of Chloroflexi (on banks) and Chlamydiae (on Mondmilch).

The phylum taxonomic profile of archaea differed in Airlock-1 compared with other rooms (Passage, Mondmilch, Diaclase), in relation to the relative prevalence of Soil Crenarchaeotic Group (2.1% in Airlock-1 vs 97.7-99.7% elsewhere), but did not differ according to stain presence (Fig. 5A).

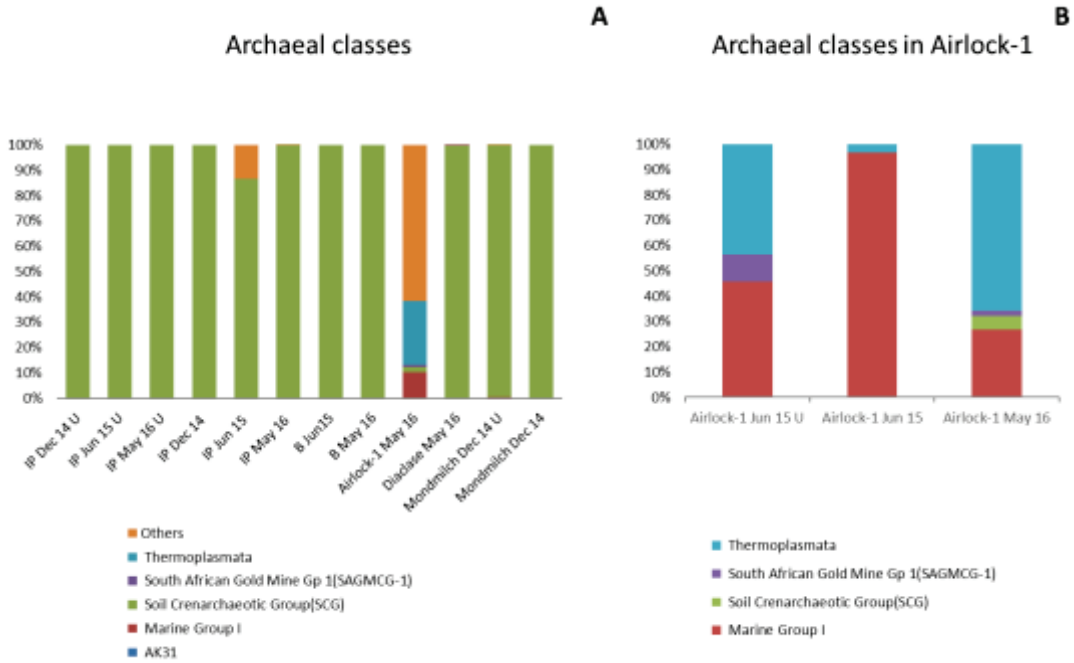

Figure 5. Archaeal community composition at class level for Passage's inclined planes on December 2014, June 2015, May 2016, Passage's banks on June 2015, May 2016, Airlock-1 and Diaclase on May 2016 and Mondmilch on December 2014 (A) and Airlock-1 samples at different dates (B). Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks), date (abbreviated month and year) and sample type (U for unstained parts).

The phylum taxonomic profile of fungi differed according to the room (Airlock-1 vs Passage vs Mondmilch vs Diaclase), the geologic substrate (banks vs inclined plane in the Passage) and the presence of stains (for Mondmilch and Passage) (Fig. 6A). For room × geologic substrate combinations (unstained samples), these differences were linked to the relative prevalence of Basidiomycota (> 41% in Diaclase) or Ascomycota (> 83% on Passage's inclined planes and banks and Mondmilch). Three fungal taxa with black pigmentation potential (*Exophiala, Ochroconis* and Herpotrichiellaceae) were found in each room, but with lower relative abundances in Airlock-1 (0.01% of fungal sequences) than in other rooms (30-67% elsewhere) when considering black stains only. Stain presence was associated with higher levels of these fungi on Passage's inclined planes and Mondmilch, but not on Passage's banks which displayed even lower *Exophiala* levels on stains than outside stains (Fig. S3).

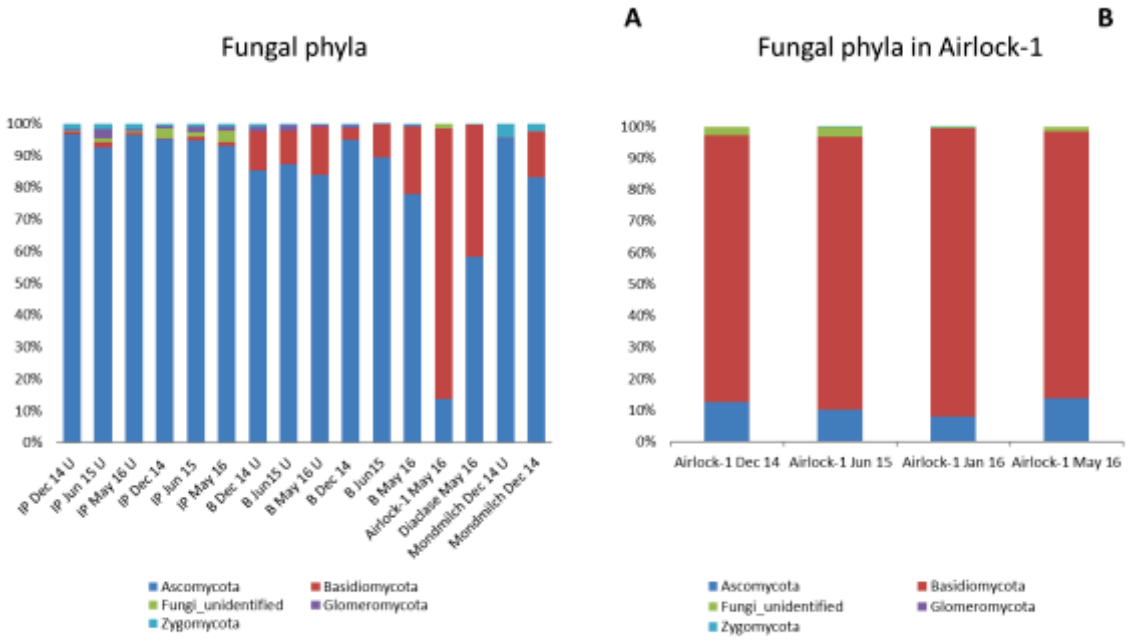

Figure 6. Fungal community composition at phylum level for Passage's inclined planes and Passage's banks on December 2014, June 2015, May 2016, Airlock-1 and Diaclase on May 2016 and Mondmilch on December 2014 (A) and Airlock-1 samples at different dates (B). Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks), date (abbreviated month and year) and sample type (U for unstained parts).

Largely similar findings were obtained when considering the phylum taxonomic profile of the micro-eukaryotic community at large, with the prevalence of Basidiomycota in Airlock-1 (>62% of sequences) but of Ascomycota in the other rooms (> 42%). For non-fungal micro-eukaryotes, higher levels of Cercozoa were found in unstained samples of Passage's inclined planes compared with samples of Passage's banks, Airlock-1, Diaclase or Mondmilch (Fig. 7).

Overall, significant differences in the taxonomic composition of microbial communities were evidenced at phylum level when comparing Lascaux rooms/geologic substrates and the effect of stains, even more so than when considering microbial community structure. It is interesting to note that fungi with pigmentation potential were more prevalent inside than outside stains on Passage's inclined planes and Mondmilch, yet they were also well established outside stains, indicating that the genetic potential for new stain formation is already well in place on these surfaces. In the case of Passage's banks, these fungi were even more prevalent outside stains, at least when considering the *Exophiala* genus, which suggests that population size is not the main factor determining whether a stain will develop. In Airlock-1, fungal taxa known for their black pigmentation potential (i.e. *Exophiala, Ochroconis* and certain Herpotrichiellaceae) were evidenced at low levels in DNA sequences from stains (i.e. 0.01% of fungal sequences), yet black fungal isolates corresponding to *Ochroconis lascauxensis*, *Exophiala angulospora*, *Exophiala* sp. and *Acremonium nepalense* were readily obtained. Fungi from the Telephorales order (which

includes ectomycorrhizal taxa) were prevalent, probably because their entrance into Airlock-1 was promoted by development of tree roots entering Airlock-1 though the cement between certain limestone blocks.

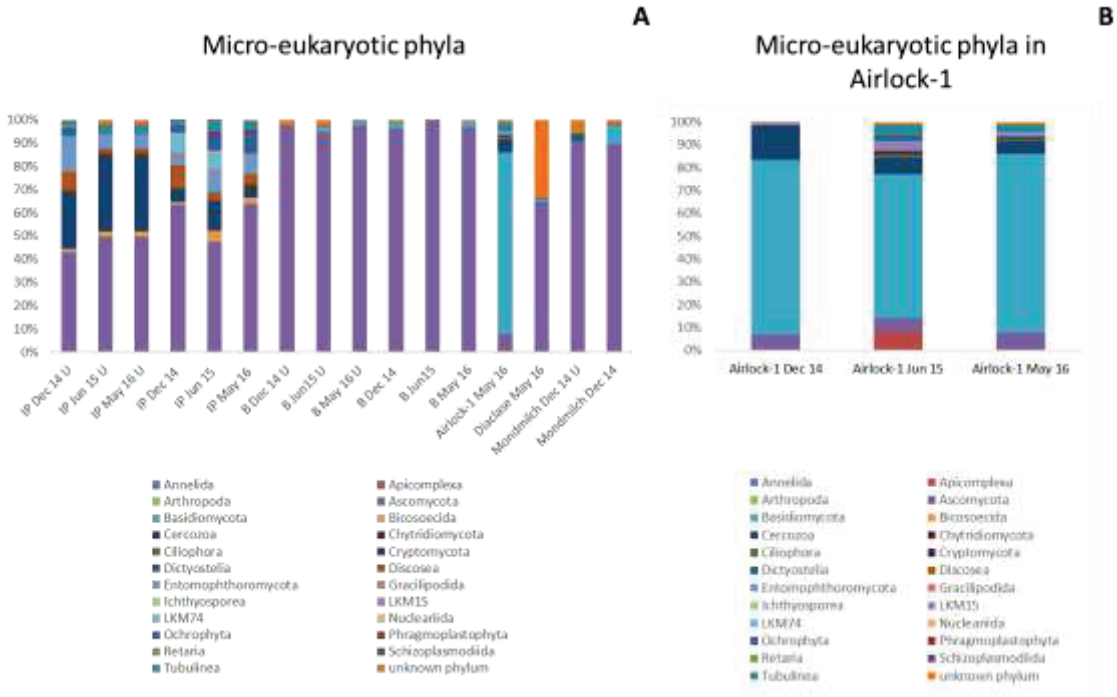

Figure 7. Micro-eukaryotic community composition at phylum level for Passage's inclined planes and Passage's banks on December 2014, June 2015, May 2016, Airlock-1 and Diaclase on May 2016 and Mondmilch on December 2014 (A) and Airlock-1 samples at different dates (B). Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks), date (abbreviated month and year) and sample type (U for unstained parts).

Metatranscriptomic data are mainly structured according to Lascaux room or geologic substrate

We obtained ~ 1.4 billion forward or reverse RNA sequences, which gave ~ 213 million mRNA sequences. Per sample, ~ 3.6 million mRNA sequences were obtained (i.e. ~ 150.000 mRNA contigs).

Multivariate analysis of raw mRNA sequences using the Simka (Soft Independent Modeling of Class Analogy) software showed a main distinction according to Lascaux room or geologic substrate, in that all Airlock-1 samples and most samples from Passage's banks, Passage's inclined plane, Mondmilch and Diaclase clustered separately (Fig. 8). The effect of stain presence was significant for Airlock-1 but not in the other locations.

This first analysis based on raw mRNA sequences provides interesting insights in terms of transcription activity. Indeed, the distinctions that could be made between conditions are reminiscent of the differences found when assessing DNA barcodes, which means that differences in community structure/composition can translate into different transcription profiles. The fact that stain presence was a significant factor only in Airlock-1 is compatible with this. The relative importance of metabolic pathways implicated in stain formation among all other metabolic activities of microorganisms is unknown and perhaps it

is rather low overall, even when considering pigmented microorganisms only. Therefore, it could be that the particular case of Airlock-1 could be explained by specific pioneer community properties and/or ongoing pigmentation activity, stains investigated in other rooms being rather ancient and stable.

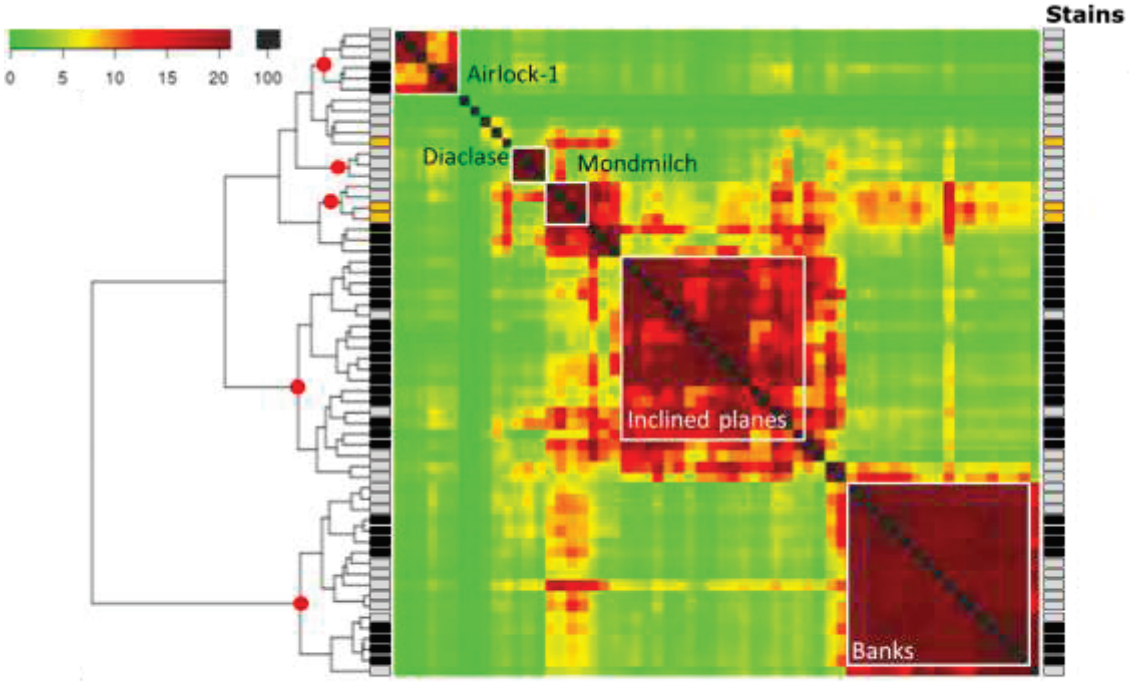

Figure 8. Identification of Probable Groups with SimkA approach on raw mRNA sequences taken in Airlock-1, Passage's banks, Passage's inclined plane, Mondmilch and Diaclase. On the left and the right of the heatmap, black boxes represent black stains, grey boxes unstained parts and yellow boxes yellow stains. Colors are used within the heatmap to indicate similarity levels (see scale).

Metatranscriptomic profiles of main gene categories did not vary significantly with Lascaux room, geologic substrate or stain presence

When considering potential genes identified from mRNA sequences in terms of the main gene categories they belonged to, differences were not significant following NMDS comparisons (i) of stains vs neighboring unstained parts for Passage's inclined planes (black stains), Passage's banks (black stains), Airlock-1 (black stains) and Mondmilch (yellow stains) or (ii) between the different rooms (Fig. 9A). In addition, there was no effect of time, based on the analysis of samples from Passage's inclined planes and banks (Fig. 9B).

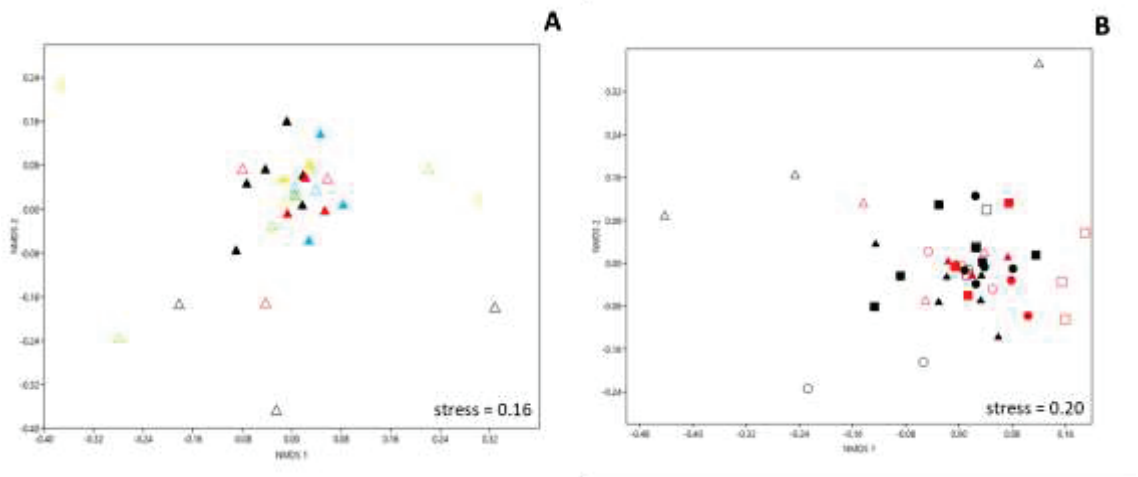

Figure 9. NMDS comparison of cave wall conditions based on transcriptomic data at the level of COG functional groups. Passage's banks (outside and inside black stains), Passage's inclined planes (outside and inside black stains), Mondmilch (outside and inside yellow stains), Airlock-1 and Diaclase on May 2016 are compared in A, and all Passage conditions across the three sampling dates in B. Passage's banks are represented in red, Passage's inclined planes in black, Mondmilch in yellow, Diaclase in green and Airlock-1 in blue in A. The three sampling dates are represented using circles for December 2014, squares for June 2015 and triangles for May 2016 in B.

Metatranscriptomic profile composition based on main gene categories varies with Lascaux room or geologic substrate, as well as stain presence

When the relative importance of the main gene categories obtained from metatranscriptomic data was assessed, significant diversity was evidenced between the means of the 3 or 6 replicates studied, regardless of whether the comparison was carried out for all ecological conditions considered at a single sampling (Fig. 10A) or for all Passage conditions across the three sampling dates (Fig. 10B). In all samples, the COG categories better represented were energy production and conversion (C), translation, ribosomal structure and biogenesis (J), and post translational modification, protein turnover, chaperones (O).

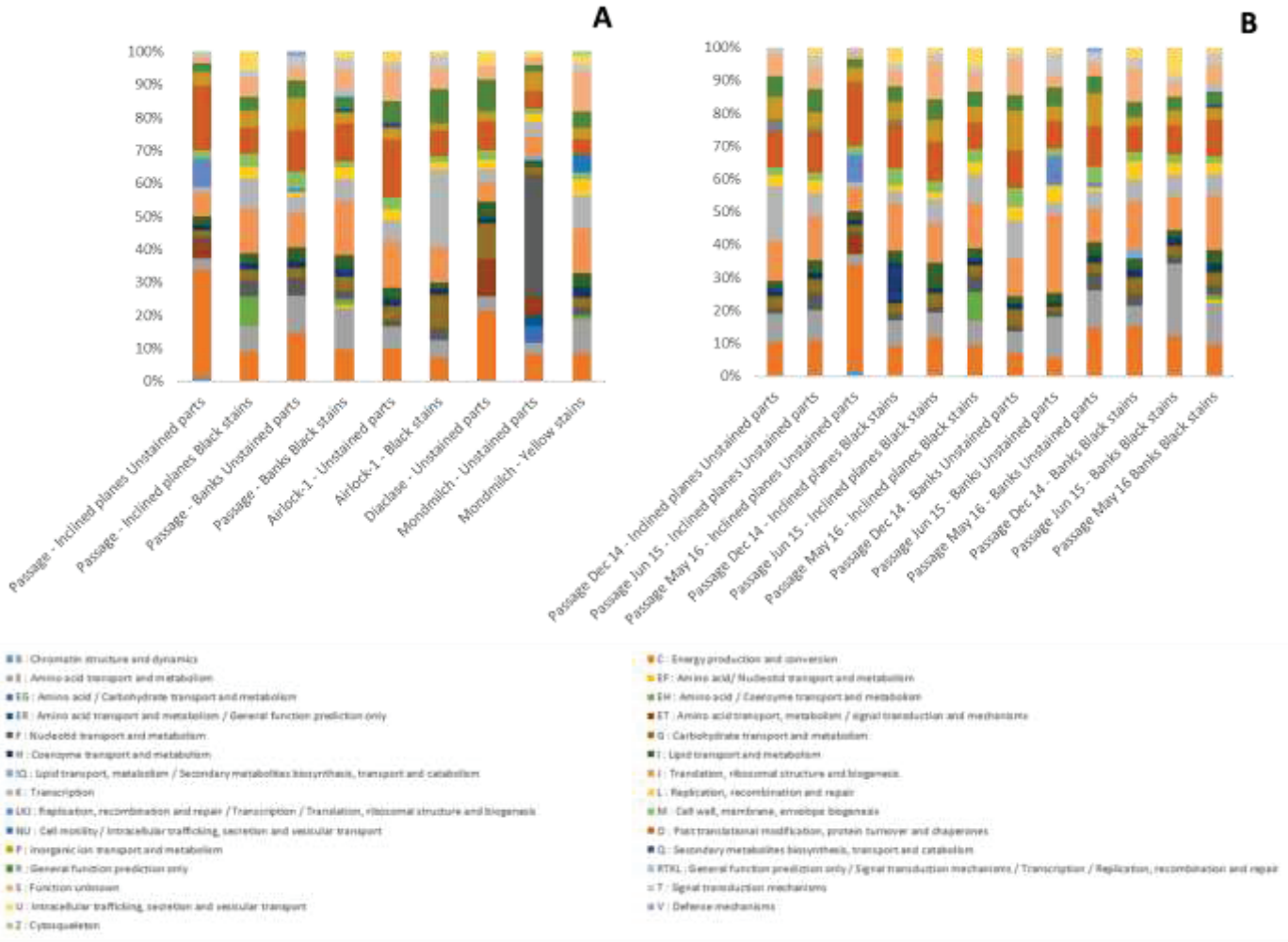

Figure 10. Transcriptomic profiles of cave wall conditions based on COG functional groups on May 2016 for Passage's inclined planes (outside and inside black stains), Passage's banks (outside and inside black stains), Airlock-1, Diaclase and Mondmilch (outside and inside yellow stains) (A) and for all Passage conditions across the three sampling dates (B). Only COG functional groups with a relative abundance higher than 1% of the sequences are represented. Data are the means of 3 or 6 replicates (see Table 1).

When focusing on treatment comparisons, however, we found that metatranscriptomic profile composition depended on the room (Airlock-1 vs Passage vs Mondmilch vs Diaclase), the geologic substrate (banks vs inclined plane in the Passage) and the presence of stain (especially for the Mondmilch and inclined planes) (Fig. 10A). In comparison, the effect of time (assessed in the Passage) was of less magnitude, especially in black stains (Fig. 10B). For room × geologic substrate combinations (unstained samples), differences were due to the relative prevalence of particular gene categories (> 30% C and > 8% LKJ on Passage's inclined planes, > 9% P and > 4% M on Passage's banks, > 9% S, > 1% Q, > 5% K and > 12% J in Airlock-1, > 5% O in the Mondmilch, > 8% R, > 10% ET, > 10% G and > 4% U on Diaclase walls). In comparison with unstained samples, the presence of stains was associated with high levels of gene categories E, EH, F, J, K, L, S and U and lower levels of C, ET, LKJ and O (on Passage's inclined planes), higher levels of J, G, L, S and U and lower levels

of P, M, F, R and C (on banks), and higher levels of E, J, K, NU, S, R and Z and lower levels of F, IQ and P (on Mondmilch).

Metatranscriptomic profiles for each condition investigated displayed substantial variability when considering the 3 or 6 replicates available (not shown), meaning that there is significant spatial variability in microbial functioning for a given cave surface condition, even with samples originating in the vicinity of one another, where surface visual properties were seemingly the same. When comparing the different conditions, these metatranscriptomic profiles evidenced differences that depended on the room, the geologic substrate (within the Passage) and stain presence. These differences are indicative of broad microbial functioning in the community, as the gene categories used are very general (e.g. C for energy production and conversion, I for lipid transport and metabolism, etc.). In relation to black stain formation, categories Q (secondary metabolism) and V (defense mechanisms) might be of particular interest, but they did not show up when considering differences with unstained parts.

Transcriptomic analysis of individual genes in vs outside stain in Passage's inclined planes and banks, Airlock-1 and Mondmilch

BCA analysis was carried out to compare gene transcription levels in stain vs unstained samples from May 2016 (Fig. 11). At that scale, BCA differences between stains and unstained parts were not statistically significant because of the small number of samples compared (n = 3 or 6), despite contrasted BCA profiles for Passage's inclined planes (Fig. 11A), Passage's banks (Fig. 11B), Airlock-1 (Fig. 11C) or Mondmilch (Fig. 11D).

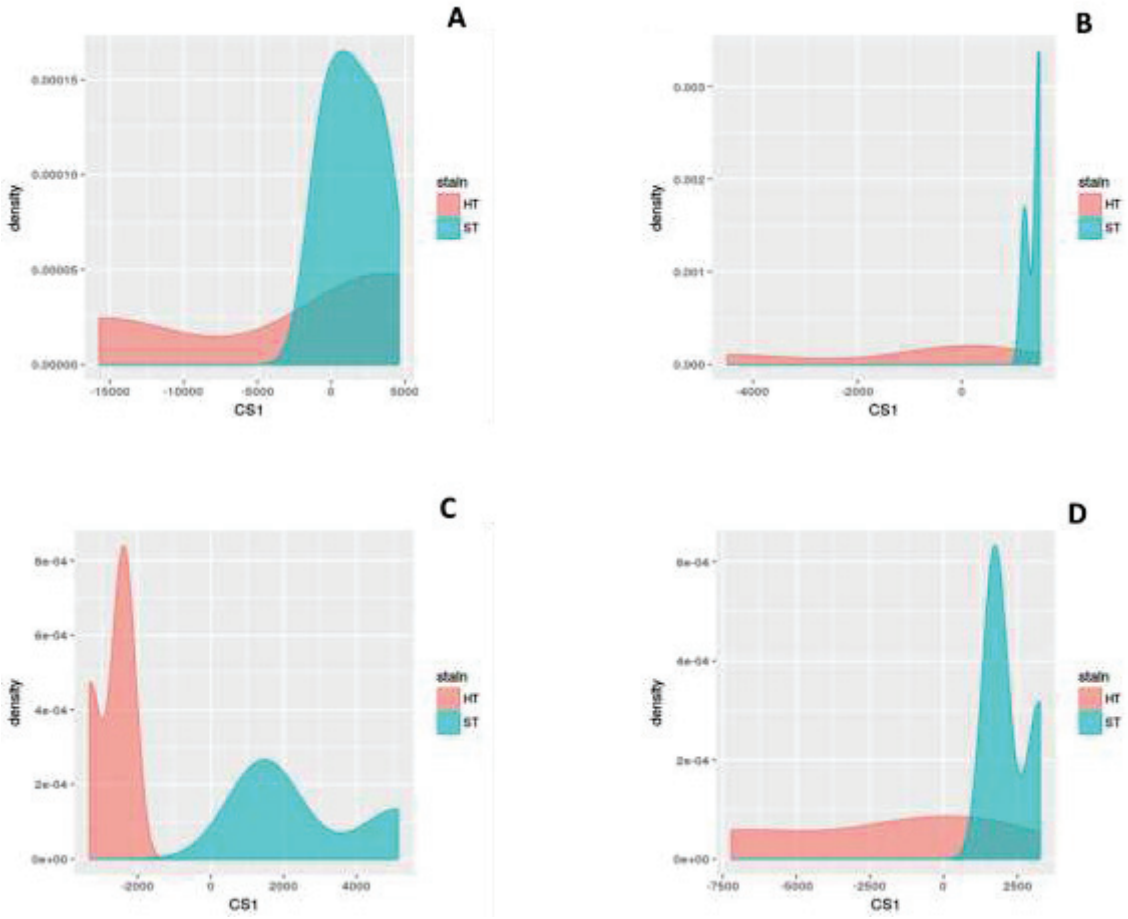

Figure 11. Comparison of predicted enzyme distribution profiles (based on mRNA sequences recovered) for stains vs unstained parts on May 2016 by between-class analysis in Passage's inclined planes (A), Passage's banks (B), Airlock-1 (C) and Mondmilch (D). See Table 2 for corresponding enzymes.

The function of predicted enzymes that explained BCA trends are listed in Table 2, based on a list of the most discriminant enzymes. Thus, when considering protein products deduced from mRNA sequences, nucleotidyltransferases [EC2.7.7] were evidenced in all situations. Oxidoreductases [i.e. EC1 enzymes] and transferases [i.e. EC2 enzymes] were associated with unstained parts of Passage's inclined planes, versus hydrolases [i.e. EC3 enzymes] for black stains of Passage's inclined planes. Oxidoreductases were associated with unstained parts of Passage's banks, versus transferases and hydrolases for stains on Passage's banks. Transferases, hydrolases and lyases [i.e. EC4 enzymes] were associated with unstained parts of Airlock-1 refreshed area, versus oxidoreductases for stains of Airlock-1. Finally, transferases and hydrolases were associated with unstained parts of Mondmilch, versus oxidoreductases for yellow stains on Mondmilch.

Predicted enzymes associated with black stains included transferases, hydrolases and/or oxidoreductases, but they correspond to broad enzyme categories and these enzymes were rather associated with unstained parts in specific conditions, i.e. certain transferases on Passage's inclined planes and in Airlock-1, hydrolases in Airlock-1, and oxidoreductases on Passage's inclined planes and banks. These results suggest different microbial functioning in relation to stain presence in different rooms or geologic substrates

of Lascaux Cave. These findings will need to be confirmed by analysis of log-transformed data to ensure lack of bias linked to higher expression levels of yet moderately-expressed genes. In addition, two types of protein-serine/threonine kinases [EC2.7.11], i.e. mitogen-activated protein kinase (MAPK) [EC2.7.11.24] on Mondmilch and mitogen-activated protein kinase kinase kinase (MAPKKK) [EC2.7.11.25] enzymes on Passage's inclined planes were found on stains but not outside stains. These enzymes play a role in signal transduction and could be related to the metabolism of melanin. Hyperphosphorylation of MAPK increased production of dark brownish pigment pyomelanin (Jain et al. 2011), and the MAPK pathway was activated in response to different environmental stimuli (Jain et al. 2011; Seong et al. 2016).

As an alternative approach, pre-identified enzymes potentially associated with melanin synthesis were sought in the entire data set and were evidenced, but they did not show up amongst the 40 most discriminant enzymes, perhaps because stains were too ancient. Sequences of laccases [EC1.10.3.2] were found in all conditions, with similar levels of expression, and sequences of tyrosinases [EC1.14.18.1] in all conditions (except Diaclase), with overexpression (log2-fold-change of 3.055) in black stains of Passage's banks compared to black stains of Airlock-1. Sequences of scytalone dehydratases [EC4.2.1.94] were found on Passage's inclined planes (on black stains and outside stains) and Passage's banks (on black stains and outside stains), all at similar levels, whereas sequences of THN reductases [EC4.2.1.94] were evidenced in all conditions (except Diaclase), and at similar levels. Further work will need to focus on more recent black stains of natural Lascaux walls to be closer to stain-forming conditions.

Table 2. Enzymes associated with unstained parts (left) or stains (right) as identified by between-class analysis of Passage's inclined planes, Passage's banks, Airlock-1 or Mondmilch on May 2016 (see Figure 11).

Inclined planes - Unstained	Inclined planes - Black stains
EC 2.7.13 : Protein-histidine kinase	EC 1.1.1 : Peroxidases
EC 2.7.7 : Nucleotidyltransferases	EC 1.15.1 : Acting on superoxide as acceptor
EC 3.6.1 : In phosphorus-containing anhydrides	EC 1.6.99 : Acting on NADH or NADPH with other acceptors
EC 3.6.3 : Acting on acid anhydrides	EC 1.9.3 : Acting on a heme group of donors with oxygen as acceptor
EC 4.4.1 : Carboxy-lyases	EC 2.4.1 : Hexosyltransferases
EC 6.2.1 : Acid thiol ligases	EC 2.5.1 : Transferring alkyl or aryl groups, other than methyl
	EC 2.7.11 : Protein-serine/threonine kinases
	EC 2.7.7 : Nucleotidyltransferases
	EC 3.4.22 : Cysteine endopeptidases
Banks - Unstained	**Banks - Black stains**
EC 2.1.1 : Methyltransferases	EC 1.1.2 : With a cytochrome as acceptor
EC 2.4.1 : Hexosyltransferases	EC 1.11.1 : Peroxidases
EC 2.7.13 :Protein-histidine kinase	EC 1.15.1 : Acting on superoxide as acceptor
EC 2.7.7 : Nucleotidyltransferases	EC 1.17.4 : With a disulfide as acceptor
EC 3.2.1 : Glycosylases	EC 1.6.5 : With a quinone or similar compound as acceptor
EC 3.6.3 : Catalyzing transmembrane movement of substances	EC 1.6.99 : Acting on NADH or NADPH with other acceptors
EC 4.2.1 : Hydro-lyases	EC 1.9.3 : With oxygen as acceptor
	EC 2.7.11 : Protein-serine/threonine kinases
	EC 2.7.7 : Nucleotidyltransferases
	EC 2.8.1 : Sulfurtransferases
	EC 3.4.21 : Serine endopeptidases
	EC 3.6.3 : Acting on acid anhydrides
	EC 3.6.4 : Catalyzing transmembrane movement of substances
	EC 5.99.1 : Other isomerases
Airlock-1 - Unstained	**Airlock-1 - Black stains**
EC 1.1.1 : With NAD(+) or NADP(+) as acceptor	EC 2.1.1 : Methyltransferases
EC 1.11.1 : Peroxidases	EC 2.3.3 : Acyl groups converted into alkyl groups on transfer
EC 1.14.15 : With reduced iron-sulfur protein as one donor, and incorporation of one atom of oxygen	EC 2.4.1 : Hexosyltransferases
EC 1.2.1 : With NAD(+) or NADP(+) as acceptor	EC 2.7.7 : Nucleotidyltransferases
EC 1.4.1 : With NAD(+) or NADP(+) as acceptor	EC 3.2.1 : Glycosylases
EC 1.6.5 : With a quinone or similar compound as acceptor	EC 3.4.16 : Serine-type carboxypeptidases

EC 1.6.99 : Acting on NADH or NADPH with other acceptors	EC 3.4.22 : Cysteine endopeptidases
EC 1.9.3 : With oxygen as acceptor	EC 3.6.1 : In phosphorus-containing anhydrides
EC 2.3.1 : Transferring groups other than amino-acyl groups	EC 4.1.2 : Aldehyde-lyases
EC 2.7.7 : Nucleotidyltransferases	EC 4.4.1 : Carbon-sulfur lyases
EC 3.6.3 : Acting on acid anhydrides	EC 5.4.2 : Phosphotransferases (phosphomutases)
EC 6.3.1 : Acid-ammonia (or amine) ligases (amide synthases)	EC 6.3.2 : Acid-amino-acid ligases (peptide synthases)
Mondmilch - Unstained	Mondmilch - Yellow stains
EC 1.2.1 : With NAD(+) or NADP(+) as acceptor	EC 2.3.1 : Transferring groups other than amino-acyl groups
EC 1.3.5 : With a quinone or related compound as acceptor	EC 2.4.1 : Hexosyltransferases
EC 1.4.1 : With NAD(+) or NADP(+) as acceptor	EC 2.7.11 : Protein-serine/threonine kinases
EC 2.7.13 : Protein-histidine kinases	EC 2.7.12 : Dual-specificity kinases (those acting on Ser/Thr and Tyr residues)
EC 2.7.7 : Nucleotidyltransferases	EC 2.7.7 : Nucleotidyltransferases
EC 3.4.24 : Metalloendopeptidases	EC 3.2.1 : Glycosylases
EC 3.6.3 : Acting on acid anhydrides	EC 3.4.19 : Omega peptidases
	EC 3.4.22 : Cysteine endopeptidases
	EC 3.6.1 : In phosphorus-containing anhydrides
	EC 5.3.4 : Transposing S-S bonds
	EC 6.3.2 : Acid-amino-acid ligases (peptide synthases)

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Supplementary data

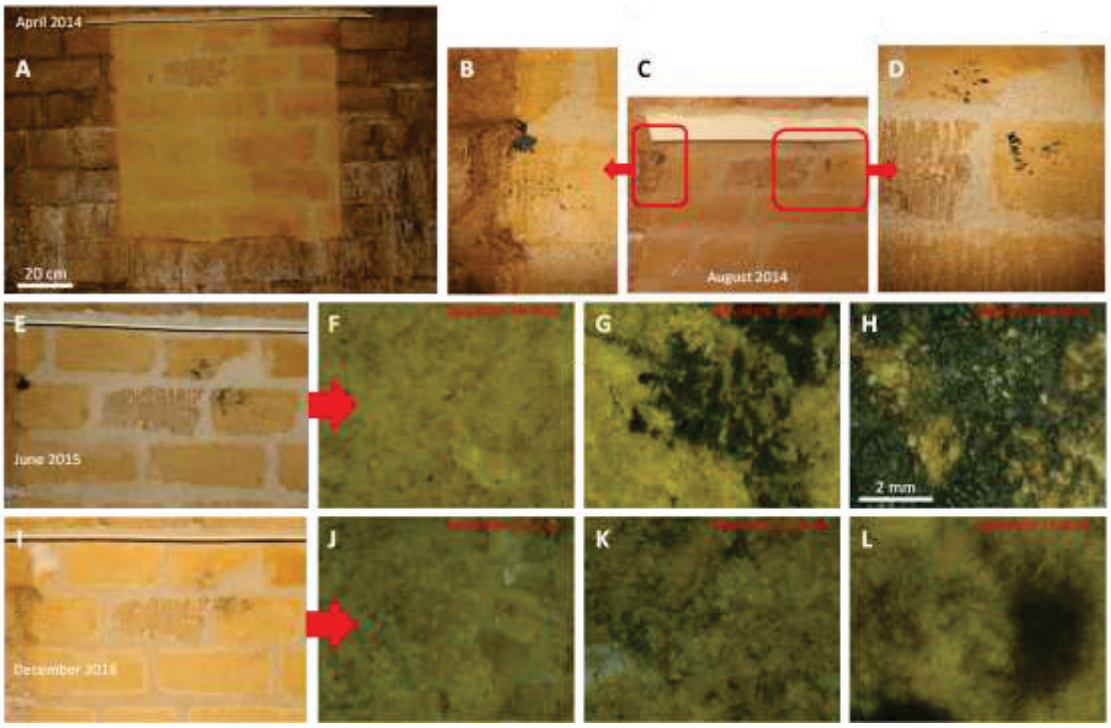

Figure S1. Photographs of Airlock-1 entrance zone (second compartment) in the 16 months following surface refreshment. Work took place on 30 April 2014 and was carried out over a 80 cm × 80 cm area (see A for photograph taken immediately afterwards). A 100-cm border was placed above to prevent drops of condensation water from higher parts of the ceiling. Details of early black stains (C) are shown in B and D. Dino-Lite microscope photographs of the area in June 2015 (E) and December 2016 (I) are shown respectively in F, G, H and J, K, L. Unstained parts are in F and J, whereas stains are shown in G, H, K and L. Black stains of different extents can be seen in June 2015 (see G and H), but stains faded during the second half of 2015 and 2016, leading to more greyish stains (I) corresponding to presumably lower development of black fungal biomass (K and L).

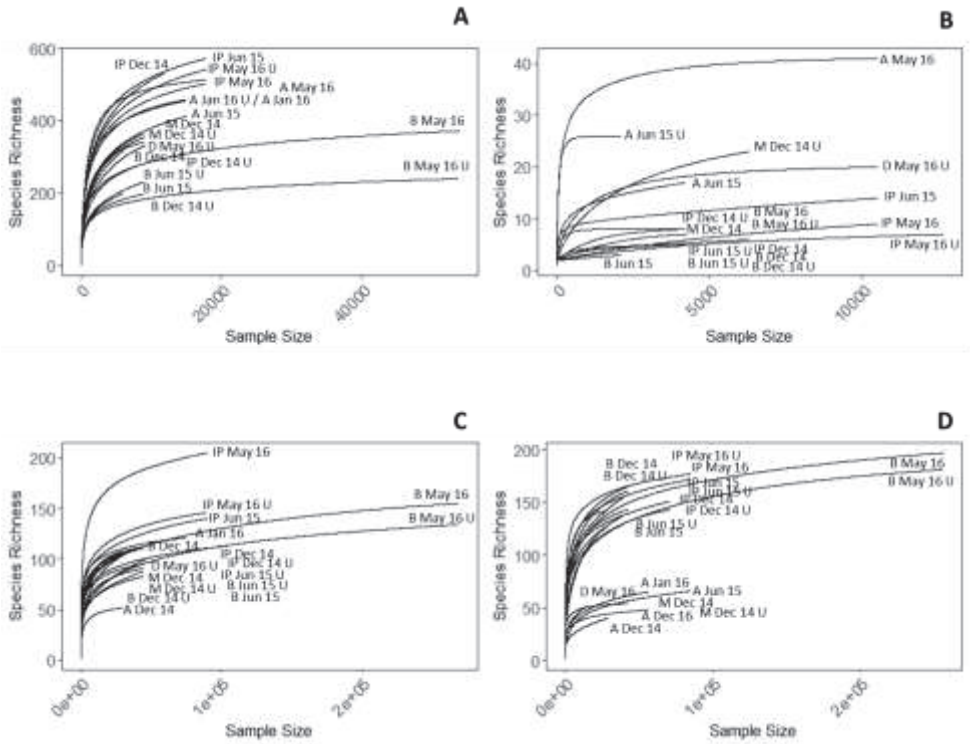

Figure S2. Rarefaction curves at OTUs level for cave samples in the case of bacteria (16S rRNA gene dataset) (A), archaea (16S rRNA gene dataset) (B), fungi (ITS dataset) (C) and micro-eukaryotes et large (18S rRNA gene dataset) (D), based on observed metabarcoding data. Rarefaction curves reached an asymptote in 11 of 20 cases (bacteria), 14 of 14 cases (archaea), 19 of 19 cases (fungi) and 18 of 18 cases (micro-eukaryotes at large). Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks, M : Mondmilch, A : Airlock-1 and D: Diaclase), date (abbreviated month and year) and sample type (U for unstained parts).

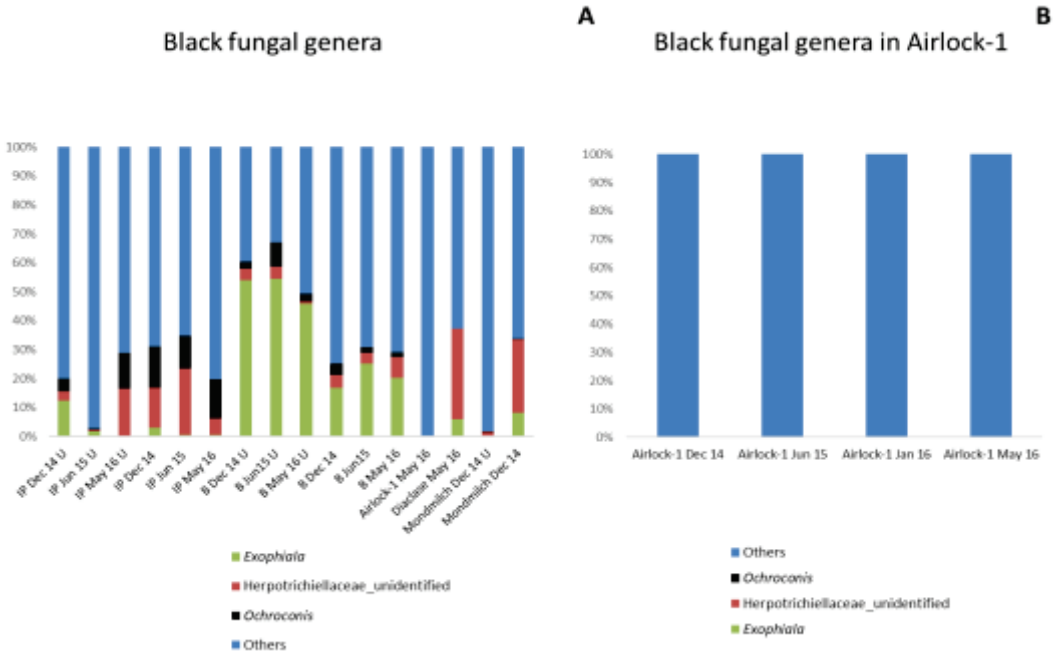

Figure S3. Prevalence of fungi with black pigmentation potential considering fungal genus composition for Passage's inclined planes, Passage's banks, Airlock-1, Diaclase on May 2016 and Mondmilch on December 2014 (A) and Airlock-1 samples at different dates (B). In B, these taxa were well below 0.1%. Treatment information includes the room/substrate (PI : Passage's inclined planes, B : Passage's banks), date (abbreviated month and year) and sample type (U for unstained parts).

Chapitre 7 : Discussion générale et perspectives

L'objectif de ces travaux de thèse était d'étudier l'hétérogénéité spatio-temporelle du microbiote de la grotte de Lascaux avec une approche globale en utilisant le séquençage à haut débit des acides nucléiques. L'utilisation de marqueurs taxonomiques ciblant les trois domaines de la vie a permis de rendre compte de la biodiversité de la grotte. Différentes hypothèses ont été émises au début de cette thèse, cette partie de conclusion permet d'une part, d'évaluer leur pertinence, et d'autre part de positionner ces travaux de recherche dans un contexte plus global et de proposer de nouvelles perspectives de recherche.

A. Retour sur les hypothèses initiales

A1. Première hypothèse

La première hypothèse était que la diversité microbienne est liée au niveau d'anthropisation, et son évaluation correspond aux travaux du chapitre 3 « Relation entre anthropisation et communautés microbiennes des grottes, en comparant Lascaux et d'autres grottes de Dordogne ». Nos travaux visaient à comparer la communauté microbienne de grottes anthropisées et non anthropisées, Lascaux étant a priori davantage anthropisée que toutes les autres. Les résultats montrent que (i) la diversité des bactéries et des archées est plus faible dans les grottes anthropisées que dans les grottes non anthropisées, (ii) la composition taxonomique des communautés est grotte spécifique, (iii) la structure de la communauté microbienne des grottes est impactée par l'anthropisation, et (iv) celle de la grotte de Lascaux se distingue de toutes les autres, donc le caractère anthropisé ainsi que le niveau d'anthropisation impactent la communauté microbienne. Ainsi, l'ensemble de ces résultats valident notre hypothèse de départ.

Ces résultats étaient attendus, car la littérature montre que les perturbations anthropiques affectent les communautés bactériennes (Ager et al. 2010) et les communautés fongiques (Cruz et al. 2017). En effet, une diminution de la richesse microbienne peut être observée après un évènement de pollution, qui en plus de la perte d'espèces s'accompagne d'une modification de la structure de la communauté (Ager et al. 2010). Par contre, le fait que le niveau d'anthropisation puisse lui aussi influencer la structure de la communauté, puisque la grotte de Lascaux présente une structure particulière en comparaison avec les grottes anthropisées, est un résultat nouveau.

L'anthropisation impacte donc la structure de la communauté des bactéries, des archées et des micro-eucaryotes, mais les distances représentant les coefficients de dissimilarité (NMDS) entre les grottes anthropisées et non anthropisées pour la communauté des micro-eucaryotes sont plus petites que celles pour les communautés bactériennes et d'archées, donc l'anthropisation semble avoir un impact plus important sur les bactéries et les archées que sur les micro-eucaryotes. Ce type d'analyse prenant en compte les trois communautés est nouveau pour des grottes, limitant les possibilités de comparaison. En revanche, d'autres études se sont intéressées à l'effet de facteurs de stress et notamment des températures suboptimales, montrant que les champignons et les

bactéries sont affectés de la même façon en condition de laboratoire (Barceras-Moreno et al. 2009), mais que les champignons s'adaptent mieux que les bactéries aux basses températures dans des sols agricoles ou forestiers (Pietikäinen et al. 2005).

Le panel de grottes anthropisées était hétérogène, et parmi ces grottes la situation de Tourtoirac est remarquable, car c'est une grotte découverte récemment et qui subit une anthropisation depuis 2010 seulement. Cependant, la structure de sa communauté microbienne est comparable à celles des autres grottes, qui sont anthropisées depuis plusieurs dizaines d'années. Ainsi l'anthropisation impacte rapidement la communauté.

Certains phylums bactériens sont préférentiellement retrouvés dans les grottes anthropisées, comme les Bacteroidetes, ou bien dans les grottes non anthropisées, comme les Nitrospirae. Ces observations rejoignent celles faites dans d'autres études (Pfendler et al. 2018 ; Tomczyk-Żak & Zielenkiewicz, 2016 ; Zhou et al. 2007), et ainsi les Bacteroidetes pourraient être proposées comme des bioindicateurs d'anthropisation et les Nitrospirae des phylums adaptés aux conditions des grottes non anthropisées.

La grotte de Lascaux a quant à elle subi de nombreux traitements chimiques, ce qui n'a pas été pratiqué dans les autres grottes, en tout cas à cette échelle. C'est la caractéristique qui différencie le plus Lascaux des autres grottes anthropisées, et ainsi cela suggère fortement que l'application des traitements chimiques est le facteur d'anthropisation prépondérant en ce qui concerne l'impact sur la communauté microbienne. Dans la grotte de Lascaux, deux salles ont été prises en compte dans cette comparaison, le Passage choisi pour sa localisation centrale et son niveau d'anthropisation élevé, et la Diaclase qui en comparaison est moins anthropisée. En effet, la Diaclase n'a jamais été ouverte aux visites touristiques et est séparée du reste de la grotte par une trappe, néanmoins un extracteur d'air y avait été installé et quelques traitements chimiques ont été effectués notamment au voisinage de la trappe. Par conséquent, cette salle représente un point de comparaison utile pour le Passage. Cependant, les communautés microbiennes de la Diaclase ne diffèrent pas beaucoup de celles du Passage, étant en position intermédiaire entre le Passage et les autres grottes anthropisées. Cela indique que la Diaclase, bien que moins soumise à l'influence humaine que le Passage, ne peut être considérée comme une zone non perturbée de la grotte, comme on le pensait jusqu'ici.

L'équipe de Claude Alabouvette (Bastian et al. 2009b) avait déjà mis en évidence que l'application de traitements chimiques avait vraisemblablement modifié la composition de la communauté microbienne de Lascaux, cependant les différences entre les zones traitées et non traitées n'avaient pas été étudiées et les méthodes disponibles à l'époque (clonage-séquençage) ne permettent pas d'évaluer l'impact de l'anthropisation sur l'ensemble de la communauté microbienne. D'autres études ont aussi analysé la diversité de communautés microbiennes (bactéries, champignons, microalgues, diatomées) dans le monde souterrain, mais en étudiant l'impact de l'anthropisation à l'échelle d'une même grotte (Leuko et al. 2017), ou en comparant différentes grottes mais sans considérer l'aspect anthropique (Pfendler et al. 2018). Cette approche comparative, à l'échelle régionale, de l'impact de l'anthropisation sur les communautés microbiennes des grottes est donc nouvelle.

A2. Deuxième hypothèse

La deuxième hypothèse était que les taches impactent plus que le type de substrat minéral et le temps la structure de la communauté microbienne. Cette hypothèse a été testée en étudiant la diversité de la communauté microbienne présente (ADN) et active (ARN) du Passage (chapitre 4). Les résultats indiquent que les variations saisonnières impactent faiblement la communauté, ce qui est en accord avec les faibles variations des conditions environnementales dans la grotte. En revanche, la nature du substrat minéral impacte fortement la structure de la communauté microbienne, et ce davantage que la présence de taches malgré le fait que les taches soient des altérations récentes. Notre hypothèse de départ est donc à revoir. Une étude de l'écologie microbienne de différentes surfaces rocheuses de tombes montre que le type de roche utilisé pour les tombes affecte plus la composition de la communauté microbienne que la localisation géographique du cimetière (Brewer & Fierer, 2017). Une autre étude révèle que les biofilms ne colonisent pas ou très peu les briques et le marbre alors qu'ils colonisent les autres surfaces (une roche à aspect alvéolaire ou tuf, mortier et plâtre) dans des catacombes de Rome (Sanchez-Moral et al. 2005). Ces études montrent que les propriétés des substrats minéraux ont un rôle important dans la sélection de communautés microbiennes particulières.

Le Passage présente plusieurs types de substrats minéraux différents, dont le sol composé de sable calcaire, les banquettes d'argile et les plans inclinés qui sont calcaires. Donc le sol et les plans inclinés ont une composition chimique proche, mais leurs caractéristiques exactes (granulométrie, pH, composition chimique) ne sont pas connues. De plus, les traitements chimiques appliqués sur les substrats minéraux étaient différents (Bastian et al. 2010). Le sol a été recouvert de chaux alors que les plans inclinés et les banquettes ont été traités par une solution de chlorure de benzalkonium et d'antibiotiques. Les résidus de traitement des parois peuvent atteindre les banquettes et le sol et par ruissellement, et l'argile est un substrat absorbant, donc l'impact des traitements sur la contamination des substrats minéraux est potentiellement différent. L'ensemble de ces facteurs contribue aux différences de composition physico-chimique entre les trois substrats minéraux étudiés et pourraient expliquer, au moins en partie, les différences de structure des communautés microbiennes.

L'étude sur le Passage indique que les réseaux de co-occurrence des taxons microbiens varient en fonction du temps, du substrat minéral et de la présence des taches. Les réseaux de co-occurrence permettent d'identifier des interactions microbiennes potentielles, ainsi que des consortiums microbiens possibles. Les conditions environnementales dans Lascaux varient peu dans le temps, donc l'obtention de réseaux différents à chaque échantillonnage est surprenante. En revanche, le substrat minéral impacte la structure des communautés microbiennes et la composition microbienne diffère sur taches et en dehors des taches, donc des différences de réseaux étaient potentiellement attendues. Ainsi, les interactions microbiennes potentielles ne semblent pas déterminées par un facteur en particulier (temps, substrat minéral, présence de tache). Les réseaux de co-occurrence obtenus étaient

très complexes, c'est-à-dire composés de nombreux taxons, ce qui indique que beaucoup de taxons ont des dynamiques communes en ce qui concerne leurs effectifs, et le nombre d'interactions potentielles entre ces taxons est susceptible de varier en fonction des conditions testées. Les consortiums microbiens seraient différents en fonction du temps, du substrat minéral et de la présence de taches. De plus, la majorité des interactions identifiées étaient positives, donc il y a peut-être davantage de phénomènes de coopération que de compétition ou d'antagonisme entre les microorganismes présents.

A3. Troisième hypothèse

La troisième hypothèse était que la communauté microbienne des taches noires est différente de la communauté microbienne des zones sombres, mais que dans les deux cas les collemboles se nourrissent de ces microorganismes et ont un rôle dans la dissémination de ceux-ci. Cette hypothèse est testée dans le chapitre 5, qui comprend deux parties.

Dans la première partie, nos résultats montrent que la communauté microbienne des taches noires et des zones non tachées est différente. De plus, le champignon *Ochroconis* et d'autres champignons noirs sont prévalents dans les taches noires alors que le genre *Pseudomonas* a une abondance relative beaucoup plus faible dans les taches noires que dans les zones non tachées. Comme les *Pseudomonas* peuvent inhiber *in vitro* la croissance des champignons noirs, il est possible que ces bactéries limitent l'extension des taches noires. Cependant, les collemboles présents sur les taches noires (et identifiés comme *Folsomia candida*) peuvent se nourrir des taches noires, et ils ont la capacité d'assimiler et de disséminer les champignons noirs. Cela suggère que l'extension d'une tache noire implique de manière prépondérante les collemboles, qui permettent aux champignons noirs de coloniser de nouvelles surfaces malgré le potentiel inhibiteur des *Pseudomonas*. De plus, les collemboles ont la capacité de consommer des *Pseudomonas*, ce qui contribue potentiellement à leur élimination, facilitant alors la colonisation par les champignons noirs.

Les résultats pointent vers une interaction tripartite entre les champignons noirs, les *Pseudomonas* et les collemboles dans l'Abside. Les collemboles se nourrissent de bactéries (dont des d'actinobactéries), d'algues et principalement de champignons (Caravaca & Ruess, 2014) et ont une préférence pour les champignons noirs (Böllmann et al. 2010 ; Scheu & Simmerling, 2004). Les collemboles et les champignons noirs sont plus abondants (visuellement et par séquençage, respectivement) sur les zones tachées que les zones non tachées, suggérant un lien entre ces organismes, qui avait déjà été proposé par l'équipe Alabouvette. En revanche, les résultats de l'analyse isotopique effectuée par le LEHNA valident de manière quantitative le potentiel de consommation des taches noires et des champignons noirs par les collemboles. Aussi, la prépondérance des *Pseudomonas* en dehors des zones tachées et leur faible présence dans les taches rajoutent une dimension nouvelle, car cela suggère qu'il faille ajouter les *Pseudomonas* comme un acteur

supplémentaire pour comprendre l'interaction entre les collemboles et les champignons noirs.

Dans la seconde partie, les résultats montrent que les taches noires, les zones sombres et les zones non tachées adjacentes possèdent des communautés microbiennes distinctes. Au niveau des champignons par exemple, les *Cordycipitaceae* et le genre *Kazachstania* sont prépondérants sur les zones sombres mais pas sur les taches noires. Notre hypothèse est donc vérifiée. Les taches noires et les zones sombres ont été prélevées dans l'Abside mais sur des plans différents, ce qui implique que les zones non altérées adjacentes aux taches noires ou aux zones sombres ont aussi été prélevées sur des plans différents, et se pose la question de savoir si la différence de distribution spatiale pourrait être un facteur expliquant la différence de composition de la communauté microbienne. D'ailleurs, si les taches noires et les zones sombres diffèrent clairement des zones adjacentes (sans altérations), les communautés microbiennes des taches noires et des zones sombres présentent des similarités. De plus, plusieurs zones sombres ont évolué pendant la durée du projet, avec la formation de taches noires au centre de certaines zones sombres. Il serait intéressant de poursuivre le suivi des zones sombres pour déterminer dans quelle mesure ces zones sombres pourraient constituer des stades intermédiaires dans la formation de taches noires dans les conditions actuelles de fonctionnement de l'Abside.

A4. Quatrième hypothèse

La quatrième hypothèse est que les gènes impliqués dans le métabolisme de la mélanine ou la synthèse d'autres pigments noirs sont surexprimés sur les taches noires en comparaison avec les zones non tachées. Le chapitre 6 permet de tester cette hypothèse en étudiant la dynamique fonctionnelle de la communauté microbienne en ciblant les transcrits, et en recherchant les séquences potentiellement impliquées dans la production de pigments noirs.

Pour cela, la structure et la composition de la communauté microbienne ont été étudiées dans un premier temps, pour déterminer si les différences de transcription escomptées correspondent à des différences de colonisation microbienne (avec des taxons différents) ou des différences de fonctionnement de communautés par ailleurs similaires. Cela a aussi permis de déterminer quels facteurs (temps, salle, présence de tache) façonnent cette communauté. Les résultats montrent que (i) la diversité microbienne varie principalement en fonction de la salle ou du substrat minéral, et dans une moindre mesure avec la présence de taches, et (ii) la structure de la communauté diffère elle-aussi en fonction de la salle ou du substrat minéral, ainsi que de la présence de taches.

Ce type de résultat a été retrouvé aussi pour le Passage et l'Abside. En effet, dans le Passage le substrat minéral structure davantage la communauté microbienne que la présence de taches, et dans le Passage comme dans l'Abside, la composition de la communauté diffère avec la présence de taches. Ainsi, ces résultats semblent généralisables à l'ensemble de la grotte.

La zone test du Sas-1 suivie sur l'ensemble du projet de thèse a montré une modification rapide de l'aspect visuel des taches. Cette salle se distingue des autres à la fois par la structure et la composition de sa communauté microbienne. Par exemple, les taxons de champignons noirs rencontrés ailleurs sont présents sur les taches de la zone test du Sas-1, mais à des niveaux significativement plus faibles en comparaison avec les taches noires des autres salles. Cela suggère que d'autres champignons seraient à l'origine de la production de pigments noirs dans le Sas-1. Ainsi, les taches noires du Sas-1 représentent un stade pionnier mais ne sont pas représentatives des taches noires stabilisées du reste de la grotte. De plus, cette salle est composée de calcaire mais a été construite par l'Homme, et c'est la salle la plus proche de l'entrée de la grotte, ce qui constitue des spécificités qui pourraient expliquer la microbiologie particulière de cette salle.

Dans un second temps, l'analyse métatranscriptomique a été réalisée. L'analyse Simka basée sur le comptage de k-mers (toutes les sous-séquences possibles de longueur k), à partir d'une lecture du jeu de données de métatranscriptomique, montre que les salles de la grotte sont le facteur explicatif principal. Ce résultat est donc comparable à ceux issus des analyses de l'ADN.

Les profils métatranscriptomiques basés sur les catégories fonctionnelles de gènes COG variaient en fonction du temps, de la salle/substrat minéral et de la présence de taches. L'analyse inter-classe (BCA) a mis en évidence des profils spécifiques lors de la comparaison des taches et des zones non tachées, et divers types d'enzymes discriminantes pour les deux conditions. L'ensemble du jeu de données n'ayant pas été encore totalement analysé à ce jour, l'hypothèse ne peut être confirmée ou infirmée.

Certaines enzymes potentiellement impliquées dans le métabolisme de la mélanine, comme les laccases, les tyrosinases ou la scytalone déshydratase, sont retrouvées dans toutes les conditions (salles, substrats minéraux), avec des niveaux comparables en présence ou absence de tache noire, donc cela n'apporte pas d'information déterminante quant à la formation des taches.

Actuellement dans la littérature, aucune donnée sur la métatranscriptomique dans les grottes n'est disponible, donc ces travaux sont nouveaux et permettront de mieux comprendre la dynamique fonctionnelle des microorganismes dans la grotte de Lascaux.

B. Réponses aux problématiques de la grotte de Lascaux

B1. Pourquoi y a-t-il des taches à Lascaux ?

Les modifications environnementales dans la grotte de Lascaux ont entrainé l'apparition d'altérations microbiennes sur les parois, et les traitements antibiotiques et chimiques appliqués pour éliminer ces altérations ont perturbé la composition de la communauté microbienne de la grotte de Lascaux (Bastian et al. 2009b).

Certains microorganismes produisent des pigments tels que la mélanine (Eisenman & Casadevall, 2012 ; Nosanchuk & Casadevall, 2006) comme mécanisme de défense vis-à-vis

d'un stress environnemental comme l'oxydation ou la radiation par ultra-violet. Le centre d'étude des substances naturelles de l'UMR Ecologie microbienne a identifié différents types de mélanines dans les taches noires et les champignons noirs isolés à partir de prélèvement de la grotte de Lascaux, ce qui confirme l'origine de la coloration noire des taches.

Comme indiqué précédemment, il semblerait que les collemboles jouent un rôle important dans la formation des taches par leurs interactions avec les champignons noirs et les bactéries *Pseudomonas*, et ce en favorisant les champignons noirs. Malgré le fait que les collemboles consomment les champignons noirs, ils les disséminent via leurs fèces ou leurs déplacements, puis ils se nourrissent aussi des *Pseudomonas* qui inhibent la croissance des champignons noirs, ainsi ils contribuent à la diminution des *Pseudomonas*, antagonistes des champignons noirs. Actuellement, un suivi du nombre de collemboles (comptages) est réalisé par le personnel de Lascaux afin d'évaluer la variation de cette population au cours du temps.

Nos travaux montrent que certains champignons noirs colonisent aussi les zones non tachées mais avec une abondance faible, d'ailleurs deux de ces champignons noirs *Ochroconis lascauxensis et Ochroconis anomala* sont endémiques à la grotte de Lascaux ce qui indique que la grotte de Lascaux possède une communauté microbienne particulière. Il apparait donc que la présence de champignons noirs n'est pas suffisante pour engendrer la formation de taches, qui est peut-être déclenchée par un ensemble de facteurs comprenant les interactions entre les champignons noirs et d'autres microorganismes partenaires.

B2. Pourquoi les altérations de la grotte sont-elles réparties de façon hétérogène ?

La grotte de Lascaux présente plusieurs salles composées de différents substrats minéraux et les altérations sont réparties de façon hétérogène dans la grotte. Nous avons montré que des champignons noirs se trouvent en dehors des zones tachées, ces zones sont donc susceptibles de former ultérieurement une tache. Les points à prendre en compte sont la présence et donc en amont l'arrivée de champignons noirs (dissémination par l'air ou les collemboles), ainsi que leur capacité à proliférer et à synthétiser des mélanines, nécessitant de considérer les micro-environnements sur les parois et les consortiums microbiens auxquels ils prennent part.

Lors de l'une des campagnes d'échantillonnage, l'impact de l'hétérogénéité spatiale à l'échelle métrique et centimétrique sur la structure de la communauté microbienne a été étudié (Figure 5).

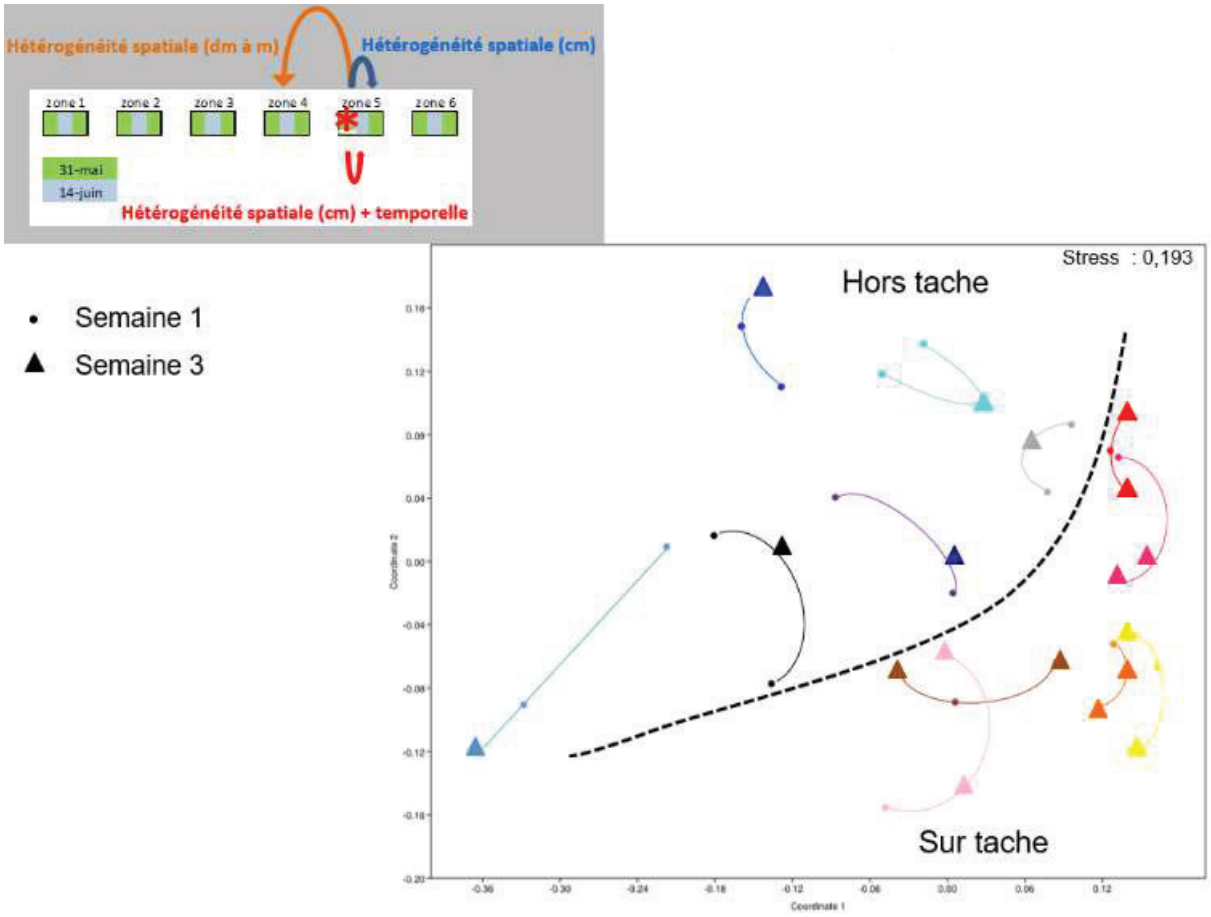

Figure 5. Comparaison de la structure de la communauté bactérienne de six taches noires et de six zones non tachées de la banquette du Passage avec une analyse NMDS, en prenant en compte la variabilité spatiale de la communauté. Chaque couleur correspond à une tache, et chaque tache a été échantillonnée sur les bords puis au centre deux semaines plus tard, ou bien au centre puis sur les bords. Ces résultats n'ont pas été inclus dans l'un des chapitres expérimentaux de la thèse.

Cette analyse montre que les 3 points de chacune des 12 zones prélevées sont relativement proches les uns des autres par comparaison avec les points des autres zones, suggérant que la proximité génétique des communautés augmente lorsque la distance entre les points échantillonnés diminue. Cela revient à dire que l'hétérogénéité spatiale impacte la structure de la communauté bactérienne.

Les réseaux de co-occurrence montrent de nombreuses interactions entre les microorganismes, cependant aucun patron spécifique est mis en avant car les consortiums microbiens étaient différents en fonction du temps, du substrat minéral ou de la présence de tache, ce qui participe à la distribution hétérogène des altérations.

Précédemment, nous avons montré que les collemboles pouvaient disséminer des microorganismes dont les champignons noirs, et la dissémination par l'air a aussi été étudiée. Dans des échantillons d'air de la grotte de Lascaux, des isolats de champignons noirs ainsi que d'autres microorganismes ont été retrouvés. L'importance de ce moyen de dissémination a été montré dans d'autres grottes (Garcia-Anton et al. 2014 ; Mulec et al. 2012 ; Wang et al. 2010).

Enfin, la répartition hétérogène des altérations dans la grotte pourrait résulter de processus stochastiques. Par exemple, toutes les conditions permettant la formation des taches pourraient être réunies à un moment particulier, sans qu'aucune tache ne se forme. Plus plausible, c'est la conjonction des différentes conditions nécessaires pour la production d'une tache qui pourrait survenir de façon aléatoire. Par exemple, les flux d'air vont permettre de manière aléatoire le contact entre les spores disséminées et une paroi, la capacité d'adhésion de ces spores sur les parois fait peut-être que quelquefois l'adhésion se fait et d'autres fois non, et ensuite les possibilités de prolifération des champignons noirs sur les parois dépend des micro-conditions biotiques et abiotiques sur la paroi. Ainsi, des taches peuvent se former ou pas, de manière peut-être aléatoire.

C. Limites de ces travaux de thèse

Notre travail est essentiellement basé sur le séquençage à haut débit des acides nucléiques. Cette technique comporte des biais, notamment des erreurs de séquençage ce qui nécessite de filtrer les séquences obtenues pour écarter les artéfacts potentiels. Dans notre étude, nous avons appliqués des filtres sur les longueurs de séquences en adéquation avec la longueur de l'amplicon attendu et éliminé les séquences contenant des bases ambiguës pour limiter les biais techniques. Aussi, le séquençage à haut débit comporte des limites car l'identification des microorganismes est basée sur l'affiliation qui s'appuie sur des bases de données, or ces bases de données sont souvent incomplètes. Ainsi, les bases de données sont la principale limite dans ce travail puisque c'est leur contenu qui conditionne les résultats d'identification taxonomiques.

Les contraintes d'échantillonnages liées à l'étude de la grotte de Lascaux, d'une part, et une faible quantité de matière pour certains échantillons d'autre part, a engendré un faible nombre d'échantillons pour certaines analyses. La durée d'échantillonnage dans la grotte de Lascaux était limitée donc le nombre d'échantillons possibles par condition aussi, et dans certains cas, la quantité de matière prélevée était trop faible ce qui n'a pas permis d'extraire suffisamment d'ADN pour l'amplifier. Ainsi certaines analyses comportent moins de répétitions que ce qui était prévu, et c'est le cas pour les zones non tachées du Sas-1 par exemple.

De plus, le fait de ne pas pouvoir prélever une grande quantité de matière ne nous a pas permis de réaliser une caractérisation physico-chimique des parois de la grotte. Par exemple, le pH impacte la diversité et la richesse des communautés bactériennes (Fierer & Jackson, 2006 ; Tomczyk-Żak & Zielenkiewicz, 2016), donc des mesures des caractéristiques du matériau et de sa surface permettraient de compléter les observations déjà acquises.

De nombreuses taches étudiées sont situées dans le Passage, or certaines de ces taches datent de 2006, et ne sont plus évolutives. La communauté microbienne étudiée ne représente peut-être pas la communauté microbienne à l'origine de la formation des taches.

D. Perspectives

Certains de nos résultats méritent d'être complétés par d'autres approches. Notre étude ciblait les communautés microbiennes et le lien avec les propriétés physico-chimiques des parois n'a pas pu être établi. Ainsi, la composition des différents substrats minéraux nécessite d'être étudiée.

Les expériences de dissémination et de nutrition des champignons par les collemboles ont été effectuées *in vitro*, ces résultats pourraient être confirmés par des expériences *in situ*, par exemple dans des grottes dites « laboratoire », sans valeur patrimoniale, et dans lesquelles des expériences d'introduction d'organismes exogènes pourraient être réalisées.

Des études montrent que les collemboles peuvent avoir des préférences alimentaires (Böllmann et al. 2010 ; Scheu & Simmerling, 2004). Comme ils consomment des champignons noirs et des bactéries *Pseudomonas*, une expérience de préférence alimentaire par mesure isotopique pourrait être menée afin de mieux caractériser l'interaction entre ces trois types d'organismes.

Les *Pseudomonas* isolés d'échantillons de taches et de zones non tachées dans la grotte de Lascaux n'ont pas pu être caractérisés au niveau de l'espèce, car par manque de temps nous avons utilisé seulement le gène de l'ARNr 16S, qui n'est pas un marqueur taxonomique suffisamment résolutif pour identifier en détail les *Pseudomonas*. En revanche, l'utilisation complémentaire des gènes *rpoD* et *gyrB* permettrait d'identifier l'espèce des isolats. Concernant les séquences de *Pseudomonas* retrouvées dans le jeu de données de séquençage Illumina, celles-ci pourraient être caractérisées plus finement avec des paires d'amorces différentes mises au point par Garrido-Sanz (2016) pour affilier des *Pseudomonas* de façon précise aux différents sous-groupes du complexe d'espèces de *P. fluorescens*.

Les zones sombres de l'Abside sont les altérations les plus évolutives actuellement. Une étude métatranscriptomique de ces zones sombres ainsi que des zones non tachées adjacentes permettrait de caractériser la dynamique fonctionnelle de ces nouvelles altérations.

Différentes mélanines se trouvent dans les champignons noirs, cependant nous n'avons pas pu caractériser les gènes responsables de leur synthèse. Une analyse du génome de ces champignons noirs apporterait des informations importantes sur leur biologie, et potentiellement sur leur capacité de prolifération, leur résistance aux biocides et la régulation de la synthèse des mélanines.

Cette thèse a permis de caractériser la diversité, la structure, la composition et les activités transcriptionnelles de la communauté microbienne de la grotte de Lascaux. Ces informations permettent de mieux comprendre le fonctionnement microbien de la grotte et d'alimenter la réflexion sur les stratégies à mettre en place pour optimiser la conservation de ce site paléolithique majeur.

Références

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Annexe

Tableau des échantillons prélevés pour le projet Ecologie microbienne de la grotte de Lascaux

	Déc 14 (14D)				Juin 15 (15J)				Janv 16 (16J)				Mai 16 (16M)				Déc 16 (16D)				Fév 17 (17F)				Mai 17 (17M)			
Autres grottes	44				37				0				44				0				54							
Sas-1 - paroi - hors taches	0	*0*	0	R	3	*0*	0	R	6	*0*	0	R	6	*0*		3	R	6	*6*	0	R								
Sas-1 - paroi - taches noires	6	*0*	0	*	9	*0*	0		6	*0*	0		6	*0*		3		6	*6*	0									
Sas-2 - paroi - hors glaires	3																											
Sas-2 - paroi - glaires	3																											
Sas-2 - paroi - hors vermiculations	4				4				6																6			
Sas-2 - paroi - vermiculations	4				4				6																6			
Taureaux - paroi - hors taches																γ												
Taureaux - paroi - taches noires				*												γ												
Taureaux - banquettes - hors glaires																					6							
Taureaux - banquettes - glaires																					6							
Taureaux - gours - hors vermiculations									6																			
Taureaux - gours - vermiculations									6																			
Taureaux - paroi - hors vermiculations									6																			
Taureaux - paroi - vermiculations									6																			
Diverticule axial - paroi - taches noires				*	1			*αβ																				
Passage - sol - hors taches	3				3				6				6			γ		6										
Passage - banquettes - hors taches	3	*0*	3		3	*0*	3		6	*6*	0		18	*0*		3	γ	6	*6*	0					6			
Passage - banquettes - taches noires	3	*0*	3		3	*0*	3		6	*6*	0	*	18	*0*		3		6	*6*	0					6			
Passage - plan incliné - hors taches	3	*0*	3		3	*0*	3		6	*6*	0		6	*0*		3		6	*6*	0		6			6			
Passage - plan incliné - taches noires	6	*0*	6		6	*0*	6		6	*6*	0	*	6	*0*		6		6	*6*	0								
Passage - voûte - hors taches	3	*3*	1																									
Passage - voûte - taches noires	3	*3*	1																									
Passage - voûte - taches blanches	3	*3*	1																									
Nef - Mondmilch - hors taches	3															3	γ											
Nef - Mondmilch - taches jaunes-grises	3			*												3												
Nef - Mondmilch - hors taches																					6							
Nef - Mondmilch - taches noires chapelet																					6							
Nef - plan incliné - hors taches																					6							
Nef - plan incliné - taches noires piqûre																					6							
Nef - hors 'ZS'																				*	6							
Nef - 'ZS'																				*	6							
Abside - paroi - hors taches													6					8		*				*				
Abside - paroi - taches				*	13			*αβ					8			γ		8		*				*				
Abside - paroi - glaires					5			*αβ																				
Abside - paroi - hors zones sombres					2			*α	12				6					16		*				*				
Abside - paroi - zones sombres					9			*α	12				8					16		*				*				
Diaclase - paroi - hors taches	3												6			3									6			
Diaclase - paroi - taches noires	2			*									6															
Félins - hors taches																					6							
Félins - anciennes taches																					6							
Félins - taches noires																					6							
Air - Sas-1	1			*	1				3				1					1				1			1			
Air - Taureaux	1			*	1				3				1					1				1			1			
Air - Passage	1			*	1				3				1					1				1			1			
Arthropodes - taches noires													8					8				5			8			
Arthropodes - zones sombres									12				8					16				2			8			
Eau - racines	6				6				6				6					6										
Eau - écoulements	3				0				1				4									4						
	mars-15								janv-16				juin-16									févr-17						
	114	9	18		114	0	15		130	24	0		179	0	0	30		123	36	0		140	0	0		55	0	0	

	Déc 14 (14D)	Juin 15 (15J)	Janv 16 (16J)	Mai 16 (16M)	Déc 16 (16D)	Fév 17 (17F)	Mai 17 (17M)
Tot Passage:	***54***	***33***	***54***	***69***	***54***	***6***	***18***

1ère colonne : **ADNg**
2ème colonne : ARNtot
3ème colonne : **ARNm**
R : racines
* : isolement
α : arthropodes
β : isotopie
γ : dosage carbone et azote